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Influência da osteoporose induzida por glicocorticoide na perda óssea alveolar em ratos com periodontite experimental e o efeito do tratamento com Atorvastatina

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Dissertação apresentada ao Programa de Pós-graduação em Ciências da Saúde da Universidade Federal do Ceará, como requisito para a obtenção do Título de Mestre em Ciências da Saúde.

Orientadora: Profa. Dra. Paula Goes.

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LUZIA HERMINIA TEIXEIRA DE SOUSA

UNIVERSIDADE FEDERAL DO CEARÁ
CAMPUS SOBRAL
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

ATA DA 10ª SESSÃO DE DEFESA DA DISSERTAÇÃO DE MESTRADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE DA UNIVERSIDADE FEDERAL DO CEARÁ-*CAMPUS* DE SOBRAL, REALIZADA NO DIA QUINZE DE FEVEREIRO DE DOIS MIL E DEZESSEIS.

Às oito horas do dia quinze de fevereiro de dois mil e dezesseis no Departamento de Patologia e Medicina Legal da Universidade Federal do Ceará - *Campus* de Fortaleza realizou-se a sessão de defesa da Dissertação de Mestrado da aluna **Luzia Herminia Teixeira de Sousa**. O trabalho tinha como título "Influência da osteoporose induzida por glicocorticóide na perda óssea alveolar em ratos com periodontite experimental e o efeito do tratamento com Atorvastatina". Compuseram a banca examinadora as professoras doutoras Paula Goes Pinheiro Dutra (Orientadora), Delane Viana Gondim (Examinadora Externa), Virgínia Cláudia Carneiro Girão (Examinadora Externa). A sessão foi aberta pela orientadora, que apresentou a banca examinadora e passou a palavra à aluna para que desse início aos trabalhos. Após apresentação, seguiu-se o processo de arguição da mestranda. Em seguida, a banca examinadora se reuniu reservadamente a fim de avaliar o desempenho da candidata. Por unanimidade, a banca examinadora considerou **APROVADO** o trabalho da mestranda. Nada mais havendo a relatar, a sessão foi encerrada. E eu, Paulo Roberto Santos, Vice-Coordenador do Programa de Pós-Graduação em Ciências da Saúde (PPGCS), lavrei a presente Ata, que depois de lida e aprovada, será assinada por mim e pelos membros da banca examinadora. Sobral, quinze de fevereiro de dois mil e dezesseis.

Profa. Dra. Paula Goes Pinheiro Dutra
(Orientadora)

Profa. Dra. Delane Viana Gondim
(Examinadora)

Profa. Dra. Virgínia Cláudia Carneiro Girão
(Examinadora)

Prof. Dr. Paulo Roberto Santos
(Vice-Coordenador do PPGCS)

Aos meus pais Francisco e Socorro pelo apoio incondicional

"Se cheguei até aqui foi porque me apoiei no ombro dos gigantes"

Isaac Newton

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RESUMO

A Periodontite é uma doença infecto-inflamatória crônica que afeta as estruturas de suporte dos dentes. A osteoporose induzida por glicocorticoides (OIGC) é o efeito adverso principal do uso prolongado do fármaco. A atorvastatina (ATV), um fármaco hipolipemiante, tem se destacado por apresentar efeitos pleiotrópicos: anti-inflamatório, antioxidante e anabólico ósseo. Diante disso, essa dissertação tem como objetivo avaliar a influência da osteoporose induzida por glicocorticoide na perda óssea alveolar em ratos com periodontite experimental (PE) e o efeito anti-inflamatório, antirreabsortivo e antioxidante do tratamento com Atorvastatina. No estudo foram utilizados 48 ratos *Wistar* machos que foram divididos em grupos: *Naïve*, PE, OIGC, OIGC+PE e ATV. Para indução da osteoporose induzida por glicocorticoide, os grupos OIGC, OIGC+PE e ATV receberam injeções intramusculares de 7 mg/kg de dexametasona (DEXA) 1x/semana por 5 semanas, e os grupos *Naïve* e PE receberam injeções intramusculares de 0,5 ml de solução salina (SAL). Em seguida, os grupos PE, OIGC+PE e ATV foram submetidos a PE induzida por ligadura em torno do 2º molar superior esquerdo. O grupo ATV recebeu 27 mg/kg de ATV 30 minutos antes da ligadura e diariamente por 11 dias, os demais (*Naïve*, PE, OICG e OICG+PE), receberam 2 ml de SAL por via oral por 11 dias, quando então foram eutanasiados. Os animais do grupo *Naïve* não receberam ligadura e foram utilizados como controle. A perda óssea alveolar (POA) foi determinada por exames macroscópicos, radiográficos, micro-tomográficos e microscópicos das hemimaxilas dos animais. Os fêmures direitos dos ratos foram removidos para análise radiográfica e biomecânica. A atividade de mieloperoxidase (MPO) na gengiva, e os níveis gengivais de fator de necrose tumoral (TNF)-α, interleucina (IL)-1β, -6, -8, e -10, glutationa reduzida (GSH), superóxido dismutase (SOD) e catalase (CAT) foram analisados para investigar o estresse oxidativo e inflamação. Para avaliar a influência da via WNT/β-catenina, a expressão imunohistoquimica de RANKL, OPG, WNT10b, DKK-1 e β-catenina foram investigadas. O leucograma e os níveis séricos de transaminases, ureia, creatinina e fosfatase alcalina óssea (FAO) também foram realizadas. Os animais do grupo OICG+PE mostraram maior POA ($p<0,05$), maior redução de FAO ($p<0,05$), da densidade radiográfica da maxila e módulo de Young no fêmur ($p<0,05$) quando comparado ao grupo PE. O tratamento com ATV reduziu a POA considerando todos os parâmetros analisados. A ATV reduziu a atividade de MPO, e os níveis de TNF-α, IL-1β, -6, e -8, enquanto aumentou os níveis gengivais de IL-10, GSH, SOD e CAT. ATV reduziu a expressão de RANKL e DKK-1, e aumentou OPG, WNT10b e β-catenina. A administração de ATV aumentou a atividade de FAO no 11º dia comparado a OICG+PE. Nenhuma diferença foi vista nos níveis de transaminases, ureia e creatinina em qualquer dos grupos estudados. Assim podemos concluir que OICG piora a POA, inibe a atividade osteoblástica e altera a perda óssea sistêmica em ratos com PE, e que a ATV previne a POA em ratos submetidos à OICG+PE, por meio de efeito antirreabsortivo, anti-inflamatório e antioxidante, com participação da via de sinalização WNT.

Palavras chave: Atorvastatina. Periodontite. Osteoporose. Glicocorticoides.

ABSTRACT

Periodontitis is a chronic infectious and inflammatory disease affecting the tooth supporting structures. The glucocorticoid-induced osteoporosis (GIOP) is the major adverse effect of long-term use of the drug. Atorvastatin (ATV) a lipid-lowering drug, has been highlighted by presenting pleiotropic effects: anti-inflammatory, antioxidant and bone anabolic. Therefore, this dissertation aims to evaluate the influence of glucocorticoid-induced osteoporosis in alveolar bone loss in rats with experimental periodontitis (EP) and anti-inflammatory effect, antirreabsortivo and antioxidant treatment with Atorvastatin. For the study, 48 male *Wistar* rats were divided into groups: *Naïve*, EP, GIOP, GIOPE + EP and ATV. For induction of glucocorticoid-induced osteoporosis, the GIOP groups GIOP + EP and ATV received intramuscular injections of 7 mg / kg of dexamethasone (DEXA) 1x / week for 5 weeks, and *Naïve* and EP groups were given intramuscular injections of 0.5 ml of saline (SAL). Then the EP groups GIOP + EP and EP were subjected to ATV induced by ligature around the left upper second molar. The ATV group received 27 mg / kg ATV 30 minutes before the ligation and daily for 11 days, the other (*Naïve*, EP, GIOP and GIOP + EP) received 2 ml of SAL orally for 11 days, when they were euthanized. Animals *naïve* group received no ligation and were used as controls. Alveolar bone loss (ABL) was determined by macroscopic, radiographic, micro-tomographic and microscopic of the maxillary sides of animals. The right femurs of rats were removed for radiographic and biomechanical analysis. Myeloperoxidase activity (MPO) in the gingiva and gingival levels of tumor necrosis factor (TNF) -α, interleukin (IL) -1β, -6, -8, and -10, reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were analyzed to investigate oxidative stress and inflammation. To evaluate the influence of Wnt / β-catenin, the immunohistochemical expression of RANKL, OPG, Wnt10b, DKK-1 and β-catenin were investigated. The leucocyte count and serum levels of transaminases, urea, creatinine, and bone alkaline phosphatase (BALP) were also performed. The animals GIOP + EP group showed higher of ABL ($p < 0.05$) greater reduction of BALP ($p < 0.05$), the radiographic density of the maxilla and Young's modulus of the femur ($p < 0.05$) when compared the EP group. Treatment with ATV reduced the ABL considering all parameters. The ATV reduced MPO activity and TNF-α levels, IL-1β, -6, and -8, while gingival increased levels of IL-10, GSH, SOD and CAT. ATV reduced the expression of DKK-1 and RANKL and increased OPG Wnt10b and β-catenin. The ATV administration increased the BALP activity on the 11th day compared to GIOP + EP. No difference was seen in the levels of transaminases, urea and creatinine in any of the groups studied. Thus we conclude that GIOP worsens ABL inhibit osteoblastic activity and alters systemic bone loss in rats with EP, and ATV prevents ABL in rats subjected to GIOP + EP through antirreabsortive effect, anti-inflammatory and antioxidant with participation of the WNT signaling pathway.

Keywords: Atorvastatin. Periodontitis. Osteoporosis. Glucocorticoids.

LISTA DE ABREVIATURAS

ATV	Atorvastatina
BMP	Proteína morfogenética óssea
CBFA	*core-binding factor subunit alpha*
CAT	Catalase
DKK	*Dickkopf*
EO	Estresse Oxidativo
EROs	Espécies reativas de oxigênio
FAO	Fosfatase alcalina óssea
GCs	Glicocorticoides
GPx	Glutationa peroxidase
HMG-CoA	3-hidroxi3-metilglutaratl co-enzima A
ICAM	Moléculas de adesão intercelular
IFN	Interferon
IL	Interleucina
LEF1	Fator amplificador de linfócitos
LPS	Lipopolissacarídeos
LRP5/6	*Low-density lipoprotein receptor-related protein 5/6*
MPO	Mieloperoxidase
OIGC	Osteoporose Induzida por glicocorticoide
OPG	Osteoprotegerina
PG	Prostaglandina
PMNs	Polimorfonucleares
POA	Perda óssea alveolar
RANK	Receptor Ativador do Fator Nuclear-κB
RANKL	Ligante do Receptor Ativador do Fator Nuclear-κB
ROOH	Peróxido
RAR	Raspagem e alisamento radicular
RUNX	*Runt-related transcription fator*
SAL	Salina
SFRP	Proteína relacionada ao *frizzled* secretada
SOD	Superóxido dismutase

SOST	Esclerostina
TCF	Fator de célula T
TNF	Fator de Necrose Tumoral
VCAM	Moléculas de adesão vascular
VEGF	Fator de crescimento endotelial vascular
WIF	Fator inibidor de WNT
WNT	*Wingless*

Sumário

1. INTRODUÇÃO

1.1. PERIODONTITE

A periodontite é uma doença infecto-inflamatória dos tecidos de suporte do dente, de alta prevalência na população mundial, e é uma das principais causas de perda dentária (Petersen e Ogawa, 2005; Borrell e Papapanou, 2005).

A etiologia da periodontite é multifatorial. Estudos têm demonstrado que a presença de periodontopatógenos é fundamental para o desencadeamento dessa doença nos sítios periodontais (Teles et al., 2006; Boehm & Scannappieco, 2007; Bascones et al., 2009). Fatores de risco genéticos e ambientais podem alterar a resposta do hospedeiro, o que contribui sobremaneira para o início e progressão da periodontite (Jansson, 2006), por desequilibrar a homeostasia dos tecidos periodontais (Pihlstrom et al., 2005). A susceptibilidade do hospedeiro é determinante para o início e progressão da doença, pois a hiperresponsividade de vias imunológicas e exacerbação da inflamação com a liberação de citocinas inflamatórias, podem resultar em destruição tecidual aumentada (Pihlstrom et al., 2005).

Considerando a resposta do hospedeiro, diante da presença de periodontopatógenos, há uma maior liberação de lipopolissacarídeos (LPS) e recrutamento de neutrófilos para o sítio de infecção, os quais liberam enzimas proteolíticas que causam dano tecidual (Giannobile, 2008). A primeira linha de defesa contra os periodontopatógenos presentes no periodonto é formada pelos leucócitos polimorfonucleares (PMN). No sulco gengival, os PMNs tentam eliminar as bactérias através da fagocitose, auxiliado por substâncias inflamatórias e antimicrobianas, tais como lisozima, lactoferrina, fosfatase alcalina, hidrolases ácidas, proteínas catiônicas, espécies reativas de oxigênio (EROs) e mieloperoxidase (MPO) (Bascones et al., 2009).

Os LPS também agem ativando macrófagos, que promovem a liberação de vários mediadores pró-inflamatórios, tais como interleucina (IL)-1β, Fator de Necrose Tumoral (TNF)-α e prostaglandina (PG) E_2, importantes na destruição de tecido periodontal (Górska et al., 2003; Giannobile, 2008).

A constante infiltração e ativação de PMNs nos tecidos inflamados estimula a liberação de diversas citocinas pró-inflamatórias, entre estas podemos citar IL-1β, IL-8 e TNF-α (Van Dyke e Serhan 2003). A resposta

inflamatória torna-se cada vez mais amplificada, levando ao aparecimento de outras células da imunidade adaptativa tais como linfócitos T e B, que em conjunto acabam por repercutir no tecido ósseo aumentando a reabsorção óssea alveolar (Soedarsono et al*.,* 2006).

A MPO é uma enzima que auxilia a geração de espécies reativas de oxigênio (EROs) e está presente nos grânulos azurofílicos dos neutrófilos, e por isso pode ser considerada um indicador do acúmulo de neutrófilos nos tecidos. Fisiologicamente, participa da destruição de microrganismos, mas, em condições patológicas e causar danos teciduais, por aumentar o estresse oxidativo (EO) (Yamalik et al., 2000; Malle et al., 2006).

Consequências do EO incluem danos ou morte de células através de uma variedade de mecanismos (Halliwell e Whiteman, 2004). Mecanismos enzimáticos são responsáveis para a neutralização direta de EROs, e esses mecanismos são constituídos por enzimas que tem por objetivo manter os níveis de EROs normais (Kaklamanos e Tsalikis, 2002). Exemplos destas enzimas são superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) (Kaklamanos e Tsalikis, 2002).

A SOD é uma das mais abundantes enzimas antioxidantes no corpo humano (Akalin et al., 2007). Um de seus mecanismos de ação é a conversão de ânions superóxido em peróxido de hidrogênio (H_2O_2), operando como um antioxidante, porque evita a formação do radical hidroxila (OH-) (Scandalios et al., 2000). Os níveis de SOD tem se mostrado reduzidos em pacientes com periodontite crônica, quando comparados aos controles (Kim et al., 2010). A CAT é encontrada nos peroxissomas e é capaz de remover H_2O_2 e radicais superóxido intracelulares com grande eficácia (Battino et al., 1999). Níveis salivares de CAT foram reduzidos em pacientes com periodontite crônica quando comparados com adultos saudáveis (Trivedi et al., 2014). A GPx é responsável pela a proteção de células de mamíferos contra danos oxidativos por reduzir uma variedade de hidroperóxidos, tais como o ROOH e H_2O_2 (Chapple et al., 2002).

Fisiologicamente, o tecido ósseo encontra-se em equilíbrio dinâmico devido à ação dos osteoblastos, envolvidos na formação de matriz óssea, e osteoclastos, os quais são responsáveis pelo processo de reabsorção. Tal fenômeno é mediado pelo sistema constituído pelo Receptor Ativador do Fator

Nuclear-κB (RANK), pelo seu Ligante (RANKL) e pela Osteoprotegerina (OPG) (RANK/RANKL/OPG) (Xing et al., 2005; Brennan et al., 2007; Reid & Holen, 2009). O RANKL, pertencente à superfamília do TNF, é expresso 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, consequente, reabsorção óssea (Reid & Holen, 2009). Por outro lado, para regular o metabolismo ósseo, osteoblastos produzem e liberam a OPG que inibe a interação RANKL-RANK, por meio de ligação competitiva com RANKL (Soedarsono et al., 2006; Reid & Holen, 2009). Entretanto, durante um processo inflamatório crônico, como se observa na periodontite, o aumento dos vários mediadores pró-inflamatórios, tais como TNF-α e IL-1β, estimulam a expressão abundante de RANKL em osteoblastos, bem como em células T, B e fibroblastos, favorecendo então a osteoclastogênese (Soedarsono et al*.,* 2006).

Além do eixo RANK-RANKL-OPG, mais recentemente, foi relatado na literatura a participação do via WNT/β-catenina como um modulador do metabolismo ósseo (Wang et al., 2014). Esta via tem sido bastante explorada em estudos de proliferação celular e carcinogênese (Kikuchi, 2003; Nusse, 2005; Clevers, 2006; Sherwood, 2015), entretanto, foi demostrado que ela também é crítica para a diferenciação de células mesenquimais em osteoblastos maduros (Hartmann, 2006).

Os membros da família WNT são proteínas secretadas que após interação com seu receptor impede a destruição da β-catenina, a qual se estabiliza e é translocada para o núcleo e, acompanhada por LEF1 (fator amplificador de linfócitos)/TCF (fator de célula T), funciona como co-ativador para transcrição de genes que levam a diferenciação de osteoblastos (Moon et al., 2004). Esta via por sua vez, é controlada por proteínas, tais como proteína relacionada ao *frizzled* secretada (SFRP) ou fator inibidor de WNT (WIF) que capturam e inativam a WNT no compartimento intercelular, ou ainda Dickkopf (DKK) e Esclerostina (SOST) que bloqueiam o receptor LRP5/LRP6 (*Low-density lipoprotein receptor-related protein)* de WNT (Wend et al., 2012). Em doenças inflamatórias ósseas, como a periodontite, onde citocinas inflamatórias são produzidas, antagonistas da via WNT como DKK e SOST estão aumentados

levando a um bloqueio dessa via a qual é importante para o metabolismo ósseo levando a alterações na osteoblastogênese (Westendorf et al., 2004). Foi demostrado um aumento dos níveis de SOST e DKK no tecido gengival de pacientes com periodontite crônica evidenciando o envolvimento da via WNT/β-catenina na patogênese da perda óssea na periodontite (Napimoga et al., 2014).

Sabendo que o processo inflamatório e a perda óssea são os principais achados clínicos da periodontite, o diagnóstico desta doença na prática clínica tem sido realizado por meio de exames capazes de detectar parâmetros como: aspecto clínico do periodonto, presença de sangramento, profundidade de bolsas, nível clínico de inserção, dentre outros (Pihlstrom et al., 2005). Adicionalmente, como forma auxiliar de avaliação do grau de perda óssea periodontal, exames de imagens são frequentemente requeridos. Imagens radiográficas digitais se destacam frente às imagens convencionais, visto que apresentam maior capacidade de detecção de sítios com perdas ósseas ainda sutis, quando comparadas às imagens radiográficas convencionais (Khocht et al., 2003). O uso de imagens tomográficas vem assumindo posição de destaque na Odontologia e Periodontia, pois propiciam maior riqueza de detalhes quanto à qualidade e quantidade de suporte ósseo (Khocht et al., 2003), o que favorece, consequentemente, diagnóstico e terapia precoces.

O tratamento clássico da periodontite se baseia na remoção mecânica dos depósitos de biofilme e cálculo através da raspagem e alisamento radicular (RAR) com instrumentos manuais (Cobb, 2002). Foi demonstrado melhorias significativas nos parâmetros clínicos e microbiológicos após a terapia convencional não-cirúrgica (Badersten et al., 1984). No entanto com base nos conhecimentos atuais sobre patogênese da reabsorção óssea inflamatória, o tratamento periodontal clássico que durante muito tempo objetivou apenas o controle bacteriano (Boehm e Scannapieco, 2007), passa por mudança de paradigma (Buduneli, 2007), pois outras estratégias terapêuticas, tais como a modulação da resposta do hospedeiro, vem sendo estudadas como uma nova abordagem do tratamento adjuvante à RAR (Preshaw et al., 2004; Buduneli, 2007).

1.2. OSTEOPOROSE INDUZIDA POR GLICOCORTICOIDE

Os glicocorticoides (GCs) são uma opção terapêutica eficaz utilizada no tratamento de muitas doenças inflamatórias e auto-imunes. No entanto, mesmo em doses moderadas, os GCs apresentam sérios efeitos adversos, dentre eles a osteoporose (Grossman et al., 2010; Rizzoli e Biver, 2015).

A osteoporose induzida por glicocorticóides (OIGC) é a forma mais comum da osteoporose iatrogênica (Compston, 2010). É caracterizada por fragilidade à fraturas que ocorrem em 30% a 50% de pacientes que fazem uso de GCs sistêmicos a longo prazo (Van Staa et al., 2003).

A patogênese de OIGC é o resultado dos efeitos indiretos mediados pela alteração no metabolismo do cálcio nos rins e no intestino, redução da produção de hormônios gonodais e efeitos prejudiciais sobre o sistema neuromuscular associado aos efeitos diretos dos GCs sobre as células ósseas (Canalis et al., 2007; Hofbauer et al., 2009, 2010).

Os GCs têm ações complexas sobre a expressão gênica das células ósseas dependendo do estágio de diferenciação e crescimento dos osteoblastos. Aumentam a reabsorção óssea por efeitos diretos e indiretos sobre os osteoclastos. Estes agentes inibem aspectos específicos da diferenciação osteoblástica, como a transcrição de osteocalcina, a proteína não colágena mais abundante no osso, e a expressão do colágeno tipo I. Além de diminuírem a síntese, os GCs aumentam a degradação do colágeno por aumentarem a expressão das colagenases e inibirem a expressão do inibidor tecidual das metaloproteinases. Como o colágeno tipo I é a maior proteína estrutural da matriz óssea, uma diminuição na sua expressão e aumento da sua degradação são críticos para a ação inibitória dos GCs na matriz óssea. (Schleimer, 1993; Delany et al., 1995; Canalis, 1996; Lukert, 1996).

Os efeitos sobre os osteoclastos são bifásicos, concentrações fisiológicas são necessárias para o estágio final de diferenciação e função, porém altas doses e exposição prolongada podem inibir a replicação celular. A reabsorção óssea observada *in vivo* ocorre pelo aumento da atividade osteoclástica com aumento da quantidade de osso reabsorvido em cada sítio. Os GCs também podem aumentar a aderência dos macrófagos ao osso por alterarem a superfície dos oligossacarídeos levando uma maior reabsorção óssea osteoclástica (Delany et al., 1995; Canalis,1996; Lukert et al., 1996).

A terapia com GCs é marcada por um aumento precoce e transiente de osteoclastos (Mazziotti et al., 2006; Weinstein, 2011) seguido da inibição importante de osteoblastos (Zhou et al., 2014). Estudos *in vitro* e *in vivo* pré-clínico e clínico, mostraram que a dexametasona impede a diferenciação osteoblástica, especialmente através da inibição da via de sinalização WNT. Este efeito foi parcialmente atribuído ao aumento da expressão de DKK-1 pela dexametasona (Ohnaka et al., 2004, 2005). Os GCs inibem a expressão Runx2/Cbfa, um fator de transcrição essencial para a diferenciação de osteoblastos e sua expressão é dependente da via WNT (Chang et al.,1998).

Uma forma de avaliação da atividade de osteoblastos aplicada a clínica em pacientes com osteoporose é a dosagem de fosfatase alcalina óssea (FAO). A FAO é uma glicoproteína tetramérica específica do osso, secretada por osteoblastos com papel na mineralização óssea (Vieira, 1999). Ela está presente nos condrócitos hipertróficos e na placa de crescimento epifisária (Price, 1993; Crofton et al., 1999). Apesar da grande semelhança estrutural entre as isoformas, imunoensaios específicos foram desenvolvidos para a isoforma óssea, o que diminui a reação cruzada com a isoforma hepática em 15% a 20% (Watts, 1999).

1.3. OIGC E PERIODONTITE

A doença periodontal esta associada a várias condições sistêmicas. Foi demonstrada a relação entre periodontite e diabetes, parto prematuro, pneumonia nosocomial, doenças cardiovasculares, osteoporose, dentre outras. (Seymour et al.,2003)

O interesse na relação entre osteoporose e periodontite tem crescido consideravelmente (von Wowern et al, 1994; Wactawski-Wende et al., 1996; Tezal et al., 2000; Boyce e Xing, 2007). Elas compartilham alguns fatores de risco comuns, tais como idade avançada, história prévia de perda óssea, algumas doenças sistêmicas, medicamentos, tabagismo e genética familiar (Reddy, 2001).

Embora se tenha observado a extensão desta relação, seus mecanismos permanecem incertos. A literatura descreve detalhadamente a associação de osteoporose causada pela deficiência hormonal com a progressão da periodontite (Wactawski-Wende et al*.,* 1996; Ronderos et al., 2000; Inagaki et al.,

2005). A baixa densidade óssea em mulheres em fase pós-menopausa foi relacionada à perda alveolar interproximal, que persistiu após o tratamento periodontal convencional (Tezal et al., 2000). No entanto, a relação entre OIGC e periodontite ainda permanece pouco estudada. Alguns estudos suportam a ideia de que a OIGC pode piorar a destruição de tecidos de suporte do dente aumentando a agressividade e acelerando a taxa de progressão da doença periodontal (Wactawski-Wende et al., 1996; Nicopoulou-Karayianni et al., 2009). Alternativas terapêuticas, como a modulação da resposta do hospedeiro, vêm sendo estudadas como uma nova abordagem no tratamento de doenças inflamatórias ósseas como a periodontite (Preshaw et al., 2004; Buduneli, 2007).

1.4. ESTATINAS

As estatinas são uma classe farmacológica que atua sobre a via do mevalonato, inibindo a enzima 3-hidroxi-3-metilglutaratil co-enzima A (HMG-CoA) redutase. As estatinas são redutoras efetivas dos níveis séricos de colesterol, 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 et al., 2011) e efeito anabólico ósseo (Mundy et al., 1999; Horiuchi & Maeda, 2006; Goes et al., 2010; Dalcico et al., 2013). Também foi demonstrado o efeito antioxidante das estatinas atuando reduzindo as EROs (Bolayirli et al., 2007). Tais propriedades oferecem grande potencial para estatinas modificarem o curso de doenças inflamatórias crônicas (Barsante et al., 2005) dentre as quais podem ser incluídas a periodontite (Dalcico et al., 2013).

A atividade anti-inflamatória das estatinas baseia-se na inibição de moléculas de adesão intercelular (ICAM), vascular (VCAM), selectinas (Nawawi et al., 2003), citocinas IL-1, TNF (Waehre et al., 2003), IL-2, IL-12, e interferon (IFN)–γ (Jasińska et al., 2006). Em adição, as estatinas também estão envolvidas na expressão de estimuladores de diferenciação osteoblástica tais como proteína morfogenética óssea (BMP) -2 e fator de crescimento endotelial vascular (VEGF) (Maeda et al., 2004). Além de possuírem propriedades antioxidantes, inibindo a expressão exagerada de EROs (Bolayirli et al., 2007).

Dentre as estatinas destaca-se a Atorvastatina (ATV), pela sua característica lipofílica e seus poucos efeitos adversos quando comparada a outras estatinas, sendo, portanto amplamente utilizada na prática clínica (Plosker e Lyseng-Williamson, 2007).

Assim, diante do exposto, para um maior conhecimento acerca da relação entre OIGC e periodontite e considerando que as estatinas possuem propriedades anti-inflamatórias, anabólicas óssea e antioxidantes, o objetivo do presente trabalho foi avaliar a influência da OIGC na perda óssea alveolar e o efeito antirreabsortivo, anti-inflamatório e antioxidante do tratamento com ATV em ratos submetidos à OIGC e periodontite experiemental.

2. OBJETIVOS GERAIS

a) Avaliar a influência osteoporose induzida por glicocorticoide na perda óssea alveolar de ratos com periodontite experimental.

b) Avaliar o efeito antirreabsortivo, anti-inflamatório e antioxidante da Atorvastatina (ATV) na perda óssea alveolar de ratos submetidos à osteoporose induzida por glicocorticoide com periodontite experimental.

3. OBJETIVOS ESPECÍFICOS

a) Avaliação da POA por meio de análises macroscópicas, radiográficas, micro-tomográficas e microscópicas;

b) Avaliação da perda óssea no fêmur por meio de análises radiográficas e ensaio biomecânico

c) Avaliação da atividade anti-inflamatória através da atividade de mieloperoxidase (MPO) e quantificação dos níveis de TNF-α, IL-1β, -6, -8, -10 no tecido gengival por ELISA;

d) Avaliação da atividade antioxidante por meio da quantificação dos níveis de enzimas do estresse oxidativo (SOD, CAT e GSH) no tecido gengival por ELISA;

e) Avaliação das vias de sinalização do metabolismo ósseo por meio de análise imunohistoquímica para RANKL, OPG, WNT 10b, DKK e β-catenina;

f) Avaliação de parâmetros sistêmicos por meio do leucograma; dosagem sérica de transaminases, ureia e creatinina;

g) Avaliação da atividade osteoblástica através da dosagem sérica de FAO.

4. CAPÍTULOS

Esta dissertação esta baseada no Regimento Interno do Programa de Pós-graduação em Ciências da Saúde da Universidade Federal do Ceará – *Campus* Sobral que regulamenta o formato alternativo para dissertações de Mestrado 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 com protocolo 78/2014. (Anexo I).

4.1 ARTIGO 1

“Effect of glucocorticoid-induced osteoporosis on alveolar bone loss of rats with experimental periodontitis”

Autores: Luzia Herminia Teixeira de Sousa, Eveline Valeriano Moura, Ana Larissa Queiroz, Danielle Val, Hellíada Chaves, Mario Lisboa, Flávia Furlaneto, Gerly Anne Brito, Paula Goes

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

Qualis B1: Medicina II

3.2 ARTIGO 2

“Effects of Atorvastatin on periodontitis of rats subjected to glucocorticoid-induced osteoporosis”

Autores: Luzia Herminia Teixeira de Sousa, Eveline Valeriano Moura, Joanna Trycia Alexandre, Mario Lisboa, Flavia Furlaneto, Raul Freitas, Isabela Ribeiro, Danielle Val, Mirna Marques, Hellíada Chaves, Conceição Martins, Gerly Anne Brito, Paula Goes.

Esse artigo seguiu normas de publicação do periódico: *Journal of Periodontology*. (ISSN 0022-3492).

Qualis A2: Medicina II

Artigo 1

Effect of glucocorticoid-induced osteoporosis on alveolar bone loss of rats with experimental periodontitis

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Running title: Effect of GIOP on alveolar bone loss

ABSTRACT

Objective: To evaluate the effect of osteoporosis induced by glucocorticoid (GIOP) in rats with experimental periodontitis (EP).

Design: 48 male *Wistar* rats were divided into: Naïve, EP, GIOP and GIOP+EP groups. Rats of GIOP and GIOP+EP groups received intramuscularly 7 mg/kg of dexamethasone once a week for 5 weeks. Following, the rats from EP and GIOP+EP groups were subjected to ligature-induced EP for 11 days. Later, the animals were euthanized, and maxillae collected for macroscopic, radiographic, micro-tomographic, and microscopic analysis of alveolar bone loss (ABL). Blood samples were collected for determination of bone-specific alkaline phosphatase (BALP) serum levels and the right femurs were removed for radiographic and biomechanical analysis.

Results: EP caused ABL and reduced BALP serum levels, but did not change the architecture or biomechanics of femur when compared to Naïve. GIOP did not cause ABL compared to Naïve, but it decreased bone mineral density, bone percentage, trabecular thickness and an increase on bone porosity, compared to Naïve ($p<0.05$). GIOP significantly reduced BALP serum levels, and radiographic density and Young's module of femur, compared to Naïve. In GIOP+EP group it was seen a greater increase on ABL, when compared to EP ($p<0.05$). It also showed a greater reduction on BALP serum levels ($p<0.05$), on radiographic density and Young's module of femur, compared to EP ($p<0.05$)

Conclusions: This data suggest that GIOP aggravates ABL, inhibits the osteoblast activity and alters systemic bone loss in rats with EP.

Keywords: Periodontitis; Osteoporosis; Glucocorticoids; Alveolar bone loss

Highlights:

- GIOP worsen ABL in animals subjected to EP

- GIOP caused a greater decrease on osteoblast activity in animals subjected to EP
- GIOP caused bone loss on femur of animals subjected to EP

1. Introduction

Periodontitis is an infectious-inflammatory and high prevalent disease, characterized by destruction of connective tissue and alveolar bone loss (ABL), and it is considered the second major cause of tooth loss (Krustrup & Petersen, 2006). This disease is mainly initiated by oral biofilm however the development of an exacerbated host response plays an important role on tissue breakdown (Pihlstrom, Michalowicz & Johnson, 2005).

Osteoporosis is a common disease characterized by systemic bone loss and impaired bone microarchitecture. It can be a consequence of hormonal imbalance in postmenopausal woman (Jilka, Weinstein, Bellido, Parfitt & Manolagas, 1992), but also it can has a secondary cause, mainly as a result of drug use such as Glucocorticoids (GCs). Glucocorticoid-induced osteoporosis (GIOP) is the most common cause of secondary osteoporosis, the first cause before 50 years and the first iatrogenic cause of the disease (Kok & Sambrook, 2009). In addition, considering the continuous raise on the prevalence of GCs use in the community population (Overman, Yeh & Deal, 2013), it seems interesting understand the biological mechanism underlying GIOP.

In recent decades, numerous studies have focused on the association between osteoporosis and periodontitis at the bone level. The majority of the studies has focused on the effect of postmenopausal osteoporosis in the loss of periodontal attachment (Hernández-Vigueras, Martínez-Garriga, Sánchez, Sanz, Estrugo-Devesa & Vinuesa, 2015; Juluri, Viswanathan, Gopalakrishnan, Kathariya, Devanoorkar & Prashanth, 2015). However, a little is known about the effect of GIOP on periodontal tissue and even more during periodontal inflammation. In this way we aimed to evaluate the effect of GIOP on alveolar bone loss of animals with experimental periodontitis.

2. Material and Methods

Animals and study design

The experiments were performed on forty-eight male Wistar rats (*Rattus*

norvegicus) from central Animal Facility of Federal University of Ceará, weighing 180–220 g, kept in appropriate cages with six animals each. The animals were housed in standard conditions (12h light-dark cycles and temperature-controlled rooms) with food and water *ad libitum*. The protocols for experimental procedures and animal treatment were approved by Animal Ethics Committee (number 78/2014) of Federal University of Ceará, Brazil.

A power calculation was performed to determine the sample size. The animal was considered the study unit. The sample size was determined to provide 80% power to recognize a significant difference of 20% among groups and the standard deviation of 15% with a 95% confidence interval (p = 0.05), considering the change in alveolar bone loss (ABL) as the primary outcome variable. Therefore, a sample size of 6 animals per group was required.

After two weeks of adapting in the laboratory environment, the rats were divided into four groups (n=6): Naïve, Experimental Periodontitis (EP), Glucocorticoid-induced osteoporosis (GIOP) and GIOP+EP. Initially, rats from GIOP and GIOP+EP groups received intramuscularly injections of 7 mg/kg dexamethasone (Decadron, Aché - Guarulhos - SP, Brazil), once a week, for 5 weeks (Lucinda, Aaresturup, Peters, Reis, de Oliveira, & Guerra, 2012), and the ones from Naïve and EP groups received 0.5 ml of 0.9% of Saline Solution. Following, the rats were anesthetized with 100 mg/ml ketamin (Cetamin - Syntec®) and 20 mg/ml xylanzine (Xilazin - Syntec®) on the dose of 1 ml/kg – intramuscularly, for EP, which was induced by the placement of a sterile nylon thread ligature (3-0; polysuture NP45330, São Paulo, Brazil) around the cervix of the maxillary left second molar of rats (EP and GIOP+EP groups) (Bezerra et al., 2000). After 11 days all animals were euthanized with 20 mg/kg thiopental (0.5 g Thiopentax; Cristália, São Paulo, SP, Brazil). Rats of Naïve and GIOP group did not receive the ligature.

Micro-computed tomography (CT) scanning and Assessment of alveolar bone loss

For macroscopic analysis, the excised maxillae were fixed in 10% neutral formalin for 24 h. Both maxillary halves were then defleshed and stained with 1% aqueous methylene blue in order to differentiate bone from teeth, then, they were placed on microscope slides and photographed. The area of alveolar bone loss

(ABL) was measured by a trained and blinded observer, using an imaging software (ImageJ® National Institutes of Health, Washington, DC, USA), as previously described (Goes, Lima, Melo, Rego, & Lima, 2010).

The same specimens used for macroscopic study were radiographed using a digital system (Digora Soredex Digital System®, Portslade-East Sussex, UK). The specimens were posed over the sensor and the radiographic images were acquired using 63 kVp, 8 mA, exposure time of 0.06 s and focal distance of 30 mm. These images were evaluated using Image J® software (Goes, Lima, Melo, Rego, & Lima, 2010).

The same non-demineralized specimens were then scanned by a cone beam micro-computed tomography (CT) system (Skyscan 1172, Bruker, Kontich, Belgium). The x-ray generator was operated at an accelerated potential of 50 kV with a beam current of 200 µA and an exposure time of 560 ms per projection. Images were produced with a voxel size of 6x6x6 µm. Using an appropriated software (Data Viewer®, version 1.5.0, Bruker, Kontich, Belgium), the generated 3 dimensional models were rotated into a standard position as described previously (Lisboa, Gondim, Ervolino, Vale, Frota & Nunes, 2015). Linear measurements on ABL were performed at 3 different sites: buccal, furcation and interproximal. For the interproximal site, coronal dataset was analyzed using appropriated software (CT-Analyser®, version 1.13.5.1+, Bruker, Kontich, Belgium). Bone mineral density (BMD), bone percentage, bone porosity, and trabecular thickness (Tb.Th) were also analyzed (Lisboa, Gondim, Ervolino, Vale, Frota & Nunes, 2015). All micro-CT analyses were performed by one blinded and calibrated examiner.

Bone histology observation

Another set of experiment was performed for histopathological analysis. On day 11th day after EP the maxillae were removed and fixed in 10% neutral buffered formalin and demineralized in 10% EDTA buffered solution. Later the specimens were dehydrated, embedded in paraffin and sectioned along the molars in the mesiodistal plane for hematoxylin and eosin staining. Sections of 4 µm thickness, corresponding to the area between the first and second molars where the ligature had been placed, were evaluated by light microscopy (x40 magnification). The parameters analyzed were based on the study (Leitão,

Ribeiro, Chaves, Rocha, Lima, & Brito, 2005) using scores from 0–3. The histopathological analysis was performed by a certified histologist.

Serum levels of bone-specific alkaline phosphatase (BALP)

Blood samples were collected from the orbital plexus at the 11th day after EP. Bone-specific alkaline phosphatase (BALP), a thermosensible isoform of total alkaline phosphatase, was evaluated using a thermoactivation method (Whitby & Moss, 1975). The samples were heated to 56 °C for 10 min. Serum levels of BALP were calculated by the difference between heated alkaline phosphatase from total alkaline phosphatase in serum (Labest®, Lagoa Santa, MG, Brazil) (Goes, Melo, Dutra, Lima, & Lima, 2012; Goes, Melo, Silva, Benevides, Alencar & Ribeiro, 2014).

Biomechanical testing and Radiographic Density (RD) of Femur

The rats' right femur were collected 11 days after ligature placement and fixed, for 24 h, in 10% formaldehyde (Reagen Produtos para Laboratórios Ltda®, Rio de Janeiro-RJ, Brazil). The specimens were radiographed using Digora Soredex System® (Dental Imaging Company Ltd, Portslade-East Sussex, UK) with the same configuration as for the maxilla. These images were evaluated by Image J® software. A region of interest (ROI) was created measuring 0.5 (w) 0.5 (y), in the form of a square, with 473 x 229 pixels, and was posed in the proximal femur diaphysis, and the same evaluator drew all ROI panels. Grey tone differences from both areas were considered as a value of radiographic density. The RD analysis was done similarly to the one of maxilla (Mahl, Tonietto, Giorgi, Girotto, & Fontanella, 2009).

The same non-demineralized femurs were analyzed in a materials testing machine (InstronE10000, Instron Corporation, Norwood, MA, USA), using a three-point bending procedure. Femurs were compressed until failure at a displacement rate of 3 mm/min (span = 20 mm) with the anterior side down, and all force and displacement data were recorded until the specimen was broken. Young's modulus (MPa) were calculated, assuming the cross-sectional geometry of the femurs were elipses (Xu, Chen, Zhang, Zhai, Liu & Qin, 2014).

Statistical analysis

The data are presented as mean±standard error of the mean or as median (range), where appropriate. Normality and homoscedasticity of the data were verified. ANOVA followed by the Bonferroni test were used to compare the means, and Kruskal–Wallis and Dunn tests were used to compare the medians. The significance level was set at 5% in all tests. All calculations were performed using the Prism 5 (GraphPad Software Inc., San Diego, CA, USA). All protocols and analyses were performed in a blinded manner.

3. Results

Assessment of alveolar bone loss and Micro-computed tomography (CT) scanning

On the macroscopic analysis on hemimaxillae (Figure 1A), Naïve group (Figure 1C) presented an ABL close to zero, there was a non significant difference between the two sides of the maxilla. Eleven days of EP caused a significant ($p<0.05$) ABL compared to Naïve showing furcation lesions and root exposure (Figure 1E). The response to GIOP (Figure 1G) was similar to that of Naïve group. However, animals subjected to GIOP+EP (Figure 1I) showed greater ($p<0.05$), ABL when compared to EP.

The results of RD analysis of hemimaxillae can be evaluated on Figure 1B. Radiographs of EP animals showed ABL by 19% (Figure 1F) ($p<0.05$) when compared Naïve group (Figure 1D). In GIOP group there was no significant change in the RD of hemimaxillae (Figure 1H) compared to Naïve. However hemimaxillae of the animals subjected to GIOP+EP had lower RD, by 12% (Figure 1J) when compared to EP group ($p <0.05$)

Micro-CT analysis of linear and volumetric parameters can be evaluated on Figure 2. Considering the linear measurements, EP group showed increased ABL at buccal (Figure 2A), furcation (Figure 2B) and inteproximal (Figure 2C) sites, when compared to Naïve group ($p<0.05$). GIOP did not affect the alveolar bone level when compared to Naïve. In the other hand, GIOP+EP group presented a greater increase on ABL when compared to EP in all three analyzed sites ($p<0.05$). The assesment of volumetric parameters, revealed that EP reduced BMD (Figure 2D), bone percentage (Figure 2E), trabecular thickness (Tb.Th) (Figure 2F) and increased on bone porosity (Figure 2G), in relation to

Naïve group. No differences were seen in GIOP group compared to EP group ($p>0.05$). However, in GIOP+EP group, it was observed greater reduction on BMD, bone percentage and trabecular thickness as well as, greater increase on bone porosity when when compared to EP ($p<0.05$).

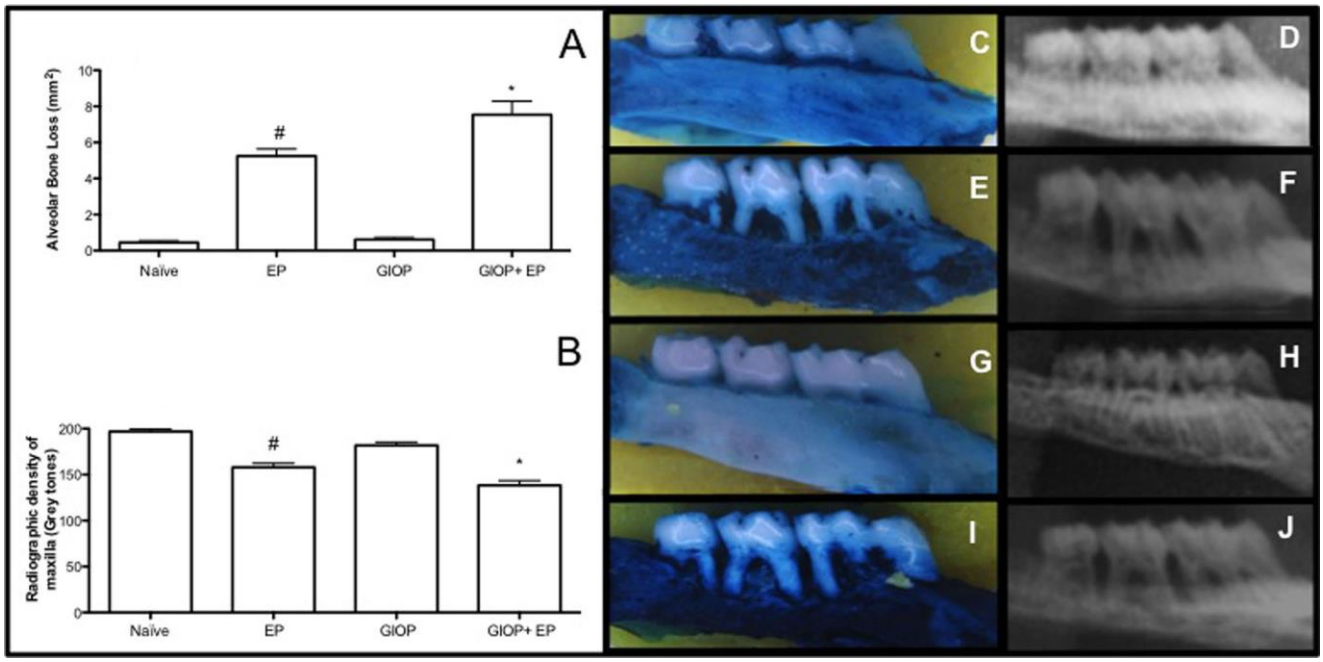

Figure 1. Effect of GIOP on alveolar bone tissue of rats with experimental periodontitis. **A**) Macroscopic analysis. **B**) Radiographic density. Bars represent the mean±SEM of 6 animals per group. #Significant difference compared to Naïve group. *Significant difference compared to EP group. ($p<0.05$; ANOVA followed by Bonferroni Test). Macroscopic and radiographic images of hemimaxillae of animals from **C** and **D**) Naïve group, **E** and **F**) EP group, **G** and **H**) GIOP group, **I** and **J**) GIOP+EP group.

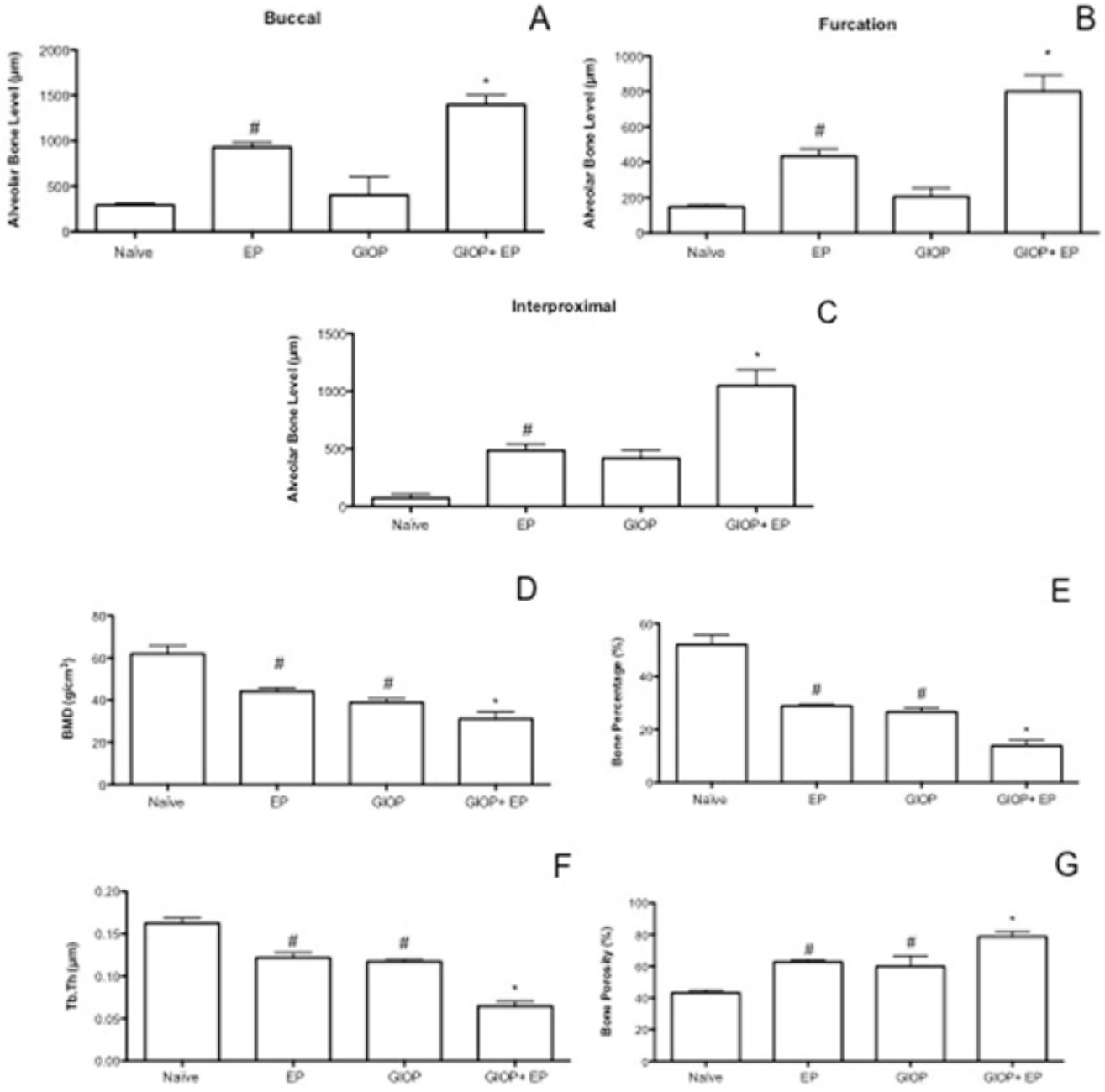

Figure 2. Effect of GIOP by micro-CT analysis of linear and bone volumetric parameters **A**) Buccal site, **B)** Furcation site, **C**) Interproximal site, **D)** Bone Mineral Density (BMD), **E)** Bone Percentage, **F)** Trabecular Thickness (Tb.Th), **G)** Bone Porosity. Bars represent the mean±SEM of 6 animals per group. #Significant difference compared to Naïve group. *Significant difference compared to EP group. ($p<0.05$; ANOVA followed by Bonferroni Test).

Bone histology

Periodontal histopathological analysis of the region between first and second molars of naïve animals shows the normal structure of gingiva, periodontal ligament (PDL), alveolar bone and cementum (Figure 3A). The periodontium of animals subjected to experimental periodontitis (EP group) demonstrated accentuated inflammatory cell infiltration, breakdown of alveolar bone, collagen fiber derangement within the periodontal ligament, and resorption of cementum, receiving a score of 3 (Figure 3B; Table 1). The periodontal tissue of animals subjected to GIOP did not any change on the architecture (Figure 3C; Table 1). However, the animals subjected to GIOP+EP showed enhanced bone loss and inflammatory infiltrate (Figure 3D; Table 1).

Table 1. Effect of GIOP on Histopathological analysis of hemimaxillae

	Naïve	EP	GIOP	GIOP+EP
Histopathological Analysis (Scores)	0	3 [#]	0	3 [*]

Data is presented as median (extreme value).

EP=experimental periodontitis; GIOP=glucocorticoid-induced osteoporosis;

[#]Significant compared to Naïve group *Significant compared to EP group; (Kruskal Wallis followed by Dunn's; $p<0.05$).

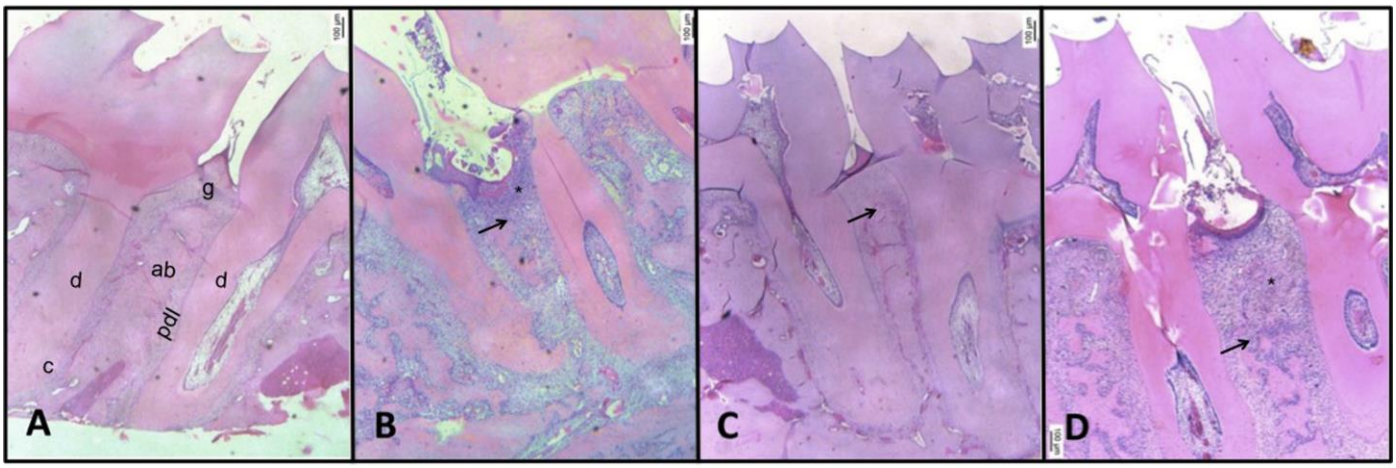

Figure 3. Effect of GIOP on the microarchitecture of alveolar bone tissue of rats with experimental periodontitis. **A**) Naïve, **B**) EP, **C**) GIOP, **D**) GIOP+EP. Region between the first and second molars of rats of normal periodontium (A), periodontium of animals submitted to ligature-induced alveolar bone resorption (B), periodontium of animals submitted to GIOP (C), periodontium of animals submitted to GIOP ad EP (D). Dentin (d); Cementum (c); Alveolar Bone (ab); Gingiva (g); Periodontal Ligament (pdl). Inflammatory infiltrate (*) and bone resorption (➔). Bars - 500 µm. Hematoxylin & eosin (H&E). (40x magnification),

Serum biochemical analysis of BALP

Serum dosage of BALP was analyzed (Figure 4). EP caused a significant decrease, by 27% on BALP serum levels (21.64±2.48 U/L) when compared to Naïve (29.52±5.14 U/L). GIOP also caused a reduction by 41% (17.4±2.65 U/L) on BALP serum levels compared to Naive ($p>0.05$). However, in GIOP+EP group it was seen a greater reduction by 55% (13.25±2.11 U/L) on BALP serum levels when compared to EP ($p<0.05$).

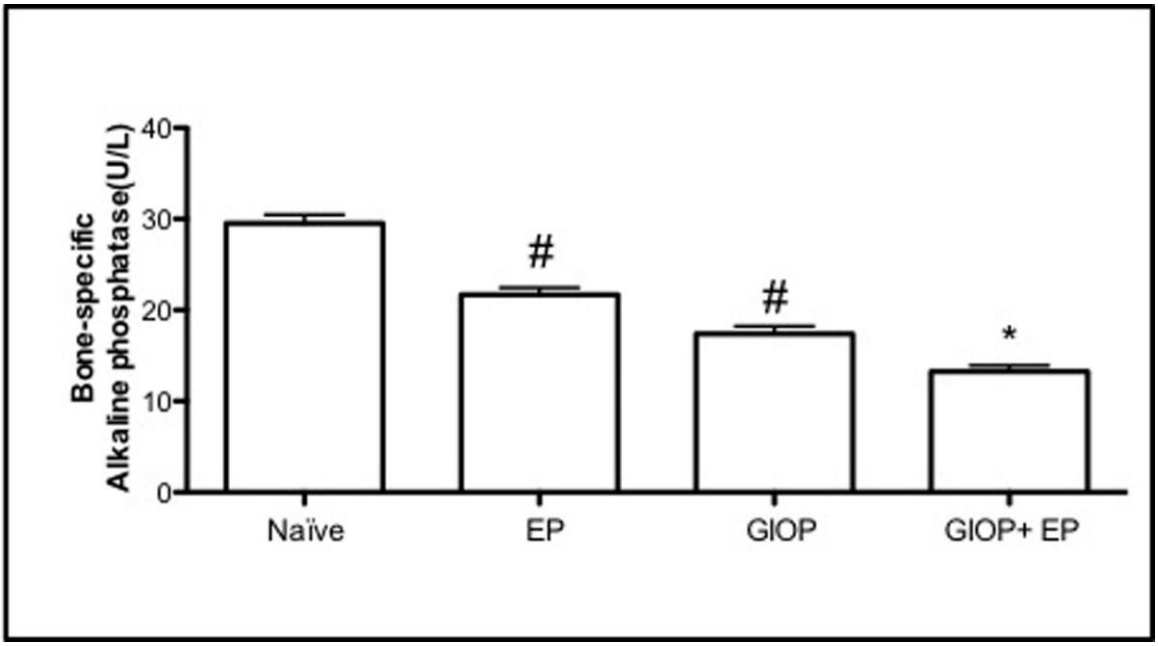

Figure 4. Effect of GIOP in BALP serum levels of rats with experimental periodontitis. Bars represent the mean±SEM of 6 animals per group. #Significant difference compared to Naïve group. *Significant difference compared to EP group. ($p<0.05$; ANOVA followed by Bonferroni Test).

Biomechanical testing and RD of Femur

Assessing RD of femur (Figure 5E), those animals undergoing only EP (Figure 5B) showed no significant difference in RD when compared to the femurs of Naïve group (Figure 5A). The GIOP caused a significant reduction of RD in femur (Figure 5C) when compared to Naïve and EP groups. In the group animals submitted to GIOP+EP there was a reduction of RD femur (Figure 5D) when compared to Naïve or EP groups ($p < 0.05$).

We further checked femur biomechanical properties GIOP and experimental periodontitis (Figure 5F). After 11 days of EP did not show difference on Young's modulus when compared to the femurs of Naïve group ($p > 0.05$). There was a significant decrease in Young's modulus in the group subjected to GIOP compared to Naïve group, as well as in the group GIOP+EP compared to EP group.

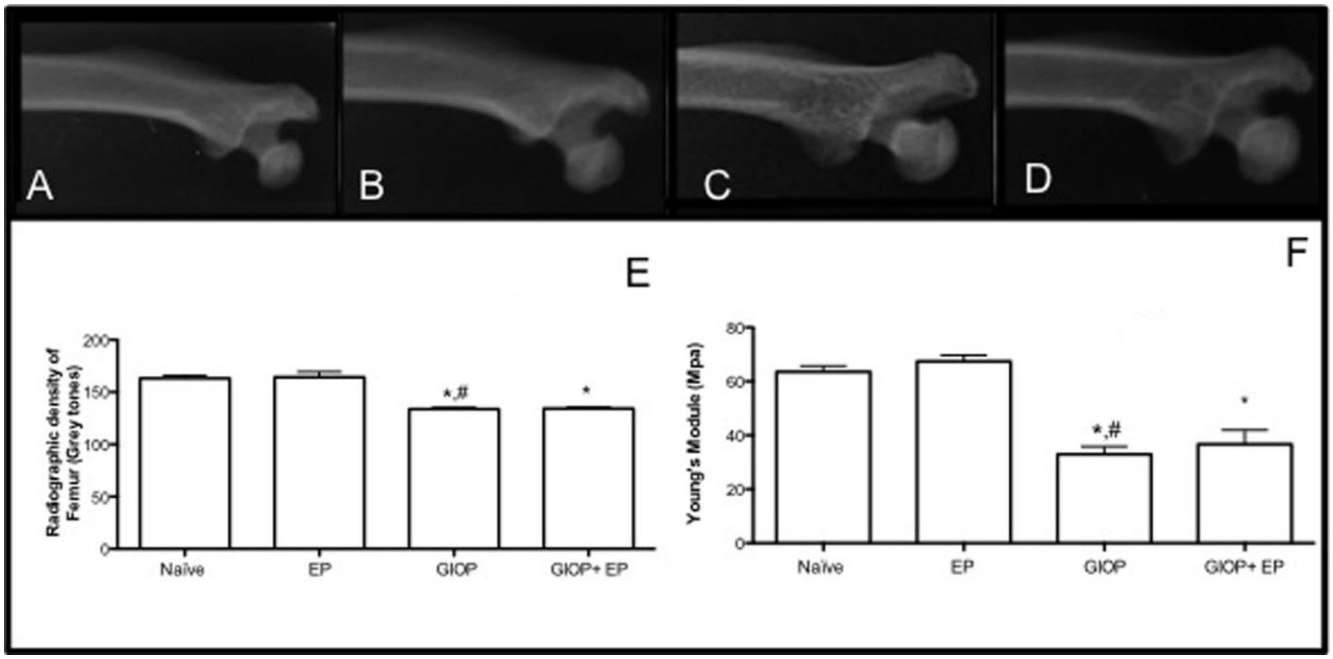

Figure 5. Effect of GIOP on femur of rats with experimental periodontitis. Radiographic images of femur of animals from **A**) Naïve group, **B**) EP group, **C**) GIOP group, **D**) GIOP+EP group. **E**) Radiographic Density of femur and **F**) Young's modulus (MPa) of femur. Bars represent the mean±SEM of 6 animals

per group. #Significant difference compared to Naïve group. *Significant difference compared to EP group. ($p<0.05$; ANOVA followed by Bonferroni Test).

4. Discussion

In this study we examined the effect on GIOP on EP. It was seen that EP caused an increase on ABL considering macroscopic, radiographic, micro-tomographic and histological analysis, and also reduced BALP serum levels, however it did not alter the architecture or biomechanics of femur when compared to Naïve group. The animals subjected to GIOP, in turn, did not show ABL when compared to Naïve group, but in micro-CT analysis of volumetric parameters of alveolar bone it was seen decrease on BMD, bone percentage, trabecular thickness and an increase on bone porosity when compared to Naïve group ($p<0.05$). GIOP promoted significant reduction on BALP serum levels and caused reduction on radiographic density and Young's module of femur when compared to Naïve group. However, in GIOP+EP group it was seen a greater increase on ABL in macroscopic, radiographic, micro-tomographic analysis, a greater reduction on BALP serum levels, and higher decrease on radiographic density and Young's module of femur when compared to EP.

There has been an increase in the use of animal models in studies of initiation and progression of bone diseases, such as osteoporosis and periodontal disease. The ligature induced periodontitis model has been used extensively as a highly reproducible model that evaluates the progression of periodontitis (Bezerra, de Lima, Alencar, Vieira, Brito & Ribeiro, 2000; Leitão, Ribeiro, Chaves, Rocha, Lima, & Brito, 2005; Goes, Melo, Silva, Benevides, Alencar & Ribeiro, 2014). As with human periodontitis, experimentally induced periodontitis in rats involves inflammatory cell recruitment with overproduction of pro-inflammatory cytokines and osteolytic enzymes, with osteoclast activation, and ultimately leads to alveolar bone and soft tissue attachment loss, which has been demonstrated and well established, by our research group, (Bezerra, de Lima, Alencar, Vieira, Brito & Ribeiro, 2000; Bezerra, Brito, Ribeiro, & Rocha, 2002; Lima, Vidal, Rocha, Brito, & Ribeiro, 2004; Leitão, Rocha, Chaves, Lima, Cunha & Ribeiro 2004; Leitão, Ribeiro, Chaves, Rocha, Lima, & Brito, 2005; de Menezes, Souza, Gomes, de Carvalho, Leitão, Ribeiro & Oliveira, 2012;; Goes,

Lima, Melo, Rego, & Lima, 2010; Goes, Melo, Dutra, Lima, & Lima, 2012; Dalcico, de Menezes, Deocleciano, Oriá, Vale & Ribeiro, 2013; Goes, Melo, Silva, Benevides, Alencar & Ribeiro, 2014; Lisboa, Gondim, Ervolino, Vale, Frota & Nunes, 2015). In the other hand, consistent with our findings, there is no evidence that periodontitis, by itself, can provoke systemic bone loss (Xu, Chen, Zhang, Zhai, Liu & Qin, 2014).

Systemic risk factors may determine acceleration on the initiation of periodontal disease (Borrell & Papapanou, 2005; Kornman, 2008), as well as, the increase on the rate of progression and severity of periodontitis (Genco & Borgnakke, 2013). Among these risk factors, osteoporosis is one of the six main factors (Genco & Borgnakke, 2013). Therefore, using reproducible experimental models, the present study investigates the influence of glucocorticoid-induced osteoporosis on ABL of rats subjected to experimental periodontitis assessing alveolar bone level, femur bone loss and osteoblastic activity.

Osteoporosis is mainly caused by postmenopausal estrogen deficiency, and its association with periodontitis it well described in literature (Hernández-Vigueras, Martínez-Garriga, Sánchez, Sanz, Estrugo-Devesa & Vinuesa, 2015; Juluri, Viswanathan, Gopalakrishnan, Kathariya, Devanoorkar & Prashanth, 2015). However, osteoporosis has also a secondary cause, related to the long-term use of glucocorticoids (GCs). Considering the periodontal tissue, from the best of our knowledge, this the first time that the effect of GIOP is evaluated on experimental periodontitis in rats.

Our results showed that GIOP worsen the ABL in animals with EP. GCs adversely affect bone tissue in a number of ways (Compston, 2010). Notably, after initiation of GCs therapy, there is an early and transient increase in bone resorption (Teitelbaum, 2015). GCs increase the expression of the macrophage colony stimulating factor (M-CSF) and RANKL, and decrease the expression of its soluble decoy receptor, osteoprotegerin, in stromal and osteoblastic cells (Canalis, Mazziotti, Giustina, & Bilezikian, 2007; Compston, 2010), resulting in osteoclastogenesis and a prolongation of the lifespan of osteoclasts (Compston, 2010). In addition, it has been reported that, when inflammatory disorders such as periodontitis are present, GCs may potentiate the resorptive process. Patients with rheumatoid arthritis showed increased bone degradation during the first three months of therapy when compared to healthy individuals (Hall, Spector,

Griffin, Jawad, Hall, & Doyle, 1993). This initial rapid and greater bone loss can reflect persistence of the prior effects of inflammatory cytokines, such as TNF-α and IL-1, as well as, the osteoclastic cytokine, RANKL (Teitelbaum, 2015). Therefore, these findings can explain the greater ABL in GIOP+EP.

GIOP is also characterized by increased apoptosis of osteoblasts. There is evidence that GCs decrease osteoblastogenesis, impair osteoblastic differentiation and maturation and decrease the number and function of osteoblasts (Canalis, Mazziotti, Giustina, & Bilezikian, 2007). GCs, also favor the differentiation of bone marrow stromal cells toward cells of the adipocyte lineage instead of the osteoblastic linage by blocking Wnt/β-catenin signaling pathway (Canalis, Mazziotti, Giustina, & Bilezikian, 2007; Compston, 2010). In addition, when associated to inflammatory bone disorders, such as periodontitis, the osteoblastic activity may also be suppressed in a greater way. TNF-α, IL-1, -6 and -17 can perturb the WNT and bone morphogenetic protein (BMP) signaling pathways, leading to lower osteoblast differentiation and activity (Walsh, & Gravallese, 2010). TNF-α can also induce osteoblasts apoptosis (Jilka, Weinstein, Bellido, Parfitt, & Manolagas, 1998; Wei, Kitaura, Zhou, Ross, & Teitelbaum, 2005). Thus these findings can justify the decrease on BALP serum levels seen on GIOP and GIOP+EP groups, since BALP is a homodimeric glycoprotein that is anchored to the membrane of osteoblasts and consequently it is considered a general indicator of the bone formation rate in skeletal tissue (Sardiwal, Magnusson, Goldsmith, & Lamb, 2013).

5. Conclusion

In summary, considering our results, we can conclude that GIOP can potentiate the destructive effect of periodontitis on bone tissue, by promoting bone resorption and reducing osteoblast activity. Nevertheless more studies are necessary in order to better understand the biochemical mechanisms of GCs on bone cells during a inflammatory status.

Conflict interests

The authors have no conflict of interest to disclose.

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Ethical approval

The experimental procedures described here were approved by the Institutional Animal Care and Use Committee (#78/2014) and performed in accordance with the Animal Care Standards of Federal University of Ceará.

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Artigo 2

Effects of Atorvastatin on periodontitis of rats subjected to glucocorticoid-induced osteoporosis.

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Keywords: atorvastatin; glucocorticoid-induced osteoporosis; periodontitis; bone loss; inflammation; oxidative stress

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Abstract

Aim: To evaluate the effect of Atorvastatin (ATV) on experimental periodontitis (EP) of rats subjected to glucocorticoid-induced osteoporosis (GIOP).

Material and Methods: male Wistar rats were divided into: Naïve, EP, GIOP+EP and ATV groups. GIOP+EP and ATV received 7 mg/kg of dexamethasone intramuscularly 1x/week for 5 weeks, the others received 2ml of Saline (SAL) intramuscularly. EP, GIOP+EP and ATV were subjected to EP by ligature around 2nd upper left molars for 11 days. ATV group received 27 mg/kg of ATV orally and the others SAL, 30 minutes before EP. Periodontium was analyzed by macroscopy, micro-tomography and histopathology; by immunohistochemical examination of RANKL, OPG, WNT10b, DKK-1 and β-catenin and by ELISA analysis of myeloperoxidase (MPO), TNF-α, IL-1β, -6, -8, and -10, reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT). Leukogram and liver and kidney enzymes and bone-specific alkaline phosphatase (BALP) serum levels were performed.

Results: ATV decreased bone loss, reduced MPO, TNF-α, IL-1β, -6, and -8, and increased IL-10, GSH, SOD and CAT levels. ATV reduced RANKL and DKK-1, increased OPG, WNT10b and β-catenin expressions and BALP activity.

Conclusions: ATV reduced inflammation, oxidative stress and bone loss in rats with EP and GIOP, with participation of WNT signaling pathway.

Clinical relevance

Scientific rationale for the study: There are conflicting clinical findings regarding the benefits of Atorvastatin in the treatment of osteoporosis and there is no study evaluating the effects of Atorvastatin on periodontitis associated with osteoporosis.

Principal finding: Atorvastatin demonstrated antiresorptive, anti-inflammatory and anti-oxidative effects in rats with GIOP and EP.

Practical implication: Atorvastatin seems to be interesting as a potential pharmacological approach for preventing bone resorption in periodontal inflammatory diseases when associated with osteoporosis.

Introduction

Periodontitis is an inflammatory chronic infectious disease that affects the supporting structures of teeth and can lead to loss of the periodontal ligament (PDL) and alveolar bone and eventually to tooth loss (Pihlstrom et al. 2005). Its pathogenesis is multifactorial involving the microbial challenge that leads to an exacerbated inflammatory response, the main cause of tissue destruction (Giannobile et al. 2009).

Recent studies have shown that several systemic diseases, which affect the host immune response, can modify the progression rate of periodontitis (Di Benedetto et al. 2013, Rosa et al., 2014). Among them, osteoporosis stands out. It is well established the correlation between postmenopausal osteoporosis and periodontitis, with emphasis on the explanation of possible relations between premature tooth loss and decrease of length and density of jaw bones (Straka et al. 2015). However, the effects of glucocorticoid (GCs)-induced bone loss, the most common form of secondary osteoporosis (Saag et al. 1994), on periodontal inflammation it is still unclear.

Statins are cholesterol-lowering drugs capable of inhibiting cholesterol synthesis in humans and animals. In addition, studies have suggested that statins may have other effects beyond lipid reducing function, due to the inhibition of the isoprenoid intermediates of the mevalonate biosynthetic pathway, so-called pleiotropic effects (Maeda et al. 2004). These effects are able to modulate inflammation by lowering cytokine concentrations and inhibiting recruitment, migration and cell adhesion to endothelium. Statins have also shown anabolic effects on bone tissue (Mundy et al. 1999).

However, there are conflicting clinical findings regarding the benefits of statins for bone diseases treatment, such as osteoporosis. Although some clinical studies show the positive effect of statins on bone mineral density (Chen et al. 2014), a recent clinical trial, which included data of 17,802 patients, does not support the role of statin in reducing the risk of fractures and bone resorption (Peña et al. 2015).

Atorvastatin (ATV) was effective in preventing alveolar bone loss (ABL) in experimental periodontitis in rats (Goes et al. 2010, de Araújo et al. 2013) and in treated patients with chronic periodontitis (Fajardo et al. 2010). However, there is

no study evaluating the effects of ATV on periodontitis experimental associated with osteoporosis.

In this study, the effect of the ATV on rats associated with glucocorticoid-induced osteoporosis (GIOP) and experimental periodontitis (EP) is addressed, analyzing its action on various parameters of bone metabolism, inflammation, and oxidative stress.

Material and Methods

Animals

All experimental protocols were approved by the Federal University of Ceará (UFC) Ethical Committee for Animal Research (number 78/2014). Surgical procedures and animal treatments were performed in agreement with the Ethical Principles for Animal Research.

Forty-eight male Wistar rats (180 to 220 g), from our animal facilities, were housed in temperature-controlled rooms and received water and food *ad libitum*.

A power calculation was performed to determine the sample size. The animal was considered the study unit. The sample size was determined to provide 80% power to recognize a significant difference of 20% among groups and the standard deviation of 15% with a 95% confidence interval (a = 0.05), considering the change in alveolar bone loss (ABL) as the primary outcome variable. Therefore, a sample size of 6 animals per group was required.

Treatment and experimental design

After two weeks of adaptation, the rats were divided into four groups (n=6): Naïve, EP, GIOP+EP and ATV. Initially, the groups GIOP+EP and ATV received injections of 7mg/kg dexamethasone (Decadron®, Aché, Guarulhos, SP, Brazil), intramuscularly (IM), once a week, for 5 weeks (Lucinda et al. 2012), whereas the others (Naïve and EP) received 0.5 ml of 0.9% Saline solution (SAL). Following, the rats were anesthetized with 100 mg/ml ketamine (Cetamin®, Syntec, Santana de Paraíba, SP, Brazil) and 20 mg/ml xylazine (Xilazin®, Syntec, Santana de Paraíba, SP, Brazil), at 1 ml/kg – IM for EP induction by placing a sterile nylon thread ligature around the cervix of the maxillary left second molar of rats. Thirty minutes before EP induction the animals from ATV group received orally 27 mg/kg of ATV (Lipitor®, Pfizer, São Paulo, SP, Brazil) and daily for 11

days, while the others received orally 2 ml of SAL. After 11 days the animals were euthanized with 20 mg/kg thiopental (Thiopentax®, Cristália, São Paulo, Brazil). The animals from Naïve group were not subjected to EP.

Measurement of ABL and Bone Structure Analysis

The maxillae of rats were excised and fixed in 10% buffered formalin. Both maxillary halves were then defleshed and stained with methylene blue (1%) to differentiate bone from teeth. The maxillae were then fixed in a piece of wax and photographed. The photographs were digitized and processed with imaging software (NIH ImageJ v.1.32, Bethesda, MD, USA) and ABL was measured using an area method (Goes et al. 2010). The ABL was measured in square millimeters, considering the difference between the buccal aspect of the maxillary left side by the buccal aspect of right maxilla (unligated control). All acquired images were compared with a well-known area (1.0 x 1.0 mm^2).

The same non-demineralized specimens were then scanned by a cone beam micro-computed tomography (CT) system (Skyscan 1172, Bruker, Kontich, Belgium). The x-ray generator was operated at an accelerated potential of 50 kV with a beam current of 200 μA and an exposure time of 560 ms per projection. Images were produced with a voxel size of 6x6x6 μm. Using an appropriated software (Data Viewer®, version 1.5.0, Bruker, Kontich, Belgium), the generated 3 dimensional models were rotated into a standard position as described previously (Lisboa et al. 2015). Linear measurements on ABL were performed at 3 different sites: buccal, furcation and interproximal. For the interproximal site, coronal dataset was analyzed using appropriated software (CT-Analyser®, version 1.13.5.1+, Bruker, Kontich, Belgium). Volumetric parameters such as: Bone mineral density (BMD), Trabecular Thickness (Tb.Th), Bone porosity, and Bone percentage were also analyzed (Lisboa et al. 2015). All micro-CT analyses were performed by one blinded and calibrated examiner.

Periodontal Histopathologic Analyses

Another set of experiment was performed and the excised maxillae were demineralized in a 10% EDTA solution, followed by dehydration and paraffin embedding. The specimens were stained with H&E. 4 μm-thick sections, in area between the first and second maxillary left molars, were evaluated under light

microscopy. Scores of 0 to 3 were considered for the analysis, as described previously (Leitão et al. 2005).

Certain specimens were also evaluated under confocal microscopy (Olympus FV1000 confocal laser scanning microscope, Olympus, Tokyo, Japan) to observe collagen fiber derangement, the PDL, ABL, and resorption of cementum. To evaluate autofluorescence, we used a 488 nm wave-length laser and emission channel for FITC-green fluorescence (Gonçalves et al. 2014).

Determination of Tumor Necrosis Factor-α, Interleukin-1β, Interleukin-6, Interleukin-1 and Interleukin-8 and Interleukin-10 Gingival Levels

TNF-α, IL-1β, -6, -8 and -10 concentrations were determined in the buccal gingiva surrounding the maxillary left molars 11 days after EP. The gingival tissue was removed and stored at -80 ^{o}C. The material was homogenized and the supernatant was used to determine the cytokine levels by ELISA (R & D Systems, Mineapolis, MN, USA). The results were shown as pictogram of cytokine/ml (Dalcico et al. 2013).

Gingival levels of reduced glutathione, enzyme catalase and enzyme superoxide dismutase

To evaluate oxidative stress, reduced glutathione (GSH), enzyme catalase (CAT) and enzyme superoxide dismutase (SOD) antioxidants were measured in the gingival tissue collected 11 days after EP and stored at -80 ^{o}C. GSH content in gingival tissue was estimated according to the methods described previously (Sedlak & Lindsay 1968). The GSH concentration was read off a standard curve and expressed as micrometers of GSH per gram of wet tissue. Superoxide dismutase (SOD) activity was assayed based on reduction of nitroblue tetrazolium (NBT) by the anion produced by xanthine-xanthine oxidase (Beauchamp & Fridvich 1971) and expressed as grams of SOD per ml. Catalase (CAT) activity has as principle the measurement of O_2 production rate and H_2O in proportion of H_2O_2 (Maehly & Chance 1954).

Myeloperoxidase activity

The extent of neutrophil accumulation, as an indicator of acute inflammation in the gingival tissue previously collected and stored at – 80 ^{0}C, was measured by myeloperoxidase (MPO) activity evaluation as described previously (Lima-Júnior et al. 2012). Analyses were made in gingival tissues collected 11 days after EP.

Immunohistochemistry for RANKL, OPG, WNT 10b, DKK-1 and β-catenin

Immunohistochemistry was performed using the streptavidin–biotin–peroxidase method in formalin fixed, paraffin-embedded tissue sections (4 µm thick) mounted on poly-L-lysine-coated microscope slides. For RANKL (RANKL antibody [N-19] goat policlonal IgG, Santa Cruz Biotechnology), OPG (OPG antibody [N-20] goat policlonal IgG, Santa Cruz Biothecnology), β-catenin (β-catenin antibody [C-18] goat policlonal IgG, Santa Cruz Biothecnology), DKK-1 (DKK-1 antibody [H-120] rabbit policlonal IgG, Santa Cruz Biothecnology) or WNT 10b (WNT 10b antibody [ab70816] rabbit policlonal IgG, Abcam), sections of the excised maxillae that had been demineralized in a 10% EDTA solution were used (Dalcico et al. 2013).

Leukogram analysis

The method for analysis of white blood cell, neutrophil and mononuclear cell counts was as follows: 20 µL of blood was taken from the rat tail and added to 380 µL of Turk's solution. Total white blood cell counts were performed using a Neubauer chamber, and the differential counts were made using smears stained by a rapid Instant Prov Stain Set (Newprov Products for Laboratory, Pinhais, PR, Brazil). White blood cell counts of the animals were performed 11 days after EP (Goes et al. 2014).

Serum levels of Transaminases, Urea and Creatinine

On day 11 after EP, blood samples were collected from the orbital plexus of anesthetized rats from all experimental groups. Liver function was evaluated through measurement of the serum levels of transaminases (AST and ALT), and serum levels of urea and creatinine measured kidney function. These analyses were performed following the manufacturer's instructions (Labtest®, Lagoa Santa, MG, Brazil) (Goes et al., 2014).

Serum levels of Bone-Specific Alkaline Phosphatase (BALP)

Blood samples were collected from the orbital plexus of anaesthetized animals on the 11th day. The BALP was evaluated using the thermoactivation method, by heating the sample at 56 ^{o}C for 10 min (Whitby & Moss, 1975), since BALP is a thermosensible isoform of total alkaline phosphatase (TALP). BALP serum levels were obtained by the subtraction of heated alkaline phosphatase from TALP serum levels. The methodology used to evaluate the enzymes' serum levels followed the manufacturers' directions (Labtest®, Lagoa Santa, MG, Brazil) (Goes et al. 2012).

Statistical Analysis

The data are presented as mean±SEM or as medians with variation range when appropriate. Analysis of variance (ANOVA) followed by Bonferroni test was used to compare means, and the Kruskal-Wallis test was used to compare medians. $P<0.05$ was set to indicate significant differences among groups.

Results

Effect of ATV on ABL and on Bone Structure

Administration of 27 mg/kg ATV, in animals with GIOP and EP caused a significant ($p<0.05$) decrease in ABL in macroscopic (Figure 1A) and in linear parameters by micro-CT analyses on: vestibular site (Figure 1B), furcation area (Figure 1C) and interproximal site (Figure 1D), when compared with GIOP+EP group. The Naïve group presented an ABL close to zero, with a nonsignificant difference between the two sides of the maxilla.

Considering the volumetric parameters analyzed in micro-CT, the animals treated with ATV 27 mg/kg showed a significant ($P<0.05$) increase in BMD (Figure 2A), Tb.Th (Figure 2B) and Bone percentage (Figure 2D), and reduction in Bone porosity (Figure 2C), when compared to group GIOP+EP group.

Periodontal histopathological analysis of the region between the first and second molars of animals subjected to experimental periodontitis (EP group) demonstrated intense inflammatory cell infiltration, breakdown of alveolar bone, collagen fiber derangement within the PDL and resorption of cementum, receiving a median score of 3, when compared to Naïve, receiving a median score of 0 ($P<0.05$). The GIOP+EP group showed similar findings to EP group

receiving a median score of 3 (P>0.05). The periodontium of rats treated with 27 mg/kg ATV showed preservation of the alveolar process and cementum, reduction of the inflammatory cell infiltration, and partial preservation of collagen fibers of the PDL, receiving a median score of 1 (range, 1 to 2). These values were statistically different (P<0.05) compared with the GIOP+EP group.

Confocal microscopy analysis of the periodontal region between the first and second molars of animals subjected to EP and GIOP+EP demonstrated considerable collagen fiber derangement within the PDL, ABL, and resorption of cementum. The periodontium of rats treated with 27 mg/kg Atorvastatin showed preservation of alveolar process and cementum and maintenance of collagen fibers of the PDL (Figure 3).

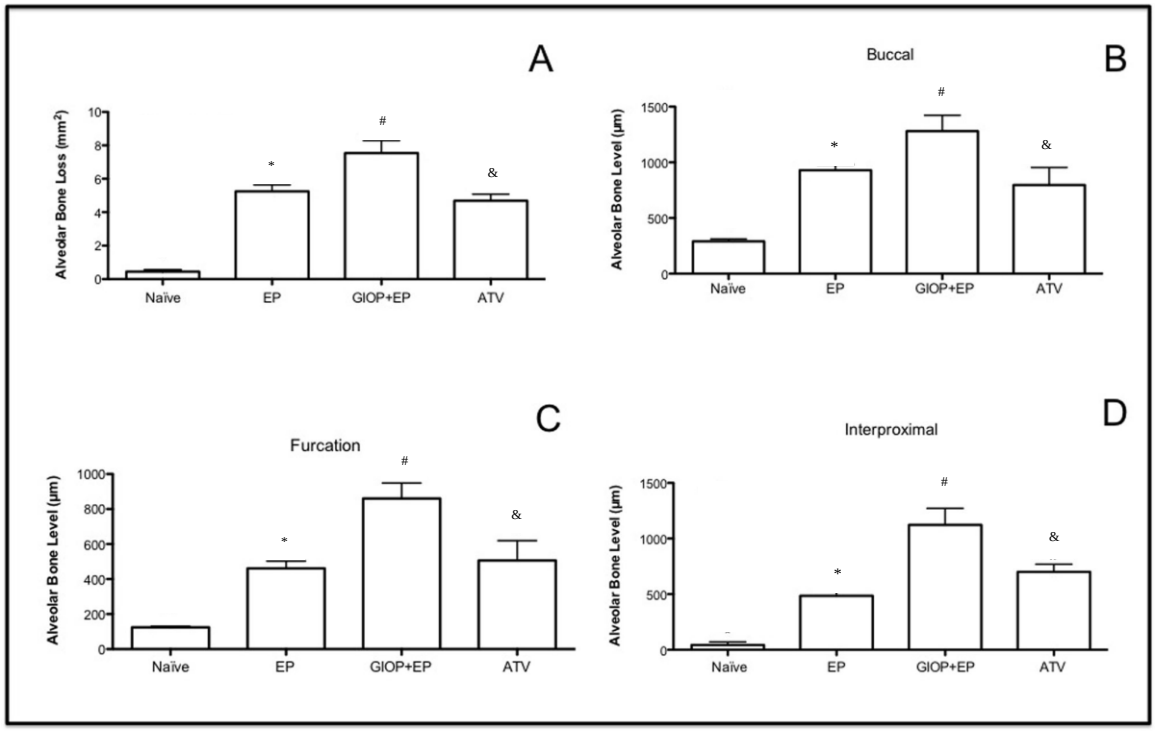

Figure 1. Effect of ATV on ABL in rats with GIOP and EP. Macroscopic Analysis (A); and Micro-CT analysis (B-D) of Vestibular site (B), Furcation area (C), Interproximal site (D). Bars represent the mean±SEM of 6 animals per group. *p<0.05 was considered to be significantly different compared with Naive. #P<0.05 was considered to be significantly different compared with EP. &P<0.05 was considered to be significantly different compared with GIOP+EP (ANOVA followed by the Bonferroni test).

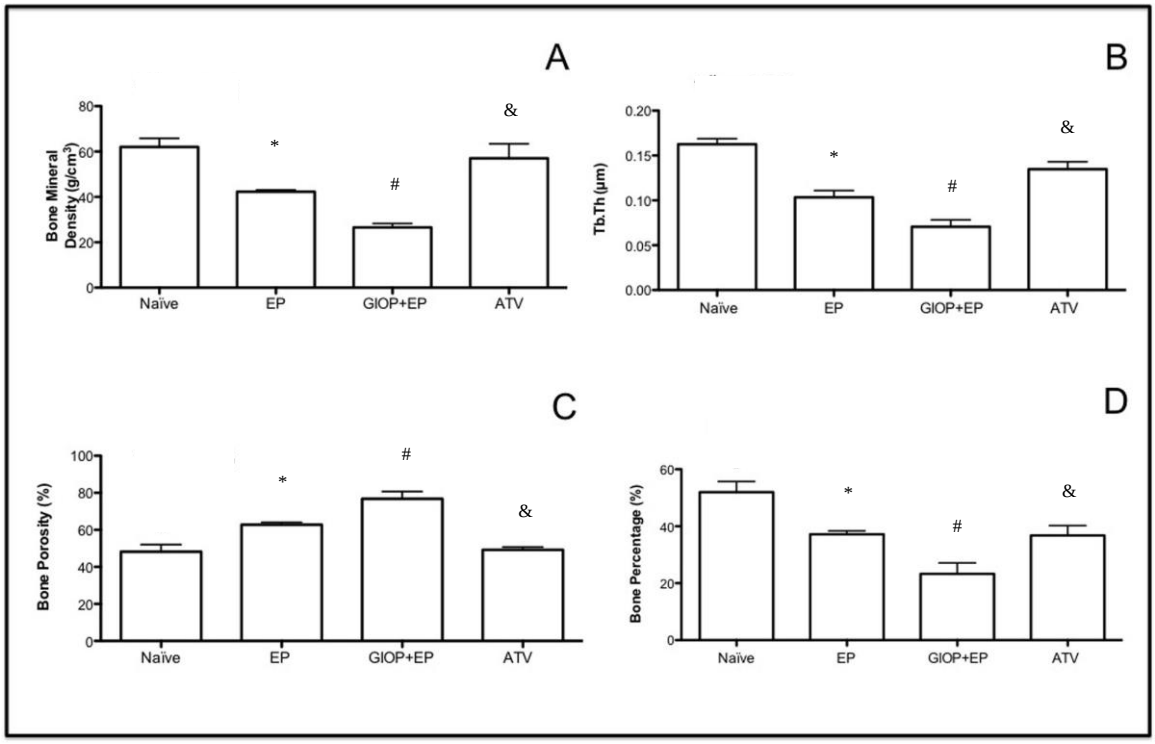

Figure 2. Micro-CT analysis of the effect of ATV on Bone Structure in rats with GIOP and EP. Bone Mineral Density (A), Trabecular Thickness (B), Bone Porosity (C), Bone Percentage (D). Bars represent the mean±SEM of 6 animals per group. *P<0.05 was considered to be significantly different compared with naive. $^{\#}$p<0.05 was considered to be significantly different compared with EP. . $^{\&}$p<0.05 was considered to be significantly different compared with GIOP+EP (ANOVA followed by the Bonferroni test).

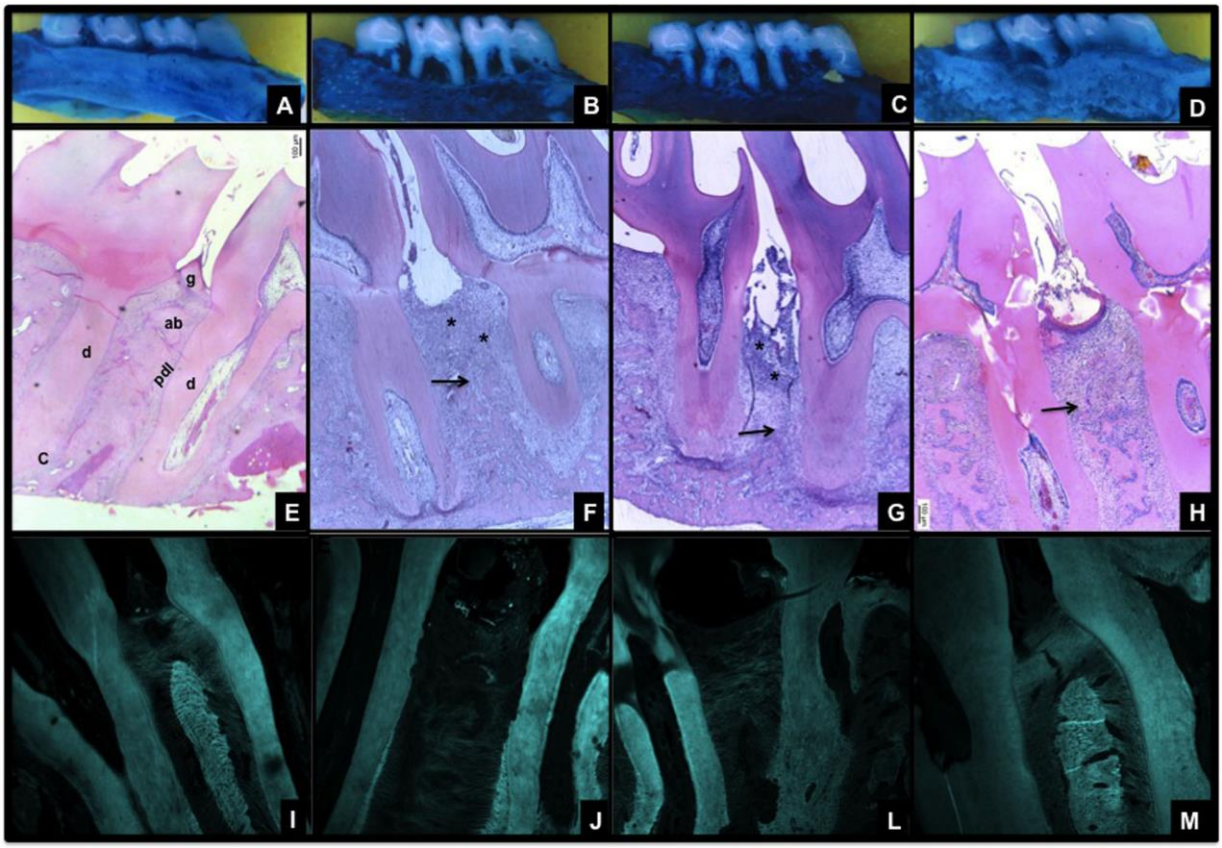

Figure 3. Effect of ATV on periodontal tissue of animals with GIOP and EP. Macroscopic (A-D, 4x magnification), Histopathologic (E-H, 40x magnification), and Confocal Microscopy (I-M, 100x magnification) of region between the first and second molars of rats of normal periodontium (A, E, I), periodontium of animals submitted to ligature-induced alveolar bone resorption, that received during the 11 days saline (B, F, J), periodontium of animals submitted to GIOP ad EP, that received during the 11 days saline (C, G, L), periodontium of animals submitted to GIOP ad EP, treated with 27 mg/kg atorvastatin (D, H, M). Dentin (d); Cementum (c); Alveolar Bone (ab); Gingiva (g); Periodontal Ligament (pdl). Inflammatory infiltrate (*) and bone resorption (→). Bars - 500 µm. Hematoxylin & eosin (H&E).

Effect of ATV on Gingival levels of TNF-α, IL-1β, IL-6, IL-8 and IL10

The animals from EP and GIOP+EP groups presented a significant increase (P<0.05) in gingival TNF-α, IL-1β, IL-6 and IL-8 levels, while it was seen a significant decrease (P<0.05) in gingival IL-10 levels, compared to Naïve. Treatment with 27 mg/kg Atorvastatin significantly (P<0.05) decreased pro-inflammatory cytokine levels (TNF-α [Figure 4A], IL-1β [Figure 4B], IL-6 [Figure 4C] and IL-8 [Figure 4D]), and increased (P<0.05) anti-inflammatory cytokine levels (IL-10 [Figure 4E]), compared to GIOP+EP group.

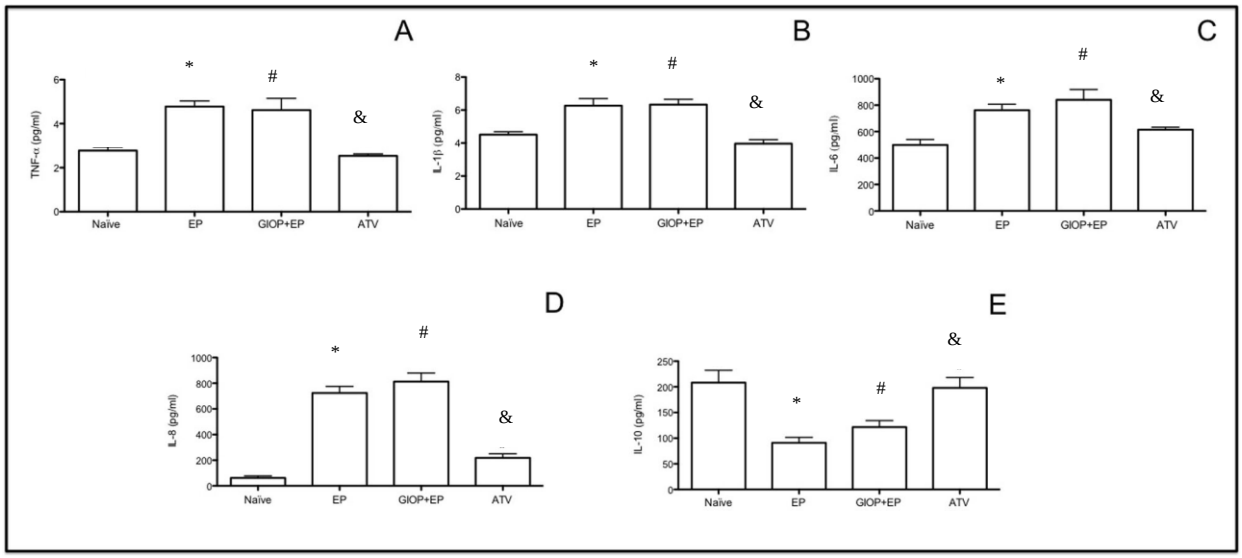

Figure 4. Effect of Atorvastatin on TNF-α (A), IL-1β (B), IL-6 (C), IL-8 (D) and IL-10 (E) concentrations in the gingival tissue of rats with GIOP and EP. Bars represent the mean±SEM. *p<0.05 was considered to be significantly different compared with Naive. #P<0.05 was considered to be significantly different compared with EP. &P<0.05 was considered to be significantly different compared with GIOP+EP. (ANOVA followed by the Bonferroni test).

Effect of ATV on MPO activity

Analysis of the buccal gingivae of animals pretreated with Atorvastatin (27 mg/kg) revealed reduction of MPO activity compared with GIOP+EP rats (P<0.05) (Figure 5A). There was also a statistical difference between the Naïve group and the groups with periodontal disease that received saline.

Effect of ATV on Gingival GSH, SOD and CAT levels

The placement of a ligature around the maxillary second molar of rats resulted in a significant reduction of the GSH (Figure 5B), SOD (Figure 5C) and CAT (Figure 5D) levels (P<0.05) in gingival tissue compared with the Naïve group. This finding indicates that oxidative stress is also present in gingival tissue subjected to periodontal disease. Similar findings were seen on GIOP+EP group when compared to EP. Administration of 27 mg/kg Atorvastatin increased gingival GSH, SOD and CAT concentration compared with GIOP+EP group.

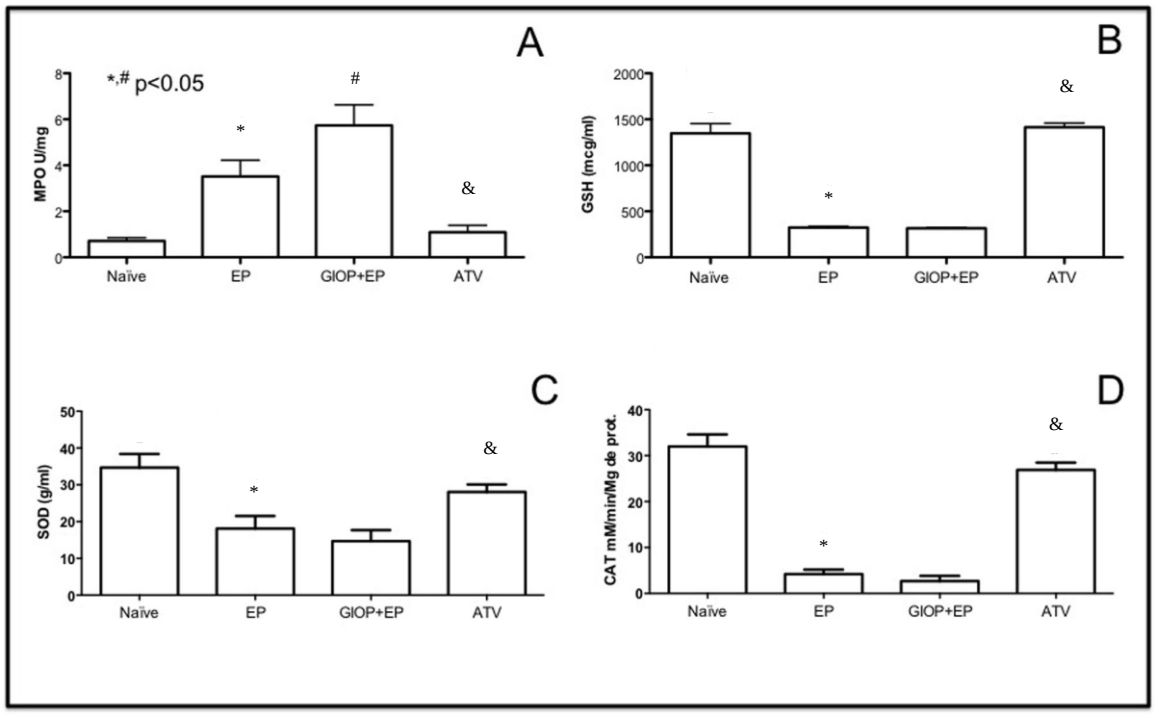

Figure 5. Effect of ATV on MPO (A), GSH (B), SOD (C) and CAT (D) concentrations in the gingival tissue of rats with GIOP and EP. Bars represent the mean±SEM. *p<0.05 was considered to be significantly different compared with Naive. #P<0.05 was considered to be significantly different compared with EP. &P<0.05 was considered to be significantly different compared with GIOP+EP. (ANOVA followed by the Bonferroni test).

Effect of ATV on RANKL, OPG, WNT 10b, DKK-1 and β-catenin

Figure 6 shows that stronger immunostaining was detected in periodontal tissues after induction of experimental periodontitis and in GIOP+EP group, in RANKL (Figure 6B; 6C) and DKK (Figure 6O; 6P) than in the Naïve group (RANKL [Figure 6A], DKK [Figure 6N]). It was seen a reduction in OPG (Figure 6F; 6G), WNT 10b (Figure 6J; 6L) and β-catenin (Figure 6S; 6T) immunostaining when compared to Naïve (OPG [Figure 6E], WNT10b [Figure 6I], β-catenin [Figure 6R]). Administration of 27 mg/kg Atorvastatin reduced RANKL (Figure 6D) and DKK (Figure 6Q) immunostaining, and increased OPG (Figure 6H), WNT 10b (Figure 6M) and β-catenin (Figure 6U) in rat periodontal tissues, compared to GIOP+EP group.

Effect of ATV on Systemic Parameters

Leukogram

In relation to total leukocytes, it was observed that EP and GIOP+EP caused leukocytosis after ligation compared with the Naïve (7.27 ±0.39). This leukocytosis ($P<0.05$) was marked by neutrophilia. Atorvastatin prevented leukocytosis ($P<0.05$) and neutrophilia ($P<0.05$) when compared with the GIOP+EP group. No change on mononuclear cell count was observed (Table 1).

Biochemical serum levels

The rats challenged for periodontitis and GIOP+EP did not show alteration on serum ALT and AST, or Urea and Creatinine compared with Naïve rats. Atorvastatin did not alter transaminases or kidney enzymes serum levels when compared to GIOP+EP group (Table 1). Animals subjected to EP showed significant reduction in BALP serum levels compared to Naïve. GIOP+EP caused a greater decrease in this bone biomarker ($P<0.05$) compared to EP. Atorvastatin prevented BALP reduction ($P<0.05$) when compared to GIOP+EP (Table 1).

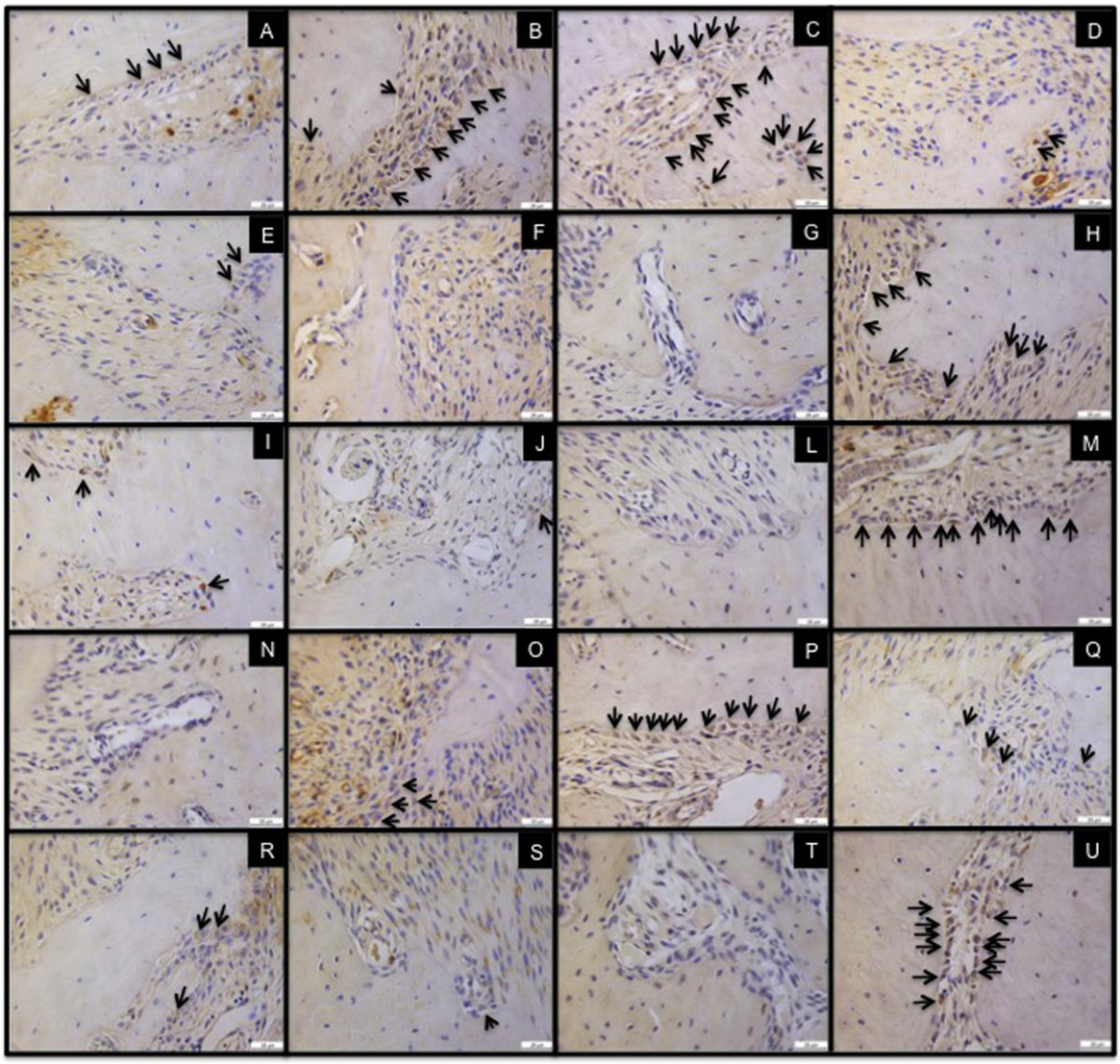

Figure 6. Photomicrographs showing immunostaining for RANKL (A, B, C, D), OPG (E, F, G, H), WNT10b (I, J, L, M), DKK-1 (N, O, P, Q) and β-catenin (R, S, T, U) in the interproximal region between 1st and 2nd molars of normal periodontium (A, E, I, N, R), periodontium of animals submitted to ligature-induced ABL (B, F, J, O, S), periodontium of animals submitted to GIOP+EP (C, G, L, P, T) or treated with ATV 27 mg/kg (D, H, M, Q, U). Immunostaining (→). (Original magnification x400).

Table 1.Effect of ATV on systemic parameters of rats with GIOP and EP.

Systemic Parameter	Naïve	EP	GIOP+EP	ATV
Leukocyte cells x 10^3 (mm^3)	7.23±0.42	17.89±1.06*	16.16±2.16	7.00±2.00$^{\&}$
Neutrophil cells x 10^3 (mm^3)	2.70±0.28	5.85±0.66*	6.33±0.46	3.10±0.53$^{\&}$
Mononuclear Cells x 10^3 (mm^3)	4.57±0.42	4.49±0.36	5.22±4.24	4.96±0.58
AST (U/L)	44.57±1.59	44.07±1.68	42.70±2.73	42.11±2.81
ALT (U/L)	24.29±1.78	23.70±2.56	21.56±2.47	21.36±3.16
Urea (mg/dl)	39.23±1.95	37.73±3.63	47.35±0.78	41.37±6.20
Creatinine (mg/dl)	0.37±0.04	0.21±0.01	0.49±0.08	0.50±0.09
BALP (U/L)	29.26±0.94	21.64±0.78*	13.10±0.62$^{\#}$	25.57±1.73$^{\&}$

Data are reported as mean±SEM for 6 rats per group
EP = experimental periodontitis; GIOP = glucocorticoid-induced osteoporosis; ATV = Atorvastatin

*p<0.05 was considered to be significantly different compared with Naive. $^{\#}$ P<0.05 was considered to be significantly different compared with EP. $^{\&}$P<0.05 was considered to be significantly different compared with GIOP+EP. (ANOVA followed by Bonferroni test) .

Discussion

There has been an increase in the use of animal models in drug-screening studies for the prevention and treatment of bone diseases, such as periodontal disease and osteoporosis. We used a well-established ABL ligature-induced model (de Lima et al. 2000) and a previously reported GIOP model (Lucinda et al. 2013) to study bone resorption, and to evaluate the effect of Atorvastatin on bone tissue. In this study, ATV prevented ABL, preserved bone structure, showed anti-inflammatory activity and reduced oxidative stress, without systemic repercussion in rats with GIOP+EP. To our knowledge, this is the first time that an *in vivo* evaluation of the ATV on alveolar bone tissue of rats with GIOP+EP is performed.

GCs are the most common cause of secondary osteoporosis and in this study GIOP caused greater ABL and destruction of bone architecture in animals with periodontitis. It has been suggested that the use of GCs concomitant with inflammatory disorders can stimulate the resorptive process by RANKL upregulation, due to the persistence of inflammatory cytokines, such as TNF-α and IL-1 (Dovio et al. 2004). Our study confirms these findings since we observed an increase in RANKL immunolabeling along with increased amount of pro-inflammatory cytokine in GIOP+EP group. ATV, in the other hand, prevented ABL and preserved bone structure in animals with GIOP+EP. An *in vitro* study found that statins increased OPG mRNA expression and decreased RANKL mRNA expression in mouse bone cell culture (Kaji et al. 2005). Statins may also inhibit osteoclastogenesis by impairing the maturation and the integrity of the osteoclast cytoskeleton as a consequence of the inhibition of prenylation of target proteins by prenyl protein transferases through a decrease in their substrates, including farnesyl pyrophosphate and geranyl pyrophosphate (Woo et al. 2000).

As skeletal exposure to GCs continues, bone formation is suppressed, contributing for ABL. GCs directly affect cells of osteoblastic lineage; cell number decreases due to an inhibition of osteoblast precursor cell replication and differentiation, and to an increase in osteoblast apoptosis (Mirza & Canalis 2015). In the presence of GCs, mesenchymal cells are directed toward cells of the adipocytic lineage instead of toward osteoblasts (Pereira et al. 2002). A mechanism by which GCs inhibit osteoblast cell differentiation is by opposing WNT/β-catenin, a critical regulator of osteoblastogenesis (Smith & Frenkel 2005,

Canalis 2013). Literature confirms our findings of reduction of WNT10b and β-catenin and increase of DKK-1 immunolabeling, a soluble inhibitor of the canonical WNT signaling pathway, in GIOP+EP group. In turn, ATV reversed WNT and β-catenin reduction and decreased DKK-1 immunostaining. DKK-1 is a direct target of the mevalonate pathway and that inhibition of the mevalonate pathway resulted in a profound suppression of DKK-1 (Rachner et al. 2014). As an inhibitor of osteoblast differentiation and function, elevated DKK-1 levels are thought to contribute to the imbalance of osteoblastic bone formation and osteoclastic bone resorption, a predominant feature in osteolytic bone disease (Guañabens et al. 2014).

The serum BALP, a biochemical indicator of bone formation secreted from osteoblast allowing the mineralization process, has been used for measuring the bone resorption in patients with bone metabolic disorders and to confirm the role of osteoblast on metabolic bone diseases (Vaisman et al. 2005). GIOP reduced BALP levels, in accordance with other authors (Elshal et al. 2013) and ATV prevented BALP serum levels reduction. ATV has been shown either to not alter or to slightly increase BALP serum levels (Majima et al. 2007), which could be explained by stimulation of bone morphogenetic protein-2 and induction of osteoblast differentiation (Mundy et al. 1999).

Atorvastatin's anti-inflammatory activity is evidenced by the reduced expression of TNF, IL-1β, -6 and -8, and increase of IL-10 in periodontium. The observed reduction in MPO levels further confirms the reduction of leukocyte migration in treated animals. These outcomes suggest that tissue destruction on GIOP+EP group involves an intricate signaling pathway. A study using the ABL ligature-induced model demonstrated that Atorvastatin (10 mg/kg) significantly reduced COX-2, MMP-2, MMP-9, MPO, IL-1β and TNF-α levels (Araújo et al. 2013).

Atorvastatin improved the gingival oxidative stress, reestablishing GSH, SOD and CAT levels on periodontal tissue, as in accordance to other studies (Dalcico et al. 2013; de Araújo et al. 2013). A study corroborated the present data by showing that the protection against osteoporosis by statins is linked to a reduction of the oxidative stress and restoration of NO formation (Yin et al. 2012).

Finally, to verify the possible systemic repercussions, over 5 weeks of GIOP and 11 days of ligature, ATV reversed leukocytosis marked by neutrophilia,

which can be explained by the anti-inflammatory effect of ATV. ATV, a hydrophobic statin, did not modify AST, ALT, urea or creatinine serum levels. It has been reported that all statins, specially the hydrophilic ones, can induce asymptomatic mild elevation of serum transaminases, but this rarely requires withdrawal of the drug (Bolego et al. 2002). In general, the elevation of serum transaminases is often self-limiting and thought to be related to alteration of the hepatocyte cellular membrane with enzyme leakage, rather than direct liver injury (Calderon et al. 2010). Additionally, Atorvastatin was administered for a short period of time (11 days), and most of the treatment regimens described in the literature used Atorvastatin for longer periods (Bonnet et al. 2008).

In summary, the present findings suggest that statins deserves additional investigation as potential pharmacological approach for preventing bone resorption in periodontal inflammatory diseases and when periodontitis is associated with GIOP. This study provides important new information on the protective effects of Atorvastatin on bone metabolism, periodontal inflammation and oxidative stress, mainly via WNT signaling pathway, which may guide the development of more efficacious and safer statin compounds targeting bone tissue in patients with Periodontitis and Osteoporosis.

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6. CONCLUSÃO GERAL

Diante do exposto, os resultados deste estudo mostraram que a osteoporose induzida por glicocorticoide agrava a perda óssea observada na doença periodontal, inibe a atividade osteoblástica e altera a perda óssea sistêmica em ratos com periodontite experimental, e que a ATV previne a POA em ratos submetidos à OICG+PE, por meio de efeito antirreabsortivo, anti-inflamatório e antioxidante, com participação da via de sinalização WNT, sugerindo que esse fármaco pode ser uma abordagem terapêutica interessante como adjuvante ao tratamento básico da periodontite quando associada à osteoporose induzida por glicocorticoides**.**

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ANEXO I

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DECLARAÇÃO

Declaramos que o protocolo para uso de animais em experimentação nº 78/2014, sobre o projeto intitulado: "**O PAPEL DA VIA WNT/ β- CATENINA NA PERDA ÓSSEA INFLAMATÓRIA EM RATAS COM OSTEOPOROSE INDUZIDA POR GLICOCORTICÓIDE E TRATADOS COM ATORVASTATINA",** de responsabilidade da Profa. Dra. Paula Goes Pinheiro Dutra e 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 5 de junho de 2014.

Fortaleza, 5 de junho de 2014

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

Profa. Dra. Nylane M. Nunes de Alencar
Coordenadora da CEPA/DFFE-MED/UFC
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