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PAULA GOES PINHEIRO DUTRA

EFEITO ANTIRREABSORTIVO DO ALENDRONATO E DA COMBINAÇÃO ENTRE ALENDRONATO E ATORVASTATINA NA PERIODONTITE INDUZIDA POR LIGADURA EM RATOS

FORTALEZA
2012

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Tese submetida à Coordenação do Programa de Pós-Graduação em Odontologia, da Universidade Federal do Ceará, como requisito parcial para a obtenção do grau de Doutora em Odontologia.

Área de Concentração: Clínica Odontológica.

Orientadora: Profª Drª Vilma de Lima

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Aprovada em 19/01/2012

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À minha avó Maria Elisa Goes (in memoriam)

"Eu tenho tanto pra lhe falar
Mas com palavras não sei dizer
Como é grande o meu amor por você..."

(Roberto Carlos)

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RESUMO

A doença periodontal é uma desordem infecto-inflamatória, e fármacos têm sido estudados como moduladores deste processo inflamatório. Neste contexto, esta tese, constituída por 3 artigos, teve por objetivo: (1) Realizar uma revisão sobre o efeito de Bisfosfonatos (BFs) na doença periodontal; (2) Investigar o efeito do Alendronato (ALD) nos níveis de Fosfatase Alcalina Óssea (FAO) na perda óssea alveolar (POA) em ratos; (3) Avaliar o efeito da combinação entre ALD e Atorvastatina (ATV) na POA em ratos. No estudo 1 buscou-se, em bases de dados, utilizando as palavras chave: "*Bisphosphonates*" e "*Periodontitis*", estudos pré-clínicos e clínicos, publicados em língua Inglesa ou Portuguesa, nos últimos 10 anos. No estudo 2, 36 ratos *Wistar* machos, submetidos à periodontite induzida por ligadura, receberam solução Salina (SAL) 0,9% ou ALD nas doses de 0,01; 0,05; 0,25 mg/kg-s.c, 30 min antes da colocação do fio e diariamente por 11 dias. Avaliou-se: POA (morfometria e histologia); níveis séricos de FAO, transaminases e Fosfatase Alcalina Total (FAT); Leucograma e Peso. No estudo 3, 78 ratos *Wistar* machos, submetidos à periodontite induzida por ligadura, receberam de forma profilática (P): SAL ou ALD (0,01; 0,25 mg/kg-s.c) ou ATV (0,3; 27 mg/kg-v.o.) ou a combinação ALD+ATV (0,25+27; 0,01+0,3; 0,25+0,3; 0,01+27 mg/kg), 30 min antes da ligadura e diariamente por 11 dias; ou ainda a combinação ALD+ATV (0,01+0,3 mg/kg) na forma terapêutica (T), ou seja administrada a partir do 5º dia após ligadura, até o sacrifício. Avaliou-se: POA [morfometria, histologia, histometria; imunohistoquímica para fosfatase ácido tártaro resistente (TRAP); mieloperoxidase (MPO); FAO, transaminases; Leucograma e Peso]. O artigo 1 mostrou que BFs apresentaram efeitos antirreabsortivo e anti-inflamatório, reduziram FAO e Telopeptídeo N-terminal de colágeno tipo I (NTx) e melhoraram os parâmetros clínicos periodontais. No artigo 2, o ALD (0,25 mg/kg) preveniu a redução de FAO e POA, não alterou níveis de transaminases, mas não preveniu redução dos níveis de FAT ($p<0,05$), preveniu neutrofilia e linfomonocitose ($p<0,05$), sem causar perda de peso importante. No 3º estudo, os tratamentos isolados, em altas doses, e todas as combinações avaliadas controlaram POA ($p<0,05$). A combinação de ALD+ATV em baixas doses controlou POA (P [38,96%] ou T [53,53%]). As análises histológicas, histométricas ($p<0,05$) e imunohistoquímicas corroboraram os achados macroscópicos. A combinação de ALD+ATV em baixas doses reduziu a atividade de MPO, preveniu redução de FAO, reduziu neutrofilia e linfomonocitose ($p<0,05$), sem alterar os níveis de transaminases e causar perda de peso. Desta forma conclui-se que os BFs apresentaram efeitos antirreabsortivo e anti-inflamatório, reduziram níveis de marcadores bioquímicos do metabolismo ósseo e melhoraram os parâmetros clínicos periodontais. O ALD, administrado isoladamente, preveniu redução de FAO, POA, sem repercussões sistêmicas e a combinação de ALD+ATV, em baixas doses, reduziu POA e inflamação periodontal, também sem causar alterações sistêmicas importantes.

Palavras-chave: Alendronato. Atorvastatina. Periodontite. Inflamação. Osso.

ABSTRACT

Periodontal disease is an infectious-inflammatory disease, and drugs have been studied as modulators of this inflammatory process. In this context, this thesis, constituted by 3 articles had by objective: (1) Perform a review about the effect of Bisphosphonates (BPs) on periodontal disease; (2) Investigate the effect of Alendronate (ALD) on Bone-specfic Alkaline Phosphatase (BALP) on alveolar bone loss (ABL) in rats; (3) Evaluate the effect of ALD and Atorvastatin (ATV) combination on ABL in rats. On study 1, we sought in data basis, using the keywords "*Bisphosphonates*" and "*Periodontitis*", pre-clinical and clinical studies, published in English and Portuguese, in the last 10 years. On study 2, 36 *Wistar* male rats, submitted to ligature-induced periodontitis, received 0.9% Saline (SAL) or ALD on the doses of 0.01; 0.05; 0.25 mg/kg-s.c., 30 min before ligature placement and daily during 11 days. It was evaluated: ABL (morphometry and histology) serum levels of Bone-specific Alkaline Phosphatase (BALP), transaminases, and Total Alkaline Phosphatase (TAP); and leukogram and corporal mass. On study 3, 78 *Wistar* male rats, submitted to ligature-induced periodontitis, received prophylactically (P): SAL or ALD (0.01; 0.25 mg/kg-s.c) or ATV (0.3; 27 mg/kg-v.o.) or the combination ALD+ATV (0.25+27; 0.01+0.3; 0.25+0.3; 0.01+27 mg/kg), 30 min before ligature and daily for 11 days; or the combination ALD+ATV (0.01+0.3 mg/kg) administered therapeutically (T), from the 5th day after ligature until the sacrifice. It was evaluated: ABL [morphometry, histology, histometry; immunohistochemistry for tartrate resistant acid phosphatase (TRAP); myeloperoxidase (MPO); BALP, transaminases; Leukogram and corporal mass]. The study 1 showed that BPs presented anti-resorptive and anti-inflammatory effects, reduced FAO and Telopeptide N-terminal of type I collagen (NTx) and improved periodontal clinical parameters. On article 2, ALD (0.25 mg/kg) prevented BALP and ABL reduction, and did not alter transaminases serum levels, but reduced TAP serum levels ($p<0.05$), it reduced neutrophilia and lymphomonocytosis ($p<0.05$), without causing important loss of weight. On the 3rd study, the isolated treatments in high doses, and all combinations controlled ABL ($p<0.05$). Low doses combination of ALD+ATV controlled ABL (P [38.96%] or T [53.53%]). The histological, histometric ($p<0.05$) and immunohistochemical analysis corroborated macroscopical findings. The low dose combination of ALD+ATV reduced MPO activity, prevented BALP reduction, reduced neutrophilia and lymphomonocytosis ($p<0.05$), without altering transaminases serum levels and without causing loss of weight. In this way, we can conclude that BPs presented anti-resorptive and anti-inflammatory effects reduced levels of biochemical markers of bone metabolism and improved periodontal parameters. ALD, administered isolated prevented BALP and ABL reduction, without causing systemic problems, and the combination of ALD+ATV, in low doses, reduced ABL and periodontal inflammation, without causing important systemic alterations as well.

Key words: Alendronate. Atorvastatin. Periodontitis. Inflammation. Bone.

LISTA DE ABREVIATURAS

3-hidroxi-3-metilglutaril co-enzima A	HMG-CoA
Alendronato	ALD
Atorvastatina	ATV
Bisfosfonatos	BF
Farnesil difosfato sintase	FPPS
Fator de ativação plaquetária	PAF
Fator de crescimento endotelial vascular	VEGF
Fator de Necrose Tumoral	TNF
Fosfatase ácida tártaro resistente	TRAP
Hidroxiapatita	HA
Interferon	IFN
Interleucina	IL
Leucotrieno	LT
Ligante do Receptor Ativador do Fator de Transcrição NF-κB	RANKL
Lipopolissacarídeo	LPS
Metaloproteinase de matriz	MMP
Mieloperoxidase	MPO
Moléculas de adesão intercelular	ICAM
Moléculas de adesão intercelular vascular	VCAM
Osteoclasto	OTC
Osteoprotegerina	OPG
Pequenas proteínas ligantes de Guanosina Tri-fosfato	GTPases
Prostaglandina	PG
Prostaglandina	PG
Proteína morfogenética óssea	BMP
Receptor Ativador do Fator de Transcrição NF-κB	RANK

SUMÁRIO

1 INTRODUÇÃO GERAL

As periodontites são desordens infecto-inflamatórias dos tecidos de suporte dental, comuns na cavidade oral, e que apresentam etiologia multifatorial. Embora a colonização periodontal por periodontopatógenos GRAM-negativo seja importante para o seu estabelecimento, a susceptibilidade do hospedeiro é determinante para o início e progressão da doença, pois a hiperresponsividade de vias imunológicas pode resultar em destruição tecidual aumentada (PIHLSTROM *et al.,* 2005).

Entre os tecidos de suporte afetados durante a progressão da periodontite, destaca-se o osso alveolar. Este tecido, que participa da sustentação e função dos dentes, caracteristicamente encontra-se em constante processo de remodelação, processo este mediado principalmente pelo eixo RANK-RANKL-OPG, ou seja, do Receptor Ativador do Fator de Transcrição NF-κB (RANK), seu Ligante (RANKL) e Osteoprotegerina (OPG) (XING *et al*., 2005; REID; HOLEN, 2009).

Na doença periodontal, com a presença de periodontopatógenos e consequentemente maior liberação de lipopolissacarídeos (LPS), há o recrutamento de neutrófilos para o sítio de infecção, os quais liberam enzimas proteolíticas que causam dano tecidual. LPS também age ativando macrófagos, que promovem a liberação de vários mediadores pró-inflamatórios, tais como interleucinas (IL)-1β, fator de necrose tumoral (TNF)-α, prostaglandina (PG) E_2, importantes na destruição de tecido periodontal (KINNEY *et al.,* 2007; GIANNOBILLE, 2008). Além da destruição de tecido conjuntivo, o alto nível destes mediadores estimula a expressão abundante de RANKL, seja ligado à superfície celular (LERNER, 2006; BOYLE *et al.,* 2003) ou clivado em forma solúvel (YAVROPOULOU; YOVOS, 2008; MIZUNO *et al.,* 2002; NAKASHIMA *et al.,* 2000) em vários tipos celulares, tais como fibroblastos e linfócitos T e B (LERNER, 2006), além dos osteoblastos. Concomitantemente, tais mediadores também induzem inibição de OPG, provocando, assim, maior desequilíbrio a favor de reabsorção óssea, devido à ativação osteoclástica (REID; HOLEN, 2009).

Após a ativação e multinucleação, os osteoclastos (OTCs) seguem para os sítios de reabsorção onde se aderem à matriz óssea, via integrina avβ3, para iniciar o ciclo reabsortivo. Primeiramente os OTCs organizam seu citoesqueleto (LAKKAKORPI *et al*., 1989; 1991) e em seguida, sofrem polarização da membrana

plasmática, com a formação de 2 sítios: a borda enrugada e o domínio secretório (YAVROPOULOU; YOVOS, 2008; VÄÄNÄNEN, 2005), sendo este último responsável pela remoção de fragmentos de cálcio, fosfato e colágeno (SALO *et al*., 1996). Já a borda enrugada, também chamada de organela reabsortiva, apresenta grande número de vesículas ácidas intracelulares que são lançadas na interface OTC/matriz óssea (PALOKANGAS *et al.,* 1997), para a dissolução de cristais de hidroxiapatita (HA) (BLAIR *et al*., 1989; TUUKKANEN; VÄÄNÄNEN, 1986; VÄÄNÄNEN *et al.,* 1990; BLAIR; ZAIDI*,* 2006).

Existe ainda nos OTCs um grupo de pequenas proteínas ligantes de GTP (GTPases), importantes para a formação da organela reabsortiva (ZHAO *et al*., 2001) e organização do tráfego vesicular intracelular. Além disso, em OTCs ativados, observa-se a presença de fosfatase ácida tártaro resistente (TRAP), um marcador de atividade osteoclástica (KAUNITZ; YAMAGUCHI, 2008).

Portanto, com base nos conhecimentos sobre patogênese da reabsorção óssea inflamatória, o tratamento periodontal clássico, que durante muito tempo objetivou apenas o controle da placa bacteriana (BOEHM; SCANNAPIECO, 2007), passa por uma mudança de paradigma (BUDUNELI *et al.,* 2007) uma vez que outras estratégias terapêuticas, tais como a modulação farmacológica da resposta do hospedeiro, vem se sobressaindo como uma nova abordagem de tratamento associada à raspagem e alisamento radicular não-cirúrgicos (BUDUNELI *et al*., 2007; PRESHAW *et al*., 2004).

Um dos moduladores da resposta do hospedeiro que agem sobre o tecido ósseo são os Bisfosfonatos (BFs). BFs são análogos estáveis dos pirofosfatos, por apresentar 2 átomos de fósforo que dividem o mesmo átomo de carbono. Os BFs são potentes inibidores da reabsorção óssea e por isso têm sido usados como agentes terapêuticos efetivos no tratamento da osteoporose (PANICO *et al.*, 2011), doença de Paget (KUSAMORI *et al.,* 2010), hipercalcemia neoplásica, mieloma múltiplo, e metástase óssea secundária a câncer de mama e de próstata (MILLER *et al.,* 2011).

A estabilidade química do BFs e sua resistência à hidrólise ácida ou enzimática são conferidas pelo átomo de carbono (RUSSELL *et al.,* 2008). Os radicais R1 e R2, aderidos lateralmente ao átomo de carbono, são responsáveis por uma grande variedade de atividades observadas entre os BFs. Substituintes de R1, tais como grupamentos hidroxila, aumentam a quimioabsorção pelo mineral (VAN

BEEK *et al.,* 1999), enquanto que os substituintes de R2 relacionam-se a diferentes potências antirreabsortivas (VAN BEEK *et al.,* 1999). Tal potência antirreabsortiva está associada à habilidade do BFs em inibir a atividade bioquímica da enzima farnesil difosfato sintase (FPPS). Assim considerando a escala de potência antirreabsortiva, dentre a classe farmacológica dos BFs, destaca-se o Alendronato (ALD) como 2º fármaco mais potente no *ranking* de afinidade por HA, e primeira escolha no tratamento da osteoporose (NANCOLLAS *et al.,* 2006; EBETINO *et al*., 2011).

Os BFs apresentam papel modulador na função de OTCs (TENENBAUM *et al.,* 2002). A inibição da via do mevalonato, via inibição da FPPS (KIMMEL, 2007), induz redução da isoprenilação de GTPases, tais como Rab, Rac, Ras e Rho, levando à redução da atividade celular, alterando a morfologia celular, organização do citoesqueleto, tráfego vesicular (PAVLOS *et al.,* 2005) e formação da borda enrugada. Tais alterações refletem em redução da adesão de OTCs à matriz óssea bem como da profundidade do sítio de reabsorção, o que consequentemente reduz o *turnover* ósseo, confirmando assim o excelente efeito antirreabsortivo dos BFs (KIMMEL, 2007).

Em adição ao efeito antirreabsortivo dos BFs, diversos estudos tem mostrado uma atividade anti-inflamatória exercida por este fármaco, tais como, inibição de células apresentadoras de antígenos (TOUSSIROT, *et al*., 2007) e redução na atividade de mieloperoxidase (MPO) (CHEN *et al*., 1996). BFs também atuam inibindo alguns mediadores inflamatórios, por exemplo, prostaglandina (PG) (LIU *et al*., 2006), leucotrienos (LT) (CARVALHO *et al*., 2006), que estão relacionados à destruição de tecido conjuntivo (HIKIJI *et al*., 2008).

O uso de BFs também tem sido relacionado a importantes efeitos adversos. A osteonecrose de maxilares (OTM) tem sido observada após a administração intravenosa de BFs, especialmente o aminobisfosfonatos tais como zoledronato e pamidronato (NICOLATOU-GALITIS *et al*., 2011). Em alguns casos, a OTM também foi observada após administração oral de BFs, tais como ALD (DROZDZOWSKA, 2011). Outras reações adversas, menos frequentes, também têm sido relatadas tais como: alergias a fosfatos ou intolerância gastrointestinal, além de, ulcera esofágica e estomacal, reação de fase-aguda e dor muscular (PAPAPETROU, 2009).

Outra abordagem farmacológica, moduladora da resposta do hospedeiro utilizada nesse estudo, foram as Estatinas. Esta classe farmacológica atua sobre a via do mevalonato, inibindo a enzima 3-hidroxi-3-metilglutaril co-enzima A (HMG-CoA) redutase. As estatinas são redutoras efetivas dos níveis séricos de colesterol e, portanto, amplamente prescritas para o tratamento da hipercolesterolemia e aterosclerose (KIM *et al.*, 2011). Além dos efeitos sobre o colesterol, estudos têm mostrado que as Estatinas apresentam efeitos pleiotrópicos, incluindo ação anti-inflamatória (DIMITROW; JAWIEŃ, 2010) e efeito anabólico sobre o tecido ósseo (MUNDY *et al.*, 1999; HORIUCHI; MAEDA, 2006; GOES *et al., 2*010). Tais propriedades oferecem grande potencial para estatinas modificarem o curso de doenças inflamatórias crônicas (BARSANTE *et al.,* 2005), dentre as quais podem ser incluídas as periodontites crônicas.

A atividade anti-inflamatória das Estatinas baseia-se na inibição de moléculas de adesão intercelular (ICAM), vascular (VCAM), selectinas (NAWAWI *et al.*, 2003), IL-1, TNF (WAEHRE *et al*., 2004), IL-2, IL-12, e interferon (IFN)-γ. (JASIÑSKA *et al*., 2007). Em adição, Estatinas também promovem a expressão de estimuladores de diferenciação osteoblástica tais como proteína morfogenética óssea (BMP-2) e fator de crescimento endotelial vascular (VEGF) (MAEDA *et al*., 2003). Dentre as várias estatinas, destaca-se a Atorvastatina (ATV), não apenas pela sua característica lipofílica, a qual está intimamente ligada aos seus efeitos pleiotrópicos (IZUMO *et al.*, 2001), mas também aos seus poucos efeitos adversos e melhor relação custo-benefício (COSTA-SCHARPLATZ *et al.,* 2008), quando comparada a outras Estatinas (NEWMAN *et al*., 2008), sendo portanto amplamente usada na prática clínica (PLOSKER; LYSENG-WILLIAMSIN, 2007).

Alguns efeitos adversos devem ser considerados quando do uso de estatinas. Alteração sobre transaminases (alanina e asparato) tem sido frequentemente relacionado ao uso de estatinas. Geralmente tais marcadores de função hepática parecem estar aumentados nos 6 primeiros meses de terapia (KAPUR *et al*., 2008). Outros achados como a mialgia sem alteração em níveis de creatinina, podendo levar a rabdomiólise, bem como alterações renais também tem sido associado ao uso de estatinas (Sakaeda *et al.,* 2011). Porém é valido salientar que dentre esta classe farmacológica, a ATV tem apresentado tais efeitos adversos com menos freqüência (SAKAEDA *et al.,* 2011).

Neste contexto, sabendo que BFs e Estatinas apresentam propriedades antirreabsortivas e anabólicas ósseas, em separado, respectivamente, mas que ambos interferem na via do mevalonato, embora em níveis diferentes, parece-nos interessante, avaliar se a combinação desses fármacos (ALD+ATV) pode ter algum efeito benéfico adicional no metabolismo ósseo dos tecidos periodontais, como proposto por Russell (2011).

2 PROPOSIÇÃO

Os objetivos do presente trabalho foram:

1. Realizar uma revisão sobre o efeito dos Bisfosfonatos na doença periodontal
2. Avaliar o efeito do Alendronato sobre a Fosfatase Alcalina Óssea e perda óssea periodontal em ratos *Wistar* através de:
    a. Dosagem sérica de Fosfatase Alcalina Óssea
    b. Análises macroscópica e histológica da perda óssea alveolar
    c. Avaliação de parâmetros sistêmicos como: Dosagens séricas de Fosfatase Alcalina Total e Transaminases, Leucograma e Variação de massa corpórea
3. Avaliar o efeito da combinação de Alendronato e Atorvastatina administrado de forma profilática ou terapêutica na periodontite induzida por ligadura em ratos *Wistar*, através de:
    a. Análises macroscópica, microscópica e histométrica da perda óssea alveolar
    b. Análise imunohistoquímica para Fosfatase Ácida Tártaro Resistente (TRAP)
    c. Atividade de mieloperoxidase (MPO)
    d. Avaliação de parâmetros sistêmicos como: Dosagens séricas de Fosfatase Alcalina Óssea e Transaminases, Leucograma e Variação de massa corpórea

3 CAPÍTULOS

Esta tese está baseada no Artigo 46 do Regimento Interno do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará que regulamenta o formato alternativo para teses de Doutorado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato.

Por se tratar de pesquisa envolvendo animais os protocolos utilizados neste trabalho foram submetidos à apreciação e foram devidamente aprovados pelo Comitê de Ética Animal (Anexo A).

Dessa forma, a presente tese é composta por três artigos científicos redigidos de acordo com as revistas científicas escolhidas.

3.1 Capítulo 1

“Efeito dos Bisfosfonatos na Doença Periodontal – Revisão da literatura”.

Autores: Goes, P, Lima V.

Este artigo seguiu normas de publicação do periódico: ***Revista de Odontologia da UNESP*** (ISSN 0101-1774).

3.2 Capítulo 2

"Effect of Alendronate on Bone-specific alkaline phosphatase on periodontal bone loss in *Wistar* rats”.

Autores: Paula Goes, Ana Patrícia Souza de Lima, Nylane Maria Nunes Alencar, Gerly Anne Castro Brito, Vilma Lima.

Este artigo seguiu normas de publicação do periódico ***Archives of Oral Biology*** (ISSN 0003-9969).

3.3 Capítulo 3

“Effect of Alendronate and Atorvastatin combination on alveolar bone loss in rats”.

Autores: Paula Goes, Caio S Dutra, Iracema M Melo, Norma M. B. Benevides, Vilma Lima.

Este artigo seguiu normas de publicação do periódico ***Journal of Bone and Mineral Research*** (ISSN 1523-4681).

3.1 Capítulo 1

EFEITO DOS BISFOSFONATOS NA DOENÇA PERIODONTAL – REVISÃO DA LITERATURA

Resumo

Este trabalho objetivou avaliar o efeito dos Bisfosfonatos (BFs) na doença periodontal através de uma revisão da literatura. Para tanto, buscou-se artigos em diversas bases de dados computadorizadas, utilizando as palavras chave: "*Bisphosphonates"* e "*Periodontitis".* Foram selecionados ensaios pré-clínicos e clínicos, publicados em língua Inglesa ou Portuguesa, envolvendo o efeito de BFs, de uso sistêmico, na doença periodontal nos últimos 10 anos. Inicialmente, 144 referências foram encontradas, em seguida os títulos e resumo foram analisados por uma única investigadora. Referências de revisões sobre o assunto, relatos de caso ou avaliações sobre doença peri-implantar foram excluídas. Finalmente, 17 artigos completos foram selecionados, sendo 11 estudos pré-clínicos em animais, e 6 ensaios clínicos em humanos. Os estudos mostraram que, de forma geral, o tratamento com BFs preveniu a perda óssea alveolar ($p<0,05$), alterando o número e morfologia de osteoclastos; modulou a inflamação, reduzindo recrutamento de neutrófilos, a atividade de mieloperoxidase, mediadores inflamatórios, metaloproteinases de matriz, bem como índices de sangramento gengival. BFs também mostraram redução dos níveis séricos de Telopeptídeo N-terminal de colágeno tipo I e Fosfatase Alcalina Óssea, marcadores do metabolismo ósseo. Em suma, o tratamento com BFs preveniu a reabsorção óssea alveolar, modulou a inflamação, e reduziu o nível sérico de marcadores bioquímicos do metabolismo ósseo, com melhora dos parâmetros clínicos periodontais, sendo, portanto, uma importante ferramenta farmacológica a ser sugerida como adjuvante à raspagem e alisamento radicular não-cirúrgico

PALAVRAS-CHAVE: Bisfosfonato; doença periodontal; inflamação

Abstract

The aim of this work was to evaluate de effect of Bisphosphonates (BPs) on periodontal disease through a review of literature. For this, we searched articles in several computadorized database, using the keywords: *Bisphosphonates* and *Periodontal disease.* It was selected pre-clinical and clinical assays, published in English or Portuguese, involving the effect of BP, administered systemically, on periodontal disease, in the last 10 years. Initially, 144 references were found, following, titles and abstracts were analyzed by only one investigator. References about reviews, case report, or peri-implantitis were excluded. Finally, 17 complete articles were selected, being 11 pre-clinical studies *in vivo*, and 6 clinical trials. The studies showed that, in general, the treatment with BPs, prevented alveolar bone loss ($p<0.05$), altering the osteoclast number and morphology; it also modulated inflammation, reducing neutrophil recruitment, myeloperoxidase activity, inflammatory mediators, matrix metalloproteinases and gingival bleeding indexes. BPs also showed reduction on serum levels of N-terminal cross-linking telopeptide of type I collagen and Bone-specific alkaline phosphatase, biochemical markers of bone metabolism. In summary, the treatment with BPs prevented alveolar bone resorption, and reduced serum levels of biochemical markers of bone metabolism, along with improvement of periodontal clinical parameters, being therefore, an important pharmacological tool suggested as an adjuvant to basic periodontal therapy.

KEYWORDS: Bisphosphonates; periodontal disease; inflammation

1. Introdução

Periodontite é uma doença de etiologia multifatorial que apesar de iniciada por biofilme bacteriano, conta com a geração de resposta inflamatória incluindo a produção de citocinas, eicosanóides, entre outros mediadores[1,2], para destruição dos tecidos de suporte dental.

Assim, de acordo com o modelo atual de patogênese da doença periodontal[3,4], o maior componente de destruição de tecidos moles e duros associado com doença periodontal é resultado da ativação da resposta imunoinflamatória do hospedeiro frente ao desafio bacteriano[2]. A expressão de moléculas de adesão intercelular em células endoteliais, e a produção de mediadores inflamatórios por neutrófilos, monócitos, linfócitos e fibroblastos caracterizam essa resposta[2].

Sabendo que a mais importante consequência da concentração de mediadores inflamatórios no periodonto é a reabsorção óssea alveolar, torna-se interessante conhecer o eixo RANK-RANKL-OPG (Receptor Ativador do fator de transcrição nuclear NF-κB – Ligante do Receptor Ativador do fator de transcrição nuclear NF-κB – Osteoprotegerina), principal mecanismo regulador da homeostasia óssea. O RANKL, presente em várias células, se liga ao RANK e induz a diferenciação de precursores de osteoclastos (OTC) em células que degradam osso, enquanto que a OPG previne a ligação RANK-RANKL, por inibição competitiva. Entretanto, diante de um processo inflamatório, no caso a periodontite, o aumento na concentração de citocinas pró-inflamatórias pode afetar diretamente a perda óssea aumentando os níveis de RANKL e ativação de osteoclastos, além de inibir a atividade de OPG[1].

Desta forma, reguladores da atividade de osteoclastos, como os Bisfosfonatos (BF), têm se destacado como estratégias farmacológicas para modulação do metabolismo ósseo. Os BFs representam uma classe de compostos químicos estruturalmente relacionados ao pirofosfato[5,6]. O pirofosfato regula a mineralização por se ligar ao cálcio (Ca^{2+}) dos cristais de hidroxiapatita (HA) *in vitro*, mas não é estável *in vivo*, pois sofre rápida hidrólise das ligações P-O-P[7]. A realocação do átomo de oxigênio pelo átomo de carbono (P-C-P) resulta na formação de uma molécula de BF, um composto quimicamente estável e resistente à hidrólise enzimática. Dado a sua afinidade de se ligar a cristais de HA, prevenir seu crescimento e dissolução, e também devido a sua habilidade de inibir

recrutamento e ativação de osteoclastos, os BFs são amplamente usados no manejo de desordens ósseas metabólicas sistêmicas[8].

Na periodontite a administração de BFs pode ter aplicações em potencial. Estudos têm demonstrado que BF promovem redução da perda óssea[9,10] e melhora em parâmetros inflamatórios[11]. Portanto o objetivo deste trabalho foi realizar uma revisão da literatura para avaliar o efeito dos bisfosfonatos na doença periodontal.

2. Metodologia

2.1 Estudos

Para esta revisão foram incluídos estudos pré-clínicos realizados *in vivo* utilizando modelos animais de periodontite, e ensaios clínicos prospectivos, controlados ou não por placebo, recebendo intervenção com tratamento periodontal não-cirúrgico de raspagem e alisamento radicular (RAR), realizados em voluntários adultos (>18 anos) com diagnóstico de periodontite.

2.1.1. Tipos de Intervenções

Foram incluídos estudos utilizando BFs de uso sistêmico, administrados em quaisquer doses, via ou duração. Nos estudos, o uso de BF foi considerado como sendo a terapia primária ou adjunta à terapia periodontal não-cirúrgica.

2.1.2. Tipos de medidas de resultados

Os estudos pré-clínicos apresentaram análises morfométricas, histológica, radiográficos e/ou laboratoriais Os ensaios clínicos avaliaram parâmetros clínicos periodontais, tais como, nível de inserção clínica (NIC), profundidade de sondagem (PS), sangramento à sondagem (SS), índice de placa (IP), recessão gengival (RG), mobilidade dental (Mob), dentre outros; radiográficos e/ou bioquímicos.

2.2. Critérios de Inclusão:

Para esta revisão foram incluídos estudos publicados em língua Inglesa ou Portuguesa, envolvendo o efeito de BFs, de uso sistêmico, na doença periodontal.

2.3. *Critérios de exclusão*

Para esse estudo, foram excluídas todas as referências relacionadas a revisões de literatura, relatos de caso ou avaliações sobre doença peri-implantar.

2.4. Estratégias de Pesquisa

Foram acessados Bancos de dados computadorizados, tais como PubMed, Lilacs, Scielo, Medline e o Centro de Registro de Ensaios Clínicos da Biblioteca Cochrane (COCHRANE/CCTR). Para a pesquisa foram utilizadas as palavras chave: "*Bisphosphonates*" e "*Periodontitis*", limitando-se ao período de 2001 até 2011.

3. Resultados

Inicialmente, 144 referências foram encontradas sobre bisfosfonatos e periodontite. Em seguida, os títulos e resumos foram analisados por uma única investigadora, e referências relacionadas a revisões sobre o assunto, relatos de caso ou avaliações sobre doença peri-implantar foram excluídas. Finalmente, 17 artigos completos foram selecionados de acordo com a Tabela 1. Destes, 11 artigos referiram-se a estudos pré-clínicos [12-22], enquanto 6 referiram-se a ensaios clínicos, sendo todos realizados em paciente com periodontite crônica[23-28]. (Figura 1)

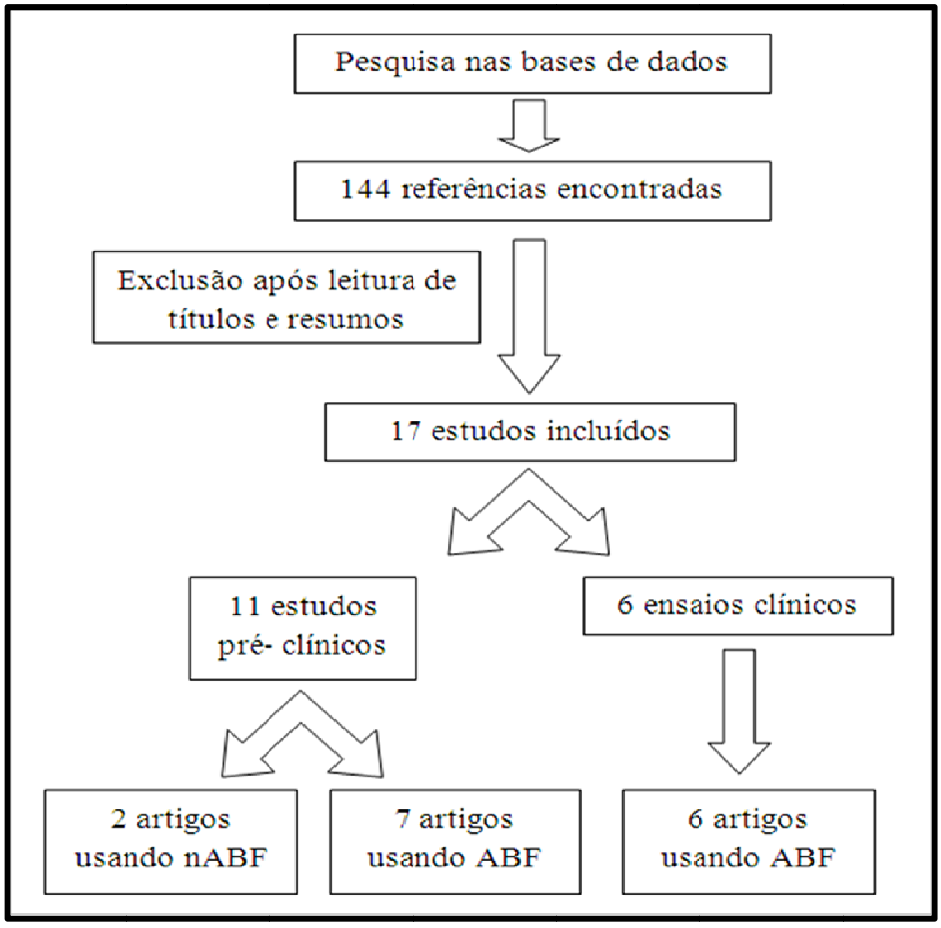

Figura 1. Fluxograma da estratégia de pesquisa e seleção dos artigos. nABF= não-aminofosfonatos; ABF = aminofosfonatos.

Tabela 1. Distribuição dos artigos selecionados quanto ao tipo: estudos pré-clínicos ou ensaios clínicos

Tipo de estudo	Autor/ano	
Pré-clínico em ratos	LLavaneras et al., 2001[12] Alencar et al., 2002[13] Tani-Ishii et al., 2003[14] Buduneli et al., 2004[15] Duarte et al., 2004[16]	Buduneli et al., 2005[17] Menezes et al., 2005[18] Anbinder et al., 2007[19] Buduneli et al., 2007[20] Spolidório et al., 2007[21] Cetinkaya et al., 2008[22]
Clínico em humanos	Rocha et al., 2001[23] El-Shinnawi, El-Tantawy, 2003[24] Rocha et al., 2004[25]	Lane et al., 2005[26] Jeffcoat et al., 2007[27] Graziani et al., 2009[28]

3.1. Estudos Pré-clínicos in vivo

A tabela 2 mostra o efeito dos BFs na periodontite em estudos pré-clínicos *in vivo*. Alencar et al.[13], avaliaram o efeito do Clodronato (CLD), um não aminobisfosfonato (nABF), em abordagens profilática (P) e terapêutica (T) e verificaram prevenção da perda óssea alveolar (POA) ($P<0,05$), redução do infiltrado celular inflamatório e do número de osteoclastos (OTC), além de preservação de cemento e processo alveolar, confirmando o efeito antirreabsortivo e anti-inflamatório deste fármaco. Quando em combinação com a Doxiciclina, uma tetraciclina quimicamente modificada (CTM-8), observou-se inibição da POA, redução da mobilidade dental (Mob) ($P<0,05$), da atividade de colagenase (MMP-1) e gelatinases (MMP-2 ou MMP-9) e elastase gengivais, especialmente na presença de APMA (ativador de pró-MMPs)[12].

Dentre o grupo dos aminobisfosfonatos (ABF), estudos avaliaram o efeito do Risedronato (RIS)[22], Ibandronato (IBD)[14], Alendronato (ALD)[16,18,19,21] e a combinação de Alendronato com Doxiciclina (DOX)[15,17,20], na doença periodontal experimental (Tabela 2). O estudo de Cetinkaya et al.[22] mostrou que o tratamento de curta duração com RIS aumentou na densidade volumétrica de osso (DVO) e de osteoblastos (DVOb) e reduziu a densidade volumétrica da medula (DVM) ($P<0,05$). O grupo de animais tratados com RIS com altas doses em longa duração apresentou redução do número de vasos sanguíneos (NVS) com correlação positiva entre NVS e DVO. Portanto, a administração de RIS de curta duração pode ser útil na inibição da POA, porém doses excessivas utilizadas por longo período de tempo

podem causar impedimento da formação de osso e dificultar a angiogênese (Tabela 2). O Ibandronato, um ABF de potência moderada, mostrou, em modelo de periodontite em ratos induzida por *P. gingivalis,* melhora significativas quanto à largura e à área do ligamento periodontal (LP) e de densidade mineral óssea (DMO) das porções cortical e trabecular após 8 semanas de uso. Os achados histológicos mostraram ainda, redução de células inflamatórias no tecido gengival, bem como organização paralela das fibras colágenas do LP[14] (Tabela 2).

O ALD, ABF de elevada potência, quando administrado de forma P ou T em ratos normossistêmicos mostrou redução da POA ($P<0,05$), com preservação do osso alveolar, cemento e fibras do LP, bem como redução do infiltrado inflamatório, da atividade de mieloperoxidase (MPO) e do recrutamento de neutrófilos, caracterizando um efeito antirreabsortivo e anti-inflamatório[18]. Neste estudo ainda observou-se inibição do crescimento de *F. nucleatum* sugerindo um possível efeito antimicrobiano deste fármaco[18] (Tabela 2). Utilizando outro modelo de indução de doença periodontal, através de ciclosporina A (CsA) (10 mg/kg/d) por via subcutânea durante 60 dias, observou-se que a combinação de CsA+ALD, provocou aumento significante dos níveis séricos de osteocalcina, bem como do volume ósseo destes animais[21]. Além disso, em estudo esteriométrico, esta combinação causou aumento do número de osteoblastos (OTB) e redução de osteoclastos (OTC)[21]. O efeito do ALD também foi avaliado em ratas ovariectomizadas, onde este apesar de não ter causado aumento de concentrações séricas de fosfatase alcalina (FA), foi capaz de proteger o osso alveolar destes animais, mesmo após a suspensão da terapia, caracterizando um efeito residual do fármaco[16]. Adicionalmente observou-se prevenção da redução da densidade radiográfica alveolar nestes animais[19].

O ALD também foi estudado em combinação com outros agentes moduladores da resposta do hospedeiro[15,17,20]. A associação entre ALD e Doxiciclina, doses subantimicrobianas, (ALD+DOX), mostrou, apesar dos altos valores de POA, inibição da expressão de mediadores inflamatórios relacionados à destruição periodontal tais como: prostaglandina $(PG)E_2$, $PGF_{2\alpha}$, leucotrieno B $(LTB)_4$, e fator de ativação plaquetária (PAF)[15]. Além disso, esta combinação provocou aumento do nível de osteocalcina (OC)[17], um marcador de formação óssea ($P<0,05$), redução da expressão de metaloproteinases de matriz (MMP)-8 e -14, endopeptidases destruidoras de tecido conjuntivo, e aumento na expressão de inibidores teciduais de metaloproteinases (TIMP)-1 ($P<0,05$)[20] (Tabela 2).

3.2. Estudos Clínicos

Todos os ensaios clínicos selecionados avaliaram o papel de ABF como adjuvantes à terapia periodontal de raspagem e alisamento radicular (RAR). Considerando a escala de potência de BFs proposta por Nancollas et al.[29] os estudos foram agrupados em ordem crescente de potência do BF utilizado: Risedronato (RIS)[26], Alendronato (ALD)[23, 24,25,27] e Neridronato (NER)[28] (Tabela 3).

O estudo de Lane et al.[26], avaliou o efeito do RIS como adjuvante à periodontite em pacientes normossitêmicos. Os resultados deste estudo mostraram que o tratamento com RIS+RAR melhorou significativamente em diversos parâmetros clínicos periodontais quando comparados à RAR isoladamente. Portanto, a terapia com RIS associada à RAR pode ter papel benéfico no *status* periodontal sendo sugerido, seu uso como adjuvante apropriado para a preservação da massa óssea periodontal (Tabela 3).

Quatro estudos avaliaram o efeito do ALD como adjuvante à terapia periodontal, sendo 2 destes em pacientes normosistêmicos[24,27], 1 em pacientes diabéticos[23] e outro em pacientes com diagnóstico de osteoporose[25] (Tabela 3). Em pacientes normossitêmicos, o tratamento com ALD adjuvante à raspagem e alisamento radicular (RAR) não-cirúrgico, promoveu melhora da densidade mineral óssea[24,27]. Em diabéticos, o uso sistêmico de ALD+RAR melhorou diversos parâmetros periodontais quando comparado à RAR isolada. Adicionalmente, a terapia farmacológica associada à RAR reduziu significativamente a distância da crista óssea alveolar à junção cemento esmalte (COA-JCE), bem como o níveis de telopeptídeo N-terminal de colágeno tipo I (NTx), um marcador de reabsorção óssea, mas sem alterar os níveis de hemoglobina glicada (HbA_{1c}) (Tabela 3)[23]. Nos casos de osteoporose, a terapia com ALD+RAR também mostrou efeitos benéficos, aumentando a densidade mineral óssea (DMO) e reduzindo significativamente a distância COA-JCE destes indivíduos. Observou-se ainda redução dos níveis de NTx e Fosfatase Alcalina Óssea (FAO), sem alteração do nível de hormônios sexuais das pacientes[25] (Tabela 3).

Mais recentemente, o estudo de Graziani et al.[28], avaliou o efeito do NER, um ABF administrado por via intra-venosa, como adjuvante à terapia periodontal. Os resultados mostraram que o uso de NER associado à RAR não resultou em melhora adicional das condições periodontais quando comparado à RAR isoladamente (Tabela 3).

Tabela 2. BF utilizado, modelo de periodontite empregado, resultados, conclusões dos estudos pré-clínicos *in vivo*.

BF utilizado	Autor/Ano	Modelo de periodontite	Resultados	Conclusões
CLD – P ou T (1, 5 ou 25 mg/kg-s.c.)	Alencar et al., 2002[13]	Induzida por ligadura em ratos	• Redução do infiltrado inflamatório e do número de OTC. • Preservação de cemento e osso	Efeito antirreabsortivo e anti-inflamatório
CLD+CMT-8 (1 mg/dia-v.o.)	Llavaneras et al., 2001[12]	Induzida por endotoxina em ratos	CMT-8+CLD • Redução da Mob, POA, da atividade de colagenase, gelatinase e elastase	Efeito antirreabsortivo e anti-inflamatório
RIS 0,1 e 1 mg/kg por 3 ou 8 sem. (v.o.)	Centikaya et al., 2008[22]	Induzida por ligadura em ratos	3 semanas - Aumento de DVO e DVOb e Redução da DVM 8 semanas - Redução de NVS	Efeito antirreabsortivo Altas doses de RIS por longo período pode impedir a osteo e angiogênese
IBD (2 mg/kg-v.o.) 2 dias/sem. 2, 4 ou 8 semanas	Tani-Ishii et al., 2003[14]	Induzida por *P. gingivalis* em ratos	• Redução da largura e área do LP • Organização paralela das fibras colágenas do LP • Melhora da DMO cortical e trabecular • Redução de células inflamatórias	Efeito antirreabsortivo e anti-inflamatório
ALD –P (0,01; 0,05; 0,25 mg/kg-s.c.) ALD – T (0,25 mg/kg-s.c.) 11 dias	Menezes et al., 2005[18]	Induzida por ligadura em ratos	• Inibição de POA (P<0,05) • Preservação do osso, cemento e fibras do LP • Redução do infiltrado inflamatório e da atividade de MPO • Redução de recrutamento de neutrófilos • Inibição de *F. nucleatum* (ALD-T)	Efeito antirreabsortivo, anti-inflamatório e antimicrobiano
ALD (0.3 mg/kg/sem – s.c.) 60 dias	Spolidório et al., 2007[21]	Induzida por CsA 10 mg/kg/d – s.c. 60 dias	CsA+ALD • aumento sérico de OC e do volume ósseo • aumento no número de OTB e redução de OTC	Efeito antirreabsortivo
ALD (5 mg/kg-s.c.) 4 dias/sem. 80 ou 40 dias	Duarte et al., 2004[16]	Induzida por ligadura em ratas ovariectomizadas	• Manutenção das concentrações séricas de FA • Proteção do osso contra a deficiência de EST	Efeito antirreabsortivo Efeito residual
ALD (2 mg/kg/d-v.o.) 35 dias	Anbinder et al., 2007[19]	Induzida por ligadura em ratas ovariectomizadas	• Prevenção da redução da DRA	Efeito antirreabsortivo
ALD+DOX	Buduneli et al., 2004[15]	Induzida por endotoxinas em ratos	• Altos valores de POA • Redução dos níveis gengivais de PGE_2, $PGF_{2\alpha}$, LTB4 e PAF	Efeito anti-inflamatório
ALD = 0,5 mg/kg-i.v. em dias alternados por 3 dias	Buduneli et al., 2005[17]		• Altos valores de POA • Altos níveis séricos de IL-1β, CRP • Níveis elevados de OC	Efeito anti-inflamatório
DOX=5 mg/kg-v.o. por 7 dias	Buduneli et al., 2007[20]		• Altos valores de POA • Reduziu MMP-14	Efeito anti-inflamatório

ALD = Alendronato;CLD = Clodronato; CMT-8 = tetraciclina quimicamente modificada; CRP = proteína C reativa; CsA = Ciclosporina A; DMO = densidade mineral óssea;DOX = doxiciclina; DRA = densidade radiográfica alveolar; DVM = densidade volumétrica da medula; DVO = densidade volumétrica óssea; DVOb = densidade volumétrica de osteoblastos; EST = estrógeno;FA = fosfatase alcalina; i.v. = intra-venoso; IBD = Ibandronato; IL-1β = interleucina 1β; LP = ligamento periodontal; LTB4 = leucotrieno B4; MMP = metaloproteinase de matriz; Mob = mobilidade dental; MPO = mieloperoxidase; NVS = número de vasos sanguíneos; OC = osteocalcina; OTB = Osteoblasto; OTC = Osteoclasto; P = Profilático; PAF = fator ativador de plaqueta; PGE_2 = prostaglandina E_2; $PGF_{2\alpha}$= Prostaglandina $F_{2\alpha}$; POA = perda óssea alveolar; RIS = Risedronato; s.c. = via subcutânea; T = Terapêutico; v.o. = via oral;

Tabela 3. BF utilizado, período de acompanhamento, parâmetros avaliados, resultados e conclusão dos ensaios clínicos

BF utilizado	Estudo	Amostra	Período de acompa-nhamento	Parâmetros avaliados	Resultados	Conclusão
RAR + RIS 5 mg/dia	Lane et al., 2005[26]	N=41	6 e 12 meses	**Parâmetro Periodontal (PP)** • NIC; PS; SS; IP. • Análise fractal e subtração radiográfica	Melhora de NIC, PS e SS (P<0,05) Sem alteração na massa óssea periodontal	Efeito antirreabsortivo e melhora de PP
RAR + Placebo		N=25				
RAR + ALD (10 mg/dia – v.o.)	El-Shinnawi, El-Tantawy, 2003[24]	N=24	6 meses	**Parâmetros periodontais (PP)** PS; NIC; IG, DMO	Melhora de DMO (P<0,001)	Efeito antirreabsortivo
RAR						
RAR + ALD (70 mg 1x/sem)	Jeffcoat et al. 2007[27]	N=335	6, 12 e 18 meses	**Parâmetros Periodontais (PP)** POA e DOA	Sem alteração na POA ou DOA. ALD reduziu POA em paciente com baixa DMO .	Efeito antirreabsortivo
RAR + placebo						
RAR + ALD (10 g/dia – v.o)	Rocha et al., 2001[23]	Pacientes diabético N=40	6 meses	**Parâmetro Periodontal (PP)** • PS;Mob; RG; SG; NIC • COA – JCE **Parâmetros Bioquímicos(PB)** • Glicemia em jejum, HbA_{1c}, NTx	Melhora de PP Redução de COA-JCE (P<0,05) Redução de NTx (P=0,05) Sem alteração em HbA_{1c}	Efeito antirreabsortivo, melhora em PP e redução em PB; não afetou o controle glicêmico dos pacientes
RAR + placebo						
RAR + ALD (10 mg/dia)	Rocha et al., 2004[25]	Paciente com Osteoporose	6 meses	**Parâmetro Periodontal (PP)** • PS; RG, PIC; Mob; SG; IP • COA – JCE e DMO **Parâmetros bioquímicos (PB)** • Glicemia em jejum, FAO, NTx, FSH, LH, hormônios esteróides	DMO (P<0,05) Redução de COA-JCE (P<0,05) Redução de NTx e FAO Sem alteração em níveis hormonais	Efeito antirreabsortivo melhora de PP e redução em PB; não afetou níveis hormonais
RAR + placebo		N=40				
RAR+NER 12,5 mg/2 ml 1 x /sem por 12 sem	Graziani et al., 2009[28]	N=60	3 e 6 meses	**Parâmetro Periodontal (PP)** • IP; RG; PS; NIC	Não houve diferença estatística	NER não resultou em melhora adicional para condições periodontais.
RAR						

ALD = Alendronato de sódio; BF = Bisfosfonato;COA-JCE = Distância entre crista óssea alveolar e junção cemento esmalte; DMO = Densidade mineral óssea; DOA = Densidade óssea alveolar; FAO = Fosfatase alcalina óssea; FSH = Hormônio folículo estimulante; HbA = Hemoglobina glicada; IG = Índice de sangramento gengival; IP = Índice de placa; LH = Hormônio luteinizante; Mob = Mobilidade dental; NER = Neridronato; NIC = Nível de inserção clínica; NTx = telopeptídeo N-terminal de colágeno tipo I; OA = osso alveolar; PIC = Perda de inserção clínica; PS = Profundidade de sondagem; POA = Perda óssea alveolar; RAR = Raspagem e alisamento radiculares; RG = Recessão gengival; RIS = Risedronato; SS = Sangramento à sondagem; v.o. = via oral.

4. Discussão

Bisfosfonatos são fármacos amplamente usados no controle de doenças metabólicas do osso devido seu potencial antirreabsortivo[30]. Na periodontite, o efeito dos BFs tem sido avaliado através de abordagem tanto pré-clínicas, como clínicas. Os resultados desta revisão mostraram que os BFs reduziram significativamente a perda óssea alveolar, a inflamação, o nível sérico de marcadores de metabolismo ósseo, com consequente melhora de parâmetros clínicos periodontais.

O efeito antirreabsortivo dos BFs foi observado em 13[12,13,14,16,18,19,21,22,23, 24,25,26,27] estudos dos 17 avaliados. Tal efeito foi marcado redução do número de osteoclastos (OTC)[13,21], culminando em redução da reabsorção óssea alveolar[12,13,16,18], confirmado por redução do nível de inserção periodontal[14] e da distância da crista óssea alveolar a junção cemento esmalte, observado em análises radiográficas e de densidade mineral óssea[14,19], além de aumento de densidade volumétrica de osso (DVO) e osteoblasto (DVOb)[22]. Clinicamente, estes achados refletiram em redução da profundidade de sondagem[23,25,26] e ganho de inserção clínica[21-23].

Os BFs apresentam papel modulador na função de OTC no metabolismo ósseo. Em nível tecidual, reduzem o *turnover* ósseo, devido à redução da reabsorção de osso e por inibir novas unidades multicelulares ósseas. Em nível celular, BFs alteram a função do OTC, reduzindo sua adesão à matriz óssea, a profundidade do seu sítio de reabsorção, bem como liberação de citocinas[31,] podendo causar inclusive apoptose de OTC[32]. Durante a reabsorção óssea, BFs parecem ser internalizados por endocitose, juntamente com outros produtos de reabsorção[30]. Após a ingestão celular, observa-se ausência de borda enrugada, como principal característica de OTC tratados com BFs, além de alteração em citoesqueleto[33]. Estas mudanças morfológicas podem ser explicadas pela redução da sinalização intracelular dependente de prenilação dentro do OTC. Adicionalmente, além de atuar principalmente em clastos maduros, os BFs também têm mostrado poder de prevenção da formação de OTC[30], garantindo, portanto, o efeito protetor ósseo dessa classe farmacológica.

Além da atividade antirreabsortiva, os BFs têm sido sugeridos como adjuvantes farmacológicos à RAR não-cirúrgica devido sua atividade anti-

inflamatória, limitando o processo de destruição óssea, observado em doenças inflamatórias como a periodontite. Nesta revisão, 10 estudos[12,13,14,18,20,23,25,26] mostraram tal efeito, através da redução do infiltrado inflamatório[13,14,18], do recrutamento de neutrófilos[18], de atividade de mieloperoxidase[18], de mediadores inflamatórios[15,17,20], metaloproteinases de matriz[20] e enzimas líticas: colagenase, gelatinase e elastase[12] Clinicamente, o efeito anti-inflamatório dos BFs foi marcado por redução em índices de sangramento gengival[23,25,26].

BFs apresentam diversos mecanismos anti-inflamatórios. BFs atuam em células apresentadoras de antígenos[34,35] e inibem o crescimento e diferenciação de células da medula em linhagem de macrófagos[35]. Aumento na liberação de óxido nítrico (NO), devido ativação de óxido nítrico sintase constitutiva (NOSc) [36-38], que por sua vez, pode regular a função de OTC e servir como regulador negativo da atividade de MPO[39].

A inibição de mediadores inflamatórios, tais como prostaglandina (PG)[15], leucotrienos (LT)[15] e Fator ativador de plaquetas (PAF)[15], também foi um mecanismo anti-inflamatório importante, visto que, estes mediadores atuam diretamente no processo de reabsorção óssea[40]. As PGs amplificam a reposta inflamatória e estimulam a produção de quimiocinas e enzimas líticas[41] além de contribuir para reabsorção óssea via regulação positiva da expressão de RANKL e inibição de OPG em células osteoblásticas[42]. Os LTs, especialmente o LTB_4, aumentam a reabsorção osteoclástica[43], promovem formação de OTCs independente de RANKL[44], afetando diretamente a reabsorção óssea por aumentar o número e atividade de OTC[40]. PAF, por sua vez, aumenta sobrevivência de OTC e ativa vias de sinalização molecular nessa linhagem celular[45].

MMPs são enzimas que atuam tanto no desenvolvimento fisiológico e na remodelação tecidual, como na destruição patológica de tecido[46]. Considerando que as MMPs necessitam de Ca^{2+} para sua atividade, a inibição desta enzima após terapia com BFs parece envolver mecanismos de quelação com o cálcio[11], pois muitos BFs formam um “gancho ósseo” ligando sua estrutura química ao Ca^{2+} dos cristais de hidroxiapatita, reduzindo assim a oferta de Ca^{2+} no meio[47].

Vale salientar que além do efeito anti-inflamatório, os BF, também podem apresentar reações inflamatórias não desejáveis, como por exemplo, o aumento de proteínas de reação de fase aguda (IL-1β e CRP)[17], em aproximadamente 10±50% de pacientes tratados[48]. Reações de fase aguda podem ocorrer porque os ABF depositam-se por longo período de tempo em baço e fígado[49] e podem induzir reações inflamatórias em vários tipos de células, órgãos, tecidos e sangue[50,51]. No entanto, a reação de fase aguda, geralmente acontece na primeira exposição ao fármaco e tipicamente não dura mais do que 72 horas. Sintomas de reações de fase aguda podem estar associados à BF de uso intravenoso ou oral[52], porém nem todos BF induzem respostas de fase aguda na mesma extensão[53].

Os resultados desta revisão mostraram ainda que os BFs podem apresentar atividade antimicrobiana, devido à redução de colônias de *F. nucleatum*[18] e índices de placa bacteriana[25,26]. *F. nucleatum*, é um dos anaeróbios GRAM-negativo mais abundantes, presentes principalmente em sítios com doença periodontal estando associado a várias formas de doença do periodonto[54,55]. O lipopolissacarídeo de *F. nucleatum* estimula macrófagos e fibroblastos a secretarem uma grande quantidade de citocinas e moléculas efetoras com capacidade inflamatória e destruidora de tecido. Linfócitos são estimulados por LPS a produzir grande número de anticorpos com diferentes especificidades, que por sua vez exacerbam a reposta inflamatória. Nas células ósseas, LPS induzem reabsorção óssea e inibem a formação de osso resultado em erosão do osso alveolar de suporte dental[56]

No entanto, apesar de benéfico, mais estudos ainda se fazem necessários para confirmar o efeito antimicrobiano dos BFs. O estudo de Menezes *et al*, 2005, realizou análise microbiológica por turbidimetria, no entanto as recomendações da Farmacopéia Brasileira[57], sugerem que a atividade antimicrobiana de uma fármaco deve ser avaliada através de análise de turbidimetria associada à difusão em ágar. Adicionamente, este estudo mostrou a presença de *Peptostreptococcus*, pertencente do complexo laranja[58] e associado periodontite, nos 3 grupos experimentais utilizados (Naive, Salina e Tratado). Por fim, nenhuma espécie do grupo complexo vermelho foi avaliada[58]

Quanto aos parâmetros bioquímicos observou-se que o tratamento com BFs causou redução de Telopeptídeo N-terminal de colágeno tipo I (NTx)[23,25] e Fosfatase Alcalina Óssea (FAO)[25]. O NTx é um marcador de reabsorção óssea, pois no processo de reabsorção, fragmentos de colágeno com terminais amino são liberados na circulação e podem ser medidos através de imunoensaios. Chesnut et al.[59], encontraram relação entre NTx na urina e a taxa de perda óssea. Sabendo que o tratamento com BFs inibe o processo de reabsorção consequentemente os níveis de NTx mostram-se também reduzidos60[60,61].

A Fosfatase Alcalina Óssea (FAO) é uma isoenzima da Fosfatase Alcalina Total (FAT) e um excelente biomarcador da atividade de osteoblastos. A FAO está localizada na membrana de precursores de osteoblastos, osteoclastos maduros e nas vesículas de matriz, as quais permitem acúmulo de íons cálcio e fosfato[62]. Esta enzima atua promovendo mineralização através da liberação de íons fosfato (Pi) liberados do ATP e também hidrolisando pirofosfato inorgânico (PPi), agente inibidor de mineralização. Entretanto observou-se nesta revisão que o tratamento com BFs provocou redução dos níveis séricos de FAO, estando de acordo com relatos prévios da literatura[63]. Sabe-se que a potência inibição da reabsorção óssea causada por BFs está relacionada ao comprimento do radical-2 ligado ao átomo de carbono, enquanto que o grupo hidroxila (-OH) posicionado no radical-1 melhora a afinidade mineral óssea, uma característica dos BFs, especialmente do Alendronato, e que quando auxiliado por grupos fosfatos, forma uma espécie de gancho ósseo[47]. Assim redução dos níveis de FAO após terapia com BFs se deve a quelação de cátions divalentes por este gancho ósseo formado[47], uma vez que a inibição da atividade de FAO pode ser revertida quando do excesso de Mg^{2+} e Zn^{2+}[47].

Em suma, o tratamento com BFs preveniu a reabsorção óssea alveolar, modulou a inflamação, reduziu a atividade de marcadores bioquímicos do metabolismo ósseo, com consequente melhora dos parâmetros clínicos periodontais, sendo, portanto, uma ferramenta farmacológica importante como adjuvante à RAR não-cirúrgica.

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3.2 Capítulo 2

EFFECT OF ALENDRONATE ON BONE-SPECIFIC ALKALINE PHOSPHATASE AND ON PERIODONTAL BONE LOSS IN WISTAR RATS.

ABSTRACT

Objective: To evaluate the effect of Alendronate (ALD) on Bone-specific Alkaline Phosphatase (BALP) serum levels and on periodontal bone loss in Wistar rats. **Design:** Periodontitis was induced by ligature around the upper second molar in 36 male Wistar rats (± 200 g). Groups of 6 animals received 0.9% Saline (SAL) or ALD (0.01; 0.05; 0.25 mg/kg-s.c.), over 11 days when they were sacrificed and their maxillae were removed to be defleshed and stained to macroscopic or histopathological analysis. Blood samples were collected for BALP, transaminases and total alkaline phosphatase (TAP) serum dosage, and hematologic study. Rats were weighted daily. **Results:** Periodontitis induction caused: intense reduction of BALP, alveolar bone loss (ABL), and cementum and periodontal ligament destruction, and intense leukocyte infiltration seen microscopically. Systemically, periodontitis induced leukocytosis, weight loss and total alkaline phosphatase (TAP) reduction. ALD (0.25 mg/kg) prevented BALP reduction (19.17±1.36 U/l) when compared to SAL (13.6±1.5), as well as prevented ABL, by 57.2%, when compared to SAL (4.74±0.19 mm^2), which was corroborated by histological finding [ALD 0.25 mg/kg=1.5 (1-2) and SAL=3 (2-3)] ($p<0.05$). ALD did not alter transaminases, but reduced TAP levels ($p<0.05$). ALD 0.25 mg/kg reduced 6^{th} hour neutrophilia (2.50±0.22 cell x $10^3/mm^3$) and 7^{th} (12.29±0.66) and 11^{th} day lymphomonocytosis (15.74±0.52) when compared to SAL (5.20±0.28; 18.24±1.05 and 23.21±1.48, respectively). ALD did not alter the weight loss. **Conclusion**: ALD prevented BALP reduction and ABL, and reduced inflammatory infiltrate, without causing systemic alterations.

KEYWORDS: Alendronate; Bone-specific Alkaline Phosphatase; Alveolar bone loss; Inflammation.

1. Introduction

Periodontitis is a chronic infectious-inflammatory disease, that if not treated, can cause tooth mobility leading to subsequent tooth loss.[1] Some mechanisms are related to etiopathogenesis of periodontitis, however, factors associated to immunoinflammatory host response are being widely studied, and the use of drugs that modulate this response may be an interesting approach for periodontitis treatment.[1]

Bone resorption is the main characteristic of periodontitis, and it is mediated by osteoclast (OTCs). These cells, originated by blood precursors from bone marrow, are activated by various mediators, especially tumor necrosis factor (TNF) and interleukin (IL)-1. After activation and fusion, OTCs, on the resorption site attach to bone matrix forming a sealing zone[2] and become polarized, exhibiting a ruffled border. By then, a great amount of acid vesicles are released on the resorption site in order to start hydroxyapatite crystals dissolution.[2]

The nitrogen-containing Bisphosphonates (nBP) are anti-resorptive that possess a chemical structure similar to the pyrophosphate[3]. Among nBPs, Sodium Alendronate (ALD) points out due to its high affinity to bone tissue. nBP mechanism of action is based on the inhibition of farnesil diphosphate synthase (FPPS)[4] which stimulates the isoprenylation of small GTPases that regulate OTC morphology, cytoskeleton arrangement, vesicle traffic and ruffled border formation[4]. Due to the inhibition on vesicular traffic and ruffled border, the activities that elicit bone resorption are also reduced[4]. However, current evidence suggests that BPs not only act on the osteoclasts, inhibiting bone resorption, but also have direct effects on osteoblasts, regulating differentiation and function of these cells.[5]

The isolation and characterization of cellular and extracellular components of the skeletal matrix have resulted in the development of biochemical markers that specifically reflect bone metabolism. These biochemical indices have greatly enriched the spectrum of analyzes used in the assessment of bone diseases. They are non-invasive, comparatively inexpensive and, when applied and interpreted correctly, are helpful tools in the diagnostic and therapeutic assessment of metabolic bone disease.[6]

Alkaline phosphatase (AP) is an important biochemical marker that has been known for many years[7]. AP is a membrane-bound metalloenzyme, distributed particularly in the liver, bowel, placenta and bone[7]. Among its isoforms, stands out Bone-specific alkaline phosphatase (BALP), which has been implicated in bone formation[7] by removal of inorganic pyrophosphate (PPi), an inhibitor of bone mineralization.[7] Therefore. BALP may be used to evaluate osteoblast activity.[8]

In this context, considering the role of nBPs in both osteoblast and osteoclast, we aimed to evaluate the effect of Alendronate on serum dosage of Bone-specific Alkaline Phosphatase and periodontal bone loss in Wistar rats.

2. Methods and Materials

2.1. Animal selection

Thirty-six male Wistar rats (*Rattus norvegicus*) weighing 180 to 220 g, from our own animal facilities, were used in this study. The animals were acclimatized for at least 1 week before the beginning of the experiment and were housed under normal laboratory conditions with laboratory chow and water available *ad libitum*. Experimental protocols were executed following ethical principles for laboratory animal use, and were approved by institutional Ethical Committee of Animal Research (Protocol nº 101/2009).

2.2. Model of Experimental Periodontitis

The rats were divided into four groups, with 6 animals each. A previously calibrated investigator induced periodontitis using the model of ligature-induced periodontitis, which consists on insertion of nylon ligature around the cervix of second left upper molar of rats anesthetized with Chloral Hydrate (Vetec®, Duque de Caxias, RJ, Brazil).[9,10] Ligature was placed through proximal space of the respective tooth, and was knotted on buccal side of the tooth, resulting in a subgingival position palatinally and in a supragingival position buccally of the ligature. The contralateral right side was used as the unligated control. Animals were observed until the 11th day, period of the most intense alveolar bone loss,[11] when they were then sacrificed. All ligature-induced periodontitis was blinded.

2.3. Experimental Groups

2.3.1. Saline Groups

This control group was constituted by 6 rats submitted to periodontitis. The animals received 0.5 ml of 0.9% sterile Saline solution subcutaneously (s.c.), 30 min. before ligature and, after that, daily, for a 11 day period, when they were then sacrificed.

2.3.2. Alendronate Group (ALD)

The animals were subdivided in 3 groups of 6 animals each, which received s.c. Alendronate (Fosamax®, Merck, São Paulo-SP, Brazil) dissolved in 0.9% sterile Saline solution on the doses of 0.01, 0.05, 0.25 mg/kg, respectively, 30 min. before ligature, and daily until the 11th day. The choose of doses was based on a previous report that showed the anti-resorptive action of ALD without development of adverse effects.[12]

2.4. Morphometric study of bone tissue

On the 11th day, after periodontitis induction, the animals were sacrificed and their maxillae were removed and fixed in 10% neutral buffered formalin (Reagen®, Rio de Janeiro, RJ, Brazil), during 24 hours. Following, maxillae were separated in half, dissected and stained with 1% aqueous methylene blue (Vetec®, Duque de Caxias, RJ, Brazil) and placed on microscope slides.[10,11] By then, they followed to photographic registration using a digital camera Nikon® (D40, Melville, NY, USA). The measurement of resorption area was made by delimited region, involving occlusal border of vestibular side of the hemimaxilla until bone border. These areas were evaluated by IMAGE J® software (Software ImageJ 1.32j, National Institute of Health; EUA) in accordance to methodology described by Goes et al.[10] All measurements and analysis were made in a blind manner.

2.5. Histological analysis of alveolar bone

Two extra groups of 6 animals with periodontitis that had received Saline or ALD (0.25 mg/kg) were sacrificed as described above and had their maxillae excised. The specimens were fixed in 10% neutral buffered formalin and were demineralized in 10% EDTA (Dinâmica Química Contemporânea®,

Diadema, SP, Brazil) for 40 days. Following, the specimens were dehydrated, embedded in paraffin, then sectioned in a buccal-lingual direction, in a mesio-distal plane, for Mallory trichrome staining. Sections of 4 μm thickness, corresponding to the area between the first and second molars were evaluated by light microscopy. Parameters such as inflammatory cell infiltration, osteoclast number, alveolar bone and cementum integrity, were determined in a single-blind manner and graded, by scores varying from 0 to 3, based on the intensity of findings, as follows: Score 0: absence of or only discrete cellular infiltration, few osteoclasts, preserved alveolar process and cementum; Score 1: moderate cellular infiltration, presence of some osteoclasts, some but minor alveolar process resorption and intact cementum; Score 2: accentuated cellular infiltration, large number of osteoclasts, accentuated degradation of the alveolar process, and partial destruction of cementum; Score 3: accentuated cellular infiltrate, total destruction of alveolar process and cementum.[11]

2.6. Serum dosage of Bone-Specific Alkaline Phosphatase (BALP)

Blood samples were collected from orbital plexus of anesthetized animals (Saline and ALD) before the experiment and on the 11th day. The BALP was evaluated using the thermoactivation method, by heating the sample at 56 °C for 10 min[13], since BALP is a thermosensible isoform of Total Alkaline Phosphatase (TAP). BALP serum levels were obtained by the subtraction of TAP from Heated Alkaline Phosphatase (HAP) serum levels. The methodology used to evaluate the enzymes serum levels followed the manufacturer orientations (Labtest®, Lagoa Santa-MG, Brazil). Biochemical analysis was made in a blind manner.

2.7. Serum dosage of Transaminases (AST and ALT) and Total Alkaline Phosphate

On the baseline and at the 11th day of the assay, blood samples were collected from orbital plexus of anesthetized animals (Saline and ALD). The liver function was evaluated through serum dosage of transaminases: Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT). Total Alkaline Phosphate (TAP) serum levels were also evaluated. Specific kits were used,

and methodology followed the manufacturer instructions (Labtest®, Lagoa Santa-MG, Brazil). Biochemical analyses were made in a blind manner.

2.8. Hematologic study

The method used to analyze white blood cell counts, as well as its subpopulation (neutrophil and mononuclear cells) was as follows: 20 μl of blood, taken from the rat tail, was added to 380 μl of Turk solution. Total white blood cell counts were performed using a Neubauer chamber and the differential counts were made using smears stained by rapid Instant Prov Stain Set (Newprov Produtos para Laboratório; Pinhais-PR, Brazil). Leukogram of the groups of animals (Saline and ALD) was performed before periodontitis induction, at the 6th hour and 2nd, 7th and 11th days after the ligature. Hematologic analysis was made in a blind manner.

2.9. Corporal mass variation

Animals from Saline and ALD groups had their body mass measured before periodontitis induction and after that daily until the 11th day. Values were expressed as body mass variation (g) compared to the initial body mass. Corporal mass variation was made in a blind manner

Statistical analysis

The data is presented as mean±standard error of the mean (SEM) or median (and range), where appropriate. Analysis of variance (Anova), followed by Bonferroni's test or Student´s t-test, were used to compare means, and Kruskal-Wallis and Dunn tests were used to compare medians. A $p<0.05$ value was considered as indicating significant differences. All calculations were performed using GraphPad Prism 5 software (GraphPad, Inc., San Diego, CA, USA).

3. Results

3.1. Morphometric study of bone tissue

The macroscopic analysis of alveolar bone showed that 11 days ligature-induced periodontitis caused intense bone resorption (Table 1), associated with root exposition and furcation lesion (Fig. 1C). ALD at the lowest

dose (0.01 mg/kg) did not protect alveolar bone ($p>0.05$) when compared to Saline. ALD at higher doses (0.05 and 0.25 mg/kg) was able to significantly inhibit bone loss by 33.5% and 57.2%, respectively, when compared to Saline ($p<0.05$). Animals treated with ALD (0.25 mg/kg) presented alveolar bone preservation similar to normal hemimaxilla (Fig. 1A), however the periodontal aspect was different from Saline (Fig. 1E).

3.2. Histological analysis of alveolar bone

For the histological analysis, another assay was performed, and then the hemimaxillae were processed for histological analysis (Table 1). It was observed that alveolar bone and cementum resorptions were associated to intense inflammatory infiltrate ($p<0.05$) on animals submitted to periodontitis (Table 1; Fig. 1D), when compared to normal periodontium (Table 1; Fig. 1B) ($p<0.05$). ALD (0.25 mg/kg) treatment significantly attenuated inflammatory infiltrate and preserved periodontal ligament, root cementum and alveolar bone (Table 1; Fig. 1F), when compared to Saline ($p<0.05$).

Table 1. Macroscopic and Histological analysis of normal hemimaxilla or submitted to periodontitis receiving Saline or ALD.

	Normal	Saline	ALD 0.01 mg/kg	ALD 0.05 mg/kg	ALD 0.25 mg/kg
Morphometric analysis Mean (mm^2)	--	4.80±0.18	4.10±0.35	3.19±0.54*	2.05±0.12*
Histological analysis Scores	0 (0-0)	3 (2-3)$^{\#}$	--	--	1.5 (1-2)*

(*) indicates statistically significant difference when compared to Saline; ($^{\#}$) indicates statistically significant difference when compared to normal hemimaxillae ($p<0.05$). (–) indicates that there was no analysis. For macroscopic analysis, values represent the mean followed by S.E.M of a minimum of 6 animals per group by Anova and Bonferroni test. For histological analysis, values represent the medians followed by scores variation (lower-higher) of a minimum of 6 animals per group by Kruskal-Wallis and Dunn test.

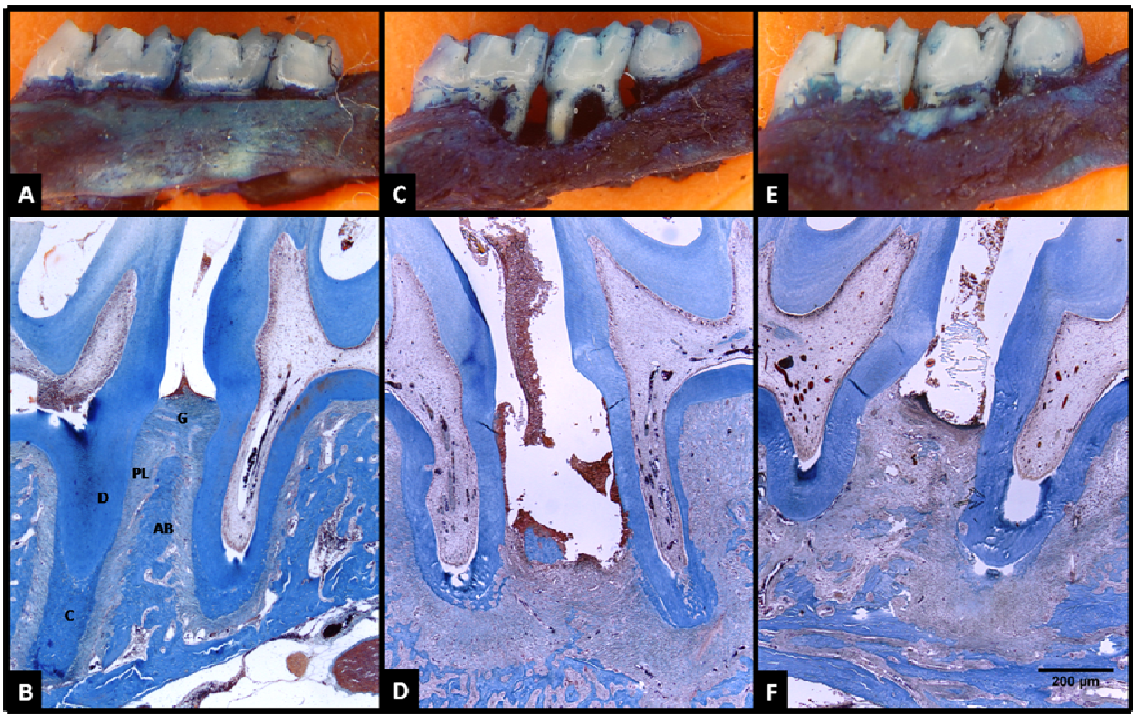

Figure 1. Macroscopic and microscopic aspect respectively of normal periodontium **(A and B)** and periodontium of rat submitted to periodontitis receiving Saline, showing macroscopic bone resorption **(C)** and alveolar bone and cementum resorption, and inflammatory cell infiltration seen in histopathology **(D). E** and **F** illustrate the reduction of inflammation and alveolar bone loss in periodontium of rats treated with ALD (0.25 mg/kg) for 11 days (Macroscopic original magnification 7x; Microscopic original magnification 40x). Letters of photomicrographs: G= gingiva; PL= periodontal ligament; D= dentin; AB= alveolar bone; C= cementum.

3.3. Serum dosage of Bone-Specific Alkaline Phosphatase (BALP)

Serum dosages of BALP were analyzed (Fig. 2). Saline presented a significant decrease by 45.6% on BALP serum levels (13.62±1.56 U/l) when compared to its baseline (25.04±1.43 U/l). The treatment with ALD (0.01 and 0.05 mg/kg) caused a reduction on BALP serum levels, although not significant ($p>0.05$), by 17.6% (19.92±2.97 U/l) and 19.5% (21.62±2.39 U/l), respectively, when compared to its respective baseline (ALD 0.01 mg/kg=24.19±1.62 U/l; ALD 0.05 mg/kg=26.67±2.15 U/l). The treatment with ALD (0.25 mg/kg) induced a significant decrease by 28.1% (19.17±1.36 U/l), on this enzyme after 11 days ligature-induced periodontitis when compared to its baseline data (26.67±2.15 U/l), however the treatment with the highest dose of ALD prevented BALP reduction, by 17.5%, when compared to SAL after 11 days of periodontitis ($p<0.05$)

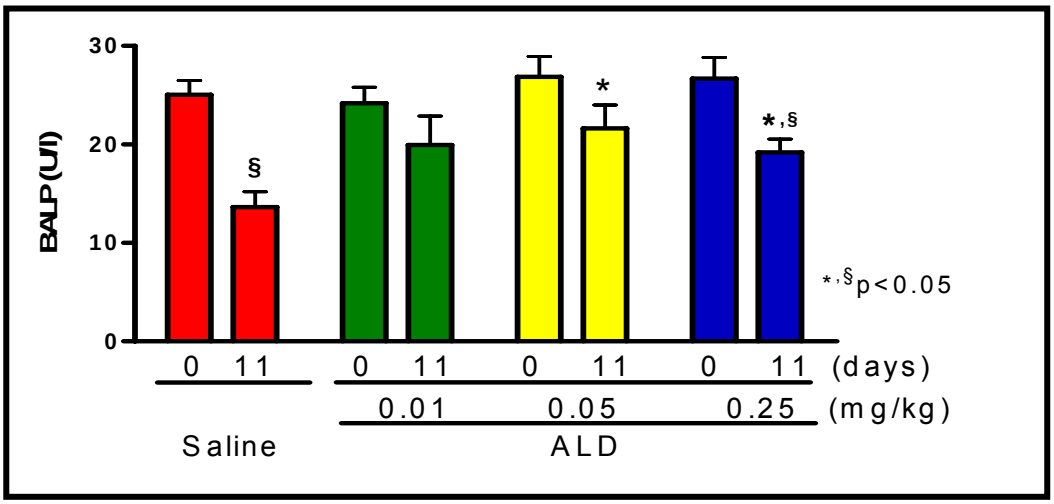

Figure 2. Effect of ALD on Bone-specific alkaline phosphatase. Bars represent Mean±SEM of BALP (U/l) of a minimum of 6 animals per group. (*) indicates statistically significant difference when compared to Saline 11 day data. (§) indicates statistically significant difference when compared to its respective baseline data. [Two-way Anova; Bonferroni test and Student´s t-test] ($p<0.05$).

3.4. Serum dosage of Transaminases and Total Alkaline Phosphatase (TAP)

Serum dosages of transaminases (AST and ALT) and TAP were analyzed in animals of Saline and ALD (Table 2). At the 11th day, for AST and ALT, there was no statistical difference in Saline when compared to its respective baseline. However, it was observed a significant decrease on TAP serum levels, of animals from SAL group after 11 days, when compared to its baseline data. The treatment with ALD did not cause significant alteration ($p>0.05$) on AST and ALT serum levels, but, it reduced ($p<0.05$) TAP serum levels when compared to its respective baseline data.

Table 2. Serum dosage of AST and ALT e TAP of animals submitted to periodontitis and receiving Saline or ALD.

	Groups / Days	Saline	ALD 0.01 mg/kg	ALD 0.05 mg/kg	ALD 0.25 mg/kg
AST (U/l)	0	44.51±2.13	40.61±2.97	45.44±3.92	47.44±3.33
	11	48.64±4.74	38.72±2.50	46.04±3.86	42.51±3.52
ALT (U/l)	0	18.44±3.89	19.19±3.81	17.36±3.27	19.32±4.18
	11	22.03±3.44	19.91±1.30	21.57±2.72	16.02±1.99
TAP (U/l)	0	95.61±1.21	96.51±1.52	97.07±1.97	93.06±1.09
	11	70.14±1.74§	77.29±1.99§	75.75±2.11§	69.64±1.71§

Values represent Mean±SEM of 6 animals per group. (§) indicates statistically significant difference when compared to its respective baseline data. [Two-way Anova; Bonferroni test and Student´s t-test]. ($p<0.05$).

3.5. Hematologic study

On total leukocyte counts it was observed that periodontitis caused leukocytosis at the 6th hour after ligature (18.77±1.66 leukocytes x 10^3/mm^3) (Fig. 3A), when compared to its baseline data (11.56±0.31). This leukocytosis was marked ($p<0.05$) by neutrophilia (5.20±0.28 neutrophil x 10^3/mm^3), when compared to its baseline (1.37±0.08) (Fig. 3B). Following, on the 2nd day, there was a decrease on total leukocyte count, however the basal cell counts was not achieved. A new leukocytosis were observed at the 7th (21.73±0.87 leukocytes x 10^3/mm^3) and 11th (25.84±1.23) days, with predomination of mononuclear cells (7th d= 18.24±1.05; 11th d= 23.21±1.48 mononuclear cells x 10^3/mm^3) when compared to its baseline (10.19±0.25) (Fig. 3C). All doses of ALD prevented neutrophilia at the 6th hour (ALD 0.01= 4.00±0.42; ALD 0.05= 2.98±0.21; ALD 0.25= 2.50±0.22), when compared to Saline (5.20±0.28) ($p<0.05$) (Fig. 3B). However, only ALD (0.25 mg/kg) prevented mononuclear cell peaks at 7th (12.29±0.66) and 11th (15.74±0.52) days (Fig. 3C).

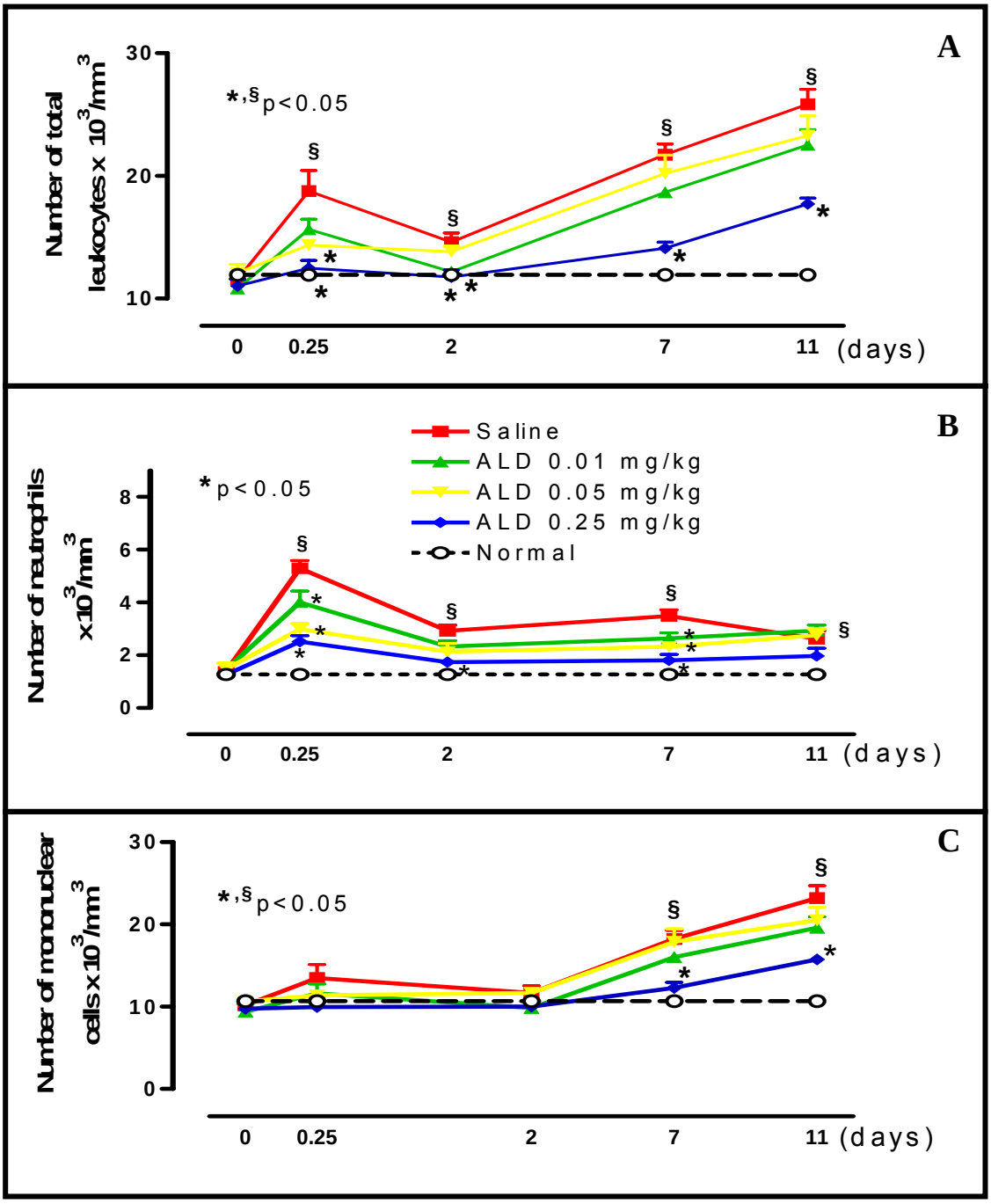

Figure 3. Effect of ALD on leukocyte counts. Points represent Mean±SEM of total leukocytes **(A)**, neutrophils **(B)**, mononuclear cells **(C)** x 10^3/mm^3 of a minimum of 6 animals per group. (*) indicates statistically significant difference when compared to Saline. (§) indicates statistically significant difference when compared to its baseline data. [Anova and Bonferroni test]. ($p<0.05$).

3.6. *Corporal mass variation*

Periodontitis caused body weight loss marked on the 3rd day after ligature placement when compared to normal animals. After that, animals showed gain of weight and a tendency to follow the normal animal corporal mass curve. Animals treated with ALD showed a similar corporal mass pattern to Saline. ALD did not alter initial loss of weight, when compared to Saline. After 3rd day, it was observed gain of mass accompanying animals from Saline group (Fig. 4).

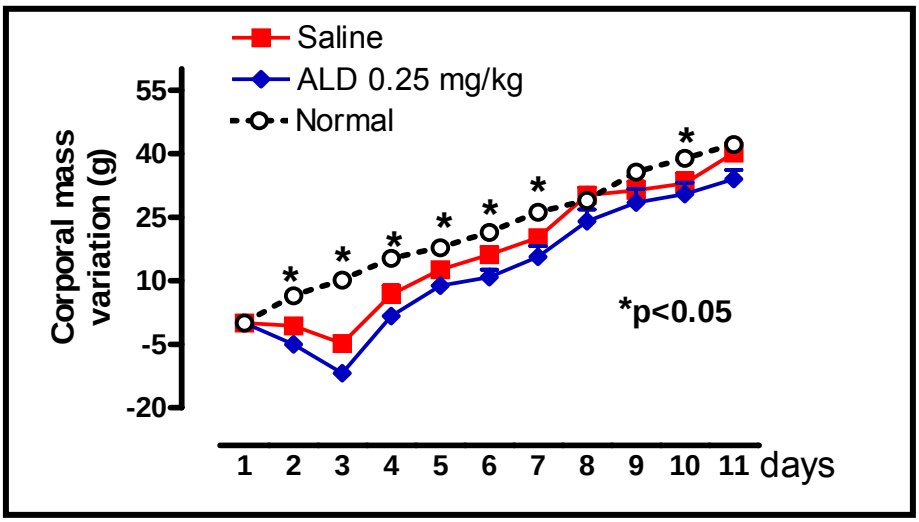

Figure 4. Effect of ALD on corporal mass variation. Points represent Mean±SEM of a minimum of 6 animals per group. (*) indicates statistically significant difference when compared to Saline. [Anova and Bonferroni's test] ($p<0.05$).

4. Discussion

In the present study, it was seen that ligature induced periodontitis caused intense alveolar bone resorption and periodontal inflammation, as demonstrated by macroscopic and histological analysis. In addition, it was observed a significant decrease on BALP and TAP serum levels, and no change on AST and ALT serum levels. Periodontitis caused leukocytosis marked by neutrophilia on the 6th hour and by lymphomonocytosis on the 7th and 11th days. In addition was observed an initial weight loss followed by tendency to accompany corporal mass curve of normal rats. Treatment with ALD prevented bone resorption of animals submitted to ligature-induced periodontitis, confirmed by in macroscopic and histological analysis, when compared to Saline. ALD, on the higher dose, prevented the reduction of BALP

serum levels when compared to Saline, and did not alter transaminases serum levels. Besides, ALD prevented 6th h neutrophilia, as well as lymphomonocytosis observed on 7th and 11th days. ALD did not prevent the initial weight loss, although the animals had showed gain of corporal mass similar to Saline corporal mass curve.

Reports from literature have shown that nBPs not only act on osteoclast but also affect the behavior and metabolism of osteoblasts.[14,15] Knowing that BALP, an isoform of total alkaline phosphatase (TAP), acts specifically as a marker of bone formation, it seemed interesting to evaluate the effect of ALD on osteoblast through serum dosage of BALP.[8] In this study we have shown that the lowest doses of ALD (0.01 and 0.05 mg/kg) prevented BALP reduction, when compared to its respective baseline data. In the other hand, the highest dose of ALD (0.25 mg/kg) prevented BALP reduction when compared to SAL after 11-days periodontitis, but its levels were significantly reduced when compared to its baseline.

The reduction of BALP serum levels after exposure to ALD may be related to 2 aspects: the chemical structure, which is closely linked to the anti-resorptive effect of this drug, and its concentration.[16,17] nBPs, like ALD, have 2 radicals linked to the carbon atom, one, called R1 that has a hydroxyl group (-OH) and improves mineral affinity, and the other one, called R2, which increases nBP potency to inhibit bone resorption.[15] This chemical structure elicits the development of a structural motif called “bone hook” that binds to the mineral by chelation of divalent cations.[17] Therefore, considering that BALP needs divalent cations to become activated and that ALD bone hook reduces the offer of these cations, our present observations suggest that the highest dose of ALD inhibited BALP activity through divalent cations chelation within the bone hook structure. This suggestion is based on previous report where BALP inhibition was be reversed by an excess of Zn^{2+} or Mg^{2+}.[14]

However, it was seen that lower doses of ALD prevented BALP reduction while the highest dose did not, when compared to baseline, therefore we can infer that ALD may have a dose-dependent effect on BALP serum levels.[16,17,18] At low concentrations, ALD, was shown to increase formation of fibroblastic colonies[16] and to stimulate BALP activity[17], suggesting a mild anabolic effect. However, at high concentrations, ALD caused a total inhibition

of colony formation[16], was toxic to osteoclast[18] and inhibited BALP activity[17]. Thus, our present observations are physiologically relevant in the context of a local action of nBPs on osteoblasts.

In order to evaluate the role of ALD on osteoclast, we analyzed its bone sparing action by morphometric and histological studies. A significant bone protection was observed only when the highest dose of ALD was used. The alveolar bone protection performed by ALD after ligature induced periodontitis has been demonstrated previously.[19,12] This anti-resorptive effect may be explained by the attraction of ALD to the bone and its interference on enzyme activity.[20,21] nBPs, like ALD inhibit FPPS, a mevalonate pathway enzyme responsible for isoprenylation of small GTPases.[22] These small GTPases are signaling proteins that, when activated, up regulate several structural properties important for osteoclast function, including morphology, cytoskeletal arrangement, vesicular trafficking and membrane ruffling.[23,24] By the time that vesicular trafficking and membrane ruffling are inhibited, bone resorption is also reduced. So, FPPS inhibition seems to be responsible for the pharmacologic effects of the nBPs at tissue level.[25]

The macroscopic aspect was corroborated by histological analysis, It was seen a partial preservation of alveolar bone, cementum and periodontal ligament as well as reduction on inflammatory infiltrate in animals receiving ALD. Beyond anti-resorptive action, it has been reported an anti-inflammatory activity associated to ALD, by inhibition of proinflammatory mediators release, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF) and of nitric oxide (NO).[26-28] This anti-inflammatory activity may also rebound on ALD anti-resorptive action, since IL-1 and TNF, mainly, stimulate expression of receptor activator of nuclear factor-κB ligant (RANKL), a TNF family cytokine, which is essential for osteoclastogenesis induction.[29]

Treatment with ALD seemed to be safe. Animals treated with ALD showed initial weight loss, similar to Saline, which may have been caused by ligature placement. After that, it was seen that ALD therapy did not induce additional loss of weight, according to previous data.[12] ALD therapy did not cause significant changes in AST and ALT serum levels, suggesting that ALD does not interfere on liver function, what was expected, since this drug is not metabolized in the liver.[30] Studies in patients that received liver transplant

demonstrated that ALD has been well tolerated without deleterious effects on liver function tests.[31] Patients taking ALD and diagnosed with primary biliary cirrhosis did not present significant hepatic effects regarding biochemical parameters of liver disease.[31] Our study also revealed significant inhibition of TAP serum levels after 11 days of periodontitis in animals receiving either saline or ALD. This inhibition may be due to the reduction on the bone isoform, since BALP represents about 90% of the TAP.[17]

We also observed that ALD prevented neutrophilia and lymphomonocytosis. These findings are in accordance with previous report in which ALD treatment induced a significant decrease on total white blood cell, neutrophil and lymphocyte counts, in patients with Paget's disease.[33] The reduction on neutrophil count may reverberate on neutrophil migration and activity, once it was seen that ALD decreased on neutrophil influx using carrageenan-induced peritonitis model and reduced mieloperoxidase activity as well.[12] In addition, the reduction on peripheral mononuclear cells, which includes monocytes and lymphocytes, it was also an important finding considering that circulating monocytes, can migrate and differentiate locally on osteoclast, thereby exerting bone resorption activity.[21] Thus, the reduction of mononuclear cells may contribute to bone sparing effect of ALD in this model.

In summary, our results demonstrated that low doses of ALD prevented BALP reduction, while high dose did not, in the other hand, only high dose of ALD prevented ABL, and reduced inflammatory infiltrate, without causing systemic alterations.

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3.3 Capítulo 3

EFFECT OF ALENDRONATE AND ATORVASTATIN COMBINATION ON ALVEOLAR BONE LOSS IN RATS

ABSTRACT

Periodontitis is chronic infectious-inflammatory disease and Alendronate and Atorvastatin have shown anti-resorptive and anti-inflammatory effects in different conditions. Therefore we aimed to evaluate the effect of Alendronate (ALD) and Atorvastatin (ATV) combination on alveolar bone loss (ABL) in experimental periodontitis. Periodontitis was induced by ligature around the upper 2nd molar in 78 Wistar rats. Groups of 6 animals received prophylactically (P), 30 min before ligature and daily until sacrifice, 0.9% Saline (SAL) or ALD (0.01 or 0.25 mg/kg) subcutaneously or ATV (0.3 or 27 mg/kg) orally. Later, groups of 6 animals submitted to periodontitis, received P the combination of ALD+ATV (0.01+0.3; 0.25+0.3; 0.01+27; 0.25+27 mg/kg). An extra group of 6 animals was submitted to periodontitis and received therapeutically (T), 5 days after ligature and daily until sacrifice, SAL or the lower doses combination (LDC) of ALD+ATV (0.01+0.3 mg/kg). On the 11th day, the animals were killed and maxillae were removed for macroscopic, histopathological, histometric and tartrate resistant acid phosphatase (TRAP) immunohistochemical analysis. Gingival samples were collected to evaluate mieloperoxidase (MPO) activity. Blood samples were collected for bone-specific alkaline phosphatase (BALP) and transaminases dosage and leukogram analysis. Rats were weighted daily. All combined therapy prevented ABL when compared to SAL or to the low-dose monotherapy with ALD or ATV ($P<0.05$). Lower doses combination prevented ABL when administered both P (39.0%) or T (53.5%), when compared to SAL. These data corroborated the decrease in bone and cementum resorption, leukocyte infiltration, immunostaining for TRAP and MPO activity. The lower doses combination prevented BALP reduction ($P<0.05$), and did not change serum transaminases, it also reduced peripheral neutrophilia and lymphomonocytosis, and did not cause weight loss, when compared to SAL. Thus, the combination of lower doses of ALD+ATV showed a protective effect on experimental alveolar bone loss.

KEY WORDS: Alendronate; Atorvastatin, Periodontitis; Alveolar bone loss; Inflammation.

INTRODUCTION

Periodontal diseases encompass multifactorial diseases involving bacterial biofilms and the generation of an inflammatory response, leading to the production of cytokines, eicosanoids, matrix metalloproteinases (MMPs), among other mediators.[1] However, chronic inflammatory diseases, such as periodontitis are frequently associated with bone loss due to the increase on bone resorption and decrease on bone formation.

It is well-known that bone remodeling cycle is controlled by a variety of mechanisms.[2,3] The discovery of osteoprotegerin (OPG)/receptor activator of nuclear factor-κB ligand (RANKL) system has given insight into a major component of remodeling cycle. RANKL is expressed on the surface of osteoblasts and its expression increases in response to a variety of pro-resorptive signals such as pro-inflammatory cytokines.[4] Therefore, inflammatory cytokine such as TNF-α, IL-1β, IL-6, IL-11 and IL-17, present on periodontal site, will stimulate osteoclastogenesis,[5] which can be clinically detected as periodontal pockets associated with loss of clinical attachment.[6]

Considering the role of host response on bone diseases, pharmacological approaches have emerged as an alternative to prevent or to treat these diseases.[7,8] Among a variety of drugs, used to modulate the host response stands out Alendronate and Atorvastatin. Alendronate (ALD), a Bisphosphonate (BP), is a stable analog of pyrophosphate and potent inhibitor of bone resorption, which has been used as effective therapeutic agent for the management of osteoporosis and other bone diseases, such as Paget's disease and bone metastasis.[9] Inhibition of bone resorption by BPs has been mainly attributed to their inhibitory effect on osteoclasts. BPs decrease the commitment of osteoclast progenitors into osteoclasts and promote apoptosis of mature osteoclasts by inhibition of farnesyl diphosphate synthase (FPPS) a key enzyme in the mevalonate pathway.[10]

Atorvastatin (ATV), a drug from Statins group, also known as 3-hydroxy-3-methyl-glutaryl- coenzyme A (HMG-CoA) reductase inhibitor, is widely used for lowering serum cholesterol levels.[11] It has been reported that statins have the so-called pleiotropic effects such as: antioxidant properties, inhibition of inflammatory responses, immunomodulatory actions and anabolic effects on bone metabolism *in vitro* and *in vivo*.[12,13] Clinical studies have

shown the beneficial effect of statins on osteoporosis.[14] In addition, it has been reported that statins affect osteoclast directly through mechanisms analogous to those of BP, because statins and BPs exert their effect by inhibiting the same mevalonate pathway.[15,16]

Therefore, considering that both ALD and ATV act on mevalonate pathway and that they have presented anti-resorptive and, anti-inflammatory and bone anabolic actions respectively, the aim of this study was to evaluate, for the first time, the effect of Alendronate and Atorvastatin combination, administered either prophylactically or therapeutically on alveolar bone loss in rats.

MATERIAL AND METHODS

Animal selection

Seventy-eight male Wistar rats (*Rattus norvegicus*), weighing 180 to 220 g, from our own animal facilities, were used in this study. The animals were acclimatized for at least 1 week before starting the experiment and were housed under normal laboratory conditions with laboratory chow and water available *ad libitum*. Experimental protocols were executed following ethical principles for laboratory animal use, and were approved by institutional Ethical Committee of Animal Research (Protocol nº 101/2009).

Model of Experimental Periodontitis

For the study, the rats were divided in groups, with 6 animals each. The model of ligature-induced periodontitis used was based on Lima and colleagues[17], which consists on insertion of a nylon ligature around the cervix of the second left upper molar of rats anesthetized with Chloral Hydrate (Vetec®, Duque de Caxias, RJ, Brazil). Ligature was placed through proximal space of the respective tooth, and was knotted on buccal side of it, resulting in a subgingival position palatinally and in a supragingival position buccally of the ligature. The contralateral right side was used as the unligated control. Animals were observed until the 11th day, when they present the most intense alveolar bone loss.[17] At this time, the rats were then sacrificed. All ligature-induced periodontitis were made in a blind manner.

Experimental Groups

Saline Group

This control group was constituted by 6 rats submitted to periodontitis. The animals received 2 ml/kg of 0.9% sterile Saline solution (SAL) orally, 30 minutes before ligature and, after that, daily, for a 11 day period, when they were sacrificed.

Sodium Alendronate Group

The animals were divided in 2 subgroups of 6 animals each, which received s.c. Sodium Alendronate (ALD) (Fosamax®, Merck, São Paulo-SP, Brazil) dissolved in 0.9% sterile Saline solution on the doses of 0.01 and 0.25 mg/kg 30 minutes before ligature, and daily until the 11th day, when they were then sacrificed. The choose of doses was based on a previous report.[18]

Atorvastatin Group

The animals were divided in 2 subgroups of 6 animals each, which received Atorvastatin (ATV) (Lipitor®, Pfizer, São Paulo-SP, Brazil) administered orally (v.o.) on the doses of 0.3 and 27 mg/kg, 30 minutes before ligature, and daily until the 11th day, when they were then sacrificed. The choose of doses was based on a previous report.[13]

Sodium Alendronate+Atorvastatin Group: prophylactic regimen

The animals were divided in 4 subgroups of 6 animals each, which received ALD combined with ATV (ALD+ATV), 30 minutes before ligature, and daily until the 11th day, when they were then sacrificed. The combinations used were with: low doses (ALD 0.01+ATV 0.3 mg/kg); high-low or low-high doses (ALD 0.25+ATV 0.3 mg/kg and ALD 0.01+ATV 27 mg/kg) or high doses (ALD 0.25+ATV 27 mg/kg).

Sodium Alendronate+Atorvastatin Group: therapeutic regimen.

An extra group of 6 animals was submitted to ligature-induced periodontitis. They received the lower doses combination of ALD+ATV (0.01+0.3 mg/kg) from the 5th day after ligature placement until 11th day, when

they were then sacrificed. Therapeutic treatment followed methodology described by Alencar and colleagues.[19]

Morphometric study of bone tissue

For macroscopic analysis, the maxillae were removed on the 11th day and fixed in 10% formaldehyde (Reagen®, Rio de Janeiro-RJ, Brazil) for 24 h. The morphometric analysis was performed in accordance to the methodology described by Goes and colleagues[13]. For this, maxillae were separated in half defleshed stained with 1% aqueous methylene blue (Vetec®, Duque de Caxias-RJ, Brazil) and placed on microscopic slides and followed to photographic registration using a digital camera (Nikon D40, Melville-NY, USA). The measurement of area resorption was made by a delimited region, involving the occlusal border of vestibular side of the teeth until bone border. Data was evaluated by IMAGE J® software (Software ImageJ 1.32j, National Institute of Health; EUA). All measurements and analyses were made in a blind manner.

Histological analysis of alveolar bone

Three extra groups, of 6 animals each, were submitted to periodontitis and received SAL or the lower doses combination (ALD 0.01+ATV 0.3), in prophylactic or therapeutic regimens. On the 11th day, the animals were sacrificed as described above and had their maxillae excised. The specimens were fixed in 10% neutral buffered formalin and demineralized in 7% formic acid (Merck®, Jacarepaguá-RJ, Brazil), for 10 days. Following, the specimens were dehydrated, embedded in paraffin, then sectioned in a buccal-lingual direction, in a mesio-distal plane, and stained for Hematoxylin and Eosin. Sections of 4 μm thickness, corresponding to the area between the first and second molars were evaluated by light microscopy (40x). Parameters such as inflammatory cell infiltration, osteoclast number, and alveolar bone and cementum integrity, were determined in a single-blind manner and graded by scores varying from 0 to 3, based on the intensity of findings, as follows: Score 0: absence of or only discrete cellular infiltration, few osteoclasts, preserved alveolar process and cementum; Score 1: moderate cellular infiltration, presence of some osteoclasts, some but minor alveolar process resorption and intact cementum; Score 2: accentuated cellular infiltration, large number of osteoclasts,

accentuated degradation of the alveolar process, and partial destruction of cementum; Score 3: accentuated cellular infiltrate, total destruction of alveolar process and cementum.[17]

Histometric study

The same slides used for histological analysis were used for the histometric study. The slide inclusion criteria were: the presence of a portion of the dental root, epithelial tissue and interproximal bone in the same section. Images of these sections were captured at 40x magnification. After excluding the first and last sections, four equally distant sections of each tooth were selected for histometric analysis.[20] A trained observer, blinded to the group to which the slide belonged, used the IMAGEJ® software to obtain 3 linear measurements (Medial [1]; Intermediate [2]; and Distal [3]) from alveolar bone crest to cemento-enamel junction (ABC - CEJ) at the buccal root of the second upper molar (Fig. 1). The distance ABC-CEJ was determined by the mean of the 3 measurements.

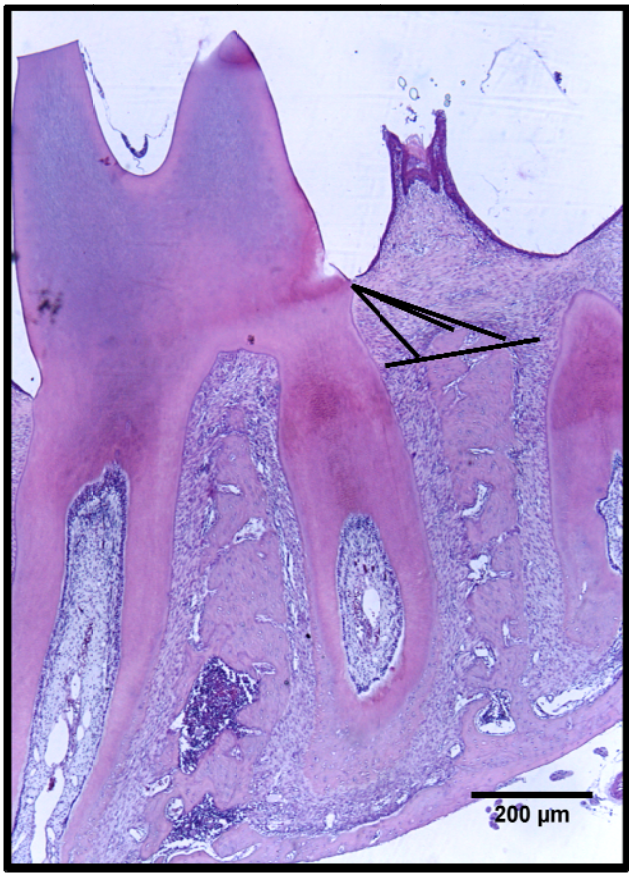

Fig. 1. Histometric analysis: Linear measurement from bone crest to cemento-enamel junction.

Tartrate resistant acid phosphatase (TRAP) immunohistochemical staining.

Histological sections from maxillae of rats submitted to periodontitis, which received prophylactically or therapeutically SAL or the lower doses combination of ALD+ATV (0.01+0.3 mg/kg), were submitted to indirect immunoperoxidase method using polyclonal antibody in order to identify TRAP. Initially, histological sections were collected, had their paraffin removed and were rehydrated. The sections were washed in sodium phosphate buffer (PB), under slow agitation and submitted to endogenous peroxidase blockade using 3% H_2O_2 in methanol for 30 min and then washed with phosphate buffered Saline (PBS). Following, histological sections were incubated with a solution containing polyclonal primary antibody obtained in goat anti-TRAP k17 from human (1:100, sc30833, Santa Cruz Biotechnology, CA, USA), diluted with PBS added to donkey normal serum (017-000-001, Jackson Immunoresearch laboratories, PA, USA) during 24 h at room temperature, over agitation. After that, the sections were rinsed with PBS and then submitted to the second incubation with anti-goat biotinylated secondary antibody done in donkey (1:200, 705-066-147, Jackson Immunoresearch Laboratories, PA, USA), and diluted in PBS added to donkey normal serum during 1 h at room temperature, under agitation. Latter, the histological sections were once again submitted to PBS wash and incubated with estreptavidin conjugated with peroxidase (1:200, Kit ABC, PK6100, Vector Laboratories, CA, USA) diluted in PB solution at room temperature during 1 h. Imunoperoxidase reaction disclosure was done in PBS solution added with 0.005% diaminobenzidine, followed by inactivation through numerous washes in PBS. These histological sections were contra-staining with Harris hematoxylin, dehydrated, diaphanyzed in xylene and assembled with hydrophobic set up medium (Erv-mount, Erviegas, SP, Brazil). All immunoperoxidase reactions were accompanied by a negative control, through primary and secondary antibody omission, followed by the procedure mentioned above.[21] TRAP analysis was made in a blind manner.

Myeloperoxidase (MPO) activity

MPO activity, a marker for neutrophil activity in inflamed tissue, was also evaluated in sample of gingival tissue, using methodology from Lima and colleagues[22]. Groups of rats submitted to periodontitis, that received SAL or the lower doses combination of ALD+ATV (0.01+0.3 mg/kg), in prophylactic and therapeutic regimens, had a sample of their challenged gingival removed on 11th day of experimentation for analysis of MPO activity. The gingiva of the contralateral hemimaxilla of rats that received SAL only was used as the normal control. The specimens were stored at -80 ^{o}C until the assay. For this, the gingiva was weighed and triturated using a Polytron Ultraturrax in ice-cold buffer solution, and the homogenate was centrifuged at 4 °C for 15 min (3,000 g). The supernatant was collected for MPO activity, determined by measuring the change on absorbance at 450 nm. MPO analysis was made in a blind manner

Systemic parameters

Serum dosage of Bone-Specific Alkaline Phosphatase (BALP)

Blood samples were collected from orbital plexus of anesthetized animals that received SAL or the lower doses combination of ALD+ATV (0.01+0.3 mg/kg), in prophylactic and therapeutic regimens, before the experiment and on the 11th day. The BALP was evaluated using the thermoactivation method, by heating the sample into 56 °C for 10 min[23] since BALP is a thermosensible isoform of Total Alkaline Phosphatase (TAP). Therefore BALP serum levels were obtained by the subtraction of TAP to Heated Alkaline Phosphatase (HAP) serum levels. Methodology to evaluate the enzymes followed manufacturer orientations (Labtest®, Lagoa Santa-MG, Brazil). Biochemical analysis was made in a blind manner

Serum dosage of Transaminases (AST and ALT).

On the baseline and at the 11th day of the assay, blood samples were collected from orbital plexus of anesthetized animals that received SAL or the lower doses combination of ALD+ATV (0.01+0.3 mg/kg), in prophylactic and therapeutic regimens. Liver function was evaluated through serum dosage of Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT).

Specific kits were used, and methodology followed manufacturer orientations (Labtest®, Lagoa Santa-MG, Brazil). Biochemical analyses were made in a blind manner

Hematologic study

The method used for the analysis of white blood cell counts, as well as its subpopulation (neutrophil and mononuclear cells) was as follows: 20 μl of blood, taken from the rat tail, was added to 380 μl of Turk solution. Total white blood cell counts were performed using a Neubauer chamber and the differential counts were made using smears stained by rapid Instant Prov Stain Set (Newprov Produtos para Laboratório; Pinhais-PR, Brazil). White blood cell counts of the groups of animals that received SAL or the lower doses combination of ALD+ATV (0.01+0.3 mg/kg), in prophylactic and therapeutic regimens, were performed before periodontitis induction, at the 6th h and on the 2nd, 7th and 11th days after ligature. Hematologic study was made in a blind manner

Corporal mass variation

Animals from group that received SAL or lower doses combination of ALD+ATV (0.01+0.3 mg/kg), in prophylactic and therapeutic regimens, had their body mass measured before periodontitis induction and after that daily, until the 11th day. Corporal mass variation was made in a blind manner

Statistical analysis

The data are presented as mean±standard error of the mean (SEM) or median (and range), where appropriate. Analysis of variance (Anova), followed by Bonferroni's test or Student´s t-test, were used to compare means, and Kruskal-Wallis and Dunn tests were used to compare medians. A $P<0.05$ value was considered as indicating significant differences. All calculations were performed using GraphPad Prism 5 software (GraphPad, Inc., San Diego-CA, USA).

RESULTS

Morphometric study of bone tissue

The morphometric study of alveolar bone demonstrated that 11 days of ligature-induced periodontitis showed intense alveolar bone loss (ABL) (Fig. 2) associated with root exposition and furcation lesion (Fig. 3C), when compared to normal periodontium (Fig. 3A). The prophylactic high-doses monotherapy of ALD or ATV prevented bone loss (ALD 0.25=2.1±0.1 mm^2; ATV 27=1.7±0.2 mm^2), when compared to Saline (SAL=4.6±0.2 mm^2) ($P<0.05$). However, the low-doses monotherapy of ALD or ATV, did not protect alveolar bone (ALD 0.01=4.2±0.4 mm^2; ATV 0.3=4.2±0.4 mm^2) (Fig. 2A), when compared to SAL.

All combinations of ALD+ATV, administrated prophylactically, protected ($P<0.05$) alveolar bone (ALD 0.25+ATV 27=2.1±0.1 mm^2; ALD 0.01+ATV 0.3=2.8±0.1 mm^2; ALD 0.01+ATV 27=2.4±0.3 mm^2; ALD 0.25+ATV 0.3=2.7±0.2 mm^2) when compared to SAL (Figs. 2B and 3E) or to the low-doses monotherapy with either ALD or ATV (Fig. 2A). Among all combination, the one using low doses stood-out, since when administered as monotherapy did not prevented ABL. Thus, the combination of lower doses of ADL+ATV was administered therapeutically, and it was observed a significant reduction of ABL (2.5±0.6 mm^2) (Figs. 2C and 3G), when compared either to SAL or to low-dose monotherapy with either ALD or ATV.

Histological analysis of alveolar bone

For microscopic study, another assay was performed, and then the hemimaxillae were processed for histological analysis (Table 1). It was observed that alveolar bone and cementum resorption were associated to an important inflammatory infiltrate ($P<0.05$) seen on periodontium of animals submitted to periodontitis (Table 1; Fig. 3D), when compared to normal periodontium (Table 1; Fig. 3B) ($P<0.05$). The combination of lower doses (ALD 0.01+ATV 0.3), administered prophylactic or therapeutically, significantly attenuated inflammatory infiltrate and preserved periodontal ligament, root cementum and alveolar bone (Table 1; Figs. 3F and 3H), when compared to Saline ($P<0.05$).

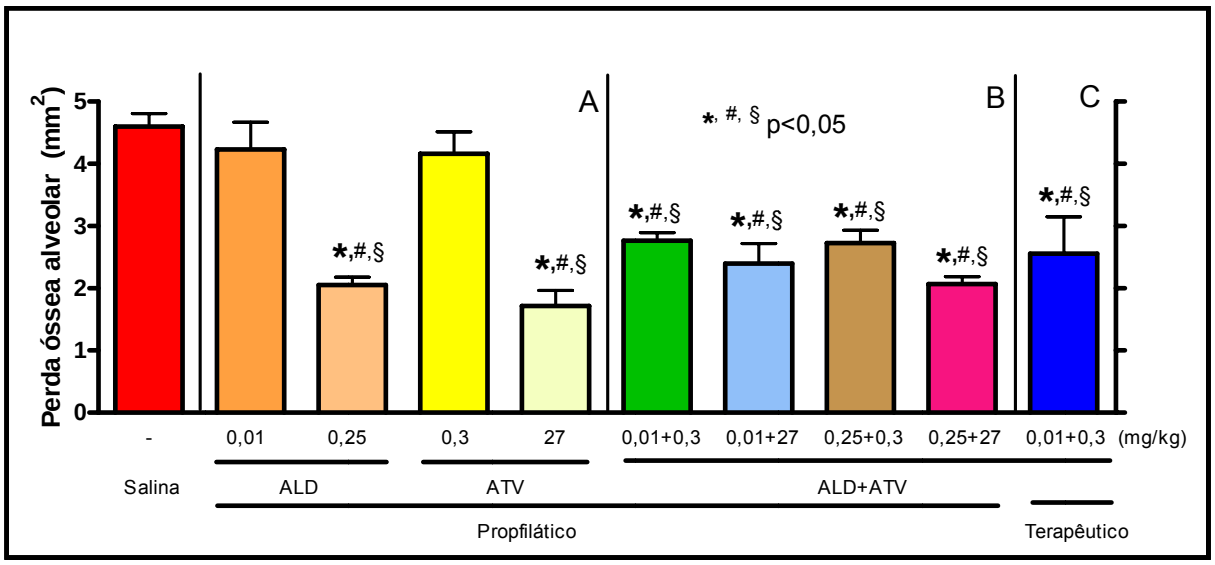

Fig. 2. Effect of ALD and ATV on bone tissue. Bars represent the Mean±SEM of, at least, 6 animals per group receiving prophylactic monotherapy of ALD or ATV in high and low doses **(A)**; prophylactic combination of ALD+ATV doses **(B);** and therapeutic combination of lower doses (ALD 0.01+ATV 0.3) **(C)**. (*) indicates statistical difference when compared to Saline; ($^{\#}$) indicates statistical difference when compared to ALD (0.01 mg/kg); (§) indicates statistical difference when compared to ATV (0.3 mg/kg). [Anova and Bonferroni's test] (P<0.05)

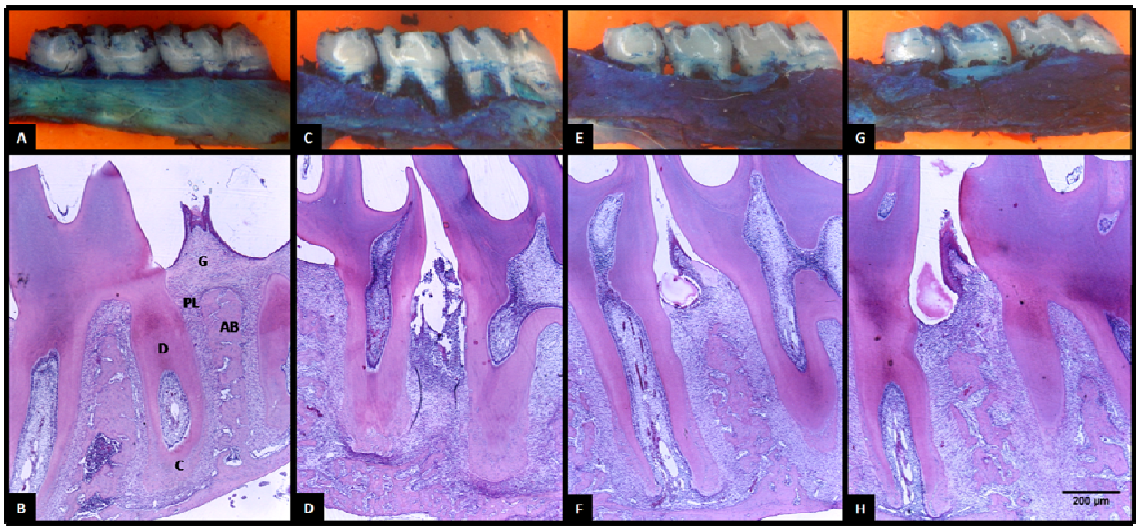

Fig. 3. Macroscopic and microscopic aspects, respectively of normal periodontium (**A** and **B**), or periodontium of animals submitted to periodontitis, receiving Saline (**C** and **D**), and prophylactic (**E** and **F**) or therapeutic (**G** and **H**) combinations of lower doses (ALD 0.01+ATV 0.3). The removed maxillae were dissected and photographed or processed for hematoxylin and eosin (H&E) staining. (Macroscopic original magnification 7x; Microscopic original magnification 40x).

G = gingiva; **PL** = periodontal ligament; **D** = Dentin; **AB** – Alveolar bone; **C** = cementum.

Table 1. Histological analysis of normal hemimaxilla or submitted to periodontitis receiving SAL or combinations of lower doses (ALD 0.01+ATV 0.3).

	Normal	Saline	ALD+ATV Prophylactic	ALD+ATV Therapeutic
Histological (Scores)	0 (0-0)	3 (2-3)$^{\#}$	1 (1-3)*	1 (1-2)*

Values represent the medians followed by scores variation (lower-higher) of, at least, 6 animals per group. (*) indicates statistical difference when compared to Saline; ($^{\#}$) indicates statistical difference when compared to normal hemimaxillae [Kruskal-Wallis and Dunn test]. ($P<0.05$).

Histometric analysis of alveolar bone

The histometric study of alveolar bone corroborated the morphometric and histological findings. It was seen that 11 days of ligature-induced periodontitis caused intense bone resorption (0.80±0.04 mm) when compared to normal periodontium (0.23±0.01 mm) ($P<0.05$). The lower doses combined therapy (ALD 0.01+ATV 0.3 mg/kg), administered either in prophylactic (0.62±0.06 mm) or therapeutic (0.50±0.02 mm) regimens, prevented and reduced ABL when compared to Saline ($P<0.05$), respectively. The animals that received the lower doses of the combined therapy (ALD 0.01+ATV 0.3 mg/kg), therapeutically, presented a significant reduction of the distance from cementum-enamel junction to bone crest, when compared to the ones that received the lower doses of the combined therapy (ALD 0.01+ATV 0.3 mg/kg) prophylactically (Fig.4)

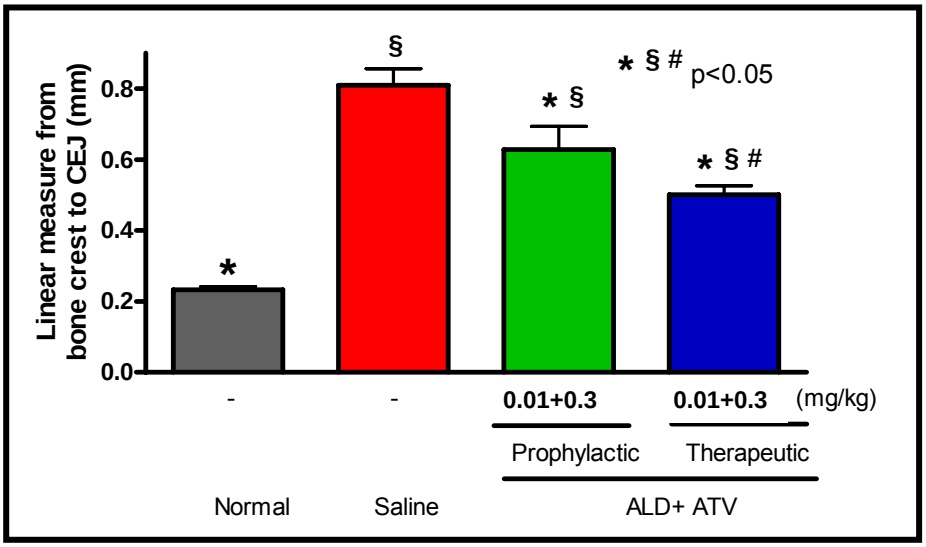

Fig. 4. Effect of prophylactic or therapeutic combination of lower doses of ALD+ATV on measurements from bone crest to cementum-enamel junction (CEJ). Bars represent the Mean±SEM of, at least, 6 animals per group. (*) indicates statistical difference when compared to Saline; ($^{\#}$) indicates statistical difference when compared to ALD+ATV administered prophylactically; (§) indicates statistical difference when compared to normal. [Anova and Bonferroni's test] (P<0.05).

Immunohistochemistry for TRAP

The immunohistochemical analysis for TRAP on the region between the first and second molars revealed intense labeling after 11 days of ligature-induced periodontitis (Fig.5B), when compared to the normal hemimaxilla (Fig. 5A). The prophylactic combination of the lower doses of ALD+ATV did not show labeling of TRAP (Fig. 5C), while the therapeutic regimen revealed mild immunostaining when compared to Saline (Fig. 5D).

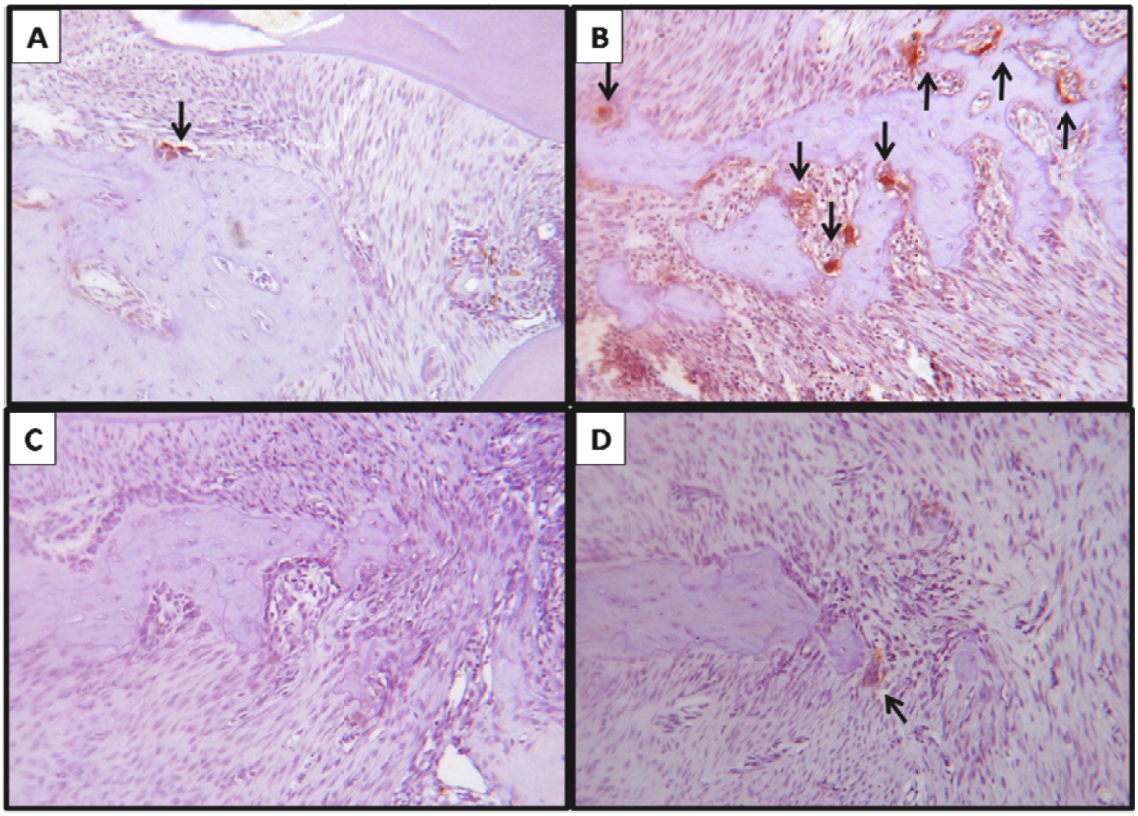

Fig. 5. Immunohistochemical aspects for detection of TRAP expression. Low TRAP expression (arrow) in normal periodontium (**A**). Intense labeling (arrows) on the periodontium of animals submitted to periodontitis, receiving Saline (**B**). No labeling on the treatment using combination of lower doses (ALD 0.01+ATV 0.3) administered prophylactically (**C**), or mild immunostaining (arrow) when administered therapeutically (**D**). (Original magnification 250x).

Myeloperoxidase (MPO) activity

MPO activity was evaluated on gingival tissue of animals submitted to ligature-induced periodontitis receiving Saline or pharmacological treatments. Fig. 6 shows that animals submitted to 11 days of periodontitis presented a significant increase on MPO activity in gingival tissue (53.2±12.7 MPO activity/g of tissue), when compared to normal animals (0.7±0.2). On the other hand, the combinations of lower doses of ALD+ATV, administered prophylactically (14.2±4.4) or therapeutically (10.9±3.2), prevented MPO activity after 11 days of ligature-induced periodontitis, when compared to Saline (P<0.05) (Fig. 6).

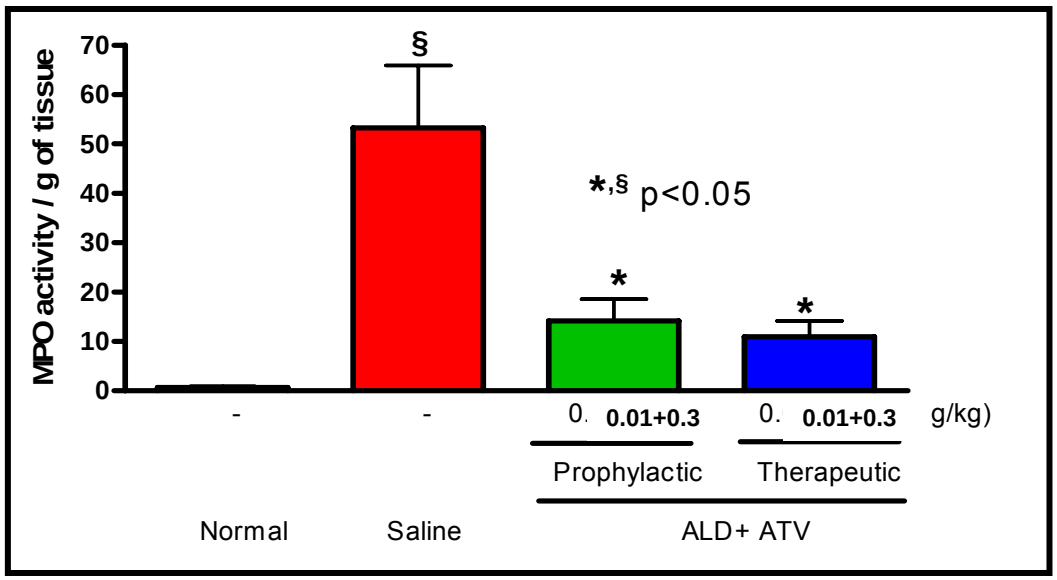

Fig. 6. Effect of prophylactic or therapeutic combination of lower doses of ALD+ATV on MPO activity. Bars represent Mean±SEM of MPO activity/g of gingival tissue of, at least, 6 rats per group. (*) indicates statistical difference when compared to Saline; (§) indicates statistical difference when compared to Normal. [Anova and Bonferroni's test] (P<0.05)

Serum dosage of Bone-specific Alkaline Phosphatase (BALP).

Serum dosages of BALP were analyzed (Table 2). The Saline group presented significant decrease by 51.9% on BALP serum level in the 11th day when compared to its baseline. The prophylactic and therapeutic combinations of lower doses (ALD 0.01+ATV 0.3), both prevented ($P<0.05$) the reduction of BALP serum levels when compared to data from Saline group, 11 days after periodontitis.

Serum dosage of Transaminases (AST and ALT) activity

Serum dosages of transaminases were analyzed, in animals that received Saline or the combination of ALD+ATV (Table 2). For AST and ALT serum levels there was no statistical difference in Saline group when data from day 11 were compared to its respective baseline. The prophylactic and therapeutic combinations of lower doses (ALD 0.01+ATV 0.3) did not cause any significant alteration on AST or ALT serum levels ($P>0.05$) (Table 2).

Hematologic study

Leukocytosis was observed at 6th h (23.4±1.0 cell x10^3/mm^3) after ligature (Fig. 7A), when compared to Normal animals not submitted to ligature-induced periodontitis (11.9±0.8). At this time, the observed leukocytosis was marked by neutrophilia (6.2±0.8) (Fig. 7B). Following, cells count at 2nd day achieved similar basal cell counts (13.1±0.8) ($P>0.05$), and new leukocytosis was observed at the 7th (16.2±0.8) and 11th (24.6±1.4) days, with predomination of mononuclear cells (Fig. 7C). The combination of lower doses (ALD 0.01+ATV 0.3) administered prophylactically (1.7±0.3) or therapeutically (1.5±0.2) prevented the neutrophilia at the 6th h when compared to Saline ($P<0.05$) (Fig. 7B). The combination the lower doses of ALD+ATV, in both regimens (Prophylactic= 15.7±2.2; Therapeutic= 14.9±1.5), also reduced ($P<0.05$) mononuclear cell counts at the 11th d when compared to Saline group (23.1±1.2) (Fig. 7C).

Table 2. Serum dosage of BALP, AST and ALT of animals submitted to periodontitis and receiving Saline or prophylactic or therapeutic combinations of lower doses (ALD 0.01+ATV 0.3).

	Groups / Days	Saline	ALD+ATV Prophylactic	ALD+ATV Therapeutic
BALP (U/L)	0	25.5±3.4	24.5±2.8	22.9±3.3
	11	13.2±1.7[§]	20.3±2.4*	28.1±3.2*
AST (U/L)	0	36.2±1.7	36.2±2.1	31.9±1.0
	11	36.9±2.7	41.6±1.7	40.3±3.0
ALT (U/L)	0	27.9±2.5	25.1±1.3	32.5±3.1
	11	25.3±3.2	28.6±2.4	32.9±2.0

Values represent Mean±SEM of, at least, 6 animals per group. ([§]) indicates statistical difference when compared to its respective baseline. (*) indicates statistical difference when compared to Saline 11 day data [Two-way Anova and Bonferroni test and Student´s t-test]. ($P<0.05$).

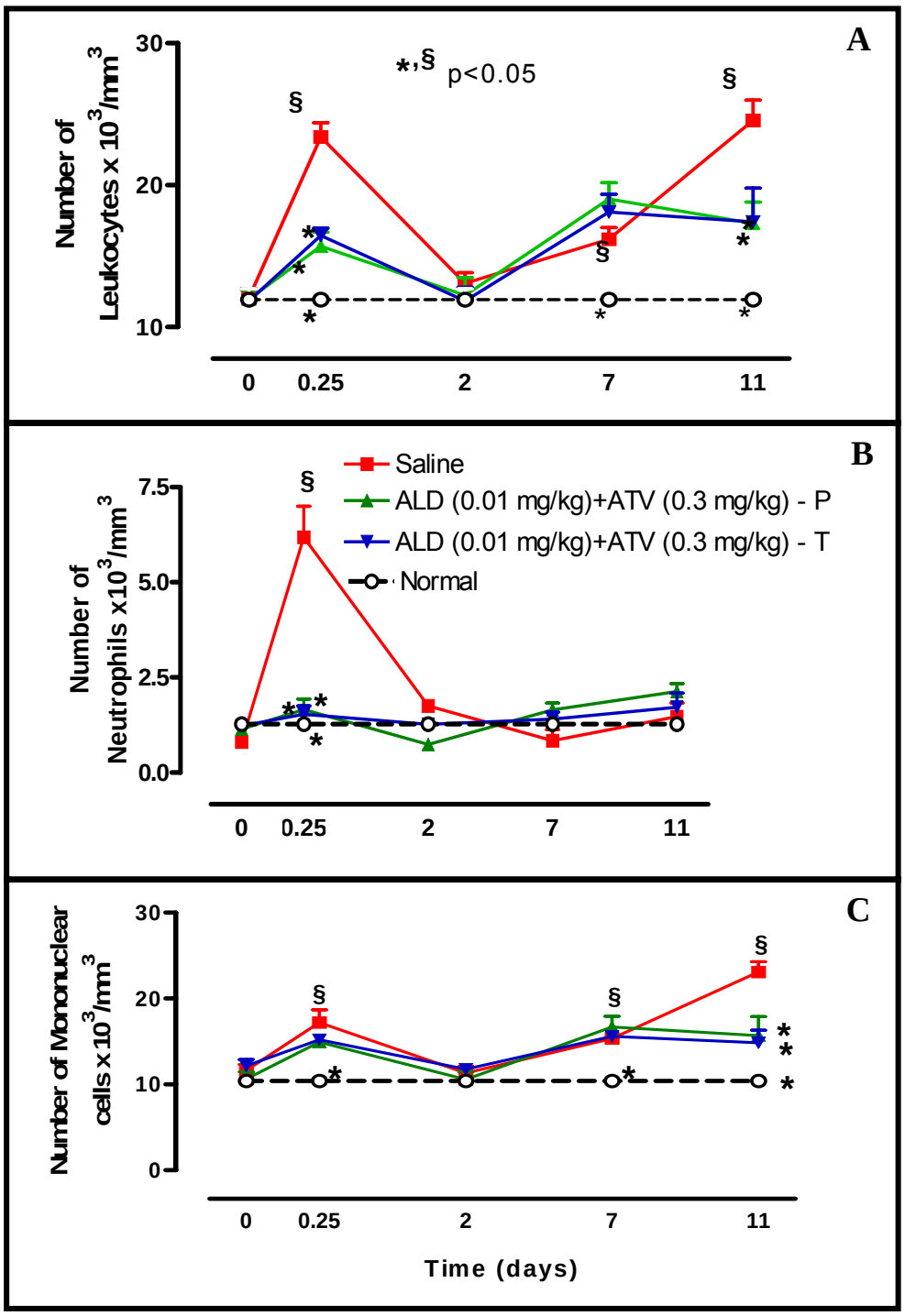

Fig. 7. Effect of prophylactic or therapeutic combinations of lower doses (ALD 0.01+ATV 0.3) on leukocyte counts. Points represent Mean±SEM of total leukocytes (**A**), neutrophils (**B**), mononuclear cells (**C**) x $10^3/mm^3$ of a minimum of 6 animals per group. (*) indicates statistical difference when compared to Saline group. (§) indicates statistical difference when compared to baseline [Anova and Bonferroni's test] (P<0.05)

Corporal mass variation

The periodontitis induction caused body weight loss starting on the first day, and lasting until the 3rd day (P<0.05). After that, animals gained weight with kinetic curve similar to that observed for normal animals. The prophylactic and therapeutic combinations of lower doses (ALD 0.01+ATV 0.03) did not prevent the initial body weight loss. However, the prophylactic combination initiated a greater weight gain of animals from the 8th day until the last day of the experiment (P<0.05), when compared to Saline group (Fig. 8).

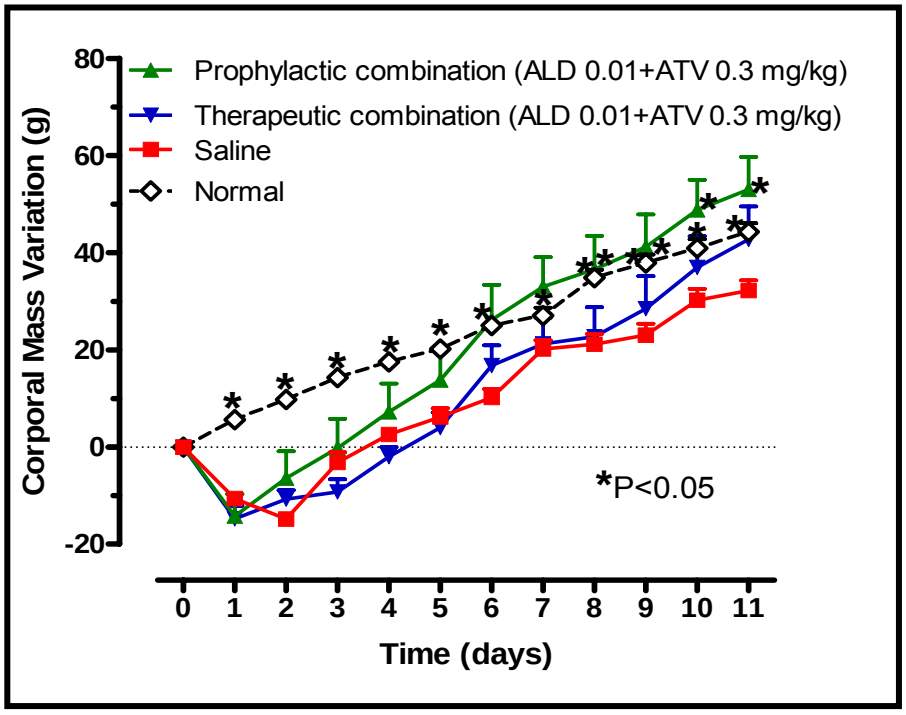

Fig. 8. Effect of prophylactic or therapeutic combinations of lower doses (ALD 0.01+ATV 0.3) on corporal mass variation. Points represent Mean±SEM of corporal mass variation of, at least, 6 animals per group. (*) indicates statistical difference when compared to Saline [Anova and Bonferroni's test] (P<0.05).

DISCUSSION

In this study, it was seen that the placement of a ligature caused intense alveolar bone loss (ABL). This finding was corroborated by microscopic and biochemical analysis. The histopathology showed an intense alveolar bone resorption, cementum loss and cell influx into the periodontium, added by the raise on MPO activity in the gingival tissue. Measurements in the proximal area, demonstrated augmentation on the distance between cementum-enamel junction and alveolar bone crest. Moreover, it was noted an intense immunostaining for TRAP, and decrease of BALP serum levels. These findings are in agreement with other authors,[24,20,25,26] which demonstrated that the induction of periodontitis caused loss of alveolar bone[24], cementum and periodontal ligament[24], and intense inflammatory infiltrate[24], decreased bone crest height[20] and increase on TRAP staining[25] and MPO activity[26]. Systemically, the periodontitis induction did not caused any liver alterations in animals, evaluated by transaminases serum levels. Leukocytosis, with significant neutrophilia at the 6th h and lymphomonocytosis at the 11th day, was also seen. An initial weight loss was observed, probably due to the trauma of ligature placement, followed by tendency to keep up with the rat normal weight curve. Taken together, this periodontitis model reproduced the changes previously reported in rats, with severe local inflammatory reaction and alveolar bone loss, coupled with leukogram alteration.[17,24 27-30]

Considering the role of the inflammation on bone loss, we decided to evaluate the effect of ALD and ATV in ligature-induced periodontitis in rats, a well-establish model to study periodontitis.[13,17,22,24-26,28-30] These drug were tested because Bisphosphonates have shown to be potent inhibitors of osteoclast-mediated bone resorption,[31] and Statins, beyond its prevention of cardiovascular disease,[32] have shown important pleiotropic effects, such as, anti-inflammatory, immunomodulatory, antithrombotic properties,[33-35] and more recently, anti-resorptive by protecting alveolar bone loss.[13,36]

In this study we observed that when ALD or ATV were administered as single therapy, only high doses of each drug were effective on preventing alveolar bone loss. The protective effect of these drugs may be due to mechanisms that inhibit inflammation and bone resorption. ALD has shown anti-inflammatory activity by inhibition of antigen presentation, growth, migration,

differentiation, and viability of macrophages,[37-39], reduction of IL-1β, IL-6, TNF-α and C-reactive protein (CRP),[40] and colagenase activity.[41] ATV has been reported to inhibit IL-6,[42] monocyte chemoattractant protein (MCP)-1 secretion,[43] inducible nitric oxide synthase (iNOS) immunostaining,[44] as well as cyclooxygenase (COX)-2 expression and matrix metalloproteinase (MMP)-9 activity.[45]

On bone tissue, nitrogen-containing Bisphosphonates (nBP) exert a well-known bone sparing effect by inhibition of farnesyl diphosphate synthase (FPPS), a key enzyme in the mevalonate pathway, that cause a shortage in farnesyl diphosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). This shortage in isoprenoids prevents isoprenylation of small GTPases like Ras, Rac and Rho,[46] which is believed to play a critical role in osteoclast-mediated bone resorption.[31] In the other hand, Statins has shown anabolic bone properties, preserving alveolar bone by stimulation of vascular endothelial growth factor (VEGF) expression in osteoblasts,[47] and bone morphogenetic protein (BMP)-2,[12] increasing RANKL/OPG ratio,[48] and OPG mRNA levels.[49] In addition to these effects, ALD and ATV, by acting on mevalonate pathway, interfere simultaneously, on cholesterol biosynthesis,[32,46] resulting on the inhibition of cholesterol and its metabolites. This effect may also contribute to alveolar bone protection, because cholesterol decreases osteoblasts activity and consequently avoids bone mineralization.[50]

Therefore, considering that BPs and Statins have a well-known anti-resorptive and anabolic bone properties in separate, and that both interfere on mevalonate pathway,[51] we speculated if the combination of these drugs could have any effect on bone metabolism.[52] From our knowledge, this is the first time that it has been reported the effect of Alendronate and Atorvastatin combination on alveolar bone loss.

In this study, all combinations of ALD+ATV showed significant alveolar bone protection when compared do SAL or low-doses monotherapy. Among these combinations, stood-out the one using the lower doses, which showed bone protection, that was not seen when these low doses were administered as monotherapy. In addition, low doses combination of ALD+ATV, did not cause important systemic alterations, so, we can infer that low doses combination of ALD+ATV, may be advantageous not only on controlling the

inflammation and the bone loss underlying periodontitis, but also in systemic parameters, such as transaminases, leukogram and corporal mass variation. This systemic safety was an important finding, since literature has pointed-out rhabdomyolysis as a side effect of ALD+ATV combination,[53] due to the potential that nBPs may have in enhancing the effect of Statins on lowering cholesterol, leading to abnormal membrane behaviors, affecting intracellular signaling and reducing mitochondrial respiratory function.[54] Nevertheless, this important side effect was suggested to have a dose-response relationship.[55] Therefore, the use of reduced doses, beyond therapeutic benefits, may also be important on the minimization of adverse effects.

Low doses combinations of ALD and ATV, administered prophylactically, showed significant bone protection when compared to Saline or low-dose monotherapy. When administered therapeutically, this chosen combination also prevented alveolar bone resorption. On the histometric study, the animals treated therapeutically, showed an even greater bone protection than the ones treated prophylactically, which may be related to the less amount of stress induced by manipulation or drug administration. Previous report have shown that stress can significantly increased bone loss[56,57] by a local increase in proinflammatory, such as IL-1β, -6 and IFNγ, and pro-resorptive factors (RANKL).[56]

In this study, the histological analysis showed that the combination of lower doses significantly preserved alveolar bone and reduced inflammatory infiltrate. In inflammation, the initial step is the leukocyte migration to the challenged site, and then, neutrophils, the first cell to migrate, release enzymes, as myeloperoxidase (MPO). MPO assay has been used as an index of neutrophil infiltration and as a marker for acute inflammation in various injuries when polymorphonuclear cell infiltration occurs.[58] According to our findings, we can say that the inflammatory infiltrate in the periodontitis observed by histology may be due to the presence of neutrophils in the periodontal tissue, and when the combination of drugs reduced the MPO activity, probably, it occurred because of the neutrophils reduction in the area. In fact, the anti-oxidative effect of Alendronate has been previously reported. It has been shown that non-chorinated BP, such as ALD, decreased MPO activity[59] and reduced neutrophil influx into rats gingiva submitted to *Porphyromonas gingivalis*-induced

periodontitis.[60] Atorvastatin has shown to improve abnormalities in the free radical system and supporting the antioxidative defense mechanisms *in vitro* and *in vivo*.[61-64] Cadirci and colleagues[65] have shown that reduction on MPO levels, after ATV-therapy, was attended by the concomitant decrease in the activities of antioxidant enzyme, superoxide dismutase (SOD). Statins also cause a dose-dependent inhibition on multiple steps of leukocyte recruitment and migration[66-68] which may reflect on MPO reduction.[44,69]

Taken together, we also observed that the combination of these drugs caused an important reduction on TRAP labeling. It has been described that TRAP is expressed by activated osteoclasts, and recently on macrophages, dendritic cells and a number of other cell types.[70] Therefore, TRAP assays have often been used to assess bone resorption.[71] It was demonstrated that the combined therapy administered whether prophylactically or therapeutically, markedly reduced TRAP expression. Then, our results are in accordance to other studies that have shown a reduction of TRAP labeling after ALD therapy,[72] because ALD is internalized by osteoclasts and inhibit bone resorption by indirectly prevention on protein isoprenylation, that cause osteoclast inactivation and apoptosis.[46,73] Statins had also reduced TRAP-positive multinucleated cells,[74] which indicate that the degree of bone formation is superior to that of bone resorption under the situation of low bone turnover in lower doses combination group.[75]

Considering BALP levels, this study revealed that treatment with lower doses combination prevented the decrease of this enzyme after 11 days of ligature-induced periodontitis. BALP is a enzyme highly expressed on osteoblastic differentiation and it is concentrated on the membranes of matrix vesicles, which appear to be required for the initiation of mineralization.[76] It is well-documented that BPs like ALD cause reduction of BALP serum levels,[77-80] however this effect is dose dependent, since lower doses of BPs can stimulate BALP activity.[82,83] On the other hand, Atorvastatin has been show to either do not alter[84] or to slightly increase BALP serum levels,[85,86] which may be explained by stimulation on BMP-2,[12] resulting in osteoblast differentiation.[49,86]

The BPs, as Alendronate, and Statins, as Atorvastatin, are drugs widely prescribed on clinical practice. Bisphosphonates are used to treat

metabolic bone disease, such as osteoporosis.[31] Statins competitively inhibit hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase and are used to lower blood LDL cholesterol levels, being important in the prevention of cardiovascular diseases.[32] Therefore, we sought to evaluate the possible systemic implications of the use of ALD and ATV combination. The combination of lower doses (ALD 0.01+ATV 0.3) did not prevent the loss of body mass observed in this study, which was probably due to the ligature trauma,[17] since it was seen that these drugs did not induced additional loss of weight, according to previous data.[18,87] Also, this combination administered either prophylactically or therapeutically did not change transaminases serum level. In fact, it has been demonstrated that ALD is not metabolized in liver.[88] Studies in patients with liver transplant demonstrate that ALD was well tolerated without deleterious effects on liver function tests (AST and ALT).[89] Patients taking ALD and diagnosed with primary biliary cirrhosis showed no significant effects regarding biochemical parameters of liver disease.[90] For Atorvastatin, the literature has reported that statins can induce asymptomatic mild elevation of serum transaminases, although it rarely requires withdrawal of therapy,[91] which support our idea of using combinations of low doses of these drugs.

Regarding the leukogram changes, the combination of drugs also inhibited 6th h neutrophilia, as well as 11th day lymphomonocytosis, observed on treated animals. ALD has shown to induce significant decrease on total white blood cells, neutrophil and lymphocyte counts in patients with Paget's disease.[92] ATV has also demonstrated to significantly reduce neutrophil migration.[93] Actually, it has been described that the recruitment and activation of polymorphonuclear neutrophils constitute the front line in the acute host inflammatory response, representing the main source of PGE_2, and promoting the initiation of bone metabolism breakdown by stimulating osteoclasts.[94] Therefore, the ability to reduce neutrophilia, seemed to be important to reduce inflammatory bone loss. The reduction on circulation mononuclear cells, which includes monocytes, it is also an important finding considering that circulating monocytes may differentiate locally to osteoclast, thereby exerting bone resorbing activity.[95] Thus, mononuclear cells reduction may contribute to bone sparing effect of lower doses combination in this model. Additionally, oral

treatment with Atorvastatin has shown to reverse hematological changes induced by inflammatory process.[96-98]

In summary, animals submitted to periodontitis and treated with the combination of lower doses of ALD and ATV, administered either prophylactically or therapeutically, showed reduction of the periodontal inflammation and alveolar bone loss, without important systemic changes, which may be an interesting approach as an adjuvant treatment of periodontitis.

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4 CONCLUSÃO GERAL

Em suma, os resultados deste estudo mostraram que considerando a literatura pertinente, o tratamento com os diversos BFs preveniu a reabsorção óssea alveolar, modulou a inflamação, reduziu a atividade de marcadores bioquímicos do metabolismo ósseo, com consequente melhora dos parâmetros clínicos periodontais.

O ALD, especificamente mostrou que apesar preveniu a redução de FAO, da POA e do infiltrado inflamatório, sem causar alterações sistêmicas.

O tratamento usando baixas doses da combinação (ALD+ATV), administrado profilática ou terapeuticamente, mostrou redução da inflamação periodontal e da POA, sem causar repercussões sistêmicas, sugerindo que esta combinação possa ser uma abordagem terapêutica interessante como adjuvante ao tratamento da periodontite.

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ANEXO

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DECLARAÇÃO

Declaramos que o protocolo para uso de animais em experimentação n°101/2009, sobre o projeto intitulado: **"EFEITO DA ASSOCIAÇÃO ENTRE ATORVASTATINA E ALENDRONATO DE SÓDIO NA PERIODONTITE POR LIGADURA EM RATOS."**, de responsabilidade de Paula Goes Pinheiro, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA).

Declaramos ainda que o referido projeto foi aprovado pela Comissão de Ética em Pesquisa Animal – CEPA – em reunião realizada em 07 de abril de 2010.

Fortaleza, 08 de abril de 2010.

Profa. Dra. Nylane Maria Nunes de Alencar
Coordenadora da Comissão de Ética em Pesquisa Animal – CEPA