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EFEITO ANTIINFLAMATÓRIO DA ATORVASTATINA NA PERIODONTITE INDUZIDA POR LIGADURA EM RATOS

FORTALEZA
2009

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Dissertação submetida à Coordenação do Programa de Pós-Graduação em Odontologia, da Universidade Federal do Ceará, como requisito parcial para a obtenção do grau de Mestre em Odontologia. Área de Concentração: Clínica Odontológica.

Orientadora: Profª Drª Vilma de Lima

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Dedicatória

À Valeria Goes (minha mãe)

“ ...Vê-la me faz crescer
Vê-la me faz ter fé
Vê-la me faz viajar
Vê-la me faz pensar em tanta coisa que eu nunca vim pensar
Vê-la me faz viver, vê-la me faz querer mudar
Ela é quem me dá asas pra voar...”
(Jorge Vercilo)

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Lista de Abreviaturas

RESUMO

Efeito Antiinflamatório da Atorvastatina na Periodontite induzida por ligadura em ratos. PAULA GOES PINHEIRO. Dissertação apresentada ao Curso de Pós-graduação em Odontologia do Departamento de Clínica Odontológica da Faculdade de Farmácia Odontologia e Enfermagem da Universidade Federal do Ceará, como pré-requisito para a obtenção do título de Mestre em Odontologia. Aprovação em 30 de Janeiro de 2009. Orientadora: Profª Drª Vilma de Lima.

A periodontite é uma doença caracterizada por infiltração de leucócitos, perda de tecido conjuntivo e reabsorção óssea. Estatinas são fármacos amplamente usados para o tratamento da hiperlipidemia, com destaque à Atorvastatina (ATV), dada seus efeitos pleiotrópicos importantes, como atividade antiinflamatória e capacidade anabólica óssea, com potencial para modificação do curso de doenças inflamatórias crônicas. O objetivo desse trabalho foi avaliar o efeito antiinflamatório da ATV, utilizando modelo de periodontite induzido por ligadura em ratos *Wistar* machos, distribuídos em grupos experimentais: controle (Salina a 0,9%), e 5 subgrupos (ATV 0,3; 1; 3; 9 ou 27 mg/kg), administrados por via oral, diariamente, 30 min antes da colocação do fio de náilon 3.0 em torno dos segundos molares superiores esquerdos dos animais, durante 11 d, quando, então, foram sacrificados, e os seguintes parâmetros, analisados: 1) Perda Óssea Alveolar (POA), avaliada através de estudos morfométrico, histológico e radiográfico; 2) Avaliação Sistêmica através de: a) Leucogramas realizados antes e após a ligadura (6 h; 2, 7 e 11 d); b) Variação de massa corpórea; c) Análises hepáticas e renais, por dosagens séricas bioquímicas e estudo histológico; e d) Avaliação sérica de Fosfatase Alcalina Óssea (FAO). Os animais submetidos a 11 d de periodontite apresentaram intensa reabsorção óssea. Baixa dose de ATV (0,3 mg/kg) não foi capaz de prevenir a POA ($p>0,05$), contudo, todas as demais (ATV 1, 3, 9 ou 27 mg/kg) foram, de forma significante, capazes de reduzir a POA em 35%, 39%, 53%, 56%, respectivamente. Tal inibição foi corroborada pela análise histopatológica, onde se observou que a ATV (27 mg/kg) causou maior preservação do tecido periodontal [Mediana: 1,5 (0-2)], quando comparada à Salina [Mediana: 3 (2-3)]. Adicionalmente, animais submetidos a 11 d de periodontite apresentaram redução significante de densidade radiográfica periodontal (58%). ATV (1, 3 ou 9 mg/kg) preservou tal densidade em 5%, 9% e 20%, respectivamente. O leucograma dos animais com periodontite apresentou pico de leucocitose na 6ª h, mediado por neutrófilos, e nova leucocitose a partir do 7º d, à custa de mononucleares. ATV (27 mg/kg) foi capaz de reduzir a leucocitose, reduzindo o número de neutrófilos ou mononucleares, respectivamente ($p<0,05$), bem como foi capaz de reduzir a perda inicial de massa corpórea vista na periodontite. As análises bioquímicas séricas e histológicas de fígado e rins dos animais com 11 d de periodontite tratada (ATV 27 mg/kg) ou não (Salina) não apresentaram alterações ($p>0,05$). Observou-se aumento nas variações de dosagens séricas de FAO dos animais com 11 d de periodontite (Salina: 63,4±10,8 U/l), enquanto que ATV (27 mg/kg) previniu tal aumento (13,6±3,5 U/l) ($p<0,05$). Dessa forma, os resultados demonstram que o modelo de periodontite em ratos reproduziu os principais aspectos da doença em humanos, e ATV reduziu a destruição periodontal, sem causar alterações significantes hepáticas ou renais, além de manter os níveis de FAO, o que sugere que a ATV pode ser uma abordagem farmacológica importante como adjuvante à terapia periodontal a ser ensaiada clinicamente, devido a sua eficácia e segurança.

Palavras-chave: Periodontite, Atorvastatina, Inflamação, Radiografia, Ratos.

ABSTRACT

Antiinflammatory Effect of Atorvastatin on Ligature-Induced Periodontitis in rats. PAULA GOES PINHEIRO. Dissertation presented to Dentistry Post-graduation course from Clinical Dentistry Department of Pharmacy, Dentistry and Nursing Faculty of Federal University of Ceara, as pre-requisite for Master Degree on Dentistry. Approved in January 30th 2009. Supervisor: Prof. Dr. Vilma de Lima.

Periodontitis is a disease characterized by leukocyte influx, loss of connective tissue and bone resorption. Statins are drugs widely used to hyperlidemia treatment, in which stand out Atorvastatin (ATV) due to its important pleiotropic effects, such as antiinflammatory activity and anabolic bone capacity, with great potential to modify chronic inflammatory disease course. In this way the aim of this work was to evaluate the aniinflammatory effect of ATV, through ligature-induced periodontitis model in rats. *Wistar* male, located in experimental groups: control (0.9% Saline), and 5 subgroups (ATV 0.3, 1, 3, 9 or 27 mg/kg), given orally daily, 30 min before nylon thread 3.0 aroud cervix of second left upper molars during 11 d, when then, rats were sacrified, and the following parameters were analyzed: 1) alveolar bone loss (ABL), evaluated through morphometric, histologic and radiographic studies; 2) Sistemic evaluation through a) leucograms performed before and after ligature (6h and 2, 7, and 11 d); b) corporal mass variation; c) of liver and kidney analysis, by serum biochemical dosage and histological study; and d) serum evaluation of Bone-Specific Alkaline Phosphatase (BALP). Animals submitted to 11 d periodontitis presented intense bone resoption. Low dose of ATV (0.3 mg/kg) was not able to prevent ABL ($p>0.05$), meanwhile the other dose ATV (1, 3, 9 or 27 mg/kg) were , in a significant way able to reduce ABL by 35%, 39%, 53%, 56%, respectively. Such inhibition was corroborated by histological analysis where was observed that ATV (27 mg/kg) caused greater periodontal tissue preservation [Mean 1.5 (0-2)], when compared to Saline [Mean 3 (2-3)] . In addition, animals submitted to periodontitis presented a significant reduction on periodontal radiographic density (58%). ATV (1, 3 ou 9 mg/kg) preserved such density in 5%, 9% e 20%, respectively. The leucogram of animals submitted to periodontitis presented leukocytosis peak on the 6th h mediated by neutrophils and new leukocytosis after 7th d due mononuclear cells. ATV (27 mg/kg) was able to reduce leukocytosis, decreasing neutrophils or mononuclear cells respectivelly ($p<0.05$), as well as, it was able to reduce initial corporal mass loss seen in periodontitis. Serum biochemical and histological analysis of liver and kidneys of animals with 11 d periodontitis treated with (ATV 27 mg/kg) or not (Saline), did not show alterations ($p>0.05$). It was observed a raise on serum BALP dosage variation of animals with 11 d periodontitis (Saline: 63.4±10.8 U/l), while ATV (27 mg/kg) prevented that increase (13.6±3.5 U/l) ($p<0.05$).In this way, the results demonstrated that this periodontitis model in rats reproduced the main aspects of periodontal disease in humans, and ATV reduced periodontal destruction, without cause significant alterations on liver and kidneys, besides of keeping BALP activitys, what suggests that ATV may be an important pharmacological approach as an adjuvant to periodontal therapy, to be evaluated clinically, due to its efficacy and safety.

Keywords: Periodontitis, Atorvastatin, Inflammation, Radiography, Rats

SUMÁRIO

1. INTRODUÇÃO GERAL

A doença periodontal encontra-se entre as duas maiores doenças orais que afetam a população humana e, em todo o mundo, apresenta-se com altas taxas de prevalência (PETERSEN & OGAWA, 2005). Esse termo usualmente se refere a uma variedade de patologias que acometem tecidos de proteção, como a gengiva, ocasionando as chamadas gengivites, e os tecidos de sustentação, que incluem o osso alveolar, cemento radicular e o ligamento periodontal, determinando as variadas formas de periodontites (PIHLSTROM *et al.,* 2005).

À medida que a periodontite evolui, pode-se observar destruição progressiva de tecidos e conseqüente perda dentária. Diversos estudos têm sido realizados, e atualmente está bem descrito que para o desencadeamento destes eventos é fundamental a presença de periodontopatógenos nos sítios periodontais (TELES *et al.*, 2006). Embora a colonização periodontal por bactérias destruidoras de tecido seja importante para o estabelecimento da periodontite, sabe-se, contudo, que a susceptibilidade do hospedeiro é extremamente necessária para o aparecimento dos sinais clínicos da doença, inerentes ao desequilíbrio nos processos de homeostase óssea (PIHLSTROM *et al.,* 2005).

O processo de remodelagem óssea compreende um equilíbrio dinâmico entre osteoblastos, envolvidos na formação óssea, e osteoclastos, os quais são responsáveis pela reabsorção óssea. Tais fenômenos são mediados pelo sistema constituído pelo Receptor Ativador do Fator Nuclear-κB (RANK), pelo Ligante do Receptor Ativador do Fator Nuclear-κB (RANKL) e pela Osteoprotegerina (OPG) (RANK/RANKL/OPG) (XING *et al.*, 2005; REID & HOLEN, 2009).

A OPG, uma glicoproteína produzida por osteoblastos, pertence à superfamília de Receptores do Fator de Necrose Tumoral (TNFR). O RANKL, uma citocina da família TNF, é expressa por osteoblastos como uma proteína transmembrana e se liga ao seu receptor RANK na superfície de osteoclastos e de precursores de osteoclastos. Isso resulta na ativação de vias de sinalização que conduzem à formação, diferenciação e ativação de osteoclastos e, conseqüente, reabsorção óssea (REID & HOLEN, 2009). Para regular o

balanço entre formação e reabsorção ósseas, a interação RANKL-RANK é inibida pela OPG, visto que a OPG atrai receptor e se liga como homodímero à estrutura homotrimérica de RANKL, prevenindo, assim, a ativação osteoclástica. Em outras palavras, a homeostase óssea é mantida através da OPG que inibe a osteoclastogênese por meio de ligação competitiva com RANKL (REID & HOLEN, 2009; SOEDARSONO *et al*., 2006).

Entretanto, durante um processo inflamatório crônico, onde se observam, dentre vários mediadores químicos, altos níveis de citocinas como TNF, ocorre a expressão abundante de RANKL. A superexpressão de RANKL determina uma inibição da ação da OPG, provocando, portanto, um maior desequilíbrio a favor de reabsorção óssea (REID & HOLEN, 2009). A periodontite é uma doença inflamatória crônica, onde se observa uma intensa desordem, conduzindo à perda óssea em torno dos dentes. Estudos têm demonstrado, inclusive, que um dos principais agentes causais da doença, a *Porphyromonas gingivalis*, é capaz de liberar proteases que tem sido relacionadas à degradação da forma recombinante de OPG (KOBAYASHI-SAKAMOTO *et al*., 2004).

O processo inflamatório periodontal tem como objetivo inicial a eliminação de bactérias e toxinas presentes nos tecidos subjacentes, diminuindo os danos decorrentes da inflamação mantida ou não controlada. Paradoxalmente, nas formas crônicas de periodontite, observa-se a superexpressão de mediadores químicos e a exacerbação das respostas imunoinflamatórias, o que conduz à destruição de tecido de suporte dentário e a alterações potencialmente irreversíveis (SHAPIRA *et al.,* 2005).

Vários são os mediadores químicos secretados quando o estímulo inflamatório é difundido no periodonto (MADIANOS *et al.,* 2005), tais como TNF (NILSSON & KOPP, 2008) e interleucina (IL)-1 ou IL-6 (FERREIRA *et al.,* 2008; NIBALI *et al.*, 2008), metaloproteinases de matriz (MMPs) (GENDRON *et al.,* 2004), prostaglandinas (PGs) (INABA *et al.*, 2008) e óxido nítrico (NO) (DI PAOLA *et al.,* 2004). Estes mediadores são encontrados em abundância no fluido crevicular e têm sido fortemente relacionados à destruição tecidual e, quando associados a moléculas quimiotáticas (MCP), estimulam a expressão de selectinas (E e P) e moléculas de adesão intercelular e vascular (ICAM e

VCAM) na parede endotelial (HUANG *et al.*, 2008), as quais medeiam a migração de leucócitos para o sítio infectado (KANTERS *et al.*, 2008). Ainda no periodonto, os neutrófilos contribuem para a destruição tecidual, induzindo novamente a produção de espécies reativas de oxigênio (ROS), como NO, e outras citocinas, que amplificam a resposta inflamatória (SALVEMINI *et al*., 2003).

Um aspecto importante relacionado às periodontites consiste em seu diagnóstico, tratamento, controle e manutenção. Assim, exames clínicos, especialmente associados a exames complementares auxiliares, são imprescindíveis para o diagnóstico precoce e preciso sobre o estágio da gravidade da doença. Parâmetros diversos como presença de sangramento à sondagem periodontal, profundidade de bolsas, cálculo dentário, biofilme bacteriano, bem como aspecto clínico do periodonto e o nível clínico de inserção, dentre outros, podem ser observados através do exame de sondagem e análise visual (PIHLSTROM *et al.,* 2005).

Entretanto, como forma auxiliar de se avaliar o grau de perda óssea periodontal, imagens radiográficas podem ser obtidas, visto que apresentam e propiciam maior riqueza de detalhes quanto à qualidade e quantidade de suporte ósseo (KHOCHT *et al.,* 2003). Entre os tipos de exames, as imagens radiográficas digitais vêm assumindo posição de destaque na odontologia e, principalmente, na periodontologia (VAN DER STELT, 2005), pois apresentam maior capacidade de detecção de sítios com perdas ósseas ainda sutis, quando comparadas às imagens radiográficas convencionais (KHOCHT *et al.,* 2003), o que favorece, conseqüentemente, diagnóstico e terapia precoces.

Durante muito tempo, a base do tratamento periodontal objetivou o controle da placa bacteriana (BOEHM & SCANNAPIECO. 2007). No entanto, em alguns casos de periodontite tratados de forma convencional, através de controle de placa bacteriana juntamente com raspagem e alisamento radiculares, não se mostram com prognóstico favorável ao controle da progressão da doença, requerendo, portanto, terapias adjuvantes (BUDUNELI *et al.,* 2007). Considerando o papel proeminente do hospedeiro, como principal componente da destruição de tecidos moles e duros vista na periodontite, estratégias terapêuticas, como a modulação farmacológica da resposta do

hospedeiro, têm se sobressaído como uma nova abordagem de tratamento (BUDUNELI *et al.,* 2007; PRESHAW *et al.*, 2004).

Inibidores da enzima 3-hidroxi-3-metilglutaril coenzima A (HMG-CoA) redutase ou estatinas são fármacos amplamente utilizados no tratamento da hiperlipidemia e aterosclerose, por causar redução nos níveis sanguíneos de colesterol (KRONMANN *et al.*, 2007). Alguns estudos vêm demonstrando que as estatinas, por sua vez, também apresentam efeitos pleiotrópicos não relacionados a sua capacidade hipolipemiante (KRONMANN *et al.*, 2007), dentre eles, destacam-se a atividade antiinflamatória (NICHOLLS *et al.*, 2006) e a capacidade anabólica em tecido ósseo (MUNDY *et al.*, 1999). 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.

Os efeitos secundários das estatinas estão intimamente relacionados ao seu grau de solubilidade. Estatinas lipofílicas apresentam maior potencial osteogênico (IZUMO *et al.,* 2001), bem como, exercem maior influência na via regulatória de monócitos que regulam a produção de citocinas, induzindo uma reposta inflamatória mais controlada tanto *in vivo* como *in vitro* (KIENER *et al.*, 2001). Dentre as estatinas, a Atorvastatina (ATV) é o agente que mais tem se destacado (SCHACHTER, 2005), não apenas por sua lipofilicidade, mas, também pelos poucos efeitos adversos apresentados e melhor relação custo-benefício (COSTA-SCHARPLATZ *et al.*, 2008), justificando, portanto, o amplo uso da ATV na prática clínica (PLOSKER & LYSENG-WILLIAMSON, 2007), inclusive em doenças como, por exemplo, artrite reumatóide (McCAREY *et al.,* 2004).

Nesse contexto, parece interessante que condições que envolvam alterações ósseas, como as periodontites, artrite ou osteoporose em pacientes normossitêmicos ou que também apresentem quadro de dislipidemia, de tal forma que necessitem um tratamento de base com ATV ou outras estatinas, possam ser avaliadas sob o aspecto protetor deste agente, diante da fisiopatologia de doenças ósseas e não apenas dislipidêmicas. Dessa forma, estudos que venham a contribuir para uma maior compreensão de mecanismos específicos, dessa relação, devem ser encorajados.

2. PROPOSIÇÃO

Os objetivos do presente trabalho, segundo cada um dos artigos relacionados adiante, foram:

1. Avaliar o efeito antirreabsortivo da Atorvastatina na periodontite experimental induzida por ligadura em ratos, através de:
    a. Análises macroscópica e histológica da perda óssea alveolar
    b. Avaliação de parâmetros sistêmicos como leucograma, variação de massa corpórea, alterações hepáticas e renais, e dosagens séricas de Fosfatase Alcalina Óssea.

2. Avaliar o efeito da Atorvastatina na densidade radiográfica na perda óssea alveolar induzida em ratos, através de:
    a. Análises comparativas de densidade radiográfica e mensuração macroscópica de perda óssea alveolar.

III – ARTIGOS CIENTÍFICOS

Esta dissertação 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 dissertações de Mestrado e 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 devidamente aprovados pelo Comitê de Ética em Pesquisa com Animais da Universidade Federal do Ceará (Anexo 1).

Dessa forma, a presente dissertação é composta por dois artigos científicos redigidos de acordo com as revistas científicas escolhidas para as devidas publicações, como apresentados adiante:

- ✓ **Artigo 1:**

"Anti-resorptive Effect of Atorvastatin on Ligature-induced Periodontitis in Rats". Goes P, Lima APS, Lima NA, Melo IM, Benevides NMB, Brito GAC, Alencar NMN, Rego ROCC, Lima V.

Este artigo seguiu normas de publicação do periódico ***European Journal Oral Science*** (ISSN 1600-0722).

- ✓ **Artigo 2:**

"Effect of Atorvastatin in Radiographic Density on Alveolar Bone Loss in Rats". Goes, P, Lima APS, Melo IM, Rego ROCC, Lima V.

Este artigo seguiu normas de publicação do periódico ***Brazilian Oral Research*** (ISSN 1806-8324).

ARTIGO 1

Anti-resorptive Effect of Atorvastatin on Ligature-induced Periodontitis in Rats.

Paula Goes[1], Ana Patrícia Souza Lima[1], Neiberg Alcântara Lima[2], Iracema Matos Melo[2], Norma Maria Barros Benevides[3], Gerly Anne de Castro Brito[4], Nylane Maria Nunes Alencar[2], Rodrigo Otávio César Citó Rêgo[5], Vilma Lima[2,*].

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Running title: Atorvastatin effect on rat periodontitis

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ABSTRACT

Periodontitis is an inflammatory disease, characterized by alveolar bone loss (ABL). Atorvastatin (ATV), or HMG-CoA reductase inhibitor, is widely used on hyperlipidemia treatment, and has shown pleiotropic effects, as antiinflammatory and anabolic bone activity. This study aimed to evaluate the anti-resorptive effect of ATV on periodontitis. Periodontitis was induced by ligature in molar of rats for 11d. Animals received orally 0.9% Saline (0.5 ml) or ATV (0.3, 1, 3, 9 and 27 mg kg^{-1}). ABL was evaluated through morphometric and microscopical analysis. To verify possible systemic repercussions, leukogram, corporal mass variation, liver and kidneys conditions, as well as, serum bone-specific alkaline phosphatase (BALP) activity were analyzed. Animals submitted to periodontitis presented intense ABL on 11^{th} d. The low dose of ATV (0.3 mg kg^{-1}) did not show bone protection ($p>0.05$), however ATV (1, 3, 9 or 27 mg/kg) significantly reduced ABL, by 35%, 39%, 53%, 56%, respectively. This inhibition was corroborated by histological analysis. ATV (27 mg/kg) also reversed leukocytosis, maintained the serum BALP activity, did not affect either liver and kidney, or body mass weight. In conclusion, ATV efficiently and safety, reduced ABL, suggesting that ATV may be an important tool as an adjuvant on periodontal therapy.

Key-words: Atorvastatin; bone loss; inflammation; periodontitis; animal model.

INTRODUCTION

Periodontitis is an inflammation that extends deep into the tissues and causes loss of supporting connective tissue and alveolar bone (1). This disease is one of the two major dental problems that affect human population at high prevalence rates (2). Nowadays, periodontal disease is understood as a result of a complex interplay between bacterial infection and host response, modified by behavioral factors (3). Periodontal inflammatory process initially has a protective role against bacterial invasion, but then, becomes destructive due to prolonged overexpression of harmful mediators (4), such as interleukin (IL-1) and tumoral necrosis factors (TNF) (5,6), and reactive oxygen species (ROS) (7), among others. These mediators, therefore, stimulate expression of leukocyte adhesion molecules. Selectins, intercellular and vascular adhesion molecules (ICAM and VCAM) act promoting neutrophil transmigration (8) that contributes to tissue destruction inducing de novo production of ROS and cytokines, which further amplify inflammatory response (9).

Statin or 3-hidroxy-3-methylglurayl coenzyme A (HMG-CoA) reductase inhibitor is a well-established pharmaceutical agent that effectively lower serum cholesterol levels, being therefore, widely prescribed for hypercholesterolemia treatment and atherosclerosis (10,11). In fact, recent studies have focused on the ability of statins to modulate chronic inflammatory diseases, such as multiple sclerosis (12). In animal models of the latter condition, atorvastatin, a statin of long duration, prevented or reverted chronic and relapsing paralysis and inhibited the secretion of cytokines IL-2, IL-12, TNF-α, and IFN-γ (13).

Moreover, statins do more than just reduce the burden of atherosclerosis and its consequences (10). They have pleiotropic effects, including antiinflammatory action and anabolic effect on bone tissue (14). It has been demonstrated that the basis of antiinflammatory activity of statins are the inhibition of ICAM, VCAM and selectins (15), cytokines, as IL-1 and TNF (16), besides ROS (17). Additionally, these drugs also promote expression of osteoblastic differentiation stimulators, as bone morfogenetic protein-2 (BMP-2), and other bone anabolic factors like vascular endothelial growth factor (VEGF)

(18). In the midst of various statins, Atorvastatin (ATV) stand out not only due to its lipofilicity, which is closely linked to pleiotropic effects (19), but also due to few adverse effects and better cost-effectiveness relationship (20) when compared to other statins (21) being, therefore, widely used on clinical practice (22).

Considering mainly pleiotropic effects of statins, this study was designed to evaluate the anti-resorptive effect of Atorvastatin on the inflammatory response and bone loss in an experimental model of periodontitis in rats.

MATERIAL AND METHODS

Animals

Forty-eight male *Wistar* rats (±200 g) (*Rattus novergicus*) from the Federal University of Ceará were used in this study. Animals were maintained on specific cages in temperature-controlled rooms, with free access to food and water during the whole experiment. All procedures and animal treatment conducted in order to reduce the number of animals and their suffering, were approved by Institutional Ethics Committee of Federal University of Ceará (Protocol number 74/07).

Experimental Protocol

Periodontitis Model

A model for experimental periodontal disease in rats was used as described previously (23). Briefly, rats were anesthetized with chloral hydrate (300 mg kg^{-1}, i.p.), and a nylon (000) (Point Suture,Point Suture do Brasil Fortaleza-CE, Brazil) thread ligature was placed around the cervix of the second left upper molar. The ligature was then knotted on the vestibular side of the tooth. The contralateral right side was used as the unligated control. Rats were weighed daily until to 11^{th} day, which demonstrated the apex of alveolar bone loss, and then, animals were killed (23).

Experimental Groups

The animals were divided into 2 groups, both submitted to

periodontits. One of them (control) received 0.9% Saline solution (0.5 ml; v.o.) 30 min before the ligature. The other one (test) was subdivided into 5 more groups, which received Atorvastatin, on doses of 0.3, 1, 3, 9 and 27 mg kg^{-1}, respectively, given orally 30 min before ligature. After this procedure, both groups received daily Saline or Atorvastatin, respectively, until the 11^{th} d. Atorvastatin (Lipitor®, Pfizer; São Paulo-SP, Brazil), presented as 10 mg tablet, was macerated and dissolved in distilated water.

1. Alveolar bone structure loss

1.1 Morphometric analysis of alveolar bone

On the 11^{th} day, animals were sacrificed under anesthesia (10% Chloral Hydrate), and had their maxillae removed and fixed in 10% neutral formallin for 24 h. Following, maxillae were splited in half, dissected, and stained with 1% methylene blue in order to differentiate bone from teeth (6, 23) In order to quantify alveolar bone loss (ABL), hemimaxillae were adjusted in microscope slides to be photographed with digital camera (Sony Cyber-Shot® model DSC-W80; Hong Kong, China). The acquired image was sent to the computer programm Image J® (ImageJ 1.32j, National Institute of Health; EUA) for horizontal alveolar bone loss analysis, which was measured using a modification of the area method of KUHR *et al.,* 2004 (24). For this, measurements were made along the region between the molar cusp tip and the alveolar bone crest (Fig 1B), and subtracted from the respective area of contralateral normal hemimaxilla (unligated control) (Fig. 1A). All obtained images were compared to well-known area (0.5x0.5 mm^2).

1.2. Histological analysis of alveolar bone

Extra groups of 6 animals with periodontitis that had received Saline or ATV (27 mg kg^{-1}) were sacrificed as described above and had their maxillae excised. The specimens were fixed in 10% neutral buffered formallin and demineralized in 10% nitric acid. Following this, the specimens were dehydrated, embedded in paraffin, and sectioned along the molars 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 (x40). Parameters such as inflammatory cell infiltration, osteoclast number, and alveolar bone and cementum integrity, were determined in a single-blind manner and graded, on a score of 0–3 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 (23).

2. Sistemic Parameters Analysis

2.1. Hematologic Study and Corporal Mass Variation

The method used for the analysis of white blood cell counts was as follows: 20 μl of blood, taken from the rat tail, was added to 380 μl Turk solution. Total and differential white blood cell counts were performed using a Neubauer chamber and stained smears by rapid Instant Prov Stain Set (Newprov Produtos para Laboratório; Pinhais-PR, Brazil), respectively. Leukogram of the groups of animals (Saline and ATV 27 mg kg^{-1}) was performed before periodontitis induction, 6 h and 2, 7 and 11 d after the ligature. Also, animals from group Saline and ATV 27 mg kg^{-1} had their body mass measured before periodontitis induction, and after that daily until the 11^{th} d. Values were expressed as body mass variation (g) compared to initial body mass.

2.2. Evaluation of Liver and Kidney Function

A. Serum Biochemical Parameters

On the zero time (basal levels) and at the 11^{th} d of the assay, blood samples were collected from orbital plexus of anesthetized animals (Saline and ATV 27 mg kg^{-1}). Liver function was evaluated through serum dosage of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Total Alkaline Phosphatase (TAP). Activity of Urea and Creatinin were evaluated as renal function markers. Specific kits were used, and methodology followed manufactor orientations (Labtest®; Lagoa Santa-MG, Brasil). Values were

expressed as serum dosage variation obtained on 11th d and compared to baseline of each animal.

B. Histological analysis of liver and kidney

On the 11th day, the animals (Saline and ATV 27 mg kg^{-1}) killed for maxillae removal, also had their liver and kidney collected and fixed in 10% neutral formallin for 24-48 h period. Specimens were included in paraffin and serial sections of 4 μm thickness were obtained for hematoxillin and eosin (H&E) staining. Analyses were made through optical microscopy.

Liver parameters based on presence and amout of collagen fibers were determined in a single-blind manner and graded, on a score of 0-4 based on the findings intensity, as follows: *Score 0:* normal; *Score 1:* fibrosis present, collagen fibers present that extend from the portal triad or central vein to the peripheral region; *Score 2:* mild fibrosis, some extended collagen fibers present without compartmental formation; *Score 3:* moderate fibrosis, moderate amounts of collagen fibers present with some pseudolobe formation; *Score 4*: severe fibrosis, abundant collagen fibers present with a thickening of the partial compartments and frequent pseudolobe formation (25).

Kidneys parameters, such as protein/cellular casts in proximal tubule; cortical proximal convoluted tubule necrosis; pallor of outer stripe of proximal tubule; intracellular mineralization; nuclear pyknosis; interstitial nephritis were also determined in a single-blind manner and graded, on a score of 0-3 based on the findings intensity, as follows: *Score 0:* normal; *Score 1:* Mild; *Score 2:* Moderate; *Score 3:* Severe (26).

2.3. Serum dosage variation of Bone-Specific Alkaline Phosphatase (BALP) activity

Blood samples were collected from orbital plexus of anesthetized animals (Saline and ATV 27 mg kg^{-1}) before the experiment and on the 11th d. The Bone-Specific Alkaline Phosphatase (BALP) was evaluated using the thermoactivation method, by heating the sample into 56 ℃ for 10 min (27), since BALP is a thermosensible isoform of Total Alkaline Phosphatase (TAP). When TAP is subtrated from Heat Alkaline Phosphatase (HAP), it results in

Bone Alkaline Phosphatase (BALP). Methodology to evaluate the enzymes followed the manufactor orientations (Labtest®: Lagoa Santa-MG, Brasil). Values were expressed as serum variation activity compared to baseline.

3. Statistical Analysis

The data are presented as means±standard error of the mean (SEM) or medians (and range), where appropriate. Univariate analysis of variance (Anova), followed by Bonferroni's test, was used to compare means, and Kruskal-Wallis and Mann Whitney tests were used to compare medians. A *P*-value of <0.05 was considered as indicating significant differences.

RESULTS

1. Alveolar bone structure

1.1 Morfometric analysis of bone tissue

In preliminary experiments, we confirmed that the bone changes observed peaked at 11 days of periodontitis (data not shown), as showed by other authors (23). Therefore, for the analysis of drug treatment, alveolar bone loss in the buccal side was measured at this time. Analysing of bone tissue through morphometric measurement (Fig. 2) we showed that ligature-induced periodontits in rats receiving only Saline during 11 d caused intense alveolar resorption (4.19±0.3 mm^2) (Fig. 2), when compared to the normal hemimaxillae (Fig. 3A). The hemimaxilla submitted to ligature (Saline group) presented classical clinical signs of periodontitis such as root exposure, furcation lesion, intense alveolar resorption, and lack of proximal contact (Fig. 3C). In the other hand, Atorvastatin (ATV 1, 3, 9, or 27 mg kg^{-1}) treatment tended to elicited a significant (*P*<0.05) alveolar bone protection in a dose-dependent manner (Figs. 2 and 3E), reducing alveolar bone loss by 35%, 39%, 53%, 56%, respectively. The minor dose of ATV (0.3 mg kg^{-1}) .ATV 0.3 mg kg^{-1} of ATV was not able to protect alveolar bone (4.16±0.3 mm^2) (Fig. 2).

1.2. Histopathological analysis of alveolar bone

Hemimaxillae from 6 animals per group that received Saline or ATV (27 mg kg^{-1}) submitted to ligature-induced periodontitis and the Normal ones

were processed for histopathological analysis (Table 1). Some alterations (P<0.05) were observed on Saline group, characterized by alveolar bone and cementum resorption, associated to important inflammatory influx of leukocytes, [Median score of 3 (2-3)] (Table 1; Fig. 3D), when compared to normal periodontium [Median score of 0 (0-0)] (Table 1; Fig. 3B). ATV (27 mg kg^{-1}) treatment significantly (P<0.05) showed an absence cellular inflammatory infiltration, and a preservation of the periodontal ligament, alveolar process and cementum [Median score of 1.5 (0-2)] (Table 1; Fig. 3F), when compared to the Saline group.

2. Sistemic Parameters Analysis

2.1. Hematologic Study and Corporal Mass Variation

In order to verify a possible systemic repercussion of ligature-induced periodontitis, leukocyte counts were performed, and body weight was measured. All experimental groups presented the similar leukocyte levels or corporal mass on day 0 (P>0.05) (Figs. 4 and 5). It was observed that ligature-induced periodontitis caused a leukocytosis (P<0.05), at 6 h (19.5±0.9 leukocytes x 10^3 mm^{-3}) (Fig. 4A), marked by neutrophils (6.8±0.9 x 10^3 mm^{-3}) (Fig. 4B). On the 2^{nd} d leukocytes tended to normal levels (Fig. 4A). Following, the new leucocytosis (P<0.05), at 11^{th} d (18.8±1.5 leukocytes x 10^3 mm^{-3}) was represented by mononuclear cells (15.8±0.6 x 10^3 mm^{-3}) (Fig. 4C). ATV (27 mg kg^{-1}) reduced (P<0.05) the leukocytosis at 6 h occurring in rats submitted to periodontitis (14.8±1.5 leukocytes x 10^3 mm^{-3}), as well as neutrophil cells (4.4±0.4 x 10^3 mm^{-3}) (Figs. 4A and B)., and also reduced the mononuclear cells at 11^{th} d (12.1±1.3 x 10^3 mm^{-3}), when compared to Saline (Figs. 4A and C). Figure 5 shows that periodontitis caused a significant loss (P<0.05) in body weight starting on day 2, which persisted during the 11 d of observation, in comparison to normal animals. ATV did not alter the loss in body weight observed in animals submitted to periodontitis.

2.2. Serum Biochemical Parameters and Histological analysis of liver and kidney

Animals submitted to 11 d ligature-induced periodontitis had their

serum biochemical dosage variation analyzed either in liver (AST and ALT) or kidney (Urea and Creatinin) activity. ATV 27 mg kg^{-1} did not alter these serum dosages in animals submitted to periodontitis, when compared to non-treated rats (Saline), except for serum Creatinin activity (Table 2). So, to confirm the non-toxicity of ATV, histological analysis of liver and kidneys were performed. It was observed that any alterations were found, since that evaluating serial slices of both organs after 11 d of ATV (27 mg kg^{-1}) therapy, it as possible to notice total absence of liver fibrosis, as well as the normal aspect of kidney, when compared to Saline or normal animal organs (data not shown).

Besides, serum TAP activity variation after 11 d periodontitis showed, although not significant, lower variation, when compared to baseline.

2.3. Serum dosage of Bone-Spefic Alkaline Phosphatase (BALP)

To confirm that the minor alteration in serum TAP activity, serum BALP activity were evaluated in both groups of animals (Saline and ATV 27 mg kg^{-1}) submitted to 11 d periodontitis (Table 2). Non-treated animals presented a great variation from day 0 to day 11 (63.35±10.76 U/l), while animals treated with ATV 27 mg kg^{-1} showed low minor variation (13.60±3.462 U/l). When treated group was compared to Saline it was possible to see verify a statistical significance.

TABLES

Table 1. Microscopic analysis of rat hemimaxillae submitted to periodontitis.

	Normal	**Saline**	**ATV 27** (mg kg^{-1})
Scores	0 (0-0)	3 (2-3)*	1.5 (0-2)$^{\#}$

Ligature-periodontitis was induced in rats. Animals were examined at day 11. Data represent the median values (and range) of microscopic scores in 6 animals per group. *P<0.05 compared to normal contralateral; $^{\#}P$<0.05 compared to Saline (Data were analysed by using Kruskal-Wallis and Mann Whitney tests).

Table 2. Serum variation of biochemical dosages of animals submitted to ligature-induced periodontitis.

Biochemical dosages	**Unit**	**Saline**	**ATV 27** (mg kg^{-1})
AST	U/l	19.89±9.07	13.29±5.33^{ns}
ALT	U/l	0.61±2.84	1.43±1.35 ^{ns}
TAP	U/l	50.47±6.22	37.15±9.64^{ns}
Urea	mg/dl	0.66±1.47	3.53±1.25 ^{ns}
Creatinin	mg/dl	0.23±0.05	0.07±0.10*
BALP	U/l	63.35±10.76	13.60±3.46*

Ligature-periodontitis was induced in rats. Animals were examined at day 11. Activity of Aspartate Amino Transferase (AST), Alanina Aminotransferase (ALT), Total Alkaline Phosphatase (TAP), Urea, Creatinin, and Bone-specific Alkaline Phosphatase (BALP). *P<0.05 compared to Saline; ns= non-significant (Data were analysed by using *t*-Student test).

FIGURES

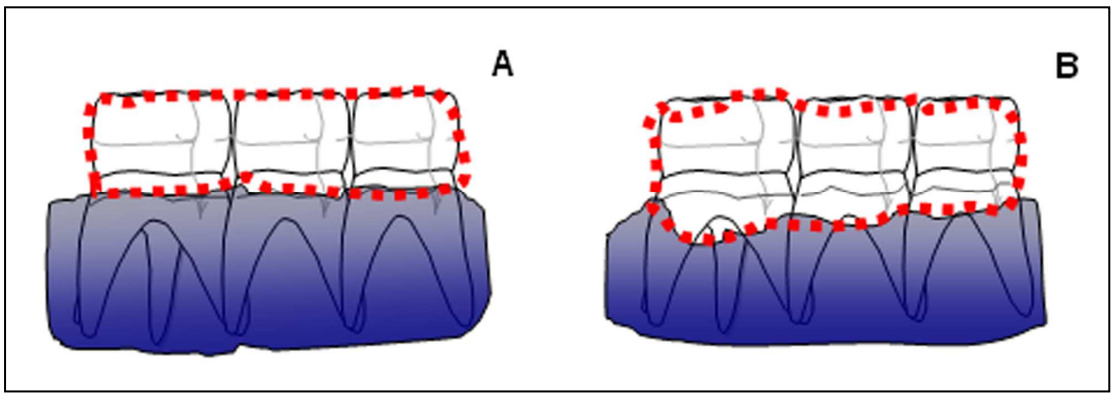

Fig. 1. Representation of the demarked area for alveolar bone resorption measurement. Area of contralateral hemimaxilla (A) and its hemimaxilla with periodontitis (B), whose difference was considered as value of alveolar bone resorption (mm^2).

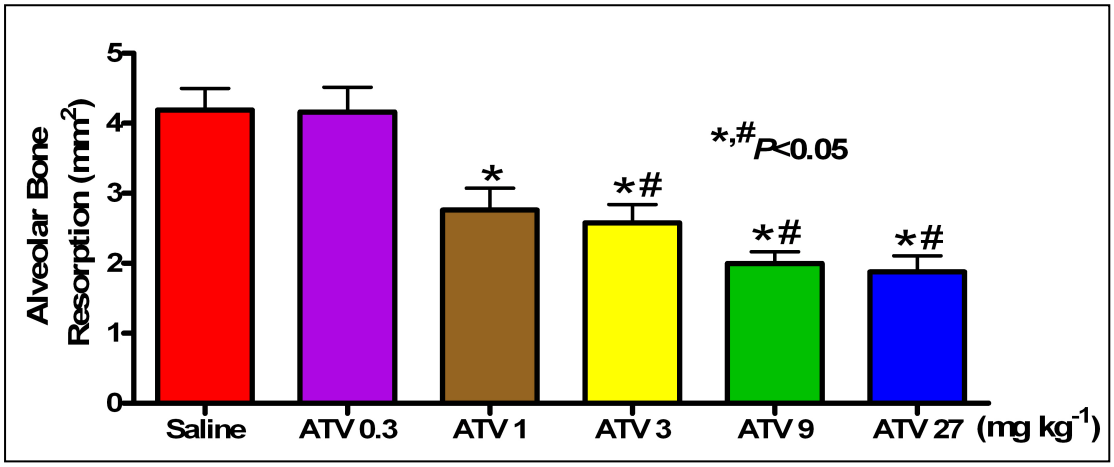

Fig. 2. Effect of Atorvastatin (ATV) on Alveolar Bone Resorption of rats submitted to periodontitis. Periodontitis was induced by ligature around second right upper molars. Animals received orally (v.o.) ATV (27 mg kg^{-1}) or 0.5 ml Saline 30 min before periodontitis induction, and daily for 11 d. The hemimaxillae were dissecated and photographed after the animal was killed, and measured for alveolar bone resorption (mm^2). Barrs represents the mean value ± standard error of the mean (SEM). *P<0.05 represents statistical differences compared to the group with ligature-induced periodontitis receiving Saline; $^{\#}P$<0.05 indicates statistical diference from animals which received ATV (1, 3, 9, or 27 mg kg^{-1}) when compared to animals treated with ATV 0.3 mg kg^{-1}. The number of animals in each group was at least six [data were analysed by using analysis of variance (ANOVA) and Bonferroni tests].

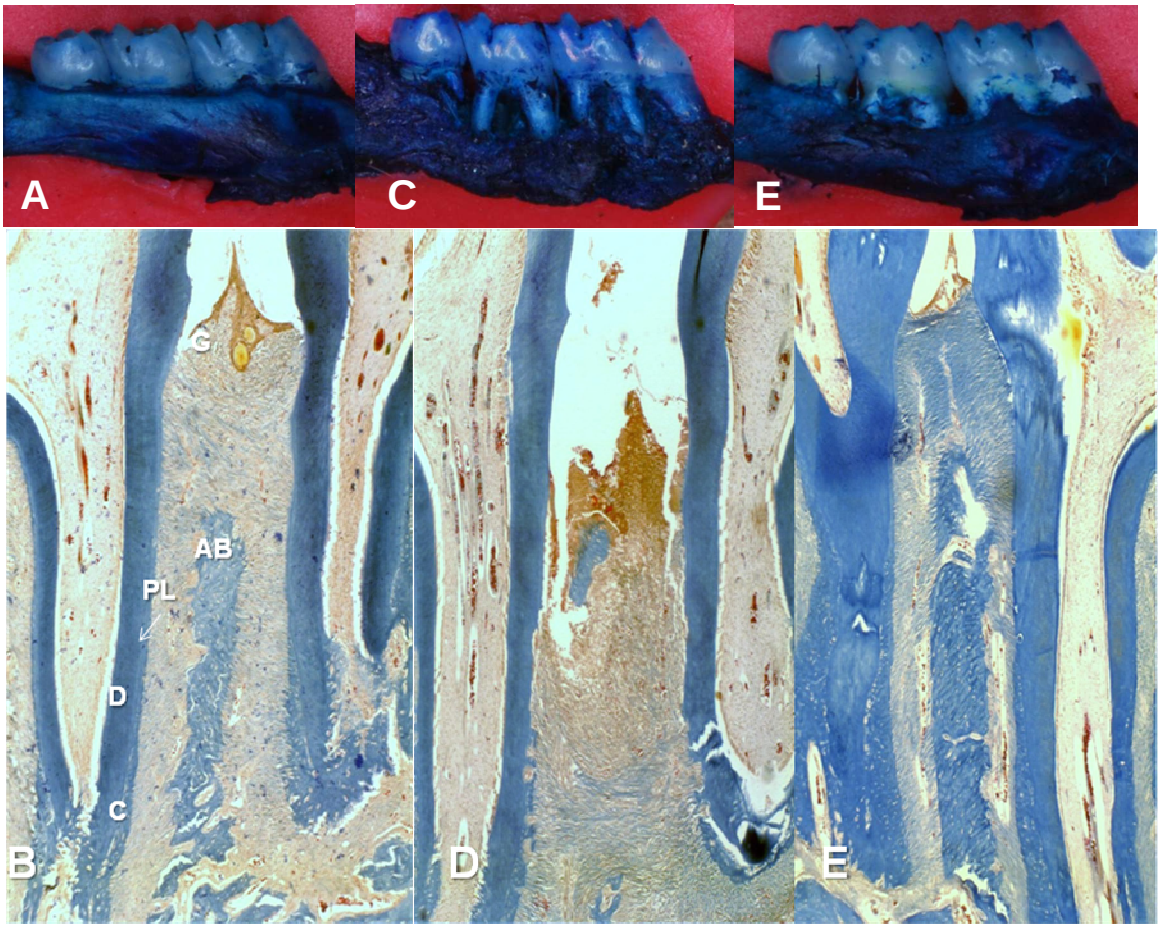

Fig. 3. Macroscopic and microscopic aspects of normal hemimaxillae (A and B) or hemimaxillae of rats submitted to periodontitis, receiving Saline (C and D) or 27 mg kg^{-1} Atorvastatin (ATV) (E and F), respectivelly. Periodontitis was induced by ligature around second right upper molars. Animals received orally (v.o.) ATV (27 mg kg^{-1}) or 0.5 ml Saline 30 min before periodontitis induction, and daily for 11 d. D=dentin; C=cementum; AB=alveolar bone; G=gingival and PL=periodontal ligament. The hemimaxillae were dissecated and photographed, or processed for hematoxylin & eosin (H&E) staining (x 40) after the animal was killed.

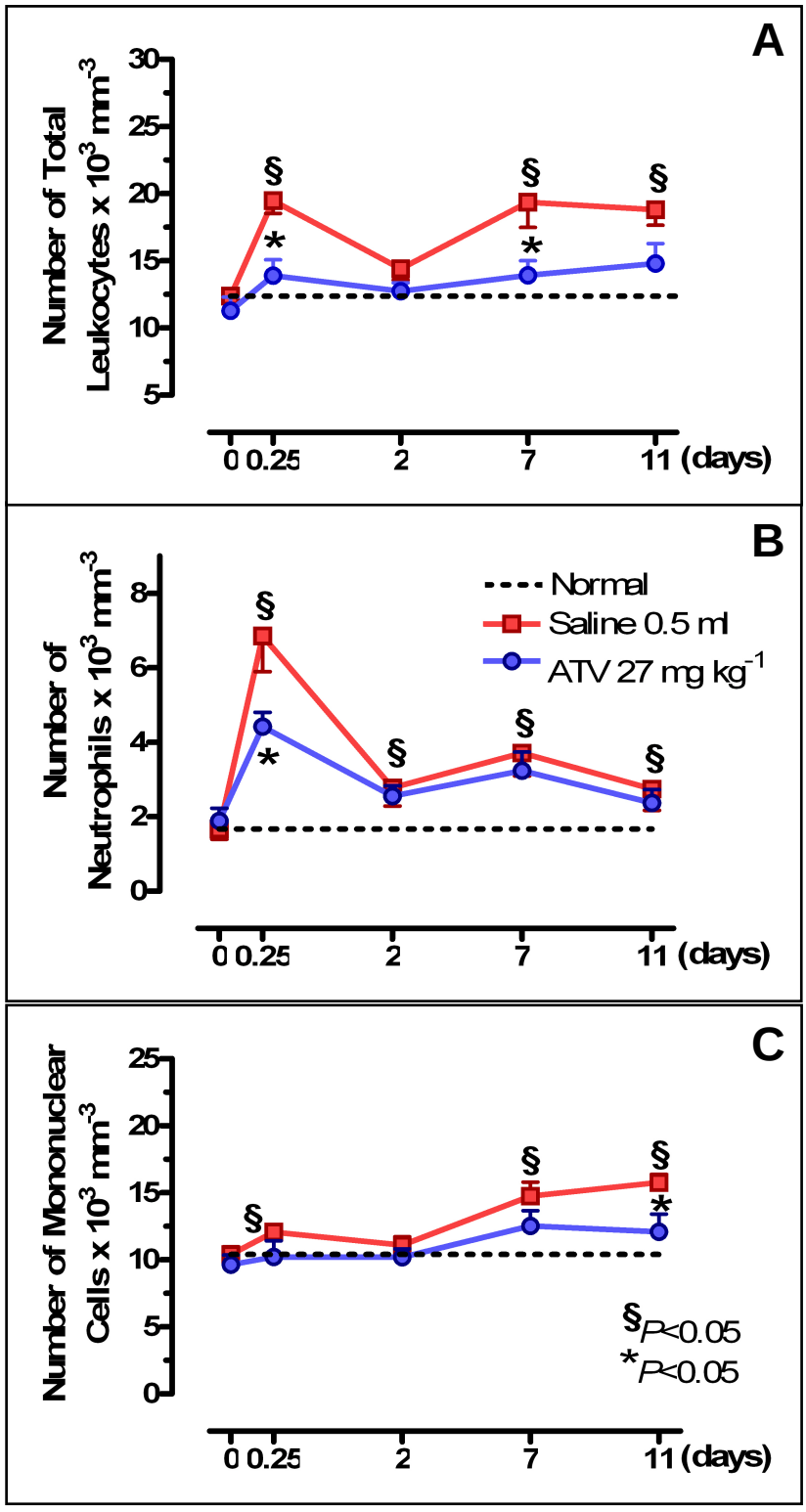

Fig. 4. Effect of Atorvastatin (ATV) on leukocyte counts of rats. Saline (0.5 ml) or ATV (27 mg kg^{-1}) was injected orally daily for 11 d after ligature placement. Blood was taken from the rat tail immediately before experimental periodontitis and afterwards at 6 h and 2, 7 and 11 d. Each point represents the mean value ± standard error of the mean (SEM) of total leukocytes (A), neutrophils (B) and mononuclear cells (C) x 10^3 mm^{-3} of group. *P<0.05 represents statistical differences compared to the group with ligature-induced periodontitis receiving Saline. The number of animals in each group was at least six [data were analysed by using analysis of variance (ANOVA) and Bonferroni tests].

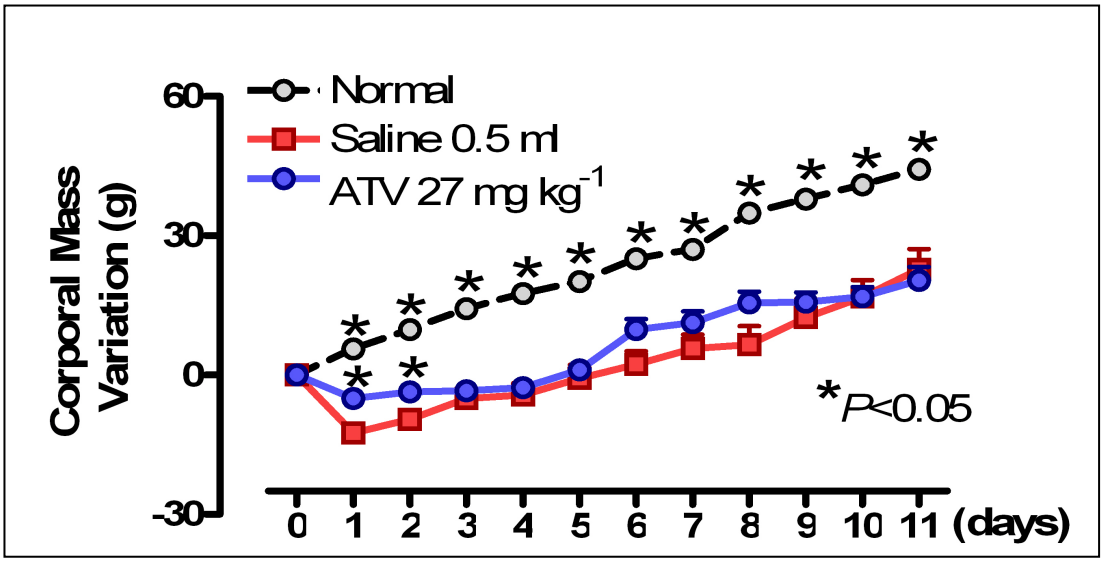

Fig. 5. Atorvastatin (ATV) does not reduce weight loss in rat with periodontitis. Periodontitis was induced by ligature around second right upper molars. Animals received orally (v.o.) ATV (27 mg kg^{-1}) or 0.5 ml Saline 30 min before periodontitis induction, and daily for 11 d. Data are expressed as the mean value ± standard error of the mean (SEM) of body weight variation (g). **P*<0.05 represents statistical differences compared to the group with ligature-induced periodontitis receiving Saline. The number of animals in each group was at least six [data were analysed by using analysis of variance (ANOVA) and Bonferroni tests].

DISCUSSION

Periodontitis is considered the second major oral pathology that most affect human population world wide (2). For this reason, a better understanding about its origin, development, diagnosis and treatment is necessary in order to lower this disease prevalence rates. Several aetiological factors have been associated to periodontitis, nevertheless recent research about its pathogenesis have shown an important paradigm shift on disease progression and severity (28). Although bacterial biofilms have been shown to be primary aetiological factor to periodontal destruction, its presence alone accounts for a relatively small proportion being to sufficent to explain periodontitis progression and severity (29). Therefore, the major component of soft- and hard-tissue destruction associated with periodontitis is the result of activation of the host immuno-inflammatory response to bacterial challenge (29).

In fact, the undelying biological mechanisms of this response are characterized by expression of endothelial cells and intercellular adhesion molecules (29), as well as excessive production and persistence of inflammatory mediators, like tumor necrosis factor (TNF) and interleukin-1 (IL-1) (30), and -6 (31), matrix metalloproteinases (MMPs) (32); nitric oxide (NO) (33), prostaglandins (PGs) (34), along with inflammatory cells like, neutrophils monocytes, lymphocytes and fibroblasts (29), that lead to destruction of periodontal tissue and results in irrevesible pathological changes such as clinical condition of periodontitis (4).

There are several ways to treat periodontitis, depending on its severity. The goal of periodontitis treatment is to thoroughly clean the pockets of bacteria and to prevent more damage. However, a number of patients not response favorably to all conventional treatment, so additional approaches, besides more studies about this disease, are both necessary. Periodontitis can be studied in different ways. Nevertheless, due to the disease prolonged time-course and involved ethical principles, animal models have been widely used. Among the different models and animals, ligature-induced periodontitis model in rats stand out because of the easiness technique and access, low costs, and mainly due to periodontal tissue similarity between humans and rats (6, 35). Ligature-induced periodontits model can reproduce the main characteristics of

human periodontitis, and is already well-stablished in literature (1).

Nowadays, we have used a periodontitis model described by LIMA *et al.*(6), with some modification with respect to the taking of macrocospic measures of the bone loss. Similarly to other studies (6, 23, 36, 37), our results have shown that the placement of nylon thread during 11 d caused intense alveolar bone destruction, root exposition and lack of proximal contact. Although the exact role of the bacteria or the host response in periodontal destruction observed in rodents is not clear yet, it has been proposed that accumulus of bacterial plaque on nylon thread induces host response that leads to inflammatory cell infiltration, osteoclast formation, bone loss and loss of attachment (4, 38). Corroborating these findings, our histopathological analysis of periodontitis showed alveolar bone resorption, intense inflammatory cellular infiltration, cementum injury and soft tissue destruction. In fact, these data are in accordance with previous reports (39, 40), and probably can be explained by release of several inflammatory mediators (4, 38).

In normal situations, inflammation produces little or no destruction of host tissues because the inciting agent is rapidly removed, and production of inflammatory mediators is attenuated. On the other hand, if the inflammatory responses are not effective in removing the iniciating agent, or are not effectively down-regulated, host tissues are destroyed because of chronic activation of leukocytes. Considering that the recruitment and activation of lymphocytes and phagocytic leukocytes are an important component of inflammation, we decided to evaluate leukogram of animals, besides other parameters that could affect the systemic conditions of the animals (41). It was found that periodontitis caused leukocytosis marked by neutrophilia and lymphomonocytosis on the 6^{th} h and 7^{th} and 11^{th} d, respectively. At sites of inflammation, leukocytes roll along the endothelium of postcapillary venules, collect inflammatory signals, arrest and then transmigrate (42). Several mediators orchestrate white cells recruitment, once, TNF and IL-1 induce selectin and ICAM expression on endothelial cells, Platelet-activating factor (PAF) promotes pro-adhesive process, Leucotriene B4 elicits chemotactic responses on leukocytes and Complement protein C5a, is a powerful chemoattractant (43). Therefore we can suggest that inflammatory process,

accompained by chemical mediator release caused by ligature placement was responsible for leukocytes peak.

In the present and unpublished study, we aimed to demonstrate that Atorvastatin (ATV), an agent known to have pleiotropic effects, including antiinflammatory action and anabolic effect on bone tissue, could alter the evolution of a periodontitis in rats. Our resuts showed that Atorvastatin (ATV) was able to significantly reduce alveolar bone loss (ABL) in this periodontitis model. This effect was associated with a reduction of inflammatory parameters seen by histological analysis, besides ATV reversed peripheral leukocytosis in 11 days of ligature-induced periodontitis, without affect systemic parameters.

It has been described that alveolar bone protection exerted by ATV is linked to the ability of statins on increasing up to 50% new bone formation and in promoting osteoblastic differentiation and mineralization, by enhancing production of Vascular Endothelial Growth Factor (VEGF) in osteoblasts stimulating bone growth and repair (18, 44). Such bone anabolic property seems be related to the drug lipophilicity and this ATV chemical characteristic may elicit greater mineralization process (18, 19) In addition, ATV presents antiinflammatory activity, once it was shown that ATV was to be able to inhibit important mediators involved on recruitment and transmigration of leukocytes and alveolar bone resorption as IL-6 (15, 45), TNF (46), nuclear transcriptional factor-κB (NF-κB) (47), ICAM-1 and VCAM (15), monocyte chemoattractant protein 1 (MCP-1) (48) and P-selectin expression (49). Additionally, analysing corporal mass variation, we have seen that periodontitis induced important loss of weight in the first two days of experiment, probably due to ligature placement trauma, as seen in previous reports (23). Meantime, it was found that ATV prevented initial loss of weight, but was not able to recover corporal mass lost thoughout the experiment, indicanting that statin therapy does not intefere significantly on body mass index (50).

Our study demonstrated that periodontitis, as well as ATV treatment, did not cause important alteration on liver and kidney either on serum biochemical assays or in histological analysis, except by elevation serum Creatinin activity, when compared to saline. It has been reported that serum liver enzymes may increase during statin therapy specially the hydrophilic ones

(51), nevertheless ATV is a lipophilic statin, what suggests an explanation for transaminases findings in this study (52). Moreover, a recent clinical trial demonstrated that only 1% of patient presented upper levels of transaminases after 12 month-ATV therapy (53). Additionally, in spite of urea and creatinine are considered both sensitive biochemical markers employed in the diagnosis of renal damage because urea and creatinin are excreted through the kidney (54), it has been related that creatinin activity is not good enough to stabilish a definitive diagnostic of renal function, been necessary to associate more analytical exams (55, 56, 57). Although some assays had demonstrated that statin therapy does not induce tubular disfunction (58) or alter glomerular filtration even in higher doses (59), our findings suggested a significant alteration on serum Creatinin activity. It is worthy to notice that despite of creatinin reflect renal filtration (WU, 2008) (50), it is not linearly related to glomerular filtration rate, being often linked to bias (55), and being considered as low specific biomarker (56). In fact, when analyses of kidneys were performed, we observed that any macroscopic or histological alterations were seen in the rat kidneys that received ATV, there was normal arrangement of the medulla and cortex with the glomeruli and blood vessels neatly arranged, when compared to control rats. So, in this context, we considered that ATV in fact did not alter liver or kidneys.

Considering that the bone-specific alkaline phosphatase (BALP), an isoenzyme of total alkaline phosphatase (TAP), is a bone formation marker due to its linkage with osteoblastic differention (60) and mineralization of newly formed bone (61), we also aimed is enzyme as bone marker in rat periodontitis. Our data suggested that periodontitis induced great variation on serum BALP activity, from day 0 to day 11, indicated by low BALP activity, probably related to bone resorption (62). In the other hand, ATV maintained, with less variation, the serum BALP activity, corroborating with some clinical trials on hipercholesterolemic patients using ATV, which demonstrated low variation on serum BALP activity, when compared to baseline (63, 64, 65), contributing in this way to bone homeostasis.

In summary, our results demonstrated that ATV elicited alveolar bone protection, reduced local inflammatory cellular infiltration and reverted

leukocytosis in ligature-induced periodontitis in rats. Moreover, ATV therapy kept serum BALP activity, and did not promoted significant alteration on liver or kidney, nor affected, significantly, corporal mass when compared to control animals. Therefore, ATV reduced bone loss in an efficient and safety manner, suggesting that ATV may be an important tool as an adjuvant on periodontal therapy, besides it merits further investigation.

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ARTIGO 2

Effect of Atorvastatin in Radiographic Density on Alveolar Bone Loss in Rats (Periodontics Section)

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Abstract

The use of digital radiographic images has contributed in alveolar bone loss analysis. Atorvastatin (ATV), a hypolipemiant drug has demonstrated pleitropic effects, standing out an anti-inflammatory action and an anabolic bone potential. We aimed to study ATV activity on alveolar bone loss radiographic density induced in rats. Periodontitis was induced throughout ligature around the upper molar of male *Wistar* rats (±200 g). Groups of six animals received via oral gavage 0.9% Saline solution or ATV (1, 3 and 9 mg/kg), over 11 days, when were sacrificed and their maxillae were removed, dissected, stained, radiographed through Digora System®, and photographed using digital camera. It was verified that ATV (9 mg/kg) caused a significant increase over 48% on gray tone variation (ATV 9= 118.25±11.97), when compared to Saline animals (Saline= 79.79±6.22 gray tones), indicating greater radiographic density. Those data were corroborated by macroscopic findings, where ATV (9 mg/kg) lowered ABL over 47% ($p<0.05$), when compared to untreated animals (Saline). ATV was able to protect alveolar bone loss seen on periodontitis, and that bone increments, even the most delicate ones, can be well visualized by digital radiographic analysis, in a very secure manner.

Descriptors: Atorvastatin, Periodontitis, Alveolar Bone, Radiographic Density.

Introduction

The bone tissue is an organ that it is continuously in remodeling process through a coordinated activity of osteoblasts and osteoclasts. Meanwhile, in front of pathological process, this homeostasis suffers an unbalance allowing resorptive events overlap the formative ones, resulting in loss of bone structure.[1] Periodontitis is characterized by inflammatory response and alveolar bone loss, which chemical mediators, such as cytokines, prostaglandins, metalloproteinases, among others, have been identified as immunoinflammatory process regulators.[2] For this reason, this disease is among oral problems that extensively affect the human population, being one of the greatest responsible for teeth loss in adults.[3]

Different approaches have been used to treat periodontal diseases. Mechanical therapy, as scaling and root planning, and surgical procedures, reduce microbial burden being effective on the control of periodontitis progression.[4] Nevertheless, this regulation is not always satisfactory, possibly due to the prominent role of immune response on periodontal destruction and, then, adjunctive therapies may be required in some cases.[5]

Statins gather a class of agents, which inhibit Hydroxymethylglutaryl Coenzyma A (HMG-CoA) reductase enzyme, leading to reduction on cholesterol production.[6] Among theses drugs, Atorvastatin has been widely used on clinical practice with the aim of preventing cardiovascular accidents.[6] Beyond hypolipemiant function, statins have stood out by its additional secondary effects, including anti-inflammatory, immunomodulatory, antioxidant, antithrombotic and endothelium stabilization action,[7] besides of angiogenesis promotion and increase on osteoblastic differentiation, inducing formation of bone tissue.[8] Still, statins slow down atherosclerosis progression by inhibition of monocyte activation, synthesis of vascular metalloproteinases and cytokine production, such as tumor necrosis factor (TNF) and interleukins (IL)-6 and IL-1β.[9]

The determination of periodontal therapy efficacy, be mechanical or chemical, it is based on a process that promotes bone mineralization or recuperation of alveolar support. Generally, observation of therapy evolution in periodontal patients is done by clinical probing along with radiographic

images.[10] The recognition of quantitative information over any bone alteration is the main objective of radiographic images, which gain greater importance on periodontics, due to the slow progression of the disease that provokes very delicate mineralization differences.[11]

In spite of conventional radiography to be the complementary exam most used on Dentistry, this method presents some limitations due to reduced sensibility and high inter-examiners disagreement. Nowadays, digital images have taken over a notability position by presenting greater capacity on detecting discrete bone loss, besides that, they allow an wide variety of manipulation and quantification tools and determination of gray tone, promoting objective analysis,[12] which has a particular interest on periodontal diseases evaluation.

The rat is an animal that has been largely used to study the progression of periodontitis,[13] because besides its anatomical and histological junctional epithelium and connective tissue similarities when compared to humans, they also present easy handle, low maintenance cost and high reproducibility of induced lesions, among other qualities. An easy and fast method for inducing periodontitis in rats consists in ligature around the animal molar tooth, acting as a strange body unleashing an unspecific anti-inflammatory process, which is kept by bacterial plaque accumulus.[13,14]

In this way, the aim of this study was to evaluate the effect of Atorvastatin on radiographic density on alveolar bone loss induced in rats.

Material and Methods

1. Animal Selection

Twenty-four male *Wistar* rats (±200 g), from our own animal facilities, were used in this study. Experimental protocols were executed following ethical principles for laboratory animal use, and they were approved by institutional Ethical Committee of Animal Research under number 74/07. All efforts were made to reduce the number of animals, its pain, suffer and stress.

2. Model of Experimental Periodontitis

For the study the model of ligature-induced periodontitis was used based on Lima *et al.*[13], which consists on an insertion of a nylon ligature (Point

Suture,Point Suture do Brasil Fortaleza-CE, Brazil) around the cervix of the second left upper molar of rats anesthetized with Chloral Hydrate (Vetec, Duque de Caxias-RJ, Brazil). Ligature was followed by a guide used on proximal spaces of the referred tooth, and was knotted on the buccal side of the tooth, resulting in a subgingival position palatinally and in a supragingival position buccally. The contralateral right side was used as the unligated control. Animals were watched until the 11th day, lesion apex day, with intense alveolar bone loss, when then, they were sacrificed under anesthesia. All ligature-induced periodontitis was made randomly and blind.

3. Experimental Groups

3.1. Saline Group

This control group was constituted by six rats each, submitted to periodontitis. The animals received 0.5 ml of 0.9% sterile saline solution by oral gavage (v.o.), 30 minutes before ligature and, after that, daily, for 11 days period, when then were sacrificed.

3.2. Atorvastatin Groups (ATV)

The animals were subdivided in 3 groups of six animals each, which received v.o. Atorvastatin (Lipitor®, Pfizer, São Paulo-SP, Brazil) dissolved in 0.9% sterile saline solution on the doses of 1, 3 and 9 mg/kg, respectively, 30 minutes before ligature, and daily until the 11th day, when then were sacrificed.

4. Local Parameters evaluated on Experimental Periodontitis

4.1. Analysis of radiographic density of resorption area

On the 11th day, after periodontitis induction, animals were sacrificed and their maxillae were removed and fixed in 10% formaldehyde, during 24 hours. Following, maxillae were separated in half, dissected and stained in 1% methylene blue, in order to differentiate bone from teeth.[13,14] After that, specimens were analyzed about its radiographic density through digital radiography using Digora Soredex System® (Dental Imaging Company Ltd, Portslade-East Sussex, United Kingdom). Hemimaxillae were posed perpendicularly on the sensor. Radiographic images were acquired using 63 kVp, 8 mA, exposition time of 0.06 s and focal distance of 30 cm. Then, these

images were analyzed by the IMAGE J® software (ImageJ 1.32j, National Institute of Health, EUA) using 8 bits configuration. A 128 pixels interest region (Figure 1) was selected and posed under amelocemental junction from mesial to distal area of second molar in the periodontitis side (Figure 1A) and its contralateral normal side (Figure 1B). The difference of gray tones from both areas was considered as value of radiographic density. The radiographic density analysis of interest region (IR) was achieved through histogram tool of the referred program, with uses a 256 gray tone scale, where zero indicates the black color, and the value 255, the white. Data were expressed in arbitrary gray tones.[10]

4.2. Morphometric study of bone tissue

For the macroscopical bone resorption quantification, the same specimens were used from radiographic analysis. Both hemimaxillae were arranged on glass slices and followed for photographic registration with digital camera Sony Cyber-Shot® (DSC-W80 model, Sony, Hong Kong – China). Images were evaluated using IMAGE J® Software for alveolar bone loss (ABL) quantification. The calculus of resorption area was done by subtraction of the delimited region involving occlusal border of vestibular teeth until remained bone border of challenged hemimaxilla (Figure 2B)., from the respective area on the contralateral hemimaxilla, own animal control (Figure 2A). All the obtained images were compared to a well-known area of 0.25 mm^2 for posterior conversion of pixels for mm^2.

5. Statistical Analysis

Results were expressed as Mean±S.E.M., followed by ANOVA and Bonferroni's test. A $p<0.05$ value was considered as indicating significant differences.

Results

Table 1 shows radiographic density analysis determined by gray tones. Radiographies of non-treated animals submitted to periodontitis (Saline group) indicated significant rarefaction on considered region, indicating intense alveolar bone resorption ($p<0.05$), when compared its own control (contra-lateral hemimaxilla). Although low doses of Atovastatin (ATV) showed a tendency to reduce bone rarefaction ($p>0.05$), only the ATV 9 mg/kg exhibited significant gray tone raise on interest region, indicating prevention of bone loss after ligature-induced periodontitis.

Table 1: Radiographic density of rat hemimaxillae submitted to periodontitis for 11days.

	Normal	**Saline**	**ATV 1**	**ATV 3**	**ATV 9** (mg/kg)
Radiographic Density (gray tone)	189.10±3.38*	79.79±6.22	90.31±10.46	96.76±7.58	118. 25±11.97*

Values indicate Mean±SEM of radiographic density of, at least, 6 animals. (*) $p<0.05$ when compared to Saline (ANOVA, Bonferroni).

Following, macroscopical analysis of alveolar bone resorption of animals submitted to periodontitis can be seen on Table 2. It was verified that 11 days of ligature caused intense alveolar bone resorption on Saline group ($p<0.05$). At the same time, it was noted the protective effect of Atorvastatin (ATV) on the alveolar bone tissue of animals submitted to periodontitis. We can observe that low doses of ATV (1 or 3 mg/kg) presented a non significant tendency to bone protection, and also was able to reduce the alveolar bone loss when used on the higher dose and compared to Saline ($p<0.05$). Thus, we can see that macroscopical data corroborate radiographic findings, since data of both analyses had been correlated each other in a indirectly proportional manner.

Table 2: Morphometric analysis of rat hemimaxillae submitted to periodontitis for 11days.

	Saline	ATV 1	ATV 3	ATV 9 (mg/kg)
Alveolar bone resorption (mm^2)	3.91±0,26	2.96±0,27	3.00±0.24	2.25±0.22*

Values indicate Mean±SEM of alveolar bone resorption of, at least, 6 animals. (*) $p<0.05$ when compared to Saline (ANOVA, Bonferroni).

Data obtained on both analyses, radiographic and macroscopical, can be represented on Figure 3, in which images B and E indicate radiographic and macroscopical aspects, respectively, of a non-treated hemimaxilla after 11 days ligature-induced periodontitis (Saline). On the radiographic image we observe that second molar region presents rarefied bone tissue, associated to horizontal bone loss in mesial and distal area of the referred element, as well as, the initial alveolar bone structure loss in furcation area. Macroscopically, we see an intense alveolar bone destruction, root and furcation area exposition. These data are especially different when compared to normal hemimaxilla, where the periodontal structure is found naturally preserved in both radiographic and macroscopical analysis, respectively (Figure 3A and D). In order to illustrate the protective effect of Atorvastatin (9 mg/kg), Figure 3C shows its radiographic image, which indicates alveolar crest region preservation, besides of lower bone loss in furcation area as much as in proximal faces of second upper molar, keeping a preserved pattern of supportive periodontium, corroborated by the respective macroscopic aspect of alveolar bone well preserved (Figure 3F).

Discussion

Periodontitis has been correlated with several inflammatory mediators, which contribute not only to bone homeostasis, but also to tissue destruction.[15] Considering that local bone loss is a combination of immunoinflammatory exacerbated reaction and localized osteoclastogenesis,[16] we thought that the alveolar bone loss induced by ligature occurred due to abnormal activation of host immunological system, with consequent uncontrolled inflammatory response.[17]

It is known that inflammatory mediators, as the cytokines TNF, IL-1 and IL-6, induce expression of Receptor Activator of Nuclear-κB Factor

(RANK) and its ligant (RANKL), recently identified, with important role on the osteoclast development, inducing osteoclastic differentiation and maturation, promoting unbalance on bone environment, and predominating the resorption.[17] Osteoprotegerin (OPG), is a factor that naturally occurs and it is responsible for antagonism of RANKL effects, preserving bone integrity[18]. Many others osteoclast activator factors have been identified, among them there are Transforming Growth Factor (TGF)-β, Macrophage-Colony Stimulator Factor (M-CSF), Hepatocytes Growth Factor (HGF), Matrix metalloproteinases (MMPs), Macrophage Inflammatory Protein (MIP)-1α.[17]

It has been described that Atorvastatin may act in important steps on exacerbated inflammatory response, promoting reduction on neutrophil influx,[19] by diminishing expression on neutrophil adhesion molecules, such as ICAM-1, VCAM-1[20,21] and E-selectins. In addition, the expression of several inflammatory mediators, as IL-1, IL-6 and TNF,[19] overproduction of NF-κB,[20] production of cytotoxic nitric oxide (NO), as well as activity of other reactive oxygen species,[21] were also attenuated by Atorvastatin action.

Nevertheless the anti-inflammatory mechanisms are not completely well-understood, it is recognized that the role of Atorvastatin has important clinical benefits in inflammatory disorders, interfering consequently, on the development of bone resorptive processes. Thus, we can consider that the 47% of alveolar bone loss protection promoted by ATV (9 mg/kg) were due to its anti-inflammatory action, besides its capacity to promote bone anabolism.

Several bone formation markers are used on laboratory practice, such as measurement of Bone Morphogenic Protein 2 (BMP-2) expression, whose studies in rat calvaria have shown the ability of statins on improving trabecular bone volume on these animals[22] or even, in clinical trials, where Bone-specific Alkaline Phosphatase (BALP) has been used as bone formation marker, coinciding to the raise of Bone Mineral Density (BMD) in osteoporic women after Atorvastatin treatment.[23] Thus, in some inflammatory disorders, as periodontitis, which present varied severity and evolution velocity, the use of faithful diagnose tools for early local alteration on bone tissue becomes fundamental.

In that sense, radiographic conventional studies are still widely used

on the initial evaluation on periodontal patients, because such images show obvious bone alterations. However, when bone anabolic events are still subtle, conventional biochemical and radiographic markers not always are sensitive enough to reveal such subtle alterations. So, digital radiographic images present an additional vantage, because they demonstrate capacity to reveal larger number of early sites with bone loss.[24]

Considering that the radiographic analysis also evidenced the protecting effect of ATV in the bone loss, when compared to the macroscopic analysis, it is supposed that digital images can be a quite reliable tool in situations where the bone loss are subtle, as observed them in this animal model.

Thus, we can suggest that even subtle anti-inflammatory or anabolic effects of ATV can be well-visualized by the use of digital radiographies with full reliability. Therefore, digital images can become useful for future studies of the periodontitis, especially in patients who present medical alterations that require the use of ATV.

Conclusion

Atorvastatin was able to promote protection of alveolar bone loss determined by both radiographic and macroscopical analysis, which suggests that subtle bone increments can be verified in clinical practice by digital radiographic exams with great reliability.

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Figures and Illustration legends

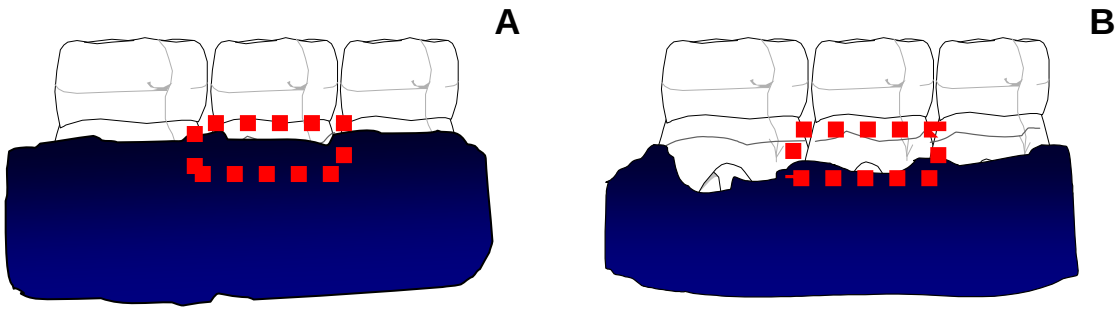

Figure 1: Representation of interest region (IR; demarked area). IR in a hemimaxilla submitted to periodontitis (A) and in its normal contralateral hemimaxilla (B) whose difference was considered as value of radiographic density (gray tones).

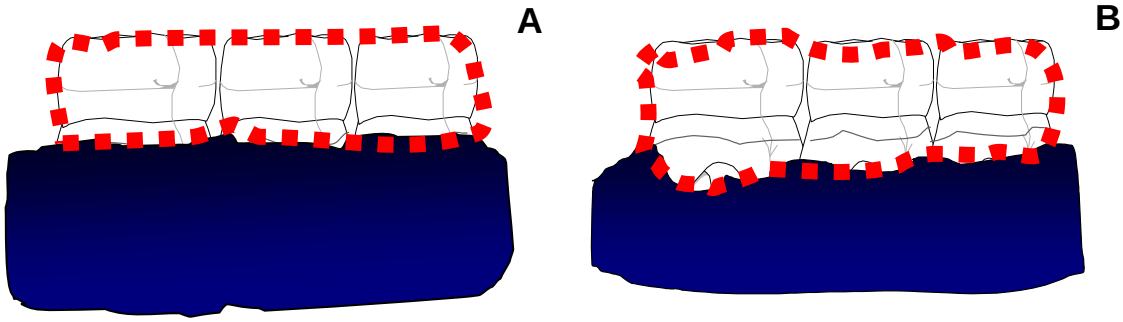

Figure 2: Representation of the demarked area for alveolar bone resorption measurement. Area of normal contralateral hemimaxilla (A) and the hemimaxilla with periodontitis (B), whose difference was considered as value of alveolar bone resorption (mm^2).

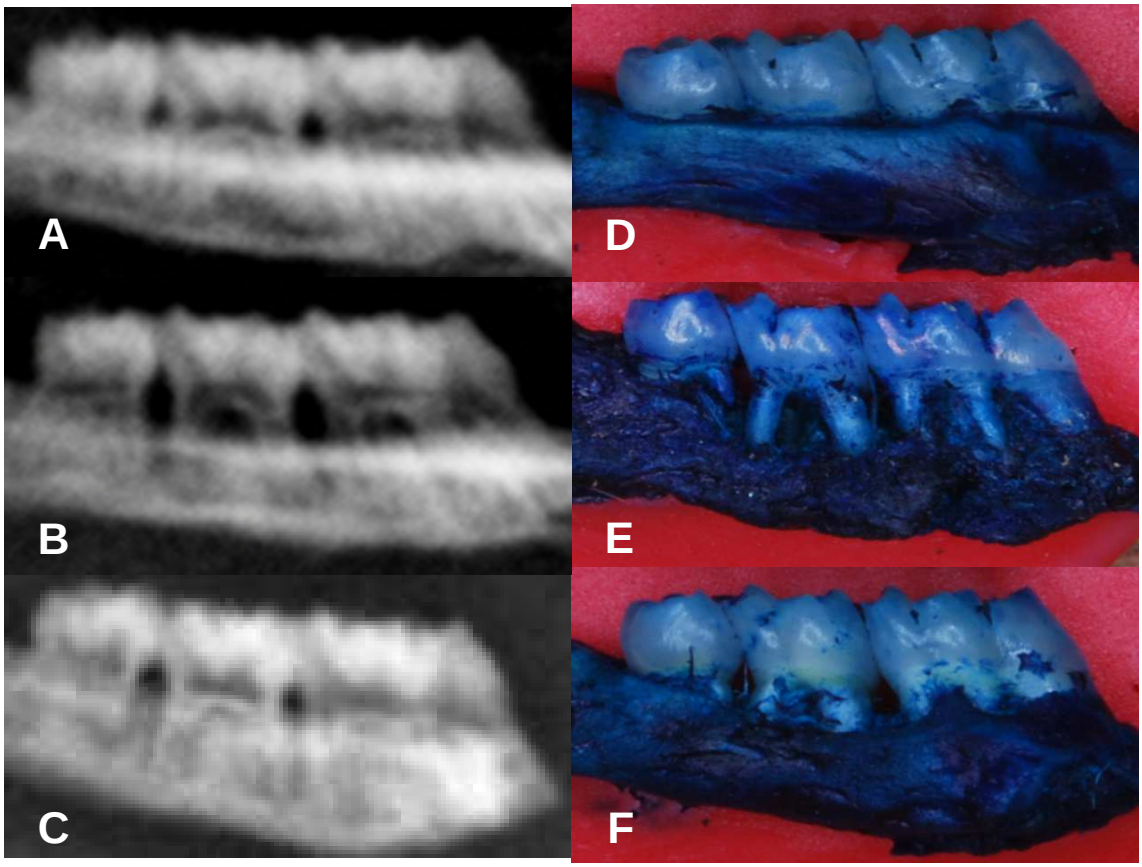

Figura 3: Radiographic and macroscopic aspects of hemimaxillae. (**A**) and (**D**) Normal hemimaxilla; (**B**) and (**E**) hemimaxilla of animal submitted to periodontitis that received Saline; (**C**) and (**F**) hemimaxilla of animal submitted to periodontitis that received ATV (9 mg/kg).

5. DISCUSSÃO GERAL

A doença periodontal corresponde à segunda maior patologia bucal que mais afeta a população humana no mundo (PETERSEN & OGAWA, 2005). Por esta razão, um melhor entendimento sobre sua etiologia, patogênese, diagnóstico e tratamento faz-se necessário à mudança deste quadro epidemiológico, especialmente porque estudos recentes vêm demonstrando uma mudança interessante de paradigma dentro da periodontologia (PRESHAW *et al.,* 2004). Durante muito tempo, o biofilme bacteriano foi entendido como o fator etiológico primário para a destruição periodontal. Entretanto, quando de forma isolada, a presença deste agente tem se mostrado insuficiente para explicar a progressão e severidade da periodontite (SALVI & LANG, 2005). Assim, o papel do hospedeiro na etiologia das doenças periodontais ganha destaque, pois o maior componente responsável pela destruição tecidual periodontal resulta da ativação da resposta imunoinflamatória do hospedeiro, como conseqüência ao desafio microbiano (MADIANOS et al., 2005).

De fato, bactérias periodontopatogênicas estimulam células que induzem os tecidos periodontais a expressar vários mediadores inflamatórios tais como interleucinas (IL)-6 (RADVAR et al., 2008) e IL-1, fator de necrose tumoral (TNF) (ASSUMA et al., 1998), óxido nítrico (NO) (DI PAOLA et al., 2004), ou o ligante de receptor ativador do fator nuclear (RANKL). Subseqüentemente, estes mediadores podem ativar a produção de metaloproteinases de matriz (ACHONG *et al.,* 2003) e prostaglandinas (ALPAGOT *et al.,* 2007), além de induzir o recrutamento de células inflamatórias, como neutrófilos, monócitos, linfócitos (SALVI & LANG, 2005), e a diferenciação de osteoclastos, resultando em destruição irreversível de tecido conjuntivo e reabsorção óssea alveolar (HONDA *et al.,* 2006).

Várias são as maneiras de se estudar a periodontite. Porém, o longo curso da doença e os princípios éticos envolvidos fazem dos modelos animais meios interessantes para o entendimento da patogênese e a determinação de novas estratégias terapêuticas para essa doença (WEINBERG & BRAL, 1999). Manipulação da dieta (ROBINSON *et al.,* 1991), o uso de ligadura em molares

(SALLAY *et al.,* 1982) ou inoculação de bactérias periodontopatogênicas (JORDAN *et al.,* 1972; FIEHN *et al.,* 1992) estão entre os principais modelos utilizados que, por sua vez, têm sido realizados em macacos, ratos, cachorros e hamsters. O modelo de periodontite induzido por ligadura em ratos tem se sobressaído principalmente pela facilidade na técnica de indução da doença, acessibilidade e custos reduzidos para sua realização. Contudo, a mais importante vantagem deste modelo baseia-se na grande similaridade dos tecidos periodontais entre humanos e ratos (WEINBERG & BRAL*,* 1999). Atualmente temos utilizado o modelo de periodontite desenvolvido por LIMA *et al.* (2000; 2004), acrescido de algumas modificações, tais como modificação no método de obtenção da área de reabsorção

Nesse estudo, a periodontite, induzida pela colocação de um fio de náilon (000), em torno dos segundos molares superiores esquerdos dos ratos, causou intensa destruição óssea alveolar, ao final dos 11 dias, avaliada através de estudo macroscópico. Tais achados foram confirmados pela análise histopatológica, onde se observou que 11 dias de ligadura causou reabsorção completa do processo alveolar, intenso infiltrado inflamatório e dano no cemento radicular. Ainda corroborando com os achados prévios desse estudo, a análise radiográfica mostrou uma redução significante da densidade radiográfica na região da ligadura.

Estes resultados estão de acordo com aqueles publicados por outros autores, os quais demonstram que, através de estudo morfométrico, ratos submetidos à periodontite de forma semelhante apresentaram reabsorção óssea alveolar significante (LIMA *et al.,*2000, 2004; CAVAGNI *et al.,* 2005; NAPIMOGA *et al.,* 2008). À análise histológica, também foi visto que a periodontite induz intensa reabsorção óssea, com presença marcante de infiltrado inflamatório (JIN *et al.,* 2007; BEZERRA *et al.,* 2008; CAI *et al.,* 2008), e, no estudo radiográfico, tal dano ósseo decorrente da indução da periodontite foi caracterizado pela perda de densidade radiográfica (CÉSAR-NETO *et al.,* 2005; HWANG *et al*., 2008).

A homeostase óssea relaciona-se estreitamente com o processo inflamatório. Linfócitos e macrófagos, mantidos pela inflamação, produzem citocinas, tais como TNF e IL-1, dentre outras, que recrutam e ativam células

inflamatórias adicionais (XING *et al.*, 2005). A superexpressão destes mediadores, por sua vez, acaba por desempenhar papel importante na patogênese da periodontite, ativando o sistema RANK/RANKL e inibindo OPG, o que promove intensa osteoclastogênese e reabsorção óssea (XING *et al.*, 2005). Além desses, muitos outros fatores ativadores de osteoclastos têm sido identificados como participantes do processo de reabsorção, tais como: fator transformador de crescimento (TGF)-β, fator estimulador de colônia de macrófagos (M-CSF), fator de crescimento de hepatócitos (HGF), metaloproteinases de matriz (MMPs) e proteína inflamatória de macrófagos (MIP)-1α (TAKAYANAGI, 2005)

Com relação aos achados hematológicos, nossos resultados mostraram que a periodontite alterou a contagem total e diferencial dos leucócitos no sangue periférico dos animais. A leucocitose observada foi marcada por neutrofilia, na 6ª hora e posteriormente por linfomonocitose nos 7º e 11º dias. Essas observações estão de acordo com alguns autores que já demonstraram ocorrer leucocitose na presença de periodontite induzida em ratos (SAMEJIMA *et al.*, 1990; LIMA *et al.*, 2000; BEZERRA *et al.,* 2000).

Quanto à variação de massa corpórea, verificou-se que os animais submetidos à periodontite apresentaram perda de massa corpórea nos dois primeiros dias após colocação da ligadura, provavelmente devido ao trauma durante a instalação do fio, pois o estabelecimento e progressão da perda óssea alveolar em ratos não sofre influência da massa corpórea (SIMCH *et al.,* 2008*)*. Posteriormente, apesar do ganho de massa corpórea, estes ratos não conseguiram acompanhar a curva de perda de peso de animais normais, corroborando achados de outros estudos (LIMA *et al.,* 2000; 2004).

Uma vez verificados os efeitos locais da ligadura dos molares dos animais, seguiram-se as avaliações no intuito de se verificar possíveis repercussões sistêmicas. Assim, os animais submetidos à periodontite foram também avaliados quanto a possíveis alterações hepáticas e renais. Neste estudo observou-se que esta doença não induz lesões nesses órgãos, uma vez que as respectivas enzimas séricas apresentaram poucas variações entre os dias 0 e 11. Tais achados foram corroborados pelas análises histológicas realizadas. Contudo, apesar da proteção observada, os níveis de fosfatase

alcalina total (FAT), considerados um forte indicador de doenças hepáticas, mostraram variações importantes, provavelmente porque alterações de suas concentrações plasmáticas podem refletir outros problemas de origens diversas (FERNANDEZ & KIDNEY, 2007), como por exemplo, patologias ósseas (GIANINNI, *et al.,* 2005).

Assim, para confirmar os achados prévios sobre nível sérico de FAT, buscou-se avaliar o comportamento da isoenzima óssea da fosfatase alcalina (FAO). De fato, animais submetidos a 11 dias de periodontite mostraram uma variação dos níveis de FAO bastante importante, indicando uma redução da concentração sérica esta isoenzima, 11 dias após o estímulo inflamatório, o que foi corroborado por outros estudos (KELES *et al.,* 2005; SHOJI *et al.,* 2006).

Dado o proeminente papel do processo inflamatório na patogênese da periodontite, o presente trabalho buscou utilizar uma ferramenta farmacológica que permita a modulação de mediadores, e conseqüentemente a resposta do hospedeiro, sobressaindo-se como uma nova abordagem de tratamento (BUDUNELI *et al.,* 2007; PRESHAW *et al.*, 2004). Assim, a Atorvastatina (ATV), fármaco indicado para o tratamento da hiperlipidemia, mas que também apresenta efeitos secundários importantes, foi utilizada (KRONMANN *et al.,* 2007).

Neste estudo, observou-se que animais, submetidos a 11 dias de periodontite induzida por ligadura e tratados com ATV diariamente, apresentaram proteção significante dos tecidos de sustentação dentária. Macroscopicamente, animais tratados com ATV (1, 3, 9 e 27 mg/kg) demonstraram redução da destruição óssea de 35%, 39%, 53% e 56%, respectivamente. A análise histológica confirmou os achados macroscópicos, uma vez que animais com periodontite, tratados com ATV (27 mg/kg), apresentaram preservação do processo alveolar e cemento, associado ao discreto infiltrado inflamatório. Ainda, corroborando os achados prévios deste estudo, as densidades radiográficas da região de segundo molares de animais submetidos à periodontite e tratados com ATV (1, 3, 9 mg/kg) mostraram-se preservadas em 5%, 9% e 20%, respectivamente, sendo apenas a maior dose estatisticamente significante.

Nossos resultados, em consonância aos publicados na literatura, podem ser explicados pelo efeito anabólico ósseo exercido pela ATV. Este fármaco promove aumento na produção de osteoprotegerina (OPG) (VIERECK *et al.,* 2005), e na transcrição dos genes de fator de crescimento endotelial vascular (VEGF) e Cbfa1 (KAJINAMI *et al.*, 2003), presentes em células osteoblásticas (MAEDA *et al.,* 2003). Essas células, por sua vez, são responsáveis pela diferenciação e mineralização do tecido ósseo (MAEDA *et al.,* 2003), induzindo assim o aumento da densidade óssea, vista em animais (KAWANE *et al.,* 2004) ou em humanos (PÉREZ-CASTRILLÓN *et al.,* 2008) após o uso de ATV. Desta forma, destaca-se o papel estabilizador da ATV em osso.

Efeito adicional pleiotrópico da Atorvastatina, também relacionado a processos reabsortivos, consiste em sua atividade antiinflamatória. Estudos mostram que a ATV inibe a expressão de marcadores de estresse oxidativo, causadores de destruição tecidual, como isoprostanos, óxido nítrico sintetase induzida (NOSi) e peroxinitritos (NAWAWI *et al.,* 2003; MATTHEWS *et al.,* 2007; CANGEMI *et al.,* 2007; LEE *et al.,* 2007; HEEBA *et al.,* 2007). Em adição, diversos marcadores pró-inflamatórios, tais como: ICAM, IL-6 (NAWAWI *et al.,* 2003); IL-1 (WAEHRE *et al.,* 2004); TNF, proteína C-reativa (ARNAUD *et al.,* 2005; MOZAFFARIAN *et al.,* 2005); NF-κB, bem como RNAm de proteína quimioatraente para monócitos (MCP-1) (ORTEGO *et al.,* 1999; TANIMOTO *et al.,* 2007) e proteínas inflamatórias de macrógrafos (MIP-1α e MIP-1β), IL-8 e seus receptores (CCR1 e CCR2), TNF-α e IL-1β (RIAD *et al.,* 2007) sofrem redução da sua expressão após o uso de ATV. Assim, esses achados sugerem que a ATV possui um importante papel na modulação da resposta inflamatória, o que pode explicar os resultados do estudo hematológico, uma vez que os animais submetidos à periodontite e tratados com ATV tiveram revertidos os picos de leucocitose, vistos nos animais do grupo Salina. Portanto, a ATV foi capaz, de certa forma, de modular a resposta inflamatória.

Analisando a variação de massa corpórea dos animais submetidos à periodontite e tratados com ATV, foi possível observar que o tratamento farmacológico reverteu a perda de peso inicial após a ligadura, vista no grupo Salina, mas não foi capaz recuperar a massa corpórea perdida durante o

experimento, o que pode ser explicado pelo fato de que a terapia com estatinas não interfere, de forma significante, no índice de massa corpórea (GEORGESCU & GEORGESCU, 2007).

Considerando que a utilidade clínica de um fármaco baseia-se, além de sua eficácia, também na segurança, buscou-se avaliar o tratamento com ATV em relação a possíveis danos hepáticos ou renais. Foi observado que a ATV não provocou alterações importantes nestes órgãos, quando analisada através de dosagens bioquímicas séricas, com exceção dos níveis de creatinina. No entanto, análises histológicas confirmaram o perfil de segurança da ATV utilizada nesse estudo.

Este foi um achado interessante, pois a elevação na concentração sérica de transaminases muito se associa ao uso de estatinas, principalmente as hidrofílicas (DALE *et al*., 2007), porém, a ATV é uma estatina lipofílica, o que provavelmente explica os achados obtidos em relação as transaminases (STOLLEY & ITO, 1999). Dosagens séricas de fosfatase alcalina total (FAT) também foram realizadas, com intuito de corroborar os resultados sobre integridade hepática, uma vez que uma injúria induzida por fármacos, neste órgão, em geral apresenta um padrão de colestático, caracterizada por aumento desta enzima (GIANNINI, *et al.,* 2005). Entretanto, foi observado que os animais tratados com ATV mantiveram os níveis de FAT, confirmando a segurança da ATV (KIYICI *et al.,* 2003; STOJAKOVIC *et al.,* 2007).

Em relação à atividade renal, embora alguns ensaios tenham demonstrado que a terapia com estatinas não induz disfunção tubular (PAULSEN *et al.,* 2008) ou altera filtração glomerular mesmo em altas doses (EPSTEIN *et al.,* 2007), nosso achados sugeriram uma alteração significante induzida pela ATV apenas nos níveis séricos de creatinina. Contudo, apesar de a creatinina refletir filtração renal (WU & PARIKH, 2008), esta não está linearmente relacionada à taxa de filtração glomerular, sendo freqüentemente associada a vieses (SOLOMON & SEGAL, 2008) e considerada, portanto, como um biomarcador de baixa especificidade (VAIDYA *et al.,* 2008). Assim, as análises histológicas dos rins foram preponderantes para a determinação de que a ATV não induziu lesão renal.

Analisando as variações dos níveis de FAO dos animais submetidos

à periodontite e tratados com ATV, observou-se que esta isoenzima pouco mostrou alteração em suas concentrações entre os dias 0 e 11, indicando manutenção dos níveis de FAO, mesmo após o estabelecimento da lesão periodontal. A FAO é considerada um marcador bioquímico de formação óssea (KELES *et al.,* 2005), e o aumento na sua expressão (KAJINAMI *et al.*, 2003; MAJIMA *et al.,* 2007), bem como de outros relacionados à formação óssea, tais como a osteocalcina e o gene da proteína morfogênica óssea-2 (BMP-2) (KAJINAMI *et al.*, 2003; RUIZ-GASPA *et al.,* 2007) também foi observado após o uso de ATV, confirmando, assim, nossos achados.

6. CONCLUSÕES GERAIS

Em suma, os resultados deste estudo mostraram que a ATV promoveu proteção dos tecidos periodontais, avaliada através de análise macroscópica, histológica e radiográfica. Além disso, o tratamento com ATV mostrou-se seguro, pois reverteu a leucocitose, não causou alterações significantes em fígado e rins, manteve os níveis de FAO e não afetou, significativamente, a massa corporal, quando comparada a animais controle. Portanto, sugere-se que a ATV pode ser uma importante ferramenta farmacológica a ser ensaiada clinicamente como adjuvante à terapia periodontal.

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ANEXO 1 – Aprovação do Comitê de Ética em Pesquisa Animal

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DECLARAÇÃO

Declaramos que o protocolo para uso de animais em experimentação nº 74/07, sobre o projeto intitulado: **"AVALIAÇÃO DA ATIVIDADE DE ESTATINAS NA PERIODONTITE INDUZIDA POR CORPO ESTRANHO EM RATOS** de responsabilidade da Profa. Vilma de Lima, 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 agosto de 2007.

Fortaleza, 07 de agosto 2007

Profa. Dra. Geanne Matos de Andrade Cunha
Coordenadora da Comissão de Ética em Pesquisa Animal - CEPA