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PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
NÍVEL DOUTORADO
ÁREA DE CONCENTRAÇÃO EM CLÍNICA ODONTOLÓGICA / ODONTOPEDIATRIA

EFEITOS DA ATORVASTATINA SOBRE A OSSIFICAÇÃO ENDOCONDRAL DE FÊMURES, REMODELAÇÃO ÓSSEA E MOVIMENTAÇÃO DENTÁRIA INDUZIDA, ESTUDO EM RATOS

GABRIEL SCHMIDT DOLCI

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Biomateriais e Técnicas Terapêuticas em Odontologia

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Este trabalho é dedicado ao meu filho Vicente.

"Meu bocado de gente, ouvir o teu riso me remete aos mais genuínos sentimentos, enche minha vida de alegria e amor"

(texto modificado de **Antoine de Saint-Exupéry**)

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“Há três métodos para ganhar sabedoria: primeiro, por reflexão, que é o mais nobre; segundo, por imitação, que é o mais fácil; e terceiro, por experiência, que é o mais amargo”

Confúcio (551 a.C. - 479 a.C)

RESUMO

As estatinas são medicamentos comumente prescritos para a prevenção da hiper-lipidemia. Além da redução do colesterol, tais medicamentos parecem estimular a osteogênese e suprimir a reabsorção óssea, o que poderia afetar a movimentação dentária induzida (MDI) e a recidiva ortodôntica. Assim, o objetivo deste estudo foi determinar se a atorvastatina (ATV) pode afetar a MDI, a recidiva e a osteoclastogênese, por meio da modulação da expressão das moléculas: - ligante do receptor ativador de NFκB (RANKL) e osteoprotegerina (OPG). Ainda foram analisados os potenciais efeitos adversos da ATV sobre a ossificação endocondral e sobre o *turnover* de ossos longos. No primeiro experimento, 36 ratos foram sujeitos a MDI durante 21 dias, quando o aparelho foi removido. Aos animais, foram administrados, diariamente, ATV ou solução salina (SAL), via gavagem. Após 7, 14 e 21 dias de administração de ATV / SAL, a recidiva dentária foi mensurada, e foram obtidos os cortes histológicos da maxila e fêmur, os quais foram submetidos às seguintes colorações: - H&E – para análise histomorfométrica; - fosfatase acida tartrato resistente (TRAP) – para contagem de osteoclastos e; - imunohistoquímica para RANKL e OPG. A atorvastatina resultou numa inibição da recidiva ortodôntica ($p < 0,05$), e numa transiente redução do número de osteoclastos ($p < 0,05$); havendo uma correlação positiva e significativa ($p < 0,01$) entre o estes dois fatores (número de osteoclastos e a taxa de recidiva). A administração de estatinas também aumentou significativamente a expressão de OPG ($p < 0,01$), mas não a de RANKL. Além disso, após 21 dias de administração de ATV, a espessura da cartilagem da placa de crescimento e da zona hipertrófica condrocítica foi significativamente aumentada. Já no segundo experimento, 24 ratos começaram a receber diariamente ATV ou solução salina (SAL), via gavagem. Duas semanas mais tarde, a MDI foi iniciada. O deslocamento do dente foi medido após 7, 14 e 21 dias, enquanto que os cortes histológicos da maxila e do fêmur foram obtidos após 14 e 21 dias de MDI; sendo então submetidos às colorações de H&E e TRAP, para avaliação histomorfométrica e contagem de osteoclastos. A administração de atorvastatina gerou um menor movimento dentário ($p <0,05$) e uma redução transitória do número de osteoclastos ($p <0,05$).

No grupo SAL, após 14 dias de MDI, ocorreu um aumento no número de osteoclastos, assim reduzindo a taxa de volume ósseo, quando comparado com as maxilas controle (sem movimento dentário), deste mesmo grupo. Contudo, tal comportamento não foi observado no grupo ATV. Interessantemente, depois de 35 dias, a atorvastatina não afetou a remodelação óssea nas maxilas controle, nem a ossificação endocondral em fêmures. Logo, guardando as devidas limitações deste estudo pré-clínico, nossos resultados sugerem que a administração sistêmica de atorvastatina é capaz de minimizar a MDI e recidiva ortodôntica. No entanto, os seus efeitos celulares sobre a ossificação endocondral e remodelação óssea durante a MDI e recidiva, parecem ser limitados a um curto período de tempo, o que aparentemente necessita de investigações futuras. Finalmente, nossos resultados lançam luz sobre a superexpressão OPG induzida por estatinas, o que representa um alvo molecular para modular o metabolismo do ósseo e, assim minimizar a recidiva ortodôntica.

Palavras-chave: Movimento dentário; osteoprotegerina; ligante do receptor ativador do fator nuclear kappa B; atorvastatina.

ABSTRACT

Statins are drugs commonly prescribed for prevention of hyper-lipidemia. In addition to the cholesterol-lowering, these medicines seem to enhance osteogenesis and suppress bone resorption, which could affect orthodontic tooth movement (OTM) and relapse. Therefore, the aim of this study was to determine whether atorvastatin (ATV) might affect the orthodontic relapse or tooth movement and osteoclastogenesis, through the modulation of the following molecules: receptor activator of nuclear κ B ligand (RANKL) and; - osteoprotegerin (OPG). Furthermore, we analyzed potential adverse effects of ATV on long bone turnover and endochondral ossification. In the first experiment, 36 rats were subjected to OTM for 21 days, when the appliance was removed. After, the animals were administered daily with ATV (15mg/Kg) or saline (SAL), via gavage (n=18, per group). Up to 7, 14 and 21 days of ATV/SAL administration the tooth relapse was measured while maxillary and femur histologic sections were obtained and prepared to: - H&E staining – used in histomorphometric analysis, tartrate resistant acid phosphatase histochemical staining (TRAP) – used to osteoclasts counting and, immunohistochemistry to RANKL and OPG. Atorvastatin resulted in a decreased tooth relapse ($p<0.05$), and a transient reduction of osteoclasts number ($p<0.05$). There was a positive and significant correlation ($p<0.01$) between these two parameters (osteoclasts number and relapse rate). The statin administration increased significantly the OPG ($p<0.01$), but not the RANKL expression. Furthermore, after 21 days of ATV administration, the thickness of growth plate cartilage and chondrocytic hypertrophic zone was enhanced. In the second experiment, 24 rats started to be administered daily with ATV or SAL, via gavage. Two weeks later, the OTM started. The tooth displacement was measured after 7, 14, and 21 days, while the maxillary and femur histologic sections were obtained only after 14 and 21 days of OTM. At these times, the sections were prepared to H&E and TRAP, intending to perform the histomorphometric analysis and osteoclasts count. Atorvastatin administration promoted a decreased tooth movement ($p<0.05$), and a transient reduction of osteoclasts number ($p<0.05$). In the SAL group, after 14 days of OTM, the increased number of osteoclasts was

associated to a reduced bone volume rate, when compared to its control maxillae. However, this trend was not obvious in ATV group. Interestingly, after 35 days, statins did not affect the bone turnover and endochondral ossification. The big picture of our study suggests that systemic administration of statins is able to minimize OTM and relapse. However, its cellular effects on endochondral ossification and bone turnover during OTM or relapse seem to be limited to a short period, apparently requiring further investigations. Finally, our results shed light on OPG overexpression induced by statins, which represents a molecular target modulating maxillary bone metabolism thus inhibiting orthodontic relapse.

Keywords: Tooth movement; osteoprotegerin; receptor activator of nuclear factor-kappa B ligand; atorvastatin.

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LISTA DE ABREVIATURAS E SIGLAS

ATV - atorvastatina

AVC – acidente vascular cerebral

BMP – proteínas morfogenéticas ósseas

Ca^{++} - cálcio

CCL – ligante de quimiocina CC (ondeC=cisteína)

c-fms – receptor do fator estimulador de colônia para macrófagos

CXCL – ligante de quimiocina C X C (onde C=cisteína e X= aminoácido)

EGF – fator de crescimento endotelial

FDA – órgão regulador americano de drogas e alimentos

FGF – fator de crescimento fibroblástico

GPCTh – espessura do disco epifisário

GTPase - guanosinatrifosfatase

H^{+} - hidrogênio

HMG Co-A redutase - 3-hidroxi-3-methyl-glutaril Co-enzima A redutase

HzTh – espessura da zona hipertrófica

IGF – fator de crescimento insulínico

IL – interleucina

KDa – quilodaltons

LDL-C – lipoproteína de baixa densidade

LRP5 – proteína 5 relacionada ao receptor de lipoproteínas de baixa densidade

LRP6 - proteína 6 relacionada ao receptor de lipoproteínas de baixa densidade

MCSF – fator estimulador de colônia para macrófagos

MDI – movimento dentário induzido

MEC – matriz extracelular

MMP – metaloproteinase de matriz

mRNA – ácido ribonucleico mensageiro

NFκB – fator nuclear kappa B

OPG – osteoprotegerina

OH^- – hidroxila

p-cofilin – cofilina fosforilada

pH – potencial de hidrogênio

PO_4^{---} – fosfato

PTH - paratormônio

RANK – receptor ativador de NFκB nuclear

RANKL – ligante do receptor ativador de NFκB nuclear

Rho – GTPase de baixo peso molecular (~21kDa)

Rock – proteína quinase associada à Rho

sRANKL - ligante do receptor ativador de NFκB nuclear extra celular, solúvel

TGF – fator de crescimento tumoral

TIMP – inibidor tecidual de metaloproteinase

TNF – fator de necrose tumoral

TRANCE - receptor ativador de NFκB nuclear

V-ATPase – adenosinatrifosfatase vacuolar

VEGF – fator de crescimento do endotélio vascular

VLDL-C - lipoproteína de muito baixa densidade

Wnt – Weingless (drosófilas mutantes sem asas) + Int (gene mutante que causa a ausência das asas)

1. INTRODUÇÃO E REVISTA DA LITERATURA

A Ortodontia contemporânea tem se caracterizado por um notável avanço nas áreas de materiais dentários e técnicas utilizadas. Por outro lado, pouco se sabe sobre os fatores moleculares e celulares que regem o movimento dentário induzido (MDI) e, nesta área, grande parte das afirmações ainda são empíricas. Tal cenário parece evidenciar um *gap* entre a clínica e a biologia da movimentação dentária, o que tem fomentado a realização de pesquisas em áreas básicas da saúde voltadas para este tema.

Clinicamente, o movimento dentário induzido por forças mecânicas (MDI) é caracterizado por três fases: 1) A fase inicial, na qual se observa uma rápida movimentação dentária; 2) Um período de platô, onde pouco movimento ou nenhum movimento dentário ocorre e; 3) A fase final, na qual ocorre um movimento dentário gradual ou, um repentino movimento dentário de maior magnitude.[1] Já quanto aos aspectos biológicos dos tecidos periodontais durante o MDI, estes são caracterizados por uma resposta inflamatória aguda asséptica nos estágios iniciais e, por uma inflamação crônica asséptica transiente nos estágios tardios. Quimiocinas, citocinas várias e fatores de crescimento, em especial, são as principais moléculas que orquestram tais reações inflamatórias, culminando com a reabsorção óssea osteoclástica e neoformação óssea osteoblástica.[2] O estado atual do conhecimento científico permite-nos vislumbrar uma plausível modulação farmacológica do *turnover* ósseo periodontal, então estimulando ou inibindo a movimentação ortodôntica, o que tem sido demonstrado em diversos estudos pré-clínicos.[3; 4; 5; 6; 7; 8]

Neste contexto, as estatinas são uma classe de drogas usadas para reduzir os níveis séricos de colesterol, por meio da inibição da HMG-CoA redutase, uma enzima crucial na via do mevalonato, ou seja, na via de biossíntese do colesterol[9; 10] (Figura 1). Em adição à redução do colesterol, estudos apontam para o fato dessas drogas também atuarem sobre o *turnover* ósseo[3; 11; 12; 13; 14; 15; 16; 17; 18], sugerindo um anabolismo ósseo promovido pelas estatinas, o que parece ser regulado por uma promoção da osteogênese e, supressão da osteoclastogênese.[19] Mundy et al.[20] ao realizarem um estudo pré-clínico *in vitro* e *in vivo*, foram os primeiros autores a relatar os efeitos das

estatinas sobre o tecido ósseo, observando que tais drogas são hábeis na indução da função osteoblástica, o que foi relacionado com uma maior expressão da proteína BMP-2. Já a inibição da osteoclastogênese mediada pelas estatinas parece envolver três diferentes mecanismos: - inibição da via NFκB, inibição da via do mevalonato e ação anti-inflamatória. Outros estudos também confirmaram a relação direta entre administração de estatinas, redução do número de osteoclastos, estimulação da diferenciação osteoblástica e osteogênese aumentada.[11; 12; 13; 17; 18; 20] Embora os efeitos das estatinas sobre o tecido ósseo sejam amplamente demonstrados em estudos laboratoriais[3; 11; 12; 13; 14; 15; 16; 17; 18], seus efeitos clínicos ainda não são convincentes[21], sendo esta uma área de controversa discussão.

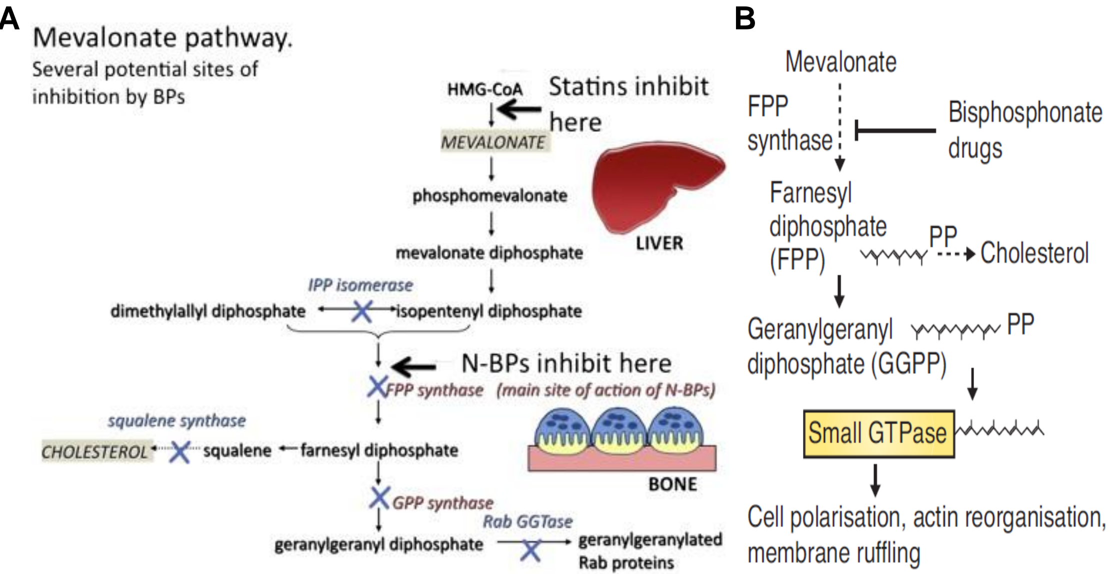

Figura 1 - (a) As estatinas atuam sobre a HMG CoA redutase, inibindo a via do mevalonato e consequentemente a síntese de colesterol. (b) Observa-se que tal via (mevalonato) é de suma importância na síntese de GTPases de baixo peso molecular, as quais estão associadas a polarização, reorganização da actina e formação do bordo em escova dos osteoclastos. BPs – Bifosfanatos.(Buhaescu [22], Crockett[23])

De acordo com Mercado[24], um total de 36,7% dos adultos norte americanos ou, 78,1 milhões de pessoas com idade ≥21 anos, estão em tratamento ou são elegíveis para o tratamento com estatinas. Assim, considerando que as estatinas são fármacos comumente prescritos para a prevenção de hiperlipidemias e doenças cardiovasculares[9; 10], seus efeitos sobre o MDI parecem ser um tema relevante na prática ortodôntica. Estudos recentes demonstraram que a administração sistêmica destas drogas, em

ratos, irá gerar uma significativa redução do movimento dentário e recidiva ortodôntica. Han et al.[3] ressaltaram a capacidade das estatinas em minimizar o deslocamento dentário, durante o período de recidiva, sugerindo uma ação da droga sobre a neoformação óssea, assim acelerando a estabilidade do dente e auxiliando durante a fase de contenção. No mesmo sentido, MirHashemi et al.[16] concluíram que a adiministração diária de atorvastatina inibiu o movimento dentário induzido em ratos Wistar. Dessa forma, guardando as devidas limitações da translação de dados obtidos em estudos laboratoriais para a prática clínica, sugere-se que a administração de estatinas possa inibir o movimento dentário em indivíduos submetidos ao tratamento ortodôntico, o que ocorreria pela modulação medicamentosa do tecido ósseo periodontal. Contudo, os aspectos celulares e moleculares envolvidos a estes significativos efeitos clínicos ainda não foram desvendados, necessitando de maiores investigações.

Embora estudos clínicos tenham demonstrado que as estatinas são bem toleradas por adultos e crianças[25; 26; 27], seus efeitos adversos sobre o tecido ósseo não foram amplamente analisados, sendo necessários apropriados acompanhamentos a longo prazo.[27; 28] De acordo com Macpherson[25], o tratamento de crianças e adolescentes com estatinas, ao longo de 2 anos, não teve impacto sobre sua altura, peso, massa corporal e maturação sexual. Por outro lado, investigações pré-clínicas, em animais, *in vivo* e *in vitro*, sugerem que essas drogas estimulam a proliferação de condrócitos e o crescimento longitudinal de ossos longos.[29; 30], o que poderia contraindicar o seu uso clínico em populações pediátricas. Embora as estatinas tenham aprovação da *Federal Drug Association* (FDA) para serem usadas em criança (≥ 10 anos)[31], essas discrepâncias entre os resultados obtidos em ensaios clínicos e estudos *in vitro* e *in vivo*[25; 26; 27; 28; 29; 30] parecem ser uma incógnita. Maiores investigações com o intuito de desvendar os efeitos adversos das estatinas sobre o *turnover* ósseo e sobre a ossificação endocondral parecem necessárias. Somente assim, seu uso tornar-se-á uma alternativa plausível na prática ortodôntica.

Os avanços da biologia celular e molecular traçam novas perspectivas para o controle da movimentação dentária induzida com auxílio de medicamentos. A possibilidade de acelerar o *turnover* ósseo com consequente

redução do tempo de tratamento ortodôntico, o provável tratamento medicamentoso da recidiva ortodôntica e, até mesmo, o movimento dentário dificultado em indivíduos que consumam determinado tipo de droga, fazem deste um tópico de especial interesse na literatura ortodôntica. Assim, para que haja um melhor entendimento dos efeitos das drogas, em especial das estatinas, sobre a movimentação ortodôntica, faz-se necessário compreender alguns conceitos referentes à biologia do tecido ósseo, à biologia do movimento dentário induzido e à modulação medicamentosa do *turnover* ósseo, conforme descrito a seguir.

1.1. Biologia do Tecido Ósseo

O tecido ósseo é considerado um tipo especializado de tecido conjuntivo, formado por células e matriz extracelular (MEC) calcificada – a matriz óssea. A MEC deste tecido é composta por uma porção inorgânica, a qual representa 50% do peso total de sua matriz, sendo os íons fosfato e o cálcio, os mais encontrados nessa. Também são observados íons bicarbonato, magnésio, potássio, sódio e citrato. O cálcio e o fósforo formam cristais com a estrutura de hidroxiapatita, com a seguinte composição: $Ca_{10}(PO_4)_6(OH)_2$. Os íons da superfície destes cristais são hidratados (capa de hidratação), o que facilita a troca iônica entre os cristais e o líquido intersticial.[32; 33]

Em relação à parte orgânica da matriz, esta é formada principalmente por fibras colágenas constituídas de colágeno do tipo I e pequena quantidade de glicosaminoglicanas, proteoglicanas e glicoproteínas específicas (osteonectina, osteocalcina, osteopontina, fosfoproteínas, sialoproteínas).[33; 34] Acredita-se que estas tenham alguma atuação sobre a mineralização da matriz, uma vez que outros tecidos ricos em colágeno tipo I, que não contêm tais glicoproteínas, normalmente não se calcificam. A associação das fibras colágenas com a hidroxiapatita confere ao osso dureza e resistência.[32; 33]

Dentre os elementos celulares que compõem o tecido ósseo, citam-se: os osteoblastos - secretores de matriz óssea orgânica, os osteócitos - que se situam em lacunas no interior da matriz, as células de revestimento ósseo - que são osteoblastos em repouso e, os osteoclastos - células gigantes móveis e

multinucleadas que reabsorvem o tecido ósseo, participando dos processos de remodelação óssea.[32; 35]

Conforme observado na Figura 2, células de revestimento ósseo, osteoblastos e osteócitos são derivados de células mesenquimais pluripotentes e representam vários estágios de um mesmo tipo celular. Segundo Wozney et al.[36], tais células mesenquimais diferenciar-se-ão em osteoblastos quando expostas a BMP, contudo o conhecimento sobre o processo de diferenciação osteoblástica ainda é muito limitado.

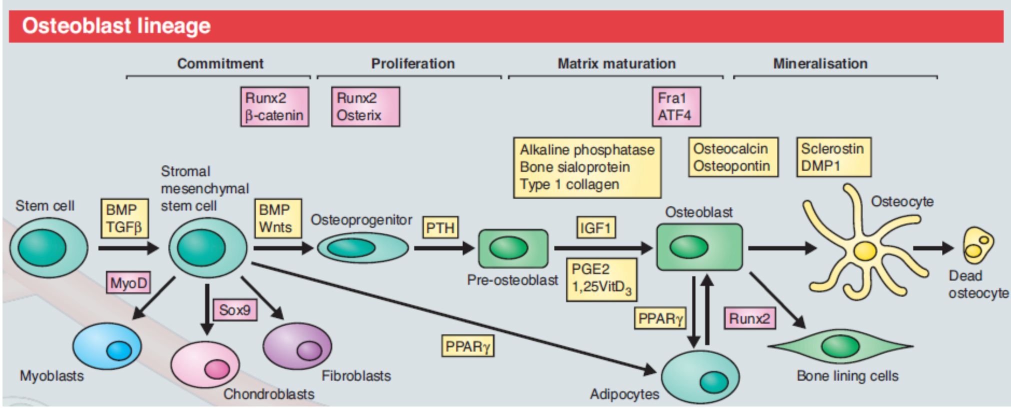

Figura 2 – As diferentes etapas da diferenciação osteoblástica: do comprometimento celular à mineralização. Observe que osteoblastos, ósteócitos e células de revestimento ósseo compartilham a mesma via de diferenciação, que ocorre a partir da célula mesenquimática pluripotente do estroma. (Crockett[37])

Quando maturas, os osteoblastos são as células que sintetizam a parte orgânica da matriz óssea (colágeno tipo I, proteoglicanas, glicoproteínas e glicosaminoglicanas) e, além disso, são capazes de concentrar fosfato de cálcio, participando da mineralização da matriz. Quanto a sua localização, situam-se na superfície óssea, num arranjo que lembra o epitélio simples e, quando em intensa atividade sintética, são cubóides com citoplasma muito basofílico. Por outro lado, quando estão em estado pouco ativo, tornam-se achatados e a basofilia citoplasmática diminui. Além disso, os osteoblastos em fase de síntese mostram características ultra-estruturais de células produtoras de proteínas, sendo que a matriz óssea recém formada, adjacente aos osteoblastos ativos e que ainda não está calcificada, denomina-se osteóide.[32; 33]

A formação do osso, processo denominado de osteogênese, envolve a proliferação e migração das células osteoprogenitoras e a diferenciação destas

em osteoblastos. Tais células irão depositar a Matriz óssea inorgânica (osteóide) e, após, atuarão na mineralização da mesma. Fatores como a fosfatase alcalina, osteocalcina e outras matrizes de proteínas não colágenas, estimulam a diferenciação em osteoblastos. Este processo é controlado por uma cascata de eventos combinados a uma programação genética com a regulação de genes por fatores sistêmicos e locais, entre eles os hormônios, os fatores de crescimento e outras citocinas.[32; 33]

Os osteoblastos, nos seus diferentes estágios de diferenciação, produzem fosfatase alcalina, colágeno tipo I, osteocalcina, osteopontina, citocinas várias, fatores estimulantes de colônias, colagenase, TIMP (tecido inibidor de metaloproteinases) e ativador de plasminogênio. Estes fatores autócrinos e parácrinos, os quais incluem os fatores de crescimento, ajudam a regular o metabolismo celular.[33]

Uma vez aprisionado na matriz óssea sintetizada, o osteoblasto passa a ser chamado de osteócito. Os osteócitos são encontrados no interior da matriz óssea, em lacunas das quais partem canalículos (canalículos calcóforos), onde se encontram os prolongamentos destas células. Tais prolongamentos e a escassa matriz extracelular, que os envolve, constituem uma via de transporte de nutrientes e metabólitos entre os vasos sanguíneos e os osteócitos.

Embora as características estruturais indiquem baixa atividade sintética, os osteócitos são essenciais para a manutenção da matriz óssea, sendo sua morte sucedida por reabsorção da matriz.[32; 33]

Além disso, os osteócitos sintetizam a esclerostina, uma proteína secretada que inibe formação óssea por ligação aos co-receptores LRP5/LRP6, assim inibindo a ativação da via de sinalização Wnt.[38] A esclerostina é negativamente regulada pelo paratormônio (PTH), o que parece explicar em parte o a função anabólica do PTH sobre o osso, em parte, através deste mecanismo.[39]

Por fim, estudos pioneiros sobre osteócitos levantaram a possibilidade de essas células estarem associadas à regulação da homeostase ionica sérica de fosfato. A superfície dos osteócitos é, aproximadamente, 100 vezes maior

do que a superfície óssea trabecular e tem o potencial para contribuir significativamente para a homeostase mineral.[40] Atualmente, acredita-se que os osteócitos, ao contrário de osteoclastos, não reabsorvem quantidades significativas de osso, mas sim modificam a composição da matriz mineral nas áreas perilacunares.[41]

As células de revestimento ósseo representam os osteoblastos inativos que recobrem as superfícies ósseas. Possuem poucas organelas de síntese e secreção de proteínas e formam uma camada contínua de células interconectadas, com capacidade de manter a homeostasia do tecido ósseo. São estas células que regulam a concentração plasmática de cálcio por mecanismos de estímulo à remodelação óssea. Além disso, em determinadas situações, estas células poderão se diferenciar novamente em osteoblastos e assim contribuírem para a deposição da matriz.[23; 32; 33]

Já os osteoclastos diferenciam-se a partir de células hematopoiéticas mononucleares, utilizando uma via de diferenciação que também é compartilhada pelos monócitos /macrófagos. Logo, como poderia se esperar, o fator estimulador de colônia para macrófagos (M-CSF) exerce importante papel na regulação destes estágios compartilhados de diferenciação osteoclástica e de monócitos/macrófagos (Figura 3).[42]

Na gênese dos osteoclastos, destaca-se o papel de suas células precursoras provindas da medula óssea, pertencentes à linha monócito-macrófago. Estudos têm apontado para o potencial de diferenciação celular em osteoclastos, a partir de precursores em diferentes estágios de maturidade: blastos jovens, blastos mielóides ou monócitos. Além disso, ainda relatam-se diferenças nos potenciais osteoclastogênicos de células provindas da medula de ossos longos e de ossos maxilares.[43; 44]

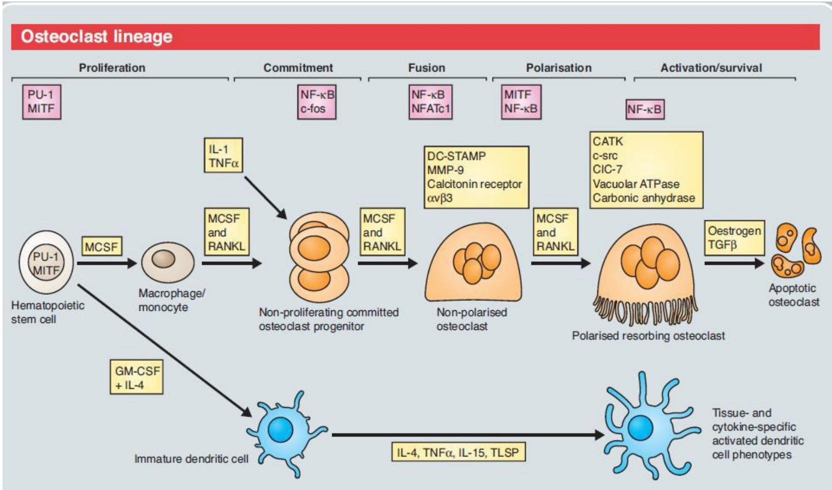

Figura 3 – As diferentes etapas da diferenciação osteoclástica: da diferenciação à ativação/sobrevivência osteoclástica Uma pré-condição inicial para esta diferenciação é a ação do estimulador formador de colônia para macrófagos (M-CSF) e, a sinalização da via NFκB, pela ligação RANK/RANKL, na célula hematopoiética pluripotente (pré-osteoclasto). Note que o macrófago compartilha dos estágios iniciais desta via de diferenciação. (Crockett[37])

Recentemente, a identificação do sistema RANK/RANKL/OPG como mediador final dominante da osteoclastogênese, representou um importante avanço para a biologia celular. No tecido ósseo, o ligante do receptor ativador de NFkB nuclear (RANKL) é sintetizado e secretado pela linhagem de células osteoblásticas e exerce sua função ligando-se ao receptor ativador de NFkB nuclear (RANK) na linhagem de células osteoclásticas. Esta ligação irá promover a ativação da via de NFkB (Figura 3), assim gerando uma rápida diferenciação das células precursoras de osteoclastos em osteoclastos maturos. Já a osteoprotegerina (OPG) tem uma ação de competição com RANK para a ligação ao RANKL. Sendo assim, os efeitos biológicos da OPG sobre as células ósseas incluem a supressão da ativação de osteoclastos, inibindo sua função reabsortiva e estimulando a apoptose dos mesmos (Figura 3). Logo, pode-se considerar que a remodelação óssea é controlada por um balanço entre a ligação RANK-RANKL e a produção de OPG.[37; 45; 46]

RANK, também conhecida como Receptor TRANCE, é uma proteína transmembrana do tipo I, com 616 aminoácidos, expressa primariamente nas células da linhagem monócito-macrófago, incluindo as células pré-osteoclásticas, células T e B, células dendríticas e fibroblastos.[47]

RANKL é pertencente a superfamília da Fatores de Necrose Tumoral (membro11), sendo uma proteína com 317 aminoácidos. Quando RANKL é

expresso por células de linhagem osteoblástica, é aderido à célula e, quando expresso por linfócitos T, é solúvel. (sRANKL).[23; 46] RANKL exerce sua função ligando-se ao receptor RANK na linhagem de células osteoclásticas, ligação esta que irá gerar uma rápida diferenciação de células precursoras de osteoclastos em osteoclastos maturos, como já foi dito.

OPG é um membro da superfamília dos receptores do Fator de Necrose Tumoral (TNF) e representa uma proteína matura, com 380 aminoácidos. O RNA mensageiro da OPG (mRNA OPG) é expresso em vários tecidos, sendo esta proteína OPG secretada apenas de forma solúvel, principalmente por células osteoblásticas e por células de outros tecidos, tais como pulmões, coração, rins, intestino, cérebro, estômago, fígado, medula espinhal, glândula tireóide e osso.[48] A ação da OPG está associada ao fato desta proteína neutralizar a ligação RANK/RANKL, atuando como um receptor antagonista da RANKL. Sendo assim, os efeitos biológicos da OPG sobre as células ósseas incluem a supressão da ativação de osteoclastos, inibindo a função reabsortiva dos mesmos e estimulando sua apoptose.[37] De acordo com Oshiro et al.[37], RANKL OPG e BMPs (Proteínas Morfogenéticas Ósseas) são secretados por osteócitos presentes no osso alveolar, fibroblastos e osteoblastos presentes no ligamento periodontal.

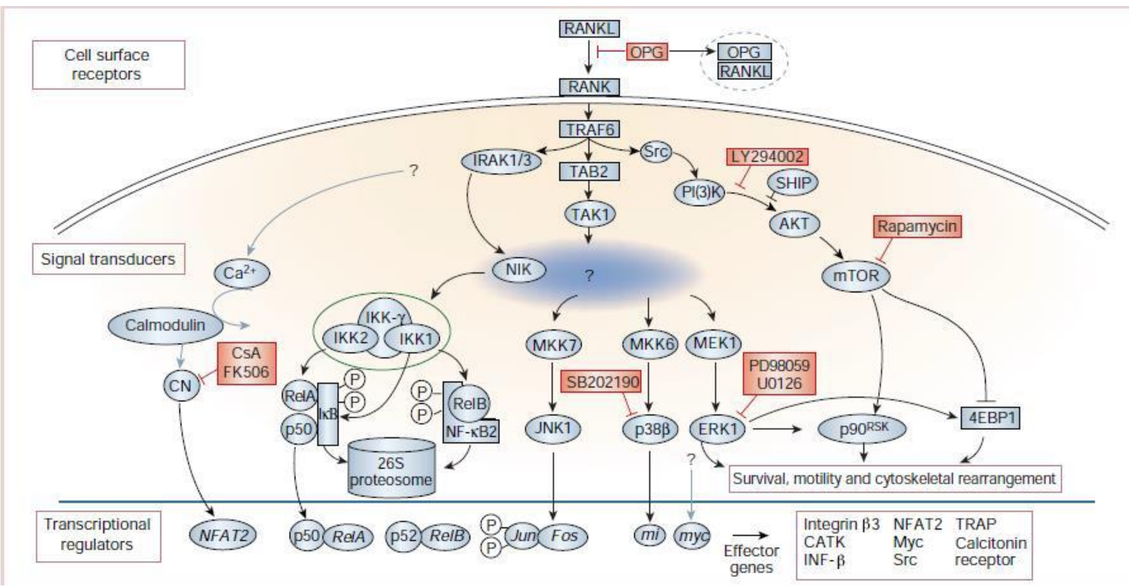

Figura 4 – Sinalização da via NFκB por meio da ligação RANKL/RANK. A proteína OPG é um receptor chamariz, que poderá se ligar a RANKL, assim impedindo a interação via RANK/RANKL. No microambiente ósseo periodontal, a proteína RAKL é expressa na membrana de células osteoblásticas, enquanto a OPG é produzida por osteoblastos fibroblastos do ligamento. (Boyle, Simonet & Lacey[49])

Hakeda et al.[50] detectaram na membrana plasmática de osteoclastos maturos isolados de coelho, uma proteína de ligação à OPG de 140 kDa. A ligação de OPG a esta proteína presente na membrana de osteoclastos, reduziu ou rompeu a formação de anéis de F-actina em osteoclastos isolados. A F-actina é uma estrutura citoesqueletal que está correlacionada com a atividade de reabsorção óssea, por ser importante elemento da estrutura celular osteoclástica. Estes achados demonstram que a OPG pode diretamente inibir a função osteoclástica, por meio de um mecanismo independente de RANKL.

Para Crocktett[23; 51], a diferenciação de osteoclastos, inicialmente depende de sinalização através de c-fms, o receptor para o fator estimulador de colónias de macrófagos, em células precursoras mononucleares, o que irá regular positivamente a expressão de RANK. Desta forma, a sinalização através RANK e c-fms, em precursores mononucleares, seria o principal regulador da diferenciação osteoclástica.

Quando em seu estado ativo, os osteoclastos são células gigantes, móveis, ramificadas, com partes dilatadas que contém de seis a 50 ou mais núcleos. Como os cortes histológicos revelam apenas pequenas porções dos osteoclastos, a morfologia geral destas células é melhor evidenciada por microscopia eletrônica. Sob este aspecto, o osteoclasto ativo é uma célula contendo projeções citoplasmáticas (borda pregueada) e, circundando esta região, no citoplasma, se encontra a zona clara, que é desprovida de organelas e apresenta um sistema de microfilamentos contendo actina.[52] A zona clara é um local de adesão do osteoclasto com a matriz óssea, propiciando um microambiente fechado onde ocorre a reabsorção do tecido ósseo.[32]

A superfície ativa dos osteoclastos denomina-se borda pregueada, ou borda em escova, e apresenta prolongamentos vilosos irregulares na porção em contato com o tecido ósseo que está sendo reabsorvido. A borda em escova é uma organela reabsortiva, formada da fusão de vesículas citoplásticas ácidas. Ainda em relação à polarização do osteoclasto ativo, em cada célula, ocorrerá a divisão de dois domínios basolaterais e um terceiro domínio central e apical, também conhecido como domínio funcional secretor,

não havendo evidências de barreiras estruturais entre os 3 domínios. Frequentemente, nas áreas de reabsorção do tecido ósseo, encontram-se porções dilatadas dos osteoclastos colocadas em depressões da matriz escavadas por atividade osteoclástica, as quais são conhecidas como lacunas de Howship.[23; 32; 33; 35; 53]

De acordo com Vaananen et. al.[52], a sequência de eventos celulares necessários para a reabsorção óssea é chamado "ciclo de reabsorção", o qual requer uma série de atividades para a sua ocorrência, tais como: 1) migração dos osteoclastos ao local de reabsorção, ativação e adesão ao osso; 2) polarização da célula clástica e formação de novos domínios de membrana; 3) dissolução da hidroxiapatita e degradação de matriz orgânica; 4) remoção de produtos de degradação do processo reabsortivo e apoptose dos osteoclastos, ou o seu retorno para o estágio inicial (previamente à fase de reabsorção).

Após a migração do osteoclasto a um local de reabsorção, a zona de selamento (zona clara) forma-se sob o osteoclasto, na sua periferia.[53] As interações moleculares entre a membrana plasmática e a matriz óssea na zona de selamento ainda são desconhecidas. As moléculas envolvidas na adesão do osteoclasto ao osso são integrinas, expressas na superfície celular e funcionam como receptores de proteínas da matriz óssea (colágeno tipo I, osteopontina, sialoproteínas, trombospondina e fibronectina).[23; 35; 52]

Assim, no osteoclasto ativo, por ação de enzimas (V–ATPase), ocorre bombeamento de prótons (H^+) para fora da célula, especificamente na região delimitada pela zona de selamento, reduzindo o pH e facilitando a degradação da matriz óssea.[35; 54] As enzimas V-ATPases concentram-se em vesículas citoplasmáticas no bordo em escova osteoclástico, estando associadas com o citoesqueleto de actina.[55] Holiiday et. al.[56] relatam que a atividade de ligação da V-ATPases aos microfilamentos de actina é imprescindível para o transporte destas enzimas ao bordo em escova, sendo este um processo necessário à reabsorção óssea.

No processo de degradação da matriz óssea pelo osteoclasto, a dissolução mineral ocorre devido à redução do pH no interior da lacuna formada, como descrito anteriormente. Depois da solubilização da fase mineral,

diversas enzimas proteolíticas degradam a matriz orgânica. Neste processo, têm sido estudadas com maior frequência: Proteinases de Cisteínas Lisossômicas (catepsinas) e as Metaloproteinases da Matriz (MMPs – colagenase e gelatinases). Sugere-se que, o pH de ação da colagenase varie de 6 -7.5, fato que determina um papel protagonista das catepsinas, que atuam em pH entre 4.5 – 5, na degradação da matriz óssea orgânica.[35; 57]

De acordo com Nesbitt & Horton[57] os produtos provenientes da degradação da matriz óssea serão transportados, por transocitose, dos bordos em escova até o domínio de membrana central secretor, região na qual ocorrerá a exocitose dos mesmos. Contudo, não se sabe ao certo qual o percentual dos produtos degradados que permanecem extracelulares e qual percentual transportado via intracelular.

Por fim, todo este processo de reabsorção osteoclástica é expressivamente induzido por uma ação de osteoblastos, a qual está associada a mecanismos específicos:[35]

a) Toda superfície óssea, exceto as áreas de reabsorção, é revestida por uma matriz não calcificada, denominada osteóide. Os osteoclastos são incapazes de reabsorver o tecido ósseo se esta camada osteóide estiver intacta. Assim, quando estimulado pelo hormônio da paratireoide (PTH), por prostaglandinas (modulador do processo inflamatório) ou por citocinas como o a interleucina 1 (IL-1) e fatores de necrose tumoral (TNF), os osteoblastos têm sua homeostasia alterada e liberam metaloproteinases, como a colagenase (MMP-1) e a estromalisina (MMP-3). Estas acabam por promover a degradação da osteóide, assim expondo a matriz óssea calcificada. Logo, o contato da matriz mineralizada com os osteoclastos estimulará o início da reabsorção óssea;

b) Muitos fatores associados à reabsorção óssea exercem sua função indiretamente sobre os osteoblastos. Estes fatores induzem os osteoblastos a liberarem mediadores químicos solúveis, que ativam a reabsorção óssea por estímulo da diferenciação de células da linhagem precursora de osteoclastos em osteoclastos maturos, gerando um aumento na população destas células.

1.2. Biologia do Movimento Dentário Induzido

A teoria “Pressão – Tensão” explica a biologia da movimentação dentária induzida por considerar que, ao aplicarmos uma força sobre um elemento dentário, será gerada uma zona de pressão e outra de compressão no ligamento periodontal[58; 59], como observado na Figura 5. A partir daí, ocorre um distúrbio na homeostasia tecidual, estimulando a remodelação do osso alveolar e do ligamento periodontal, assim movimentando o dente. Salienta-se que uma pré-condição deste evento é a ocorrência de um processo inflamatório nos tecidos periodontais adjacentes ao dente e, neste contexto, alterações vasculares e celulares induzidas por mediadores químicos, característicos da inflamação, possibilitarão o movimento dentário induzido.

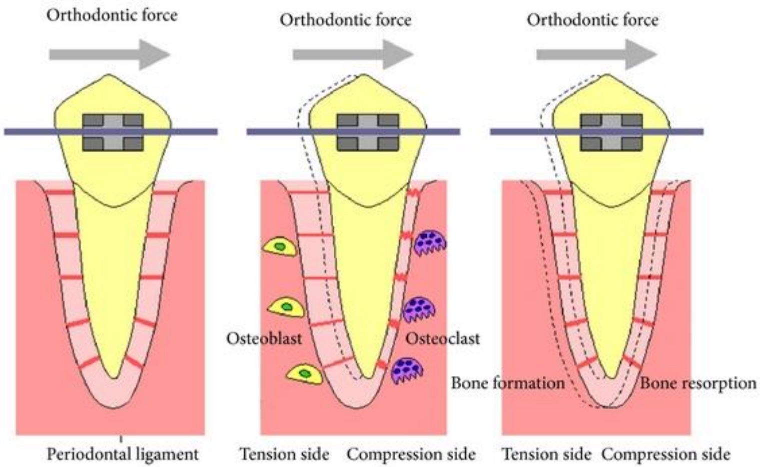

Figura 5 – Zonas de pressão e tensão geradas durante o movimento dentário induzido. Flecha indica a direção e o sentido da força aplicada. Tal estímulo mecânico induzirá a diferenciação celular em osteoclastos e osteoblastos nas zonas de pressão e tensão, assim promovendo a reabsorção e a neoformação óssea, conforme ilustrado. (Kitaura et al[60])

Considera-se que, após a aplicação de uma força ao dente, este irá se mover por certa distância, havendo uma mudança quase imediata no fluxo de fluidos do ligamento periodontal, nos lados de pressão e tensão, o que estimularia a transmissão de fluidos entre os osteócitos. Quanto a isso, duas alternativas foram propostas: 1) a transmissão de fluidos seria estimulada por um agente mecânico externo e; 2) durante o movimento dentário induzido ocorreriam micro lesões no osso, que estimulariam a transmissão de fluidos intraósseos. Assim, tanto a deformação da matriz, como a transmissão de

fluidos no ligamento periodontal, causarão uma deformação nas células deste microambiente, induzindo a sinalização de integrinas e de outros caminhos de transdução, por meio dos quais mediadores serão produzidos e ativarão diversos tipos celulares.[61]

De acordo com Tadei et al.[2], quimiocinas, citocinas e fatores de crescimento, oriundos de um processo inflamatório asséptico, são as principais moléculas envolvidas no recrutamento, ativação, proliferação, diferenciação e sobrevivência de células ósseas,durante a movimentação ortodôntica. Estas moléculas estimulam as células do ligamento periodontal a se diferenciarem em osteoclastos, na zona de pressão, promovendo a reabsorção óssea e, em osteoblastos na zona de tensão, aí promovendo a neoformação óssea. As principais moléculas que parecem estar envolvidas neste processo são: - Fator estimulador de colônia para macrófagos (M-CSF); - RANK, RANKL, OPG; - Fator de necrose tumoral alfa (TNF); - Interleucinas 1, 6, 8 e 11; - Quimiocinas CCL2, CCL3, CCL5, IL-8 (CXCL8), CXCL12; - Fator de crescimento vascular endotelial (VEGF); - Fator de crescimento de transformação beta (TGFβ); - Fatores de crescimento insulínico (IGF); - Fator de crescimento fibroblástico 2 (FGF2) e; - Fator de crescimento epidérmico (EGF). Os autores descrevem que, durante o movimento dentário induzido, o processo inflamatório, é iniciado quando hipoxia local aumenta a expressão de IL-1β, IL-6, IL-8, TNF- α e VEGF em fibroblastos do ligamento periodontal. O VEGF, fator angiogênico, irá aumentar o fluxo e a permeabilidade vascular, levando ao extravasamento de leucócitos plasmáticos por meio do processo de diapedese. Estes leucócitos recrutados interagem direta ou indiretamente com toda a população celular nativa do periodonto, aumentando a produção de quimiocinas, citocinas e fatores de crescimento específicos, todos envolvidos na reabsorção óssea. Desta forma, uma fase aguda da inflamação é substituída por um processo crónico, permitindo que leucócitos e precursores de osteoclastos continuem sua migração para os tecidos periodontais.

Já Garlet el al.[62] atentam para o fato da distinta expressão de citocinas nas zonas de pressão e tensão geradas durante o movimento dentário em humanos. Segundo os autores, a expressão de TNFα é significativamente maior no lado de pressão, o qual é caracterizado pela reabsorção óssea e uma

maior expressão de RANKL. Os autores também observaram um aumento da expressão da interleucina 10 (IL10), no lado de tensão, a qual estava positivamente correlacionada com a expressão de osteocalcina, assim sugerindo que tal citocina desempenha importante papel sobre a neoformação óssea. Outra citocina observada, nos lado de tensão, foi o TGFβ, sugerindo-se que esta deva exercer um efeito anabólico sobre o periodonto, induzindo a formação de tecido conjuntivo mineralizado e não mireral.

Ainda considerando os aspectos celulares e moleculares do microambiente ósseo durante a MDI, na zona de pressão, ocorre uma maior expressão das proteínas RANKL e menor expressão de OPG, as quais parecem estar associadas à regulação da diferenciação em osteoclastos, como tem sido demonstrado *in vitro*[63] e *in vivo.*[64]

Diversos autores têm observado a expressão das proteínas RANKL e OPG nos tecidos periodontais e no fluido crevicular, durante o movimento dentário induzido (MDI). Kawasaki et al.[65] relataram que a redução na quantidade de movimento dentário observada em decorrência do aumento da idade, estava associada a uma redução na razão RANKL/OPG, no fluido crevicular, durante os estágios iniciais de movimentação dentária. Já Shiotani et al.[64] observaram a presença de RANKL nos tecidos periodontais durante a movimentação ortodôntica, em molares de ratos. Os autores sugeriram que a expressão desta molécula seja regulada por citocinas inflamatórias no periodonto, durante o MDI.

Os esperados efeitos celulares destas alterações moleculares do microambiente ósseo apontam para uma maior diferenciação osteoclástica na zona de pressão, durante a movimentação ortodôntica. Segundo Ren et al.[66], ao avaliar o movimento dentário em ratos jovens e adultos, os autores observaram que houve um aumento significativo de osteoclastos em ambos grupos de ratos, sendo que o pico de células ocorreu após duas semanas. Além disso, os autores concluíram que há uma correlação significante e positiva entre o número de células osteoclásticas e a quantidade de movimento dentário, em ratos jovens.

1.2. Recidiva Ortodôntica

Sob o prisma clínico, após a movimentação dentária induzida e consequente correção da má oclusão, o tratamento ortodôntico entra na fase denominada “contenção”. Neste momento, há uma tendência dos dentes retornarem às suas posições iniciais, sendo tal processo denominado “recidiva ortodôntica”. Por isso, faz-se necessário o uso de aparelhos que inibam tal ocorrência indesejada. Diversos estudos relatam as possíveis causas da recidiva ortodôntica sendo a grande maioria destes relatos de caso ou estudos longitudinais.[67; 68; 69; 70] Contudo, tais estudos clínicos não consideram a hipótese de haver fatores etiológicos da recidiva relacionados à biologia do tecido periodontal.

Neste sentido, recentemente, alguns pesquisadores têm utilizado modelos animais para um melhor entendimento e tentativa de controle da remodelação óssea no período posterior a movimentação dentária induzida (recidiva ortodôntica).[3; 4; 8; 71; 72; 73] Yoshida et al.[73] sugeriram que a remodelação do osso alveolar é uma das principais causas de recidiva após o movimento do dente em ratos.

Já Franzen et al.[71], relataram que, após 7 dias, o primeiro molar continuou a recidiva, embora as fibras transeptais estivessem normalmente estiradas, assim concluindo que o alongamento destas fibras podem não desempenhar um papel central na etiologia deste processo. Os autores ainda ressaltaram que a recidiva ortodôntica, considerando ratos como modelo animal, ocorre rapidamente, logo após a remoção do dispositivo ortodôntico, e neste período a remodelação óssea do alvéolo desempenha um papel central.

Autores [3; 4; 71; 72; 73] têm trabalhado no estabelecimento de protocolos para o estudo da recidiva ortodôntica em animais. Nestes ensaios laboratoriais utilizaram-se períodos de 7, 21 e 28 dias para o movimento dentário induzido e, 0, 1, 4, 5, 7,8,10,16 ,21 e 24 dias para o controle da recidiva ortodôntica. Tais estudos objetivaram minimizar a ocorrência da recidiva por meio da administração de drogas via intraperitoneal ou local (mesial do primeiro molar), ou simplesmente estudar os aspectos biológicos periodontais durante a recidiva ortodôntica. Quanto ao movimento dentário induzido, este fora realizado por meio da aplicação de uma força de magnitude variando de 35 a

50g, sobre a coroa do primeiro molar maxilar, uni ou bilateralmente. Os estudos avaliaram a quantidade do movimento dentário por meio de modelos de gesso, os quais foram escaneados ou medidos diretamente com paquímetro (em lupa). Além disso, para o estudo da remodelação óssea, as regiões de interesse selecionadas foram as zonas de pressão e tensão adjacentes a raiz mesial do molar em questão. Outras análises foram incluídas nestes estudos, tais como: imunohistoquímica para RANK, RANKL e OPG; avaliação da densidade e grau de mineralização óssea e níveis séricos de OPG e TRAP5b.

1.3. Estatinas, Modulação Medicamentosa do Tecido Ósseo e a Movimentação Ortodôntica

Os efeitos das drogas classificadas como estatinas têm sido relatados como influentes sobre os processos de reabsorção e neoformação óssea. [3; 11; 17; 20; 74; 75; 76] A medicina contemporânea tem usado tais drogas para abaixar os níveis de colesterol no sangue, também estabilizando a placa ateromatosa e evitando AVCs.(Acidente Vascular Cerebral). Dentro dessa classe de drogas, insere-se a Atorvastatina (ATV). Quanto ao seu mecanismo de ação, esse medicamento (ATV) é um inibidor da enzima HMG-CoA redutase, uma precursora dos esteróis, inclusive do colesterol (Figura 1).

No que concerne a Odontologia, ensaios clínicos tem relatado uma associação direta entre a administração de estatinas (atorvastatina e sinvastatina) e a inibição do processo de reabsorção óssea periodontal. Segundo Lyndy et al.[74] os indivíduos que realizavam o uso destas drogas apresentavam 37% menos bolsas periodontais patológicas, quando comparados ao grupo de pessoas que não administravam a droga. Os autores concluíram que as estatinas reduziram os sinais de periodontite.

Neste sentido, considerando a ação das estatinas sobre o tecido ósseo, Góes et al.[75] realizaram estudo com intuito de avaliar os efeitos da ATV sobre a perda óssea alveolar induzida em ratos. Os métodos usados para tal avaliação foram a análise da densidade óssea radiográfica e a análise óssea morfométrica. Os autores concluíram que, baseados no resultado do estudo, a droga gerou a proteção do tecido ósseo alveolar.

Em relação aos efeitos das estatinas sobre a reabsorção óssea, estes ainda não são bem compreendidos, contudo, sugerem-se três plausíveis hipóteses:

1) Efeitos antiinflamatórios – Rosendo et. al.[77] salientaram que as estatinas podem reduzir o risco cardiovascular por outros mecanismos, além dos efeitos na redução do colesterol, tais como: a melhora da disfunção endotelial (aumentando a liberação de óxido nítrico derivado do endotélio), efeitos antioxidantes diretos (inibindo a oxidação da LDL-C e VLDL-C) e indiretos, ação anti-inflamatória (demonstrada pela redução da Proteína C Reativa, redução de moléculas de adesão e, inibição da proliferação de células do músculo liso na placa aterosclerótica) e efeitos imunomodulatórios. Assim parte do benefício do tratamento com inibidores da HMG-CoA redutase seria atribuído a este efeito anti-inflamatório, observado clinicamente pela diminuição dos marcadores de atividade inflamatória.

Durante e após o movimento dentário induzido, o microambiente periodontal é habitado por células inflamatórias agudas e/ou crônicas. Neste contexto, espera-se que drogas com ação antiinflamatória possam afetar, de certa forma, a expressão de moléculas envolvidas na remodelação óssea induzida por uma força ortodôntica. Dentre os efeitos anti-inflamatórios das estatinas, estas parecem atuar especialmente sobre os linfócitos T, os quais representam 65-75% dos linfócitos do sangue, sendo responsáveis pela resposta imune do tipo celular (produção e liberação de citocinas). Autores sugerem que as estatinas são capazes de suprimir a ativação das células T[78] e a expressão de RANKL por estas células, assim inibindo a reabsorção óssea.[79]

2) Inibição da ativação das GTPases de baixo peso molecular, cruciais para a sobrevivência de osteoclastos (Figura 1). De acordo com Staal et al.[11], a capacidade destas drogas em inibir a HMG-CoA redutase está positivamente correlacionada com a sua capacidade para inibir a reabsorção óssea. Do mesmo modo, Grasser et al.[17] verificaram*, in vitro*, de que o tratamento com lovastatina resulta em uma diminuição no número de osteoclastos, o que pode estar relacionado com a privação da via do mevalonato, impedindo, assim, a ativação de proteínas de ligação ao GTP (por

exemplo, Rho , Rab e Rac), cruciais para a formação do bordo em escova e sobrevivência de células osteoclásticas.

Neste sentido, Pan et al.[80] cultivaram células periodontais humanas e as submeteram a um protocolo de tensionamento *in vitro, c*onstatando um aumento na expressão das proteínas GTPase Rho, Rock (proteína quinase associada a Rho) e p-coffilin (cofilina fosforilada), sob tais condições de estresse físico. Ainda observou-se uma direta relação entre a expressão da proteína RhoA e a formação de anéis de F-actina. Sugeriu-se que superexpressão de RhoA irá promover a polimerização da actina citoplasmática e, consequentemente, rearranjará o citoesqueleto celular, assim permitindo, segundo os autores, o movimento dentário induzido.

Dessa forma, pode-se esperar que a inibição da prenilação de GTPases de baixo peso molecular, induzida pelas estatinas, tenha um efeito direto sobre a fisiologia do movimento dentário ortodôntico.

3) Inibição da via NFκB[3; 14; 81], um alvo à distância da ligação RANK / RANKL, na célula precursora de osteoclastos (Figura 2). De acordo com Han et al.[3] há uma redução significativa da recidiva ortodôntica em ratos tratados com sinvastatina, após 7 e 28 dias da remoção do aparelho fixo. Os autores sugeriram que, durante a recidiva, as estatinas atenuam a expressão de RANKL e aumentam a expressão de OPG, impedindo assim a ligação RANK / RANKL e, consequentemente, inibem a recidiva ortodôntica. Tais achados parecem confirmar o efeito da droga sobre a reabsorção óssea, assim acelerando a estabilidade do dente durante a fase de contenção. Além disso, Jin et al.[76] confirmaram que a sinvastatina aumenta o volume ósseo em ratos afetados pela doença periodontal, que foi associado a uma reduzida expressão RANKL.

Por fim, vale ressaltar que a plausível aplicação das estatinas como alternativa terapêutica para a Ortodontia clínica, deve passar por uma criteriosa avaliação de seus efeitos colaterais sobre o tecido ósseo. Embora estudos clínicos tenham indicado que as estatinas são bem toleradas na população adulta e jovem[25; 26; 27], os dados de longo prazo sobre o impacto da terapia com estatinas no crescimento e desenvolvimento são limitados[27; 28]. De acordo com

Macpherson[25], o tratamento de pacientes jovens com estatinas, ao longo de 2 anos, não teve impacto sobre a altura, peso, índice de massa corporal e maturação sexual. Por outro lado, Leem et al.[29], observaram que a lovastatina aumenta o crescimento longitudinal do osso e os níveis de BMP-2 na placa de crescimento de fêmures de ratos, o que poderia ter consequências negativas para o tratamento de crianças. Já Yamashita et al.[30], observaram que as injeções intraperitoneais diárias de rosuvastatina aumentaram significativamente o comprimento ântero-posterior de crânios, ulnas, fêmures e tíbias de ratos geneticamente modificados, com acondroplasia. Os autores também observaram, *in vitro*, que o tratamento com estatinas estimulou a proliferação e a maturação de condrócitos.

Em vista do exposto, parece evidente que a biologia celular e molecular ainda nos permitirá testemunhar grandes avanços na clínica odontológica. Por isso, propusemo-nos a realizar este trabalho, onde se observou o efeito da astorvastatina sobre a biologia do tecido ósseo, analisando seus possíveis impactos sobre a dinâmica ortodôntica.

2. OBJETIVOS

2.1. Geral:

Investigar, *in vivo*, uma plausível ação da atorvastatina sobre a ossificação endocondral, o *turnover* ósseo e o deslocamento dentário, durante a recidiva e a movimentação ortodôntica.

2.2. Específicos:

Para atingir tal objetivo, iremos:

1) Estudar o efeito da atorvastatina sobre o deslocamento dentário, durante a recidiva e a movimentação ortodôntica;
2) Avaliar o efeito da atorvastatina sobre o número de células osteoclásticas e sobre o volume ósseo, durante e após o movimento dentário induzido;
3) Avaliar o efeito da atorvastatina sobre a expressão das proteínas RANKL e OPG, nos tecidos periodontais, durante a recidiva ortodôntica;
4) Estudar os efeitos da administração de atorvastatina, em curto e longo prazo, sobre a ossificação endocondral, osteoclastogênese e volume ósseo de fêmures.

3. Manuscrito 1

Pharmacological induction of OPG overexpression reduces orthodontic relapse

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Institutional Review Board Approval

All procedures for treatment and maintenance of the animals were conducted in keeping with internationally accepted guidelines - Guide for the Care and Use of Laboratory Animals. This research was approved by Ethics Committee of the School of Dentistry of Rio Grande do Sul Federal University (CEUA 23145).

Pharmacological induction of OPG overexpression reduces orthodontic relapse

Abstract

Introduction: The statin class of drugs enhances osteogenesis and suppresses bone resorption, which could represent a plausible biological mechanism for mitigation of orthodontic relapse. We aimed to determine whether atorvastatin (ATV) might affect orthodontic relapse and osteoclastogenesis by modulating expression of RANKL and OPG, crucial molecules involved in bone turnover. Furthermore, we analyzed the adverse effects of ATV on femur turnover and endochondral ossification. *Methods:* Wistar rats were subjected to orthodontic tooth movement (OTM) for 21 days, followed by removal of the appliance and start of ATV or saline administration. Up to 7, 14, and 21 days of ATV administration, tooth relapse was measured and maxillary and femur sections were obtained and prepared for H&E, TRAP, and immunohistochemical (RANKL and OPG) staining. *Results:* ATV decreased tooth relapse (p=0.03) and osteoclast count (p=0.04), which were positively correlated (p=0.006). Statin administration increased periodontal expression of OPG (p=0.008), but not of RANKL protein. ATV administration also enhanced growth plate cartilage thickness. *Conclusions:* Statin-induced OPG overexpression reduces relapse after OTM, in a phenomenon correlated with decreased osteoclast counts. This phenomenon sheds light on OPG as a molecular target that modulates maxillary bone metabolism and orthodontic relapse.

Keywords

Tooth movement; osteoprotegerin, osteoclasts; atorvastatin; rats.

1. Introduction

Despite the clinical relevance of orthodontic relapse, the cellular and molecular mechanisms involved in this event are not fully understood.[1; 2; 3] Yoshida et al.[2] proposed that remodeling of the periodontal ligament (PDL) fibers and alveolar bone are the main causes of relapse. In addition, Franzen et al.[1] found that orthodontic relapse and orthodontic tooth movement (OTM) are associated with similar cellular adaptations, such as increased osteoclast differentiation in compression areas. Given this background, one could argue that endogenous or pharmacological bone modulation to inhibit osteoclast resorption and promote osteoblast neoformation may have clinically relevant effects on the regulation of OTM and relapse.[3; 4; 5; 6; 7; 8]

Statins are inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase, the rate-limiting enzyme within the mevalonate pathway of cholesterol biosynthesis.[9; 10] In addition to their cholesterol-lowering properties, statins have a series of pleiotropic and anti-inflammatory effects.[11] Studies have suggested that statins are able to influence bone turnover, enhancing osteogenesis and suppressing bone resorption.[3; 12; 13; 14; 15; 16; 17; 18; 19] These effects involve modulation of the receptor activator of nuclear kappa B (RANK), receptor activator of nuclear kappa B ligand (RANKL), and osteoprotegerin (OPG), ultimately promoting suppression of osteoclastogenesis.[3; 15; 17] In the bone system, RANKL is expressed on the osteoblasts and, as it binds to the RANK receptor expressed on hematopoietic osteoclast precursors, it induces rapid differentiation of these cells to mature osteoclasts. OPG is a decoy receptor produced by fibroblasts, osteoblasts, and even osteoclasts. This molecule will compete with RANK for RANKL binding, thus inhibiting differentiation of osteoclasts and inducing their apoptosis.

Apparently, the effects of statins on orthodontic relapse have been less explored. Han et al.[3] observed that the ability of simvastatin to minimize tooth displacement was associated with decreased RANKL and increased OPG expression. The authors suggested an effective drug stimulation of bone neoformation, thus accelerating tooth stability and assisting the retention phase. Furthermore, Jin et al.[20] found that simvastatin increases bone volume in rats

affected by periodontal disease, with decreased RANKL expression apparently involved. Although statins seems to be well tolerated in adult and young patients[21; 22; 23], long-term undesirable effects must be considered in clinical practice.[24] For instance, *in vitro* and *in vivo* preclinical studies have suggested that statins increase chondrocyte proliferation and longitudinal bone growth[25; 26], which may preclude their use as an orthodontic pharmacological strategy in children.

In this study, we hypothesized that short-term ATV treatment in rats might reduce orthodontic relapse and osteoclastogenesis through modulation of RANKL and OPG expression. We also analyzed the adverse effects of ATV on long-bone turnover and endochondral ossification.

2. Material and Methods

2.1. Animals

Thirty-six male Wistar rats, age 6 weeks, weighing approximately 330-340 g, were used in the experiments. The animals were housed four to a cage, under a 12-h light/dark cycle, at a constant temperature of 23°C, and provided food and water *ad libitum*. All animal handling and care procedures were conducted in keeping with internationally accepted guidelines (*Guide for the Care and Use of Laboratory Animals*)[27] and were approved by the ███ ███ School of Dentistry Ethics Committee (CEUA 23145).

2.2. Experimental tooth movement

After induction of anesthesia with ketamine (80 mg/kg) and xylazine (5 mg/kg), a superelastic closed nickel-titanium coil spring exerting a force of 50 cN was inserted unilaterally, between the upper right first molar and upper incisors, as described in the split-mouth design.[1; 17; 28; 29; 30] Our protocol was based on previous demonstrations that 50cN provides substantial tooth movement.[1; 3; 4; 8; 30] The device was kept in place for 21 days to generate mesial displacement of the first molar (M1). Whereas the right maxillary molar was used for experimental tooth movement, the left maxillary molar served as the internal control (Co), in which there was no orthodontic tooth movement.

Throughout the study, animals were evaluated weekly for weight gain or loss, appliance breakage, and signals of gingival or other soft tissue inflammation. After 20 days of OTM, animals were randomly divided into two groups: control (SAL, n=18) and experiment (ATV, n=18). ATV, 15mg/Kg, was given daily ([redacted]), via gavage, for 7, 14, or 21 days (Figure 1A). Elewa[31],suggested that the atorvastatin peak plasma concentration achieved after administering 15 mg/kg by oral route, in rats, was similar to that reported after a dose of 80 mg/day atorvastatin is given to humans. Rats in the control group received 0.1 mL of phosphate-buffered saline (PBS), daily, via gavage. On day 21, the appliance was removed, marking the start of the relapse phase (Figure 1A). The experimental time points were set at 7, 14, and 21 days after appliance removal (Re7, Re14, and Re21, respectively).

2.3. Measurement of tooth movement

Using dental stone (Durone, Dentsply®, PA, USA), precise plaster models of the maxilla were obtained from impressions made with silicone material ([redacted]). Impressions were obtained every 7 days, under anesthesia (Figure 1A), in both groups. The occlusal surfaces were photographed (DSC#H10, Sony®, Tokyo, Japan) at 300 dpi and magnified (4x) using Image J® software (version 1.44, National Institute of Health, 2011). A 100-mm ruler was placed next to the casts to calibrate measurements. The mean distance between the distal surface of M1 and the mesial surface of the second molar (M2), measured at three distinct points on each photo, was considered for analyses (Figure 1B). Total tooth movement during the 21 days of orthodontic treatment was recorded as “D0”. At 7, 14, and 21 days after appliance removal, the distance between M1 and M2 was measured and recorded as DFinal. Based on D0 and DFinal, the percentage of relapse in each animal was calculated and the values averaged for each group, as described in previous studies.[3]

2.4. Tissue preparation

At each experimental time point, 12 animals (6 per group) were euthanized with an overdose of ketamine and xylazine. Maxillae and the distal left fêmures were immediately dissected and fixed by submerging for 24 h in

10% buffered formalin. The specimens were demineralized in 10% EDTA (pH 7) for 30-60 days. The samples were then dehydrated through an ethanol series, embedded in paraffin, cut into 5-μm longitudinal sections, and prepared for hematoxylin and eosin (H&E), tartrate-resistant acid phosphatase (TRAP), and immunohistochemical (RANKL and OPG) staining.

2.5. TRAP staining

Briefly, for TRAP staining, histologic sections were selected and incubated in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma®, St. Louis, Missouri, USA), Fast Red Violet LB Salt (Sigma®), and 50 mM sodium tartrate. The sections were counterstained with hematoxylin.

2.6 Histomorphometry

Histomorphometric analyses of maxilla specimens were performed considering the following subgroups: ATV+OTM, experimental hemimaxillae from ATV animals; - CoATV, control hemimaxillae from ATV animals; SAL+OTM, experimental hemimaxillae from SAL animals; and CoSAL, control hemimaxillae from SAL animals. Comparisons across subgroups were performed at each time point (7, 14, and 21 days) or by analysis of overall means. For evaluation of femur specimens, the sample was divided into two groups: ATV (n=18) and SAL (n=18). In this case, all comparisons between groups were done considering overall mean values.

H&E- and TRAP-stained sections were visualized in a Nikon Eclipse 90i microscope (Nikon Co., Tokyo, Japan) coupled to a Coolsnap EZ camera (Photometrics, AZ, USA). Microphotographs were captured using the NIS Elements Imaging 3.10 Sp2 software (Nikon Co.). All histomorphometric measurements and terminology were in accordance with the American Society for Bone and Mineral Research recommendations[32].

2.6.1 Maxillary bone turnover – Molar-supporting structures were evaluated using the NIS Elements Imaging 3.10 Sp2 software. Under high magnification (×100), the number of osteoclasts was counted. The region of interest (ROI) consisted of the periodontal tissues at the distal surface of the mesial root of M1[4; 5] (Figure 1C). Cells were considered to be osteoclasts if they

were TRAP-positive, multinucleated, and located on the bone surface or residing in Howship's lacunae[30].

The maxillary bone volume ratio (BV/TV), expressed as the ratio of cancellous bone volume (BV) to total tissue volume (TV), was defined in the ROI of H&E sections (Figure 1C) using Adobe® Photoshop® CS6 software (Adobe Systems, San Jose, CA, USA) and ImageJ, following the method suggested by Egan et al.[33] The total osteoclast count and bone volume were calculated for each animal and averaged for each group.

2.6.2 Femur bone turnover – The number of osteoclasts was counted in 10 randomly selected fields of view within the metaphyseal regions of the femur specimens[34], using the same software described above. The BV/TV ratio of five rectangular areas of subchondral bone tissue was calculated according to the protocol outlined by Ho et al.[19] Again, the total osteoclast count and bone volume were calculated for each animal and averaged for each group.

2.6.3 Endochondral ossification - Growth plate cartilage and hypertrophic zone thickness (GPC.Th and HpZ.Th, respectively) were calculated as the mean of 10 different measurements performed at randomly selected locations in femur H&E sections[35; 36], using NIS Elements Imaging 3.10 Sp2 software.

2.7. Immunohistochemistry

Immunohistochemical staining was performed on each hemimaxilla section (with and without OTM) after mounting on silanized slides (DAKO® A/S, Golstrup, Denmark). After deparaffinization and dehydration, endogenous peroxidase activity was blocked using H_2O_2 30 vol. and methanol (1:1) for 10 min. Antigen retrieval was performed using trypsin (0.25%) for 20 min, at room temperature. Sections were then incubated overnight with primary antibodies in a humidified chamber at 4ºC. The antibodies and conditions were as follows: RANKL, rabbit polyclonal antibody (Bioss Inc., USA, bs-0747R, dilution 1:200); OPG, rabbit polyclonal antibody (Abcam Inc,. USA, ab73400, dilution 1:200). Immunodetection was performed using the EnVision + Dual Link system-HRP (K4063, Dako® A/S) and diaminobenzidine (DAB) as substrate-chromogen

system (K3468, Dako® A/S). Harris hematoxylin (Sigma–Aldrich®) was used as counterstain. A negative control, which consisted of primary antibody omission, was included in all reactions, as was an internal positive control (bone marrow).

Digital images representative of the periodontal ROI were obtained at a magnification of ×200. For each image, the color deconvolution method was used to isolate RANKL and OPG positive DAB-stained cells/stroma. DAB and hematoxylin nuclear staining were digitally separated using ImageJ software and an ImageJ plugin for color deconvolution [37], which calculated the contribution of DAB and hematoxylin. Following deconvolution, the DAB image was transformed into an 8-bit format and the threshold set to black and white, with the black pixels considered as positive DAB staining. The total value of black pixels observed in the ROI was calculated for each animal and then averaged for each group, ATV (n=18) or SAL (n=18). The overall mean RANKL- and OPG-stained areas were compared between the SAL and ATV groups in the experimental and control hemimaxillae (with and without OTM, respectively).

All procedures were performed by a single blinded and calibrated examiner. To determine random intra-individual error, 10% of the sample (plaster models and histologic sections) was randomly selected for re-evaluation 15 days after the first analysis. The Dahlberg formula was applied and acceptable values were obtained (<10%).

2.8. Statistical analysis

Data are presented as mean ± standard deviation or, when appropriate, standard error (SD or SE, respectively). Relapse was calculated as a percentage per group, and a linear mixed model with repeated measures for time was used. At each time point, between-subgroup comparisons of maxillary histomorphometric parameters were analyzed with one-way ANOVA followed by the LSD multiple comparison test (for homogeneous variances) or the Games-Howell test (for heterogeneous variances). Pearson correlation coefficients were used to verify the association between orthodontic relapse and osteoclast count. When comparisons were performed between two groups, the independent Student *t*-test was used. Results were processed in PASW

Statistics for Windows, Version 18.0 (SPSS Inc., Chicago, IL, USA). The significance level was set at 5% ($p < 0.05$).

3. Results

3.1. Animal status

There was no between-group difference ($p > 0.05$) in weight gain or loss during the OTM period (day 0 to 21) or during the relapse phase (day 21 to 42). Mean (SD) weight at the end of the relapse phase was 311.67 (66.15) g for the SAL group and 349.17 (30.44) g for the ATV group.

3.2. Atorvastatin reduced orthodontic relapse

SAL animals reached maximal relapse at day 7 (31.91%), and percent relapse decreased gradually thereafter through days 14 to 21 (29.36% and 28.40%, respectively). ATV animals exhibited reduced relapse at 7, 14, and 21 days (9.59%, 20.59% and 7.94%, respectively) when compared to the SAL group, indicating that ATV administration prevents orthodontic relapse independently of the time point of assessment (Figure 2A).

3.3. Atorvastatin acts on osteoclastogenesis, bone volume, and OPG expression

ATV administration significantly reduced osteoclast counts and increased BV/TV ratio during the orthodontic relapse period (Figures 2B and 2C). However, when the different time points of the relapse period were analyzed separately (7, 14, and 21 days), we observed a transient osteoclast inhibition in association with statin administration. Only after 7 days of relapse (Re7) did atorvastatin promote a marked decrease in the number of TRAP+ cells ($p < 0.05$) when compared to SAL+OTM animals and to the control hemimaxillae (without OTM) of both groups (SAL and ATV) (Figure 3A and 3B). A positive and significant correlation between osteoclast count and orthodontic relapse was confirmed ($r = 0.452$; $p = 0.006$; $r^2 = 0.204$). Control hemimaxillae (without OTM) from ATV animals also exhibited reduced osteoclast counts when compared to control hemimaxillae from the SAL group; however, this difference was not significant ($p > 0.05$).

In the absence of mechanical force (hemimaxillae without OTM), the BV/TV ratio was higher in ATV than in SAL animals (Figures 3C and 3D), indicating an effect of ATV administration on bone turnover under physiological conditions. When analyzing the BV/TV ratio at each time point of the relapse phase (7, 14, and 21 days), a statistical increase in bone volume in ATV specimens when compared to the control hemimaxillae of SAL animals was observed only after 14 days (Figures 3C and 3D).

At the molecular level, atorvastatin affected OPG expression in periodontal tissues. Overexpression of OPG was observed in both ATV subgroups (CoATV and ATV+OTM) when compared to their corresponding SAL controls (CoSAL and SAL+OTM), as shown in Figures 4A and 4B. Our observational data suggest that fibroblasts, osteoblasts, bone lining cells, and even osteoclasts presented cytoplasmic OPG labeling (Figure 4A). Interestingly, cytoplasmic expression of OPG in osteoclasts was observed especially in the control hemimaxillae (i.e., in the absence of OTM) of ATV animals. During orthodontic relapse, these cells appeared to lose cytoplasmic OPG labeling capability (Figure 4A). Furthermore, both during orthodontic relapse and under physiological conditions, RANKL expression was decreased in the ATV groups when compared to SAL animals (Figure 4C), although the difference was not statistically significant (Figure 4D).

3.4. Endochondral ossification was affected by ATV

In femoral specimens, there was no significant difference between the SAL and ATV groups in either of the parameters of interest (osteoclast count and BV/TV) (Figures 5A and 5B). Regarding endochondral ossification, ATV animals exhibited greater growth plate thickness and a larger hypertrophic zone when compared to SAL animals ($p < 0.05$), as shown in Figures 5C, 5D, 5E, and 5F.

4. Discussion

Prevention of orthodontic relapse appears to be a plausible application for statins. It should be considered that these drugs are currently used in adults, but not in children or adolescents. In clinical trials, statins have been well

tolerated by the pediatric population.[21; 22; 23; 24] Nevertheless, the adverse effects of this class have not been analyzed in depth in this population, and appropriate long-term monitoring is necessary. *In vitro* and *in vivo* preclinical investigations have suggested that statins affect endochondral ossification, increasing chondrocyte proliferation and longitudinal bone growth[25; 26], which may have implications for its clinical use in children and adolescents. Atorvastatin is FDA-approved for use in children[38], but, to the best of our knowledge, its effects on endochondral ossification have yet to be characterized.

To evaluate the effect of ATV on orthodontic relapse, we used a linear mixed model with repeated measures for the “time” factor, which calculated the power of the test for each factor independently, and the interaction between factors (group and time). Our data indicate that ATV will promote a reduction of orthodontic relapse regardless of the timing of administration (7, 14 or 21 days). In agreement with other authors[1; 2; 3; 30], we found a greater relapse rate in the SAL group immediately after appliance removal, which gradually decreased up to 21 days. This trend was not observed in ATV animals, which presented reduced relapse rates from the earliest (Re7) to the latest (Re21) time points of assessment. Similarly, Han et al.[3] reported a significant reduction of tooth relapse in rats treated with simvastatin after 7 and 28 days of appliance removal. MirHashemi et al.[17] concluded that ATV administration (5 mg/kg via gavage) decreases the rate of tooth movement in rats. However, our data showed that the drug also affected endochondral long bone ossification, what could limit its clinical use in the pediatric population, as discussed below. Thus, further explorations of the molecular and cellular mechanisms associated with bone turnover during orthodontic relapse are still required.

Our findings suggest that daily ATV administration affects the bone resorption during orthodontic relapse, as demonstrated by the significant decrease in overall osteoclast count in the ATV group as compared to SAL animals (Figure 2B). In contrast, other authors[3; 17] have reported that statins did not affect the number of osteoclasts during orthodontic tooth movement or during the relapse phase. Therefore, our study is the first to associate statin administration to a reduction in osteoclast counts during orthodontic relapse in rats. Several plausible explanations—including the type and dosage of statin,

the tooth movement/relapse protocol employed, and, especially, the use of an appropriate histochemical method for osteoclast counting (TRAP)—may explain this contradictory result at least in part.

When the different time points of assessment during the relapse period were analyzed separately (7, 14, and 21 days), we observed that osteoclast inhibition was transient, occurring only after 7 days of relapse. Other authors have demonstrated the early effect of statins on bone turnover[3; 11], but the lack of effects after 14 and 21 days (at Re14 and Re21 respectively) is puzzling. One hypothesis for this phenomenon is the presence of an *in vivo* compensatory mechanism able to stimulate osteoclasts to overcome statin blockade at Re14 and Re21, as suggested by Staal et al.[12] Furthermore, after 7 days of relapse the periodontal microenvironment is still partially inhabited by chronic inflammatory cells, but at Re14 and Re21, physiological periodontal turnover is being gradually reestablished. Thus, the anti-inflammatory mechanisms of ATV would act only at the early phase of the relapse period (Re7), emphasizing the inhibitory effect of statins on T-cell activation[39] and T-cell RANKL expression, ultimately preventing bone resorption[40]. However, even considering this transient effect of statins on osteoclastogenesis, our results highlight the clinical relevance of this finding, as orthodontic relapse was inhibited.

The present study also demonstrated that ATV increases the BV/TV ratio during orthodontic relapse (Figure 2C), an effect that seems to be related to the reduced osteoclast counts observed in this group (Figure 2B). As noted above, osteoclast inhibition in the ATV group occurred only after 7 days of relapse (Re7). This was followed by a higher bone volume ratio at Re14 (Figure 3D). In agreement, other authors have also observed a direct relation between statin administration, decreased osteoclast counts, and increased bone volume.[11; 12; 13; 14; 18; 19; 20] Moreover, under physiological conditions, in maxillae without tooth movement, ATV administration increased the BV/TV ratio, but not the osteoclast count, when compared to the SAL group (Figure 3D). We suggest that the BV/TV ratio was affected through an increase in osteoblast-mediated bone neoformation induced by statin administration. However, we did not assess

osteoblast function or any dynamic bone volume parameters, which could be construed as a limitation of our study.

The exact mechanisms involved in the inhibition of osteoclasts by statins is unknown[3; 12; 13; 15; 18]. A plausible hypothesis involves inhibition of the NF-κB pathway[3; 15; 41], a downstream target of RANK/RANKL binding. According to Han et al.[3], during orthodontic relapse, statins attenuate RANKL and increase OPG expression, thereby preventing RANK/RANKL interaction and tooth displacement. Conversely, our data showed a slight reduction of RANKL expression in the ATV group when compared to SAL animals, but this result did not reach statistical significance. It is important to emphasize the differences in methodology between these two studies, including type, dosage, and duration of statin administration. This is a topic of controversy in the literature[3; 13; 15; 20; 42; 43] and further studies are still needed to confirm statin modulation of RANKL expression.

On the other hand, we found increased expression of OPG in both ATV groups (without and with tooth movement) when compared to their respective SAL controls (Figure 4). After 7 days of relapse, it was reasonable to presume that, in the ATV group, the increase in OPG and slight reduction in RANKL expression was the major mechanism involved in osteoclastogenesis inhibition. However, after 14 and 21 days of relapse, although statin administration induced high OPG expression (data not shown), osteoclast counts were similar in the ATV and SAL groups. This result suggests that mechanisms other than statin-mediated OPG regulation are involved in osteoclastogenesis. In addition, our observational data (Figure 4A) show that atorvastatin induced OPG expression in the osteoclast cytoplasm in maxillae without OTM, suggesting an autoregulatory mechanism of these cells that plays a negative role in osteoclastogenesis and probably induces osteoclast apoptosis.[44] This finding could explain why the high OPG expression observed in control maxillae (not subjected to orthodontic tooth movement) in the ATV group was not associated with a reduction in osteoclast count, as the clastic cells themselves were contributing to higher OPG expression. In short, it seems that atorvastatin is able to modulate OPG expression during orthodontic relapse, which is a major mechanism of osteoclast inhibition.

When considering overall osteoclast number and relapse findings, we observed that SAL animals had high relapse rates and osteoclast counts, whereas ATV animals exhibited the opposite profile. Although a significant positive correlation between the rate of tooth movement and osteoclast count has been described in a previous study[45], no correlation analysis was performed regarding orthodontic relapse. We found a significant positive correlation between osteoclast count and percentage of relapse. In this regard, Yoshida et al.[2] suggested that alveolar bone remodeling is one of the main causes of relapse after tooth movement in rats. Franzen et al.[1], reported that, after 7 days, the first molar continued to relapse, although the trans-septal fibers were normally stretched. The authors concluded that stretching of these fibers may not play a central role in the etiology of relapse, while bone remodeling is a major factor to be considered. In agreement, our data indicate that osteoclast count and orthodontic relapse are directly correlated, highlighting the importance of bone resorption during the relapse phase. However, Pearson correlation yielded a coefficient of determination (R^2) of 0.2, which suggests that other variables, such as periodontal fiber stretching, are influencers of relapse.[6; 46; 47]

Besides assessing the local effects of ATV on tooth relapse and periodontal bone physiology, this study also provides insights regarding some of its undesirable effects, namely, on long bone turnover and endochondral ossification. We observed an increase in thickness of the growth plate and hypertrophic zone, corroborating previous results described in the literature.[25; 26] According to Leem[25] , lovastatin increases longitudinal bone growth and BMP-2 (bone morphogenetic protein-2) levels in the growth plate of rats, which may have negative implications for the treatment of children. Yamashita et al.[26] observed that daily intraperitoneal injections of rosuvastatin significantly increased the anteroposterior lengths of skulls, ulnas, fêmures, and tibiae in rats with achondroplasia. However, wild-type animals showed an increase in skull length only when compared to the control group. The authors also observed, *in vitro*, that statin treatment stimulated chondrocyte proliferation and maturation. The main limitation of these studies, including ours[25; 26], was the short duration of statin administration.

Taken together, these data suggest that osteoclastogenesis inhibition by statins can be a plausible target in the search for a pharmacologic therapy to minimize orthodontic relapse. However, further research evaluating the adverse effects of statins on long bone turnover and growth are apparently needed before they can be considered for this clinical indication in the pediatric population. Furthermore, considering that statins are among the most commonly prescribed pharmaceutical agents for prevention of hyperlipidemia and cardiovascular diseases[9; 10], their clinical effects on tooth movement seem to be a relevant concern in orthodontic practice.

5. Conclusions

Within the limitations of this study, drug modulation of bone remodeling appeared to provide a feasible strategy to decrease orthodontic relapse. The cellular and molecular mechanisms underlying this effect involve decreased osteoclastogenesis and increased OPG protein expression. The positive correlation between orthodontic relapse and osteoclast count highlights osteoclastogenesis as a potential mediator of orthodontic relapse.

6. References

1. Franzen, T. J.; Brudvik, P.; Vandevska-Radunovic, V. Periodontal tissue reaction during orthodontic relapse in rat molars. Eur J Orthod, v. 35, n. 2, p. 152-9, Apr 2013. ISSN 1460-2210 (Electronic)
2. Yoshida, Y. et al. Cellular roles in relapse processes of experimentally-moved rat molars. J Electron Microsc (Tokyo), v. 48, n. 2, p. 147-57, 1999. ISSN 0022-0744 (Print)
3. Han, G. et al. Effects of simvastatin on relapse and remodeling of periodontal tissues after tooth movement in rats. Am J Orthod Dentofacial Orthop, v. 138, n. 5, p. 550 e1-7; discussion 550-1, Nov 2010. ISSN 1097-6752 (Electronic)
4. Hudson, J. B. et al. Local delivery of recombinant osteoprotegerin enhances postorthodontic tooth stability. Calcif Tissue Int, v. 90, n. 4, p. 330-42, Apr 2012. ISSN 1432-0827 (Electronic)
5. Zhao, N. et al. Local osteoprotegerin gene transfer inhibits relapse of orthodontic tooth movement. Am J Orthod Dentofacial Orthop, v. 141, n. 1, p. 30-40, Jan 2012. ISSN 1097-6752 (Electronic)
6. Hirate, Y.; Yamaguchi, M.; Kasai, K. Effects of Relaxin on Relapse and Periodontal Tissue Remodeling after Experimental Tooth Movement in Rats. Connective Tissue Research, v. 53, n. 3, p. 207-219, 2012. ISSN 0300-8207.
7. Hassan, A. H.; Al-Hubail, A.; Al-Fraidi, A. A. Bone inductive proteins to enhance postorthodontic stability. Angle Orthod, v. 80, n. 6, p. 1051-60, Nov 2010. ISSN 1945-7103 (Electronic)
8. Schneider, D. A. et al. Locally limited inhibition of bone resorption and orthodontic relapse by recombinant osteoprotegerin protein. Orthod Craniofac Res, v. 18 Suppl 1, p. 187-95, Apr 2015. ISSN 1601-6343.

9. Pedersen, T. R. et al. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). 1994. Atheroscler Suppl, v. 5, n. 3, p. 81-7, Oct 2004. ISSN 1567-5688 (Print)
10. Baigent, C. et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet, v. 366, n. 9493, p. 1267-78, Oct 8 2005. ISSN 1474-547X (Electronic)
11. Mundy, G. et al. Stimulation of bone formation in vitro and in rodents by statins. Science, v. 286, n. 5446, p. 1946-9, Dec 1999. ISSN 0036-8075. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10583956 >.
12. Staal, A. et al. The ability of statins to inhibit bone resorption is directly related to their inhibitory effect on HMG-CoA reductase activity. J Bone Miner Res, v. 18, n. 1, p. 88-96, Jan 2003. ISSN 0884-0431 (Print)
13. Kim, J. Y. et al. Atorvastatin inhibits osteoclastogenesis by decreasing the expression of RANKL in the synoviocytes of rheumatoid arthritis. Arthritis Research & Therapy, v. 14, n. 4, 2012. ISSN 1478-6354.
14. Maeda, T. et al. Simvastatin promotes osteoblast differentiation and mineralization in MC3T3-E1 cells. Biochem Biophys Res Commun, v. 280, n. 3, p. 874-7, Jan 26 2001. ISSN 0006-291X (Print)
15. De Araujo Junior, R. F. et al. Atorvastatin decreases bone loss, inflammation and oxidative stress in experimental periodontitis. PLoS One, v. 8, n. 10, p. e75322, 2013. ISSN 1932-6203 (Electronic)
16. Ferreira, L. B. et al. Evaluation of bone repair of critical size defects treated with simvastatin-loaded poly(lactic-co-glycolic acid) microspheres in rat calvaria. J Biomater Appl, Sep 10 2014. ISSN 1530-8022 (Electronic)
17. MirHashemi, A. H. et al. Effect of atorvastatin on orthodontic tooth movement in male wistar rats. J Dent (Tehran), v. 10, n. 6, p. 532-9, Nov 2013. ISSN 1735-2150 (Print)
18. Grasser, W. A. et al. Regulation of osteoclast differentiation by statins. J Musculoskelet Neuronal Interact, v. 3, n. 1, p. 53-62, Mar 2003. ISSN 1108-7161 (Print)
19. Ho, M. L. et al. Simvastatin increases osteoblasts and osteogenic proteins in ovariectomized rats. Eur J Clin Invest, v. 39, n. 4, p. 296-303, Apr 2009. ISSN 1365-2362. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19292885 >.
20. Jin, J. et al. Simvastatin inhibits LPS-induced alveolar bone loss during metabolic syndrome. J Dent Res, v. 93, n. 3, p. 294-9, Mar 2014. ISSN 1544-0591.
21. Miller, M. L.; Wright, C. C.; Browne, B. Lipid-lowering medications for children and adolescents. J Clin Lipidol, v. 9, n. 5 Suppl, p. S67-76, 2015 Sep-Oct 2015. ISSN 1933-2874.
22. Macpherson, M. et al. Population pharmacokinetics of rosuvastatin in pediatric patients with heterozygous familial hypercholesterolemia. Eur J Clin Pharmacol, v. 72, n. 1, p. 19-27, Jan 2016. ISSN 1432-1041.
23. AVIS, H. J. et al. Efficacy and safety of rosuvastatin therapy for children with familial hypercholesterolemia. J Am Coll Cardiol, v. 55, n. 11, p. 1121-6, Mar 2010. ISSN 1558-3597.
24. Eiland, L. S.; Luttrell, P. K. Use of statins for dyslipidemia in the pediatric population. J Pediatr Pharmacol Ther, v. 15, n. 3, p. 160-72, Jul 2010. ISSN 1551-6776.
25. Leem, K. et al. Lovastatin increases longitudinal bone growth and bone morphogenetic protein-2 levels in the growth plate of Sprague-Dawley rats. Eur J Pediatr, v. 161, n. 7, p. 406-7, Jul 2002. ISSN 0340-6199.
26. Yamashita, A. et al. Statin treatment rescues FGFR3 skeletal dysplasia phenotypes. Nature, v. 513, n. 7519, p. 507-11, Sep 2014. ISSN 1476-4687.
27. In: (Ed.). Guide for the Care and Use of Laboratory Animals. Washington (DC), 1996. ISBN 0309053773.
28. Verna, C.; Dalstra, M.; Melsen, B. The rate and the type of orthodontic tooth movement is influenced by bone turnover in a rat model. Eur J Orthod, v. 22, n. 4, p. 343-52, Aug 2000. ISSN 0141-5387 (Print)

29. Shirazi, M. et al. Pantoprazole, a proton pump inhibitor, increases orthodontic tooth movement in rats. Iran J Basic Med Sci, v. 17, n. 6, p. 448-53, Jun 2014. ISSN 2008-3866 (Print)
30. Franzen, T. J. et al. The influence of low-level laser on orthodontic relapse in rats. Eur J Orthod, Oct 6 2014. ISSN 1460-2210 (Electronic)
31. Elewa, H. F. et al. Early atorvastatin reduces hemorrhage after acute cerebral ischemia in diabetic rats. J Pharmacol Exp Ther, v. 330, n. 2, p. 532-40, Aug 2009. ISSN 1521-0103. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19478137 >.
32. Parfitt, A. M. et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res, v. 2, n. 6, p. 595-610, Dec 1987. ISSN 0884-0431.
33. Egan, K. P.; Brennan, T. A.; Pignolo, R. J. Bone histomorphometry using free and commonly available software. Histopathology, v. 61, n. 6, p. 1168-73, Dec 2012. ISSN 1365-2559.
34. El Khassawna, T. et al. Effects of multi-deficiencies-diet on bone parameters of peripheral bone in ovariectomized mature rat. PLoS One, v. 8, n. 8, p. e71665, 2013. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23977109 >.
35. Aybar Odstrcil, A. C. et al. Effect of arsenic in endochondral ossification of experimental animals. Exp Toxicol Pathol, v. 62, n. 3, p. 243-9, May 2010. ISSN 1618-1433.
36. Fan, C. M. et al. Prevention of bone growth defects, increased bone resorption and marrow adiposity with folinic acid in rats receiving long-term methotrexate. PLoS One, v. 7, n. 10, p. e46915, 2012. ISSN 1932-6203.
37. Ruifrok, A. C.; JOHNSTON, D. A. Quantification of histochemical staining by color deconvolution. Anal Quant Cytol Histol, v. 23, n. 4, p. 291-9, Aug 2001. ISSN 0884-6812.
38. FDA. LIPITOR safely and effectively. Initial U.S. Approval: 1996. http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/020702s056lbl.pdf
39. Yilmaz, A. et al. Differential effects of statins on relevant functions of human monocyte-derived dendritic cells. J Leukoc Biol, v. 79, n. 3, p. 529-38, Mar 2006. ISSN 0741-5400.
40. Rattazzi, M. et al. Atorvastatin reduces circulating osteoprogenitor cells and T-cell RANKL expression in osteoporotic women: implications for the bone-vascular axis. Cardiovasc Ther, Oct 2015. ISSN 1755-5922.
41.Kim, J. Y. et al. Atorvastatin inhibits osteoclastogenesis by decreasing the expression of RANKL in the synoviocytes of rheumatoid arthritis. Arthritis Res Ther, v. 14, n. 4, p. R187, 2012. ISSN 1478-6354 (Print)
42. Liu, S. et al. Effect of simvastatin on the osteogenetic behavior of alveolar osteoblasts and periodontal ligament cells. Hum Cell, v. 25, n. 2, p. 29-35, Jun 2012. ISSN 1749-0774.
43. Stein, S. H. et al. Statins regulate interleukin-1β-induced RANKL and osteoprotegerin production by human gingival fibroblasts. J Periodontal Res, v. 46, n. 4, p. 483-90, Aug 2011. ISSN 1600-0765.
44. Kang, J. H. et al. Osteoprotegerin expressed by osteoclasts: an autoregulator of osteoclastogenesis. J Dent Res, v. 93, n. 11, p. 1116-23, Nov 2014. ISSN 1544-0591.
45. Ren, Y.; Kuijpers-jagtman, A. M.; Maltha, J. C. Immunohistochemical evaluation of osteoclast recruitment during experimental tooth movement in young and adult rats. Arch Oral Biol, v. 50, n. 12, p. 1032-9, Dec 2005. ISSN 0003-9969 (Print)
46. Reitan, K. Clinical and histologic observations on tooth movement during and after orthodontic treatment. Am J Orthod, v. 53, n. 10, p. 721-45, Oct 1967. ISSN 0002-9416 (Print)
47. Young, L. et al. Fiberotomy enhances orthodontic tooth movement and diminishes relapse in a rat model. Orthod Craniofac Res, v. 16, n. 3, p. 161-8, Aug 2013. ISSN 1601-6343 (Electronic)

8. Figure Captions

Figure 1 – (A) Experimental study design. Right first molars were displaced mesially during active orthodontic tooth movement (OTM). After 21 days, the orthodontic device was removed and the molars allowed to relapse (relapse phase). Saline solution or atorvastatin (15 mg/kg) were administered daily, via gavage, from days 20 through 42. Clinical evaluation of relapse and histological analysis (H&E, TRAP, and immunohistochemistry) were performed at 3 distinct time points: Re7, Re14 and Re21 (7, 14 and 21 days after appliance removal). (B) Occlusal photography of a dental cast. The distance between first and second molars at three points (A, B, and C) was measured and averaged for each animal. (C) H&E section of experimental maxilla (magnification ×40). The region of interest (ROI) was composed of the periodontal tissues at the distal surface of the first molar mesial root. The yellow arrow indicates the direction of relapse force.

Figure 2 – (A) Comparison between overall mean relapse percentage in ATV and SAL groups. A linear mixed model indicated a significant main effect of group ($p = 0.033$), demonstrating that atorvastatin reduced the rate of relapse at all time points. The interaction between group and time was not statistically significant ($p = 0.731$). All data are mean ± SE. (B) Comparison between overall mean osteoclast counts in the ATV+OTM and SAL+OTM groups. An independent Student *t*-test indicated reduced osteoclast counts in the atorvastatin group. (C) Comparison between overall mean bone volume ratio (BV/TV) in the ATV+OTM and SAL+OTM groups. An independent Student *t*-test indicated increased bone volume in the atorvastatin group. All data are mean ± SD.

Figure 3 — (A) TRAP staining of control and experimental maxillae (without and with OTM, respectively). In general, during orthodontic relapse, ATV animals exhibited fewer TRAP-positive cells as compared with SAL animals. (B) Comparison of osteoclast counts during the relapse phase across subgroups at each experimental time point (7, 14, and 21 days of relapse). ATV+OTM subgroup exhibited significantly decreased osteoclastogenesis only at Re7 (ANOVA followed by least significant difference test). (C) H&E sections of

control and experimental maxillae of ATV and SAL animals. Atorvastatin increased the BV/TV ratio as compared to SAL. (D) Atorvastatin increased the BV ratio in the absence of OTM, as well as after 14 days of relapse (Re 14), when compared to control maxillae of SAL animals (ANOVA followed by Games-Howell test). Small arrows indicate presence of osteoclasts. Scale bars represent 100 µm, at ×200 magnification. Yellow arrow represents the direction of relapse force. Bo, bone; Ro, root. All data are mean ± SD.

Figure 4 – (A) Sections of control and experimental maxillae of SAL and ATV animals, stained immunohistochemically for OPG. Osteoclast OPG expression was commonly found in the control maxillae of ATV animals, contrasting with the absence of such expression in experimental maxillae of the same group, as demonstrated at higher magnifications (*,×1000). (B) Comparison of OPG expression in control vs. experimental maxillae of ATV and SAL animals, demonstrating OPG overexpression in both control and experimental maxillae of ATV animals. (C) Sections of experimental maxillae of SAL and ATV animals, stained immunohistochemically for RANKL. (D) Comparisons between the ATV and SAL groups indicated similar RANKL expression in both groups, in control and experimental maxillae alike. Scale bars represent 100 µm at ×200 magnification. Yellow arrow represents the direction of relapse force. Bo, bone; Ro, root. All data are mean ± SD.

Figure 5 – (A) Femur osteoclast counts and (B) bone volume were not affected by atorvastatin administration. (C) Thickness of hypertrophic zone and (D) growth plate were increased in the ATV group, as shown in H&E sections of femur growth plate from SAL and ATV animals (E and F, respectively). Scale bars represent 100 µm at ×200 magnification. GPCTh, growth plate cartilage thickness. All data are mean ± SD.

9. Figures

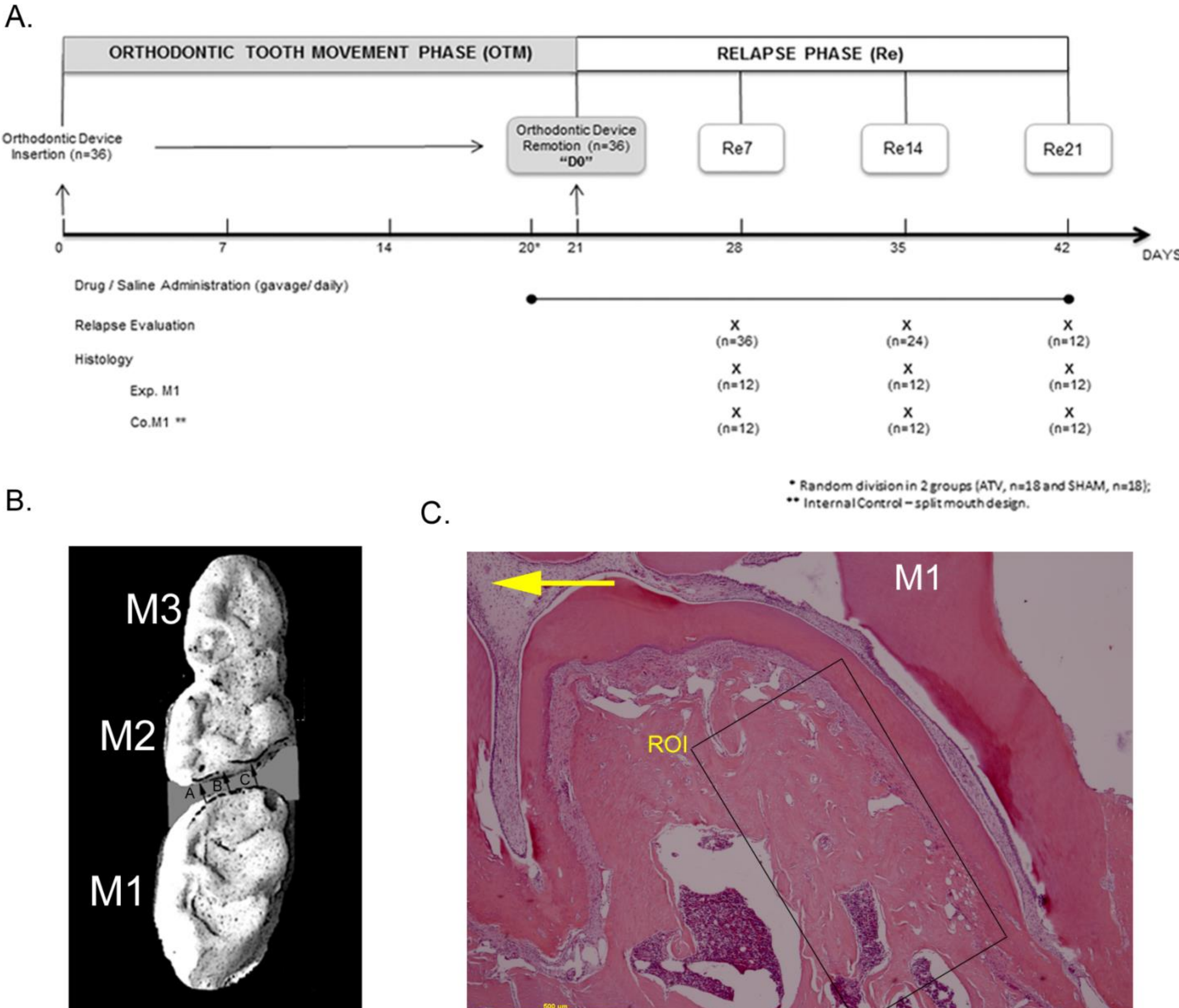

Figure 1

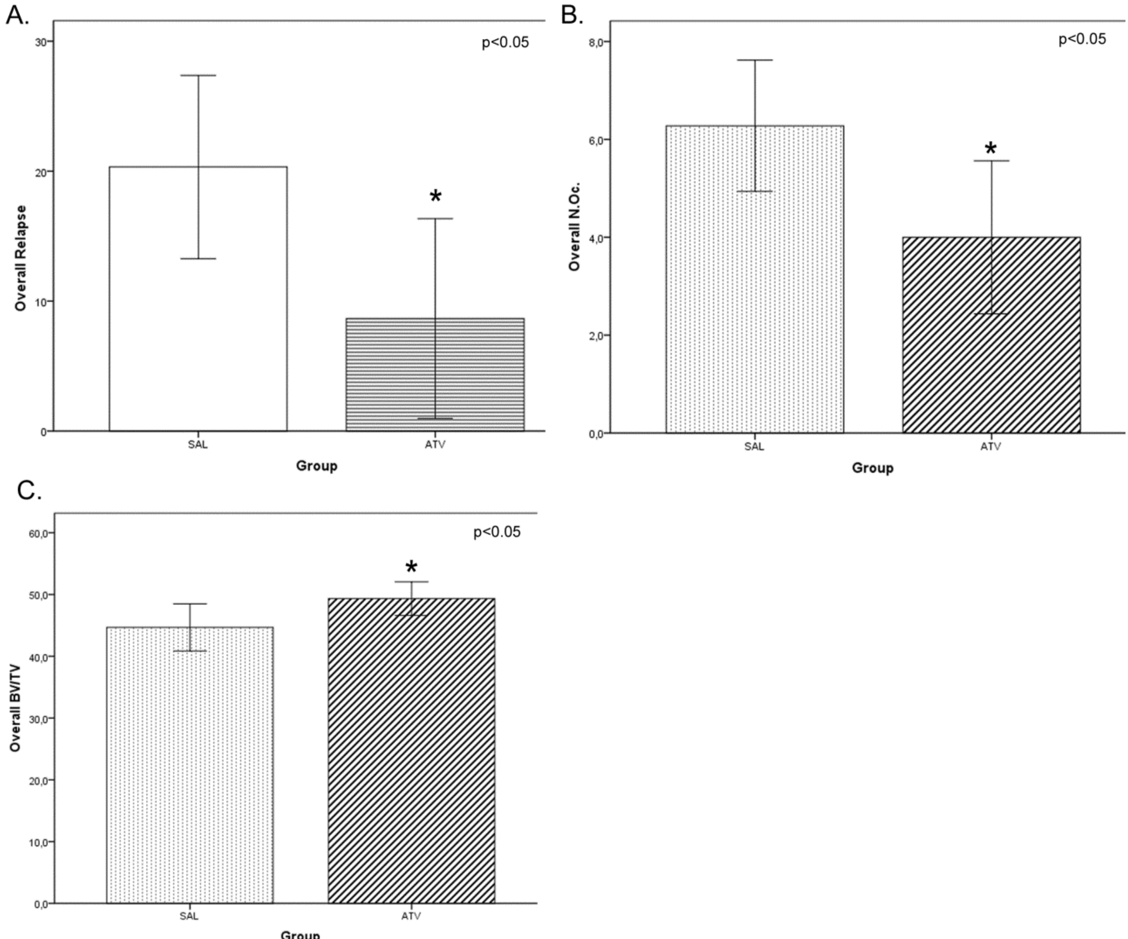

Figure 2

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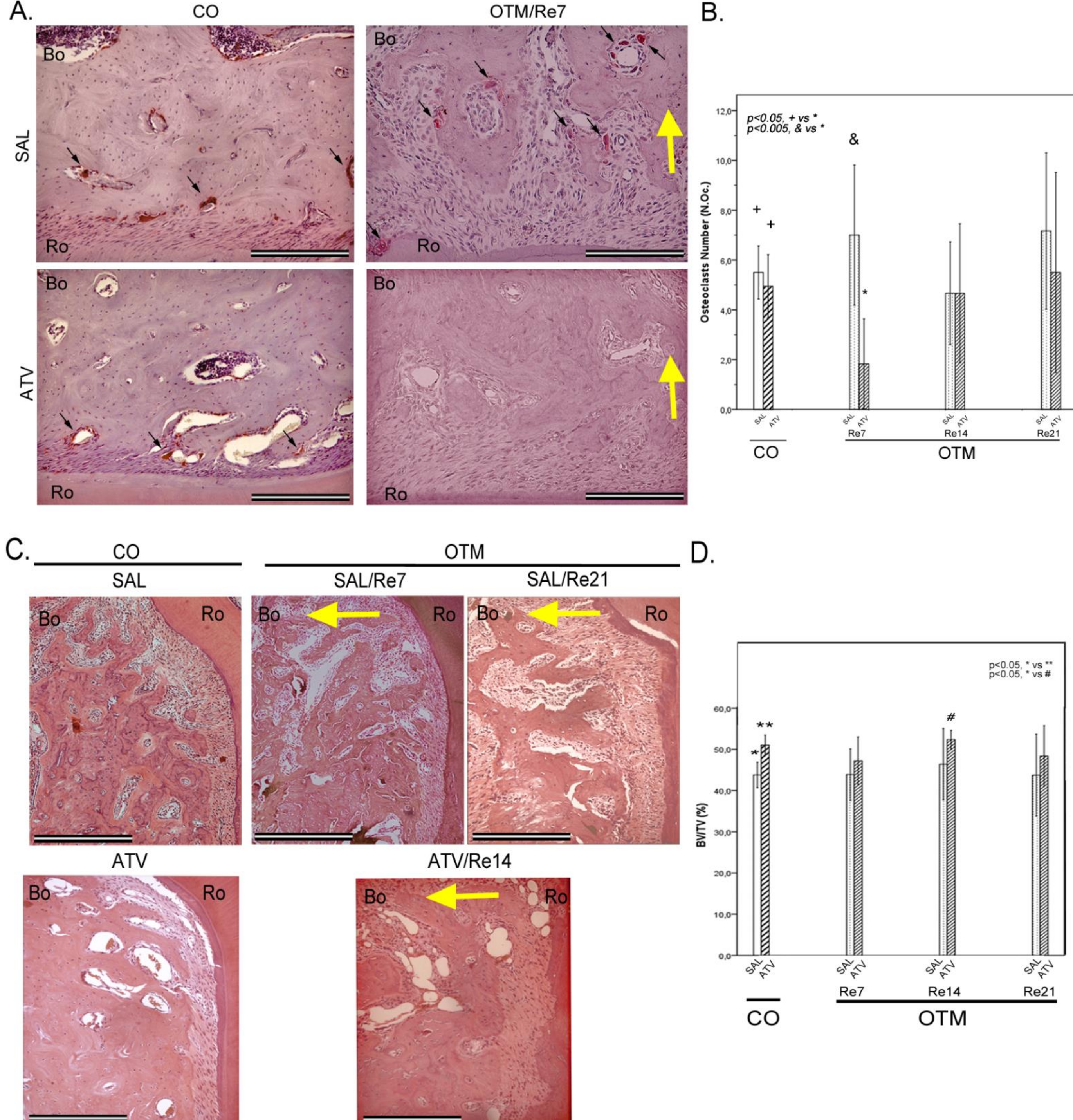

Figure 3

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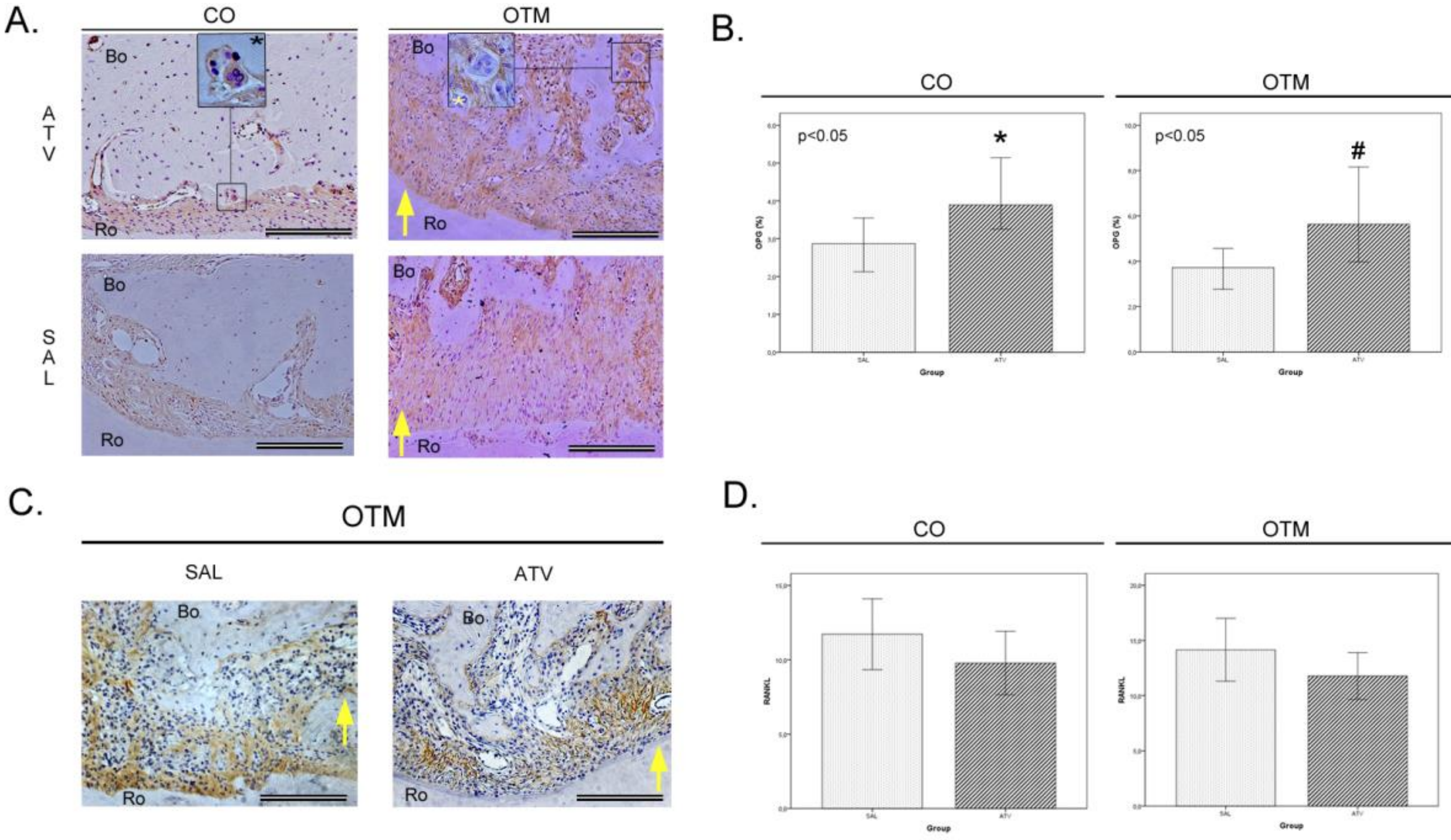

Figure 4

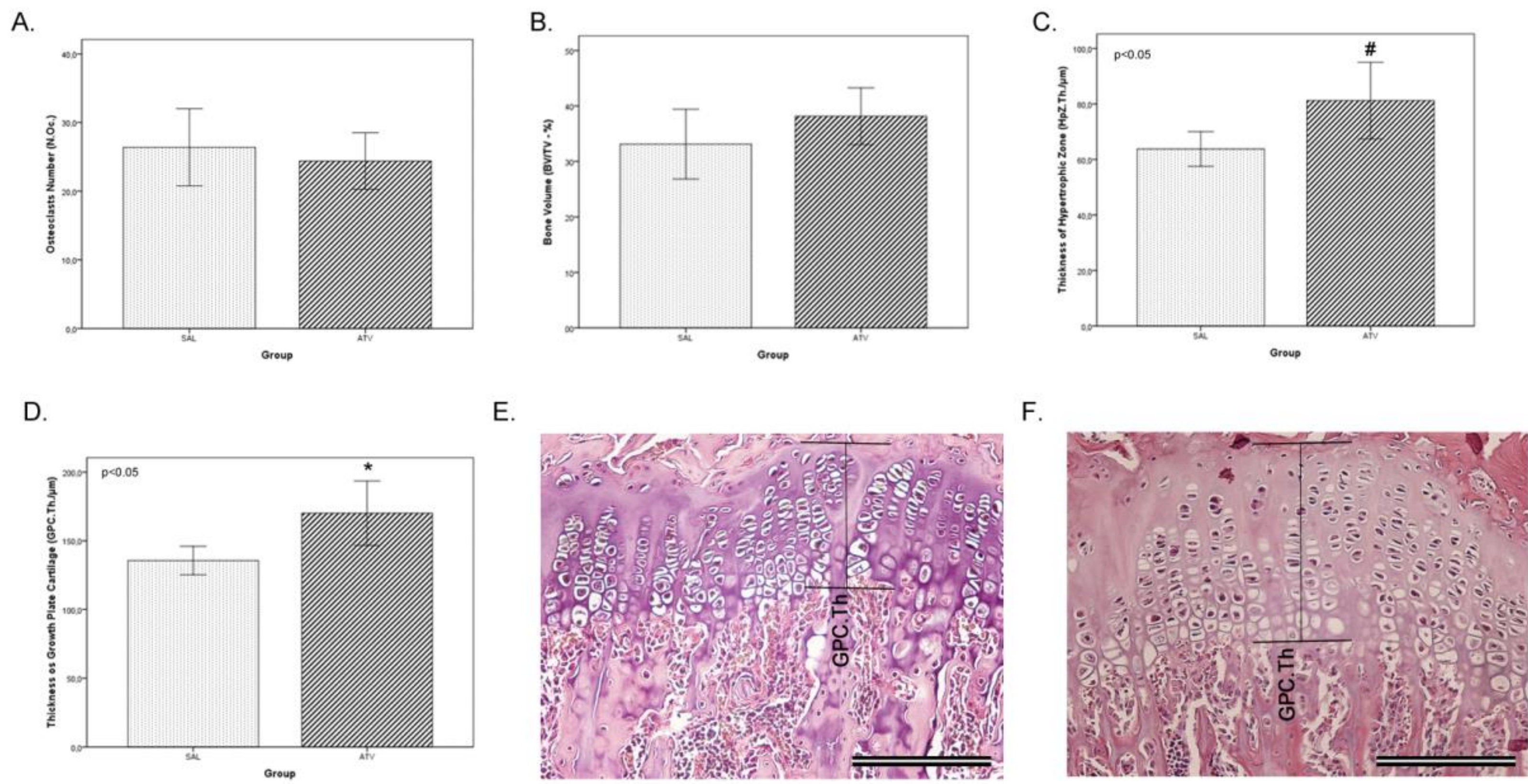

Figure 5

4. Manuscrito 2

Pharmacological bone modulation through osteoclasts inhibition arrests tooth movement

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Institutional Review Board Approval

All procedures for treatment and maintenance of the animals were conducted in keeping with internationally accepted guidelines - Guide for the Care and Use of Laboratory Animals. This research was approved by Ethics Committee of the School of Dentistry of Rio Grande do Sul Federal University (CEUA 28401).

Pharmacological bone modulation through osteoclasts inhibition arrests tooth movement

Abstract

Introduction: In addition to the cholesterol-lowering, a class of drugs namely statins seems to enhance osteogenesis and suppress bone resorption, which could represent a clinical concern during orthodontic treatment. This study aimed to determine whether atorvastatin (ATV) might affect the orthodontic tooth movement (OTM) through osteoclasts inhibition. Furthermore, we analyzed potential adverse effects of ATV on long bone turnover and endochondral ossification. *Methods:* Rats started to be administered with ATV (15mg/Kg) or saline, via gavage (n=12, per group), two weeks prior to the initial OTM. The tooth displacement was measured after 7, 14, and 21 days, while the maxillary and femur histologic sections were obtained after 14 and 21 days of OTM. The sections were prepared to H&E and TRAP staining, then histomorphometric analysis was performed *Results:* Atorvastatin resulted in a significant decrease of tooth movement ($p<0.05$), and osteoclasts number ($p<0.05$). Independently of drug administration, the OTM increased number of osteoclasts and reduced bone volume rate, when compared to the control maxillae, without OTM. Furthermore, it seems that long-term statins administration did not affect femur bone turnover and endochondral ossification. *Conclusions:* Considering the methodology used in this study, we found that atorvastatin is able to minimize orthodontic tooth movement through osteoclasts inhibition, which could represent a clinical concern. These results shed light on osteoclasts as a cellular target modulating maxillary bone metabolism and orthodontic tooth movement.

Keywords: Tooth movement; atorvastatin; osteoclasts; rats

1. Introduction

Epidemiologic studies have shown a significant increase on the prevalence of diseases such as obesity and hyperlipidemia in adults[1,2], the latter being regarded as the main cause of coronary atherosclerosis.[3] According to Mercado[2], overall 36.7% of U.S. adults or 78.1 million persons aged ≥21 years were on or eligible for lipid lower treatment, among whom 55.5% were taking cholesterol-lowering medication. In this regard, statins are a class of drugs widely used to lower cholesterol levels by the inhibition of HMG-CoA reductase, the rate-controlling enzyme of mevalonate pathway.[4,5] In addition to the cholesterol lowering, studies suggested that statins may influence bone turnover, enhancing osteogenesis and suppressing bone resorption.[6-14] Researchers have attempted to determine the mechanism of bone anabolism regulated by statins, suggesting three aspects: promotion of osteogenesis, inhibition of osteoblast apoptosis and suppression of osteoclastogenesis.[15] Although the statins effects on bone anabolism are widely demonstrated in laboratory studies[6-14], their clinical effects are not so convincing.[16]

Biological aspects of periodontal tissues, during orthodontic tooth movement (OTM), are characterized by an aseptic acute inflammatory response in the early stages, followed by an aseptic and transitory chronic inflammation. Chemokines, cytokines, and growth factors are the main molecules that orchestrate this inflammatory response, followed by osteoclastogenesis and bone resorption, and by osteoblast and new bone formation.[17] The current biological knowledge applied to clinical practice, raises the possibility of pharmacological modulation of these periodontal cellular and molecular responses, thus affecting the orthodontic tooth movement, as shown in different experimental models[11,18-22] Previous investigations demonstrated that statins reduces orthodontic tooth movement and relapse, however, the biological mechanisms behind these clinical effects are unrevealed.[11,12] Therefore, considering that statins are among the most commonly prescribed pharmaceutical agents for prevention of cardiovascular diseases,[4,5] their plausible clinical effects arresting tooth movement, seem to be a concern to orthodontic practice.

Besides, clinical trials had shown that statins are well tolerated in adult and young population [23-25], but long-term data regarding the impact of statin therapy on growth and development are limited.[25,26] According to Macpherson[23], the treatment of young patients with statins, over the course of 2 years, had no impact on height, weight, body mass index and sexual maturation. On the other hand, pre-clinical studies suggested that these drugs increase the chondrocytes proliferation and longitudinal bone growth [27,28], what could have implications for its clinical use in the pediatric population. These discrepancies between clinical trials, *in vitro* and *in vivo* studies[23-28] seem to be a puzzle and further researches are apparently necessary to investigate the adverse effects of statins on bone turnover and endochondral ossification.

In an attempt to try to mimic the clinical perspective of orthodontic treatment in patients taking statins, we developed a study design in which the drug administration started prior to OTM, in a high dosage, simulating a protocol used in humans. Thus, it was hypothesized that atorvastatin (ATV) treatment in rats may reduce the OTM through osteoclastogenesis inhibition. Furthermore, we analyzed the potential drug adverse effects on long bone turnover and endochondral ossification. So, we found that statins bone modulation could arrest the tooth displacement during orthodontic treatment, thus representing a relevant subject in the clinical practice.

5. Methods

2.1. Animals and Experimental tooth movement

Twenty four male Wistar rats, 6 weeks old, weighting approximately 330-340g, were used in the experiment. The animals were housed in separate cages (four per cage) in a 12 h light/dark cycle, at a constant temperature of 23°C and provided with food and water *ad libitum.* All procedures for treatment and maintenance of the animals were conducted in keeping with internationally accepted guidelines - Guide for the Care and Use of Laboratory Animals,[29] and were approved by Ethics Committee of the School of Dentistry of [redacted] Sul (CEUA 28401).

The animals were randomly divided in two groups: - Experimental (ATV, n=12;) and; -Control (SAL, n=12). In the experimental and control groups, respectively, the animals received, daily, via gavage, 15mg/Kg of atorvastatin ( ), or 0,1mL of Phosphate-Buffered Saline (PBS). The saline/drug administration continued until the animals were killed (Figure 1).

After 14 days of saline or drug administration, the animals were anesthetized with ketamine and xilazyne (80mg/kg and 5mg/kg, respectively), and the orthodontic device was placed. This procedure consisted in the insertion of a superelastic closed nickel-titanium coil spring between the upper right first molar and upper incisors, as previously described in the literature [12,30-33] Our protocol was based on previous studies, which demonstrate that 50cN is a good strength for providing a substantial tooth movement[11,22,32,33]. The device was maintained for 21 days (Figure 1A), in order to generate a mesial movement of the first molar (M1). Whereas the right maxillary side in each rat served as experimental orthodontic tooth movement, the left maxillary side, without tooth movement, served as internal control (Co). Through the study, the animals were evaluated weekly for weight gain/loss, appliance breakage, and gingival or other soft tissue inflammation.

2.2. Measurement of tooth movement

Precise plaster models of the maxilla were obtained from impressions made with silicone material ( ) and dental stone (Durone, Dentsply®, PA, USA). Impressions were made every 7 days, under anesthesia (Figure 1A). The occlusal surfaces, together with a 100mm ruler placed next to the casts, were photographed (DSC#H10, Sony®, Tokyo, Japan) at 300 dpis and magnified (4x) using Image J® software (version 1.44, National Institute of Health, 2011). The ruler was used to calibrate the casts' measurements. Then, the mean of the distance between the distal surface of M1 and the mesial surface of the second molar (M2), measured at 3 distinct points on each plaster, was calculated for each animal and averaged for the groups SAL and ATV.

The evaluation of tooth displacement, was performed after 7, 14, and 21 days of OTM (T7, T14, and T21, respectively), while the histological analysis was done after 14, and 21 days of orthodontic appliance insertion (T14 and T21, respectively), as demonstrated in Figure 1.

2.3. Tissue Preparation and TRAP staining

At each experimental time (T14 and T21), 12 animals, 6 per group, were sacrificed by an overdose of ketamine and xilazyne hydrochlorides. Maxillae and the distal left fêmures were immediately dissected and immersed for 24h in 10% buffered formalin to fixation. The specimens were demineralized in 10% EDTA (Ethylenediaminetetraacetic acid), pH 7, for 30-60 days. Then, the samples were dehydrated through an ethanol series, embedded in paraffin, cuted in 5μm longitudinal sections and prepared to hematoxylin and eosin (H&E) and Tartrate Resistant Acid Phosphatase (TRAP). This molecule (TRAP) is expressed in mature osteoclasts, and in their precursors, which develops an osteoclastic phenotype at early stages and also are able to promote bone resorption.[34] Briefly, for TRAP staining, histologic sections were selected and incubated in acetate buffer (pH 5.0) containing naphtol AS-MX phosphate (Sigma®, St. Louis, Missouri, USA), Fast Red Violet LB Salt (Sigma®), and 50mM sodium tartrate. The sections were counterstained with hematoxylin.

2.4 Histomorphometry

Histomorphometric analyses of maxilla was performed, considering 4 subgroups: - ATV+OTM – Experimental hemi maxillae (with OTM) of ATV group; - CoATV - Control hemi maxillae (without OTM) of ATV group; - SAL+OTM – Experimental hemi maxillae (with OTM) of SAL group and; - CoSAL - Control hemi maxillae (without OTM) of SAL group. Comparisons between the subgroups were done at each studied time (14 and 21 days) or, analyzing their overall mean. To femur evaluation, the sample was divided in two groups: ATV (n=18) and SAL (n=18). In this case, the comparisons between the groups were done considering their overall mean.

H&E and TRAP sections were visualized in a Nikon Eclipse 90i microscope (Nikon Co., Tokyo, Japan) coupled to a Coolsnap EZ camera

(Photometrics, AZ, USA). The microphotographs were captured using the software NIS Elements Imaging 3.10 Sp2 (Nikon Co.). All histomorphometric measurements and terminology were in accordance with the American Society for Bone and Mineral Research recommendations[35].

Maxillary Bone Turnover - Dental supporting structures of the molars were evaluated using the software NIS Elements Imaging 3.10 Sp2. Under high magnification (×100), the number of osteoclasts (N.Oc.) was counted on the most mesial root of the first molars. The Region of Interest (ROI) was the periodontal tissues at the distal surface of the mesial root of first molar [18,19], as described in our previous study. Cells were considered to be osteoclasts if they were TRAP-positive, multinucleated, and were located on the bone surface or residing in Howship's lacunae[33].

The maxillary bone volume ratio (BV/TV), expressed as the ratio of cancellous bone volume (BV) to total tissue volume (TV), was defined in the in the ROI of H&E sections, using the software Photoshop® (CS6; Adobe Systems, San Jose, CA, USA) and Image J, following the method suggested by Egan et al. [36]. The total osteoclasts number and bone volume were considered for each animal and after averaged for each group.

Femur Bone Turnover - The number of osteoclasts (N.Oc.) was counted in 10 fields randomly chosen within the femur metaphyseal region[37], using the software NIS Elements Imaging 3.10 Sp2. The BV/TV ratio of five rectangular areas of subchondral bone tissue was calculated, according to the protocol outlined by Ho et al.[14] The total N.Oc. and BV/TV were considered for each animal and after averaged for each group,

Endochondral Ossification- - Growth Plate Cartilage and Hypertrophic Zone Thickness (GPC.Th and HpZ.Th, respectively) were calculated as the mean of ten different measurements performed at locations randomly chosen in the H&E sections[38,39], using the software NIS Elements Imaging 3.10 Sp2.

All procedures were done by a single blinded calibrated examiner. In order to determine the random intra-individual error, 10% of the sample (plaster

models and histologic sections) were randomly chosen and evaluated twice (15 days between the first and the second evaluation). Dahlberg's equation was applied and acceptable values were obtained (<10%).

2.5. Statistical Analysis

Data are presented as mean values ± standard deviation or, when appropriated, standard error (SD or SE, respectively). A Linear Mixed Model with repeated measures to the factor time was used to evaluate the main effects and interactions of the Group (ATV or SAL) and the Time (T7, T14 andT21) on the orthodontic tooth movement. Between-group comparisons of maxillary histomorphometric parameters were performed using one way ANOVA followed by the LSD multiple comparison test (for homogeneous variances) or, Games-Howell test (for heterogeneous variances). When comparisons were performed between two groups, the independent student t test was used. Results were processed with SPSS software (version 18, SPSS, Chicago, Ill) and a significance level of 5 % ($p < 0.05$) was considered in all cases.

3. Results

3.1. Animals

There was no difference in weight gain/loss between the groups during the experimental period. The mean weights at the end of the study were 327.18 (SD ± 86.54) for SAL group and, 344.56 (SD ± 55.44) for ATV group.

3.2. Atorvastatin reduced orthodontic tooth movement (OTM)

After 7, 14 and 21 days of tooth movement, the displacement of first molars in the SAL group was 310.13±14.79, 381.38±33.33 and, 485.85±68.94µm , respectively, while in the ATV group it was 263.53±14.79, 334.78±33.33, and 439.25±68.94µm , respectively. Statistical analysis indicated that drug administration provides a significant decrease in the tooth movement ($p<0.05$), independently of the studied time (Figure 2A).

3.3. Atorvastatin inhibited osteoclastogenesis

During orthodontic tooth movement, atorvastatin administration reduced the overall number of osteoclasts, but not affected the overall bone volume rate, when compared to SAL group (Figure 2B and 2C). However, when analyzing the drug effect at each time point separately, only after 14 days of orthodontic tooth movement (T14), atorvastatin promoted osteoclasts inhibition (Figure 3A and 3B). At this time of OTM (T14), the number of TRAP+ cells in ATV group was statistically smaller than that observed in the experimental maxillae of SAL group (with OTM) and, equal to that observed in the control maxillae of SAL group (without OTM). During tooth movement there was a trend to increase the number of osteoclasts, only in in SAL group when compared to the its respective control maxillae (without OTM), as observed in Figure 3A and 3B. After 21 days, there were no statistical differences between the osteoclasts number among all groups.

Regarding to osteoclasts distribution, our observational data indicated that, independently of drug or saline administration, these cells were observed in the periphery around the bone blood vessels (Havers Canals), or sparse through the periodontal ligament (Figure 3A).Also, after 14 days, it was observed the presence of chronic inflammatory cells in the ROI, while after 21 days, apparently, there was resolution of the cellular infiltrate, increasing amount and maturation of bone. However, in some cases the width of the PDL continued to be enlarged, especially in the SAL group (Figure 3D).

After 14 and 21 days of OTM, bone volume (BV/TV) was significantly reduced in the SAL and ATV groups, when compared to their respective controls (without OTM). However, after 14 days of OTM, the BV/TV rate in the ATV group was statistically similar to that observed in the control maxillae of SAL group (without OTM), as demonstrates Figure 3B and 3C. In the absence of orthodontic force (maxillae without OTM), the number of osteoclasts and the bone volume were statistically similar between ATV and SAL groups (Figure 3B and 3D).

Long bone turnover and endochondral ossification were not affected by statins.

As observed in Table 1, atorvastatin did not affect femur osteoclastogenesis and bone volume. The thickness of growth plate cartilage and hypertrophic zone was increased in ATV group when compared to SAL; however, there was no statistical significance between the groups, at a level of 5% (p=0.259 and p=0.09, respectively).

4. Discussion

In a previous research conducted in our laboratory, we demonstrated that, in alveolar bone of rat molars, the number of osteoclasts was inhibited by statins administration, what was correlated to a decreased orthodontic relapse. However, this former study also showed that atorvastatin was able to affect endochondral ossification of long bones, since it elongated the thickness of growth plate cartilage and chondrocytic hypertrophic zone of femurs, after a short-term therapy (7-21 days). These results highlighted the clinical relevance of pharmacological bone modulation during orthodontic treatment and, the statin adverse effects on femoral growth plate, what seems to limit its use as an orthodontic pharmacological strategy for children. So, in the current study, we developed an experimental design to confirm the plausible statins effects on orthodontic tooth movement (OTM) through osteoclasts inhibition and, at the same time, analyze its potential adverse effects on endochondral ossification, now considering a longer-term therapy (28 -35 days).

Our study was conducted in Wistar rats, which are widely used in pre-clinical researches intending to evaluate the cellular aspects of OTM, being a recognized and adequate model with some translational potential.[9,11,12,19,30,32,33] Therefore, to reproduce the statin oral usage preconized in humans, we performed a daily administration, via gavage, at a dose of 15mg/Kg. Differing from others[9,11,12,40], we elect a high statin dosage, because it is expected that 73% of this oral dose would be excreted in bile.[41] Besides, according to Elewa[42], the atorvastatin peak plasma concentration achieved after administering 15 mg/kg by oral route, in rats, was similar to that reported after a dose of 80 mg/day atorvastatin is given to humans.[43] Finally, our study protocol was developed to allow the full potency of statins, once we started the drug administration two weeks before OTM. Considering that each day in the mouse

life represents 30 days of the human life[44], the period of two weeks seems to signify a drug administration for 1 year, in humans. In this regard, Reamy[45] emphasized that the clinical use of statins will achieve its full potency after 6 weeks but can require up to 12 weeks, in some patients.

The present study demonstrated that the major total molar movement in both groups occurred within the first 7 days, however, there were significant differences between the groups SAL and ATV, after 7, 14 and 21 of orthodontic tooth movement. In the SAL group, we observed a crescent tooth displacement through day 7 to 21, while the ATV group presented a reduced OTM from the earliest to the latest evaluated periods. These findings corroborate previous studies, in which it was described a significant prevention of orthodontic tooth movement and relapse after statin administration.[11,12] MirHashemi et al.[12], observed that ATV, administered in a dosage of 5 mg/Kg/day, via gavage, decreases the rate of tooth movement in Wistar rats, after 21 days of OTM. Also, Han et al.[11] reported a significant reduction on tooth relapse in rats treated with simvastatin (2.5mg/KG/day), via intraperitoneal, after 7 and 28 days of appliance removal. Based on that, if we extrapolate this potential statin action to humans bone turnover, a hindered orthodontic tooth movement should be considered in patients taking these drugs. Therefore, our results could suggest an anabolic bone effects of statins, increasing osteogenesis and suppressing osteoclastogenesis, as previously described.[6-14] Although further studies are apparently necessary to confirm this hypothesis, we suggest that statins administration is a relevant data to be included in the anamnesis form, thus allowing the clinical observation of plausible deviations from the expected tooth movement.

On the other hand, the pharmacological inhibition tooth movement observed in this study also may represent a clinical advance, since it could be used to improve the tooth anchorage during orthodontic treatment. Regarding to this, different strategies have been investigated to prevent undesirable tooth movement.[11,12,18,19,21,22,33] However, considering this purpose, it is suggested a study design electing a local drug application, in contrast with the oral administration performed in the current study.

Another important finding of our study was the statin osteoclasts inhibition. The overall number of TRAP positive cells was significantly decreased in ATV group, when compared to SAL group. In disagreement, authors suggested that these drugs are not able to affect osteoclastogenesis during orthodontic tooth movement or relapse.[11,12] On the other hand, our research used an appropriate histochemical technique to osteoclasts counting (TRAP), while the others used H&E sections, which could explain these significant differences between results. According to Kirstein, Chambers & Fuller[46], TRAP expression in osteoclasts is directly correlated with bone resorptive activity, moreover, agents that stimulate or inhibit bone resorption also stimulated and inhibits TRAP osteoclastic expression, respectively. During the bone resorption process, it is proposed that TRAP enzyme generates free radicals that help to dissolve collagen fragments, intracellularly, in vacuoles of the osteoclastic cell.[47]

In fact, the statins osteoclasts inhibition is widely supported in the literature,[6,7,9,11,13], but the exact mechanisms involved in this process are far to be known. It is suggested that statins ability to inhibit HMG CoA reductase, thus suppressing the mevalonate pathway, is positively correlated with their ability to inhibit bone resorption, since it will prevent the activation of GTP-binding proteins, crucial molecules involved to the osteoclasts development and function (actin cytoskeleton regulation, apoptosis, membrane ruffling and vesicular trafficking).[6,13] Pan et al.[48] conducted a study to further understanding of the role of small GTPase-Rho signaling pathway in the regulation of periodontal tissues while responding to mechanical stimuli. It was observed, *in vitro,* that human periodontal cells, when submitted to a cyclic strain protocol, will present an upregulation of Rho. The authors suggest that this molecular mechanism promotes actin polymerization, which may be responsible for cytoskeletal rearrangement, thus stimulating orthodontic tooth movement and alveolar bone remodeling. Therefore, it is reasonable to suppose that statins downregulation of small GTPases would affect the osteoclastogenesis during orthodontic tooth movement.

Other plausible hypothesis is the prevention of the activation of NFκB pathway[9,11,49], which involves modulation of the cell receptor activator of nuclear

kappa B (RANK), the extra-cellular (EC) and cell receptor activator of nuclear kappa B ligand (RANKL), and the EC decoy receptor osteoprotegerin (OPG), ultimately promoting suppression of osteoclastogenesis.[9,11,12] The current research attempted to investigate the cellular effects of long-term atorvastatin administration on bone turnover, disregarding molecular aspects, what seems to be a limitation. Further studies associating the cellular and molecular atorvastatin effects on bone system during orthodontic tooth movement are apparently necessary.

In the SAL group, a maximum peak of osteoclasts number was observed after 14 days of OTM (T14), which had dropped down after 21 days, agreeing with others authors.[50] However, this trend was not obvious in the ATV group, which presented, only at this time (T14), a reduced number of osteoclasts, when compared to the SAL group. It seems that this transient osteoclasts inhibition may be due to the reduction of statins effects on alveolar bone over time. In this regard, it is suggested an *in vivo* compensatory mechanism able to stimulate osteoclasts to overcome statins blockade at T21.[6] Moreover, it should be expected that statin anti-inflammatory effects will reduce the acute inflammation observed at early stages of OTM, thus inhibiting the recruitment, maturation and activation of osteoclast during the tooth movement. However, even reporting a transient statin bone effect, our results highlighted the osteoclastogenesis as a cellular candidate target modulated by ATV, which prevented tooth movement in rats, thereby providing some mechanistic clues for future studies in humans

Remarkably, although atorvastatin had affected the osteoclasts number, our observational data showed a quite similar distribution of these cells among all the studied groups. Most osteoclasts were localized around blood vessels, in the prospective Haversian canals. These findings seems to confirm that, during orthodontic tooth movement, early mononuclear precursors in the bone marrow differentiate into committed mononuclear precursors, fuse, and migrate into the PDL, where they differentiate into active multinuclear osteoclasts[51]. Also, it should be emphasized the importance of angiogenesis during bone remodeling. It has been shown that microvascular pericytes, which comprises undifferentiated cells located around the periphery of the blood vessels[52], have

pluripotent potential to differentiation into osteoblastic bone cells and chondroblasts.[52-54] Apparently, further preclinical investigations are crucial to confirm the plausibility of osteoclasts differentiation from pericytes, which could explain some observations of this study (Figure 3A).

To assess the effects of ATV administration on periodontal tissues under physiological conditions, a split-mouth design was used, in accordance with other studies [12,30,32,33]. Our data indicated that the long-term Atorvastatin administration did not affect the bone remodeling cycle on control maxilla (without OTM), since the overall osteoclasts number and bone volume rate (BV/TV) were statistically similar between the groups ATV and SAL. During orthodontic tooth movement, the overall BV/TV rate was similar between ATV and SAL groups. Considering this result as an unexpected data, once the ATV administration had inhibited tooth displacement and osteoclastogenesis, we had analyzed the BV/TV rate at each time point, separately. .After 14 days of tooth movement, in the SAL group, there was a trend toward a markedly decrease on alveolar bone volume (BV/TV), when compared to the control maxillae (without tooth movement), as already described by others[18,22].This finding seems to be related with the higher osteoclasts number observed in this group, during OTM (Figure 3B), corroborating with other authors[50]. On the other hand, in the ATV group, after 14 days of OTM, the BV/TV rate was similar to that observed in control maxillae of SAL group without tooth movement, apparently confirming that statins osteoclasts inhibition was able to prevent bone resorption and/or promote bone formation, thus maintaining bone volume. In short, our results seem to confirm that the alveolar bone microenvironment is modified during OTM, ultimately promoting a higher osteoclastic activity and a reduction on alveolar bone volume. However, the atorvastatin administration had affected this bone modulation, thus arresting OTM.

In the current research, the long-term statin administration did not demonstrate effects adverse on long bone turnover and endochondral ossification, in agreement with several studies.[23-25] Although a reduced thickness of hypertrophic zone and growth plate cartilage was observed in animals that received ATV, there were no statistical differences between ATV and SAL groups, at significance of 5%. In contrast, a previous research

conducted in our laboratory showed that atorvastatin elongates the growth plate cartilage and chondrocytic hypertrophic zone, after a short-term therapy (7-21 days), which seems to be a major clinical limitation of its use in children and adolescents.[27,28] These divergences between results are apparently associated to the period of ATV administration, which comprised, in the current study, a mean time of 31.5 days, while in the former research it was equal to 14 days. Consequently, in the present research, the mean age of the animals at the sacrifice time was greater than in our previous investigation; what seems to be associated with the physiologic reduction in the thickness of growth plate cartilage and hypertrophic zone, normal for this age. In accordance, Walker & Kember [55] verified that over time, the cellular proliferation slows in the growth plate, causing the rate of longitudinal bone growth to decrease and approach zero as the organism approaches its adult size. Therefore, further preclinical studies aiming to confirm the statins effects on endochondral ossification are apparently necessary, thus confirming its safety use in children and adolescents.

Taken together, our data suggest that osteoclastogenesis inhibition by statins is able to minimize orthodontic tooth movement (OTM), in rats. Whether these findings will translate to the clinical practice is not known, but considering the widespread use of statins[4,5], clinical trials should be performed to evaluate this bone-anabolic role of statins during tooth movement. Finally, among the expected statins effects applied to orthodontic practice and science, we underline two conflicting aspects: 1) a hindered tooth displacement in patients taking these drugs, which represents a clinical concern and; 2) an additional tooth anchorage obtained through the pharmacological bone modulation, which represents a clinical aid.

5. Conclusion

In summary, considering the methodology applied in this study, we can conclude that the drug modulation of bone turnover through osteoclasts inhibition is able to minimize orthodontic tooth movement, in rats. Furthermore, under physiological conditions, atorvastatin did not affect the bone turnover and endochondral ossification.

6. Bibliography

1. Kim HJ, Kim Y, Cho Y, Jun B, Oh KW. Trends in the prevalence of major cardiovascular disease risk factors among Korean adults: results from the Korea National Health and Nutrition Examination Survey, 1998-2012. Int J Cardiol 2014;174:64-72.
2. Mercado C, DeSimone AK, Odom E, Gillespie C, Ayala C, Loustalot F. Prevalence of Cholesterol Treatment Eligibility and Medication Use Among Adults - United States, 2005-2012. MMWR Morb Mortal Wkly Rep 2015;64:1305-1311.
3. LaRosa JC, Hunninghake D, Bush D, Criqui MH, Getz GS, Gotto AM et al. The cholesterol facts. A summary of the evidence relating dietary fats, serum cholesterol, and coronary heart disease. A joint statement by the American Heart Association and the National Heart, Lung, and Blood Institute. The Task Force on Cholesterol Issues, American Heart Association. Circulation 1990;81:1721-1733.
4. Pedersen TR, Kjekshus J, Berg K, Haghfelt T, Faergeman O, Faergeman G et al. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). 1994. Atheroscler Suppl 2004;5:81-87.
5. Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet 2005;366:1267-1278.
6. Staal A, Frith JC, French MH, Swartz J, Gungor T, Harrity TW et al. The ability of statins to inhibit bone resorption is directly related to their inhibitory effect on HMG-CoA reductase activity. J Bone Miner Res 2003;18:88-96.
7. Kim JY, Lee EY, Lee EB, Lee YJ, Yoo HJ, Choi J et al. Atorvastatin inhibits osteoclastogenesis by decreasing the expression of RANKL in the synoviocytes of rheumatoid arthritis. Arthritis Research & Therapy 2012;14.
8. Maeda T, Matsunuma A, Kawane T, Horiuchi N. Simvastatin promotes osteoblast differentiation and mineralization in MC3T3-E1 cells. Biochem Biophys Res Commun 2001;280:874-877.
9. de Araujo Junior RF, Souza TO, de Moura LM, Torres KP, de Souza LB, Alves Mdo S et al. Atorvastatin decreases bone loss, inflammation and oxidative stress in experimental periodontitis. PLoS One 2013;8:e75322.
10. Ferreira LB, Bradaschia-Correa V, Moreira MM, Marques ND, Arana-Chavez VE. Evaluation of bone repair of critical size defects treated with simvastatin-loaded poly(lactic-co-glycolic acid) microspheres in rat calvaria. J Biomater Appl 2014.
11. Han G, Chen Y, Hou J, Liu C, Chen C, Zhuang J et al. Effects of simvastatin on relapse and remodeling of periodontal tissues after tooth movement in rats. Am J Orthod Dentofacial Orthop 2010;138:550 e551-557; discussion 550-551.
12. MirHashemi AH, Afshari M, Alaeddini M, Etemad-Moghadam S, Dehpour A, Sheikhzade S et al. Effect of atorvastatin on orthodontic tooth movement in male wistar rats. J Dent (Tehran) 2013;10:532-539.
13. Grasser WA, Baumann AP, Petras SF, Harwood HJ, Jr., Devalaraja R, Renkiewicz R et al. Regulation of osteoclast differentiation by statins. J Musculoskelet Neuronal Interact 2003;3:53-62.
14. Ho ML, Chen YH, Liao HJ, Chen CH, Hung SH, Lee MJ et al. Simvastatin increases osteoblasts and osteogenic proteins in ovariectomized rats. Eur J Clin Invest 2009;39:296-303.
15. Ruan F, Zheng Q, Wang J. Mechanisms of bone anabolism regulated by statins. Biosci Rep 2012;32:511-519.
16. Yue J, Zhang X, Dong B, Yang M. Statins and bone health in postmenopausal women: a systematic review of randomized controlled trials. Menopause 2010;17:1071-1079.
17. Jr. IA. Inflammation and Tooth Movement: The Role

of Cytokines, Chemokines, and GrowthFactors. In: Taddei SRA, editor. Seminars in Orthodontics: © 2012 Elsevier Inc.; 2012: p. 257-269.
18. Hudson JB, Hatch N, Hayami T, Shin JM, Stolina M, Kostenuik PJ et al. Local delivery of recombinant osteoprotegerin enhances postorthodontic tooth stability. Calcif Tissue Int 2012;90:330-342.
19. Zhao N, Lin J, Kanzaki H, Ni J, Chen Z, Liang W et al. Local osteoprotegerin gene transfer inhibits relapse of orthodontic tooth movement. Am J Orthod Dentofacial Orthop 2012;141:30-40.
20. Hirate Y, Yamaguchi M, Kasai K. Effects of Relaxin on Relapse and Periodontal Tissue Remodeling after Experimental Tooth Movement in Rats. Connective Tissue Research 2012;53:207-219.
21. Hassan AH, Al-Hubail A, Al-Fraidi AA. Bone inductive proteins to enhance postorthodontic stability. Angle Orthod 2010;80:1051-1060.
22. Schneider DA, Smith SM, Campbell C, Hayami T, Kapila S, Hatch NE. Locally limited inhibition of bone resorption and orthodontic relapse by recombinant osteoprotegerin protein. Orthod Craniofac Res 2015;18 Suppl 1:187-195.
23. Macpherson M, Hamrén B, Braamskamp MJ, Kastelein JJ, Lundström T, Martin PD. Population pharmacokinetics of rosuvastatin in pediatric patients with heterozygous familial hypercholesterolemia. Eur J Clin Pharmacol 2016;72:19-27.
24. Avis HJ, Hutten BA, Gagné C, Langslet G, McCrindle BW, Wiegman A et al. Efficacy and safety of rosuvastatin therapy for children with familial hypercholesterolemia. J Am Coll Cardiol 2010;55:1121-1126.
25. Miller ML, Wright CC, Browne B. Lipid-lowering medications for children and adolescents. J Clin Lipidol 2015;9:S67-76.
26. Eiland LS, Luttrell PK. Use of statins for dyslipidemia in the pediatric population. J Pediatr Pharmacol Ther 2010;15:160-172.
27. Leem K, Park SY, Lee DH, Kim H. Lovastatin increases longitudinal bone growth and bone morphogenetic protein-2 levels in the growth plate of Sprague-Dawley rats. Eur J Pediatr 2002;161:406-407.
28. Yamashita A, Morioka M, Kishi H, Kimura T, Yahara Y, Okada M et al. Statin treatment rescues FGFR3 skeletal dysplasia phenotypes. Nature 2014;513:507-511.
29. Guide for the Care and Use of Laboratory Animals. Washington (DC); 1996.
30. Verna C, Dalstra M, Melsen B. The rate and the type of orthodontic tooth movement is influenced by bone turnover in a rat model. Eur J Orthod 2000;22:343-352.
31. Shirazi M, Alimoradi H, Kheirandish Y, Etemad-Moghadam S, Alaeddini M, Meysamie A et al. Pantoprazole, a proton pump inhibitor, increases orthodontic tooth movement in rats. Iran J Basic Med Sci 2014;17:448-453.
32. Franzen TJ, Brudvik P, Vandevska-Radunovic V. Periodontal tissue reaction during orthodontic relapse in rat molars. Eur J Orthod 2013;35:152-159.
33. Franzen TJ, Zahra SE, El-Kadi A, Vandevska-Radunovic V. The influence of low-level laser on orthodontic relapse in rats. Eur J Orthod 2014.
34. Takahashi N, Udagawa N, Tanaka S, Murakami H, Owan I, Tamura T et al. Postmitotic osteoclast precursors are mononuclear cells which express macrophage-associated phenotypes. Dev Biol 1994;163:212-221.
35. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 1987;2:595-610.
36. Egan KP, Brennan TA, Pignolo RJ. Bone histomorphometry using free and commonly available software. Histopathology 2012;61:1168-1173.
37. El Khassawna T, Böcker W, Govindarajan P, Schliefke N, Hürter B, Kampschulte M et al. Effects of multi-deficiencies-diet on bone parameters of peripheral bone in ovariectomized mature rat. PLoS One 2013;8:e71665.

38. Aybar Odstrcil AC, Carino SN, Ricci JC, Mandalunis PM. Effect of arsenic in endochondral ossification of experimental animals. Exp Toxicol Pathol 2010;62:243-249.
39. Fan CM, Foster BK, Hui SK, Xian CJ. Prevention of bone growth defects, increased bone resorption and marrow adiposity with folinic acid in rats receiving long-term methotrexate. PLoS One 2012;7:e46915.
40. Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G et al. Stimulation of bone formation in vitro and in rodents by statins. Science 1999;286:1946-1949.
41. Black AE, Hayes RN, Roth BD, Woo P, Woolf TF. Metabolism and excretion of atorvastatin in rats and dogs. Drug Metab Dispos 1999;27:916-923.
42. Elewa HF, Kozak A, El-Remessy AB, Frye RF, Johnson MH, Ergul A et al. Early atorvastatin reduces hemorrhage after acute cerebral ischemia in diabetic rats. J Pharmacol Exp Ther 2009;330:532-540.
43. Lins RL, Matthys KE, Verpooten GA, Peeters PC, Dratwa M, Stolear JC et al. Pharmacokinetics of atorvastatin and its metabolites after single and multiple dosing in hypercholesterolaemic haemodialysis patients. Nephrol Dial Transplant 2003;18:967-976.
44. Andreollo NA, Santos EF, Araújo MR, Lopes LR. Rat's age versus human's age: what is the relationship? Arq Bras Cir Dig 2012;25:49-51.
45. Reamy B. Hyperlipidemia Management for Primary Care: An Evidence-Based Approach. Springer; 2008.
46. Kirstein B, Chambers TJ, Fuller K. Secretion of tartrate-resistant acid phosphatase by osteoclasts correlates with resorptive behavior. J Cell Biochem 2006;98:1085-1094.
47. Halleen JM, Räisänen S, Salo JJ, Reddy SV, Roodman GD, Hentunen TA et al. Intracellular fragmentation of bone resorption products by reactive oxygen species generated by osteoclastic tartrate-resistant acid phosphatase. J Biol Chem 1999;274:22907-22910.
48. Pan J, Wang T, Wang L, Chen W, Song M. Cyclic strain-induced cytoskeletal rearrangement of human periodontal ligament cells via the Rho signaling pathway. PLoS One 2014;9:e91580.
49. Kim JY, Lee EY, Lee EB, Lee YJ, Yoo HJ, Choi J et al. Atorvastatin inhibits osteoclastogenesis by decreasing the expression of RANKL in the synoviocytes of rheumatoid arthritis. Arthritis Res Ther 2012;14:R187.
50. Ren Y, Kuijpers-Jagtman AM, Maltha JC. Immunohistochemical evaluation of osteoclast recruitment during experimental tooth movement in young and adult rats. Arch Oral Biol 2005;50:1032-1039.
51. Xie R, Kuijpers-Jagtman AM, Maltha JC. Osteoclast differentiation during experimental tooth movement by a short-term force application: an immunohistochemical study in rats. Acta Odontol Scand 2008;66:314-320.
52. Chim SM, Tickner J, Chow ST, Kuek V, Guo B, Zhang G et al. Angiogenic factors in bone local environment. Cytokine Growth Factor Rev 2013;24:297-310.
53. Doherty MJ, Ashton BA, Walsh S, Beresford JN, Grant ME, Canfield AE. Vascular pericytes express osteogenic potential in vitro and in vivo. J Bone Miner Res 1998;13:828-838.
54. Diaz-Flores L, Gutierrez R, Lopez-Alonso A, Gonzalez R, Varela H. Pericytes as a supplementary source of osteoblasts in periosteal osteogenesis. Clin Orthop Relat Res 1992:280-286.
55. Walker KV, Kember NF. Cell kinetics of growth cartilage in the rat tibia. II. Measurements during ageing. Cell Tissue Kinet 1972;5:409-419.

8. Figure Captions

Figure 1 – Experimental study design. Saline solution or atorvastatin (15 mg/kg) were administered daily, via gavage, since day 0 through day 35. At day 14, the right first molars started to be mesialy moved. The tooth displacement was evaluated at 7, 14 and 21 days of OTM (T7, T14 and T21, respectively), while the histological analysis (H&E and TRAP) were performed at T14 and T21. The left maxilla served as internal control (without tooth movement).

Figure 2 – (A) A liner mixed model was used compare overall tooth displacement in SAL and ATV groups, indicating a significant reduction of tooth displacement in the ATV group. The data are mean ± SE. (B) Occlusal photographic views of representative saline and atorvastatin (ATV) administered animals. Note the difference in interdental distance (overall mean) between the first and second molar teeth in saline versus ATV treated animals. (C) An independent student t-test indicated that the overall mean of osteoclasts number in ATV group was decreased, in comparison to SAL group. The data are mean ± SD. (D) Independent t-test showed that the overall bone volume rate (BV/TV) was not affected by atorvastatin administration ($p>0.05$). The data are mean ± SD.

Figure 3 — (A) TRAP staining of Control maxilla (without tooth movement) and Experimental maxilla (after 14 of OTM), in ATV and SAL groups. The images supported that, during OTM, osteoclasts were inhibited by statins administration. Also, these cells were frequently observed in the periphery of blood vessels, independently of the studied time or drug administration. (B) Osteoclasts number in the control maxilla (without tooth movement) and experimental maxilla (after 14 and 21 days of OTM) of ATV and SAL groups. One-way ANOVA was performed at each time (14 and 21 days), indicating that Atorvastatin did not affect the osteoclasts number under physiological conditions (control maxillae, without tooth movement). After 14 days of OTM, the ATV group presented a reduced number of TRAP positive cells. (C) H&E sections of control and experimental maxilla of ATV and SAL groups.

Descriptive analysis, indicated that ATV did not affect the bone volume rate (BV/TV) under physiological conditions (control maxilla, without tooth movement). During OTM, ATV administered animals seemed to present a slight increase in the BV/TV rate. (D) Statistical comparisons between bone volume rate (BV/TV) between ATV and SAL groups (one way ANOVA). The OTM reduced the bone volume in both groups (ATV and SAL), when compared to their controls, without tooth movement (CoATV and CoSAL). Atorvastatin prevented bone loss after 14 days of OTM, once the BV/TV rate of ATV group was statistically equal to that observed in control maxilla of SAL group. Small arrows indicate the presence of osteoclasts. The scale bars represents 100μm, at a magnification of 200x. Yellow arrow represents the direction of relapse force; Bo – bone; Ro – root. The data are mean ± SD.

Table 1 – Considering the femurs' histomorphometric analysis, independent-student t test was performed to compare osteoclasts number, bone volume, thickness of hypertrophic zone and growth plate cartilage between the groups ATV and SAL. All the parameters were not affected by atorvastatin administration ($p > 0.05$). NS – not significant. The data are mean ± SD.

9. Figures

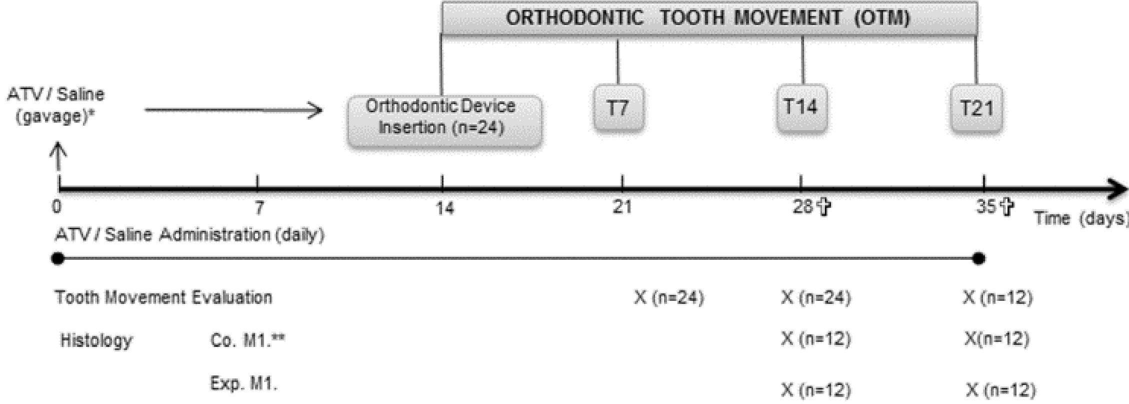


Figure 1

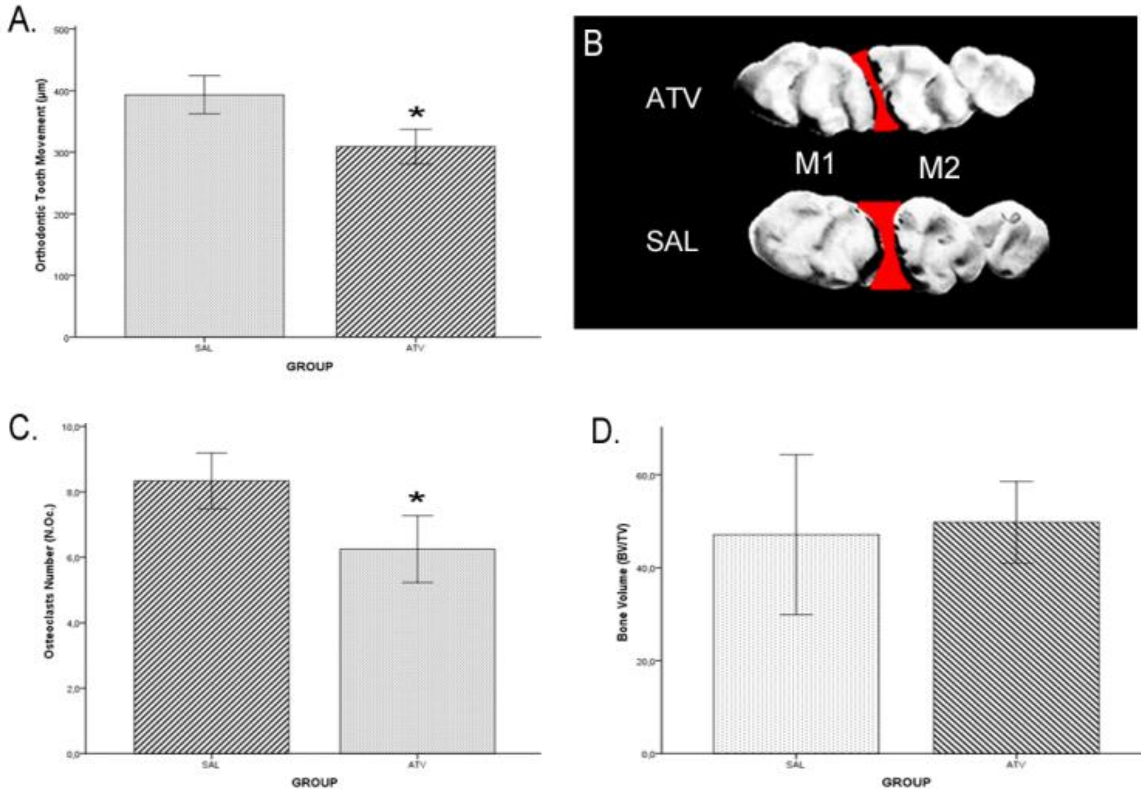


Figure 2

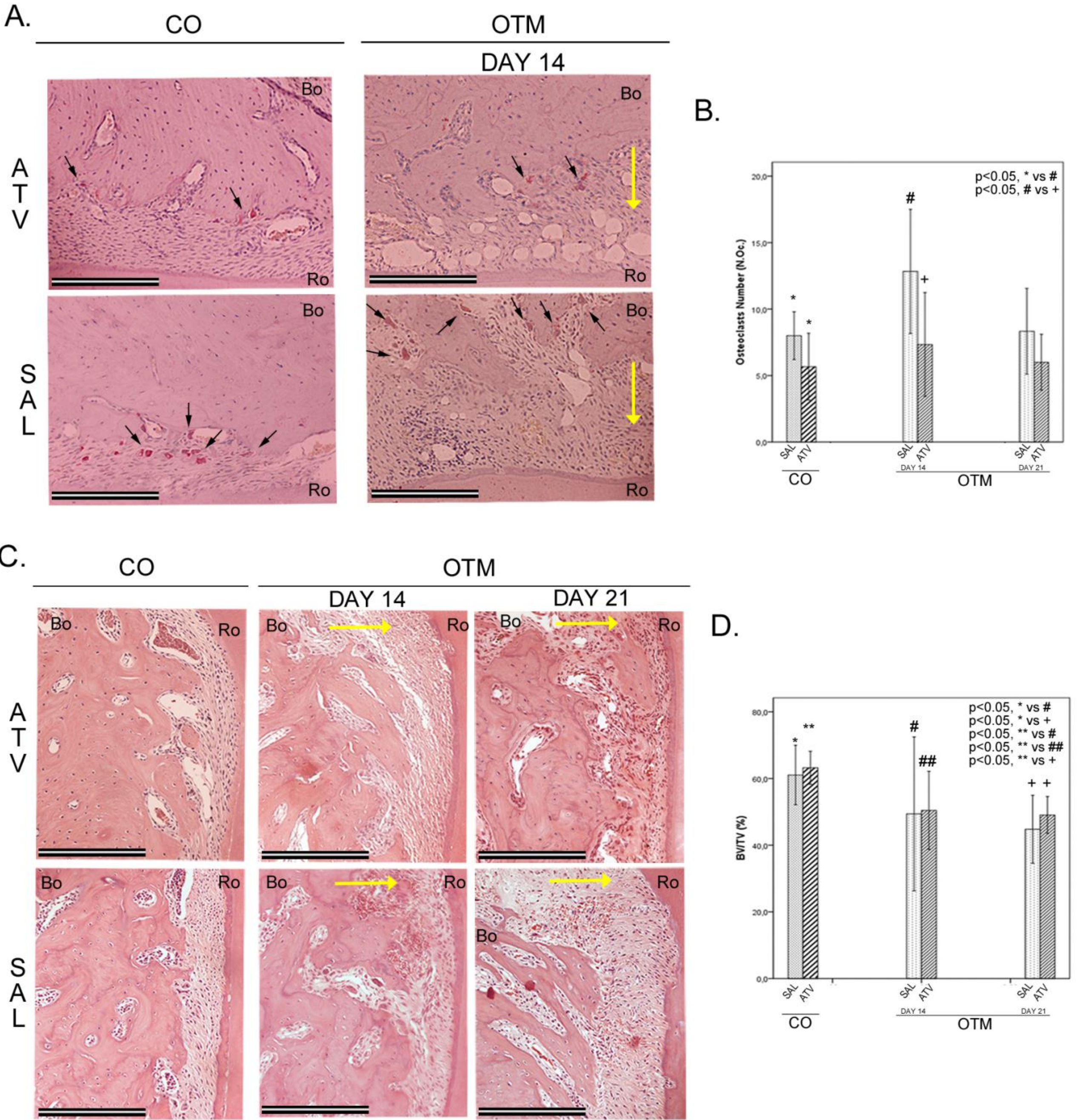


Figure 3

Table 1

	Group		P
	ATV	SAL	
Osteoclasts Number (Oc.N.)	26.33±9.14	20.25±8.90	NS
Bone Volume (BV/TV) (%)	27.10±9.91	20.97±7.73	NS
Thickness of hypertrophic zone (HpZ.Th) (µm)	67.20±12.09	58.25±12.64	NS
Thickness of growth plate cartilage.GPC.Th (µm)	136.70±24.50	124.29±27.81	NS

5. Considerações Finais

Durante o movimento dentário induzido, o microambiente ósseo é caracterizado pela presença de quimiocinas, citocinas várias e fatores de crescimento, os quais estimularão células a se diferenciarem em osteoclastos na zona de pressão, e osteoblastos na zona de tensão. No caso da osteoclastogênese, esse processo dependerá, impreterivelmente, da sinalização através da ligação RANKL/RANK em células pré-osteoclásticas, o que poderá ser inibido pela ação da mólécula OPG, que competirá com RANK à ligação com RANKL, impedindo a sinalização via RANKL/RANK. Tal mecanismo regulador da diferenciação osteoclástica é amplamente discutido em estudos laboratoriais, inclusive sugerindo-se a possibilidade de controle biológico do movimento dentário induzido. Apesar das limitações ao transladar esses dados laboratoriais para a prática diária, interessantes aplicações clínicas da biologia celular e molecular têm sido descritas na literatura.

Drogas anabolistas do tecido ósseo, tais como as estatinas, parecem inibir a reabsorção osteoclástica por três distintas vias: (a) efeitos antiinflamatórios, (b) inibição da ativação da via de NFκB e, (c) inibição de GTPases de baixo peso molecular. Sob esta perspectiva, nossos resultados evidenciaram que a modulação farmacológica do tecido ósseo alveolar, induzida pela administração sistêmica da droga atorvastatina, provou ser uma estratégia plausível para reduzir a recidiva ortodôntica. Um dos principais mecanismos biológicos envolvidos neste importante achado foi a inibição da osteoclastogênese, associada a superexpressão da osteoprotegerina (OPG) nos tecidos periodontais. Outro dado importante, embora observacional, foi a expressão de OPG no citoplasma de osteoclastos, o que aparentemente confirma um papel autorregulatório exercido por este tipo celular. Já quanto a expressão proteína RANKL durante o movimento denário induzido, não houve diferença significativa entre os animais que administraram ou não atorvastatina, apesar de ter se observado uma suave redução desta proteína no periodonto de animais tratados com a referida droga.

Por outro lado, em um experimento paralelo, também observamos que a administração de atorvastatina previamente ao início do movimentação ortodôntica, reduziu o deslocamento dentário em ratos Wistar, via inibição da

osteoclastogênese. Ao inferir que tal resultado possa ser reproduzido em humanos, devemos observá-lo sob dois prismas: (1) considerando-o um problema clínico - visto que indivíduos em tratamento da hipercolesterolemia, com o uso continuado de estatinas, poderiam ter o movimento dentário dificultado durante o tratamento ortodôntico e; (2) considerando-o um avanço clínico – visto a possibilidade de induzir uma redução localizada da movimentação dentária, assim permitindo um aumento de ancoragem em regiões específicas do arco dentário, por exemplo.

Já na análise descritiva dos dados, observamos que a maior concentração de células osteoclásticas ocorreu na periferia de vasos sanguíneos, o que parece confirmar que o comprometimento e a diferenciação de pré-osteoclastos ocorre na medula óssea, processo sucededido pela a migração destas células ao ligamento periodontal. Além disso, levantou-se a hipótese de que a presença de células pluripotentes na região perivascular (pericitos), poderiam estar associadas a este achado.

Interessantemente, em ambos experimentos, seja durante a recidiva ortodôntica ou seja durante a fase ativa da movimentação dentária, a atorvastatina teve um efeito biológico muito semelhante, inibindo a osteoclastogênese especialmente nos períodos iniciais dos experimentos (após7 dias de recidiva e após 14 dias de movimento dentário). Sugere-se que o fato da inibição osteoclástica não ter sido observada nos tempos subsequentes, provenha de um mecanismo compensatório observado *in vivo*, capaz de estimular as células osteoclásticas a superar a sua inibição via estatinas, o que ocorreria após determinado tempo. Outra hipótese é que as estatinas poderiam atuar apenas nos estágios iniciais do processo inflamatório, durante a recidiva e movimento dentário ortodôntico. À medida que o microambiente ósseo reestabelecesse sua condição fisiológica, a droga perderia seus efeitos sobre a osteoclastogênese. Ressalta-se que, mesmo considerando este transitório efeito biológico, a atorvastatina foi capaz de promover significativos resultados clínicos, conforme descrito anteriormente.

Além disso, a redução do número de osteoclastos observado nos grupos ATV, durante o movimento dentário e recidiva ortodôntica, parece estar associada ao aumento do volume ósseo alveolar, quando comparados aos grupos SAL. Outra hipótese para explicar tal achado é um plausível estímulo da

neoformação óssea promovido pelo uso da droga, sendo necessárias maiores investigações nesta área.

Em relação ao efeitos colaterais da atorvastatina, preocupamo-nos em estudar a ação destas drogas sobre a ossificação endocondral e *turnover* de ossos longos (fêmures). Para uma melhor avaliação destes dados, devemos tomar os resultados de nossos dois exprimentos em conjunto. Dessa forma, o amplo aspecto de nosso desenho experimental contempla a análise de fêmures de animais com admnistração de estatinas pelos períodos de 7, 14, 21, 28 e 35 dias. Os três primeiros tempos (7, 14 e 21 dias), foram considerados como "administração por curto prazo" (média de 14 dias), enquanto os dois últimos tempos (28 e 35 dias) foram denominados "administração por longo prazo" (média de 31,5 dias). De forma inesperada, a administração da droga por curto prazo promoveu um aumento da espessura do disco epifisário (GPCTh) e da zona hipertrófica de condrócitos (HzTh), apontando para um efeito anabólico sobre a ossificação endocondral. Já, quando considerado um maior tempo de administração das drogas, também houve um incremento nas medidas destes mesmos parâmetros (GPCTh e HzTh), mas estas não foram estatisticamente maiores do que aquelas observadas em animais sem administração de estatinas. Esta intrigante diferença de ação da droga sobre a ossificação endocondral, entre o curto e o longo prazo, parece evidenciar um efeito do fator tempo: a esse respeito, pode-se supor que o aumento de espessura verificado retratasse apenas um momento fisiológico da cartilagem, o que foi ultrapassado em momento posterior. Por outro lado, tem sido sugerido na literatura uma crível ação das estatinas estimulando a ossificação endocondral e aumentando o comprimento de ossos longos, o que parece ser de grande valia clínica.

Finalizando, de um modo geral, este estudo aponta para promissoras perspectivas, onde a aplicação da biologia celular e molecular deve modificar definitivamente algumas condutas ortodônticas diagnósticas e terapêuticas. Nesse cenário, a modulação medicamentosa dos tecidos periodontais poderá se tornar uma realidade, devendo ser rotineiramente usada para distintas finalidades clínicas

6. Bibliografia

[1] BURSTONE, C. J.; TANNE, K. [Biomechanical basis of tooth movement]. **Nihon Kyosei Shika Gakkai Zasshi,** v. 45, n. 4, p. 541-51, Dec 1986. ISSN 0021-454X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/3505570 >.

[2] JR., I. A. **Inflammation and Tooth Movement: The Role**

of Cytokines, Chemokines, and GrowthFactors. TADDEI, S. R. A. Seminars in Orthodontics: © 2012 Elsevier Inc. 18**:** 257-269 p. 2012.

[3] HAN, G. et al. Effects of simvastatin on relapse and remodeling of periodontal tissues after tooth movement in rats. **Am J Orthod Dentofacial Orthop,** v. 138, n. 5, p. 550 e1-7; discussion 550-1, Nov 2010. ISSN 1097-6752 (Electronic)

0889-5406 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21055594 >.

[4] HUDSON, J. B. et al. Local delivery of recombinant osteoprotegerin enhances postorthodontic tooth stability. **Calcif Tissue Int,** v. 90, n. 4, p. 330-42, Apr 2012. ISSN 1432-0827 (Electronic)

0171-967X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22382900 >.

[5] ZHAO, N. et al. Local osteoprotegerin gene transfer inhibits relapse of orthodontic tooth movement. **Am J Orthod Dentofacial Orthop,** v. 141, n. 1, p. 30-40, Jan 2012. ISSN 1097-6752 (Electronic)

0889-5406 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22196183 >.

[6] HIRATE, Y.; YAMAGUCHI, M.; KASAI, K. Effects of Relaxin on Relapse and Periodontal Tissue Remodeling after Experimental Tooth Movement in Rats. **Connective Tissue Research,** v. 53, n. 3, p. 207-219, 2012. ISSN 0300-8207. Disponível em: < <Go to ISI>://WOS:000303326700003 >.

[7] HASSAN, A. H.; AL-HUBAIL, A.; AL-FRAIDI, A. A. Bone inductive proteins to enhance postorthodontic stability. **Angle Orthod,** v. 80, n. 6, p. 1051-60, Nov 2010. ISSN 1945-7103 (Electronic)

0003-3219 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20677954 >.

[8] SCHNEIDER, D. A. et al. Locally limited inhibition of bone resorption and orthodontic relapse by recombinant osteoprotegerin protein. **Orthod Craniofac Res,** v. 18 Suppl 1, p. 187-95, Apr 2015. ISSN 1601-6343. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25865548 >.

9 PEDERSEN, T. R. et al. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). 1994. **Atheroscler Suppl,** v. 5, n. 3, p. 81-7, Oct 2004. ISSN 1567-5688 (Print)

1567-5688 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15531279 >.

10 BAIGENT, C. et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. **Lancet,** v. 366, n. 9493, p. 1267-78, Oct 8 2005. ISSN 1474-547X (Electronic)

0140-6736 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16214597 >.

11 STAAL, A. et al. The ability of statins to inhibit bone resorption is directly related to their inhibitory effect on HMG-CoA reductase activity. **J Bone Miner Res,** v. 18, n. 1, p. 88-96, Jan 2003. ISSN 0884-0431 (Print)

0884-0431 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12510809 >.

12 KIM, J. Y. et al. Atorvastatin inhibits osteoclastogenesis by decreasing the expression of RANKL in the synoviocytes of rheumatoid arthritis. **Arthritis Research & Therapy,** v. 14, n. 4, 2012. ISSN 1478-6354. Disponível em: < <Go to ISI>://WOS:000314974600031 >.

13 MAEDA, T. et al. Simvastatin promotes osteoblast differentiation and mineralization in MC3T3-E1 cells. **Biochem Biophys Res Commun,** v. 280, n. 3, p. 874-7, Jan 26 2001. ISSN 0006-291X (Print)

0006-291X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11162604 >.

14 DE ARAUJO JUNIOR, R. F. et al. Atorvastatin decreases bone loss, inflammation and oxidative stress in experimental periodontitis. **PLoS One,** v. 8, n. 10, p. e75322, 2013. ISSN 1932-6203 (Electronic)

1932-6203 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24130702 >.

15 FERREIRA, L. B. et al. Evaluation of bone repair of critical size defects treated with simvastatin-loaded poly(lactic-co-glycolic acid) microspheres in rat calvaria. **J Biomater Appl**, Sep 10 2014. ISSN 1530-8022 (Electronic)

0885-3282 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25209881 >.

16 MIRHASHEMI, A. H. et al. Effect of atorvastatin on orthodontic tooth movement in male wistar rats. **J Dent (Tehran),** v. 10, n. 6, p. 532-9, Nov 2013. ISSN 1735-2150 (Print)

1735-2150 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24910664 >.

[17] GRASSER, W. A. et al. Regulation of osteoclast differentiation by statins. **J Musculoskelet Neuronal Interact,** v. 3, n. 1, p. 53-62, Mar 2003. ISSN 1108-7161 (Print)

1108-7161 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15758366 >.

[18] HO, M. L. et al. Simvastatin increases osteoblasts and osteogenic proteins in ovariectomized rats. **Eur J Clin Invest,** v. 39, n. 4, p. 296-303, Apr 2009. ISSN 1365-2362. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19292885 >.

[19] RUAN, F.; ZHENG, Q.; WANG, J. Mechanisms of bone anabolism regulated by statins. **Biosci Rep,** v. 32, n. 6, p. 511-9, Dec 2012. ISSN 1573-4935. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22799752 >.

[20] MUNDY, G. et al. Stimulation of bone formation in vitro and in rodents by statins. **Science,** v. 286, n. 5446, p. 1946-9, Dec 1999. ISSN 0036-8075. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10583956 >.

[21] YUE, J. et al. Statins and bone health in postmenopausal women: a systematic review of randomized controlled trials. **Menopause,** v. 17, n. 5, p. 1071-9, Sep-Oct 2010. ISSN 1530-0374 (Electronic)

1072-3714 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20473231 >.

[22] BUHAESCU, I.; IZZEDINE, H. Mevalonate pathway: a review of clinical and therapeutical implications. **Clin Biochem,** v. 40, n. 9-10, p. 575-84, Jun 2007. ISSN 0009-9120. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17467679 >.

[23] CROCKETT, J. C. et al. Bone remodelling at a glance. **J Cell Sci,** v. 124, n. Pt 7, p. 991-8, Apr 1 2011. ISSN 1477-9137 (Electronic)

0021-9533 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21402872 >.

[24] MERCADO, C. et al. Prevalence of Cholesterol Treatment Eligibility and Medication Use Among Adults - United States, 2005-2012. **MMWR Morb Mortal Wkly Rep,** v. 64, n. 47, p. 1305-11, 2015. ISSN 1545-861X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26633047 >.

[25] MACPHERSON, M. et al. Population pharmacokinetics of rosuvastatin in pediatric patients with heterozygous familial hypercholesterolemia. **Eur J Clin Pharmacol,** v. 72, n. 1, p. 19-27, Jan 2016. ISSN 1432-1041. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26387811 >.

[26] AVIS, H. J. et al. Efficacy and safety of rosuvastatin therapy for children with familial hypercholesterolemia. **J Am Coll Cardiol,** v. 55, n. 11, p. 1121-6, Mar 2010. ISSN 1558-3597. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20223367 >.

[27] MILLER, M. L.; WRIGHT, C. C.; BROWNE, B. Lipid-lowering medications for children and adolescents. **J Clin Lipidol,** v. 9, n. 5 Suppl, p. S67-76, 2015 Sep-Oct 2015. ISSN 1933-2874. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26343214 >.

[28] EILAND, L. S.; LUTTRELL, P. K. Use of statins for dyslipidemia in the pediatric population. **J Pediatr Pharmacol Ther,** v. 15, n. 3, p. 160-72, Jul 2010. ISSN 1551-6776. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22477808 >.

[29] LEEM, K. et al. Lovastatin increases longitudinal bone growth and bone morphogenetic protein-2 levels in the growth plate of Sprague-Dawley rats. **Eur J Pediatr,** v. 161, n. 7, p. 406-7, Jul 2002. ISSN 0340-6199. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12174823 >.

[30] YAMASHITA, A. et al. Statin treatment rescues FGFR3 skeletal dysplasia phenotypes. **Nature,** v. 513, n. 7519, p. 507-11, Sep 2014. ISSN 1476-4687. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25231866 >.

[31] FDA. LIPITOR safely and effectively. Initial U.S. Approval: 1996. http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/020702s056lbl.pdf

[32] JUNQUEIRA L. C., C. J. **Histologia Basica**. 10ª. Rio de Janeiro: Guanabara Koogan S.A., 2004. 487

[33] CATE, T. **Histologia bucal : desenvolvimento, estrutura e função**. Rio de Janeiro: Guanabara Koogan, 2001. 439

[34] NANCI, A. Content and distribution of noncollagenous matrix proteins in bone and cementum: relationship to speed of formation and collagen packing density. **J Struct Biol,** v. 126, n. 3, p. 256-69, Jun 30 1999. ISSN 1047-8477 (Print)

1047-8477 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10441531 >.

[35] DANTAS, S. **Mecanismos Celulares e Moleculares da Inflamação**. Rio de Janeiro: MEDSI - Editora Médca Científica, 2000. 238 ISBN 85-7199-211-8.

[36] WOZNEY, J. M. et al. Novel regulators of bone formation: molecular clones and activities. **Science,** v. 242, n. 4885, p. 1528-34, Dec 1988. ISSN 0036-8075. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/3201241 >.

[37] OSHIRO, T. et al. Osteoclast induction in periodontal tissue during experimental movement of incisors in osteoprotegerin-deficient mice. **Anat Rec,** v. 266, n. 4, p. 218-25, Apr 1 2002. ISSN 0003-276X (Print)

0003-276X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11920384 >.

[38] TEN DIJKE, P. et al. Osteocyte-derived sclerostin inhibits bone formation: its role in bone morphogenetic protein and Wnt signaling. **J Bone Joint Surg Am,** v. 90 Suppl 1, p. 31-5, Feb 2008. ISSN 1535-1386. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18292354 >.

[39] BELLIDO, T. et al. Chronic elevation of parathyroid hormone in mice reduces expression of sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis. **Endocrinology,** v. 146, n. 11, p. 4577-83, Nov 2005. ISSN 0013-7227. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16081646 >.

[40] TETI, A.; ZALLONE, A. Do osteocytes contribute to bone mineral homeostasis? Osteocytic osteolysis revisited. **Bone,** v. 44, n. 1, p. 11-6, Jan 2009. ISSN 1873-2763. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18977320 >.

[41] TAZAWA, K. et al. Osteocytic osteolysis observed in rats to which parathyroid hormone was continuously administered. **J Bone Miner Metab,** v. 22, n. 6, p. 524-9, 2004. ISSN 0914-8779. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15490261 >.

[42] ROODMAN, G. D. Cell biology of the osteoclast. **Exp Hematol,** v. 27, n. 8, p. 1229-41, Aug 1999. ISSN 0301-472X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10428500 >.

[43] DE SOUZA FALONI, A. P. et al. Jaw and long bone marrows have a different osteoclastogenic potential. **Calcif Tissue Int,** v. 88, n. 1, p. 63-74, Jan 2011. ISSN 1432-0827 (Electronic)

0171-967X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20862464 >.

[44] DE VRIES, T. J. et al. Myeloid blasts are the mouse bone marrow cells prone to differentiate into osteoclasts. **J Leukoc Biol,** v. 85, n. 6, p. 919-27, Jun 2009. ISSN 1938-3673 (Electronic)

0741-5400 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19304896 >.

[45] SIMONET, W. S. et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. **Cell,** v. 89, n. 2, p. 309-19, Apr 1997. ISSN 0092-8674. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9108485 >.

[46] SCHOPPET, M.; PREISSNER, K. T.; HOFBAUER, L. C. RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. **Arterioscler Thromb Vasc Biol,** v. 22, n. 4, p. 549-53, Apr 1 2002. ISSN 1524-4636 (Electronic)

1079-5642 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11950689 >.

[47] ANDERSON, D. M. et al. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. **Nature,** v. 390, n. 6656, p. 175-9, Nov 13 1997. ISSN 0028-0836 (Print)

0028-0836 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9367155 >.

[48] SIMONET, W. S. et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. **Cell,** v. 89, n. 2, p. 309-19, Apr 18 1997. ISSN 0092-8674 (Print)

0092-8674 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9108485 >.

[49] BOYLE, W. J.; SIMONET, W. S.; LACEY, D. L. Osteoclast differentiation and activation. **Nature,** v. 423, n. 6937, p. 337-42, May 2003. ISSN 0028-0836. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12748652 >.

[50] HAKEDA, Y. et al. Osteoclastogenesis inhibitory factor (OCIF) directly inhibits bone-resorbing activity of isolated mature osteoclasts. **Biochem Biophys Res Commun,** v. 251, n. 3, p. 796-801, Oct 1998. ISSN 0006-291X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9790989 >.

[51] CROCKETT, J. C. et al. New knowledge on critical osteoclast formation and activation pathways from study of rare genetic diseases of osteoclasts: focus on the RANK/RANKL axis. **Osteoporos Int,** v. 22, n. 1, p. 1-20, Jan 2011. ISSN 1433-2965. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20458572 >.

[52] VAANANEN, H. K. et al. The cell biology of osteoclast function. **J Cell Sci,** v. 113 (Pt 3), p. 377-81, Feb 2000. ISSN 0021-9533 (Print)

0021-9533 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10639325 >.

[53] VAANANEN, H. K.; HORTON, M. The osteoclast clear zone is a specialized cell-extracellular matrix adhesion structure. **J Cell Sci,** v. 108 (Pt 8), p. 2729-32, Aug 1995. ISSN 0021-9533 (Print)

0021-9533 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/7593313 >.

[54] BLAIR, H. C. et al. Osteoclastic bone resorption by a polarized vacuolar proton pump. **Science,** v. 245, n. 4920, p. 855-7, Aug 1989. ISSN 0036-8075. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/2528207 >.

[55] LEE, B. S.; GLUCK, S. L.; HOLLIDAY, L. S. Interaction between vacuolar H(+)-ATPase and microfilaments during osteoclast activation. **J Biol Chem,** v. 274, n. 41, p. 29164-71, Oct 1999. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10506172 >.

[56] HOLLIDAY, L. S. et al. Osteoclast polarization and orthodontic tooth movement. **Orthod Craniofac Res,** v. 12, n. 2, p. 105-12, May 2009. ISSN 1601-6343. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19419453 >.

[57] NESBITT, S. A.; HORTON, M. A. Trafficking of matrix collagens through bone-resorbing osteoclasts. **Science,** v. 276, n. 5310, p. 266-9, Apr 1997. ISSN 0036-8075. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9092478 >.

[58] PROFFIT. **Ortodontia Contemporânea**. 2a. Rio de Janeiro: Guanabara Koogan, 1995. 596

[59] BISHARA. **Ortodontia**. São Paulo: Livraria Santos LTDA, 2004. 593 ISBN 85-7288-345-2.

[60] KITAURA, H. et al. Effect of cytokines on osteoclast formation and bone resorption during mechanical force loading of the periodontal membrane. **ScientificWorldJournal,** v. 2014, p. 617032, 2014. ISSN 1537-744X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24574904 >.

[61] HENNEMAN, S.; VON DEN HOFF, J. W.; MALTHA, J. C. Mechanobiology of tooth movement. **Eur J Orthod,** v. 30, n. 3, p. 299-306, Jun 2008. ISSN 1460-2210 (Electronic)

0141-5387 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18540017 >.

[62] GARLET, T. P. et al. Cytokine expression pattern in compression and tension sides of the periodontal ligament during orthodontic tooth movement in humans. **Eur J Oral Sci,** v. 115, n. 5, p. 355-62, Oct 2007. ISSN 0909-8836. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17850423 >.

[63] NOMURA, S.; TAKANO-YAMAMOTO, T. Molecular events caused by mechanical stress in bone. **Matrix Biol,** v. 19, n. 2, p. 91-6, May 2000. ISSN 0945-053X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10842092 >.

[64] SHIOTANI, A.; SHIBASAKI, Y.; SASAKI, T. Localization of receptor activator of NFkappaB ligand, RANKL, in periodontal tissues during experimental movement of rat molars. **J Electron Microsc (Tokyo),** v. 50, n. 4, p. 365-9, 2001. ISSN 0022-0744. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11592682 >.

[65] KAWASAKI, K. et al. Effects of aging on RANKL and OPG levels in gingival crevicular fluid during orthodontic tooth movement. **Orthod Craniofac Res,** v. 9, n. 3, p. 137-42, Aug 2006. ISSN 1601-6335. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16918678 >.

[66] REN, Y.; KUIJPERS-JAGTMAN, A. M.; MALTHA, J. C. Immunohistochemical evaluation of osteoclast recruitment during experimental tooth movement in young and adult rats. **Arch Oral Biol,** v. 50, n. 12, p. 1032-9, Dec 2005. ISSN 0003-9969 (Print)

0003-9969 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15963453 >.

[67] SHIELDS, T. E.; LITTLE, R. M.; CHAPKO, M. K. Stability and relapse of mandibular anterior alignment: a cephalometric appraisal of first-premolar-extraction cases treated by traditional edgewise orthodontics. **Am J Orthod,** v. 87, n. 1, p. 27-38, Jan 1985. ISSN 0002-9416. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/3855348 >.

[68] NANDA, R. S.; NANDA, S. K. Considerations of dentofacial growth in long-term retention and stability: is active retention needed? **Am J Orthod Dentofacial Orthop,** v. 101, n. 4, p. 297-302, Apr 1992. ISSN 0889-5406. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1558058 >.

[69] BISHARA, S. E.; CHADHA, J. M.; POTTER, R. B. Stability of intercanine width, overbite, and overjet correction. **Am J Orthod,** v. 63, n. 6, p. 588-95, Jun 1973. ISSN 0002-9416. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/4513447 >.

[70] WOODSIDE, D. G.; ROSSOUW, P. E.; SHEARER, D. Postretention mandibular incisor stability after premolar serial extractions. **Semin Orthod,** v. 5, n. 3, p. 181-90, Sep 1999. ISSN 1073-8746. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10860070 >.

[71] FRANZEN, T. J.; BRUDVIK, P.; VANDEVSKA-RADUNOVIC, V. Periodontal tissue reaction during orthodontic relapse in rat molars. **Eur J Orthod,** v. 35, n. 2, p. 152-9, Apr 2013. ISSN 1460-2210 (Electronic)

0141-5387 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22023883 >.

[72] FRANZEN, T. J. et al. The influence of low-level laser on orthodontic relapse in rats. **Eur J Orthod**, Oct 6 2014. ISSN 1460-2210 (Electronic)

0141-5387 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25287057 >.

[73] YOSHIDA, Y. et al. Cellular roles in relapse processes of experimentally-moved rat molars. **J Electron Microsc (Tokyo),** v. 48, n. 2, p. 147-57, 1999. ISSN 0022-0744 (Print)

0022-0744 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10356787 >.

[74] LINDY, O. et al. Statin use is associated with fewer periodontal lesions: A retrospective study. **BMC Oral Health,** v. 8, p. 16, 2008. ISSN 1472-6831. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18482446 >.

75 GOES, P. et al. Effect of Atorvastatin in radiographic density on alveolar bone loss in wistar rats. **Braz Dent J,** v. 21, n. 3, p. 193-8, 2010. ISSN 1806-4760 (Electronic)

0103-6440 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21203699 >.

76 JIN, J. et al. Simvastatin inhibits LPS-induced alveolar bone loss during metabolic syndrome. **J Dent Res,** v. 93, n. 3, p. 294-9, Mar 2014. ISSN 1544-0591. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24352501 >.

77 ROSENDO, A. B. et al. [Pharmacogenetics and anti-inflammatory effect of HMG-CoA reductase inhibitors]. **Arq Bras Endocrinol Metabol,** v. 51, n. 4, p. 520-5, Jun 2007. ISSN 0004-2730. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17684611 >.

78 YILMAZ, A. et al. Differential effects of statins on relevant functions of human monocyte-derived dendritic cells. **J Leukoc Biol,** v. 79, n. 3, p. 529-38, Mar 2006. ISSN 0741-5400. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16387846 >.

79 RATTAZZI, M. et al. Atorvastatin reduces circulating osteoprogenitor cells and T-cell RANKL expression in osteoporotic women: implications for the bone-vascular axis. **Cardiovasc Ther**, Oct 2015. ISSN 1755-5922. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26506085 >.

80 PAN, J. et al. Cyclic strain-induced cytoskeletal rearrangement of human periodontal ligament cells via the Rho signaling pathway. **PLoS One,** v. 9, n. 3, p. e91580, 2014. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24619024 >.

81 KIM, J. Y. et al. Atorvastatin inhibits osteoclastogenesis by decreasing the expression of RANKL in the synoviocytes of rheumatoid arthritis. **Arthritis Res Ther,** v. 14, n. 4, p. R187, 2012. ISSN 1478-6354 (Print)

1478-6354 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22901757 >.

82 In: (Ed.). **Guide for the Care and Use of Laboratory Animals**. Washington (DC), 1996. ISBN 0309053773.

83 VERNA, C.; DALSTRA, M.; MELSEN, B. The rate and the type of orthodontic tooth movement is influenced by bone turnover in a rat model. **Eur J Orthod,** v. 22, n. 4, p. 343-52, Aug 2000. ISSN 0141-5387 (Print)

0141-5387 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11029824 >.

84 SHIRAZI, M. et al. Pantoprazole, a proton pump inhibitor, increases orthodontic tooth movement in rats. **Iran J Basic Med Sci,** v. 17, n. 6, p. 448-53, Jun 2014. ISSN 2008-3866 (Print)

2008-3866 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25140207 >.

85 ELEWA, H. F. et al. Early atorvastatin reduces hemorrhage after acute cerebral ischemia in diabetic rats. **J Pharmacol Exp Ther,** v. 330, n. 2, p. 532-40, Aug 2009. ISSN 1521-0103. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19478137 >.

86 PARFITT, A. M. et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. **J Bone Miner Res,** v. 2, n. 6, p. 595-610, Dec 1987. ISSN 0884-0431. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/3455637 >.

87 EGAN, K. P.; BRENNAN, T. A.; PIGNOLO, R. J. Bone histomorphometry using free and commonly available software. **Histopathology,** v. 61, n. 6, p. 1168-73, Dec 2012. ISSN 1365-2559. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22882309 >.

88 EL KHASSAWNA, T. et al. Effects of multi-deficiencies-diet on bone parameters of peripheral bone in ovariectomized mature rat. **PLoS One,** v. 8, n. 8, p. e71665, 2013. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23977109 >.

89 AYBAR ODSTRCIL, A. C. et al. Effect of arsenic in endochondral ossification of experimental animals. **Exp Toxicol Pathol,** v. 62, n. 3, p. 243-9, May 2010. ISSN 1618-1433. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19447590 >.

90 FAN, C. M. et al. Prevention of bone growth defects, increased bone resorption and marrow adiposity with folinic acid in rats receiving long-term methotrexate. **PLoS One,** v. 7, n. 10, p. e46915, 2012. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23071661 >.

91 RUIFROK, A. C.; JOHNSTON, D. A. Quantification of histochemical staining by color deconvolution. **Anal Quant Cytol Histol,** v. 23, n. 4, p. 291-9, Aug 2001. ISSN 0884-6812. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11531144 >.

92 LIU, S. et al. Effect of simvastatin on the osteogenetic behavior of alveolar osteoblasts and periodontal ligament cells. **Hum Cell,** v. 25, n. 2, p. 29-35, Jun 2012. ISSN 1749-0774. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22399266 >.

93 STEIN, S. H. et al. Statins regulate interleukin-1β-induced RANKL and osteoprotegerin production by human gingival fibroblasts. **J Periodontal Res,** v. 46, n. 4, p. 483-90, Aug 2011. ISSN 1600-0765. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21463327 >.

[94] KANG, J. H. et al. Osteoprotegerin expressed by osteoclasts: an autoregulator of osteoclastogenesis. **J Dent Res,** v. 93, n. 11, p. 1116-23, Nov 2014. ISSN 1544-0591. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25256714 >.

[95] REITAN, K. Clinical and histologic observations on tooth movement during and after orthodontic treatment. **Am J Orthod,** v. 53, n. 10, p. 721-45, Oct 1967. ISSN 0002-9416 (Print)

0002-9416 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/5233926 >.

[96] YOUNG, L. et al. Fiberotomy enhances orthodontic tooth movement and diminishes relapse in a rat model. **Orthod Craniofac Res,** v. 16, n. 3, p. 161-8, Aug 2013. ISSN 1601-6343 (Electronic)

1601-6335 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23323644 >.

[97] KIM, H. J. et al. Trends in the prevalence of major cardiovascular disease risk factors among Korean adults: results from the Korea National Health and Nutrition Examination Survey, 1998-2012. **Int J Cardiol,** v. 174, n. 1, p. 64-72, Jun 2014. ISSN 1874-1754. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24742812 >.

[98] LAROSA, J. C. et al. The cholesterol facts. A summary of the evidence relating dietary fats, serum cholesterol, and coronary heart disease. A joint statement by the American Heart Association and the National Heart, Lung, and Blood Institute. The Task Force on Cholesterol Issues, American Heart Association. **Circulation,** v. 81, n. 5, p. 1721-33, May 1990. ISSN 0009-7322. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/2184951 >.

[99] TAKAHASHI, N. et al. Postmitotic osteoclast precursors are mononuclear cells which express macrophage-associated phenotypes. **Dev Biol,** v. 163, n. 1, p. 212-21, May 1994. ISSN 0012-1606. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8174777 >.

[100] BLACK, A. E. et al. Metabolism and excretion of atorvastatin in rats and dogs. **Drug Metab Dispos,** v. 27, n. 8, p. 916-23, Aug 1999. ISSN 0090-9556. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10421619 >.

[101] LINS, R. L. et al. Pharmacokinetics of atorvastatin and its metabolites after single and multiple dosing in hypercholesterolaemic haemodialysis patients. **Nephrol Dial Transplant,** v. 18, n. 5, p. 967-76, May 2003. ISSN 0931-0509. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12686673 >.

[102] ANDREOLLO, N. A. et al. Rat's age versus human's age: what is the relationship? **Arq Bras Cir Dig,** v. 25, n. 1, p. 49-51, 2012 Jan-Mar 2012. ISSN 2317-6326. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22569979 >.

103 REAMY, B. **Hyperlipidemia Management for Primary Care: An Evidence-Based Approach**. Springer, 2008. 227

104 KIRSTEIN, B.; CHAMBERS, T. J.; FULLER, K. Secretion of tartrate-resistant acid phosphatase by osteoclasts correlates with resorptive behavior. **J Cell Biochem,** v. 98, n. 5, p. 1085-94, Aug 2006. ISSN 0730-2312. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16475168 >.

105 HALLEEN, J. M. et al. Intracellular fragmentation of bone resorption products by reactive oxygen species generated by osteoclastic tartrate-resistant acid phosphatase. **J Biol Chem,** v. 274, n. 33, p. 22907-10, Aug 1999. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10438453 >.

106 XIE, R.; KUIJPERS-JAGTMAN, A. M.; MALTHA, J. C. Osteoclast differentiation during experimental tooth movement by a short-term force application: an immunohistochemical study in rats. **Acta Odontol Scand,** v. 66, n. 5, p. 314-20, Oct 2008. ISSN 1502-3850. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18720051 >.

107 CHIM, S. M. et al. Angiogenic factors in bone local environment. **Cytokine Growth Factor Rev,** v. 24, n. 3, p. 297-310, Jun 2013. ISSN 1879-0305. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23611723 >.

108 DOHERTY, M. J. et al. Vascular pericytes express osteogenic potential in vitro and in vivo. **J Bone Miner Res,** v. 13, n. 5, p. 828-38, May 1998. ISSN 0884-0431. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9610747 >.

109 DIAZ-FLORES, L. et al. Pericytes as a supplementary source of osteoblasts in periosteal osteogenesis. **Clin Orthop Relat Res**, n. 275, p. 280-6, Feb 1992. ISSN 0009-921X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1735226 >.

110 WALKER, K. V.; KEMBER, N. F. Cell kinetics of growth cartilage in the rat tibia. II. Measurements during ageing. **Cell Tissue Kinet,** v. 5, n. 5, p. 409-19, Sep 1972. ISSN 0008-8730. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/4639299 >.

7. Anexos

Materiais Suplementares

Anexo I – Aprovação do Comitê de Ética no Uso de Animais (CEUA).

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 23145
Título: EFEITOS DA ATORVASTATINA SOBRE A REMODELAÇÃO ÓSSEA APÓS MOVIMENTAÇÃO DENTÁRIA INDUZIDA EM RATOS

Pesquisadores:

Equipe UFRGS:

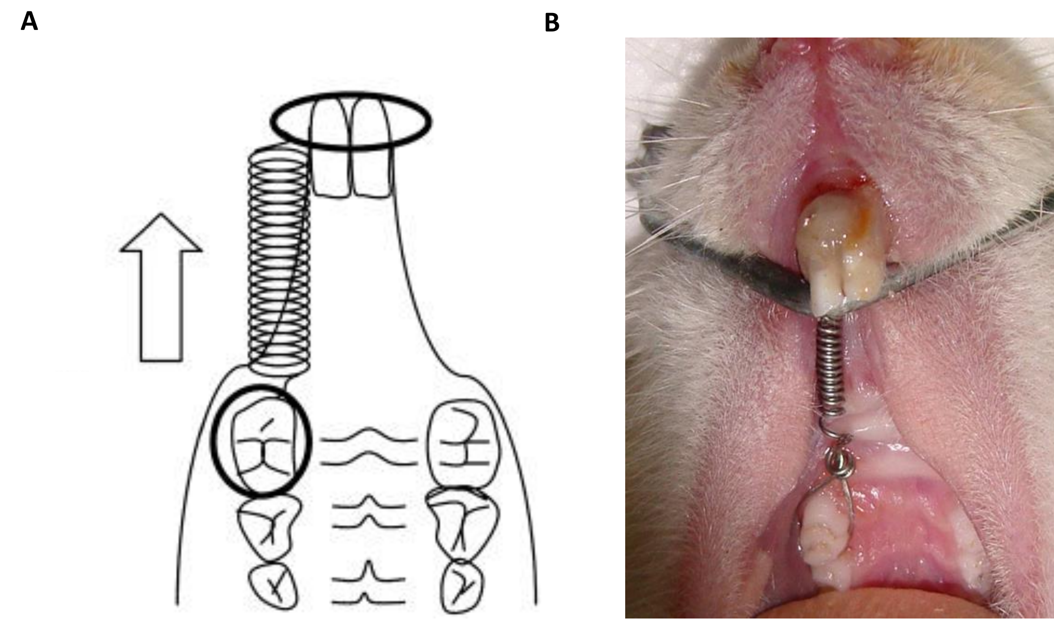
ANNA CHRISTINA MEDEIROS FOSSATI - coordenador desde 09/04/2012
DIOGO ONOFRE GOMES DE SOUZA - pesquisador desde 09/04/2012
Gabriel Schmidt Dolci - pesquisador desde 09/04/2012

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 18/03/2013 - Sala de Reuniões do 2° andar do Prédio da Reitoria - Campus Central., em seus aspectos éticos e metolodológicos, para a utilização de 54 ratos Wistar machos, de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Quinta-Feira, 28 de Março de 2013

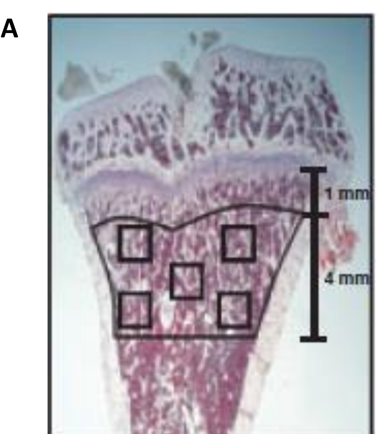
STELA MARIS KUZE RATES
Coordenador da comissão de ética

Anexo II – Método da boca dividida, usado para a movimentação do primeiro molar superior direito, em ratos Wistar. O lado contralateral (primeiro molar superior esquerdo) serviu como controle interno. (a) A força foi mensurada com um tensiomêtro, numa magnitude de 50cN. (b) Foto do dispositivo ortodôntico instalado.

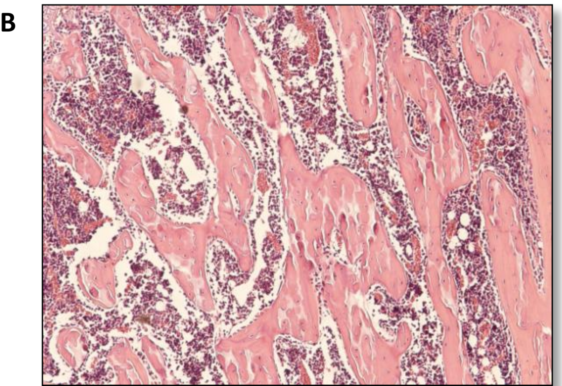


Anexo III – Método usado para a análise do volume ósseo, em secções de H&E de fêmures. (a) Foram selecionadas cinco regiões, na metáfise dos ossos longos, conforme método descrito por Ho et al[18]. Magnificação de 4x. (b) Nestas regiões, procedeu-se a avaliação do volume ósseo (200x). (c) Representação da imagem mostrada em “b” digitalmente manipulada. Os pixels pretos representam o volume ósseo (BV), o qual era dividido pelo volume total da imagem (TV). Para cada fêmur, considerava-se o valor total das cinco medidas realizadas. Após, era calculada a média por grupo.

A



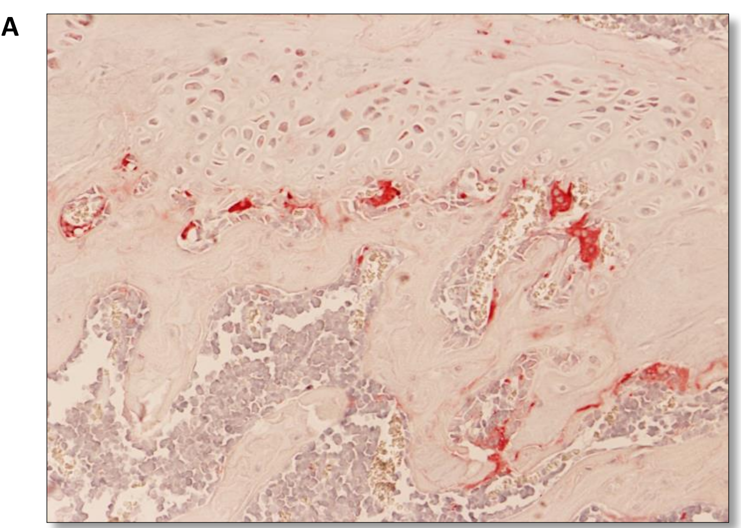
B



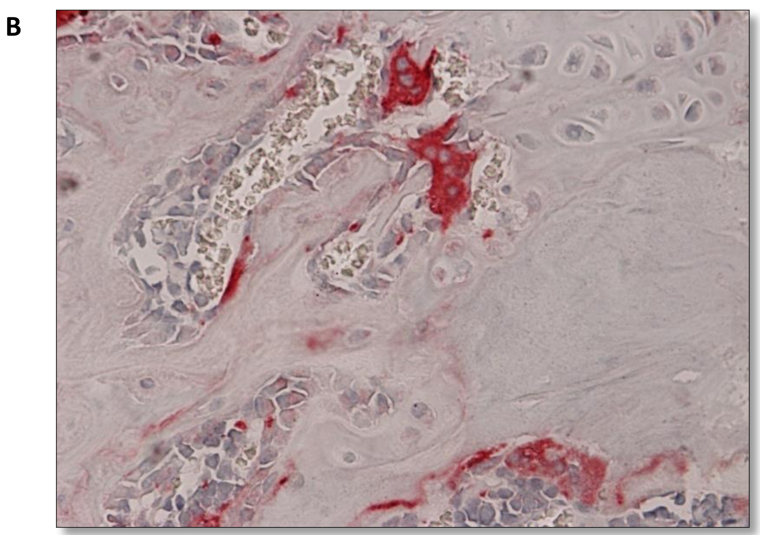
C

Anexo IV – (a) As células TRAP positivas foram contadas em 10 regiões subcondrais distintas, aleatóriamente selecionadas, em fêmures (magnificação de 200x). (b) Detalhe da coloração TRAP positiva (magnificação 400x), indicando a presença de osteoclastos.

A



B



Anexo V – (a) A ossificação endocondral foi avaliada em 10 regiões subcondrais aleatoriamente selecionadas, representativas de toda extensão do disco epifisário (magnificação 100x). (b) Sob um aumento de 200x, foram realizadas as medições. (c) Detalhe da região de interesse (magnificação de 400x).