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PAULO GOBERLÂNIO DE BARROS SILVA

EXPRESSÃO DE MARCADORES INFLAMATÓRIOS NA OSTEONECROSE DOS MAXILARES INDUZIDA POR BISFOSFONATOS E EFEITO DO TRATAMENTO CRÔNICO COM ÁCIDO ZOLEDRÔNICO NOS TECIDOS GENGIVAL E ÓSSEODENTÁRIO DE RATOS

FORTALEZA
2016

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, como um dos requisitos para a obtenção do título de Doutor em Odontologia.

Área de Concentração: Clínica Odontológica

Orientadora: Profa. Dra. Ana Paula Negreiros Nunes Alves

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*A **Deus, Minha Mãe, Meu Pai, Shaila e meus amigos,** por me fazerem sorrir todos os dias.*

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“*Entia non sunt multiplicanda praeter necessitatem*”

(William de Ockam)

RESUMO

Introdução: O Ácido Zoledrônico (AZ) é um bisfosfonato (BF) capaz de causar desregulação imune-inflamatória estando associado à Osteonecrose dos Maxilares induzida por Bisfosfonatos (OMB). O objetivo deste estudo foi determinar o perfil celular e a imunoexpressão de marcadores inflamatórios do osso mandibular em modelo murino de OMB e nos tecidos gengival e osseodentário de ratos tratados cronicamente com AZ. **Materiais e Métodos:** Ratos Wistar machos (180-220g) foram submetidos a quatro infusões venosas de solução salina estéril ou AZ nas doses de 0,04, 0,20 ou 1,00 mg/kg, sendo o lado esquerdo submetido a exodontia do primeiro molar inferior (dia 49). Após 70 dias, realizou-se eutanásia e as mandíbulas dos animais foram hemisseccionadas (lados esquerdo e direito) e submetidas a análise histológica e técnica de imuno-histoquímica pelo método da estreptavidina-biotina-peroxidase para CD68, Fator de Necrose Tumoral alfa (TNF-α), Interleucina(IL)-1β, Óxido nítrico sintase induzida (iNOS), Fator Nuclear kappa B (NF-kB) e IL-18 bp (bindding protein). No tecido ósseo do lado submetido à exodontia (lado esquerdo), contaram-se as lacunas de osteócitos (vazias e preenchidas), osteoclastos (normais e apoptóticos), polimorfonucleares neutrófilos (PMN), mastócitos e células com marcação positiva para os marcadores supracitados. A polpa dentária e o periodonto do primeiro molar inferior direito (lado contralateral) foram analisados por microscopia de luz convencional quanto à presença de animais exibindo vasos dilatados/ectásicos (somente polpa dentária) e células inflamatórias. O estudo imuno-histoquímico foi realizado por meio da mesma técnica através de escores. O experimento foi repetido (infusão de salina versus AZ 0,20 mg/kg) para dosagem de TNF-α, IL-1β, mieloperoxidase (MPO), malanoaldeído (MDA) e glutationa (GSH) das gengivas excisadas cirurgicamente no dia da eutanásia dos animais. **Resultados:** a OMB foi observada nos animais tratados com 0,20 e 1,00 mg/kg de AZ com aumento do percentual de lacunas de osteócitos vazias ($p<0.001$) e osteoclastos apoptóticos ($p<0.001$), número total de osteoclastos ($p=0.003$), PMN ($p=0.009$) e células com positividade citoplasmática para CD68 ($p<0.001$), TNF-α ($p=0.001$), IL-1β ($p=0.001$), iNOS ($p<0.001$) e NF-kB ($p=0.006$), e positividade nuclear NF-kB ($p=0.011$). A imunoexpressão de IL-18bp foi reduzida de forma dose-dependente ($p=0.005$). Na polpa dentária, houve incremento do número de mononucleares CD68+ ($p=0.026$) e imunoexpressão de TNF-α ($p=0.020$), IL-1β ($p=0.027$), iNOS ($p=0.001$) em odontoblastos e IL-1β ($p=0.027$) em células não odontoblastos. No periodonto, observaram-se, nos grupos tratados com AZ, um aumento do

número de células inflamatórias ($p=0.001$) e imunoexpressão citoplasmática para TNF-α ($p=0.003$), IL-1β ($p=0.004$), iNOS ($p=0.008$) e NF-kB ($p=0.025$). Os níveis de MPO ($p<0.001$), TNF-α ($p=0.002$), IL-1β ($p<0.001$) e GSH ($p=0.005$) estavam significantemente aumentados na gengiva dos animais tratados com 0,20 mg/kg de AZ. **Conclusão:** Conclui-se que há superexpressão de marcadores inflamatórios no sítio de exodontia não cicatrizado e com instalação da OMB. A infusão crônica de AZ mostrou-se capaz de aumentar a expressão de citocinas inflamatórias e do infiltrado inflamatório nos tecidos gengival e osseodentário de ratos.

Palavras-chave: Osteonecrose da arcada osseodentária associada a bisfosfonatos, Fator de Necrose Tumoral alfa, Interleucina-1, Óxido nítrico sintase induzida tipo 2, NF-kappa B, IL-18 binding proteins, Ácido Zoledrônico, Polpa Dentária, inflamação, Bisfosfonatos, Periodonto, Inflamação, Estresse Oxidativo, Glutationa.

ABSTRACT

Introduction: The Zoledronic Acid (ZA) is a bisphosphonate (BF) that can lead to immune-inflammatory dysregulation leading to Bisphosphonate-related Osteonecrosis of the Jaws (BRONJ). The objective of this study was to determine the cellular profile and the immune expression of inflammatory markers in mandibular bone of a BRONJ experimental model and in gingival and tooth of rats chronically treated with ZA. **Materials and Methods:** Male Wistar rats (180-220 g) were chronically infused (four times) venously with sterile saline or 0.04, 0.20 or 1.00 mg/kg of ZA and the left hemimandible was subjected to first molar exodontia (day 49). After 70 days we performed the euthanasia and the hemimandibles (right and left) were histologically and immunohistochemically analyzed (CD68, Tumor Necrosis Factor alpha (TNF-α), Interleukin(IL)-1β, inducible Nitric Oxide Synthases (iNOS), Nuclear Factor kappa B (NF-kB) and IL-18(bidding protein) bp). In bone subjected to exodontia (left hemimandible) we counted the viable and empty osteocyte lacunae, the viable and apoptotic osteoclasts, the polymorphonuclear neutrophils cells (PMN), mast cells and positive cytoplasmic cells for cited markers. Dental pulp and periodontium of first right molar (contralateral hemimandible) was histologically analyzed by presence of inflammatory cells, dilated/ectasic blood vessels (pulp only) and immunohistochemically by scores. We repeated the protocol (saline versus 0.20 mg/kg ZA) to dosage of TNF-α, IL-1β, myeloperoxidase (MPO), Malondialdehyde (MDA) e Glutathione (GSH) of gingiva of animals. **Results:** the BRONJ was observed in 0.20 mg/kg and 1.00 mg/kg ZA-treated animals that showed increase in percentage of empty osteocyte lacunae ($p<0.001$) and percentage of apoptotic osteoclast ($p<0.001$), total osteoclasts number ($p=0.003$), PMN ($p=0.009$), cytoplasmic positive cells for CD68 ($p<0.001$), TNF-α ($p=0.001$), IL-1β ($p=0.001$), iNOS ($p<0.001$) e NF-kB ($p=0.006$), and nuclear positivity for NF-kB ($p=0.011$). The immune expression of IL-18bp decreased dose dependently ($p=0.005$). In dental pulp there was augment in number of mononuclear CD68+ cells ($p=0.026$) and immunostaining for TNF-α ($p=0.020$), IL-1β ($p=0.027$), iNOS ($p=0.001$) in odontoblasts and IL-1β ($p=0.027$) in non odontoblasts dental pulp cells. In periodontium ZA treated animals showed an increase in number of animals showing inflammatory cells ($p=0.001$) and cytoplasmic immunostaining for TNF-α ($p=0.003$), IL-1β ($p=0.004$), iNOS ($p=0.008$) and NF-kB ($p=0.025$). MPO ($p<0.001$), TNF-α ($p=0.002$), IL-1β ($p<0.001$) and GSH ($p=0.005$) levels were significantly raised in 0.20 mg/kg ZA treated gingiva animals. **Conclusion:** There is overexpression of inflammatory markers in exodontia

area with establishing of BRONJ. The chronic infusion of ZA raised the expression of inflammatory markers and cells in gingiva, periodontium and pulp tissue of rats.

Keywords: Bisphosphonate-Associated Osteonecrosis of the Jaw, Tumor Necrosis Factor-alpha, Interleukin-1, Nitric Oxide Synthase Type II, NF-kappa B, IL-18 binding proteins, Zoledronic acid, Dental Pulp, Inflammation; Diphosphonates; Periodontium; Inflammation; Oxidative Stress; Glutathione.

LISTA DE ABREVIATURAS

ABC: Avidin Biotin Complex (complexo avidina biotina)

ANOVA: analysis of variance (análise de variância)

APC: Antigen Presenting Cells (células apresentadoras de antígeno)

AZ: Ácido Zoledrônico

BF: Bisfosfonato

BRONJ: Bisphosphonate-related osteonecrosis of the jaw (Osteonecrose dos Maxilares Induzida por Bisfosfonatos)

CCL20: C-C motif chemokine ligand 20 (ligante tipo 20 do receptor tipo C-C)

CCR6: C-C chemokine receptor type 6 (receptor de quimiocinas tipo 6)

CD68: receptor scavenger CD68

CEPA: Comitê de Ética em Pesquisa Animal

CEUA: Comissão Ética no Uso de Animais

CTX: telopeptídeo carboxiterminal do colágeno tipo I

DAB: 5,5-diaminobenzidine tetrahydrochloride (5,5 diaminobenzidina tetrahidroclorada)

DTNB: acid 5',5'-dithio-bis(2-nitrobenzoic) acid (ácido 5’,5’-di-tio-bis(2-nitrobenzoico))

EDTA: Ethylenediaminetetraacetic acid (ácido etilenodiaminotetracético)

ELISA: elisa enzyme linked immunosorbent assays

GSH: Glutathione (glutationa reduzida)

H&E: hematoxylin & eosin (hematoxilina-eosina)

H_2O_2: peróxido de hidrogênio

HE: haematoxylin eosin (hematoxilina-eosina)

HRP: Horseradish Peroxidase (peroxidase equina)

HTAB: hexadecyltrimethylammonium (hexadeciltrimetilamonio)

IL-17: Interleukin 17

IL-18bp: Interleukin 18 binding protein (proteína ligante da Interleucina 18)

IL-1β: interleukin 1 beta (Interleucina 1 beta)

IL-6: Interleucina 6

IL-6R: Receptor da Interleucina 6

iNOS: inducible nitric oxide synthase (Óxido-nítrico sintase induzida)

KCl: Cloreto de potássio

MCP-1: Monocyte Chemoattractant Protein-1 (proteína quimioatratotora de monócitos tipo 1)

MDA: Malondialdehyde (malanoaldeído)

MIP: Macrophage Inflammatory Protein

MPO: myeloperoxidase (myeloperoxidase)

NF-κB: nuclear factor kappa B (fator nuclear kappa B)

OMB: Osteonecrose dos Maxilares Induzida por Bisfosfonatos

OPD: o-phenylenediamine (o-fenilenidiamina)

OPG: osteoprotegerin (osteoprotegerina)

PBS: Phosphate buffered saline (solução tampão de fosfato)

PD: periodontal disease (doença periodontal)

PDGF (Fator de Crescimento Derivado de Plaquetas).

R1: Radical 1 ligado ao átomo central de carbono da molécula de bisfosfonato

R2: Radical 2ligado ao átomo central de carbono da molécula de bisfosfonato

RANK: receptor activated nuclear factor kappa b (receptor ativador do fator nuclear kappa B)

RANKL: receptor activated nuclear factor kappa b ligand (ligante do receptor ativador do fator nuclear kappa B.

ROS: reactive oxigen species (espécies reativas de oxigênio)

SEM: standard error (erro padrão)

TNF-α: tumor necrosis factor alpha (Fator de Necrose Tumoral alfa)

TNFR: Receptor do Fator de Necrose Tumoral

TRAP: tartarate resistant alkalin phosphatase (fosfatáse ácida resistente ao tartarato)

ZA: Zoledronic acid

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1. Introdução Geral

Os Bisfosfonatos (BFs) são fármacos inibidores da reabsorção óssea, constituídos por uma estrutura similar ao pirofosfato endógeno. Os BFs foram desenhados com base na molécula de pirofosfato devido a sua semelhança e interação com essa molécula. Diferente do pirofosfato, no entanto, os BFs contêm dois grupos fosfonatos ligados um a um ao átomo de carbono central (estrutura P-C-P), não oxigênio, o que lhes confere alta resistência à degradação enzimática e maior tempo de meia vida (ROGERS et al., 2000; RUSSELL, CROUCHER, ROGERS, 2007).

Diversos grupos químicos podem ser acrescentados à estrutura dos BFs por meio de duas ligações covalentes adicionais ao átomo de carbono central, dando origem a diversos compostos (ROGERS et al., 2000) e conferindo diferentes propriedades a esses fármacos. O grupo R1 determina a afinidade dos BFs pelos cristais ósseos, enquanto o grupo R2 é responsável pela potência e atividade farmacológica (FLEISCH, 1998) (Figura 1).

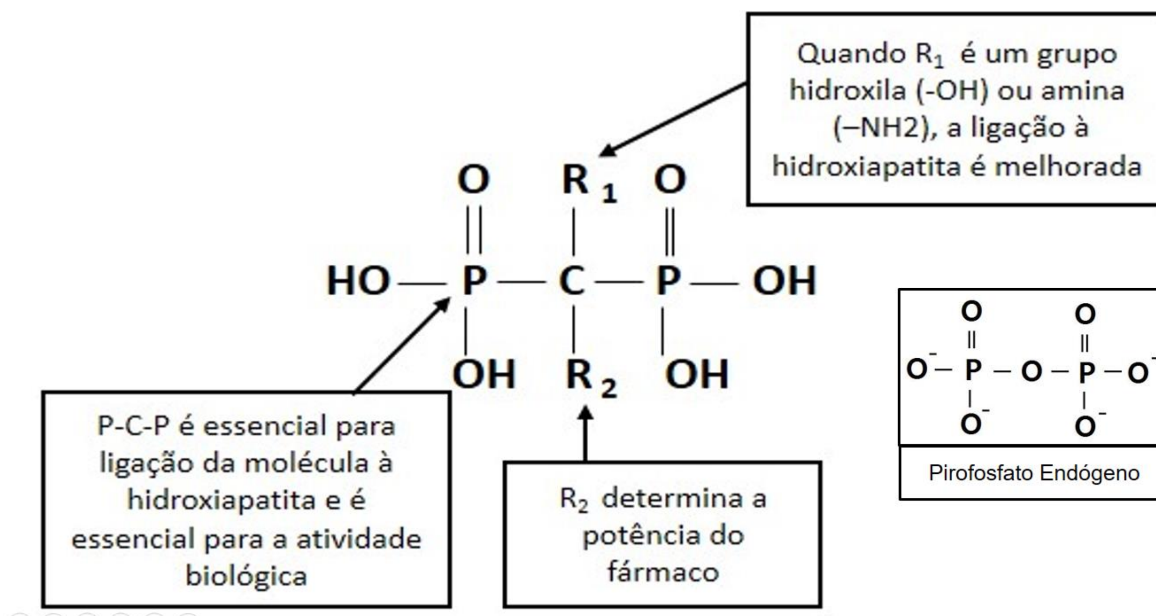

Figura 1: Representação esquemática da estrutura química geral dos bisfosfonatos e do pirofosfato endógeno. Fonte: Própria.

Os BFs podem ser divididos em duas classes, os não-amino-BFs e os amino-BFs. Estes dois subgrupos diferem na presença de um átomo de nitrogênio ligado direta ou

indiretamente ao átomo de carbono central, o que modifica sua potência e seu mecanismo de ação (WOO et al., 2006).

Os não-amino-BFs têm uma potência reduzida em relação aos amino-BF. Seu mecanismo de ação envolve a ligação à Adenosina-Trifosfato e a sua inativação, com consequente inibição da função celular e indução de apoptose. (AAPRO et al., 2008; HARVEY et al., 2007; WOO et al., 2006)

Os amino-BFs possuem um ou mais átomos de nitrogênio adicionados ao carbono central do análogo do pirofosfato (HARVEY et al., 2007). Com isso os amino-BFs passam a apresentar mecanismos de ação adicionais. Durante o processo de reabsorção óssea e desenvolvimento da borda em escova, clássico sinal histológico indicativo de atividade osteoclástica, o osteoclasto ativado reduz o pH no meio externo adjacente à matriz óssea mineralizada. Com isso, ocorre liberação do BF aderido à hidroxiapatita, internalização nesta célula e indução da produção de um análogo da adenosina trifosfato, que leva à apoptose osteoclástica. Os novos amino-BFs inibem a farnesil difosfato sintase, ocorrendo interrupção da via do mevalonato, um importante precursor da produção de colesterol, com subsequente redução de sua síntese. Devido à falta de colesterol, ocorre desregulação do transporte intracelular, desorganização citoesquelética e inibição da proliferação osteoclástica. Além disso, os amino-BFs reduzem o recrutamento de osteoclastos e induzem osteoblastos a produzirem fatores de inibição de osteoclastos (Figura 2) (WOO et al., 2006).

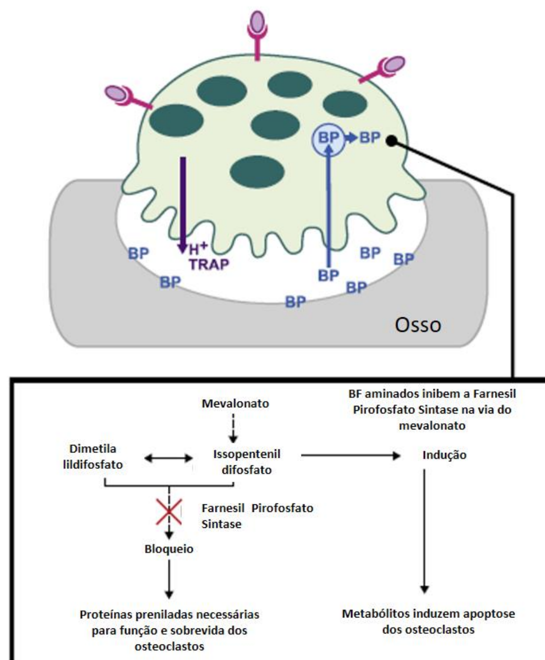

Figura 2: Absorção celular e mecanismo de ação do amino-BF no osteoclasto. Imagem de BARON, FERRARI e RUSSEL (2011).

Em decorrência da potência aumentada dos novos amino-BFs estes se tornaram medicamentos de escolha para a prevenção e tratamento de desordens ósseas com alta taxa de reabsorção, as metástases ósseas (HARVEY et al., 2007), assim como doenças ósseo-degenerativas, como a osteoporose e o hiperparatireoidismo primário (BRANDÃO et al., 2008; McCLUNG et al., 2008; MARTIN et al., 2007).

Os não-amino-BFs existentes e com uso liberado são o etidronato e o clodronato e os amino-BFs são o pamidronato, alendronato, ibandronato, risedronato e o zoledronato. De todos estes, o zoledronato, ou Ácido Zoledrônico (AZ) é o que possui a maior potência antirreabsortiva (Figura 3).

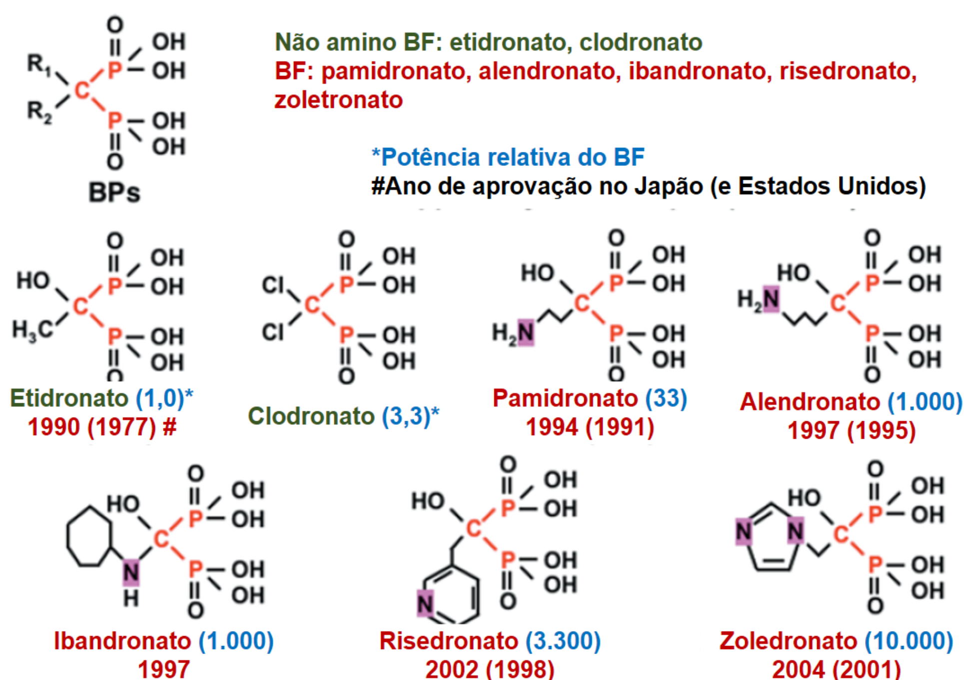

Figura 3: Estrutura dos BFs aminados (NBPs) e não-aminados (non-NBPs), potência relativa, e ano de aprovação no Japão e nos Estados Unidos, respectivamente (*BF de referência para cálculo da potência relativa). Imagem adaptada de OIZUMI et al., 2009.

O AZ é um amino-BF derivado imidazólico heterocíclico de terceira geração. Esse fármaco é administrado unicamente por via endovenosa, em uma infusão de 4 mg por cerca de 15 minutos. Ao final da administração, sua concentração plasmática eleva-se

rapidamente, seguida de um declínio, apresentando, após 24 h, concentração plasmática de 1%. Cerca de 56% do AZ liga-se a proteínas plasmáticas e este, praticamente, não é acumulado no organismo, com uma única exceção: o sistema esquelético (RUSSEL et al., 1999; FERNANDES, LEITE, LANÇAS, 2005; LYSENG-WILLIAMSON, 2008).

Como outros amino-BFs, seu mecanismo de ação envolve a formação de um análogo da adenosina trifosfato osteoclástica, que induz a apoptose e a inibição da via do mevalonato na síntese de colesterol por inativação da farnesil difosfato sintase, com consequente desregulação do transporte intracelular, desorganização citoesquelética e inibição da proliferação osteoclástica (WOO *et al.,* 2006). Além de possuir o mecanismo de ação inerente aos amino-BFs, o AZ destaca-se por sua ação antitumoral, antiangiogênica (CLÉZARDIN, 2005) e possui excelentes resultados na redução de atraso da morbidade esquelética e das complicações decorrentes da doença óssea metastática, sendo, por conseguinte, utilizado, por mais de 15 anos, em pacientes com metástases ósseas de tumores sólidos (AAPRO et al., 2008).

No entanto, esse fármaco possui uma toxicidade considerável, com diversos eventos pró-inflamatórios, os quais também se associam ao principal efeito colateral deste: a Osteonecrose dos Maxilares Induzida por Bisfosfonatos (OMB).

1.2. Osteonecrose dos Maxilares Induzida por Bisfosfonatos

1.2.1. Características clínicas

A Sociedade Acadêmica Americana de Cirurgiões Oral e Maxilo-Facial e a Sociedade Americana de Pesquisa Mineral e Óssea definem a OMB como uma exposição de osso necrótico na região maxilo-facial, por mais de oito semanas, em pacientes sob uso de BFs sem história prévia de radioterapia. A OMB pode estar associada à dor e, principalmente, à infecção que compromete a qualidade e expectativa de vida do paciente usuário (SILVERMAN et al., 2009).

Recentemente, em meados de 2015, devido ao aumento do número de fármacos relacionados ao desenvolvimento da osteonecrose dos maxilares, a OMB passou a fazer parte de uma condição patológica maior denominada osteonecrose dos maxilares associada ao uso de antirreabsortivos. Sua definição passou a elencar o desenvolvimento de osteonecrose nos

ossos gnátios, independentemente do tipo de antirreabsortivo utilizado e do tempo de desenvolvimento, mas a condição excludente de radioterapia de cabeça e pescoço continua fazendo parte da sua denominação (OLIVEIRA et al., 2016).

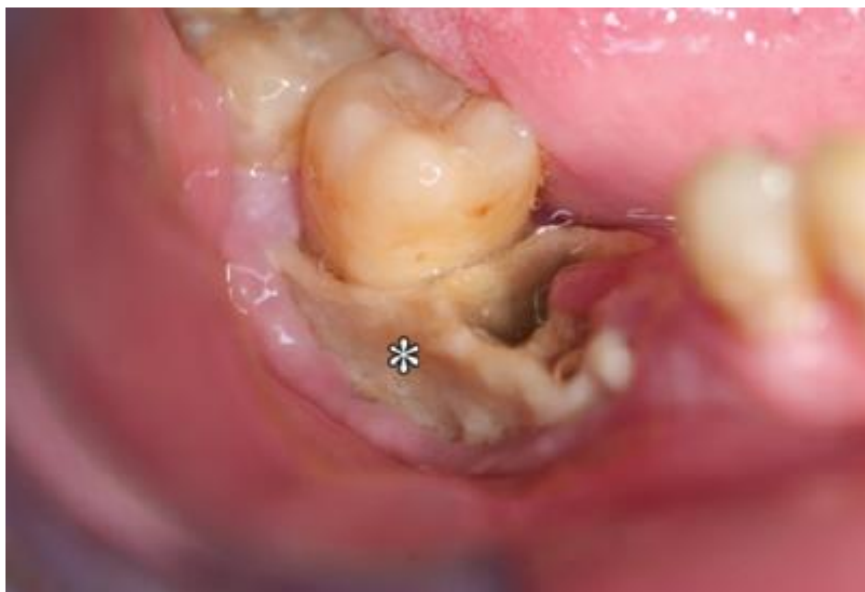

Figura 4: Aspectos clínico da OMB. Imagem de RUGGIERO et al., 2006.

Apesar de não ser possível determinar a incidência de OMB devido às inconsistências nos relatos de casos publicados (RUGGIERO et al., 2006), a OMB está mais fortemente relacionada ao uso da forma endovenosa do que à administração oral. Apesar disso, as OMBs dependentes da forma de administração oral têm apresentado incidência crescente (DEL CONTE et al., 2010; POZZI et al., 2007). A forma endovenosa é a de principal escolha para tratamento oncológico e, devido à grande concentração de fármaco absorvida, é a principal via associada à OMB. A administração oral é mais utilizada para tratamento de osteoporose e outras desordens metabólicas ósseas como a Doença de Paget, mas devido à baixa taxa de reabsorção intestinal (cerca de 3%) é fracamente associada ao desenvolvimento da OMB (DEL CONTE et al., 2010; SILVERMAN et al., 2009; POZZI et al., 2007). Cerca de 95% de casos de OMB são relacionados aos pacientes submetidos a tratamento endovenoso, sendo mais comum a sua associação ao uso de AZ (WOO *et. al*, 2006).

O diagnóstico da OMB é predominantemente clínico-imaginológico, e os estádios podem ser divididos a depender da sintomatologia e da análise das imagens conforme a tabela 1.

Tabela 1: Estádios de apresentação clínica da OMB.

Estádio	Característica Clínica
Pacientes de risco	Sem osso exposto/necrótico em pacientes que foram tratados com bisfosfonatos orais ou endovenosos.
Estádio 1	Osso exposto/necrótico em pacientes assintomáticos e sem evidência de infecção.
Estádio 2	Osso exposto/necrótico associado com infecção e evidência de dor e eritema na região do osso exposto com drenagem purulenta.
Estádio 3	Osso exposto/necrótico em pacientes com dor, infecção e um ou mais dos seguintes sinais: fraturas patológicas, fístula extraoral ou osteólise se estendendo às bordas da lesão.

Adaptado de RUGGIERO et al., 2009.

O diagnóstico por imagem pode ser realizado por radiografia panorâmica, tomografia computadorizada, ressonância magnética ou cintilografia óssea por tecnécio 99m. Os principais achados sugestivos de OMB são mostrados na tabela 2.

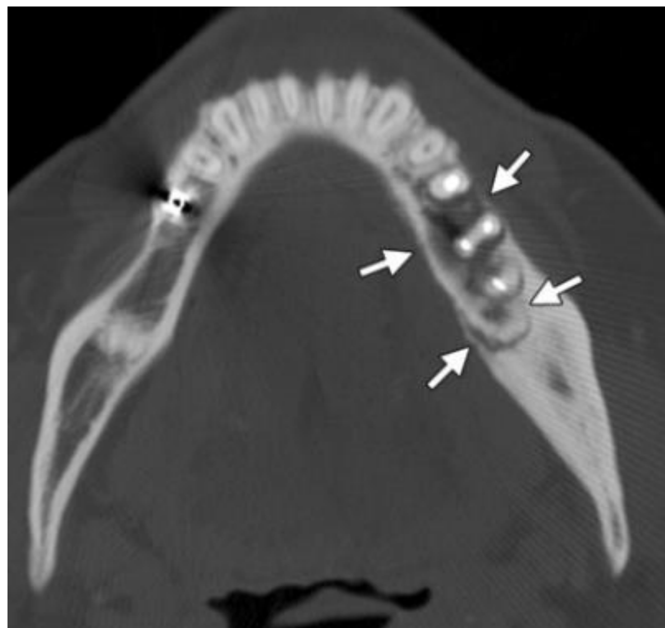

Figura 5: Aspecto imaginológico (tomografia computadorizada de feixe cônico, corte axial) da OMB. Imagem de RUGGIERO et al., 2006.

Tabela 2: Características imaginológicas da OMB.

Sistema	Característica
Radiografia Panorâmica e Tomografia Computadorizada de Feixe Cônico	Osteólise, lesões escleróticas, reação periosteal, estreitamento do trabeculado ósseo, envolvimento do canal alveolar inferior e fraturas patológicas
Ressonância Magnética	
Contraste T1	Tipicamente diminui a intensidade do sinal
Contraste T2	Variável: pequeno ou moderado aumento na intensidade do sinal nas lesões jovens com aumento ou descréscimo em lesões antigas
Material de contraste	Variável: pode se correlacionar com o decréscimo da intensidade do sinal nas imagens com contraste T1 e tipicamente apresenta interrupções no contraste T2 sugestivo de sequestros ósseos
Cintilografia com tecnécio 99m	Áreas de baixa captura podem se apresentar nas lesões jovens; em estágios avançados há um aumento da captura do radio-isótopo com interrupções focais centrais.

Adaptado de MORAG et al., 2009.

Até o presente momento, não foi descrito adequado exame preditor de OMB. A dosagem sérica do telopeptídeo carboxiterminal do colágeno tipo I (CTX), uma forma indireta de medir atividade osteoclástica, tem sido sugerida como biomarcador preditor de OMB. Ao reabsorver tecido ósseo, o osteoclasto deixa como remanescente esse resíduo (CTX) que, na corrente sanguínea, indica atividade osteoclástica. É um biomarcador que tem sido um exame utilizado para avaliar doenças osseodegenerativas, mas possui baixíssima sensibilidade como fator preditor de OMB, tendo em vista que há alteração direta no metabolismo osteoclástico (FLEISHER et al., 2010).

1.2.2. Fatores de risco

A identificação de fatores de risco tem sido considerada o método mais seguro em predizer o desenvolvimento de OMB. Quando o uso de BFs é somado a algum procedimento (cirúrgico, por exemplo), ou doença com alteração metabólica óssea ou inflamatória, ocorre aumento do risco de desenvolver a OMB (BARASCH et al., 2011; TSAO et al., 2013).

O principal fator de risco à OMB é o trauma cirúrgico. Procedimentos odontológicos cruentos são diretamente associados à elevada prevalência de OMB. A exodontia é o principal procedimento associado a essa condição, aumentando a prevalência de OMB de 17 até 299 vezes a mais, a realização de implantes osseointegrados em até nove vezes, e alguns casos de OMB, após a realização de alveoloplastias, têm sido recentemente publicados. Até mesmo procedimentos odontológicos não invasivos, como o tratamento endodôntico, aumentam o risco de desenvolvimento dessa doença (BARASCH et al., 2011).

A doença periodontal, apesar de não se relacionar a trauma cirúrgico, apresenta um forte caráter inflamatório, similar ao que acontece fisiologicamente com as exodontias, constituindo um importante fator de risco à OMB. E, adicionalmente, casos de OMB espontânea decorrentes de próteses mal ajustadas também já foram ocasionalmente publicados (TSAO et al., 2013; DINIZ-FREITAS et al., 2012; BARASCH et al., 2011).

O uso de corticoides, a presença de doenças como o diabetes mellitus, osteoporose e anemia e condições relacionadas, como radioterapia de cabeça e pescoço, são os principais fatores de risco associados. O tempo de uso dos BFs e a idade (faixa de corte de 60 anos) também são diretamente proporcionais ao aumento do risco de OMB (BARASCH et al., 2011).

A infecção também tem sido citada como um fator de risco ao surgimento de OMB, no entanto, a patogênese parece estar mais fortemente relacionada à desregulação imune-inflamatória (LÓPEZ-JORNET et al., 2010). Os BFs aumentam a adesão microbiana na hidroxiapatita, o que potencializa os eventos pró-inflamatórios supracitados induzidos pelos BFs (NORTON et al., 2012). O problema é que a desregulação imune-inflamatória é independente da presença de microrganismos e, talvez por isso, a antibioticoterapia, isoladamente, tem pouca ou nenhuma eficácia no tratamento da OMB.

Todos esses fatores de risco, independente se locais ou sistêmicos, possuem em comum um forte caráter inflamatório, o que mostra ter relação com a OMB.

Devido aos eventos pró-inflamatórios associados à infusão endovenosa de amino-BFs, o papel da inflamação na OMB tem sido pesquisado, recentemente, como um potencial fator etiológico associado ao desenvolvimento dessa condição, tanto em modelos experimentais (KUIPER et al., 2012; NORTON et al., 2012) como em estudos com seres humanos (TSAO et al., 2013; ROSSINI et al., 2012; LESCLOUS et al., 2009). Esses estudos parecem apontar uma relação direta entre OMB e inflamação, porém a natureza dessa relação ainda é incerta. Segundo SILVERMAN & LANDESBERG (2009) e ALLEN (2011), existem, na literatura, muito mais hipóteses de mecanismos fisiopatológicos de OMB do que evidências científicas propriamente ditas, o que faz desta linha de pesquisa uma forte candidata a explicar a patogênese da OMB.

1.2.3. Terapêutica

Nenhuma modalidade terapêutica proposta para a OMB tem total eficácia para ser amplamente utilizada. Metade dos pacientes tratados com descontinuação dos BFs, antibioticoterapia local ou sistêmica, oxigênio-terapia hiperbárica ou debridamento cirúrgico mantém quadro clínico inalterado, e apenas 3% dos casos tratados com uma dessas modalidades evoluem para cura (DEL CONTE et al., 2010; POZZI et al., 2007).

Quando da necessidade de procedimentos cirúrgicos invasivos em pacientes em uso de BF, a profilaxia e a cobertura antibiótica têm sido sugeridas com a finalidade de prevenir o desenvolvimento de necrose, porém, diversos estudos apontam que a abordagem não é eficaz (ROMAGNA et al., 2008; RUPEL et al., 2014).

A ausência de uma terapêutica adequada torna a OMB uma condição ainda mais complexa, pois a exposição óssea aumenta a possibilidade de infecção local, cujo perfil microbiológico não é alterado pelo tratamento com antibióticos (JI et al., 2011), podendo evoluir para septicemia em pacientes imunocomprometidos, decorrente do tratamento oncológico (MIYAZAKI et al., 2012).

A profilaxia antibiótica pré-operatória tem se constituído uma prática comum em cirurgia oral com o objetivo de diminuir a incidência de infecção pós-operatória em decorrência de a cavidade oral ser um meio altamente contaminado. O seu uso é justificado em pacientes com desordens do sistema imunológico e risco elevado de desenvolvimento de endocardite bacteriana, pois há a diminuição da incidência de infecção. Além disso, o uso da profilaxia antibiótica minimiza os custos com cuidados em saúde e o surgimento de bactérias

resistentes (PIECUCH *et. al*, 1995) e, mesmo não havendo um padrão de indicação e prescrição bem definidos, a maioria dos cirurgiões-dentistas usa essa modalidade terapêutica para diminuir a possibilidade de infecção em pacientes com algumas alterações sistêmicas (ROMAGNA *et. al*, 2008).

A profilaxia antibiótica parece ser um método interessante a fim de prevenir OMB, porém, como forma terapêutica, as taxas de sucesso da antibioticoterapia isolada não passam de um terço dos casos. Na verdade, considera-se como sucesso terapêutico o fechamento da ferida de tecido mole, e nenhuma abordagem terapêutica conseguiu sucesso no que diz respeito a remodelação óssea. O único tratamento para a OMB com alguma taxa de eficácia em tecido mole é o debridamento cirúrgico. RUPEL et al., em 2014, por meio de uma revisão sistemática de ensaios clínicos, delinearam as taxas de eficácia dos tratamentos para a OMB sugeridos até o presente momento, segundo o estádio em que esta se encontra (Tabela 3). Eles observaram que a abordagem não cirúrgica apresenta baixas taxas de sucesso e que o tratamento cirúrgico com extensão até o osso sadio tem demonstrado o melhor prognóstico. Apesar disso, quase um quinto dos casos recidiva, sendo necessária nova abordagem cirúrgica (RUPEL et al., 2014).

Tabela 3: Descrição das abordagens terapêuticas e suas taxas de sucesso da OMB nos seus diferentes estádios de apresentação clínica.

Estádio da OMB	**Abordagem não cirúrgica**	**Cirurgia conservadora**	**Cirurgia extensiva**	**Cirurgia a laser**
Estádio 0	-	-	89%	-
Estádio 1	33%	72%	87%	100%
Estádio 2	24%	79%	96%	83%
Estádio 3	0%	27%	81%	100%

Adaptado de RUPEL et al., 2014.

As cirurgias extensivas aparentam ter o melhor prognóstico, no entanto, o procedimento cirúrgico realizado a laser, provavelmente devido à potência anti-inflamatória da abordagem, apresenta taxas também promissoras. Apesar disso, reafirma-se que esse sucesso é tido com base no fechamento da ferida de tecido mole, pois a melhora do tecido

ósseo não é observada em nenhum estudo. Assim, não há regressão total do quadro tratado de nenhum paciente com OMB (RUPEL et al. 2014).

A oxigênio-terapia hiperbárica é uma modalidade terapêutica francamente utilizada no tratamento da osteorradionecrose. A osteorradionecrose é uma condição patológica diretamente associada aos efeitos hipovasculares e hipocelulares diretos da radioterapia no tecido ósseo. Como a patogênese da OMB parece não envolver mecanismo similar (apesar de o AZ ter cunho antiangiogênico), esse tipo de tratamento é contraindicado nos pacientes com OMB em tratamento oncológico devido à possibilidade de progressão tumoral e, mesmo nos casos associados aos BFs orais, parece não haver grande alteração do prognóstico (DINIZ-FREITAS et al., 2012). Basicamente, acompanhamento a longo prazo e excelente saúde oral são indicados em todos os casos, a fim de se evitar ou prevenir as recidivas ou reincidências (RUPEL et al., 2014).

A OMB ainda é uma condição de patogênese incerta, diagnóstico complexo e tratamento oneroso, invasivo e de eficácia questionável. Investir recursos em estudos experimentais laboratoriais a fim de se compreender o mecanismo de ação dessa condição é indispensável para o delineamento de condutas clínicas baseadas em evidências sólidas.

1.3. Processo inflamatório e metabolismo ósseo

A inflamação tecidual se constitui como um evento fisiológico necessário ao reestabelecimento morfológico e funcional de um tecido lesado. No tecido ósseo, o processo inflamatório é um episódio necessário ao reparo tecidual óssea após trauma, sendo, na Odontologia, mais comum o trauma decorrente da exodontia. Fatores locais e sistêmicos, como condições gerais do paciente e uso de agentes farmacológicos, podem modificar o perfil inflamatório local, gerando alteração no metabolismo ósseo e déficit ou aceleração do processo de cicatrização (JOSEPH PIECUCH., 2012; FREITAS et al., 2007).

A remodelação óssea é um fenômeno que ocorre fisiologicamente, sendo fundamental para a renovação do esqueleto e manutenção de sua qualidade. Nele, a reabsorção é seguida da formação óssea em ciclos constantes orquestrados pelas células do tecido ósseo (osteoclastos, osteoblastos e osteócitos) (HANLEY et al., 2000).

Imediatamente após um trauma ósseo, como em exodontias, ocorre a formação de um hematoma, que é constituído principalmente por células do sangue periférico e intramedulares, como as células da medula óssea. A lesão inicia uma resposta inflamatória,

necessária para o processo de cicatrização. A resposta inflamatória, juntamente com o hematoma coagulado entre e ao redor das extremidades do defeito ósseo e dentro da medula, formam um modelo para a formação do calo ósseo, um tecido fibroso reparador no qual será depositada a matriz óssea (GERSTENFELD et al., 2003).

A resposta pró-inflamatória inicial envolve a secreção do fator de necrose tumoral-α (TNF-α) e de interleucinas como IL-1, IL-6, IL-11 e IL-18, por macrófagos, células inflamatórias e células de origem mesenquimais. O pico de concentração dessas citocinas pode ser observado em 24 horas, e estas retornam aos valores normais dentro de 72 horas após o trauma, levando posteriormente à remodelação óssea. Esses fatores, quando sistematicamente produzidos dentro do período e intensidade adequados, recrutam células inflamatórias, aumentam a síntese da matriz extracelular e estimulam a angiogênese (GERSTENFELD et al., 2003).

O processo de diferenciação das células da linhagem osteoclástica é controlado pelas células osteoblásticas, por meio de um eixo de regulação comum, conhecido por RANKL (Ligante do Receptor de Ativação do Fator Nuclear Kappa B)/RANK (Receptor de Ativação do Fator Nuclear Kappa B) /OPG (Osteoprotegerina). O equilíbrio entre formação e reabsorção óssea está na dependência das flutuações locais do equilíbrio entre RANKL/OPG (ROBLING et al., 2006).

A secreção rápida e bem regulada de moléculas pró-inflamatórias, após uma lesão aguda, é fundamental para a reparação do tecido (HANLEY et al., 2000). Elas estimulam a osteoclastogênese e a reabsorção do tecido lesado e posterior neoformação óssea. Todavia, sabe-se que uma expressão prolongada e crônica de citocinas inflamatórias tem um efeito negativo no osso (GERSTENFELD et al., 2003). Essa superexpressão pode ser modulada por condições locais, como a periodontite (KIM et al., 2012) ou ainda por fármacos como o AZ.

1.3. Desregulação imune-inflamatória provocada pelo AZ: Alvos celulares

Os amino-BFs, como o AZ, são fármacos que se ligam irreversivelmente à hidroxiapatita do tecido ósseo constituindo-se o principal fator associado à OMB. Devido a sua ligação de alta afinidade à hidroxiapatita, esses fármacos podem exercer seu efeito tóxico durante longos períodos (SILVERMAN e LANDESBERG, 2009). Tais fármacos proporcionam um efeito tóxico direto em muitos grupos celulares, como osteoclastos, células epiteliais, fibroblastos (SILVERMAN e LANDESBERG, 2009; SCHEPER et al., 2009),

osteoblastos (NAIDU et al., 2008), macrófagos (SCHELLER et al., 2011), neutrófilos (KUIPER et al., 2012), células endoteliais (MISSO et al., 2012), e impedem a maturação de células de linhagem mieloide (WOLF et al., 2006). Possuem efeitos de teratogenicidade em dentes, alteram a erupção de molares e a formação da matriz dental, estimulam a reabsorção odontoclástica e induzem a formação de dentículos e de odontoma (MASSA et al., 2006; HIRAGA et al., 2010).

Um dos grandes problemas da toxicidade direta nos elementos celulares são os eventos pró-inflamatórios. Em estudos *in vitro* com culturas de macrófagos, tem sido observado que a incubação com diferentes concentrações de AZ é capaz de ativar a óxido-nítrico sintase induzida (iNOS) de forma concentração-dependente. A ativação ocorre por uma via dependente do Fator Nuclear kappa B (NF-κB) e da consequente expressão de Interleucina(IL)-1β, Fator de Necrose Tumoral (TNF)-α e IL-6 (MURATSU et al., 2013). Até mesmo clinicamente há evidências de que o AZ é um forte indutor de alterações do sistema imune com consequente desregulação inflamatória. Diversos modelos experimentais (SONIS et al., 2009; ALI-ERDEM et al, 2011; LÓPEZ-JORNET et al., 2011; MAAHS et al., 2011; MARINO et al., 2012) e estudos clínicos (LESCLOUS et al., 2009) têm demonstrado associação entre o uso de BFs e eventos pró-inflamatórios.

In vivo, os amino-BFs aumentam de forma dose-dependente o número de leucócitos circulantes e induzem anemia associada a inflamação quando administrados cronicamente (SILVA et al., 2015). Em cavidade peritoneal de ratos, NORTON et al. (2012) observaram que a infusão de BFs aminados, inclusive o AZ, aumenta o número de neutrófilos e monócitos por uma via dependente de Interleucina 1β (IL-1 β) produzido por mastócitos residentes.

Estudos clínicos mostraram aumento de marcadores pró-inflamatórios como a IL-1β em saliva (BAGAN et al., 2013) e fluido gengival (TSAO et al., 2013), e IL-6 em saliva e sangue (BAGAN et al., 2014) de pacientes sob tratamento crônico com AZ, além de modificação do perfil celular sanguíneo com incremento de células inflamatórias e citocinas circulantes (ROSSINI *et al*, 2012; WELTON *et al*, 2013).

Sabe-se que, quando elementos celulares são submetidos a algum tipo de estresse fisiológico, há aumento da síntese de mediadores pró-inflamatórios (YU et al., 2011). A toxicidade do AZ e de outros amino-BFs é importante na migração e na ativação de neutrófilos e macrófagos (GRAVES e JIAGN, 1995). Neutrófilos possuem uma sobrevida encurtada na presença de AZ (KUIPER et al., 2011), o que induz dano tecidual e incrementa o

potencial de fagocitose de macrófagos. Mononucleares e outros fagócitos também apresentam atividade e número aumentados na presença de todas essas citocinas pró-inflamatórias, com consequente aumento na síntese de mediadores pró-inflamatórios.

Além disso, esses mediadores pró-inflamatórios, associados ao simples tratamento com BF, também induzem osteoclastogênese (ASSUMA et al., 1998) e elevam a síntese de espécies reativas de oxigênio (ROS), que podem aumentar a extensão do dano e estimular ainda mais a migração de células inflamatórias (HALLEEN et al., 1999; WIMALAWANSA, 2010; ALMEIDA e O'BRIEN, 2013).

Como citado anteriormente, o AZ é uma molécula que se adere irreversivelmente à hidroxiapatita do tecido ósseo (ENDELE et al., 2005) e, também, ao dente (MASSA et al., 2006; HIRAGA et al., 2010). Sua constante presença nos ossos gnátios e tecidos mineralizados é importante na modificação desse perfil inflamatório local. Porém, permanece obscuro como se dá o início dessa desregulação imunológica, o seu comportamento ao longo do tempo e o real papel na OMB.

Neste contexto, o presente grupo de pesquisadores desenvolveu um modelo experimental de OMB em ratos, que mimetiza clínico-radiográfica e histopatologicamente a condição em seres humanos (SILVA et al., 2015). Apoiado neste modelo, objetivou-se avaliar a expressão de marcadores inflamatórios na OMB e o efeito do tratamento crônico com AZ nos tecidos osseodentários de ratos.

2. Proposição

2.1. Objetivo Geral

- Avaliar a expressão de marcadores inflamatórios em modelo de OMB e o efeito do tratamento crônico com AZ nos tecidos gengival e osseodentários de ratos.

2.2. Objetivos Específicos

- Determinar o perfil celular e a imunoexpressão do Fator Nuclear kappa B (NFkB), IL-1β, Fator de Necrose Tumoral alfa (TNF-α), iNOS e IL-18 bidding protein (IL-18bp) na OMB em modelo murino com ratos;
- Verificar a imunoexpressão do NFkB, IL-1β, TNF-α, iNOS e IL-18bp na polpa dentária de ratos submetidos a tratamento crônico com AZ;
- Investigar a expressão de marcadores inflamatórios no periodonto de ratos submetidos a tratamento crônico com AZ.

3. Capítulos

Esta tese está baseada no Artigo 46 do Regimento Interno do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará, que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado e permite a inserção de artigos científicos de autoria ou coautoria do candidato (Anexo A) e exige certificação de línguas (Anexos B, C, D e E). Por se tratar de pesquisas envolvendo animais, ou partes deles, o projeto de pesquisa deste trabalho foi aprovado pela Comissão Ética no Uso de Animais, da Faculdade de Medicina da Universidade Federal do Ceará, sob protocolo de N.º 25/15 (Anexo F) intitulado "Estudo dos Mecanismos e Mediadores Envolvidos na Patogênese da Osteonecrose dos Maxilares induzida por Bisfosfonatos em Modelo Experimental". Assim sendo, esta tese é composta de três capítulos contendo: um artigo científico previamente publicado, um aprovado para publicação e outro em processo de submissão em idioma inglês devidamente certificado, nos periódicos "**Oral Diseases**" (Anexo G), "**International Endodontic Journal**" (Anexo H) e "**Journal of Periodontology**" (Anexo I), respectivamente, conforme descrito abaixo:

Immune cellular profile of bisphosphonate-related osteonecrosis of the jaw. (Anexo G)
Barros Silva PG, de Oliveira CC, Brizeno L, Wong D, Lima Júnior R, Gonçalves RP, Sousa FB, Mota M, de Albuquerque Ribeiro R, Alves A. Oral Diseases. Status: Publicado. Doi: 10.1111/odi.12513.

Immune cell profile of dental pulp in rats treated with zoledronic acid. (Anexo H)
Barros Silva PG, Lima Verde MEQ, Brizeno L, Wong D, Lima Júnior R, Sousa FB, Mota M, Alves A. International Endodontic Journal. Status: Aprovado para Publicação na data 14 de março de 2016.

Chronic treatment of Zoledronic Acid increases inflammatory markers in periodontium of rats. (Anexo I)
Barros Silva PG, Ferreira Junior ACE, Oliveira CC, Brizeno L, Wong D, Lima Júnior R, Sousa FB, Mota M, Alves A. Journal of Periodontology Status: Processo de Submissão iniciado na data 11 de agosto de 2016.

3.1. Capítulo 01: Immune-cellular profile of bisphosphonate-related osteonecrosis of the jaw.

Tittle Page

Original Article

Immune-cellular profile of bisphosphonate-related osteonecrosis of the jaw

Running Head

Pathogenesis: Bisphosphonate-related osteonecrosis

Authors and name affiliations

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Abstract

OBJECTIVES: To characterize the cell-profile and immunostaining of proinflammatory markers in an experimental model of bisphosphonate-related osteonecrosis of the jaw (BRONJ).

MATERIALS AND METHODS: Male Wistar rats (n=6-7) were treated chronically with saline or Zoledronic Acid (ZA) at 0.04, 0.20, or 1.00 mg/kg (1.4×10^{-7}, 6.9×10^{-6} or 3.4×10^{-5} mol/kg) and the first left inferior molar was extracted. We performed counting of viable and empty osteocyte lacunae, viable and apoptotic osteoclasts, polymorphonuclear neutrophil, mast cells (toluidine blue) and of positive cells for CD68, Tumor Necrosis Factor alpha (TNF-α), IL(interleukin)-1β, inducible Nitric Oxide Synthase (iNOS), Nuclear Factor kappa B (NF-kB) and IL-18 binding protein (IL-18bp).

RESULTS: BRONJ was showed in ZA-treated with 0.20 and 1.00 mg/kg. There is an increase dose-dependently of percentage of empty osteocyte lacunae ($p<0.001$) and apoptotic osteoclasts ($p<0.001$), counting of total osteoclasts ($p=0.003$), polymorphonuclear neutrophil cells ($p=0.009$), cytoplasmic positive cells for CD68 ($p<0.001$), TNF-α ($p=0.001$), IL-1β ($p=0.001$), iNOS ($p<0.001$) and NF-kB ($p=0.006$), and nuclear positive cells for NF-kB ($p=0.011$). There is no difference in mast cells ($p=0.957$) and IL-18bp immunostaining decrease dose-dependently ($p=0.005$).

CONCLUSIONS: BRONJ is characterized by increases in immunostaining for proinflammatory markers and NF-kB, and inversely associated with cells exhibiting IL-18bp.

Keywords: Bisphosphonate-Associated Osteonecrosis of the Jaw, Tumor Necrosis Factor-alpha, Interleukin-1, Nitric Oxide Synthase Type II, NF-kappa B, IL-18 binding proteins.

Introduction

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a complicated adverse effect of treatment with bisphosphonates (BF), especially in patients undergoing oncology therapeutics. Some characteristics of this condition have been discovered and elucidated, but the pathogenesis is still uncertain (Allen and Burr, 2009).

The main studied mechanisms of BRONJ involve inhibitory effects of BF on bone turnover and angiogenesis. Although, these purported mechanisms *per se* do not adequately explain the pathogenesis of BRONJ (Allen and Burr, 2009). BF have anti-angiogenic and anti-vasculogenic effects *in vitro* and *in vivo* (Ziebart *et al*, 2011; Misso *et al*, 2012), and this could contribute to the deficient prognosis of BRONJ (Lescaille *et al*, 2014). However, experimental murine models are not consistent with an association between BRONJ and the inhibition of vascularization or vascular proliferation markers (Sonis *et al*, 2009; Aguirre *et al*, 2010; Kobayashi *et al*, 2010; Marino *et al*, 2012).

On the other hand, infection is strongly associated with BRONJ, and antimicrobial approaches are generally utilized by dentistry clinics in association with surgical debridement. Although BF increase the adhesion of bacteria to the hydroxyapatite bone (Ganguli *et al*, 2005; Kobayashi *et al*, 2010), the isolated use of antimicrobial agents without removal of necrotic bone does not promote healing of BRONJ (Hoefert and Eufinger, 2011). The participation of infection in the pathogenesis of BRONJ has not been observed in any experimental model addressing this parameter (Sonis *et al*, 2009; Kobayashi *et al*, 2010; Maahs *et al*, 2011; Conte Neto *et al*, 2013). Despite the use of antimicrobial agents shows conflicting efficacy in the modification of serum and the local microbial profile of patients with BRONJ (Ji *et al*, 2012), why does antibiotic prophylaxis seems to be an acceptable option to forewam BRONJ in patients treated with high doses of BF (Montefusco *et al*, 2008)?

Lopez-Jornet *et al* (2011) did not observe a relationship between BRONJ and infection, but the authors reported that infected animals had intense inflammatory infiltrated in exodontic locations, implying the development of BRONJ. Acute inflammation induces osteocyte apoptosis and inhibits the vitality and activity of osteoblasts (Gilbert *et al*, 2000; Cheung *et al*, 2011; Huang *et al*, 2011; Kawai *et al*, 2012). Proinflammatory cytokines also induce osteoclastogenesis (Assuma *et al*, 1998) and increase important modulators of the vitality of bone cells such as reactive oxygen species (ROS) (Halleen *et al*, 1999; Wimalawansa , 2010; Almeida and O'Brien, 2013).

Several experimental models (Sonis *et al*, 2009; Ali-Erdem *et al*, 2011; López-Jornet *et al*, 2011; Maahs *et al*, 2011; Marino *et al*, 2012) and clinical studies (Lesclous *et al*, 2009) have demonstrated associations between inflammation and BRONJ. BF increase production of acute inflammatory mediators in vitro (Muratsu *et al*, 2013) and in vivo (Norton *et al*, 2012), modify the immunological and cellular profile of patients undergoing chronic treatment (Rossini *et al*, 2012; Welton *et al*, 2013). Therefore, a consistent characterization of the immune and cellular profile of BRONJ has not been described (Vasconcelos *et al*, 2012).

Thus, the aim of this study was to conduct histomorphometric and immunohistochemical studies to characterize the cellular profile and immunoreactivity of certain chemical mediators.

Materials and Methods

Animals, doses and experimental protocols

Male Wistar rats (n=6-7) were treated (three consecutive weeks) with intravascular (penile access) saline or zoledronic acid (ZA, molar mass = 290.1) at 0.04, (1.4 x 10^{-7} mol/kg), 0.20 mg/kg (6.9 x 10^{-6} mol/kg) and 1.00 mg/kg (3.4 x 10^{-5} mol/kg) (days 00, 07, 14, respectively). Four weeks after the last infusion of ZA or saline (day 42), the first left inferior molar was extracted (using Hollemback 3s and Lecron Zalle spatulas). During the following week (day 49), an additional ZA dose was infused. The animals were euthanized on the 70th experimental day, and their hemimandibles were fixed in neutral formalin 10% (Silva *et al*, 2015).

The approval for experimental use of laboratory animals was obtained from the local Ethics Committee on Animal Use (CEUA, former CEPA) (protocol 26/2013) and is in compliance with the Federal Law No. 11794 of October 8, 2008, and the Decree nº 6,689, July 15, 2009 that regulated the law in 11,794, available from http: www.planalto.gov.brccivil03Ato2007- 20102008LeiL11794.htm.

Histomorphometric and histochemical analysis

After decalcification (ethylenediaminetetraacetic acid 10%, pH 7.3), microscopic slides (4 µm) were prepared (conventional hematoxylin eosin (HE) method) and qualitatively

and histomorphometrically analyzed. Afterward, ten microscopic fields (400x) were used for counting of viable and empty osteocyte lacunae, total osteoclasts, viable and apoptotic osteoclasts and polymorphonuclear neutrophil cells (Yamashita *et al*, 2011; Shaker *et al*, 2013).

Hydrated tissue sections (4 µm) were immersed in 0.1% toluidine blue solution (in 0.9% sodium chloride) per 60s for histochemical assessment (Gurgel *et al*, 2013) and mast cell counting using the same methodology (Shaker *et al*, 2013).

Immunohistochemical analysis

After deparaffinization and rehydratation, the tissue sections (2.5 µm) were also submitted to immunohistochemical assessment. Antigenic recovery was performed by heating in citrate pH 6.0 solution. After cooling, the slides were submitted to peroxidase blocking with H_2O_2 3% solution diluted in PBS (phosphate buffered saline) or with methanol solution according to the antibody's specificity (30 minutes).

After protein blocking (albumin) (1 h), we performed incubation with primary antibody CD68 (Dako®), tumor necrosis factor alpha (TNF-α) (Abcam®), IL (interleukin)-1β (Abcam®), inducible nitric oxide synthase (iNOS) (Abcam®), nuclear factor kappa B (NF-kB) (SantaCruz®) or IL-18 binding protein (IL-18bp) (SantaCruz®) using the dilutions and times shown in Table 1.

Envision System Plus-HRP (Dako®) (ready to use), Simple Stain Rat MAX PO (Multi) Universal Immuno-peroxidase Polymer (anti-mouse and -rabbit) (Histofine®) (ready to use) or secondary biotinylated antirabbit polyclonal IgG and ABC (avidin-biotin-peroxidase) System (SantaCruz Biotechnology®) (in diluted solutions) were utilized for the secondary antibody incubation. The visualization system was 5,5-diaminobenzidine tetrahydrochloride (DAB) (Dako®) (Table 1).

Ten microscopic fields (400x) were used to count CD68 immunostaining mononuclear cells; cells exhibiting cytoplasmic positivity for TNF-α, IL-1β, iNOS, IL-18bp or NF-kB; and nuclear positivity for NF-kB in exodontic sites (Kim *et al*, 2012).

Statistical analysis

Kolmogorov-Smirnov Normality testing was performed, and we utilized analysis of variance (ANOVA) followed by Tukey's post-test for group comparisons. The data were expressed as the mean and standard error of the mean (Mean±SEM).

Results

ZA induced experimental BRONJ

Twenty-eight days after tooth extraction, the saline solution group showed viable osteocytes and rare osteoclasts at the site of the exodontia´s new bone (Figure 1A and 1E). The group treated with 0.04 mg/kg ZA presented delayed deposition of osteoid; however, the development of BRONJ was not observed, nor were there any inflammations signs or damaged osteoclasts (Figure 1B and 1F). The dose of 0.20 (Figure 1C and 1G) or 1.00 mg/kg (Figure 1D and 1H) ZA led to large bone sequesters associated with intense inflammatory infiltrate, osteoclasts with intracytoplasmic vacuolization, nuclear hyperchromatism, and necrotic bone with irregular peripheral resorption. No signs of healing or deposition of the mineralized bone matrix were observed.

Cellular profile of BRONJ

The groups treated with 0.20 (32.5±1.1%) or 1.00 (26.8±1.8%) mg/kg ZA showed a statistical increase in percentage of empty osteocyte lacunae compared with the saline group (6.4±1.0%) and 0.04 (14.2±3.1%) mg/kg ZA groups ($p<0.001$) (Figure 1).

The total osteoclast number was greater in these two groups. Groups treated with 0.20 (31.4±7.9) or 1.00 (39.0±3.5) mg/kg ZA had a higher osteoclast number than the saline group (6.3±1.3) ($p=0.003$). There was no significantly difference between the saline and 0.04 mg/Kg (20.5±3.2) groups. This augmentation was accompanied by the number of osteoclasts with apoptotic signs. Both groups with 0.20 (87.4±2.0%) and 1.00 (79.8±1.1%) mg/kg ZA showed a higher percentage of apoptotic osteoclasts than the saline (5.0±5.0%) or 0.04 mg/Kg (11.8±5.7%) group ($p<0.001$) (Figure 1).

Regarding the cellular inflammatory profile, an increase number of polymorphonuclear neutrophils was observed in the groups treated with 0.20 (213.3±122.4) or 1.00 mg/kg (255.0±45.0) ZA compared with the saline (0.0±0.0) and 0.04 mg/kg ZA groups

(9.5±8.3) (p=0.009). The number of mononuclear CD68 positive cells was significantly higher in the 0.20 (205.0±31.3) and 1.00 (240.7±11.3) mg/kg ZA groups than in the 0.04 mg/Kg ZA (86.5±18.7) or saline (66.7±5.8) group (p<0.001). There was no statistically significant difference in mast cell number among the groups. (Saline: 30.5±6.7; 0.04 mg/kg ZA: 30.3±7.0; 0.20 mg/kg ZA: 34.0±5.93; 1.00 mg/kg ZA: 29.8±3.7) (p=0.957) (Figure 1).

Immune inflammatory profile of BRONJ

There was a significant increase of cells exhibiting immunostaining for Th1 inflammatory markers (Figure 2).

The immunostaining for TNF-α was greater in the groups treated with 0.20 (3312.0±180.8) or 1.00 (2912.0±179.7) mg/kg ZA than in the saline group (1811.0±145.0) (p=0.001). However, there was no difference between the 0.04 (2325.0±141.8) mg/kg ZA and saline groups (Figure 2).

The number of positive cells for IL-1β was higher in the 0.20 (5386.0±459) and 1.00 (6359±512.4) mg/kg groups than the saline (2695.0±104.2) group (p=0.001). Nevertheless, a difference between the saline and 0.04 mg/Kg (3589.0±477.6) groups was not observed. Similarly, positive cells for iNOS were augmented in the 0.20 (5436.0±827.5) and 1.00 (6356.0±523.7) mg/kg ZA groups compared with the saline (933.3±396.1) or 0.04 ZA mg/Kg (1825.0±159.4) group (p<0.001) (Figure 2).

Cytoplasmic and nuclear positivity for NF-kB were higher in the groups with BRONJ. In the cytoplasm, the groups treated with 0.20 mg/Kg ZA (576.5±86.7) or 1.00 mg/kg ZA (597.7±135.6) showed elevated numbers of cells exhibiting positivity compared with the saline group (52.5± 45.5) (p=0.011). There was no difference in immunostaining for cytoplasmic NF-kB between the saline and 0.04 mg/Kg ZA (157.0±77.7) groups. Nuclear staining for NF-kB was significantly increased in the groups treated with 0.20 mg/kg ZA (84.3±9.9) or 1.00 mg/Kg ZA (285.7±115.4) compared with the saline group (8.7±4.3) (p=0.006). There was no significant difference between the saline group and the 0.04 mg/Kg (19.7±7.1) or 0.20 mg/Kg ZA (84.3±9.9) groups (Figure 2).

Immunostaining for IL-18BP was increased in the group treated with 0.04 mg/kg ZA (418.6±119.1) compared with the saline group (12.5±6.6). A difference between the groups with 0.04 mg/kg ZA and 0.20 mg/kg ZA (203.3±93.5) was not observed. Nevertheless, there was a significant decrease in positive cells for IL-18bp in the group

treated with 1.00 mg/kg ZA (105.8±31.7) compared with the 0.04 mg/kg ZA (418.6±119.1) group (p=0.005) (Figure 2).

Discussion

The participation of inflammation in BRONJ has been described since the first reports and research on the disease. However, the focus of the studies has been centered on the role of bacterial infection (Hinson *et al*, 2014). Although infection is importantly associated with BRONJ, antimicrobial therapy has a low level of success compared with the surgical approach in the treatment of this condition (Rupel *et al*, 2014).

More than 81.6% of cases of BRONJ are associated with inflammation. This is a value discreetly higher than the association between BRONJ and infection (80.3%) (Hinson *et al*, 2014). In experimental models with rats, BRONJ was not associated with infection but with intense inflammation (López-Jornet *et al*, 2011). Thus, immune dysregulation may play a key role in BRONJ pathogenesis.

BRONJ is directly associated to proinflammatory condition. This is a dose-dependent pathology associated with leukocytosis and changes of erythrocytes (Silva *et al*, 2015). It is demonstrated that low doses (0.0225 mg/kg) of ZA not induce BRONJ (Sonis *et al*, 2009) despite proinflammatory stimulus is added (Marino *et al*, 2012). *In vivo* (classical peritonitis experiment), amino-bisphosphonates acutely upregulate proinflammatory cytokine production mediated by mast cells and the IL-1 receptor (IL-1R) (Norton *et al*, 2012). This cytokine, IL-1, is directly associated with BRONJ in the salival and gingival fluid of patients (Bagan *et al*, 2013; Tsao *et al*, 2013) such as oxidative stress markers (Bagan *et al*, 2014).

In this experimental model, mast cells were not augmented in number (Figure 3). In bone, these cells can release proinflammatory cytokines by degranulation, but they are not necessarily increased in number (Huang *et al*, 2013). On other hand, there were increases of some acute inflammatory markers such as TNF-α, IL-1β, iNOS, NF-kB activation (Figure 2) and numbers of neutrophils and macrophages (positive CD68 cells) but not of mast cells. ZA is a strong myelotoxic drug (Kuiper *et al*, 2012), but it increases cytokine production by macrophages through NF-kB activation (Muratsu *et al*, 2013).

TNF-α (Cheung *et al*, 2011), IL-1β (Bonewald 2004; Bakker *et al*, 2009) and high oxidative stress from iNOS activity (Almeida and O'Brien, 2013) modulate the increase of osteocyte apoptosis. The formation of apoptotic bodies in osteocytes is a stimulus for the

recruitment and activation of macrophages and osteoclasts (Kogianni, Mann and Noble, 2008), which are important scavenger cells involved in recognition of these apoptotic bodies for phagocytosis and empty bone resorption (Oizumi *et al*, 2009; Harre *et al*, 2012).

In this study, the increase in empty osteocyte lacunae was accompanied by increases of macrophages, osteoclasts, TNF-α, IL-1β and iNOS positive cells, supporting these previous studies. However, as ZA strongly induces osteoclast apoptosis, interrupting physiological bone resorption (Oizumi *et al*, 2009), there was also an increase of apoptotic osteoclasts.

ZA and other BF (alendronate) inhibit TRAP function and infiltration of CD68-positive cells in bone (Xiong *et al*, 2010; Yamashita *et al*, 2011). However, BRONJ is a chronic condition strongly associated with tooth extraction. After a tooth is extracted, increases TNF-α expression, osteoclastogenesis, bone resorption and bone matrix deposition occur with a reduction of proinflammatory cytokines to basal levels after ten days (Kim *et al*, 2012). Nevertheless, the osteoclast apoptosis induced by ZA adds proinflammatory effects in connective tissue cells and seems to perpetuate and upregulate these proinflammatory mediators, leading to deregulated osteocyte apoptosis (Bonewald, 2004; Bakker *et al*, 2009; Cheung *et al*, 2011; Almeida and O'Brien, 2013), macrophage infiltration and activation (Kogianni *et a*l, 2008; Harre *et al*, 2012) and more cytokine production (Muratsu *et al*, 2013).

Conversely, IL-18bp shows an inverse association with the dose-dependent proinflammatory effects of ZA in bone of rats submitted to tooth extraction (Figure 2). IL-18bp inhibits IL-8, a chemokine involved in the migration of neutrophils (Novick *et al*, 1999), which is a cellular group that can increase cytokine levels and cause damage to some tissues when it is augmented in number (Lima-Júnior *et al*, 2012).

IL-18bp has anti-neutrophil effects and modulates the immune response. This mediator is a member of a binding protein family with anti-inflammatory and anti-osteoclastogenic effects, such as OPG. IL-18bp prevents binding to the IL-18R receptor (31% similarity with IL-1R (Nakanishi *et al*, 2011), a receptor directly involved in BRONJ patients (Bagan *et al*, 2013) that mediates the immune transition of the Th1/Th2 response (Novick *et al*, 1999). In Th2 responses, osteoclastogenesis and bone resorption are inhibited (Horwood *et al*, 2001; Yamada *et al*, 2002; Morita *et al*, 2010). This process can attenuate bone diseases such as periodontitis (Orozco *et al*, 2007) and decrease inflammatory mediators and high oxidative stress (Shaker *et al*, 2013).

IL-18bp is produced by plasma cells and B lymphocytes. It inhibits NF-kB activity (Novick *et al*, 1999) by interrupting the interaction between IL-18 and IL-18R. Afterward, there is a breakdown activation cascade of MyD88, IRAK1 and IRAK2, TRAF6, NIK, and IKK and a dissociation of IkB and NF-kB in the cytoplasm (Nakanishi *et al*, 2001). This is the major mechanism of ZA-induced proinflammatory cytokine production in macrophages (Muratsu *et al*, 2013).

There are no experimental models demonstrating an association between IL-18 and IL-18bp in BRONJ. However, the late mediators that modulate the immune response seem to participate in BRONJ pathogenesis, such as overexpression of IL-17 (Li *et al*, 2013). IL-17 is a proinflammatory cytokine related to increases of some others mediators such as IL-1β, TNF-α and iNOS produced by macrophages and other connective tissue cells (Lee, 2013). Other experimental models showed no alterations in inflammatory bone markers (RANK/RANKL/OPG axis) in animals treated with ZA (Vasconcelos *et al*, 2012). Thus, ZA seems modify most strongly general inflammatory markers (TNF-α, IL-1β, IL-6 (Muratsu *et al*, 2013) and IL-17 (Li et al, 2013)) than inflammatory bone markers. Accordingly, the role of inflammatory cells and their general mediators seems to constitute a possible direction for understanding the pathogenesis of BRONJ.

In conclusion, BRONJ is characterized by increases in empty osteocyte lacunae; osteoclast numbers (total and apoptotic); polymorphonuclear neutrophils; CD68 mononuclear positive cells; immunostaining for TNF-α, IL-1β, iNOS, and NF-kB (nuclear and cytoplasmic) but not mast cells. BRONJ is also inversely associated with cells exhibiting IL-18bp. More studies are needed to show that BRONJ is dependent on immune modulation.

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Table 1 Parameters and Methodologies of Immunohistochemistry evaluation

Antibody	Production	Antigenic recuperation	Peroxidase blocking	Primary antibody		Secondary antibody		Revelatin	Cell Count
		Methodology (solution)	H_2O_2 concentration (solution dilution)	Dilution	Time of incubation	System and/or dilution (Production)	Time of incubation	System (time)	
CD68	Dako®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:500	Overnight	Envision System Plus-HRP (Dako®)	30’	DAB (5’)	Mononuclear positive cells
TNF-α	Abcam®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:50	1 hour	Universal Immuno-peroxidase Polymer (Histofine®)	30’	DAB (5’)	Positive cells
IL-1β	Abcam®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:100	1 hour	Universal Immuno-peroxidase Polymer (Histofine®)	30’	DAB (5’)	Positive cells
iNOS	Abcam®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:200	Overnight	Universal Immuno-peroxidase Polymer (Histofine®)	30’	DAB (5’)	Positive cells
NF-kB	SantaCruz®	Heat (Citrate pH 6.0)	H_2O_2 3% (Methanol)	1:200	Overnight	Secondary biotinylated antirabbit IgG 1:500 + ABC System (SantaCruz®)	30’ + 30’	DAB (10’’)	Positive cells in cytoplasm and nucleus
IL-18bp	SantaCruz®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:100	Overnight	Secondary biotinylated antirabbit IgG 1:200 + ABC System (SantaCruz®)	30’ + 30’	DAB (45’’)	Positive cells

TNF-α = Tumor Necrosis factor-alpha; IL-1β = Interleukin-1beta; iNOS = induced Nitric Oxide Synthase; NF-kB = Nuclear Factor-kappa B; IL-18bp = Interleukin-18 binding protein; HRP = Horseradish Peroxidase; ABC = Avidin Biotin Complex; DAB = 5,5-diaminobenzidine tetrahydrochloride.

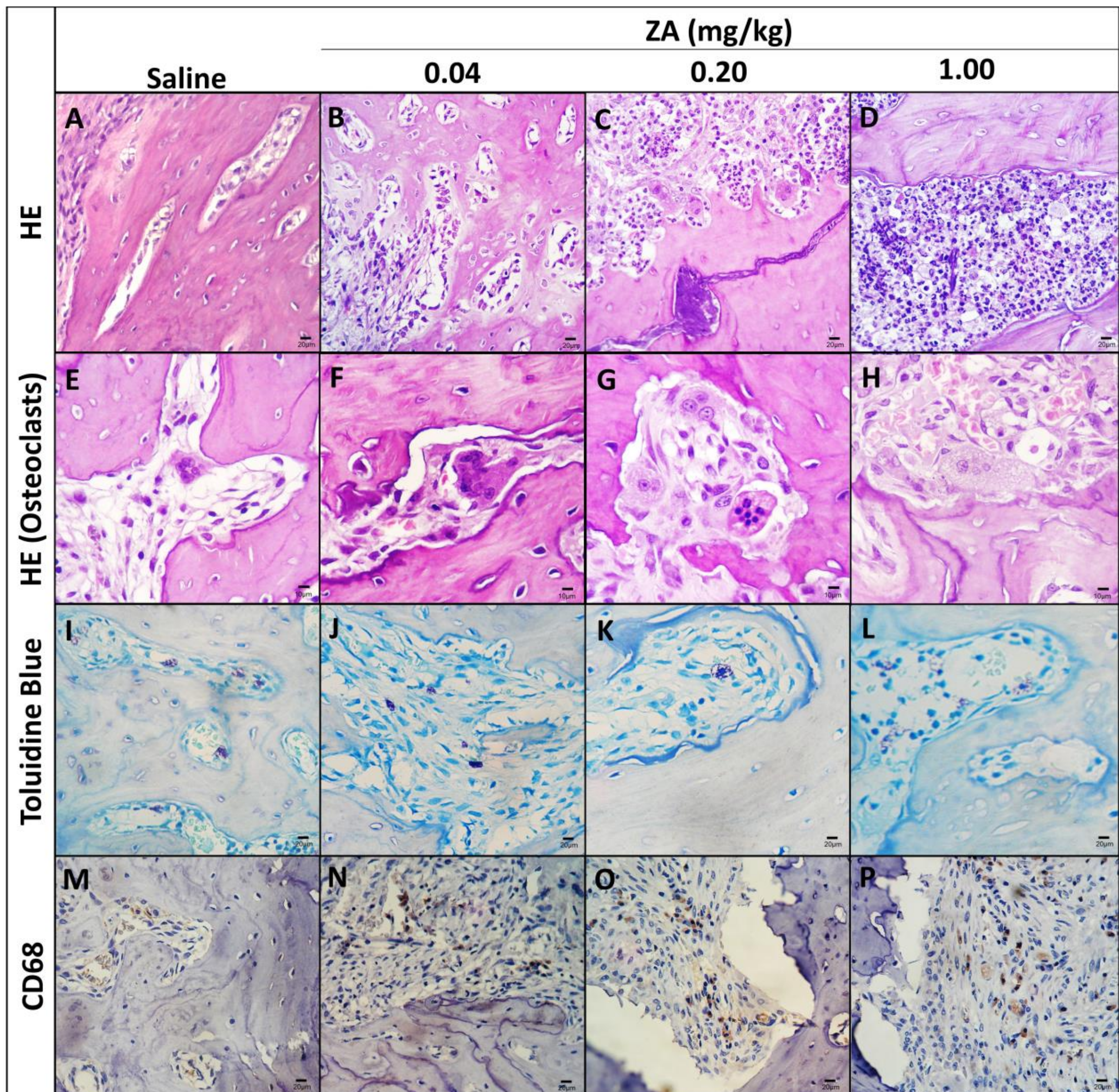

Figure 1 Cellular profile of BRONJ showing reduction in bone formation and viable osteocytes (A-D), increased number of osteoclasts (E-H) with apoptotic signs, no alteration in mast cells (I-L) and increased number of mononuclear CD68 positive cells (M-P) dose dependently (400x).

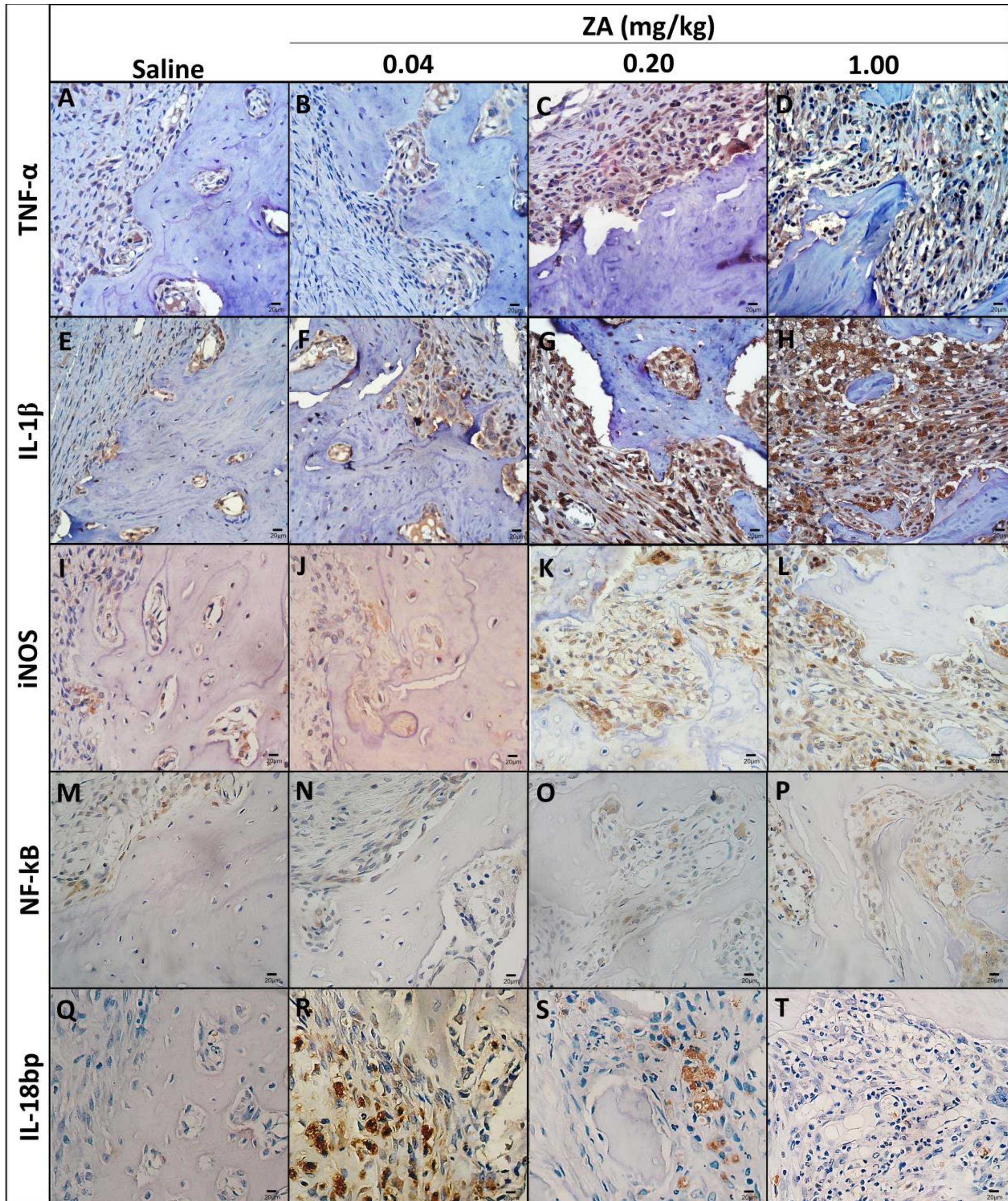

Figure 2 Cytokine profile showing dose-dependent increases in the number of TNF-α (A-D), IL-1β (E-H), iNOS (I-L) and NF-kB (nucleus and cytoplasm) (M-P) positive cells. An increase in the number of IL-18bp positive cells was seen in the 0.04 mg/kg ZA group in comparison with the saline group, and a significant reduction was seen with a higher ZA dose (Q-T) (400x).

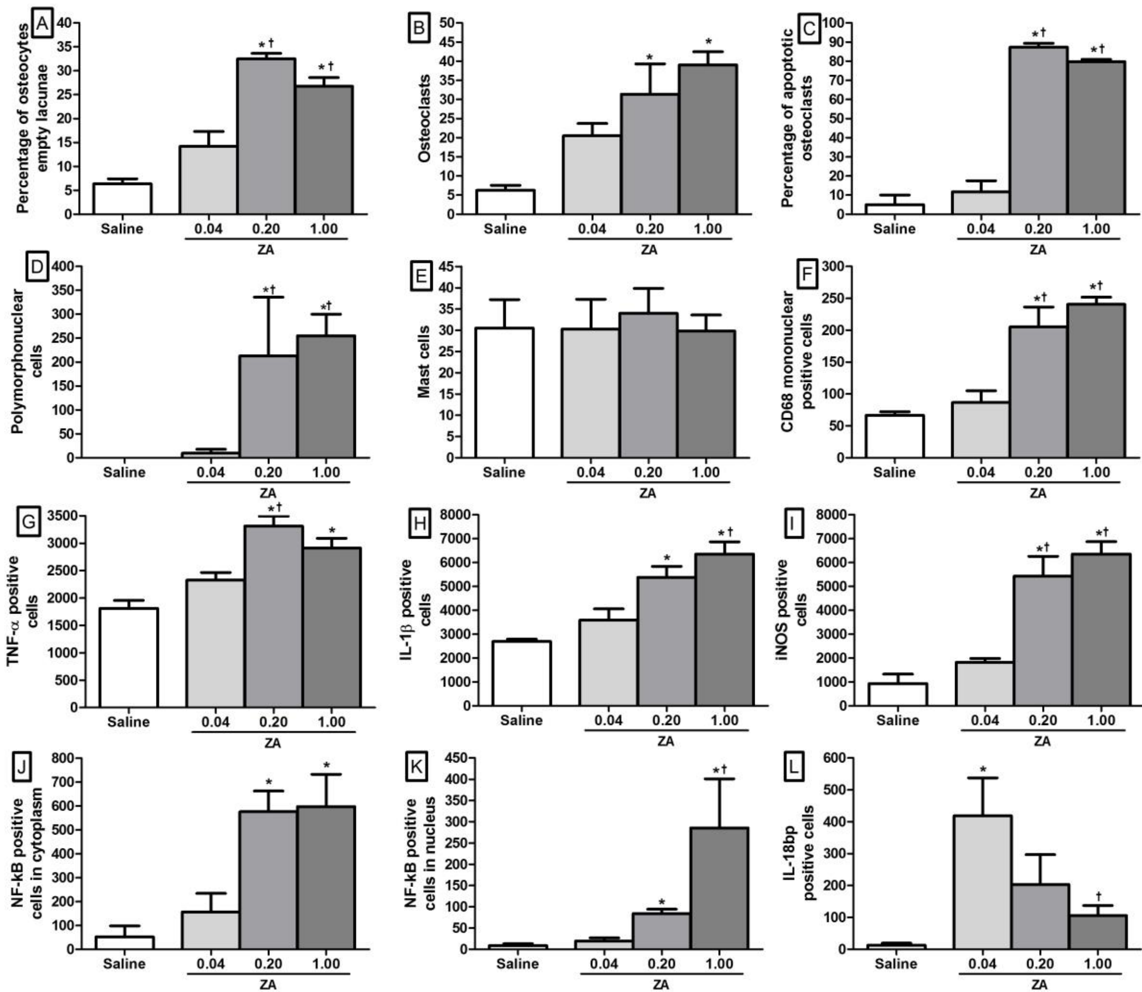

Figure 3 Cellular and cytokine profile analyses. *$p<0.05$ versus saline, †$p<0.05$ versus 0.04 mg/kg ZA (ANOVA test followed by Tukey's post-test; Mean±SEM).

3.2. Capítulo 02: Immune cell profile of dental pulp in rats treated with zoledronic acid.

Tittle Page

Tittle

Immune cell profile of dental pulp in rats treated with zoledronic acid

Running Head

Zoledronic acid and pulp inflammation markers

Authors

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Key Words

Zoledronic acid, Dental Pulp, Inflammation

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Abstract

Aim To characterize the pulp immune cell profile in the teeth of rats treated with Zoledronic Acid (ZA).
Methodology Male Wistar rats (n=6 per group) received four intravenous infusions of ZA at doses of 0.04, 0.20 or 1.00 mg/kg ZA or saline (control). On the 70th experimental day, they were euthanized. The first right molar was examined microscopically and submitted to toluidine blue reaction and immunohistochemical for CD68, Tumor Necrosis Factor (TNF)-α, Interleukin(IL)-1β, Inducible Nitric Oxide Synthase (iNOS), Nuclear Factor kappa B (NF-kB) and IL-18 binding protein (IL-18bp). The presence of ectasic/dilated vessels and inflammatory cells was analyzed and mast cells and mononuclear CD68 positive cells were counted along with the intensity of immunostaining (0 to 3) for inflammatory markers in odontoblasts and non-odontoblasts pulp cells. The Kruskall-Wallis/Dunn's test (scores or quantitative data) and the chi-square test (categorical data) (GraphPad Prism 5.0, $p < 0.05$).
Results There was no differences in number of animals exhibiting dilated/ectasic blood vessels (p=0.242), inflammatory cells (p=0.489) or in the number of mast cells (p=1.000). However, there was an increase in mononuclear CD68 positive cells (p=0.026), immunostaining of TNF-α (p=0.020), IL-1β (p=0.027), iNOS (p=0.001) in odontoblasts and IL-1β (p=0.013) in non odontoblast pulp cells dose-dependently. NFkB (nucleus and cytoplasm) and IL-18bp did not differ between groups. **Conclusion** ZA modified the immune cell profile in the dental pulp, increasing the number of macrophages and expression of pro-inflammatory markers independent of NFkB.

Keywords: Zoledronic acid, Dental Pulp, Acute-phase reaction, Inflammation

Introduction

Zoledronic acid (ZA) is a third-generation amino bisphosphonate. It is an analog of endogenous pyrophosphate and has an antiresorptive power more than 1000 times greater than that of etidronate, the first bisphosphonate used (Oizumi *et al.* 2009). Due to its potency, high-dose ZA is used for the treatment of diseases such as metastatic cancers of the bone (Silverman and Landesberg 2009). However, this drug has considerable toxicity and is associated with bisphosphonate-related osteonecrosis of the jaws (BRONJ).

ZA is directly toxic to several groups of cells, such as epithelial cells, fibroblasts (Scheper *et al.* 2009), osteoblasts (Naidu *et al.* 2006), macrophages (Scheller *et al.* 2011), neutrophils (Kuiper *et al.* 2012), and endothelial cells (Misso *et al.* 2012), and also impairs the maturation of myeloid cells (Wolf *et al.* 2006). Nevertheless, little is known about its effect on dental pulp cells.

ZA, like sodium alendronate (Hiraga *et al.* 2010), can lead to dental teratogenicity, and it alters molar eruption and tooth matrix formation, stimulates odontoclastic resorption and induces denticle and odontoma formation (Massa *et al.* 2006). At low concentrations, ZA increases collagen type I expression. At high concentrations, it affects phosphatase alkaline synthesis and alters the cellular morphology of odontoblasts (Basso *et al.* 2013). ZA also time-dependently reduces cellular viability, proliferation, and protein synthesis in pulp cells (Cvikl *et al.* 2011).

Tooth physiology involve several events that depend on complex interactions between inflammatory cytokine and protein levels (Rakian *et al.* 2013). In classical experimental models, amino bisphosphonates increase levels of TNF-α and IL-1β (Norton *et al.* 2013), which are important cytokines the pathogenesis of pulpitis (ElSalhy *et al.* 2013).

Pulpitis is greatly influenced by variations in cytokine levels. Increases in proinflammatory cytokines (Pezelj-ribaric *et al.* 2002) are related to the development of pulpitis and necrosis (Huang *et al.* 1999, ElSalhy *et al.* 2013). This is particularly important when there is a systemic proinflammatory stimulus, such as pharmacological treatments or dental caries (Zadik *et al.* 2010).

Odontoblasts and non-odontoblast cells can respond to caries (Horst *et al.* 2011) by increasing production of IL-8, TNF (Veerayutthwilai *et al.* 2007) and matrix

metalloproteinase (MMP) (Accorsi-Mendonça *et al.* 2013). When this process is added to ZA infusion (Cvikl *et al.* 2011), there may be further increases in cytokine production, culminating in pulpitis.

Caution is suggested during the endodontic treatment of patients prescribed bisphosphonates due to the risk of BRONJ (Moinzadeh *et al.* 2013). Endodontic treatment elevates the risk of BRONJ 5.5-fold (Barasch *et al.* 2011), and there are some case reports of BRONJ induced by pulp and periapical diseases (Katz *et al.* 2005, Wigler *et al.* 2013). The major risk factor for BRONJ is tooth extraction, but ZA infusion generates a paradox: ZA can elevate the risk of developing pulpitis, which requires endodontic treatment, which is a conservative approach in the prevention of BRONJ. However, endodontic treatment also increases the risk of BRONJ. There are no studies characterizing the immune cell profile in the dental pulp of rats treated with bisphosphonates.

In light of the role of ZA-dependent cytokine overproduction and ZA's ability to modulate the immune response *in vivo,* the objective of this study is to characterize the pulp immune cell profile in the teeth of rats treated with ZA, through a histological and immunohistochemical study.

Materials and Methods

Sample size calculation

Using the research of Cvikl *et al.* (2011), that showed a reduction in the rate of protein synthesis in dental pulp-derived cells treated with ZA 30µmol/mL by 24h (75.2±7.1%) or 48h (44.1±19.3%), a power of 90% and a confidence of 95% was adopted to define a sample of five animals (t test). This calculation was based in the hypothesis that ZA chronic infusion modifies the biology of the dental pulp. Due to the possibility of sample loss during the study a 20% increase in the number of animals was performed. So six animals in each group were used (n=6/group).

Animals, doses and experimental protocols

Rats (n=6/group) received three consecutive weekly intravascular (penile access) infusions of saline or 0.04, 0.20 or 1.00 mg/kg of ZA. These doses were calculated by

software Dose Calculator provided free by the Food and Drug Administration (http://www.accessdata.fda.gov). Body weight and surface area were the parameters used for pharmacological conversion of the human dose of ZA for the animals. The mensal dose (4mg) used to treat multiple myeloma was calculated to be 0.60 mg / kg for the Wistar rats, and divided into three weekly administrations of 0.20 mg / kg. Then, a dose response curve was calculated with three values: 0.20 mg / kg, five times greater (1.00 mg/kg) and five times less (0.04 mg/kg) (Silva *et al.* 2015).

The infusion were performed on days 00, 07, 14, on day 49, an additional dose was given, and three weeks later (day 70), the animals were sacrificed, and the hemi mandibles were fixed in 10% neutral buffered formalin (Ethics Protocol: 26/13).

After fixation (24 h), the hemi mandibles were decalcified (Ethylenediaminetetraacetic acid 10%, pH 7.3) for 30 days to prepare the tissue for microscopic slides.

Histological and histochemical assays

Microscopic slides (4 µm) were deparaffinized, dehydrated and cored by the conventional haematoxylin & eosin (H&E) method for histological analysis. Hydrated tissue sections (4 µm) were immersed in a 0.1% toluidine blue solution (in 0.9% sodium chloride) for 60 s for histochemical assays.

Immunohistochemical assay

After deparaffinization and rehydration, tissue sections (2.5 µm) were used in immunohistochemical assays. Antigenic recuperation was performed by heat in citrate solution (pH 6.0). After reaching room temperature, the slides were blocked in peroxidase with 3% H_2O_2 and diluted in PBS (phosphate buffered saline) or methanol solution (only for NF-kB) for 30 minutes.

After blocking with albumin for 1 h, the slides were incubated with the following primary antibodies: CD68 (Dako®, 1:500 overnight), Tumor Necrosis Factor (TNF)-α (Abcam®, 1:50 for 1 h), Interleukin (IL)-1β (Abcam®, 1:100 for 1 h), Inducible Nitric Oxide Synthase (iNOS) (Abcam®, 1:200 overnight), Nuclear Factor kappa B (NF-kB) (Santa Cruz®, 1:200 overnight) and IL-18 binding protein (IL-18bp) (Santa Cruz®, 1:100 overnight).

Universal Immune-peroxidase Polymer (Histofine®; for Dako® or Abcam® primary antibodies; 30 minutes) or Secondary biotinylated anti-rabbit IgG (for primary antibodies Santa Cruz®; 30 minutes) plus ABC System (Santa Cruz®; 30 minutes) were used. 5,5-diaminobenzidine tetra hydrochloride (DAB) was used to identify positive cells (Dako®).

Histological, histochemical and immunohistochemical analysis

The right mandibular first molar of each rat was analyzed by optic microscopy at 400x magnification (five microscopic fields per tooth). To characterize the cell profile of the pulp by H&E the presence of ectasic/dilated vessels and inflammatory cells was evaluated. The total CD68-positive mononuclear cells (IHC) and mast cells in this tooth were also counted.

To characterize the inflammatory profile of the pulp, the right mandibular first molar were evaluated, and the percentage of odontoblast and non-odontoblast cells (mesenchymal cells, such as fibroblasts and inflammatory cells) with cytoplasmic (and nuclear for NF-kB) expression of each antibody was characterized as (0) no positive cells; (1 - mild) 1-33% of positive cells; (2 - moderate) 34-66% of positive cells; (3 - intense) 67 - 100% positive cells. The final score was that agreed upon by two observers (kappa = 0.921) (Etemad-Moghadam *et al.* 2009).

Statistical analysis

Kruskall-Wallis and Dunn's post-test were used for scores (Median (Minimum-Maximum)) or mean (mean ± standard mean error) analysis; the chi-square test (absolute and percentage frequency of the animals) was used for categorical analysis in GraphPad Prism 5.0 software ($p < 0.05$).

Power Size Calculation

Based in the mean number of mononuclear CD68+ positive cells that were found in the dental pulp of the 1.00 mg/kg ZA-treated group (2.3±1.7) in relation to the saline group (0.0±0.0) and considering the sample of six animals per group (n=6), a power of 91.2% to reject the null hypothesis of this study was calculated (t test).

Results

Effect of ZA in the dental pulp

In the pulp of animals treated with saline or ZA (0.04, 0.20 or 1.00 mg/kg), there was no significant differences between the four groups. The number of animals exhibiting dilated and ectasic blood vessels (p=0.242) or inflammatory cells (p=0.489) was similar in all groups (Table 1, Figure 1).

The group given the highest dose of ZA (1.00 mg/kg) (2.3±1.7) had significantly more mononuclear CD68 positive cells than the saline group (0.0±0.0) (p=0.001) although there is no difference in the number of these cells in the groups treated with 0.04 mg/kg (0.7±0.3) or 0.20 mg/kg (0.5±0.4) of ZA versus saline. No teeth had mast cells in the pulp (p=1.000) (Table 1, Figure 1).

Effect of ZA in odontoblasts

The groups treated with ZA exhibited high levels of TNF-α expression in the cytoplasm of odontoblasts. The saline group had a median of 0 (0-1) TNF-α-positive cells, but the groups treated with 0.04 mg/kg (3, 2-3), 0.20 mg/kg (3, 2-3) or 1.00 mg/kg (3, 1-3) ZA exhibited a median of 3 TNF-α-positive cells, which was significantly higher than the saline group (p=0.020) (Table 1, Figure 2).

The number of IL-1β-positive odontoblastic cells did not differ between the saline (3, 2-3) or 0.04 mg/kg ZA groups (3, 2-3). However, the number of IL-1β-positive odontoblasts was significantly higher in the 0.20 mg/kg (3, 3-3) and 1.00 mg/kg (3, 3-3) ZA groups compared with the saline group (p=0.027) (Table 1, Figure 2).

iNOS immunoexpression was increased in all groups treated with ZA. The number of iNOS-positive odontoblasts in the saline group (0, 0-1) was significantly lower than in the 0.04 mg/kg (3, 2-3), 0.20 mg/kg (3, 3-3) and 1.00 mg/kg (3, 3-3) ZA-treated groups (p=0.001) (Table 1, Figure 2).

Odontoblasts showed no immunostaining for IL-18bp (p=0.572) or nuclear immunostaining for NF-kB (p=1.000). However, the odontoblasts in all groups had 100% cytoplasmic immunostaining for NF-kB (p=0.507) (Table 1, Figure 2).

Chronic treatment with ZA increases IL-1β in non-odontoblast pulp cells

In non-odontoblast pulp cells, the levels of immunostaining for TNF-α were equal for groups treated with saline (0, 0-2) and 0.04 mg/kg (1.5, 0-3), 0.20 mg/kg (1, 1-2) and 1.00 mg/kg ZA (2, 1-3) (p=0.162) (Table 1, Figure 2).

The IL-1β–positive non-odontoblast cells did not differ between the saline (1, 1-2) or 0.04 mg/kg (3, 2-3) or 0.20 mg/kg (3, 2-3) ZA-treated groups. However, the median number of these cells was significantly higher in the 1.00 mg/kg (3, 3-3) group (p=0.013) (Table 1, Figure 2).

There was no difference in iNOS in non-odontoblast pulp cells in the four groups (p=0.250), and none of these cells was positive for IL-18bp (p=1.000) or NF-kB (nucleus, p=1.000; cytoplasm, p=1.000) (Table 1, Figure 2).

Discussion

ZA is a toxic drug that is used at high doses for the treatment of bone metastases. ZA can deregulate the immune system and increase the number of inflammatory cells and levels of cytokines (Rossini *et al.* 2012a, 2012b, Norton *et al.* 2013, Welton *et al.* 2013).

Mast cells are poorly visualized in the dental pulp by histochemical methods, and their presence is therefore uncertain. The role of mast cells in pulp diseases is unclear (Bruno *et al.* 2010), but macrophages and dendritic cells, which are mononuclear and CD68-positive, are common cells that appear in the development and normal physiology of the pulp (Iwasaki *et al.* 2011). In this study, there were a significant number of macrophages in the pulp of rats treated with the highest dose of ZA (1.00 mg/kg). Macrophages have scavenger receptors (Harre *et al.* 2012) that recognize apoptotic cells, and they have an important role in defense against caries: macrophages accumulate in the pulp adjacent to caries-affected regions (Kamal *et al.* 1997) and phagocyte apoptotic cells (Nishikawa *et al.* 1997). These cells can serve as Antigen Presenting Cells (APC) and migrate to the apical region of teeth (Rungvechvuttivittaya *et al.* 1998), where they accumulate in response to local overexpression of MCP-1/CCR6 and generate apical granulomas (Liu *et al.* 2014).

An increase in TNF-α and IL-1β levels was observed. These cytokines are important constitutively expressed markers in the pulp, and they can activate the death domains in pulp cells, stimulating macrophage infiltration and activation (Ohazama *et al.*

2003, Paula-Silva *et al.* 2009). Therefore, these cytokines may play a role in the increased macrophage number in one of the ZA-treated groups.

There was no significant difference in iNOS expression in non-odontoblast pulp cells between treatment groups, but the odontoblasts of ZA-treated rats were associated with iNOS overexpression. iNOS is only present in inflamed pulps and not in healthy pulps (Di Nardo Di Maio *et al.* 2004). Odontoblasts are important cells that activate this enzyme in response to caries (Veerayutthwilai *et al.* 2007, Farges *et al.* 2015). The iNOS staining may reflect hyperaemia or a state of pulpitis (Di Nardo Di Maio *et al.* 2004, Veerayutthwilai *et al.* 2007, Farges *et al.* 2015). TNF-α and IL-1β (increased in this study) may be partially responsible for the modulation of iNOS activity (Bakker *et al.* 2009).

ZA did not cause direct toxicity to dental pulp cells. High concentrations of this drug are needed to cause this effect, but tissue damage increases with time (Cvikl *et al.* 2011).

In the present *in vivo* study the ZA dose was converted from human dose to rats. Cvikl *et al.* (2011) used *in vitro* empirical doses of 30 µmol/mL or 100 µmol/mL to identify the toxic dose dependent effect of ZA in dental pulp cells. So, the comparison of these two protocols is not possible due to the difficulty in demonstrating the real concentration of ZA in the pulp cells of rat's teeth.

The free (not incorporated) ZA is the most associated with ZA toxicity in dental pulp cells (Cvikl *et al.* 2011). In bone, the toxicity of bisphosphonates appears while osteoclasts demineralization releases the incorporated drug (Baron & Russel, 2011). The odontoblastic metalloproteinases in inflammatory conditions can explain partially this mechanism. The process of dentin matrix degradation and liberation of bisphosphonates by metalloproteinases can be similar in bone, although slower.

ZA infusion in peritonitis model leads to IL-1β overproduction (Norton *et al.* 2012). IL-1 is the most important cytokine described in gingival fluid and saliva of BRONJ (Tsao *et al.* 2013, Bagan *et al.* 2014). Furthermore, IL-1β is an important cytokine involved in pulpit (Veerayutthwilai *et al.* 2007) and it was altered in this study. IL-1β can strongly active odontoblastic metalloproteinases (Hiyama *et al.* 2013) and augment bisphosphonate liberation in dental pulps raising IL-1β overexpression (included in non-dental pulp cells as showed in this study) perpetuating this process.

Odontoblasts and non-odontoblast pulp cells respond by increasing pro-inflammatory cytokine synthesis, and caries can contribute to the rapid development of pulpitis in patients who use bisphosphonates (Horst *et al.* 2011).

ZA infusion chronically has been associated with increase TNF-α (Cheung *et al.* 2011), IL-1β (Bonewald 2004, Tan *et al.* 2008) and high oxidative stress by iNOS activity (Almeida *et al.* 2010). High levels of these mediators modulate excessive apoptosis and act as a stimulus for the recruitment and activation of macrophages (Kogianni *et al.* 2008, Muratsu *et al.* 2013). Thus, ZA may contribute to an increase in these proinflammatory markers.

TNF-α and IL-1β are produced by NF-κB activation, but there was no difference in NF-κB levels. NF-κB is constitutively expressed in odontoblasts and is important for the production of collagen type I and dentine sialoproteins. Carious stimuli can modulate the immune response in odontoblasts, leading to overexpression of TNF-α, IL-1β and CCL20 (binder of CCR6) (Veerayutthwilai *et al.* 2007), which participate in macrophage infiltration (Liu *et al.* 2014).

NF-κB activation is the primary driver of increased TNF-α, IL-1β and iNOS activity, but treatment with ZA did not alter NF-κB immunostaining. However, physiological expression of NF-κB in odontoblasts, accompanied by TNF-α and IL-1β overexpression, leads to pulp cell death (Hozhabri *et al.* 2015). In the pulp, there are complex ways to regulate these interactions, and other proteins can be involved in this process (ABCF1, FOS, IRF3, SP1, STA3, STAT1, FOXO, ERK1, TNFR and many others) (Horst *et al.* 2011). Cytokine production in dental pulp is increased in the presence of lipopolysaccharide (LPS), but a reduction in IκB-α activity, even in the presence of LPS, leads to an increase in TNF-α, IL-1β and IL-6 (Muratsu *et al.* 2013); thus, other mechanisms are responsible for this alteration in the dental pulp of rats treated with ZA.

ZA increased the expression of proinflammatory cytokines, independent from LPS. The addition of caries may further increase the production of these mediators, modifying the immune profile in the pulp and leading rapidly to an inflammatory disease with pulp damage and irreversible pulpitis and necrosis (ElSalhy *et al.* 2013).

Chemotherapies that alter the immune system can lead to odontalgia (Zadik *et al.* 2010) and it has been shown that high doses of ZA used chronically in chemotherapy protocols can modulate the immune response in dental pulps.

Conclusion

ZA modified the immune cell profile in the dental pulp, increasing the number of macrophages and expression of pro-inflammatory markers (TNF-α, IL-1β and iNOS)

independentely of NF-kB immunostaining. This is the first study *in vivo* showing this relationship. However, more studies are needed to investigate the ways that ZA can increase cytokine expression in the dental pulp.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Table 1 Histological, histochemical and immunohistochemical profile of molar dental pulp in rats treated with ZA or saline.

	Saline	ZA (mg/kg)			p-Value
		0.04	0.20	1.00	
Ectasic/Dilated blood vessels	6 (100.0%)	4 (66.7%)	5 (83.3%)	6 (100.0%)	0.242[a]
Inflammatory cells	3 (50.0%)	5 (83.3%)	2 (33.3%)	3 (50.0%)	0.489[a]
Mononuclear CD68+	0.0±0.0	0.7±0.3	0.5±0.4	2.3±1.7[†]	0.026[b]
Mast cells	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.000[b]
Odontoblasts					
TNF-α	0 (0-1)	3 (2-3)[†]	3 (2-3)[†]	3 (1-3)[†]	0.020[b]
IL-1β	3 (2-3)	3 (2-3)	3 (3-3)[†]	3 (3-3)[†]	0.027[b]
iNOS	0 (0-1)	3 (2-3)[†]	3 (3-3)[†]	3 (3-3)[†]	0.001[b]
IL-18bp	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-1)	0.572[b]
NF-kB					
Nucleus	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	1.000[b]
Cytoplasm	3 (2-3)	3 (3-3)	3 (3-3)	3 (3-3)	0.507[b]
Non-odontoblasts					
Dental pulp cells					
TNF-α	0 (0-2)	1.5 (0-3)	1 (1-2)	2 (1-3)	0.162[b]
IL-1β	2 (1-2)	3 (2-3)	3 (2-3)	3 (3-3)[†]	0.013[b]
iNOS	0 (0-1)	0.5 (0-1)	2 (1-3)	1 (1-2)	0.250[b]
IL-18bp	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	1.000[b]
NF-kB					
Nucleus	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	1.000[b]
Cytoplasm	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	1.000[b]

[a] Chi-square (data showed as absolute and percent frequency), *$p < 0.05$ versus Saline;

[b] Kruskall-Wallis/Dunn Test (Median (Minimum - Maximum). [†]$p < 0.05$ versus Saline.

Scores = (0) no positive cells; (1 - mild) 1-33% of positive cells; (2 - moderate) 34-66% of positive cells; (3 - intense) 67 - 100% positive cells.

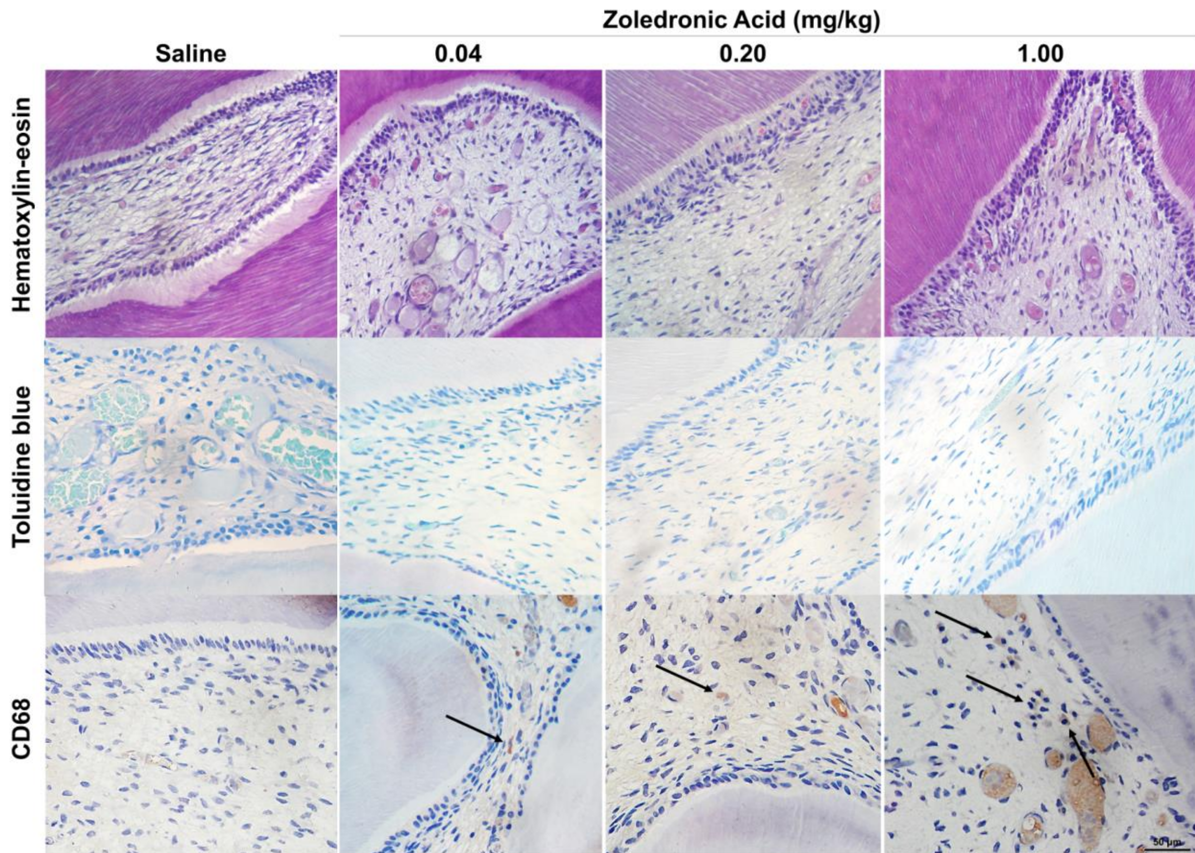

Figure 1 Cellular profile of rats dental pulp chronically treated with different doses of ZA shows no modification in histological profile or number of mast cells but an increase in the number of mononuclear CD68 positive cells in the group treated with 1.00 mg/kg ZA when compared with saline (400x).

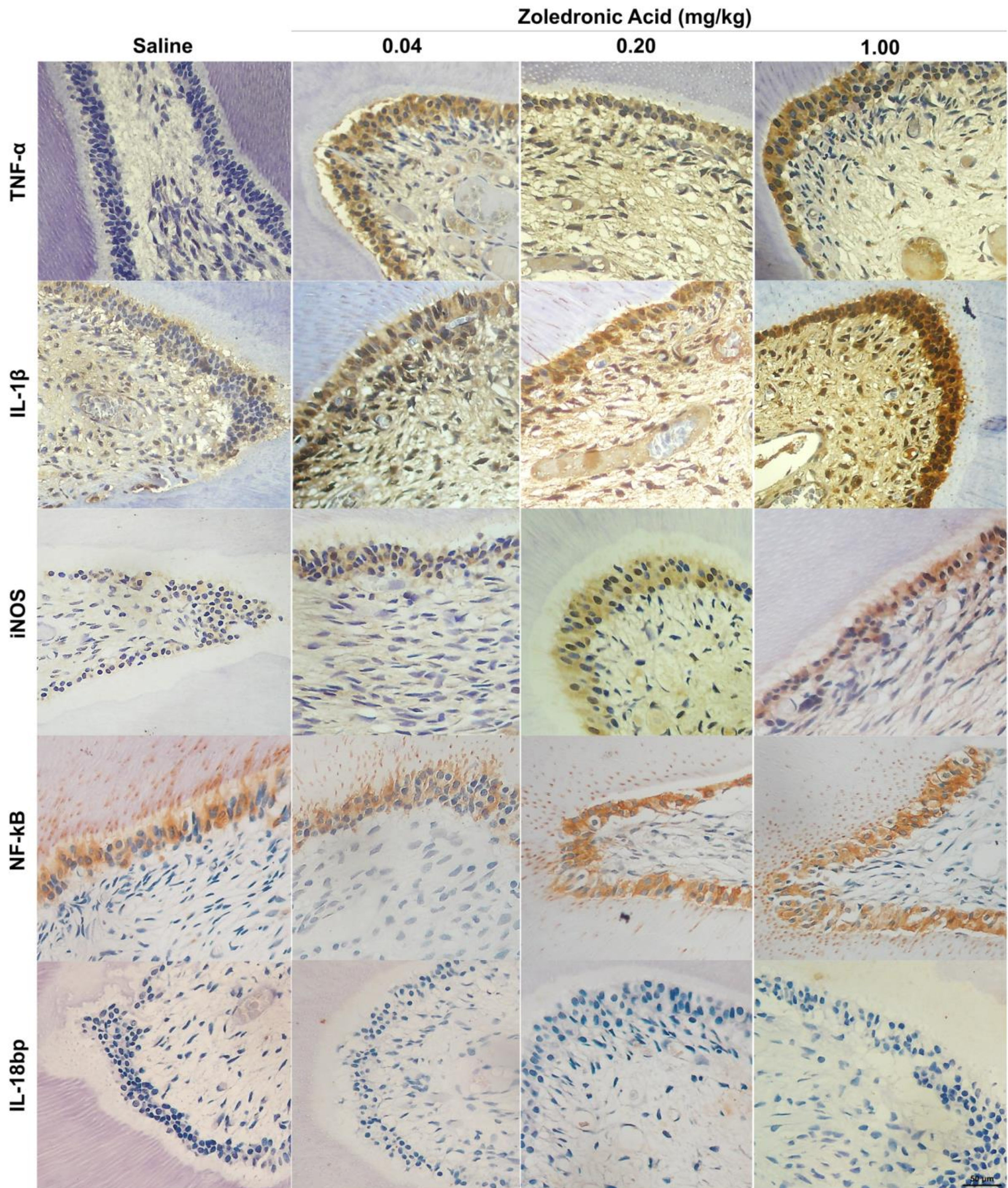

Figure 2 Cytokine profile shows an increase in the number of TNF-α, IL-1β and iNOS positive immunostained odontoblasts and non-odontoblast dental cells in ZA-treated groups, no changes in constitutive cytoplasmic NF-kB expression in odontoblasts and an absence of IL-18bp in all groups (400x).

3.3. Capítulo 03: Chronic treatment of Zoledronic Acid increases inflammatory markers in periodontium of rats

Tittle Page

Tittle

Chronic treatment of Zoledronic Acid increases inflammatory markers in periodontium of rats

Running Head

Zoledronic acid and periodontal inflammatory markers

Authors

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Key Words

Diphosphonates; Periodontium; Inflammation; Oxidative Stress; Glutathione.

Conflict of interest

The authors deny any conflict of interest

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Abstract

BACKGROUND: Bisphosphonates (BF) rises proinflammatory markers and irreversibly binds to bone. Chronically BF can lead to an inflammatory status and can increase the local oxidative stress in periodontium. Therefore, the objective of this study wasto evaluated if the chronic infusion of Zoledronic Acid (ZA) increases inflammatory markers in periodontium of rats.

METHODS: We infused chronically ZA (0.04, 0.2 or 1 mg/kg versus saline) in four doses in over a 70-day period to analyses of periodontium of first right inferior molar using histologic, histochemical (toluidine blue) and immunohistochemical (CD68, Tumor Necrosis Factor-α (TNF-α), Interleukin-1beta (IL-1β), Inducible Nitric Oxide Synthase (iNOS) and Nuclear Factor kappa B (NF-kB)) methods. The experiment was repeated (ZA 0.2 mg/kg versus saline) for myeloperoxidase (MPO) assay and TNF-α, IL-1β, Malondialdehyde (MDA) and Glutathione (GSH) dosages in gingiva of the same tooth.

RESULTS: Despite there is no alteration in mast cells ($p=0.608$) and CD68-mononuclear positive cells ($p=0.351$), in the periodontium of the ZA-treated group, we observed and increase presence of inflammatory cells ($p=0.001$) and cytoplasmic immunostaining for TNF-α ($p=0.003$), IL-1β ($p=0.004$), iNOS ($p=0.008$) and NF-kB ($p=0.025$). Levels of MPO ($p<0.001$), TNF-α ($p=0.002$), IL-1β ($p<0.001$) and GSH ($p=0.005$) were augmented in gingiva of ZA-treated group but MDA ($p=0.993$) levels and NF-kB nuclear staining ($p=0.923$) were not altered.

CONCLUSION: Chronic treatment with ZA increase proinflammatory cytokines and the number of inflammatory cells in periodontium of rats and GSH are expressed probably in a compensatory manner.

Keywords

Diphosphonates; Periodontium; Inflammation; Oxidative Stress; Glutathione.

Introduction

Bisphosphonates (BF) are potent antirreabsortive drugs and inhibit osteoclast differentiation and maturation. They have therapeutic potential for treatment of bone loss in experimental models of rats subjected to ligature (Menezes et al., 2005; Ödzemir et al., 2012; Said et al., 2012), but these drugs in high doses leads to bisphosphonate-related osteonecrosis of the jaw (BRONJ) (Silva et al., 2015).

BF are related to proinflammatory dysregulation in a dose dependent manner (Norton et al., 2012), and an increase in proinflammatory cytokines are directly associated with BRONJ (Barros Silva et al., 2016). Periodontal disease (PD) is strongly related with BRONJ (Tsao et al., 2015) and these two conditions have a common characteristic: both PD and BRONJ, have a proinflammatory characteristics (Lima et al., 2000; Barros Silva et al., 2016).

Chronic treatment with BF leads to cellular toxicity in various cell types (Scheper *et al*. 2009, Naidu *et al*. 2006, Scheller *et al*. 2011, Kuiper *et al*. 2012, Misso *et al*. 2012, Wolf *et al*. 2006), through an increase in the the synthesis of cytokines (Scheller *et al*. 2011). The BF bind to hydroxyapatite irreversibly (Oizumi et al., 2009) and the high time of contact between BF and BF-toxicity-susceptive cells increases the toxic effects of BF in these cells and increases the number of dead cells (Otto et al., 2010).

Furthermore, there is a reduction in the phagocytotic activity of macrophages that is induced by BF (Scheller *et al*. 2011), and the elimination of injured cells can be compromised and lead to high oxidative stress (Yu et al., 2011). Then, infiltration of neutrophils can occur leading to high oxidative stress and bone necrosis (Barros Silva et al., 2016).

It has been reported that periodontium has basal levels of proinflammatory markers (Almaghlouth et al., 2014), however chronic BF treatment augment cytokine levels in crevicular fluid (Tsao et al., 2012). Barros Silva et al. (2016) suggested the relationship between inflammation and BRONJ, so if is it true, why are not all patients treated with BF present BRONJ?

Mammalian have a complex antioxidant system responsible for homeostasis of free radicals (Huber et al., 2008). The reduction of oxidative stress attenuates the PD, reduces cytokine overproduction and inflammatory cells migration and decreases osteocyte loss (Menezes AM et al., 2012). However, the relationship between chronic BF and the expression of inflammatory and pro-oxidant markers in rats "not periodontally compromised" is not known.

Therefore, the objective of this study is to evaluate the changes in inflammatory markers and oxidative stress in healthy periodontal tissue of rats chronically treated with ZA.

Materials and Methods

Animals, doses and experimental protocols

We treated male Wistar rats (n=6/group) (180-200 g; 60 days old) with three weekly consecutive doses through the intravascular (penile access) infusion of saline and 0.04, 0.20 or 1.00 mg/kg of ZA (days 00, 07, 14). Then, on day 49, an additional dose was infused, and three weeks later (day 70) the animals were sacrificed. Their hemimandibles were fixed in neutral formalin 10% (Ethics Protocol: 26/13). After fixation (24 h), the hemimandibles were submitted to decalcification (ethylenediaminetetraacetic acid 10%, pH 7.3) for 30 days; histological slides were prepared (Barros Silva et al., 2015).

Histological and immunohistochemical analysis were performed, and the experimental protocol was repeated with the infusion of saline or 0.20 mg/kg ZA (n=6/group). We removed the gingiva of the animals to perform a myeloperoxidase (MPO) assay and to dose the tissue with tumor necrosis factor (TNF)-α, IL (interleukin)-1β, Malondialdehyde (MD) and glutathione (GSH).

Histological and histochemical reaction

Histological slides (4µm) were deparaffinized, dehydrated and stained by conventional haematoxylin eosin (H&E) technique or toluidine blue (0.1% for 60 s) (mast cells evaluation).

Immunohistochemical protocol

We performed an immunohistochemical assay for CD68, TNF-α, IL-1β, inducible nitric oxide synthase (iNOS) and nuclear factor kappa B (NF-kB). Antigenic recuperation was performed by heat (18 min) in a citrate solution with a pH of 6.0. The slides were submitted to peroxidase blocking with a 3% H_2O_2 solution diluted in PBS (phosphate buffered saline) or in a methanol (NF-kB) solution for 30 minutes. Subsequently, protein blocking was performed (albumin) (1 h). The fragments were incubated with primary antibodies CD68 (Dako®, 1:500, overnight), TNF-α (Abcam®, 1:50, 1 h), IL-1β (Abcam®, 1:100, 1 h), iNOS (Abcam®, 1:200, overnight) and NF-kB (SantaCruz®, 1:200, overnight).

Universal immune-peroxidase polymer (Histofine® for Dako® or Abcam® primary antibodies; 30 minutes) or secondary biotinylated anti-rabbit IgG (for primary antibodies Santa Cruz®; 30 minutes) plus ABC system (Santa Cruz®; 30 minutes) was used. The revelation system in all cases was 5,5-diaminobenzidine tetra hydrochloride (DAB) (Dako®).

Histological, histochemical and immunohistochemical analysis

The entire area of periodontium of the first right inferior molar of each rat was analyzed using optical microscopy at 400x magnification. To determine the inflammatory profile of the periodontium trough H&E, we evaluated dichotomically the presence of inflammatory cells, mast cells using toluidine blue and mononuclear CD68-positive cells using immunohistochemistry.

The inflammatory profile of the periodontium was evaluated using the following scores defined by the frequency of percentage of cells with cytoplasmic (for TNF-α, IL-1β, iNOS and NF-kB) and nuclear (for NF-kB) expression of each antibody: (0) no positive cells; (1 - mild) 1-33% of positive cells; (2 - moderate) 34-66% of positive cells; and (3 - intense) 67 - 100% positive cells (Brizeno et al., 2016). The final score was agreed upon by two observers (kappa = 0.892).

MPO assay

The gingiva of the lower first right molar of the rats (n=6/group) was homogenized in $NaPO_4$ (0.2 M, pH = 4.7) buffer and centrifuged (3,000 rpm for 15 minutes). The supernatant was separated to measure the cytokines, and 1 ml of lysing solution NaCl (0.2%) was added to the pellets. The samples were vortexed (30 s), and centrifuged (3,000 rpm for 15 minutes), and after discarding the supernatant, we added 250 µL of hexadecyltrimethylammonium bromide (HTAB) buffer. We homogenized the samples (30 s, 4ºC), centrifuged the suspension (10,000 rpm/15 minutes) and measured the change in absorbance at 450 nm using a reading solution (5 mg *O*-dianisidine, 15 µL of 1% H_2O_2, 3 ml phosphate buffer, 27 ml H_2O). The change in absorbance was recorded and plotted on a standard curve of neutrophil density with the obtained data expressed as myeloperoxidase activity (MPO/mg of tissue) (Lima Junior et al., 2011).

ELISA assay (TNF-α e IL-1β)

TNF-α and IL-1β concentrations were determined by ELISA. Microtiter plates were coated overnight at 4°C with the following mouse antibodies: anti-TNF-α and anti-IL-1β (Dako®, 1:1,000, (bovine serum albumin) BSA 1%). After washing (three times) and blocking the plates (BSA 1%, 2 h), the samples (obtained from the MPO assay supernatant) and standard at various dilutions were incubated at room temperature for 1 h. The plates were washed three times with buffer, and we added the polyclonal secondary antibody (Sigma®, 1:1,000, BSA 1%). After further incubation at room temperature for 1 h, the plates were washed, and 50 µl of avidin-HRP (Abcam®, 1:5,000) was added. The color reagent o-phenylenediamine (OPD; Biosystems®, 50 µl) was added 15 min later, and the plates were incubated in the dark at 37°C for 45 min for TNF-α and 20 min for IL-1β. The enzyme reaction was terminated with H_2SO_4, and the absorbance was measured at 490 nm. The results are expressed as pg/mg of tissue and are reported as the mean ± SE (Lima Junior et al., 2011).

Malondialdehyde assay (MDA)

The gingiva of first right inferior molar of rats (n=6/group) was homogenized in KCl (1.15%) buffer (100mg of tissue/1ml). Then, the samples were centrifuged (4,500 rpm, 15 minutes). We added 100µL of 7% sodium dodecyl sulfate (SDS) to Eppendorf tubes and

incubated the tubes in a water bath (37ºC, 30 min). Thiobarbituric acid 0.6% (200 μL) was added, and the samples were placed in a water bath at 100ºC for 50 min. After cooling in ice (5 min), we added 100 μL of n-butanol, vortexed (30 s) and centrifuged for 20 min (5.000 rpm, 4ºC).

The supernatant of samples (200 μL) and the standard were pipetted in microtiter plates, and the absorbance was measured at 535 nm. The results are expressed such as nmol/mg of tissue and reported such as mean ± SE (Martins et al., 2016).

Glutatione (GSH)

The gingiva of first right inferior molar of rats (n=6/group) was homogenized in EDTA 0.02M buffer (100mg of tissue/1ml). Then, we transferred 100 μL of the homogenate into different Eppendorf tubes, added 80 μL of H_2O and 20 μL of 50% trichloroacetic acid and vortexed (30 s).

The sample were centrifuged (3,000 rpm, 15 minutes), and 100 μL of supernatant was transferred to other Eppendorf tubes along with 100 μL of 0.4 M Tris-EDTA (pH=8.9) and 10μL of 0.01 M DTNB (acid 5',5'-dithio-bis(2-nitrobenzoic acid)) and was vortexed (40 s).

The mixture (200 μL) and the standard were pipetted into microtiter plates, and the absorbance was measured at 412 nm. The results are expressed as mg of GSH/g of tissue and are reported as the mean ± SE (Martins et al., 2016).

Statistical analysis

Statistical analyses were performed using the Kruskall-Wallis test/Dunn's test or Mann-Whitney test for scores (Median (Minimum-Maximum)) and dosage (mean ± standard mean error) analysis (non-parametric data) and the chi-square test (absolute and percentage frequency) for categorical analysis. All the tests were performed using the GraphPad Prism 5.0 software, and a significance level of 5% was used.

Results

ZA Chronic treatment increases the number of rats showing inflammatory cells in periodontium.

The groups treated with 0.20 mg/kg (n=5, 83.3%) and 1.00 mg/kg (n=6, 100.0%) ZA showed a significant number of rats with inflammatory cells in the periodontium (p=0.001) compared with saline (n=1, 16.7%) and the 0.04 mg/kg ZA (n=0, 0.0%) treated groups. There was no difference in animals showing mast cells (p=0.608) or mononuclear CD68 positive cells in periodontal ligament (p=0.351) (Table 1, Figure 1).

Chronic treatment with ZA augment the cytoplasm immunostaining for TNF-α, IL-1β, iNOS and NF-kB in periodontium of rats.

The groups treated with ZA exhibited high levels of cytoplasmatic TNF-α expression in periodontal cells. The median of TNF-α for the positive cells was 1 (0-2) in saline group, but the groups treated with 0.20 mg/kg (3, 2-3) or 1.00 mg/kg (3, 3-3) ZA exhibited a median of 3 for TNF-α-positive cells, which was significantly greater than in the saline group (p=0.003) (Table 1, Figure 2). ZA-treated group with 0.04 mg/kg (Median = 2, 1-3) did not differ from the saline group (Table 1, Figure 2).

All groups treated with ZA exhibited high levels of cytoplasmatic IL-1β expression in the periodontal cells (Median = 3, 3-3) which was significantly greater than in the saline group (Median = 2, 2-3) (p=0.004) (Table 1, Figure 2).

iNOS immunoexpression was increased in the 0.20 mg/kg and 1.00 mg/kg ZA groups (Median = 3, 3-3) when compared with the saline group (Median = 1, 1-2). The number of iNOS-positive periodontal cells in the 0.04 mg/kg ZA treated group (1.5, 1-2) did not differ from the saline group (Table 1, Figure 2).

There are no difference in the nuclear expression of NF-kB (p=0.923), but the cytoplasmatic positivity cells for NF-kB in the periodontium of rats treated with 0.20 mg/kg (Median = 1, 1-1) and 1.00 mg/kg (Median = 1, 0-2) was significantly greater compared with saline group, in which none of the animals showed immunostaining for this marker (p=0.025) (p=0.025) (Table 1, Figure 2).

Chronic treatment with ZA augment MPO activity and levels of TNF-α e IL-1β in gingiva of rats

The number of neutrophils (5.7±1.2 x10³ neutrophils/mg of tissue) and the TNF-α (281.4±25.6 pg/mg of tissue) and IL-1β (115.9±35.4 pg/mg of tissue) levels were significantly augmented in the gingiva of rats treated with 0.20 mg/kg of ZA compared with saline (MPO = 0.0±0.0 x10³ neutrophils/mg of tissue, $p<0.001$; TNF-α = 10.8±5.3 pg/mg of tissue, $p=0.002$; IL-1β = 3.4±1.7 pg/mf of tissue, $p<0.001$) (Figure 3).

Chronic treatment with ZA increase in GSH levels but not altered MDA levels

The MDA levels did not differ in rats treated with 0.20 mg/kg of ZA (MDA = 9.4±4.4x10³ nmol/mg of tissue) when compared with the saline group (MDA = 9.5±3.7x10³ nmol/mg of tissue, $p=0.993$). However, the GSH levels were increased in 0.20 mg/kg of ZA group (283.7±106.9 µg/g of tissue) compared with the saline group (1.5±1.4 µg/g of tissue) ($p=0.005$) (Figure 3).

Discussion

BF treatment leads some cellular types to die or interferes in their metabolism (Scheper *et al*. 2009, Naidu *et al*. 2006, Scheller *et al*. 2011, Kuiper *et al*. 2012, Misso *et al*. 2012, Wolf *et al*. 2006). The cellular toxicity increases in parallel to cytokines overexpression (Muratsu et al., 2013) and the fibroblasts of periodontium are not an exception (Tripton et al., 2011). Gingival tissue produced high levels of proinflammatory mediators in response to BF, as shown in this study.

The chronic infusion of ZA increases the number of inflammatory cells and TNF-α and IL-1β levels significantly. This alteration was observed in periodontal cells and in gingiva of the rats treated with ZA along with an augmentation of neutrophils. The MPO activity was also significantly enhanced in gingiva of ZA-treated groups likely because TNF-α and IL-1β are important in the chemotaxis of neutrophils (Graves and Jiang, 1995). The induction of some chemokines in a TNF-α- and IL-1β-dependent manner contributes to an augment of neutrophils and damage locally (Vasconcelos et al., 2016).

Neutrophils produce high levels of reactive oxygen species (ROS) that contribute to more inflammatory cell migration. Additionally, ZA impairs neutrophil function and reduces neutrophil survival in vitro (Kuiper et al., 2011). When these cells die (after

phagocytosis or after their short cell cycle), they liberate ROS, causing vasodilatation (Kuwabara et al., 2015).

However, the augmentation of proinflammatory markers was accompanied by an increase in GSH levels preventing an increase in MDA formation. Bagan et al. (2014) showed that cancer patients treated with intravascular BF have increased oxidized glutathione, but not GSH itself. Nevertheless, these patients have some malignances that increase the production of ROS *per se* (Liou and Storz, 2010).

Complex antioxidants agents maintain homeostasis of aerobic metabolism. Because the pro-oxidant agents did not exceed the antioxidant systems, there are compensatory mechanisms, such GSH (Halliwell, 2006).

BRONJ is a condition directly associated with proinflammatory (TNF-α, IL-1β, IL-17) markers and iNOS activity (Barros Silva et al., 2016; Li *et al*, 2013), and it appears that macrophages and the nuclear activity of NF-kB are necessary for the pathogenesis of this condition (Barros Silva et al., 2016; Muratsu et al., 2013). In this study, an increase in the nuclear expression of NF-kB and number of rats showing mononuclear CD68 positive cells were not shown in the "healthy periodontium", but we demonstrated the rise of GSH levels.

Overproduction of antioxidants, such GSH, repress cell apoptosis (Ran et al., 2004) and NF-kB activation (Post et al., 1998). So, the upregulated compensatory antioxidant system can protect the periodontal cells which are suffering damage (Yu et al., 2011) by ZA-induced cytokines (Muratsu et al., 2011). BF enhance proinflammatory cytokines (TNF-α and IL-1β) but these drugs increase the synthesis of important antioxidant molecules (Tripton et al., 2011).

Periodontal cells treated with BF produce more OPG (osteoprotegerin) (Tripton et al., 2011) that is directly associated with GSH protection activity (Romagnoli et al., 2013; Zhang et al., 2014). Additionally, osteoblasts treated with risendronate increase other antioxidant proteins as Hsp90 (Rommanelo et al., 2006). Hsp90 is an important antioxidant molecule that decrease ROS formation and peroxidation (Kim et al., 2015). In eukaryotic cells Hsp90 is linked to GSH and it is necessary the cleavage of two proteins to release GSH (Nemoto et al., 1995).

Nevertheless, the metabolism of ROS in bone is complex, occasionally paradoxical and need many studies to comprise the rule of oxidative stress ZA-mediated in pathogenesis of cellular damage ROS-induced (Wimalawansa, 2010).

Thus, we showed that chronic infusion of ZA in rats can lead to inflammatory alteration with an increase in proinflammatory markers in gingiva and periodontium. Additionally, we found a significant increase in compensatory GSH levels in gingiva of ZA treated groups. These data contribute to the hypothesis that the ZA can lead to immune inflammatory disruption and to facilitate the installation of BRONJ.

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Table 1: Histological and immunohistochemical aspects of the periodontium of animals treated with different doses of Zoledronic Acid.

		ZA			
	Saline	**0.04**	**0.20**	**1.00**	**p-Value**
Inflammatory cells	1 (16.7%)	0 (0.0%)	5 (83.3%)*	6 (100.0%)*	0.001[a]
Mast cells	2 (50.0%)	4 (66.7%)	5 (83.7%)	3 (50.0%)	0.608[a]
Mononuclear CD68+	0 (0.0%)	2 (33.3%)	3 (50.0%)	3 (50.0%)	0.351[a]
TNF-α	1 (0-2)	2 (1-3)	3 (2-3)†	3 (3-3)†	0.003[b]
IL-1β	2 (2-3)	3 (3-3)†	3 (3-3)†	3 (3-3)†	0.004[b]
iNOS	1 (1-2)	1.5 (1-2)	3 (3-3)†	3 (3-3)†	0.008[b]
NF-kB					
Nucleus	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0.923[b]
Cytoplasm	0 (0-0)	0.5 (0-1)	1 (1-1)†	1 (0-2)†	0.025[b]

[a] Chi-square test (absolut and percentual frequence), *p < 0.05 versus Saline and 0.04 mg/kg ZA groups;

[b] Kruskall-Wallis/Dunn test (Median (minimum - maximum)). †p < 0.05 versus Saline. Scores = (0) no positive cells; (1 - mild) 1-33% of positive cells; (2 - moderate) 34-66% of positive cells; (3 - intense) 67 - 100% positive cells (Brizeno et al., 2016).

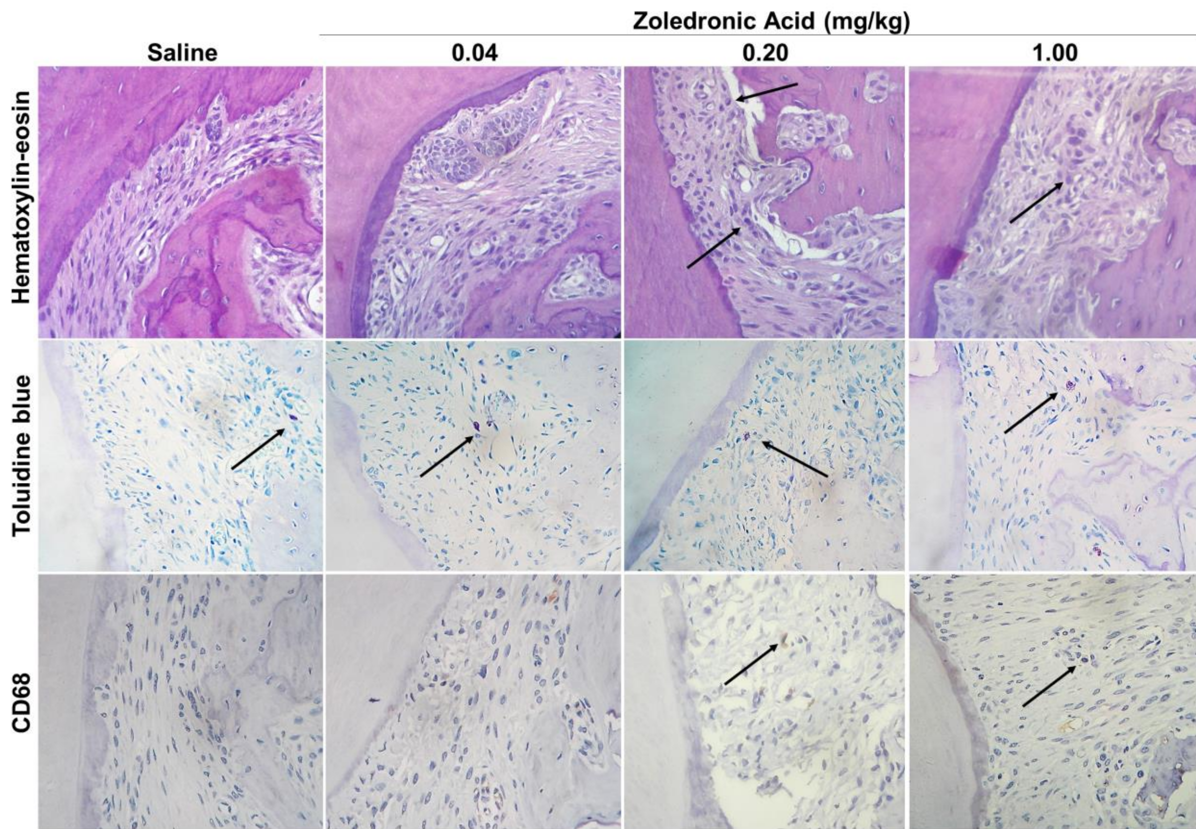

Figure 1: Periodontium of rats treated with ZA chronically showing increase of the number of animals with inflammatory cells, however without augment of mast cells or CD68 positive cells.

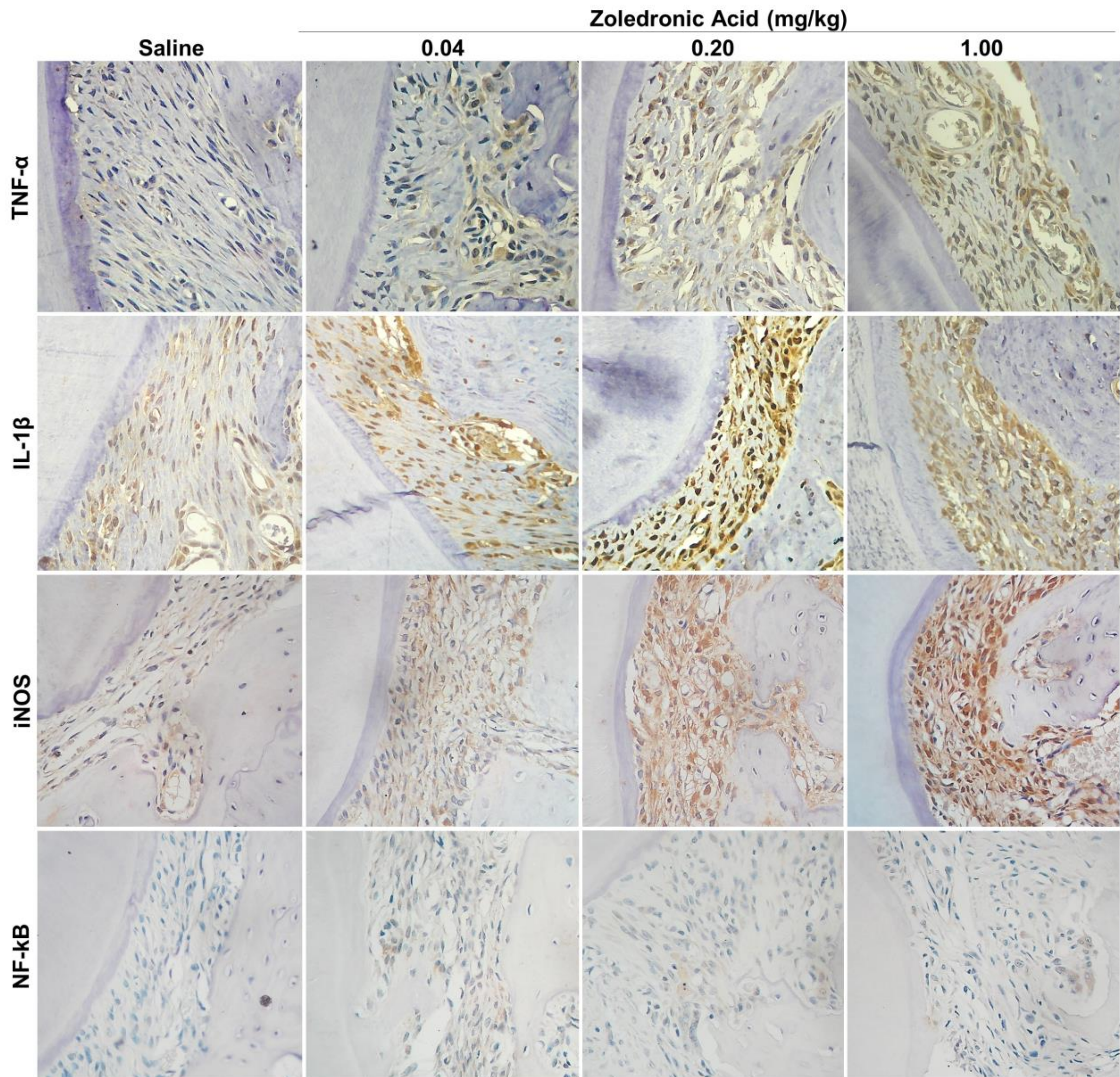

Figure 2: Inflammatory profile of rats periodontium treated with ZA chronically showing increase of cytoplasmic expression of TNF-α, IL-1β, iNOS and NF-kB. No augment of nuclear NF-kB staining was observed.

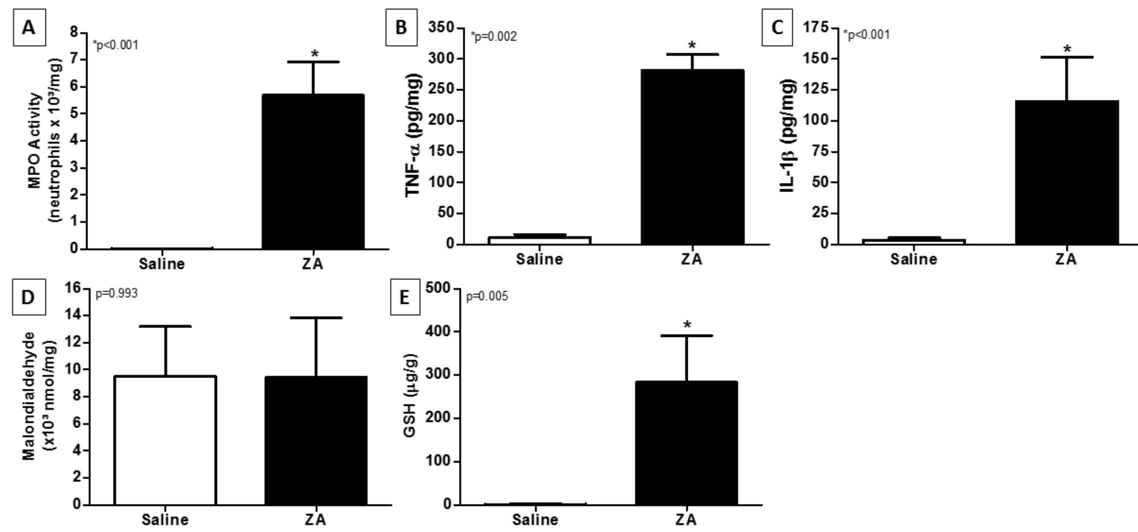

Figure 3: Inflammatory and biochemical gingival profiles of the animals treated with ZA chronically demonstrating increase of the MPO activity (A) and TNF-α (B), IL-1β (C) and GSH (E) without variation in MDA (D) levels.

4. Discussão Geral

No presente estudo, pôde-se observar que a infusão crônica de AZ causou um estado hiperinflamatório na polpa dentária, na gengiva e no ligamento periodontal dos ratos de forma dose-dependente. Entretanto, esse estado hiperinflamatório não foi o suficiente para o desencadeamento da OMB. Para sua instalação, foi necessária a realização do procedimento de exodontia que culminou com a exacerbação de todos os mediadores estudados.

No entanto, apesar de o estado hiperinflamatório ser geral (NORTON et al., 2012; ROSSINI et al., 2012), diferenças peculiares podem ser notadas nos tecidos estudados. Se, por um lado, o aumento dos níveis de TNF-α, IL-1β e a imunoexpressão para iNOS foi observada em todos os modelos (polpa, periodonto e modelo de OMB), por outro lado, o tipo celular envolvido em cada um diferiu notadamente.

Na polpa dentária, houve maior número de mononucleares CD68+; no periodonto, maior atividade de MPO, ambos sem alteração da expressão de NF-kB; e, no osso, neutrófilos e macrófagos estão dispostos em maior escala, juntamente com o aumento do número de células com positividade nuclear para NF-kB, forma ativa desse fator de transcrição.

A polpa dentária, que mostrou relação direta com o maior número de mononucleares CD68+, é um tecido que está diretamente relacionado a macrófagos. Na pulpite dentária, há elevação de diversas citocinas inflamatórias. TNF-α, IL-6, IL-8 e IFN-γ e atividade da iNOS estão diretamente associadas à transição de uma polpa sadia a um estado de pulpite reversível (DI NARDO DI MAIO et al., 2004; EL-SALHY et al., 2013). A ativação desses mediadores leva as células da polpa dentária à produção de uma série de quimiocinas específicas para monócitos e macrófagos.

MCP-1, MIP (Macrophage Inflammatory Protein)-1α e MIP-3α são importantes quimiocinas produzidas durante o processo inflamatório na polpa dentária e são potentes ativadoras da migração de monócitos e macrófagos (KIM et al., 2010, HAYASHI et al., 2015; HOSOKAWA et al., 2016). Além dessas, a CXCL14, que apresenta função similar, está aumentada na pulpite por uma via dependente da ativação dos receptores TLR4 (Receptores semelhantes a Toll tipo 4) com consequente elevação dos níveis de TNF-α, IL-1β, IFN-γ, processo normalmente observado quando o processo de cárie chega à dentina (HORST et al., 2011).

Relatos de pesquisa envolvendo cultivo de células da polpa com BF são escassos. O único trabalho *in vitro* desenvolvido foi publicado por CVIKL et al. (2011), mas não

demonstrou o perfil inflamatório, e sim as taxas de apoptose de células da polpa incubadas com AZ. Nesse estudo, os autores demonstraram redução da sobrevida de células mediante ação tóxica direta do AZ. No entanto, sabe-se que células, quando submetidas a toxicidade, normalmente expressam mediadores pró-inflamatórios (YU et al., 2011).

Em dois estudos *in vivo*, HIRAGA et al. (2010) e MASSA et al. (2006) demonstraram que fetos de ratas prenhas tratadas com bisfosfonatos desenvolvem má-formação dentária e desenvolveram má formação dentária e lesões similares a odontoma. Dentina e osso são dois tecidos mineralizados embriologicamente similares, e, apesar de ainda não ter sido descrito que os BFs podem se incorporar aos tecidos dentários, pode-se pressupor, por esses achados, que os resultados do presente estudo se devem ao efeito tóxico do AZ incorporado à dentina, o qual poderia agir em odontoblastos e em outras células da polpa, pois, mesmo após 70 dias do início do protocolo, os eventos inflamatórios se mantiveram.

ENDELE et al. (2005) determinaram um método para quantificação de ibandronato em soro, urina e osso de ratos por meio de espectrometria de massas associado a cromatografia gasosa. Porém, além da técnica nunca ter sido aplicada para o AZ, tampouco em dentes, a necessidade de maceração e dissolução ácida dos tecidos para a realização do método impede determinar se o AZ se incorpora na dentina, esmalte ou cemento.

Trabalhos buscando avaliar o efeito do AZ e outros BFs em fibroblastos gengivais e periodontais e queratinócitos da gengiva inserida são mais comuns. Tem sido descrito, recentemente, que o AZ reduz a taxa de proliferação e síntese de colágeno em culturas celulares de fibroblastos periodontais (KOMATSU et al., 2016), aumentando sua taxa de apoptose celular (SCHEPER et al., 2009; COLLI et al., 2015). Queratinócitos da gengiva inserida também sofrem com o efeito tóxico do AZ (BASSO et al., 2013) e, no geral, esse efeito tóxico parece ser dependente de estresse oxidativo (COLLI et al., 2015).

Fibroblastos do ligamento periodontal, quando incubados com AZ, elevam a síntese de espécies reativas de oxigênio por uma via dependente da NOS constitutiva, bem como da síntese de IL-6, gerando, portanto, um estresse inflamatório (COLLI et al., 2015).

No entanto, o estresse inflamatório que ocorre em gengiva gera um perfil de quimiocinas distinto do observado na polpa dentária. Enquanto a inflamação pulpar mediada por microrganismos é fortemente associada às quimiocinas ativadoras de macrófagos e monócitos, a inflamação que ocorre na gengiva é fortemente dependente de mediadores como a IL-8, uma quimiocina associada com a migração de neutrófilos (JIANG et al., 1996).

Na atual pesquisa, enquanto o estado hiperinflamatório provocado por AZ, na polpa, levou ao aumento do número de mononucleares CD68+, no periodonto, levou à elevação da atividade de MPO, uma enzima fortemente encontrada em neutrófilos (FAVORA et al., 2009).

O AZ, notadamente, apresenta toxicidade em diversos grupos celulares, mas esses efeitos tóxicos parecem estar ligados mais ao estado hiperinflamatório provocado pelo AZ do que à ação tóxica direta do fármaco. COZIN et al. (2011) demonstraram que fibroblastos gengivais têm altas taxas de apoptose celular quando incubados com AZ, mas esse processo é revertido quando as células são incubadas com PDGF (Fator de Crescimento Derivado de Plaquetas).

É provável que esse possa ser um fator determinante ao desenvolvimento de OMB, pois osteócitos são células bastante sensíveis a estresse inflamatório (WIMALAWANSA , 2010) e apenas as hemimandíbulas tratadas com exodontia desenvolveram OMB.

É importante ressaltar que a infusão crônica de AZ não modificou a imunoexpressão para NF-kB na polpa dentária e no periodonto dos animais tratados cronicamente. Mas o desenvolvimento da OMB foi diretamente relacionado ao aumento de sua expressão nuclear (forma ativa).

Para o desenvolvimento da OMB, é necessário um fator estimulador, e a exodontia é o principal fator associado (BARASCH et al., 2011), pois induz um pico de expressão de citocinas pró-inflamatórias como TNF-α e IL-6, que normalmente decai em níveis basais, após 10 dias do procedimento cirúrgico (KIM et al. 2012).

O TNF-α se liga ao seu receptor, o TNFR, estimulando uma cascata de fosforilações que culmina com a ativação do NF-kB (KON et al., 2001; BALGA, 2006). Da mesma forma, a IL-6 age similarmente por meio da interação com seu receptor transmembrana IL-6R, ativando a mesma via NF-kB (YAO et al., 2014).

Como o AZ fica irreversivelmente aderido à hidroxiapatita do tecido ósseo (ENDELE et al., 2005), o processo iniciado pela exodontia que ocorreu em tecidos que já estavam em estado de hiperinflamação perpetua-se, levando ao desenvolvimento da OMB (COMITO et al., 2016).

Assim, pôde-se observar que a infusão crônica de AZ aumentou a expressão de citocinas pró-inflamatórias em polpa e periodonto, alterando, de forma distinta, seu perfil celular (células CD68+, na polpa, e neutrófilos, no periodonto). Todavia, é necessário um

evento pró-inflamatório desencadeador (a exodontia, como visto neste estudo) para estimular a produção de quantidades ainda maiores de citocinas pró-inflamatórias e a perpetuação do quadro de inflamação com consequente desenvolvimento da OMB.

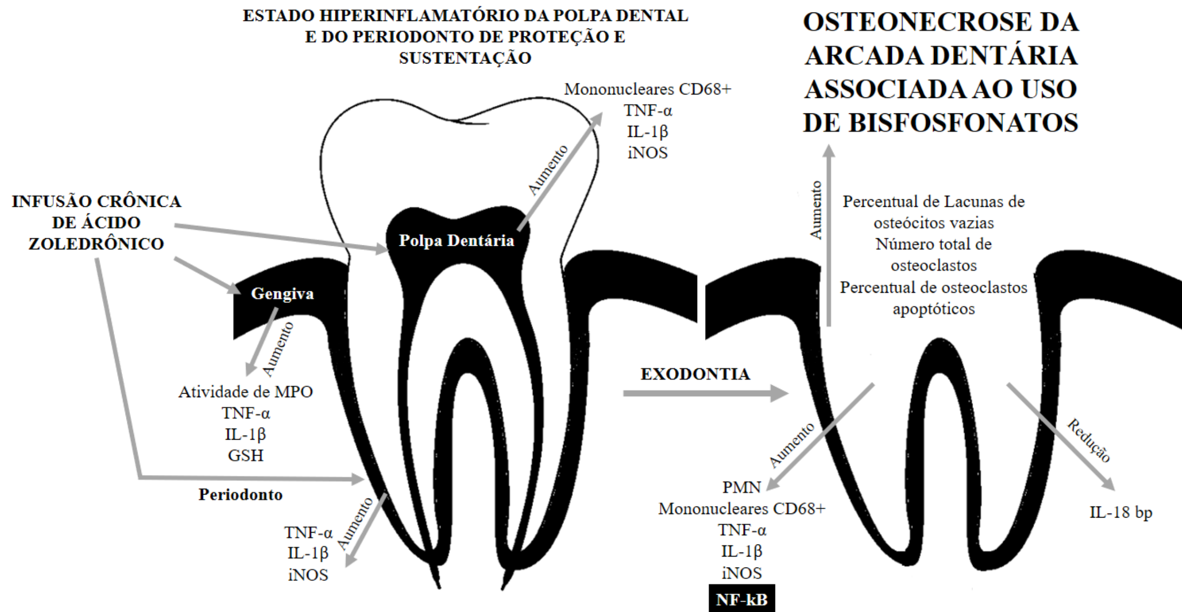

Figura 6: Desenho esquemático dos elementos celulares e mediadores químicos envolvidos no estado hiperinflamatório produzido pela infusão crônica de AZ na polpa dentária e no periodonto e na patogênese da OMB (Fonte: próprio autor).

5. Conclusão Geral

A OMB mostrou aumento do número de osteoclastos (total e apoptóticos), polimorfonucleares neutrófilos e células mononucleares CD68+, além de maior imunoexpressão de TNF-α, IL-1β, iNOS, e NF-kB (nuclear e citoplasmática). A expressão de IL-18bp, uma proteína anti-inflamatória, foi inversamente associada à concentração de AZ, reforçando a inflamação como fator importante na patogênese da OMB.

A infusão crônica de AZ elevou o número de animais exibindo células inflamatórias (mononucleares CD68+, na polpa, e neutrófilos, no periodonto) e da expressão de TNF-α, IL-1β e iNOS em ambos os tecidos, porém sem alteração na imunomarcação nuclear para NF-kB e marcadores de estresse oxidativo. Isso reforça o achado que o fármaco *per si* é capaz de provocar desregulação imune-inflamatória crônica em tecidos mineralizados, porém, é necessário um fator desencadeador para o desenvolvimento da OMB.

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Anexo A – Seguimento do Regimento Interno

Art. 46 – As dissertações e as teses apresentadas ao Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará poderão ser produzidas em formato alternativo ou tradicional. O formato alternativo estabelece: a critério do orientador e com a aprovação da Coordenação do Programa, que os capítulos e os apêndices poderão conter cópias de artigos de autoria ou co-autoria do candidato, publicados ou submetidos para

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§1° - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

§2° - A dissertação e a tese em formatos tradicionais ou formatos alternativos deverão seguir as normas preconizadas pelo Guia para Normalização de Trabalhos Acadêmicos da Biblioteca Universitária disponível no sítio http://www.biblioteca.ufc.br. As partes específicas do formato alternativo deverão ser feitas em concordância com o *Manual de Normalização para Defesa de dissertação de Mestrado e tese de Doutorado no formato Alternativo do PPGO,* disponível no sítio http://www.ppgo.ufc.br.

Anexo B – Certificação de língua portuguesa

DECLARAÇÃO

Eu, Mary Martha Studart Gurgel Mendes. RG 2009009001349. CPF 261390143-87, graduada em Pedagogia pela Universidade de Fortaleza-Unifor, diploma n.º 15458 e Letras pela Faculdade de Macapá, diploma n.º 3472, pós-graduada em Supervisão Escolar pela Universidade Salgado Oliveira, diploma n.º 972112, declaro, para os devidos fins e para comprovar junto ao Programa de Pós-Graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, que realizei a revisão de português da tese intitulada **EXPRESSÃO DE MARCADORES INFLAMATÓRIOS NA OSTEONECROSE DOS MAXILARES INDUZIDA POR BISFOSFONATOS E EFEITO DO TRATAMENTO CRÔNICO COM ÁCIDO ZOLEDRÔNICO NOS TECIDOS GENGIVAL E OSSEODENTÁRIO DE RATOS**, de Paulo Goberlânio de Barros Silva, consistindo em correção gramatical, adequação do vocabulário e inteligibilidade do texto.

Fortaleza, 15 de outubro de 2016

Mary Martha Studart Gurgel Mendes

Anexo C – Certificação de língua inglesa do artigo 01 (Immune cellular profile of bisphosphonate-related osteonecrosis of the jaw)

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Immune-cellular profile of bisphosphonate-related osteonecrosis of the jaws: a histomorphometric and immunohistochemical study

Authors:

Paulo Goberlânio de Barros Silva, Camila de Oliveira Carvalho, Luiz André Cavalcante Brizeno, Deysi Viviana Tenazoa Wong, Roberto César Pereira Lima Júnior, Romélia Pinheiro Gonçalves, Fabrício Bitú Sousa, Mário Rogério Lima Mota, Ronaldo Albuquerque Ribeiro (in memorian), Ana Paula Negreiros Nunes Alves

Date Issued:

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Manuscript title:
Immuno-cell profile of dental pulp in rats treated with zoledronic acid

Authors:
Paulo Goberlânio de Barros Silva, Maria Elisa Quezado Lima Verde, Luiz André Cavalcante Brizeno, Deysi Viviana Tenazoa Wong, Roberto César Pereira Lima Júnior, Fabrício Bitú Sousa, Mário Rogério Lima Mota, Ronaldo Albuquerque Ribeiro (in memorian), Ana Paula Negreiros Nunes Alves.

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Anexo E – Certificação de língua inglesa do artigo 03 (Chronic Infusion of Zoledronic Acid rises inflammatory markers in periodontium of rats)

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Manuscript title:
Chronic Infusion of Zoledronic Acid rises inflammatory markers in periodontium of rats

Authors:
Paulo Goberlânio de Barros Silva, Antonio Ernando Carlos Ferreira Junior, Camila Carvalho de Oliveira, Luiz André Cavalcante Brizeno, Deysi Viviana Tenazoa Wong, Roberto César Pereira Lima Júnior, Fabrício Bitú Sousa, Mário Rogério Lima Mota, Ana Paula Negreiros Nunes Alves

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Anexo F – Parecer da Comissão Ética no Uso de Animais da (Número 25/15)

Universidade Federal do Ceará
Comissão de Ética no Uso de Animal – CEUA
Rua: Coronel Nunes de Melo, 1127 Rodolfo Teófilo
Cep: 60430-970 Fortaleza-CE

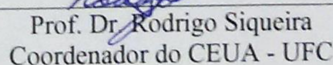
CERTIFICADO

Certificamos que o projeto intitulado **"ESTUDO DOS MECANISMOS E MEDIADORES ENVOLVIDOS NA PATOGÊNESE DA OSTEONECROSE DOS MAXILARES INDUZIDA POR BISFOSFONARTOS EM MODELO EXPERIMENTAL"**, protocolo n°25/2015, sob responsabilidade do Prof. Dr. Manoel Odorico de Moraes Filho, que envolve a produção, manutenção e/ou utilização pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica - encontra-se de acordo com os preceitos da Lei 11.794, de n°8 de outubro de 2008, do Decreto 6899 de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), foi aprovado pela Comissão de Ética no Uso de Animais (CEUA-UFC) da Universidade Federal do Ceará, em reunião em 29 de maio de 2015.

Vigência do projeto	01/01/2016 a 01/01/2018
Espécie/Linhagem	Ratos heterogênico Wistar
N° de Animais	42
Peso/Idade	180g
Sexo	Machos
Origem	Biotério Central da UFC

Fortaleza, 29 de maio de 2015

Prof. Dr. Rodrigo Siqueira
Coordenador do CEUA - UFC



Anexo G

ORAL DISEASES
Leading in Oral, Maxillofacial, Head & Neck Medicine

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ORIGINAL ARTICLE

Immune cellular profile of bisphosphonate-related osteonecrosis of the jaw

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OBJECTIVES: Characterize the cell profile and immunostaining of proinflammatory markers in an experimental model of bisphosphonate-related osteonecrosis of the jaw (BRONJ).
MATERIALS AND METHODS: Male Wistar rats (*n* = 6–7) were treated chronically with saline solution or zoledronic acid (ZA) at 0.04, 0.20, and 1.00 mg kg^{-1} (1.4×10^{-7}, 6.9×10^{-6}, and 3.4×10^{-5} mol kg^{-1}), and subsequently, the first left inferior molar was extracted. Were performed counting of viable and empty osteocyte lacunae, viable and apoptotic osteoclasts, polymorphonuclear neutrophil, mast cells (toluidine blue), and the positive presence cells for CD68, tumor necrosis factor-alpha (TNF-α), IL (interleukin)-1β, inducible nitric oxide synthase (iNOS), nuclear factor-kappa B (NF-kB) and IL-18 binding protein (IL-18 bp).
RESULTS: BRONJ was showed in ZA treated with 0.20 and 1.00 mg kg^{-1}. There is a dose dependent increase in percentage of empty osteocyte lacunae ($P < 0.001$) and apoptotic osteoclasts ($P < 0.001$), counting of total osteoclasts ($P = 0.003$), polymorphonuclear neutrophil cells ($P = 0.009$), cytoplasmic-positive cells of CD68 ($P < 0.001$), TNF-α ($P = 0.001$), IL-1β ($P = 0.001$), iNOS ($P < 0.001$), NF-kB ($P = 0.006$), and nuclear-positive cells of NF-kB ($P = 0.011$). Consequently, there is no difference in mast cells ($P = 0.957$), and IL-18 bp immunostaining decreases dose dependently ($P = 0.005$).
CONCLUSIONS: BRONJ is characterized by increases in immunostaining for proinflammatory markers and NF-kB and inversely associated with cells exhibiting IL-18 bp.

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In memorian of Ronaldo de Albuquerque Ribeiro
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Oral Diseases (2016) doi: 10.1111/odi.12513

Keywords: bisphosphonate-associated osteonecrosis of the jaw; tumor necrosis factor-alpha; interleukin-1; nitric oxide synthase type II; NF-kappa B; IL-18 binding proteins

Introduction

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a complicated adverse effect of treatment with bisphosphonates (BF), especially in patients undergoing oncology therapeutics. Some characteristics of this condition have been discovered and elucidated, but the pathogenesis is still uncertain (Allen and Burr, 2009).

The main studied mechanisms of BRONJ involve inhibitory effects of BF on bone turnover and angiogenesis. However, these purported mechanisms *per se* do not adequately explain the pathogenesis of BRONJ (Allen and Burr, 2009). BF have anti-angiogenic and antivasculogenic effects *in vitro* and *in vivo* (Ziebart *et al*, 2011; Misso *et al*, 2012), and this could contribute to the deficient prognosis of BRONJ (Lescaille *et al*, 2014). However, experimental murine models are not consistent with an association between BRONJ and the inhibition of vascularization or vascular proliferation markers (Sonis *et al*, 2009; Aguirre *et al*, 2010; Kobayashi *et al*, 2010; Marino *et al*, 2012).

On the other hand, infection is strongly associated with BRONJ, and antimicrobial approaches are generally utilized by dentistry clinics in association with surgical debridement. Although BF increase the adhesion of bacteria to the hydroxyapatite bone (Ganguli *et al*, 2005; Kobayashi *et al*, 2010), the isolated use of antimicrobial agents without removal of necrotic bone does not promote healing of BRONJ (Hoefert and Eufinger, 2011). The participation of infection in the pathogenesis of BRONJ has not been observed in any experimental model addressing

this parameter (Sonis *et al*, 2009; Kobayashi *et al*, 2010; Maahs *et al*, 2011; Conte Neto *et al*, 2013). Despite the use of antimicrobial agents shows conflicting efficacy in the modification of serum and the local microbial profile of patients with BRONJ (Ji *et al*, 2012), why does antibiotic prophylaxis seems to be an acceptable option to prevent BRONJ in patients treated with high doses of BF (Montefusco *et al*, 2008;)?

Lopez-Jornet *et al* (2011) did not observe a relationship between BRONJ and infection, but the authors reported that infected animals had intense inflammatory infiltrated in exodontic locations, implying the development of BRONJ. Acute inflammation induces osteocyte apoptosis and inhibits the vitality and activity of osteoblasts (Gilbert *et al*, 2000; Cheung *et al*, 2011; Huang *et al*, 2011; Kawai *et al*, 2012). Proinflammatory cytokines also induce osteoclastogenesis (Assuma *et al*, 1998) and increase important modulators of the vitality of bone cells such as reactive oxygen species (ROS) (Halleen *et al*, 1999; Wimalawansa, 2010; Almeida and O'Brien, 2013).

Several experimental models (Sonis *et al*, 2009; Ali-Erdem *et al*, 2011; López-Jornet *et al*, 2011; Maahs *et al*, 2011; Marino *et al*, 2012) and clinical studies (Lesclous *et al*, 2009) have demonstrated associations between inflammation and BRONJ. BF increase production of acute inflammatory mediators *in vitro* (Muratsu *et al*, 2013) and *in vivo* (Norton *et al*, 2012) and modify the immunological and cellular profile of patients undergoing chronic treatment (Rossini *et al*, 2012; Welton *et al*, 2013). Therefore, a consistent characterization of the immune and cellular profile of BRONJ has not been described (Vasconcelos *et al*, 2012).

Thus, the aim of this study was to conduct histomorphometric and immunohistochemical studies to characterize the cellular profile and immunoreactivity of certain chemical mediators.

Materials and methods

Animals, doses, and experimental protocols

Male Wistar rats (n = 6–7) were treated (three consecutive weeks) with intravascular (penile access) saline or zoledronic acid (ZA, molar mass = 290.1) at 0.04, (1.4×10^{-7} mol kg^{-1}), 0.20 mg kg^{-1} (6.9×10^{-6} mol kg^{-1}), and 1.00 mg kg^{-1} (3.4×10^{-5} mol kg^{-1}) (days 00, 07, 14, respectively). Four weeks after the last infusion of ZA or saline (day 42), the first left inferior molar was extracted (using Hollemback 3s and Lecron Zalle spatulas). During the following week (day 49), an additional ZA dose was infused. The animals were euthanized on the 70th experimental day, and their hemimandibles were fixed in neutral formalin 10% (Silva *et al*, 2015).

The approval for experimental use of laboratory animals was obtained from the local Ethics Committee on Animal Use (CEUA, former CEPA) (protocol 26/2013) and is in compliance with the Federal Law No. 11794 of October 8, 2008, and the Decree n° 6689, July 15, 2009, that regulated the law in 11 794, available from http: www. planalto.gov.brccivil03Ato2007–20102008LeiL11794.htm.

Histomorphometric and histochemical analysis

After decalcification (ethylenediaminetetraacetic acid 10%, pH 7.3), microscopic slides (4 μm) were prepared (conventional hematoxylin–eosin (HE) method) and qualitatively and histomorphometrically analyzed. Afterward, ten microscopic fields (400x) were used for counting of viable and empty osteocyte lacunae, total osteoclasts, viable and apoptotic osteoclasts, and polymorphonuclear neutrophil cells (Yamashita *et al*, 2011; Shaker *et al*, 2013).

Hydrated tissue sections (4 μm) were immersed in 0.1% toluidine blue solution (in 0.9% sodium chloride) per 60s for histochemical assessment (Gurgel *et al*, 2013) and mast cell counting using the same methodology (Shaker *et al*, 2013).

Immunohistochemical analysis

After deparaffinization and rehydration, the tissue sections (2.5 μm) were also submitted to immunohistochemical assessment. Antigenic recovery was performed by heating in citrate pH 6.0 solution. After cooling, the slides were submitted to peroxidase blocking with H_2O_2 3% solution diluted in PBS (phosphate-buffered saline) or with methanol solution according to the antibody's specificity (30 min).

After protein blocking (albumin) (1 h), we performed incubation with primary antibody CD68 (Dako®, Dopenhage, Denmark), tumor necrosis factor-alpha (TNF-α) (Abcam®, Cambridge, UK), IL (interleukin)-1β (Abcam®), inducible nitric oxide synthase (iNOS) (Abcam®), nuclear factor-kappa B (NF-kB) (SantaCruz®, Finnell Street Dallas, Texas, USA), or IL-18 binding protein (IL-18 bp) (SantaCruz®) using the dilutions and times shown in Table 1.

Envision System Plus-HRP (Dako®) (ready to use), Simple Stain Rat MAX PO (Multi) Universal Immuno-peroxidase Polymer (anti-mouse and anti-rabbit) (Histofine®) (ready to use) or secondary biotinylated anti-rabbit polyclonal IgG and ABC (avidin–biotin–peroxidase) System (SantaCruz Biotechnology®) (in diluted solutions) were utilized for the secondary antibody incubation. The visualization system used was 5,5-diaminobenzidine tetrahydrochloride (DAB) (Dako®) (Table 1).

Ten microscopic fields (400x) were used to count CD68 immunostaining mononuclear cells; cells exhibiting cytoplasmic positivity for TNF-α, IL-1β, iNOS, IL-18 bp, or NF-kB; and nuclear positivity for NF-kB in exodontic sites (Kim *et al*, 2012).

Statistical analysis

Kolmogorov–Smirnov normality testing was performed, and we utilized analysis of variance (ANOVA) followed by Tukey's post-test for group comparisons. The data were expressed as the mean and standard error of the mean (Mean±s.e.m.).

Results

ZA-induced experimental BRONJ

Twenty-eight days after tooth extraction, the saline solution group showed viable osteocytes and rare osteoclasts at the site of the exodontia's new bone (Figure 1a and 1e). The group treated with 0.04 mg kg^{-1} ZA presented delayed deposition of osteoid; however, the development of BRONJ was not observed, nor were there any inflammations signs or damaged osteoclasts (Figure 1b and 1f). The dose of 0.20 (Figure 1c and 1g) or 1.00 mg kg^{-1} (Figure 1d and 1h) ZA led to large bone sequesters associated with intense inflammatory infiltrate, osteoclasts with intracytoplasmic vacuolization, nuclear hyperchromatism, and necrotic bone with irregular peripheral resorption. No signs of healing or deposition of the mineralized bone matrix were observed.

Cellular profile of BRONJ

The groups treated with 0.20 (32.5 ± 1.1%) or 1.00 (26.8 ± 1.8%) mg kg^{-1} ZA showed a statistical increase in percentage of empty osteocyte lacunae compared with the saline group (6.4 ± 1.0%) and 0.04 (14.2 ± 3.1%) mg kg^{-1} ZA groups (P < 0.001) (Figure 1).

The total osteoclast number was greater in these two groups. Groups treated with 0.20 (31.4 ± 7.9) or 1.00 (39.0 ± 3.5) mg kg^{-1} ZA had a higher osteoclast number

Table 1 Parameters and methodologies of immunohistochemistry evaluation

Antibody	Production	*Antigenic recuperation Methodology (solution)*	*Peroxidase blocking H_2O_2 concentration (solution dilution)*	*Primary antibody*		*Secondary antibody*		*Revelation System (time)*	*Cell count*
				Dilution	*Time of incubation*	*System and/or dilution (Production)*	*Time of incubation*		
CD68	Dako®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:500	Overnight	Envision System Plus-HRP (Dako®)	30′	DAB (5′)	Mononuclear-positive cells
TNF-α	Abcam®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:50	1 h	Universal Immuno-peroxidase Polymer (Histofine®)	30′	DAB (5′)	Positive cells
IL-1β	Abcam®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:100	1 h	Universal Immuno-peroxidase Polymer (Histofine®)	30′	DAB (5′)	Positive cells
iNOS	Abcam®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:200	Overnight	Universal Immuno-peroxidase Polymer (Histofine®)	30′	DAB (5′)	Positive cells
NF-kB	SantaCruz®	Heat (Citrate pH 6.0)	H_2O_2 3% (Methanol)	1:200	Overnight	Secondary biotinylated anti-rabbit IgG 1:500 + ABC System (SantaCruz®)	30′ + 30′	DAB (10″)	Positive cells in cytoplasm and nucleus
IL-18 bp	SantaCruz®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:100	Overnight	Secondary biotinylated anti-rabbit IgG 1:200 + ABC System (SantaCruz®)	30′ + 30′	DAB (45″)	Positive cells

TNF-α, Tumor necrosis factor-alpha; IL-1β, interleukin-1beta; iNOS, induced nitric oxide synthase; NF-kB, nuclear factor-kappa B; IL-18 bp, interleukin-18 binding protein; HRP, horseradish peroxidase; ABC, avidin–biotin–complex; DAB, 5,5-diaminobenzidine tetrahydrochloride.

than the saline group (6.3 ± 1.3) (P = 0.003). There was no significantly difference between the saline and 0.04 mg Kg^{-1} (20.5 ± 3.2) groups. This augmentation was accompanied by the number of osteoclasts with apoptotic signs. Both groups with 0.20 (87.4 ± 2.0%) and 1.00 (79.8 ± 1.1%) mg kg^{-1} ZA showed a higher percentage of apoptotic osteoclasts than the saline (5.0 ± 5.0%) or 0.04 mg Kg^{-1} (11.8 ± 5.7%) group (P < 0.001) (Figure 1).

Regarding the cellular inflammatory profile, an increase in number of polymorphonuclear neutrophils was observed in the groups treated with 0.20 (213.3 ± 122.4) or 1.00 mg kg^{-1} (255.0 ± 45.0) ZA compared with the saline (0.0 ± 0.0) and 0.04 mg kg^{-1} ZA groups (9.5 ± 8.3) (P = 0.009). The number of mononuclear CD68-positive cells was significantly higher in the 0.20 (205.0 ± 31.3) and 1.00 (240.7 ± 11.3) mg kg^{-1} ZA groups than in the 0.04 mg Kg^{-1} ZA (86.5 ± 18.7) or saline (66.7 ± 5.8) group (P < 0.001). There was no statistically significant difference in mast cell number among the groups (saline: 30.5 ± 6.7, 0.04 mg kg^{-1} ZA: 30.3 ± 7.0, 0.20 mg kg^{-1} ZA: 34.0 ± 5.93, 1.00 mg kg^{-1} ZA: 29.8 ± 3.7) (P = 0.957) (Figure 1).

Immune inflammatory profile of BRONJ

There was a significant increase in cells exhibiting immunostaining for Th1 inflammatory markers (Figure 2).

The immunostaining for TNF-α was greater in the groups treated with 0.20 (3312.0 ± 180.8) or 1.00 (2912.0 ± 179.7) mg kg^{-1} ZA than in the saline group (1811.0 ± 145.0) (P = 0.001). However, there was no difference between the 0.04 (2325.0 ± 141.8) mg kg^{-1} ZA and saline groups (Figure 2).

The number of positive cells for IL-1β was higher in the 0.20 (5386.0 ± 459) and 1.00 (6359 ± 512.4) mg kg^{-1} groups than in the saline (2695.0 ± 104.2) group (P = 0.001). Nevertheless, a difference between the saline and 0.04 mg Kg^{-1} (3589.0 ± 477.6) groups was not observed. Similarly, positive cells for iNOS were augmented in the 0.20 (5436.0 ± 827.5) and 1.00 (6356.0 ± 523.7) mg kg^{-1} ZA groups compared with the saline (933.3 ± 396.1) or 0.04 ZA mg Kg^{-1} (1825.0 ± 159.4) group (P < 0.001) (Figure 2).

Cytoplasmic and nuclear positivity for NF-kB were higher in the groups with BRONJ. In the cytoplasm, the groups treated with 0.20 mg Kg^{-1} ZA (576.5 ± 86.7) or 1.00 mg kg^{-1} ZA (597.7 ± 135.6) showed elevated numbers of cells exhibiting positivity compared with the saline group (52.5 ± 45.5) (P = 0.011). There was no difference in immunostaining for cytoplasmic NF-kB between the saline and 0.04 mg Kg^{-1} ZA (157.0 ± 77.7) groups. Nuclear staining for NF-kB was significantly increased in the groups treated with 0.20 mg kg^{-1} ZA (84.3 ± 9.9) or 1.00 mg Kg^{-1} ZA (285.7 ± 115.4) compared with the saline group (8.7 ± 4.3) (P = 0.006). There was no significant difference between the saline group and the 0.04 mg Kg^{-1} (19.7 ± 7.1) or 0.20 mg Kg^{-1} ZA (84.3 ± 9.9) groups (Figure 2).

Immunostaining for IL-18 bp was increased in the group treated with 0.04 mg kg^{-1} ZA (418.6 ± 119.1) compared with the saline group (12.5 ± 6.6). A difference between the groups with 0.04 mg kg^{-1} ZA and 0.20 mg kg^{-1} ZA (203.3 ± 93.5) was not observed. Nevertheless, there was a significant decrease in positive cells for IL-18 bp in the group treated with 1.00 mg kg^{-1} ZA (105.8 ± 31.7) compared with the

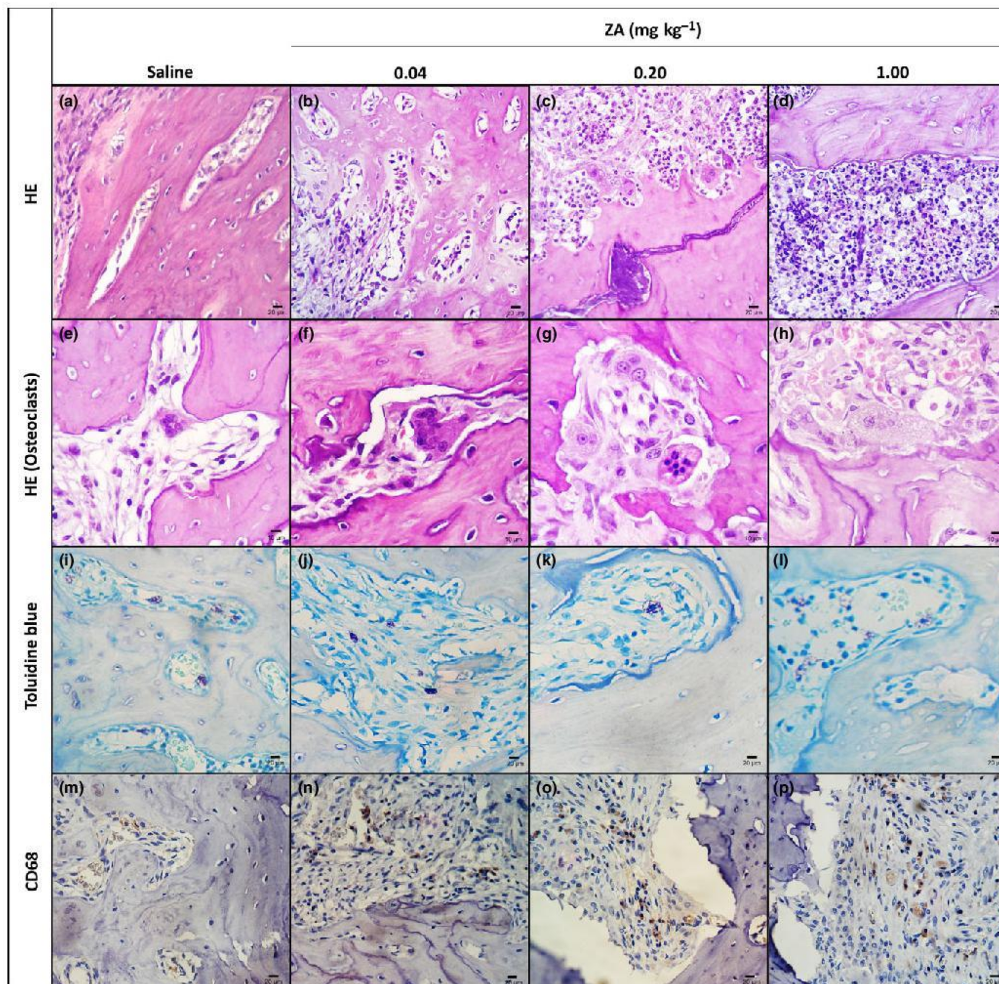

Figure 1 Cellular profile of BRONJ showing reduction in bone formation and viable osteocytes (**a**–**d**), increased number of osteoclasts (**e**–**h**) with apoptotic signs, no alteration in mast cells (**i**–**l**), and increased number of mononuclear CD68-positive cells (**m**–**p**) dose dependently (400×)

0.04 mg kg^{-1} ZA (418.6 ± 119.1) group ($P = 0.005$) (Figure 2).

Discussion

The participation of inflammation in BRONJ has been described since the first reports and research on the disease. However, the focus of the studies has been centered on the role of bacterial infection (Hinson *et al*, 2014). Although infection is importantly associated with BRONJ, antimicrobial therapy has a low level of success compared with the surgical approach in the treatment of this condition (Rupel *et al*, 2014).

More than 81.6% of cases of BRONJ are associated with inflammation. This is a value discreetly higher than the association between BRONJ and infection (80.3%) (Hinson *et al*, 2014). In experimental models with rats, BRONJ was not associated with infection but with intense inflammation (López-Jornet *et al*, 2011). Thus, immune dysregulation may play a key role in BRONJ pathogenesis.

BRONJ is directly associated to proinflammatory condition. This is a dose-dependent pathology associated with leukocytosis and changes of erythrocytes (Silva *et al*, 2015). It is demonstrated that low doses (0.0225 mg kg^{-1}) of ZA not induce BRONJ (Sonis *et al*, 2009) despite

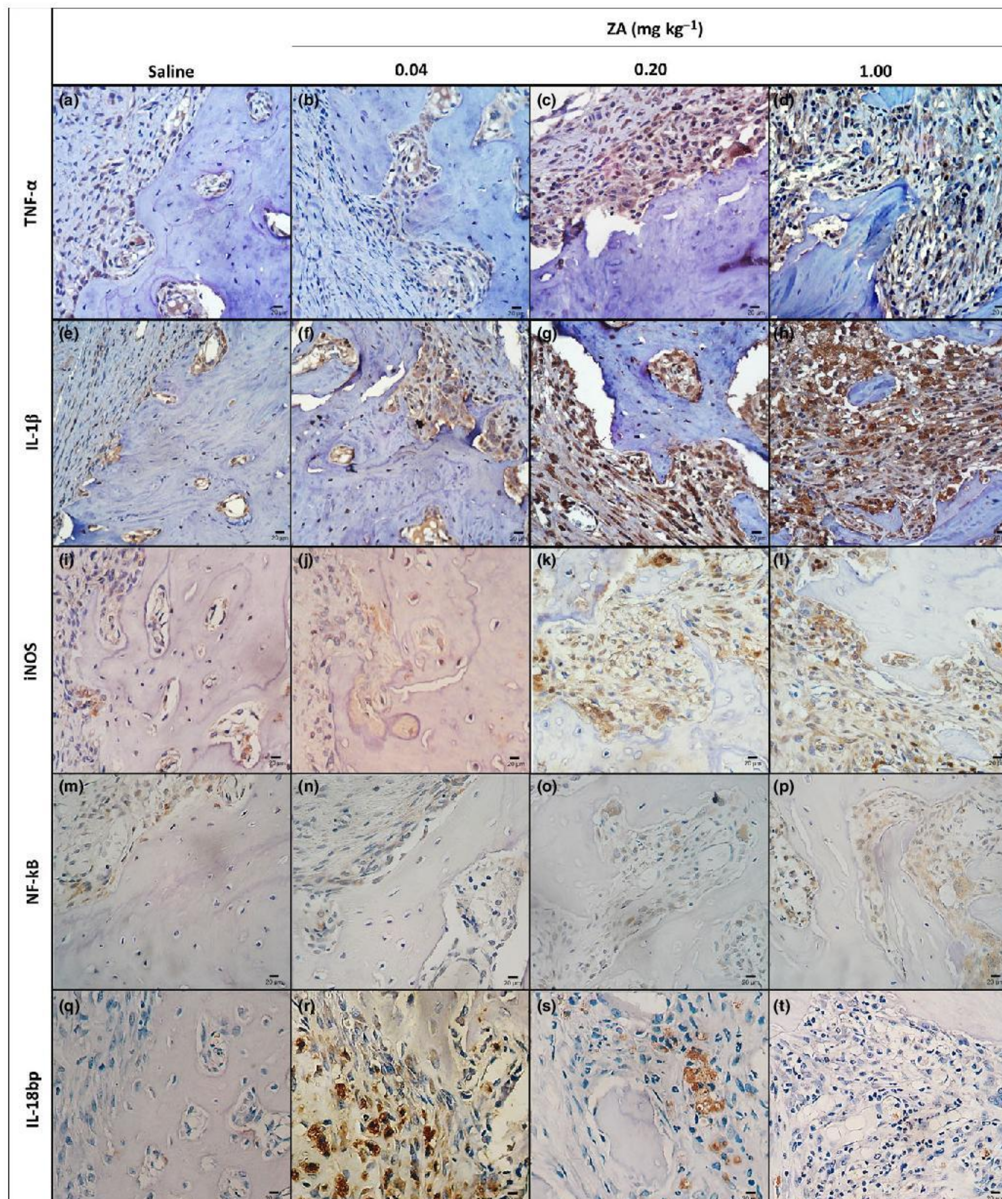

Figure 2 Cytokine profile showing dose-dependent increases in the number of TNF-α (**a**–**d**), IL-1β (**e-h**), iNOS (**i**–**l**), and NF-kB (nucleus and cytoplasm) (**m**–**p**)-positive cells. An increase in the number of IL-18 bp-positive cells was seen in the 0.04 mg kg^{-1} ZA group in comparison with the saline group, and a significant reduction was seen with a higher ZA dose (**q**–**t**) (400×)

proinflammatory stimulus is added (Marino *et al*, 2012). *In vivo* (classical peritonitis experiment) amino-bisphosphonates acutely upregulate proinflammatory cytokine production mediated by mast cells and the IL-1 receptor (IL-1R) (Norton *et al*, 2012). This cytokine, IL-1, is directly associated with BRONJ in the salival and gingival

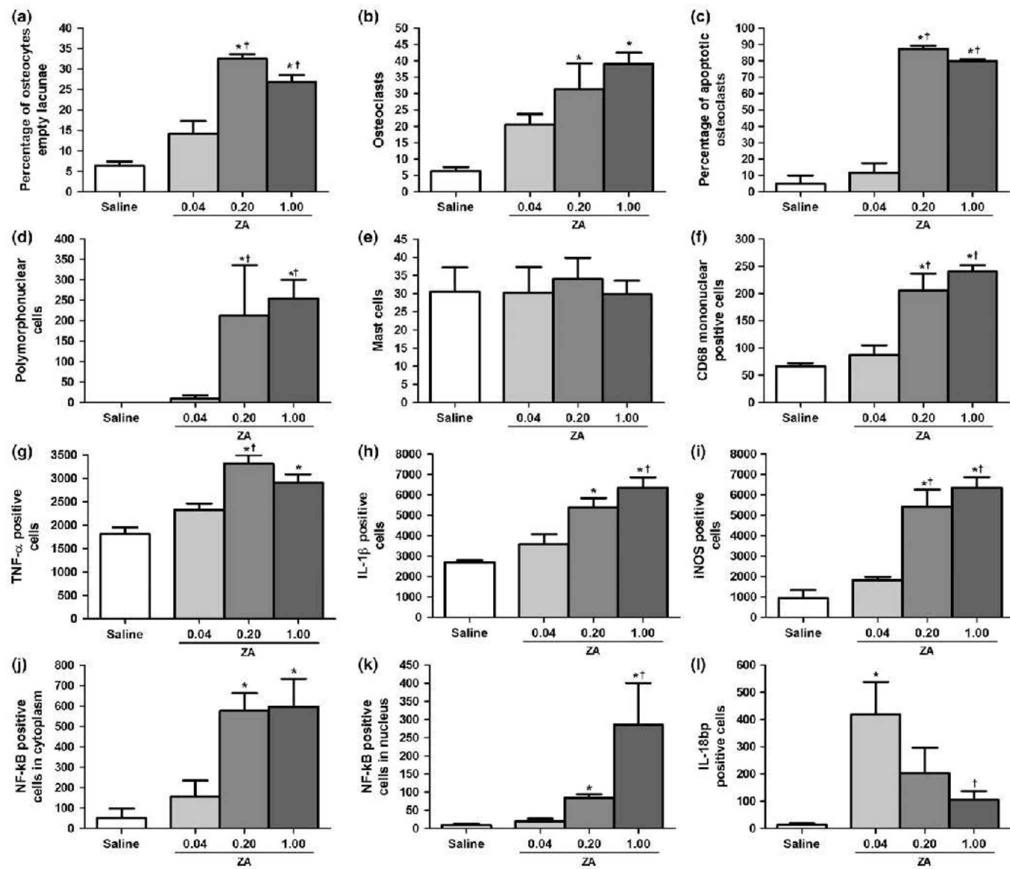

Figure 3 Cellular and cytokine profile analyses. $^{*}P < 0.05$ versus saline, $^{\dagger}P < 0.05$ versus 0.04 mg kg^{-1} ZA (ANOVA test followed by Tukey's post-test; Mean±s.e.m.)

fluid of patients (Bagan *et al*, 2013; Tsao *et al*, 2013) such as oxidative stress markers (Bagan *et al*, 2014).

In this experimental model, mast cells were not augmented in number (Figure 3). In bone, these cells can release proinflammatory cytokines by degranulation, but they are not necessarily increased in number (Huang *et al*, 2013). On the other hand, there were increases in some acute inflammatory markers such as TNF-α, IL-1β, iNOS, NF-kB activation (Figure 2), and numbers of neutrophils and macrophages (positive CD68 cells) but not of mast cells. ZA is a strong myelotoxic drug (Kuiper *et al*, 2012), but it increases cytokine production by macrophages through NF-kB activation (Muratsu *et al*, 2013).

TNF-α (Cheung *et al*, 2011), IL-1β (Bonewald, 2004; Bakker *et al*, 2009), and high oxidative stress from iNOS activity (Almeida and O'Brien, 2013) modulate the increase in osteocyte apoptosis. The formation of apoptotic bodies in osteocytes is a stimulus for the recruitment and activation of macrophages and osteoclasts (Kogianni *et al*, 2008), which are important scavenger cells involved in recognition of these apoptotic bodies for phagocytosis and empty bone resorption (Oizumi *et al*, 2009; Harre *et al*, 2012).

In this study, the increase in empty osteocyte lacunae was accompanied by increases in macrophages, osteoclasts, TNF-α, IL-1β, and iNOS-positive cells, supporting these previous studies. However, as ZA strongly induces osteoclast apoptosis, interrupting physiological bone resorption (Oizumi *et al*, 2009), there was also an increase in apoptotic osteoclasts.

ZA and other BF (alendronate) inhibit TRAP function and infiltration of CD68-positive cells in bone (Xiong *et al*, 2010; Yamashita *et al*, 2011). However, BRONJ is a chronic condition strongly associated with tooth extraction. After a tooth is extracted, it increases TNF-α expression, osteoclastogenesis, bone resorption, and bone matrix deposition occur with a reduction in proinflammatory cytokines to basal levels after 10 days (Kim *et al*, 2012).

Nevertheless, the osteoclast apoptosis induced by ZA adds proinflammatory effects in connective tissue cells and seems to perpetuate and upregulate these proinflammatory mediators, leading to deregulated osteocyte apoptosis (Bonewald, 2004; Bakker *et al*, 2009; Cheung *et al*, 2011; Almeida and O'Brien, 2013), macrophage infiltration and activation (Kogianni *et al*, 2008; Harre *et al*, 2012), and more cytokine production (Muratsu *et al*, 2013).

Conversely, IL-18 bp shows an inverse association with the dose-dependent proinflammatory effects of ZA in bone of rats submitted to tooth extraction (Figure 2). IL-18 bp inhibits IL-8, a chemokine involved in the migration of neutrophils (Novick *et al*, 1999), which is a cellular group that can increase cytokine levels and cause damage to some tissues when it is augmented in number (Lima-Júnior *et al*, 2012).

IL-18 bp has antineutrophil effects and modulates the immune response. This mediator is a member of a binding protein family with anti-inflammatory and anti-osteoclastogenic effects, such as OPG. IL-18 bp prevents binding to the IL-18R receptor (31% similarity with IL-1R (Nakanishi *et al*, 2001), a receptor directly involved in BRONJ patients (Bagan *et al*, 2013) that mediates the immune transition of the Th1/Th2 response (Novick *et al*, 1999). In Th2 responses, osteoclastogenesis and bone resorption are inhibited (Horwood *et al*, 2001; Yamada *et al*, 2002; Morita *et al*, 2010). This process can attenuate bone diseases such as periodontitis (Orozco *et al*, 2007) and decrease inflammatory mediators and high oxidative stress (Shaker *et al*, 2013).

IL-18 bp is produced by plasma cells and B lymphocytes. It inhibits NF-kB activity (Novick *et al*, 1999) by interrupting the interaction between IL-18 and IL-18R. Afterward, there is a breakdown activation cascade of MyD88, IRAK1 and IRAK2, TRAF6, NIK, and IKK and a dissociation of IkB and NF-kB in the cytoplasm (Nakanishi *et al*, 2001). This is the major mechanism of ZA-induced proinflammatory cytokine production in macrophages (Muratsu *et al*, 2013).

There are no experimental models demonstrating an association between IL-18 and IL-18 bp in BRONJ. However, the late mediators that modulate the immune response seem to participate in BRONJ pathogenesis, such as overexpression of IL-17 (Li *et al*, 2013). IL-17 is a proinflammatory cytokine related to increases in some others mediators such as IL-1β, TNF-α, and iNOS produced by macrophages and other connective tissue cells (Lee, 2013). Other experimental models showed no alterations in inflammatory bone markers (RANK/RANKL/OPG axis) in animals treated with ZA (Vasconcelos *et al*, 2012). Thus, ZA seems to modify most strongly general inflammatory markers (TNF-α, IL-1β, IL-6 (Muratsu *et al*, 2013), and IL-17 (Li *et al*, 2013)) than inflammatory bone markers. Accordingly, the role of inflammatory cells and their general mediators seems to constitute a possible direction for understanding the pathogenesis of BRONJ.

In conclusion, BRONJ is characterized by increases in empty osteocyte lacunae; osteoclast numbers (total and apoptotic); polymorphonuclear neutrophils; CD68 mononuclear-positive cells; immunostaining for TNF-α, IL-1β, iNOS, and NF-kB (nuclear and cytoplasmic) but not mast cells. BRONJ is also inversely associated with cells exhibiting IL-18 bp. More studies are needed to show that BRONJ is dependent on immune modulation.

Author contributions

Paulo Goberlânio de Barros Silva and Camila Carvalho de Oliveira performed experiments, statistical analysis, and writing of the paper. Luiz André Cavalcante Brizeno and Deysi Viviana Tenazoa Wong were responsible for histochemical and immunohistochemical reactions. Roberto César Pereira Lima Júnior and Romélia Pinheiro Gonçalves were responsible for the pharmacological study design, review of the statistical analysis, and writing of the paper. Fabrício Bitú Sousa and Mário Rogério Lima Mota were responsible for the pathological study design, review text, and writing of the paper. Ronaldo de Albuquerque Ribeiro and Ana Paula Negreiros Nunes Alves designed the model, guided experiments, and reviewed the text. All the authors read and approved the final version.

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Anexo H

International Endodontic Journal

Decision Letter (IEJ-16-00189)

From: iejeditor@cardiff.ac.uk

To: paulo_goberlanio@yahoo.com.br

CC: paulo_goberlanio@yahoo.com.br, elisalimaverde@gmail.com, luizandrebrizeno@hotmail.com, devite06@gmail.com, robertocesarpljr@gmail.com, fbitu@hotmail.com, mariolmota@yahoo.com.br, ananegreirosnunes@gmail.com

Subject: Manuscript ID IEJ-16-00189, International Endodontic Journal

Body: 19-Sep-2016

Dear Ms. Barros Silva

Manuscript ID: IEJ-16-00189

Manuscript Title: Immune cell profile of dental pulp in rats treated with zoledronic acid

I am pleased to inform you that your manuscript has been accepted for publication in the International Endodontic Journal subject to major changes being made.

The comments of the referees are included at the bottom of this letter; please revise your paper taking into account any points they have raised. Also double check that in the body of the text and in the Reference section the names of authors are spelt correctly including any non-English characters where appropriate.

You will be unable to make your revisions online using the originally submitted version of the manuscript. Instead, revise your manuscript on your PC/MAC using your word processing programme and save it on your computer. Please highlight the changes to your manuscript within the document by using the "track changes" mode in MS Word or equivalent.

To upload your revised manuscript, log on to http://mc.manuscriptcentral.com/iej and enter your Author Centre, where you will find your original manuscript title listed under "Manuscripts with Decisions". Under "Actions," click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revised manuscript, you will be able to respond to the comments made by the referees(s) in the space provided. You can use this space to document any changes you have made to the original manuscript. Please be as specific as possible in your response to the referee(s).

IMPORTANT: Your original files are available to you when you upload your revised manuscript. Please delete any redundant files that are to be replaced before completing the submission, for example, you would have to delete the file containing the main text and then upload the new version. You do not have to delete and upload files that are not being changed, for example, the Table or Figure files from the original version if they are to remain the same. **Do NOT remove the Conflict of Interest forms** these should remain with your submission throughout.

I look forward to receiving your revised manuscript.

Kind regards

Paul Dummer
Editor-in-Chief, International Endodontic Journal
iejeditor@cardiff.ac.uk

Editor comments to authors:
Editor
Comments to the Author:
This ms is of interest in principle however a major revision before publication may be considered. First off, please address all points raised by the referee. Then, please have someone proofread the paper whose first language is English and remove all first person references (We did this etc...) from the paper. The test is called Kruskal Wallis not Kruskall Wallis if you decide to resubmit.

Referee(s) comments to authors:
Referee: 1

Comments to Author
Review International Endodontic Journal
IEJ-16-00189 - Immune cell profile of dental pulp in rats treated with zoledronic acid

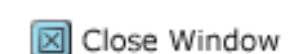
In this animal study the authors investigated the effect of intravenous infusions (four times) of a bisphosphonate in three different concentrations on the dental pulp of rats.
Overall the study design is clear and the manuscript is well defined. There are only some points that need clarification.

M&M:
In the abstract rats are mentioned and in M&M mice. Please correct this.
Why were the concentrations of ZA chosen? Is there any correlation to a therapeutic concentration in humans?
How were non-odontoblastic pulp cells defined?
Is there a possibility to investigate the if some of the ZA is bound to the dentin?
Statistic: Was a sample size calculation performed?

Discussion:
You discuss that in Cvikl et al. high concentrations of ZA cause direct toxicity. How are your concentrations in relation to the concentrations of the Cvikl et al. paper?
Furthermore there it is also described that this toxic effect disappears when ZA can bind to calcium phosphate. Please also discuss this.

Table 1:
The significance for IL-1ß is hard to follow. Would there also be a clinical significance? Please check and discuss this.

Date Sent: 19-Sep-2016



Anexo I