



**UNIVERSIDADE ESTADUAL PAULISTA  
"JÚLIO DE MESQUITA FILHO" - UNESP  
FACULDADE DE ODONTOLOGIA DE ARARAQUARA**

**Nicolau Conte Neto**

**USO DE ALENDRONATO PARA INDUÇÃO DE OSTEONECROSE  
EXPERIMENTAL: ESTUDO EM RATOS**

**Araraquara**

**2012**



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Tese apresentada ao programa de pós-graduação em Odontologia, Área de concentração Implantodontia, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista, para obtenção do título de Doutor em Odontologia.

*Orientador: Prof. Dr. Elcio Marcantonio Junior*

*Co-Orientador: Prof. Dr. Luis Carlos Spolidorio*

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**USO DE ALENDRONATO PARA INDUÇÃO DE OSTEONECROSE  
EXPERIMENTAL: ESTUDO EM RATOS**

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*Napoleon Hill*

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## **LISTA DE ABREVIATURAS**

**BFs** - Bisfosfonatos

**BPs** - Bisphosphonates

**OMAB** - Osteonecrose dos maxilares associadas aos bisfosfonatos

**BRONJ** - Bisphosphonate related osteonecrosis of the jaws

**ONJ** - Osteonecrosis of the jaws

**ALE** - Alendronato

**ALN** - Alendronate

**CTL** - Control/Controle

**CTX** - C-telopeptídeo de ligação cruzada do colágeno tipo I

**FAO** - Fosfatase alcalina específica óssea

**BALP** - Bone specific alkaline phosphatase

**BIC** - Bone-implant contact

**BAFO** - Bone area fraction occupancy

**ROI** - Region of interest

**M1** - First molars

**M2** - Second Molars

**EDTA** - Ethylenediamine tetraacetic acid

**CEJ** - Cementoenamel junction

**BV** - Bone volume

**ELISA** - Enzyme-linked immunosorbent assay

**ANOVA** - Analysis of Variance

**BRD** - Bone radiographic density

**GC** - Glucocorticoids.

**CS** - Chronic stress

**BMD** - Mineral bone density

**HPA** - Hypothalamic-pituitary-adrenal

**CRH** - Corticotrophin releasing hormone

**ACTH** - Adrenocorticotropin

**GDTC** - Gamma-delta T-cells

**IPP** - Isopentenyl pyrophosphate



Conte-Neto N. Uso de Alendronato para indução de osteonecrose experimental: estudo em ratos [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2012.

## **RESUMO**

O objetivo deste projeto foi desenvolver, em ratos, modelos experimentais de osteonecrose induzida pelos bisfosfonatos por meio da combinação de uma série de fatores de risco para esta doença, como o uso prolongado de altas doses de alendronato por via parenteral, procedimentos cirúrgicos operatórios e o estresse crônico. Os parâmetros foram estabelecidos por meio de análise histológica descritiva e por escores, análise radiográfica de alvéolos dentais, estereometria de alvéolos dentais e implantes, torque de remoção dos implantes e avaliação de marcadores do metabolismo ósseo e do estresse. No primeiro e segundo estudos foram administradas altas doses diárias ou semanais de alendronato, respectivamente, associado a exodontias dos primeiros molares inferiores. No terceiro e quarto estudos foram administradas altas doses semanais de alendronato, associado à indução de estresse crônico e instalação de implantes osseointegráveis na maxila e/ou na metáfise tibial. De um modo geral, os resultados dos estudos demonstraram que a terapia com alendronato foi associada à supressão significativa do metabolismo ósseo. Nos estudos 1 e 2, após as extrações dentais, observou-se o desenvolvimento de áreas de exposição e necrose óssea, associadas à presença de infecção significativa, especialmente na região de septo inter-dental. No estudo 3 observou-se que a indução

de estresse crônico apresentou efeitos negativos sobre o metabolismo e volume do tecido ósseo neoformado nas espiras dos implantes tibiais, os quais não foram observados nos animais tratados com alendronato. Ao contrário, nestes animais, observou-se uma melhora significativa nos parâmetros de osseointegração. Já o estudo 4 demonstrou que a administração de alendronato resultou no desenvolvimento expressivo de áreas de necrose óssea nas regiões laterais dos implantes maxilares associadas à infecção, mesmo sem evidências de exposição óssea. Conclui-se, desta forma, que a terapia com alendronato suprime o processo de remodelação óssea, apresentando, ao mesmo tempo, efeitos adversos na cavidade bucal e ações positivas sobre a osseointegração na região tibial.

**Palavras-chave:** difosfonatos; alendronato; estresse fisiológico; procedimentos cirúrgicos operatórios; osteonecrose.

Conte-Neto N. Alendronate therapy for the induction of experimental osteonecrosis: a rodents study [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2012.

## **ABSTRACT**

This study aimed to develop, in rodents, experimental models of bisphosphonates-induced osteonecrosis through the association of several risk factors to this disease, including the long-term therapy with high dosages of alendronate by parenteral route, surgical procedures and chronic stress. The parameters were established by descriptive and scored histological analysis, radiographic evaluation of alveolar sockets, stereometry of alveolar sockets and implants, torque removal of implants and biomarkers of bone metabolism and stress. In the first and second studies, it was administered daily or weekly high doses of alendronate, respectively, associated to the lower first molar extractions. In the third and fourth studies it was administered weekly high doses of alendronate plus chronic stress induction and osseointegrated implants in the maxillae and/or tibia. In general, the outcomes of this study demonstrated that alendronate therapy was associated to a markedly bone turnover suppression. In the first and second studies, after tooth extraction, it was observed the development of exposed and necrotic bone associated to a significant infectious process, especially at the inter-radicular area. In the study three the chronic stress was related to deleterious effects on the bone metabolism and volume among tibial implants threads, which weren't presented in animals treated with alendronate. On the

contrary, in these animals, it was observed a markedly increase in the osseointegration parameters. On the other hand, the fourth study showed that the alendronate treatment resulted in the substantial development of osteonecrosis regions associated to infection at lateral areas of maxillary bone implant cavity, even with absence of exposed bone areas. In this way, we conclude that the alendronate therapy suppress significantly the bone turnover, exhibiting, at the same, deleterious effects on the oral cavity and positive actions on the osseointegration at tibial region.

**Keywords:** diphosphonates; alendronate; operative surgical procedures; physiological stress; osteonecrosis

# ***1 Introdução***

Os bisfosfonatos (BFs) são compostos com estrutura química semelhante ao pirofosfato inorgânico que demonstra alta afinidade pelo tecido ósseo. Desta forma, vem sendo considerados uma das classes mais importantes de agentes anti-reabsorção, o que os tornam medicamentos indicados para o tratamento de diversas enfermidades, como osteoporose, mieloma múltiplo, doença reumáticas e neoplasias com metástases ósseas<sup>82</sup>

Dados estatísticos recentes evidenciam que as prescrições dos BFs já atingiram cifras milionárias<sup>1</sup>, o que é decorrente da alta eficiência destas drogas na estabilização da perda óssea patológica, inclusive comprovada por diversos estudos clínicos randomizados controlados<sup>2, 13, 50, 92</sup>. Entretanto, o aumento vertiginoso da utilização de fármacos torna altamente relevante o entendimento sobre os seus efeitos colaterais.

Neste contexto, os BFs são medicamentos usualmente bem tolerados, porém estão associados a diversos efeitos adversos, sendo os mais comuns a toxicidade renal e gastrointestinal, além de reações de fase aguda<sup>21</sup>. Entretanto, evidências recentes vêm associando estas drogas ao desenvolvimento de lesões graves, como o câncer gastrointestinal<sup>37, 97</sup> e a osteonecrose dos maxilares<sup>20, 96</sup>.

A osteonecrose dos maxilares associada aos bisfosfonatos (OMAB) é uma lesão agressiva que pode evoluir rapidamente e resultar em sequelas funcionais, estéticas e psicológicas importante para os pacientes. Estes aspectos são particularmente preocupantes, pois a literatura ainda permanece sob um contexto de incertezas no que se refere à patogênese, abordagens terapêuticas e preventivas para

esta doença. Por estes motivos, o desenvolvimento de modelos experimentais da OMAB em animais torna-se uma alternativa relevante e pertinente para a elucidação dos diversos aspectos relacionado a esta enfermidade.

## ***2 Revisão da Literatura***

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A revisão de literatura desta Tese é apresentada na forma de um capítulo de livro, uma seção textual e três artigos científicos, a seguir:

**2.1 Capítulo de livro** – Conte-Neto N, Bastos AS, Marcantonio RAC, Marcantonio E Jr. Osteonecrose maxilar associada aos bisfosfonatos. In: Periodontia e Implantodontia - Soluções Estéticas e Recursos Clínicos. Sallum AW, Cicareli AJ, Querido MRM, Bastos-Neto FVR. Nova Odessa: Editora Napoleão; 2010. p. 103-121.

**2.2 Seção textual complementar**- Estresse como co-factor integrante na Patogênese da OMAB.

**2.3 Artigo 1**- Conte-Neto N, Bastos AS, Spolidorio LC, Marcantonio RAC, Marcantonio E Jr. Oral bisphosphonate-related osteonecrosis of the jaws in rheumatoid arthritis patients: a critical discussion and two case reports. Head & Face Medicine. 2011; 7:1-7.

**2.3 Artigo 2** - Conte-Neto N, Bastos AS, Marcantonio RAC, Marcantonio E Jr. Is rheumatoid arthritis a risk factor for oral bisphosphonate-induced osteonecrosis of the jaws? Medical Hypotheses. 2011; 77: 905-911.

**2.4 Artigo 3** - Conte-Neto N, Bastos AS, Marcantonio RAC, Marcantonio E Jr. Epidemiological aspects of Rheumatoid Arthritis patients affected by Oral Bisphosphonate-related Osteonecrosis of the Jaws. Head & Face Medicine. 2012; 8: 2-10.



## CAPÍTULO 9

# OSTEONECROSE MAXILAR ASSOCIADA AOS BISFOSFONATOS

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Alliny de Souza Bastos<sup>2</sup>

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Os bisfosfonatos são compostos com estrutura química semelhante ao pirofosfato inorgânico, que é um regulador endógeno da mineralização óssea. Apresentam propriedade de inibição nos osteoclastos, tornando-os uma importante classe de agentes antirreabsorção utilizados para a estabilização da perda óssea ocasionada por doenças metabólicas, como osteoporose, doença de Paget, osteólise associada a neoplasias e hipercalcemia (ROGERS, GORDON, BENFORD, COXON, LUCKMAN, MONKKONEN & FRITH, 2000).

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Os efeitos benéficos expressivos dos bisfosfonatos no controle da perda óssea foram responsáveis pelo grande aumento no número de prescrições dessas medicações mundialmente. No entanto, em 2003, Marx identificou 36 casos de exposição óssea dolorosa acometendo os maxilares de pacientes que estavam fazendo uso de zolendronato e pamidronato, que são bisfosfonatos nitrogenados de alta potência administrados por via endovenosa. Diversos tratamentos foram instituídos, porém, nenhum deles resultou em remissão completa do quadro clínico. A partir de então, a literatura estava diante do mais

novo e grave efeito colateral dessas drogas, denominado osteonecrose dos maxilares associadas aos bisfosfonatos (OMAB), que vem ganhando proporções cada vez mais preocupantes em função do número crescente de casos relatados pela literatura mundial (BROOKS, GILSON, SINDLER, ASHMAN, SCHWARZ & NIKITAKIS, 2007; MAVROKOKKI, CHENG, STEIN & GOSS, 2007; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009).

## DEFINIÇÃO

Por se tratar de uma condição patológica recentemente descrita na literatura, ainda não há um consenso sobre os critérios necessários para a definição da OMAB (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007; AMERICAN COLLEGE OF RHEUMATOLOGY, 2006; KHOSLA, BURR, CAULEY *et al.*, 2007; RUGGIERO, GRALOW, MARX *et al.*, 2006; RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009). De um modo geral, a definição mais comumente aceita para OMAB envolve a presença de uma tríade de fatores, a saber: a) tratamento prévio ou atual com bisfosfonatos; b) presença de uma área de exposição óssea na região maxilofacial

que persiste por mais de oito semanas; c) ausência de tratamento radioterápico nos maxilares (KHOSLA, BURR, CAULEY *et al.*, 2007; RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009).

A importância do estabelecimento de critérios clínicos bem fundamentados reside na construção de um processo diagnóstico preciso, para exclusão de outras condições patológicas que apresentam características semelhantes, como osteomielite osteorradioneecrose, metástases tumorais, entre outros, retardando o diagnóstico correto e a instituição das medidas terapêuticas.

## INCIDÊNCIA

A partir do ano de 2003, a literatura tem relatado diversos casos de osteonecrose dos maxilares associada aos bisfosfonatos, especialmente após administração endovenosa para o controle de neoplasias malignas (BROOKS, GILSON, SINDLER, ASHMAN, SCHWARZ & NIKITAKIS, 2007; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT,

ADKINSON & BASI, 2009; MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005). Nesse grupo de pacientes, a incidência cumulativa estimada varia entre 0,8% a 12% (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009), enquanto para o uso oral de bisfosfonatos esses índices caem sensivelmente para valores que variam de 0,00038% a 0,06% dos pacientes (MAVROKOKKI, CHENG, STEIN & GOSS, 2007; AULT, 2008; FELSENBURG & HOFFMEISTER, 2006).

## ETIOPATOGENESE

Muitas teorias têm sido propostas, porém, a etiologia e a patogênese da OMAB ainda não foram elucidadas, havendo diversas indefinições no que diz respeito ao local de início (tecido ósseo ou tecido mole) e o papel dos bisfosfonatos na gênese das lesões (SILVERMAN & LANDEBERG, 2009), devido à carência de estudos prospectivos, cegos randomizados e controlados que verifiquem a relevância dessa associação. As principais teorias descritas na literatura incluem:

### SUPRESSÃO DA REMODELAÇÃO ÓSSEA

O processo de remodelação óssea é uma função fisiológica do organismo resultante de uma atuação coordenada entre os processos de reabsorção e de formação óssea, sendo fundamental para a manutenção da competência biomecânica do tecido ósseo, uma vez que substitui o tecido danificado por tecido saudável. Com a inibição dos osteoclastos pelos bisfosfonatos, o processo de reabsorção fica acentuadamente suprimido, impedindo que o osso repare os microdanos fisiológicos que ocorrem no esqueleto humano (FRITZ, FINGER & UNO, 1996).

Na cavidade bucal, essa situação é particularmente relevante dada a alta taxa de remodelação dos maxilares (ALLEN & BURR, 2008), decorrentes do estímulo constante no ligamento periodontal (MARX, SAWATARI, FORTIN & BROUMAND, 2005), da sobrecarga devido às forças mastigatórias (THEUNS, VAN DIJK, JONGEBLOED & GROENEVELD, 1983) e como resultado de procedimentos odontológicos invasivos. Dessa forma, essa demanda funcional aumentada supera a capacidade de reparo dos maxilares, culminando em acúmulo progressivo de tecido ósseo desvitalizado, originando, dessa forma, as lesões de necrose óssea.

### PROCESSO INFECCIOSO

Sedghizadeb *et al.* (2008) demonstraram em espécimes ósseos removidos de pacientes portadores de OMAB a presença de grandes áreas colonizadas com biofilme bacteriano. Embora numerosas espécies de microrganismos já tenham sido observadas, os *Actinomyces* são reconhecidos universalmente como os mais frequentes (SEDGHIZADEH, STANLEY, CALIGIURI, HOFKES, LOWRY & SHULER, 2009; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009) (Fig. 9.1).



FIG. 9.1 - Espécime ósseo removido de um paciente com OMAB que fazia uso de bisfosfonatos por via endovenosa para controle de neoplasia maligna, mostrando a presença do tecido ósseo necrótico, bem como colônias de Actinomyces.

A justificativa para essa hipótese baseia-se na capacidade do biofilme bacteriano causar reabsorção óssea patológica (SEDGHIZADEH, STANLEY, CALIGIURI, HOFKES, LOWRY & SHULER, 2009) por meio da liberação de ácidos, proteases (NAIR, MEGHJI & WILSON *et al.*, 1996) e produtos bacterianos como lipopolissacarídeos considerados mediadores da reabsorção óssea (MEGHJI, HENDERSON & NAIR *et al.*, 1997). Além disso, Kos &

Luczak (2008) observaram um aumento da adesão bacteriana ao tecido ósseo revestido pelos bisfosfonatos, por meio de uma proteína denominada componente da superfície microbiana, que reconhece moléculas de adesão da matriz e tem a capacidade de interagir com a hidroxiapatita do tecido ósseo.

Os maxilares seriam especialmente sujeitos a infecção quando comparados a outras regiões do esqueleto humano, em virtude da espessura fina de mucosa que isola o tecido ósseo do meio bucal naturalmente contaminado, suscetibilidade ao trauma e presença dos dentes (KOS & LUCZAK, 2009). No entanto, ainda não existem informações claras para elucidar se a infecção representa um evento primário ou secundário no desenvolvimento da necrose do tecido ósseo (ALLEN & BURR, 2009).

### **INIBIÇÃO DA ANGIOGÊNESE**

Estudos têm demonstrado que os bisfosfonatos apresentam propriedades antiangiogênicas, como a inibição da proliferação endotelial em cultura de células (WOO, HELLSTEIN & KALMAR, 2006), diminuição da luz do capilar e inibição do fator de crescimento endotelial vascular, *in vitro* e *in vivo* (FOURNIER, BOISSIER, FILLEUR, GUGLIELMI, CABON, COLOMBEL & CLEZARDIN, 2002), o que tem feito dessas drogas uma alternativa para conter o crescimento neoplásico por meio da supressão da angiogênese (GUISE, 2008). De acordo com essa teoria, a proliferação de células endoteliais seria inibida nos maxilares, levando à diminuição da rede vascular com consequente necrose avascular do tecido ósseo.

### **TOXICIDADE AO TECIDO MOLE**

Um dos efeitos adversos dos bisfosfonatos bem estabelecido é seu potencial de toxicidade ao tecido mole, resultando em ulcerações no trato digestivo (GONZALES-MOLES & BAGAN-SEBASTIAN, 2000). Dessa forma, altas concentrações dos bisfosfonatos no tecido ósseo dos maxilares podem resultar em toxicidade direta ao epitélio bucal, inibindo o processo cicatricial do tecido mole após procedimentos odontológicos ou outros eventos traumáticos. Assim, áreas de exposição óssea permaneceriam expostas por tempo prolongando na cavidade bucal (REID & BOLLAND, 2007; LANDESBURG, COZIN, CREMERS, WOO, KOUSTENI, SINHA, GARRETT-SINHA & RAGHAVAN, 2008). O mecanismo de comprometimento cicatricial da mucosa bucal por meio de uma interferência na sinalização molecular de fibroblastos e queratinócitos, inibindo os processos de multiplicação e migração dessas células, foi proposto por Kyrgidis *et al.* (2009).

### **HIPÓTESE GENÉTICA**

Segundo essa teoria, diferenças genéticas presentes entre os indivíduos estão associadas a respostas específicas aos bisfosfonatos em indivíduos suscetíveis (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005). Além disso, polimorfismos genéticos também têm sido implicados como fatores predisponentes (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005), como no gene da metaloproteinase 2 (MMP2), que está associado a anormalidade ósseas (LEHRER, MONTAZEM, RAMANATHAN, PESSIN-MINSLEY, PFAIL, STOCK & KOGAN, 2009) e no gene CYP2C8, que foi relacionado a um aumento de 12,5 vezes no risco de desenvolvimento de OMAB em pacientes portadores de mieloma múltiplo tratados com bisfosfonatos por via endovenosa (SARASQUETE, GONZÁLEZ, SAN MIGUEL & GARCÍA-SANZ, 2009).

## CARACTERÍSTICAS CLÍNICAS

Segundo a Associação Americana de Cirurgia Oral e Maxilofacial (AAOMS) (2009), no estágio inicial da OMAB existem apenas sinais e sintomas inespecíficos na cavidade bucal, sem evidência clínica de áreas de necrose óssea. No entanto, à medida que o quadro progride, tornam-se evidentes regiões de exposição óssea na cavidade bucal, sendo esse achado o mais frequente nos pacientes com OMAB (MARX, SAWATARI, FORTIN & BROUMAND, 2005; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; RINCÓN, RODRÍGUEZ, TAMBAY & MORENO, 2007; YAROM, YAHALOM, SHOSHANI, HAMED, REGEV & ELAD, 2007) (Fig. 9.2). A história clínica mais comum é o retardo na cicatrização de alvéolos após extrações (LEHRER, MONTAZEM, RAMANATHAN, PESSIN-MINSLEY, PFAIL, STOCK & KOGAN, 2009), entretanto, há possibilidade de ocorrência de lesões extensas de OMAB sem exposição óssea bucal, mesmo em estágios avançados (JUNQUERA & GALLEGU, 2008).

No estágio inicial, a superfície do tecido ósseo geralmente é lisa, sem a presença de margens agudas (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005). Nesse momento, usualmente não há sintomatologia dolorosa e evidência de processo infeccioso (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009), podendo permanecer assintomático por semanas ou meses (RINCÓN, RODRÍGUEZ, TAMBAY & MORENO, 2007). No entanto, à medida que a condição clínica progride, a superfície óssea se torna irregular (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005) e geralmente inicia-se a sintomatologia dolorosa, sendo esse um sintoma presente na maioria dos pacientes (MARX, SAWATARI, FORTIN & BROUMAND, 2005; LAZA-



FIG. 9.2 – Área de exposição óssea na região posterior da mandíbula de um paciente que fazia uso de bisfosfonatos por via endovenosa para controle de neoplasia maligna. Nesse paciente, as lesões desenvolveram-se de forma espontânea, uma vez que não pôde ser identificado nenhum fator causal.

ROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; YAROM, YAHALOM, SHOSHANI, HAMED, REGEV & ELAD, 2007; PAZIANAS, MILLER, BLUMENTALS, BERNAL & KOTHAWALA, 2007). A dor é usualmente decorrente da infecção secundária, que é responsável pela inflamação dos tecidos moles circunjacentes (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005), ou também pode ser devida a eventos traumáticos sobre o tecido mole (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005; KOS, KUEBLER, LUCZAK & ENGELKE, 2009). Neste estágio, também pode estar presente drenagem purulenta na cavidade bucal (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; YAROM, YAHALOM, SHOSHANI, HAMED, REGEV & ELAD, 2007).

A evolução da necrose é geralmente progressiva, havendo possibilidade de envolvimento dos dentes adjacentes, resultando em aumento da mobilidade dentária e subsequente perda do elemento (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005). Além disso, o envolvimento mandibular progressivo pode resultar em disestesia e parestesia (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007; MIGLIORATI, CASIGLIA, EPSTEIN, JACOBSEN, SIEGEL & WOO, 2006), provavelmente decorrente de compressão do nervo alveolar

inferior (MIGLIORATI, CASIGLIA, EPSTEIN, JACOBSEN, SIEGEL & WOO, 2006).

Nos estágios mais avançados da OMAB, o quadro clínico é mais grave e passa a incluir além de todos os aspectos clínicos supracitados, com presença de uma ou mais das seguintes características: fraturas patológicas, fistula extraoral, comunicação buco e nasomaxilar e osteólise que se estende para o bordo mandibular inferior ou seio maxilar (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009).

## CARACTERÍSTICAS DE IMAGEM

Os exames de imagem não representam ferramentas essenciais ao processo de diagnóstico, uma vez que não existem critérios de imagem estabelecidos para a OMAB (TREISTER, SHEEHY, BAE, FRIEDLAND, LERMAN & WOO, 2009), mas apenas achados inespecíficos que também estão presentes em outras condições como osteomielite, osteorradionecrose, metástases e doença de Paget. No entanto, esses exames são recursos importantes no sentido de fornecer informações relevantes sobre o curso, magnitude e progressão da doença (ARCE, ASSAEL, WEISSMAN & MARKIEWICZ, 2009).

Os exames de imagem mais comumente solicitados incluem as radiografias periapicais e panorâmicas (TREISTER, SHEEHY, BAE, FRIEDLAND, LERMAN & WOO, 2009; ARCE, ASSAEL, WEISSMAN & MARKIEWICZ, 2009), além de tomografias computadorizadas (YAROM, YAHALOM, SHOSHANI, HAMED, REGEV & ELAD, 2007; ARCE, ASSAEL, WEISSMAN & MARKIEWICZ, 2009; ELAD, GOMORI, BEN-AMI, FRIEDLANDER-BARENBOIM, REGEV, LAZAROVICI & YAROM, 2010). Nos estágios iniciais, é frequentemente observada uma esclerose

do tecido ósseo (ARCE, ASSAEL, WEISSMAN & MARKIEWICZ, 2009) (Fig. 2), sendo este um achado significativamente correlacionado com os aspectos clínicos da OMAB (TREISTER, SHEEHY, BAE, FRIEDLAND, LERMAN & WOO, 2009). Além disso, é comum a presença de retardo cicatricial em alvéolos pós-extração (TREISTER, SHEEHY, BAE, FRIEDLAND, LERMAN & WOO, 2009; GRO-



FIG. 9.3 – Tomografia computadorizada em cortes axiais de uma paciente que utilizou alendronato durante cinco anos, mostrando a presença de uma área de esclerose óssea na região correspondente ao segundo pré-molar inferior esquerdo. Além disso, é possível observar a presença de osteólise em torno do elemento dentário em questão.



ETZ & AL-NAWAS, 2006), espessamento do ligamento periodontal (MARX, SAWATARI, FORTIN & BROUMAND, 2005; ARCE, ASSAEL, WEISSMAN & MARKIEWICZ, 2009) e da lamina dura dentária (ARCE, ASSAEL, WEISSMAN & MARKIEWICZ, 2009), além de áreas de osteólise (RINCÓN, RODRÍGUEZ, TAMBAY & MORENO, 2007; ELAD, GOMORI, BEN-AMI, FRIEDLANDER-BARENBOIM, REGEV, LAZAROVICI & YAROM, 2010). À medida que a necrose progride, o grau de esclerose ós-

sea aumenta (ARCE, ASSAEL, WEISSMAN & MARKIEWICZ, 2009), podendo haver obliteração do canal mandibular (PHAL, MYALL, ASSAEL & WEISSMAN, 2007), reação periosteal e formações de áreas de sequestro ósseo (ELAD, GOMORI, BEN-AMI, FRIEDLANDER-BARENBOIM, REGEV, LAZAROVICI & YAROM, 2010). O envolvimento periodontal progressivo resulta em perda óssea em torno do dente envolvido (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005).

## FATORES DE RISCO

A relevância exata do papel de cofatores sobre a fisiopatologia da OMAB ainda não está bem estabelecida, mas sua presença parece desempenhar uma função importante para seu desenvolvimento (2007). Por esse motivo, é fundamental, durante o exame clínico, reconhecer e elencar todos os potenciais fatores de risco, para que mediante a realização de algum procedimento odontológico o paciente esteja informado sobre o risco de ocorrência da OMAB. Nesse sentido, os fatores que devem ser considerados incluem:

### POTÊNCIA E VIA DE ADMINISTRAÇÃO DO BISFOSFONATO

Na maioria dos casos de OMAB, os pacientes fazem uso de zolendronato e/ou pamidronato, que são bisfosfonatos nitrogenados de alta potência (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007; MAVROKOKKI, CHENG, STEIN & GOSS, 2007; MARX, SAWATARI, FORTIN & BROUMAND, 2005; THUMBIGEREMATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009; MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005; ELAD, GOMORI, BEN-AMI, FRIEDLANDER-BARENBOIM, REGEV, LAZAROVICI & YAROM,

2010). Além disso, os casos que se desenvolvem devido a medicamentos administrados por via oral, embora bem menos frequentes, usualmente estão associados ao alendronato (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007; PAZIANAS, MILLER, BLUMENTALS, BERNAL & KOTHAWALA, 2007; ELAD, GOMORI, BEN-AMI, FRIEDLANDER-BARENBOIM, REGEV, LAZAROVICI & YAROM, 2010), que é o fármaco mais potente por essa via de administração (ASSAEL, 2009). No entanto, é válido ressaltar que esses bisfosfonatos são os mais frequentemente prescritos (ASSAEL, 2009), o que obviamente aumenta o risco de ocorrência de osteonecrose induzida por essa classe.

É bem evidente a distinção entre a incidência de lesões de OMAB quando se relaciona a via de administração. Cerca de 94% dos pacientes que têm diagnósticos de OMAB fazem uso desse fármacos por via parenteral (WOO, HELLSTEIN & KALMAR, 2006). Uma das explicações para essa distinção está na biodisponibilidade da droga e nas doses utilizadas. Após administração oral, quando usualmente utilizam-se doses baixas, menos de 1% da droga é absorvida pela corrente sanguínea, ao passo que por via endovenosa estima-se que mais de 50% do fármaco esteja disponível para incorporação

pela matriz óssea (BERENSON, ROSEN, VESCIO *et al.*, 1997; 2000), culminando com um efeito inibitório potente sobre a reabsorção óssea (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005), ao passo que por via oral esses efeitos são menos severos (MARX, SAWATARI, FORTIN & BROUMAND, 2005).

Outro aspecto relevante é a indicação da via de administração dessas drogas, uma vez que a administração endovenosa é usualmente reservada para pacientes que apresentam outros cofatores possivelmente implicados na gênese da OMAB, como câncer, medicações concomitantes e morbidades médicas. Ao passo que a via oral geralmente é direcionada para a redução do risco de fratura em pacientes com osteoporose, que são indivíduos, embora mais idosos, mais saudáveis do ponto de vista de saúde geral, no que diz respeito à ausência de outros fatores de risco (ASSAEL, 2009).

## **DURAÇÃO DA TERAPIA COM BISFOSFONATO**

A duração do tratamento com os bisfosfonatos é um dos fatores mais críticos para o desenvolvimento das lesões de OMAB (BAMIAS, KASTRITIS & BAMIA *et al.*, 2005; MARX, CILLO & ULLOA, 2007), de modo que terapias de longa duração com esses fármacos têm sido associadas a riscos aumentados de necrose mandibular (BAMIAS, KASTRITIS & BAMIA *et al.*, 2005; HOFF, TOTH & ALTUNDAG *et al.*, 2006). Evidências sugerem que há uma baixa incidência de OMAB nos primeiros seis meses de tratamento com bisfosfonato (MIGLIORATI, CASIGLIA, EPSTEIN, JACOBSEN, SIEGEL & WOO, 2006), enquanto a incidência aumenta em pacientes tratados por mais de três anos com bisfosfonatos por via oral (RUGGIERO, DODSON, ASSAEL, LANDESBURG, MARX & MEHROTRA, 2009; MARX, CILLO & ULLOA, 2007). Além disso, estudos sugerem que há uma forte relação entre o tempo médio necessário para o aparecimento das lesões com via de adminis-

tração do fármaco (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007; MAVROKOKKI, CHENG, STEIN & GOSS, 2007; MARX, SAWATARI, FORTIN & BROUMAND, 2005; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009; MARX, CILLO & ULLOA, 2007). Para as drogas de uso endovenoso, como, por exemplo, o zolendronato, o tempo médio varia entre 9,4 meses e 27 meses (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007; MAVROKOKKI, CHENG, STEIN & GOSS, 2007; MARX, SAWATARI, FORTIN & BROUMAND, 2005; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009), ao passo que para os fármacos por via oral, como o alendronato, varia entre trinta e 82 meses (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007; MAVROKOKKI, CHENG, STEIN & GOSS, 2007; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009; PAZIANAS, MILLER, BLUMENTALS, BERNAL & KOTHAWALA, 2007; ASSAEL, 2009; MARX, CILLO & ULLOA, 2007).

## **AGENTE DESENCADEADOR**

Somente procedimentos cirúrgicos bucais invasivos, tais como extrações dentárias, implantes dentais e cirurgia periodontal envolvendo a manipulação de tecido ósseo podem ser considerados fatores de risco em potencial (GUTTA & LOUIS, 2007), bem como trauma decorrente de dentaduras mal adaptadas (SILVERMAN & LANDESBURG, 2009; MALDEN, BELTES & LOPES, 2009). Essa afirmação é corroborada quando se analisam os resultados dos estudos clínicos que mostram que

a vasta maioria dos casos estão associados a um procedimento odontológico invasivo, especialmente extrações dentárias (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007; MAVROKOKKI, CHENG, STEIN & GOSS, 2007; THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009; MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005; PAZIANAS, MILLER, BLUMENTALS, BERNAL & KOTHAWALA, 2007; TREISTER, SHEEHY, BAE, FRIEDLAND, LERMAN & WOO, 2009). No entanto, a OMAB também pode se desenvolver de forma espontânea, sem a identificação de uma doença bucal, tratamento ou trauma (MARX, SAWATARI, FORTIN & BROUMAND, 2005; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009).

A importância dessa distinção está no fato de que as lesões que se desenvolvem de forma espontâneas diferem clinicamente, tendendo a ser menores e mais favoráveis ao tratamento quando comparadas com as ocasionadas por procedimentos odontológicos (THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009).

A instalação de implantes osseointegrados em pacientes que fazem uso de bisfosfonatos ainda é uma questão controversa. Embora os implantes dentais tenham sido elencados como agentes desencadeadores das lesões (MARX, SAWATARI, FORTIN & BROUMAND, 2005; YAROM, YAHALOM, SHOSHANI, HAMED, REGEV & ELAD, 2007; ELAD, GOMORI, BEN-AMI, FRIEDLANDER-BARENBOIM, REGEV, LAZAROVICI & YAROM, 2010), esta parece ser uma complicação incomum após a administração oral desses fármacos, dados os altos índices de sucesso de implantes em pacientes que fazem

uso oral de bisfosfonatos (GRANT, AMENEDO, FREEMAN & KRAUT, 2008; (BELL & BELL, 2008).

## ASPECTOS ANATÔMICOS

A região posterior da mandíbula é o sítio anatômico mais afetado (MARX, SAWATARI, FORTIN & BROUMAND, 2005; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009; MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005; KOS, KUEBLER, LUCZAK & ENGELKE, 2009; ELAD, GOMORI, BEN-AMI, FRIEDLANDER-BARENBOIM, REGEV, LAZAROVICI & YAROM, 2010; MALDEN, BELTES & LOPES, 2009), especialmente a região lingual, na área da crista milo-hióide (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005); cerca de 90% das lesões que ocorrem de forma espontânea atingem essa região (THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009). A explicação para essa predileção pode ser decorrente da espessura fina de mucosa, vascularização diminuída e exostoses proeminentes frequentemente encontrada nesta área (THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009).

Alguns autores sugerem a presença de exostoses ósseas como um fator de risco, visto que há relatos de lesões de OMAB desenvolvendo-se sobre essas condições anatomicas (RUGGIERO, DODSON, ASSAEL, LANDESBURG, MARX & MEHROTRA, 2009; THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009; MALDEN, BELTES & LOPES, 2009).

## DOENÇA BUCAL

A presença de doença periodontal preexistente ou abscesso dentoalveolar crônico como fatores para o desenvolvimento espontâneo da OMAB é um aspecto controverso (RUGGIERO, DODSON, ASSAEL, LANDESBURG, MARX & MEHROTRA, 2009). No estudo realizado por Lazarovici *et al.* (2009), 83% dos casos espontâneos se desenvolveram sem a presença de uma comorbidade dental. Por outro lado, Marx *et al.* (2005) observaram que a doença periodontal estava presente em 84% dos pacientes com OMAB.

## IDADE E GÊNERO

Estudos mostram que a idade avançada é considerada um fator de risco significativo para o desenvolvimento da OMAB (BAMIAS, KASTRITIS, BAMIA, *et al.*, 2005; HOFF, TOTH & ALTUNDAG *et al.*, 2006), uma vez que a maioria dos pacientes com OMAB está acima dos sessenta anos de idade (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009; MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005; PAZIANAS, MILLER, BLUMENTALS, BERNAL & KOTHAWALA, 2007; ELAD, GOMORI, BEN-AMI, FRIEDLANDER-BARENBOIM, REGEV, LAZAROVICI & YAROM, 2010), especialmente nos casos induzidos pela administração oral de bisfosfonatos. Embora não haja uma explicação definida para essa correlação, não se pode deixar de considerar os efeitos fisiológicos nos maxilares decorrentes do envelhecimento, como a redução da vascularização e da capacidade de remodelação (MALDEN, BELTES & LOPES, 2009). Por outro lado, vale ressaltar que a maioria dos pacientes que faz uso de

bisfosfonatos por via oral é idosa, o que justificaria a não ocorrência em crianças, visto que uma pequena proporção utiliza esses fármacos (BRITISH NATIONAL FORMULARY FOR CHILDREN, 2007).

No que diz respeito ao gênero, as mulheres são frequentemente mais afetadas que os homens (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009; MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005; ELAD, GOMORI, BEN-AMI, FRIEDLANDER-BARENBOIM, REGEV, LAZAROVICI & YAROM, 2010), podendo atingir proporções de até 8:1 (PAZIANAS, MILLER, BLUMENTALS, BERNAL & KOTHAWALA, 2007). No entanto, Bamias *et al.* (2005) e Hoff *et al.* (2005) não encontraram correlação entre gênero e ocorrência de OMAB. Uma consideração relevante sobre esse assunto está no fato de que as mulheres utilizam bisfosfonatos com mais frequência que os homens (MALDEN, BELTES & LOPES, 2009), pois há uma alta incidência de câncer de mama (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007) e osteoporose entre as mulheres (PAZIANAS, MILLER, BLUMENTALS, BERNAL & KOTHAWALA, 2007).

## COMORBIDADES

De acordo com a condição sistêmica do paciente que utiliza os bisfosfonatos, podemos considerá-los de alto ou baixo risco. Os pacientes com osteoporose ou doença de Paget que são tratados com bisfosfonatos são usualmente considerados de baixo risco (MALDEN, BELTES & LOPES, 2009). Por outro

lado, o diagnóstico de câncer é um dos principais achados nos pacientes que desenvolvem a OMAB (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007; MARX, SAWATARI, FORTIN & BROUMAND, 2005; WOO, HELLSTEIN & KALMAR JR., 2006), sendo, portanto, pacientes de alto risco. No entanto, é válido ressaltar que pacientes com câncer recebem doses de doze a cinquenta vezes mais altas de bisfosfonato endovenoso quando comparados com pacientes com osteoporose que recebem esse fármaco por via oral.

Algumas condições sistêmicas têm sido associadas ao aumento adicional no risco de desenvolvimento de OMAB, como anemia, distúrbios hematológicos, imunodeficiências (ASSAEL, 2009), obesidade, diabetes (LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009) e artrite reumatóide (MALDEN, BELTES & LOPES, 2009), entre outras.

## PREVENÇÃO

### CONSIDERAÇÕES GERAIS

O estabelecimento de estratégias de prevenção, bem como da estimativa sobre o risco de desenvolvimento de OMAB, ainda representa um grande desafio, em função da ausência de critérios bem fundamentados sobre sua caracterização (SILVERMAN & LANDESBURG, 2009).

De um modo geral, tanto os pacientes que iniciarão a terapia com bisfosfonatos quanto os que já os estiverem utilizando deverão ser submetidos a uma avaliação bucal (SILVERMAN & LANDESBURG, 2009; MARX, SAWATARI, FORTIN & BROUMAND, 2005; MIGLIORATI, CASIGLIA, EPSTEIN, JACOBSEN, SIEGEL & WOO, 2006), que deve consistir de exame clínico criterioso e recursos de imagem, como radiografias periapicais e panorâmicas (MARX, SA-

### MEDICAÇÕES IMUNOSSUPRESSORAS

A maioria dos pacientes que desenvolve lesões de OMAB passa por tratamento prévio ou atual com agentes quimioterápicos (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007; MARX, SAWATARI, FORTIN & BROUMAND, 2005; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; YAROM, YAHALOM, SHOSHANI, HAMED, REGEV & ELAD, 2007; MALDEN, BELTES & LOPES, 2009), o que pode ser confirmado pelo trabalho de Marx *et al.* (2005), que concluiu que 97,5% dos pacientes apresentavam história de quimioterapia. No entanto, esses pacientes não utilizam somente agentes quimioterápicos, mas também outras medicações, como esteróides, que também estão implicados como fatores de risco (MARX, SAWATARI, FORTIN & BROUMAND, 2005; YAROM, YAHALOM, SHOSHANI, HAMED, REGEV & ELAD, 2007; MALDEN, BELTES & LOPES, 2009).

WATARI, FORTIN & BROUMAND, 2005), a fim de avaliar o estado de saúde dos maxilares e quantificar todos os fatores de risco em potenciais para cada indivíduo (CAPSONI, LONGHI & WEINSTEIN, 2006). Em todas as consultas, é imprescindível enfatizar todas as orientações sobre a manutenção da higiene bucal (SILVERMAN & LANDESBURG, 2009), incluindo técnicas de escovação, utilização de fio dental e soluções antibacterianas bucais.

No que diz respeito ao tratamento odontológico, procedimentos restauradores, protéticos e profiláticos podem ser realizados sem aumento adicional no risco de desenvolvimento das lesões OMAB. Quanto à endodontia, há certa controvérsia na literatura; embora o tratamento de canal não envolva

manipulação de tecido ósseo, já foi considerado um fator desencadeador de OMAB (THUMBIGEREMATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU, HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009).

## **PACIENTES QUE UTILIZARÃO BISFOSFONATOS**

Para os pacientes que iniciarão a terapia com bisfosfonatos, o objetivo do tratamento odontológico é a eliminação de focos infecciosos (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; MARX, SAWATARI, FORTIN & BROUMAND, 2005; MIGLIORATI, CASIGLIA, EPSTEIN, JACOBSEN, SIEGEL & WOO, 2006), evitando a necessidade de procedimentos cirúrgicos invasivos após o início da utilização desses fármacos (MARX, SAWATARI, FORTIN & BROUMAND, 2005).

Do ponto de vista periodontal, é importante a eliminação de bolsas para reduzir o acúmulo de biofilme, minimizar a inflamação periodontal crônica e as infecções periodontais agudas (MIGLIORATI, CASIGLIA, EPSTEIN, JACOBSEN, SIEGEL & WOO, 2006), uma vez que a doença periodontal foi correlacionada como um fator de risco para a OMAB (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; MARX, SAWATARI, FORTIN & BROUMAND, 2005).

Os dentes impactados completamente recobertos por tecido ósseo ou tecido mole devem ser mantidos, já os que estiverem em comunicação com a cavidade bucal devem ser removidos. Além disso, exostoses linguais pequenas podem ser mantidas, ao passo que as exostoses mandibulares grandes e palatinas medianas devem ser removidas (MARX, SAWATARI, FORTIN & BROUMAND, 2005).

No caso da realização de procedimentos odontológicos cirúrgicos, o ideal é, caso as condições sistê-

micadas do paciente permitam, aguardar o início da terapia até que haja completa epitelização ou cicatrização óssea adequada (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009). Os procedimentos cirúrgicos são usualmente associados a profilaxia antibiótica com penicilina ou quinolona mais metronidazol no caso de alergia a penicilina (MARX, SAWATARI, FORTIN & BROUMAND, 2005; YAROM, YAHALOM, SHOSHANI, HAMED, REGEV & ELAD, 2007).

## **PACIENTES EM USO DE BISFOSFONATOS**

Para os pacientes que já estão fazendo uso de bisfosfonatos, as consultas odontológicas devem ser mais frequentes, com avaliações criteriosas no sentido de identificar áreas de exposição óssea na cavidade bucal ou algum indício nos exames de imagem que possa sugerir OMAB (MARX, SAWATARI, FORTIN & BROUMAND, 2005).

Nesse momento, qualquer procedimento odontológico cirúrgico eletivo deve ser evitado (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; MARX, SAWATARI, FORTIN & BROUMAND, 2005; ASSAEL, 2009), embora, segundo a AAOMS (2009), cirurgias odontológicas eletivas não pareçam ser contraindicadas para pacientes que fazem uso oral do fármaco. No caso de dentes sem possibilidade de restauração, opte-se pelo tratamento endodôntico e amputação da coroa (MARX, SAWATARI, FORTIN & BROUMAND, 2005; MIGLIORATI, CASIGLIA, EPSTEIN, JACOBSEN, SIEGEL & WOO, 2006), e para os dentes com mobilidade grau 1 ou 2 recomenda-se a esplintagem desses elementos (MARX, SAWATARI, FORTIN & BROUMAND, 2005; CAPSONI, LONGHI & WEINSTEIN, 2006).

Para as situações em que a exodontia é indicada, recomenda-se a utilização de técnicas atraumáticas associadas à antibioticoterapia, quando

indicada, (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005) mantendo-se bochechos com gluconato de clorexidina a 0,12% até a cicatrização do alvéolo (AMERICAN DENTAL ASSOCIATION COUNCIL ON SCIENTIFIC AFFAIRS, 2006). O fechamento primário não é considerado imperativo, especialmente se houver necessidade de grandes descolamentos do periosteio (MALDEN, BELTES & LOPES, 2009). No caso de tratamento periodontal, recomendam-se apenas raspagens supragengivais (CAMPISI, DI FEDE, MUSCIOTTO, LO CASTO, LO MUZIO, FULFARO, BADALAMENTI, RUSSO & GEBBIA, 2007).

Segundo a AAOMS (2009), caso as condições sistêmicas permitam, os bisfosfonatos orais devem ser suspensos três meses antes do procedimento cirúrgico, devendo-se retornar após três meses (AAOMS, 2009). No entanto, a interrupção do fármaco é um tópico controverso entre os autores e será discutido posteriormente neste capítulo.

## TRATAMENTO

O tratamento dos pacientes com osteonecrose induzida por bisfosfonatos tem gerado uma grande discussão na literatura e atualmente ainda não existem dados científicos que justifiquem nenhum protocolo de tratamento. Diversas modalidades terapêuticas vêm sendo empregadas, havendo relatos de sucesso e insucesso para cada uma delas. As abordagens mais comumente utilizadas incluem:

### TRATAMENTO CONSERVADOR

Essa modalidade terapêutica é usualmente reservada para os estágios iniciais de OMAB (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009) e inclui a utilização de antibióticos (MARX, SAWATARI, FORTIN &

### USO DE MARCADORES BIOQUÍMICOS DO METABOLISMO ÓSSEO

Os marcadores bioquímicos do metabolismo ósseo são elementos liberados no organismo a partir dos processos de reabsorção e formação óssea. A avaliação desses elementos tem sido frequentemente utilizada para monitorar as pequenas mudanças no metabolismo ósseo. Dentre esses marcadores, o C-telopeptídeo do colágeno (CTX) é um indicador da reabsorção óssea que foi considerado por Marx *et al.* (2007) como um indicador de risco de OMAB, sugerindo que valores abaixo de 100 pg/ml representam alto risco, de 100-150 pg/ml risco intermediário e acima de 150 pg/ml baixo risco. No entanto, a ausência de dados científicos fundamentados e a presença de níveis normais do CTX em pacientes com OMAB (LEHRER, MONTAZEM, RAMANATHAN, PESSIN-MINSLEY, PFAIL, STOCK & KOGAN, 2008) compromete sua utilização como indicador confiável e de rotina para estabelecimento do risco de OMAB (DON-WAUCHOPE & COLE, 2009).

BROUMAND, 2005; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005; RINCÓN, RODRÍGUEZ, TAMBAY & MORENO, 2007) usualmente associados com soluções antibacterianas tópicas, mais comumente o gluconato de clorexidina a 0,12% (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005; RINCÓN, RODRÍGUEZ, TAMBAY & MORENO, 2007) e analgésicos, uma vez que um dos objetivos do tratamento da OMAB é o controle dos sintomas algícos e da infecção secundária (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009).

Segundo os defensores dessa abordagem, qualquer intervenção cirúrgica, como sequestrectomia e debridamento, deve ser evitada (MARX, SAWATARI, FORTIN & BROUMAND, 2005; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009), uma vez que esses procedimentos não apresentam resultados satisfatórios, levando a novas exposições ósseas, piora nos sintomas e risco de fraturas patológicas, pois na OMAB todo o tecido ósseo é afetado, sendo inviável a obtenção de margens ósseas vitais (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; MARX, SAWATARI, FORTIN & BROUMAND, 2005).

O antibiótico de escolha para os casos de OMAB é a penicilina, pois os microrganismos mais frequentes nas lesões são sensíveis ao espectro desses antibióticos (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; MALDEN, BELTES & LOPES, 2009). Para os pacientes alérgicos a penicilina podem ser utilizados quinolonas (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; MARX, SAWATARI, FORTIN & BROUMAND, 2005; YAROM, YAHALOM, SHOSHANI, HAMED, REGEV & ELAD, 2007), metronidazol (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; MARX, SAWATARI, FORTIN & BROUMAND, 2005; YAROM, YAHALOM, SHOSHANI, HAMED, REGEV & ELAD, 2007; MALDEN, BELTES & LOPES, 2009), doxiciclina (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; MALDEN, BELTES & LOPES, 2009), eritromicina (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009) e clindamicina (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; CARLSON & BASILE, 2009).

No entanto, a clindamicina não é recomendada por alguns autores, uma vez que seu espectro de ação não atinge as bactérias mais frequentemente encontradas (MARX, SAWATARI, FORTIN & BROUMAND, 2005; RINCÓN, RODRÍGUEZ, TAMBAY & MORENO, 2007).

O tempo de administração dos antibióticos pode variar de quatro semanas a meses (LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009; STANTON & BALASANIAN, 2009), devendo ser utilizados até a resolução dos sinais inflamatórios (STANTON & BALASANIAN, 2009). Para os casos refratários podem ser necessários antibióticos por via endovenosa (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009) ou a associação entre eles (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009).

## TRATAMENTO CIRÚRGICO

Não há um consenso na literatura quanto ao momento de intervenção cirúrgica, sendo indicada tanto para os casos em estágios iniciais (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005) quanto avançados da doença (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009). Segundo Ruggiero *et al.* (2004), o tratamento cirúrgico é recomendado apenas para pacientes sintomáticos, incluindo aqueles com fraturas patológicas e que não respondem ao tratamento conservador. Por outro lado, Stanton & Balasanian, (2009) acreditam que a cirurgia é benéfica não somente como alívio dos sintomas e controle da infecção, mas também para alcançar a completa resolução do processo. As modalidades cirúrgicas mais comumente utilizadas incluem debridamento, sequestrectomia (MIGLIORATI, CASIGLIA, EPSTEIN, JACOBSEN, SIEGEL & WOO, 2006) e res-



secções dos maxilares (RUGGIERO, DODSON, ASSAEL, LANDESBURG, MARX & MEHROTRA, 2009; CARLSON & BASILE, 2009).

O debridamento cirúrgico consiste na remoção do tecido ósseo necrosado de forma superficial ou mais invasiva. O debridamento superficial compreende a regularização da superfície óssea irregular, eliminando arestas ósseas agudas que possam irritar os tecidos moles (RUGGIERO, DODSON, ASSAEL, LANDESBURG, MARX & MEHROTRA, 2009; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009); RINCÓN, RODRÍGUEZ, TAMBAY & MORENO, 2007; YAROM, YAHALOM, SHOSHANI, HAMED, REGEV & ELAD, 2007; MIGLIORATI, CASIGLIA, EPSTEIN, JACOBSEN, SIEGEL & WOO, 2006). Já o debridamento mais agressivo (Fig. 9.4) requer a eliminação mais extensa do osso necrosado primando a obtenção de uma superfície óssea sangrante (STANTON & BALASANIAN, 2009; MARKOSE, MACKENZIE, CURRIE & HISLOP, 2009), optando pelo fechamento primário sempre que possível (MIGLIORATI, SHUBERT, PETERSON & SENEDA, 2005; RINCÓN, RODRÍGUEZ, TAMBAY & MORENO, 2007; CARLSON & BASILE, 2009; MARKOSE, MACKENZIE, CURRIE & HISLOP, 2009). No caso de dentes sintomáticos dentro do osso envolvido, deve ser considerada a extração, pois não há indicação de que a extração irá exacerbar o processo de necrose (RUGGIERO, DODSON, ASSAEL, LANDESBURG, MARX & MEHROTRA, 2009).

A sequestrectomia consiste na remoção de fragmentos ósseos necróticos móveis, que representam fonte constante de irritação. Essa técnica deve ser realizada independentemente do estágio da OMAB e sem a exposição do tecido ósseo vital (RUGGIERO, DODSON, ASSAEL, LANDESBURG, MARX & MEHROTRA, 2009; LAZAROVICI, YAHALOM, TAICHER, ELAD, HARDAN & YAROM, 2009).

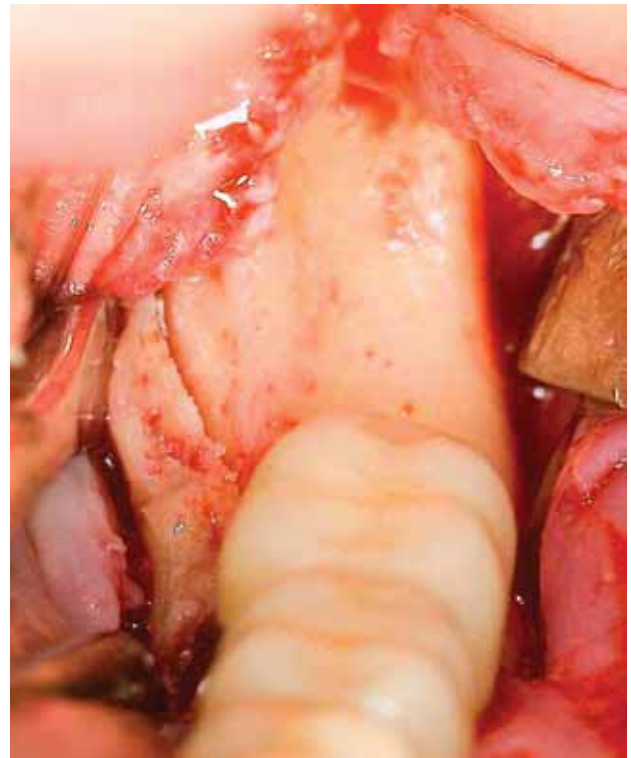


FIG. 9.4 – Debridamento cirúrgico de uma lesão de OMAB localizada na região posterior da mandíbula de um paciente que utilizava bisfosfonato por via endovenosa para controle de neoplasia maligna. Ocasionalmente, não é possível obter margens ósseas sangrantes com essa técnica, observando o aspecto avascular do tecido ósseo.

Para alguns autores, os procedimentos cirúrgicos mais conservadores, como debridamento e sequestrectomia, não apresentam resultados favoráveis, uma vez que não conseguem eliminar totalmente o osso necrótico, não alcançado margens ósseas saudáveis. Dessa forma, sugere-se que apenas procedimentos mais agressivos como a ressecção dos maxilares são efetivos na eliminação de todo o osso necrótico, obtenção de margens ósseas vitais e resolução da OMAB (CARLSON & BASILE, 2009). No entanto, para Migliorati *et al.* (2005), em estágios avançados a cirurgia óssea agressiva não está associada a resultados favoráveis, uma vez que a tentativa de estabelecer margens ósseas vitais acaba resultando no desenvolvimento de grandes defeitos de osso necrótico.

## SUSPENSÃO PROVISÓRIA OU DEFINITIVA DA DROGA

Não existem dados científicos suficientes que justifiquem a efetividade da suspensão do fármaco como medida preventiva ou terapêutica após o surgimento das lesões (VIEILLARD, MAES, PENEL, FACON, MAGRO, BONNETERRE & CORTET, 2007). Alguns estudos mostraram efeitos benéficos com a suspensão do bisfosfonato (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; MARX, CILLO & ULLOA, 2007; MARKOSE, MACKENZIE, CURRIE & HISLOP, 2009), inclusive a resolução completa das lesões de OMAB espontaneamente (MARX, CILLO & ULLOA, 2007) ou após o procedimento cirúrgico (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009), justificando com o fato de que os bisfosfonatos podem exercer efeitos em curto prazo, especialmente nos vasos sanguíneos. Por outro lado, outros estudos não observaram diferença significativa (THUMBIGERE-MATH, SABINO, GOPALAKRISHNAN, HUCKABAY, DUDEK, BASU,

HUGHES, MICHALOWICZ, LEACH, SWENSON, SWIFT, ADKINSON & BASI, 2009), ou até sugerem que a descontinuação do fármaco possa ser inútil, uma vez que essa droga persiste ligada ao tecido ósseo por vários anos (MARX, SAWATARI, FORTIN & BROUMAND, 2005).

Quanto ao tempo de suspensão dos bisfosfonatos, não há um consenso definido. Previamente aos procedimentos cirúrgicos, esse tempo varia entre dois e três meses (RUGGIERO, DODSON, ASSAEL, LANDESBERG, MARX & MEHROTRA, 2009; MALDEN, BELTES & LOPES, 2009; STANTON & BALASANIAN, 2009). Durante o pós-operatório, o paciente não deverá retornar com a medicação até que haja cicatrização total da ferida (MALDEN, BELTES & LOPES, 2009; STANTON & BALASANIAN, 2009). Desnecessário dizer que a suspensão da medicação deve ser realizada apenas se as condições sistêmicas do paciente permitirem e quando os benefícios superarem os riscos, sendo uma decisão eminentemente médica de acordo com o quadro clínico de cada paciente.

## CONCLUSÃO

A cada dia que passa, novos casos de OMAB vêm sendo relatados na literatura, e esse aumento crescente é particularmente preocupante em função do número também crescente de prescrições de bisfosfonatos mundialmente. A ausência de estratégias de prevenção e medidas terapêuticas bem fundamenta-

das reforçam a relevância desse problema, tornando-se fundamental que as classes médica e odontológica conheçam o mais grave e recente efeito adverso dos bisfosfonatos, havendo necessidade de estudos prospectivos, cegos, controlados e randomizados para elucidar todos os aspectos pertinentes a OMAB.

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### **Estresse como co-factor integrante na Patogênese da OMAB**

Estresse pode ser definido como um estado de tensão que causa uma ruptura no equilíbrio interno do organismo, que ocorre diante da necessidade de resposta à alguma demanda que ultrapassa sua capacidade adaptativa<sup>25</sup>. A importância dos agentes estressores é amplamente reconhecida, pois concorrem para o desenvolvimento de diversas condições patológicas, como disfunções metabólicas, desordens emocionais, distúrbios alimentares e doenças cardiovasculares<sup>24</sup>

No que diz respeito aos tecidos bucais, trabalhos mostram que o estresse interfere negativamente sobre a evolução da doença periodontal induzida em ratos<sup>94</sup>, além de retardar significativamente a cicatrização alveolar após exodontias em ratos<sup>78</sup>.

Neste contexto, pode ser especulado que o estresse também possa representar um co-fator para o desenvolvimento da OMAB. As justificativas para esta hipótese baseiam-se na correlação dos efeitos fisiológicos do estresse com as principais teorias que procuram explicar a patogênese da OMAB, a saber:

#### **Correlação do estresse com a teoria infecciosa**

O estresse crônico pode deprimir a resposta celular imune, por meio da modulação do sistema neural e endócrino envolvendo mecanismos diferentes que incluem a liberação de glicocorticóides (GC)<sup>87</sup>, além da ativação do sistema nervoso simpático resultando na liberação de noradrenalina e adrenalina, que, por sua vez, apresentam efeitos imunossupressores<sup>88</sup>.

Desta forma, em estados de imunossupressão, há um aumento da suscetibilidade ao desenvolvimento de processos infecciosos. Esta situação é particularmente especial nos maxilares, em virtude da espessura fina de mucosa que isola o tecido ósseo do meio bucal naturalmente contaminado, suscetibilidade ao trauma e presença dos dentes<sup>53</sup>.

### **Correlação do estresse com a teoria angiogênica**

Segundo Weinstein et al.<sup>100</sup> os GC apresentam efeitos adversos na circulação sanguínea em função de uma diminuição da angiogênese causada pela supressão da produção de VEGF. Dessa forma, a associação entre níveis elevados de GC decorrente do estresse crônico associado com os bisfosfonatos, que também apresentam propriedades anti-angiogênicas<sup>30</sup>, poderia resultar em uma supressão excessiva da vascularização, levando ao comprometimento da circulação sanguínea nos maxilares e conseqüente necrose isquêmica.

### **Correlação do estresse com a teoria da supressão da remodelação óssea**

Dentre os efeitos dos GC, destacam-se as suas ações supressoras importantes sobre a remodelação óssea, por meio do retardo na aposição óssea com diminuição do tecido osteóide, decorrente da indução de apoptose nos osteoblastos e osteócitos<sup>75</sup>. Diante disso, pode ser especulado que a associação do estresse e bisfosfonatos, que também apresentam ações supressoras sobre o metabolismo ósseo<sup>82</sup>, poderia resultar em uma inibição relevante sobre a remodelação óssea, o que poderia resultar no acúmulo de áreas de necrose óssea.



CASE REPORT

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# Oral bisphosphonate-related osteonecrosis of the jaws in rheumatoid arthritis patients: a critical discussion and two case reports

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## Abstract

**Background:** Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a clinical condition characterized by the presence of exposed bone in the maxillofacial region. Its pathogenesis is still undetermined, but may be associated with risk factors such as rheumatoid arthritis (RA). The aim of this paper is to report two unpublished cases of BRONJ in patients with RA and to conduct a literature review of similar clinical cases with a view to describe the main issues concerning these patients, including demographic characteristics and therapeutic approaches applied.

**Methods:** Two case reports of BRONJ involving RA patients were discussed

**Results:** Both patients were aging female taking alendronate for more than 3 years. Lesions were detected in stage II in posterior mandible with no clear trigger agent. The treatment applied consisted of antibiotics, oral rinses with chlorhexidine, drug discontinuation and surgical procedures. Complete healing of the lesions was achieved.

**Conclusions:** This paper brings to light the necessity for rheumatologists to be aware of the potential risk to their patients of developing BRONJ and to work together with dentists for the prevention and early detection of the lesions. Although some features seem to link RA with oral BRONJ and act as synergistic effects, more studies should be developed to support the scientific bases for this hypothesis.

## Background

Bisphosphonates (BPs) are a class of drugs commonly prescribed for bone diseases due to their osteoclast inhibition property. This class of drugs has been widely used for osteoporosis and corticosteroid-induced osteoporosis in patients with rheumatoid arthritis (RA). However, reports of bone necrosis induced by bisphosphonates (BRONJ) have generated great concern regarding the side effects of these drugs. Although RA has been considered a risk factor for this kind of osteonecrosis [1,2], the relationship between these diseases has not, until now, been completely elucidated.

The aim of this paper is to report two unpublished cases of BRONJ in non-neoplastic patients with RA and

to conduct a literature review of similar clinical cases with a view to describing the main issues related to these patients, including demographic characteristics and therapeutic approaches.

## Case 1

A 58-year-old woman presented herself at a private dental clinic in December, 2008, complaining about an intense spontaneous pain in the mandibular right side after a prosthesis replacement in an implant area that was installed sixteen years previously. The review of the patient's medical history revealed that she started a therapy with Fosamax<sup>®</sup> (alendronate sodium) 70 mg, once a week for the treatment of rheumatoid arthritis in 2004. The patient had no history of smoking, radiotherapy, infectious process or trauma in the maxillo-facial region, and the dental implant presented normally until the symptoms began.

Upon clinical examination, a mild erythema was evident in the mucosa surrounding the distally right dental

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**Figure 1 Clinical aspect of the BRONJ lesion.** Mucosal erythema surrounding the distally right implant associated with an increase on probing depth values with no gingival recession or bone exposure.

implant, without clinical evidence of purulent discharge, gingival recession or bone exposure. However, probing revealed increasing depth values and detachment of the mucosa from the periimplantar bone with biological seal loss was observed (Figure 1). A computed tomography (CT) was requested and showed a substantial radiolucency around the involved dental implant, featuring loss of the crestal bone. (Figure 2)

Periimplantitis was the primary hypothesis considered at that time, but BRONJ was also considered. The initial treatment plan was mouth-rinsing with chlorhexidine 0.12% four times a day and antibiotic therapy with Clindamycin 300 mg twice a day for 10 days, since the patient had allergy for  $\beta$ -lactam antibiotics. Surgical decontamination of the implant surface was also planned; however, upon mucosal flap incision, there was no indication of any exposition of implant threads, but there was a large zone of necrotic bone forming a sequestrum area (Figure 3a). Therefore, it was opted to removal of the implant with sequestrectomy and debridement (Figure 3b) until a bleeding bone was observed (Figure 3c). An interrupted suture was made with 4-0 silk in an attempt to close the wound primarily without

tension. After medical consensus, alendronate was suspended.

The bone specimen obtained was fixed, processed and paraffin embedded for histological analysis. Hematoxylin and eosin (H&E) staining was used for histological observation by light microscopy. The results revealed necrotic lamellar bone fragments with chronic and acute inflammatory cells, as well bacterial colonies (Figure 4).

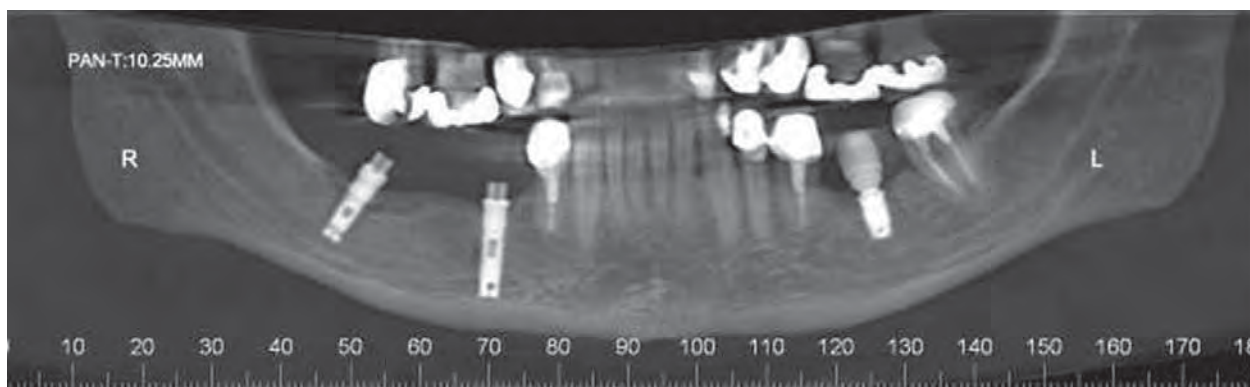
In addition, the serum C-terminal cross-linking telopeptide of collagen (CTX) test to evaluate the bone reabsorption status was solicited and revealed normal values (250 pg/mL), but this exam was performed only 4 months after surgical treatment. The healing progressed uneventfully and the patient displayed no symptoms at 8 months of postoperative time.

Observations in the clinical examination showed that the soft tissue was with normal aspect and without any signs of inflammatory or infectious processes (Figure 5)

## Case 2

A 68-year-old woman was admitted to a private dental clinic in October 2009, complaining about cold tooth sensation in the region of left mandibular second premolar. Review of the patient's medical history revealed that since 2003, she had been taking 2.5 mg of methotrexate six times a week and 70 mg of Fosamax<sup>®</sup> (alendronate sodium) once a week for the treatment of rheumatoid arthritis. Besides, she also reported steroids use during twenty years. The patient had no history of radiotherapy, infectious process or trauma in the maxillofacial region but did have a history of smoking.

During clinical examination a detachment of the marginal gingival (Figure 6a) associated with an increased probing depth value at the region of left mandibular second premolar (Figure 6b) was observed and was associated with a mild mobility without painful symptoms, purulent discharge and bone exposure. On



**Figure 2 Imaging aspect of the BRONJ lesion.** Computed tomography showing a radiolucency with aspect of loss of crestal bone around the right distally implant.



**Figure 3 Surgical approach of the BRONJ lesion.** A) Surgical exposition of the distally right implant showing a large bone sequestrum around the dental implant; B) Sequestrectomy of the bone necrosis around the dental implant; C) Surgical area after the debridement showing a bone bleeding surface associated with the dental implant removal.

periapical radiographic analysis, there was bone loss associated with osteosclerosis around the involved tooth (Figure 7a). At that time, mouth-rinsing with chlorhexidine 0.12% was prescribed and, after medical consensus, alendronate suspension was recommended. Furthermore, the serum C-terminal cross-linking telopeptide of collagen (CTX) test was solicited to evaluate the bone reabsorption status which revealed values of 33 pg/mL.

Two weeks later, during clinical examination, bone exposure was detected on the vestibular side of the left mandibular second premolar and on the disto-lingual side of the edentulous alveolar bone surrounded by inflamed soft tissue without evidence of purulent discharge or pain symptoms (Figure 8). However, the lesions progressed very quickly and, the patient complained of painful symptoms and increased tooth mobility few days later. Bone necrosis associated with mucosa ulceration involving part of the jugal mucosa was also observed (Figure 9a). On periapical radiographic analysis, it was observed increased bone loss around the involved tooth (Figure 7b) which was confirmed on computed tomography (CT) since an osteolysis area was observed around the left mandibular second premolar associated with an intense bone sclerosis (Figure 10). Given these observations, a diagnosis of BRONJ could be established.

The management of the case included the tooth extraction and bone debridement under local anesthesia

(Figure 9b and 9c), and mouth rinses with chlorhexidine plus antibiotic therapy with Clavulin 500 mg three times a day was prescribed. Within fourteen days, the formation of granulation tissue could be noted on the surgical area with no signs of inflammation or infection (Figure 11a). After two months, the debrided region was covered by normal mucosa with no painful symptoms (Figure 11b).

#### Discussion

Rheumatoid arthritis is a systemic autoimmune disease characterized by progressive joint destruction and a variety of systemic manifestations resulting from chronic inflammation [3], which has been considered a risk factor for the development of BRONJ [1,2]. Although no scientific link has been established between BRONJ and RA, some relevant factors that could link these diseases should be discussed. These factors include inflammatory alterations and drugs prescribed for these patients, including steroids and immunosuppressive agents, such as methotrexate [4], that seem to play a relevant role in the development of oral BRONJ.

The relevance of steroids and methotrexate in BRONJ pathogenesis still remains not fully understood. However, considering that the main disease theories are based on the suppression of bone remodeling, the angiogenesis-inhibitory properties of the bisphosphonate and



**Figure 4 Histological aspects of bone samples.** A) H & E stained section showing bone necrosis (Original magnification  $\times 40$ ); B) Gram stained section showing gram negative and positive bacteria (Original magnification  $\times 100$ )



**Figure 5 Clinical aspects of the BRONJ lesions after treatment.** Post operatory of 9 month showing a mucosa with normal aspect without signals of inflammatory process or bone exposure



**Figure 6 Initial clinical aspects of the BRONJ lesion.** A) Detachment of the marginal gingiva at the vestibular and distal side of # 35; B) Probing in the vestibular side of #35 showing increased probing depth values.

the infectious process [5] are factors that could be related to BRONJ; however, none of these theories have been completely accepted.

Hypothetical factors linked with BRONJ include a possible excessive suppression of bone turnover and jaw angiogenesis resulting from the association between bisphosphonates and steroids, since these drugs also reduce bone remodeling [6] and angiogenesis [7]. In addition, the immunosuppressive effects of steroids and methotrexate [8] could leave these patients more prone to infections.

In this discussion, observations that support and at the same time argue against this hypothetical association are made, especially in relation to steroid treatment. First of all, although a large number of patients with RA that develop oral BRONJ have a history of steroids and methotrexate intake [4,9-12] (as in case 2), this disease also occurs among patients with RA without the use of these drugs [9,13,14] (as in case 1). Second, it is well known that steroids can induce bone necrosis, but this necrosis differs from BRONJ because the steroids affect predominantly long bones and almost never produce bone exposure [15]. Finally, animal models of BRONJ have been



**Figure 7 Radiographic progression of bone loss in the BRONJ lesion.** A) Periapical radiographic showing bone loss associated with osteosclerosis around the #35; B) Periapical radiography showing increased bone loss around the #35.



**Figure 8 Clinical progression of the BRONJ lesions.** A) Bone exposure of the #35 on the vestibular side; B) Bone exposure on the disto-lingual side of the edentulous alveolar bone surrounded by inflamed soft tissue.

proposed to test the association of bisphosphonate and steroids [16].

Recent tendencies included BPs among the most frequently prescribed drugs in rheumatologic practice [17] especially due to the high efficiency of BPs to be a protection against generalized bone loss [18]. In this way, patients with RA have been taking BPs to the prevention and treatment of osteoporosis which is a common feature in RA for several reasons including: post-menopausal women are the main risk group for RA and are at risk for accentuated bone loss; steroid therapy is often prescribed for the treatment of RA; physical inactivity is characteristic of RA due to disease activity; and bone loss due to disease inflammatory mechanisms, such as systemic elevated cytokines [19]. For these reasons, it is reasonable to believe that the incidence of BRONJ will increase as a result of the long-term use of BPs.

Regarding the link between inflammation and BRONJ, it is well known that extraarticular structures also can be affected by the inflammatory process in RA [20]. Considering that this disease is characterized by persistent high levels of proinflammatory cytokines [21] and accumulation of inflammatory cells [20], a link factor can be hypothesized based on the observations made by Lesclous et al. [22], who stated that BRONJ is associated with inflammation and that the clinical extension of the lesions is associated with the number of inflammatory cells.

According to the cases reported in literature, patients with RA who develop BRONJ lesions after oral administration of BPs are usually women, above 60 years old, who have taken alendronate for more than 3 years. The mandible is the most common site of BRONJ in these patients. The cases reported here are in agreement with this profile, except that the patient described in case 1 is younger than 60 years old. Pazianas et al. [23] have made the interesting observation that these features have exactly the same characteristics for patients without RA that develop oral BRONJ.

Most of the oral BRONJ cases in patients with or without RA are triggered by invasive dental procedures, such as extractions and dental implants. However, other



**Figure 9** Imaging aspect of the BRONJ lesion. **A)** Computed tomography showing an irregular radiolucency at the left side of the mandible and a persistent alveolus of a molar that was extracted at least 10 years previously; **B)** Osteolysis around the left mandibular second premolar.

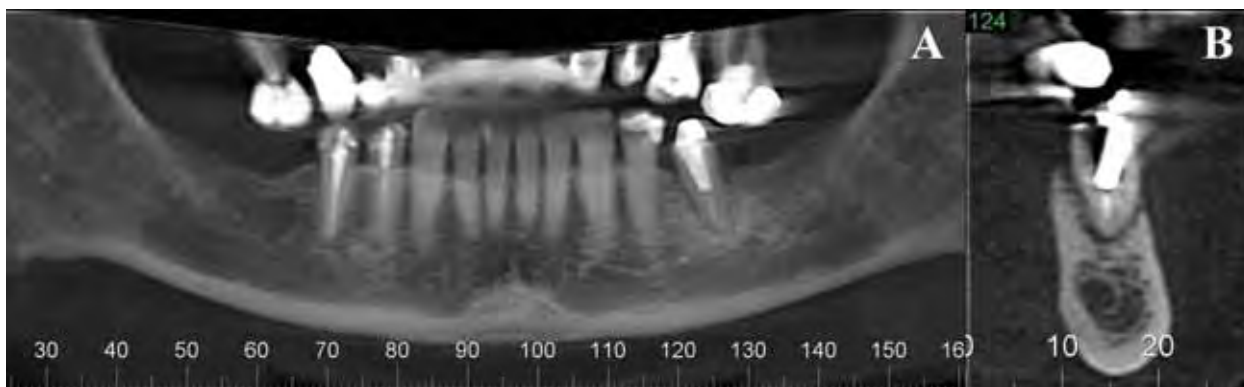
cases of BRONJ can be spontaneous [1,10,12] as seen in the cases reported in the present paper. However, some concerns should be discussed. In case 1, although no apparent precipitant factor was present, trauma may have been a trigger event [24]. An eventual occlusal overload on the prosthesis might have contributed to BRONJ, because pain symptoms appeared soon after the prosthesis replacement.

Another relevant factor is seen in case 2. Although there was no previous dentistry procedure, the patient had periodontal disease. Periodontal disease has been considered by some authors to be a trigger event [25] due to the fact that this disease could increase the potential quantity of BPs released. However, this theory is still controversial [26]. An interesting observation is that individuals with rheumatoid arthritis are more likely to experience moderate to severe periodontal disease compared to their healthy counterparts [27]. This clinical association between the two diseases might be due to a common underlying pathobiology of periodontitis and rheumatoid arthritis [28].

The main clinical aspects of patients with RA who develop oral BRONJ include bone exposure, edema, pain

and purulent discharge [9-11,13,29,30]. These features represent stage 2, as described by Ruggiero et al. [26], and indicate the lack of early attention to these patients in initial stages because these stages include nonspecific signals and symptoms in the oral cavity with no clinical evidence of bone exposure. In case 2, lesions progressed rapidly generating a great concern since in advanced stages of BRONJ lesions, paresthesia, fistula formation and pathologic fracture can also be present [9], although these features are more common in neoplastic patients [29].

According Ruggiero et al. [26], one of the diagnosis criteria of BRONJ is the presence of exposed bone in the maxillofacial region persisting for more than 8 weeks. Although most patients with RA have some kind of bone exposure, this BRONJ definition has been revised, due to some contrary observations. First, even advanced cases can also occur with no bone exposure in oral cavity [1]. Second, there is a lack of knowledge about early clinical features and their progression toward frank BRONJ [9]. This is well-illustrated in case 2, which shows the complete evolution of a BRONJ lesion in which it was possible to identify an early soft tissue necrosis and increased probing depth values that



**Figure 10** Clinical progression of the BRONJ lesions. **A)** Increasing of the bone necrosis around the #35 associated with a mucosal ulceration involving part of the jugal mucosa; **B)** Exposed bone area after the #35 extraction; **C)** Surgical area after bone debridement



progressed to exposed bone area. Another concern about this case is that the distinction of early stages of BRONJ from other diagnoses, such as localized reactivation of chronic periodontitis, may be difficult [13].

The appropriate management of patients with BRONJ remains undefined and no widely accepted treatment protocol exists. Although it has been stated that surgical procedures may achieve better outcomes in non-neoplastic patients [29], Marx et al. [25] state that surgical procedures are not effective on patients with BRONJ and that these procedures lead to further exposed bone, worsening of the symptoms and a greater risk of pathologic fracture. These effects of surgery indicate long-term antibiotics and chlorhexidine 0.12% as treatment. The literature has shown that treatment of the lesions in patients with RA using this approach along with the discontinuation of the RA drugs have mostly positive outcomes, including the complete healing of the lesions [10,12,14]. In contrast, surgical therapy literature shows more divided outcomes, including both positive [1,30] and poor outcomes [9,4,24]. In the cases reported in this paper, surgical therapy was chosen, and excellent outcomes were achieved.

The assessment of the risk of BRONJ for patients taking BPs is a challenge. Marx et al. (2007) report use of C-terminal cross-linking telopeptide of type I collagen (CTX) test as an indicator of the risk of BRONJ, suggesting that values of less than 100 pg/mL represent a high risk and more than 150 pg/mL a low risk. In this report were found both normal values for CTX test (250 pg/mL in case 1) as abnormal values (33 pg/mL in case 2). However, the patient CTX test in case 2 would be normal if the scale purposed by Lehrer et al. (2008) is considered where values ranging 32 from 580 pg/ml are considered to be normal. Moreover, normal serum bone markers also can be found in patients with BRONJ still using BPs [31]. Other relevant point is that patient 1 just did the exam 4 month after the drug suspension and after surgical treatment, which may contributed for this normal

values, as after the drug interruption there is a gradually improvement in the values of CTX test [10,31].

We acknowledge that a limitation of the present paper is the fact that it presents two BRONJ clinical cases in RA patients. Therefore, we cannot validate any hypothesis that could explain a definite association of synergistic actions of both RA and BRONJ. More studies should be developed with rigorous case ascertainment criteria, as well as appropriate documentation of risk factors and modifiers to support scientific bases for this hypothesis.

However, the present paper helps to highlight the need for a change in clinical practice or diagnostic/prognostic approaches related to BRONJ. Considering that BPs are among the most frequently prescribed drugs in rheumatologic practice [17], associated with the lack of knowledge about this disease among rheumatologists in many countries, it is reasonable to expect an increased tendency in the number of BRONJ reports involving RA patients. This fact shows the clear necessity for the improvement in the epidemiological vigilance systems of Public Health Entities, as well as a better coordination of safety-related pharmacovigilance initiatives.

## Conclusions

Although some features seem to link RA with oral BRONJ and act as synergistic effects, more studies should be developed to support the scientific bases for this hypothesis. In addition, most patients with RA and oral BRONJ are diagnosed in stage 2, which indicates the necessity for rheumatologists to be aware of the potential risk to their patients of developing BRONJ and to work together with dentists for the prevention and early detection of the lesions.

## Consent

Written informed consent was obtained from the patients for publication of these case reports and any accompanying images. A copy of the written consent form is available for review by the Editor-in-Chief of this journal.

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## Authors' contributions

NCN performed one surgery under the supervision of the corresponding author, analyzed the records, reviewed all patients' data and designed the case report. ASB drafted the manuscript and helped in writing the text. LCS and RACM drafted the manuscript and reviewed it critically. EMJ performed one of the surgical procedures and reviewed the manuscript. All authors read and approved the final manuscript.

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#### Competing interests

The authors declare that they have no competing interests.

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## Is rheumatoid arthritis a risk factor for oral bisphosphonate-induced osteonecrosis of the jaws?

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### ABSTRACT

Bisphosphonate-related osteonecrosis of the jaws is a relevant side-effect of these drugs that has been generating a great concern through increasing reports, worldwide, of this bone necrosis. Among several BRONJ hypothetical co-factors that could play a role in BRONJ pathogenesis, rheumatoid arthritis (RA) has been included as a relevant risk factor for BRONJ; however, until now the relationship between these diseases has not been fully explained. Thus, the purpose of this paper is to establish hypothetical factors that could link these two diseases, considering mainly inflammatory components and the organism effects of medicines used to treat RA, particularly steroids and methotrexate (MTX).

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### Introduction

Bisphosphonates (BPs) are stable synthetic analogs of inorganic pyrophosphate that suppress osteoclast-mediated bone resorption [1]. In this way, they have been widely used to stabilize bone loss in patients with rheumatoid arthritis (RA), especially those who develop osteoporosis, which is a common feature in this rheumatic disease [2]. However, since 2003, great concern has been generated regarding the side-effects of these drugs through increasing reports, worldwide, of bone necrosis induced by bisphosphonates (BRONJ).

There are many hypotheses regarding BRONJ pathogenesis, but none of them is completely accepted. Although there have been reports with no obvious co-morbidity factors [3,4], it is reasonable to believe that co-factors may play a relevant role in the development of these lesions, especially in patients taking oral BPs. Among these co-factors, RA has been included as a relevant risk factor for BRONJ; however, until now the relationship between these diseases has not been fully explained. For these reasons, in this paper we proposed several hypothetical pathways that could include RA as a risk factor for oral BRONJ.

### Hypothetical link factors between RA and oral BRONJ

Although there has been no scientific link factor established between BRONJ and RA, this disease has been cited as a potential

co-factor for the development of BRONJ [5,6] and, in fact, we found 26 reports of oral BRONJ in non-neoplastic patients with RA published in the literature from 2003 to 2011 [6–21].

In attempt to explain this association, it is relevant to discuss some issues that could link these diseases, such as inflammatory components and the organism effects of medicines used to treat the disease, particularly steroids and methotrexate (MTX), which are commonly prescribed for these patients and seem to play a relevant role in the development of oral BRONJ.

### Hypothetical links among inflammation, BRONJ, and RA

#### General inflammatory considerations

It is known that the inflammatory process itself has several deleterious effects in bone tissues, resulting in bone resorption and necrosis through several actions, including the release of pro-inflammatory mediators, such as prostaglandins, growth factors, and cytokines, and potential ischemic changes due to increased intramedullary pressure, and compression of sinuses and capillaries in the marrow [22].

#### Specific hypothetical links among inflammation, BRONJ, and RA

Considering that RA is an inflammatory disease, it can be theorized that the significant inflammatory nitrogen-containing bisphosphonates (N-BPs) have a potential effect which can exacerbate the arthritis process even in animal models [23], and which, associated with the inflammatory features of RA, could generate a high-level inflammation in the affected organism, therefore

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contributing to the development of BRONJ lesions according to inflammatory theory.

In this context, to discuss the relevance of this hypothesis, issues that both support and counter claims of a possible relation between RA and BRONJ will be evaluated.

#### *Pro-inflammatory considerations*

*Gamma-delta T-cells (GDTC) are implicated in both RA and BRONJ pathogenesis.* GDTC in BRONJ inflammatory pathogenesis: From the observation that the clinical extension of BRONJ lesions is linked with the number of inflammatory cells [24], it has been suggested that the entire process of BRONJ appears to be associated with an inflammatory reaction, which may be triggered in part by signals emitted by apoptotic osteocytes, as resorption is inhibited by nitrogen-containing bisphosphonates (N-BPs) [24], and/or can be the result of an infectious stimulus by bacterial colonies, which are commonly observed in histological samples of BRONJ lesions [25].

Among the possible inflammatory mechanisms involved, a recent animal model study [26] has related GDTC to BRONJ pathogenesis. In fact, it has been demonstrated that BPs can activate these cells [27], and, moreover, the BP mevalonate pathway inhibition [1] results in the accumulation of metabolic intermediates, including isopentenyl pyrophosphate (IPP) [28], which is a powerful activator of GDTC [27].

*GDTC in RA pathogenesis:* It is well-known that RA is an inflammatory disease, and scientific evidence suggests that GDTC may play a role in its pathogenesis. Some findings support this theory, including the findings that: persistent levels of these cells have been found in peripheral blood of RA patients; and the mean numbers and percentages of these cells in patients with active disease as well as in those with extra-articular manifestations were higher than those in patients in remission [29].

*GDTC have cytotoxicity effects in oral tissues.* It is well-known that GDTC may play a role in an antibody-dependent cell-mediated cytotoxic reaction toward the epithelium of the oral mucosa [30]. This mechanism is related to a sort of disease pathogenesis, including those characterized by oral mucosa ulcerations, such as recurrent aphthous stomatitis (RAS) [31], which is the most common ulcerative disease of the oral mucosa [32]. The relevance of this fact is that RA patients are also susceptible to recurrent RAS [33] and, in the presence of such ulcerations, a micro-organism-facilitated infection of the underlying bone tissue would be generated, with subsequent necrosis, culminating with the BRONJ development according to infectious and soft-tissue BRONJ theories, which will be further discussed in more detail.

In contrast, it is relevant to state that RAS ulcers are usually developed in non-keratinized oral mucosa [32], which does not coincide with the BRONJ regions. Moreover, there are no data describing the incidence of RAS concomitantly with BRONJ, especially in cases of multiple ulcers affecting both non- and keratinized oral mucosa, which could be due to the fact that it actually does not occur or because of problems related to the diagnosis and reporting of RAS.

*GDTC and vitamin D deficiency – possible interactions between RA and BRONJ.* Vitamin D deficiency has been associated with several deleterious effects on an organism, including an increased risk of developing autoimmune diseases, such as RA [34]. Curiously, a recent animal model of BRONJ was developed by vitamin D deprivation, suggesting that its pathogenesis was linked to inflammatory mechanisms, including the activating of GDTC [26], as previously stated.

According to this reasoning, if it was considered that the prevalence of vitamin D deficiency is increased among patients with RA compared with the general population, and in this situation

increased RA activity is expected, although it remains controversial [35], since a strong inversely proportional relation of these vitamin levels with baseline disease activity of RA has been shown [36], it is reasonably to be hypothesized that the probability of BRONJ occurrence in RA patients is expected to be greater, since it is naturally possible to find a RA patient with active disease and vitamin D deficiency also taking oral BPs.

*Pro-inflammatory mediators are increased in both RA and BRONJ. Pro-inflammatory cytokines in BRONJ pathogenesis:* It has been demonstrated that nitrogen-containing bisphosphonates (N-BPs) induce the release of pro-inflammatory cytokines, such as interleukin-1 (IL-1), and/or tumor necrosis factor- $\alpha$  (TNF) [23], which can induce other inflammatory reactions or necrosis through their actions or influence on several cell types [37]. Since most of patients with oral BRONJ and RA had been treated with N-BPs, it is expected that these tissue destruction reactions could be associated with inflammatory reactions of RA itself.

In contrast, it has been stated that these pro-inflammatory effects seen with N-BPs, which are responsible for acute-phase responses [38], are usually transitory, self-limiting, and occur especially after the first intravenous dose or monthly oral dose [39,40]. Therefore, it does not seem that they persist long-term, and thus would be unable to induce necrotic changes. However, Endo et al. [41] demonstrated in an animal model that a single intraperitoneal injection of an N-BP in mice resulted in a remarkably long-lasting enhancement of histidine decarboxylase (HDC) activity, which is responsible for histamine production and increased macrophage and granulocyte numbers, occurring in various tissues and possibly persisting for several days.

*Pro-inflammatory cytokines in RA pathogenesis:* It is well-known that pro-inflammatory cytokines are increased in RA, including IL-1 and TNF- $\alpha$ , which are inflammatory mediators related to tissue destruction [42] and whose intensity depends on the tissue concentrations of these mediators.

*Pro-inflammatory cytokines in RA can reach the jaws.* Although inflammatory mediators are generated in the synovial tissue, they can reach the systemic circulation and achieve distant organs and structures, including the jaws, and exert their deleterious effects locally. Jaw involvement can be confirmed by two observations: RA is the most frequent systemic inflammatory disease involving the temporomandibular joint (TMJ) [43]; and individuals with RA are more prone to develop moderate to severe periodontal disease compared with their healthy counterparts [44].

*Pro-inflammatory consequences of aging linking RA and BRONJ.* It is well-known that elderly individuals, who are the most affected by RA and BRONJ, usually present with a chronic subclinical inflammatory status [45], characterized by the high production of inflammatory mediators, such as IL-6, TNF- $\alpha$ , and C-reactive protein (CRP) [46]. Therefore, elevated levels of pro-inflammatory cytokines in the elderly could not only contribute to the overall pro-inflammatory state of RA, but could also provide a susceptibility background for the development of BRONJ.

*Oxidative stress (OS) is implicated in both RA and BRONJ pathogenesis.* It is clear that OS is related to the pathogenesis of several diseases, both systemic such as RA [47], diabetes mellitus (DM) [48], hypertension [49], and steroid-induced osteonecrosis [50], and local oral pathologies, such as oral cancer [51], RAS [52], periodontal disease [42], and oral ulcers [53].

Within this context, when one considers that soft-tissue toxicity of BPs, especially those by oral route above gastric and oral mucosa [51,52], are related to OS [51], it could be stated that the association of RA and other co-morbidities such as DM and hypertension,



which are all considered risk factors for BRONJ, with the effects of BPs could allow for a high level of OS in organisms, which could represent an additional factor that would result in BRONJ lesions.

In contrast to this assumption, it has been shown that OS is not considered extremely hazardous to the oral mucosa [54,55], and the main mechanisms implicated in soft-tissue toxicity of BPs and its ONJ theory are through apoptosis of keratinocytes and fibroblasts [56], and not by OS.

*Intensification of the inflammatory process by micro-organisms.* Is it well-known that microbial infection can trigger host immune responses [57] and therefore enhance the degree of inflammatory process acutely or through the chronic systemic elevation of pro-inflammatory cytokines. In this way, inflammation can readily spread into the jawbones via infectious dental diseases [58], which could contribute to bone necrosis. In fact, microbial colonies are commonly seen in samples retrieved from patients with BRONJ [25], but other micro-organisms can also be implicated in this bone necrosis, and they are discussed below.

#### *Anti-inflammatory considerations*

*Anti-inflammatory properties of BPs.* The biological effects of BPs *in vitro* and *in vivo* on the immune system vary considerably and are often conflicting [59]. At the same time that they have been associated with pro-inflammatory effects [23], several anti-inflammatory and even anti-arthritic effects have been described [60], including inhibition of metalloproteases (MMPs), which are important agents of tissue destruction [61] that have been related to femur osteonecrosis [62], apoptosis induction in synovial macrophages [63], and inhibition of nitric oxide (NO), TNF- $\alpha$ , and IL-1 [64].

Aside from effects on MMPs, other anti-inflammatory effects are usually related to non-aminobisphosphonates (non-N-BPs) [63,64], since they occur after therapeutic doses, while to achieve these effects with N-BPs, such as alendronate, it may be necessary to administer doses 100 times higher for its anti-resorptive effect in animal models [65]. In humans, a study also demonstrated that a high dosage of alendronate given intravenously (40 mg/daily over 90 consecutive days) in patients with RA induces a significant reduction in circulating pro-inflammatory cytokines (IL-1, IL-6, and TNF- $\alpha$ ) [66].

These observations could partially justify why only a few cases of BRONJ have been associated with non-N-BPs, although it is relevant to state that they are less often prescribed. Moreover, these high N-BP dosages correspond to doses greater than those attained during *in vivo* administration to non-neoplastic patients, such as those with RA; therefore, they are not expected in this group of patients.

*Immunosuppression and its Relation with BRONJ and RA.* Contrary to the pro-inflammatory BRONJ theory, other effects in the immune system also may be implicated with BRONJ pathogenesis – immunosuppression which is not related to the anti-inflammatory effects described previously, but to the other pathways of immunosuppression.

In fact, the possible role of immunosuppression in BRONJ pathogenesis has been attested to by experimental animal models of BRONJ achieved by the association of N-BPs and an immune impairment condition, such as steroid therapy [67] and vitamin D deprivation [26]. This hypothesis is in agreement with the infectious BRONJ theory that will be further discussed, since the main pathway involved is the impaired defense against infection, although the vitamin D deficiency deserves some relevant considerations, as previously stated.

If this theory is true, a great concern can be generated, since RA patients are related to a raised risk of bacterial infection [68],

probably due to some degree of immunosuppression that is expected in RA patients, for the following reasons: cellular immunity impairment as a consequence of RA itself, including a decline in the numbers and functions of T-suppressor and natural killer cells [69]; an elderly population, which is characteristic of RA and BRONJ, co-exists with immune dysfunction [45]; immunosuppressive effects of medicines used to treat the disease, such as steroids and MTX [70]; immunosuppressive consequences of vitamin D deficiency which have been reported in RA patients [35]; and although it appears to be irrelevant, in RA there are low levels of cytokines which suppress the immune-inflammatory response, such as IL-10 and transforming growth factor  $\beta$  (TGF- $\beta$ ), as well as specific inhibitors of IL-1 and TNF alpha, which may antagonize the pro-inflammatory effects of these cytokines [71,72].

### **Hypothetical links between steroids and MTX effects and BRONJ**

#### *General considerations*

Based on the fact that steroids have been considered the second most common cause of avascular ON [73], which is a disease that has been found in patients with RA, a possible relationship between steroid therapy and BRONJ has been proposed [74], although it remains controversial [75]. This relation is especially due to the fact that both diseases are related to some degree of vascular and bone impairment [76,77], with the same clinical avascular bone pattern. Moreover, animal models of BRONJ have been developed by the association between BPs and steroids [67], as previously stated.

In contrast, some features seem to be different in both diseases, such as in steroid-induced ON, where a predilection for long bones and rare bone exposure can be observed [78], while in BRONJ there is a high predilection for jaws and bone exposure as the most common clinical signal [4]. Moreover, most clinical studies assessing this issue [76,77] were performed in neoplastic populations who probably had other co-factors related to BRONJ pathogenesis, compromising any further association.

Discussion aside, even with no literature consensus about the inclusion of steroids and MTX therapy as a risk factor for BRONJ, if we consider the main BRONJ theories reported, including bone remodeling and vascular suppression, infection process, and soft-tissue toxicity [77], some linked factors can be related even in patients receiving oral BPs. These factors will be discussed according to BRONJ theory.

#### *Specific hypothetical links between steroids and MTX and BRONJ*

##### *Bone-remodeling suppression theory*

According to this hypothesis, strong BP osteoclast inhibition may suppress bone remodeling, which would lead to unsuccessful microdamage repair and to the accumulation of non-viable osteocytes, therefore resulting in a progressive accumulation of devitalized bone tissue [77]. To link BRONJ and RA, the following can be suggested.

*Suppression effects of steroids and MTX on bone remodeling.* It is well-known that steroids and methotrexate (MTX) have been consistently implicated in the suppression of bone turnover through a marked inhibitory effect on osteoclastogenesis [79,80], osteoblast and osteocyte apoptosis induction by steroids [81], and osteoblastic proliferation inhibition by MTX [82].

Considering these bone effects, it could be suggested that the association between and among BPs, steroids, and MTX would result in an excessive suppression of bone turnover, which is particularly relevant in the oral cavity, where the functional demand is increased by the high remodeling maxillary rate [77], masticatory

overload [82], constant stimulus from periodontal ligament [4], and, eventually, as a result of invasive dental procedures. Moreover, two observations are relevant: most patients with RA and BRONJ are elderly, as was previously mentioned, and it is known that alveolar bone turnover *in vivo* decreases rapidly in the elderly [83]; and estrogen replacement therapy has been associated with an increased risk of BRONJ [84], probably related to estrogen-induced suppression of bone turnover [85], and the peak incidence of RA in women coincides with the time of menopause [86]. In this general context, these two factors also can contribute to the suppression of bone remodeling, thus acting in favor of BRONJ development.

In spite of this theory, it is relevant to state that RANKL blocker agents, such as denosumab, are often selected as therapeutic drugs in RA, and, curiously, denosumab also has been correlated with ONJ in patients with no BP treatment [87], as well as in patients with a history of short-term use of alendronate [88]. The concern about this issue is that although patients are unlikely to receive concomitant denosumab and BPs in clinical practice, this association was made [89], and the possibility of BPS treatment synergistically enhancing denosumab inhibition of osteoclastic activity toward ONJ development cannot be disregarded.

#### Angiogenic theory

It has been demonstrated that BPs have anti-angiogenic properties, through angiogenesis inhibition, decreased capillary tube formation, and inhibition of vascular endothelial growth factor [90]. According to this theory, endothelial cell proliferation may be inhibited in the jaws, leading to loss of blood vessels and avascular necrosis [4]. To link these diseases, the following mechanisms can be suggested.

*Effects of vascular synergies between steroids, MTX, and BPs.* In this context, it has been demonstrated that steroids and MTX also have anti-angiogenic properties [78,91], although for MTX, a non-significant influence on angiogenesis has also been reported [92]. In this way, it is possible to suggest that the association between and among BPs, steroids, and MTX could result in an excessive suppression of jaw angiogenesis, with consequent reduction of blood flow in the jaws and ischemic necrosis. In fact, it was demonstrated that the combination of BPs and anti-angiogenic factors induces ONJ more frequently than BPS alone in neoplastic patients [93].

*Hypoxia, RA, and BRONJ.* As stated previously, BRONJ could be related to vascular impairment with subsequent ischemia and tissue hypoxia [4]. When one considers all these situations, which potentially offer moments of tissue hypoxia, it is possible to relate some conditions that could act in favor of BRONJ development. First, RA itself is characterized by tissue hypoxia and several microvascular alterations which also are implicated in this disease pathogenesis. Although it seems to be a localized process, it is further believed that the microcirculatory abnormality may be generalized, considering the systemic manifestations often seen in RA [94].

Second, other co-morbidities with vascular implications and hypoxic potential also commonly found in RA patients have coincidentally also been implicated in BRONJ pathogenesis: advanced age [6,95,96], which has been associated with an exponential increase in inferior alveolar artery intimal thickness with subsequent stenosis after the sixth decade compared with that seen in the younger period [97]; peripheral vascular disease [98], probably related to pro-thrombotic effects [99] and accelerated atherosclerosis in the carotid arteries [100] that are responsible for primary and collateral blood supply to the jaws [101]; and anemia [102], which naturally results in tissue hypoxia.

In fact, there are no valuable scientific data assessing the vascular pattern in BRONJ, but only reports that found different patterns,

ranging from an intact [103] vasculature to vessel obliteration [105], as well as an increased vascularity that could be seen in BRONJ samples of RA patients [8]. Therefore, it is not possible to state that vascular impairment is the main pathway related to BRONJ development.

#### Infectious process theory

Unlike the aseptic pattern of BRONJ that was previously described, other authors suggest that this bone necrosis is more similar to osteomyelitis [105], which is justified by the common presence of micro-organism colonies in histological samples retrieved from BRONJ patients, especially *Actinomyces* species [6103,104,106].

The possible micro-organism mechanisms implicated in BRONJ pathogenesis include: possibility of direct and indirect damage to bone matrix by bacterial products [107], such as porins, acids, proteases, and lipopolysaccharides [108] and direct invasion of osteoblasts by bacteria, resulting in impairment of bone remodeling processes [109]; oxidative stress (OS), which has been implicated in BRONJ pathogenesis, often caused by inflammation associated with bacterial infections [76]; and increased bacterial adhesion to BP-containing bone through a protein named 'microbial surface component', which recognizes matrix adhesion molecules and has the potential to interact with bone hydroxyapatite [25]. To link these diseases, the following can be suggested.

*Increased infection risk in RA.* Within this context, RA can be associated with an increased risk of bacterial infection [68], which might be related to impaired defense against infection [110], due to the reasons already described. In fact, steroids and MTX, which are among the most commonly drugs prescribed to RA patients, have been associated with an increased incidence of infections in clinical trials [111,112]. Moreover, *Actinomyces*, found in BRONJ samples, is a bacterium that usually becomes pathogenic when immunosuppression occurs [25], as in RA, and is commonly found in the oral cavity [113].

*Other concomitant infections relating RA and BRONJ.* *Periodontal disease (PD):* PD is a multifactorial etiology disease related to microbiological factors [42] and has been considered a relevant risk factor itself or a trigger event to BRONJ [4]. Curiously, it has been shown that individuals with RA are more prone to develop moderate to severe periodontal disease compared with their healthy counterparts, showing a higher prevalence of periodontal bone loss. Moreover, it is plausible to believe that ongoing periodontitis could trigger RA in genetically susceptible individuals [42]. All these observations allow us to suggest that individuals with RA have bacteria exerting pathogenic activities in the oral cavity that can be related to BRONJ pathogenesis.

*Osteomyelitis:* Osteomyelitis, which has been a common feature in patients with RA [114], has shared some similarities with BRONJ [104], as stated previously. In contrast, other characteristics do not support this connection, especially the fact that primary chronic osteomyelitis of the jaws is a very rare entity, and clinical features such as abscess or fistula formation and sequestration are secondary events [115]. In this way, it is reasonable to believe that bacterial presence within ONJ lesions could be not an etiologic factor in the pathogenesis of BRONJ, but rather a secondary infection of the necrotic tissues [8].

#### Soft-tissue toxicity theory

According to this theory, toxic levels of BPs can reach the oral mucosa due to physical disruption of the bone during dental intervention or through secretion in the saliva or gingival crevicular fluid [77], resulting in direct toxicity to oral epithelium that can lead to ulcer formation and areas of exposed bone which can

persist for a long time in the oral cavity [116], allowing for rapid microbial colonization [117].

The mechanisms implicated in this oral mucosa healing impairment include: interference with the molecular signaling of fibroblasts and keratinocytes, resulting in apoptosis of these cells; and GDTC actions [30] and oxidative stress effects on oral mucosa [51], as stated previously.

Moreover, it is relevant to state that oral mucosal lesions are more commonly seen in patients with RA, including ulcerative lesions, than in matched healthy controls [118]. To link RA and BRONJ, the following can be suggested.

**MTX toxicity to oral tissues.** It is well-known that MTX, even in low doses, has a certain oral epithelial toxicity [119], with oral mucosal ulceration being the most common adverse effect [120] that also often affects alveolar mucosa [121], which, in turn, is also the area most frequently affected by BRONJ lesions. Frequently, these lesions appear within 2 weeks of administration but may also develop very late [122] and can persist for several months [119].

Considering that these MTX-induced oral ulcerations are mainly mediated by folate antagonism [123], and a pre-existing folate deficiency increases MTX toxicity toward the oral mucosa [124], patients with folate deprivation and in long-term treatment with MTX are at high risk for ulcer development. In clinical practice, most of the patients have been taking long-lasting MTX, and it has also been observed that RA patients develop deficiencies of folic acid concentrations in plasma and red blood cells [125,126], although this appears to be rare [127]. Furthermore, it has been found that patients on MTX had a significantly lower intake of dietary folic acid than those on other therapies [128].

**GDTC cell actions.** Considering that these cells are increased in both RA [26] and in BRONJ [24], and their cytotoxic effects are potentially enhanced by BPs [27,29] and vitamin D deficiency [26], it is reasonable to believe that these factors at least favor the development of soft-tissue necrosis.

## Discussion

Although this paper has in fact shown that inflammatory issues, as well as the medicines used to treat RA, could really represent potential pathways to link both diseases, especially acting synergistically, some doubtful aspects of these issues mitigate against any further association.

First, the exact role of inflammation in the sequence of BRONJ events remains poorly understood, so that it is not clear whether the inflammation is due to osteonecrosis or if it appears during or after the development of lesions. Therefore, scientific studies are necessary to explore the role of inflammation and inflammatory mediators in BRONJ and their correlation with infection.

Second, based on the controversies in the literature and on the analysis of this cohort of patients who have been treated with steroids and MXT and also have other co-morbidities associated with RA, many contradictory facts become evident, for example: General lesions developed at the same length of exposure between patients with and those without a history of steroid intake; Maiden and Pai [5] reported a case, who is below the mean age for BRONJ incidence with no associated co-morbidities unless affected with RA, developed lesions very early when compared with the mean length of exposure before lesion detections; and Kwon et al. [17] reported a case, who, even though of advanced age, with osteoporosis, DM, and a history of steroid use, developed lesions 3 years after alendronate treatment. For these reasons, it is impossible to state that these medicines or other co-morbidities are really related to an increased risk of BRONJ development.

Thus, it is reasonable to suggest that we should not consider the isolated effects of each of the factors discussed as unique agents that could contribute to the development of oral BRONJ lesions in patients with RA, but we must take into account the potential role that each one of them could present, sometimes even acting together in the entire disease process. Moreover, currently, the association of possible synergistic actions of both RA and BRONJ is only a hypothesis, and more studies including randomized, controlled, prospective clinical trials that assessed jaw osteonecrosis (ONJ) risks in patients with RA who were receiving oral BPs should be developed with rigorous case ascertainment criteria, as well as appropriate documentation of risk factors and risk modifiers to support scientific bases for this hypothesis.

## Conflict of interest statement

None declared.

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REVIEW

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# Epidemiological aspects of rheumatoid arthritis patients affected by oral bisphosphonate-related osteonecrosis of the jaws

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## Abstract

This literature review aims to evaluate the epidemiologic profile of patients with rheumatoid arthritis (RA) that developed a bisphosphonate-related osteonecrosis that affect the jaws (BRONJ), including demographic aspects, as well as clinical and therapeutic issues. A search of PUBMED/MEDLINE, Scopus, and Cochrane databases from January 2003 to September 2011 was conducted with the objective of identifying publications that contained case reports regarding oral BRONJ in RA patients. Patients with RA who develop oral BRONJ are usually women above 60 years taking steroids and long-term alendronate. Most of them have osteoporosis, and lesions, triggered by dental procedures, are usually detected at stage II in the mandible. Although there is no accepted treatment protocol, these patients seem to have better outcomes with conservative approaches that include antibiotic therapy, chlorhexidine, and drug discontinuation.

**Keywords:** Rheumatoid arthritis, Bisphosphonate, Jaws, Osteonecrosis

## Background

Bisphosphonates (BPs) are stable synthetic analogs of inorganic pyrophosphate that suppress osteoclast-mediated bone resorption [1]. In this way, they have been widely used to stabilize bone loss in patients with rheumatoid arthritis (RA), especially those who develop osteoporosis, which is a common feature in this rheumatic disease [2]. However, since 2003, great concern has been generated regarding the side-effects of these drugs through increasing reports, worldwide, of a bisphosphonate-related osteonecrosis that affect the jaws (BRONJ).

There are many hypotheses regarding BRONJ pathogenesis, but none of them is completely accepted. Although there have been reports with no obvious co-morbidity factors [3,4], it is reasonable to believe that co-factors may play a relevant role in the development of these lesions, especially in patients taking oral BPs. Among these co-factors, RA has been included as a relevant risk factor for

BRONJ; however, until now the relationship between these diseases has not been fully explained.

Due to the greater number of patients taking oral BPs for the treatment of RA and osteoporosis, it is very important for risks to be assessed. Thus, the purpose of this extended literature review is to evaluate relevant issues of patients with RA who developed oral BRONJ, including demographic, clinical, and treatment aspects, with the goal of establishing comparative associations with patients without RA who developed oral BRONJ.

## Methods

We performed a computerized search to identify all papers published in English from January 2003 to September 2011 in PUBMED/MEDLINE, Scopus, and Cochrane databases. Case reports, case series, and retrospective studies were included, while short communications and letters to the Editor were excluded. The studies were approved by the Ethics in Human Research Committee and were in compliance with the ethical principles of the Helsinki Declaration. Literature reviews and systematic reviews also were considered with the objective of identifying cases already reported. The key MeSH (Medical Subject

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Headings) terms used were: diphosphonates, bisphosphonate, jaw osteonecrosis, rheumatoid arthritis, and rheumatologic.

The primary objective was to identify all articles containing clinical reports with the following inclusion criteria:

- Diagnosis of RA and oral BRONJ
- Patients with no prior history of radiation to the craniofacial region
- Non-neoplastic patients
- At least five areas of the specific demographics were collected, including gender, age, bisphosphonate type and length of exposure, concomitant medications, trigger event, clinical features, staging, lesion site, imaging and histological features, as well as management and outcomes. The stage of the lesions was retrieved directly from the papers or it was defined based on the available clinical data and according to the classification system by Ruggiero et al. [5].

- Papers published in English

All titles and abstracts from the results of the literature search were reviewed by the authors for potential inclusion in our study. We also searched the related links of all relevant papers. Furthermore, all relevant papers were checked to avoid multiple inclusions of the same patients in this study.

## Results

The search strategy yielded 1606 titles/abstracts from the databases analyzed. After title, abstract screening, and/or paper analyses, 331 potentially relevant studies were identified and screened for retrieval. Of these, 312 studies were excluded due to non-compliance with the inclusion criteria, one paper was excluded due to duplicity of one patient and, finally, 17 were included in the present review, consisting of 27 patients and 29 BRONJ lesions (Tables 1, 2, 3, and 4).

### Patient characteristics

The patients from this literature review ranged in age from 55 to 79 yr and the mean age was 67 years. Among the 22 patients whose age was reported, 17 (77.3%) were above 60 years old [6,8,10-12,14-16,18-21], 25 patients reported the gender and only one was male [12] (Table 1). Furthermore, osteoporosis was the most common co-morbidity observed in this review, being present in 18 patients (66.6%) [4,9-11,14-16,18-21], followed by hypertension (29.6%) [6,16,18-20] and diabetes mellitus (14.8%) [8,15,20] (Table 1).

### Characteristics of bisphosphonate treatment

Among the 24 patients that reported the use of BPs, 22 (91.6%) affirmed to use alendronate [4,6-9,11,12,15, 16,18-21], while 2 other patients were using ibandronate

[14] or risedronate [10] (Table 1). The mean duration of BP therapy was 48 months, ranging from 6 months to 10 years, and most of the patients (73%) were using BPs for 3 or more years [4,6,9,12,15-17,19-21] (Table 1).

### BRONJ characteristics

Most of the oral BRONJ cases in this literature review were triggered by dental surgery procedures (51.7%), such as tooth extraction and dental implants, observed in 13 patients [4,5,7-9,12,14,17-20]. Of note, a large proportion of BRONJ lesions appeared spontaneously (41.38%) [9,10,12,13,15,19-21] (Table 2).

BRONJ lesions were located most commonly in the lower jaw (72.4%), especially in the posterior area [4,6,9,12-15,17,19,21]. The main signs and symptoms reported in the studies included: bone exposure in 82% of the lesions [4,6,7,9,11-18,20,21], followed by pain (78.5%) [4,6,7,10-14,16-21], edema (35.7%) [6,7,10,12,15,17-19,21] and purulent discharge (35.7%) [6,11,13-15,17,19,21]. Moreover, according to a staging system [9], 28 lesions were diagnosed at stage II or III, and only one was at stage I (Table 2). Furthermore, most of the patients (62.5%) presented a lytic pattern observed on image exams [4,12, 16-19,21], followed by bone sclerosis that was reported in six patients (37.5%) [7,12,17,19,21].

Regarding the management of the BRONJ lesions, the studies showed the treatment either by conservative therapy alone (48%) [9,10,12,14,17,19,20] or associated with surgical procedures (52%) [4,6,7,12,13,15-19,21]. The treatment only with conservative therapy was associated with the most positive outcomes, including the complete healing of the lesions (33.3%) [9,12], partial and general positive results (58.3%) [10,14,19,20] and only one patient presented a non-healed lesion (8.33%) [17] (Table 4).

The association of conservative and surgical therapy showed more diverse results, with complete healing in 3 lesions (25%) [13,21], partial and general positive results (41.6%) [6,7,12,18,19] and 4 lesions were classified as non-healed (33.4%) [4,6,15,17] (Table 4).

## Discussion

Until the present moment, there is no randomized, controlled, prospective clinical trial that assessed jaw osteonecrosis (ONJ) risk in patients with rheumatoid arthritis who were using oral BPs. In the absence of this data, the present paper performed a critical literature review to investigate the current status of the epidemiological aspects of patients with rheumatoid arthritis affected by BRONJ.

Considering the prevalence of BRONJ lesions in patients with RA, we considered only the raw number of oral BRONJ reports in RA patients, since the accurate number of non-neoplastic patients treated with oral BPs

**Table 1 Patients and Bisphosphonate Therapy Characteristics**

Authors/Study	Gender	Age	Comorbidities	Medications (Years)
Marunick et al. [4] CS Patient 1	F	59	OST	STE + METX + AL (3)
Yarom et al. [6] RS				
Patient 2	F	73	HTN	STE + METX + AL (7), infliximab
Patient 3	F	76	HTN + Arrhythmia + hypercholesterolemia	AL (1.5)
Malden & Pai [7] CS Patient 4	F	56	NO	STE + AL (1), leflunomide
Khamaisi et al. [8] RS Patient 5	F	73	DM	AL (> 0,5)
Marx et al. [9] CS				
Patient 6	F	NA	OST	STE + METX + AL (3.1)
Patient 7	F	NA	OST	STE + METX + AL (6.3)
Patient 8	F	NA	OST	STE + METX + AL (3.3)
Hamada [10] CR				
Patient 9	F	68	OST	STE + RISE (4)
Barrow [11] CR				
Patient 10	F	70	OST	STE + AL (< 3)
Junquera et al. [12] CS				
Patient 12a/12b	M	73	NO	STE + METX + AL (3.8)
Mehanna et al. [13] CR				
Patient 13	F	55	NO	STE + Leflunomide + oral BPs (1)
Favia et al. [14] CS				
Patient 14a/14b	F	67	OST	STE + IBAN (1)
Kwon et al. [15] CR				
Patient 15	F	71	OST + DM	STE + AL (3); OH
Sedghizadeh et al. [16] RS				
Patient 16	F	63	OST + HTN	STE + chemotherapy + AL (3)
Lo et al. [17] RS				
Patient 17	NA	NA	NO	oral BPs (4.8)
Patient 18	NA	NA	Hip Osteonecrosis	STE + oral BPs (2.6)
Shin et al. [18] CR				
Patient 19	F	67	OST + HTN	AL (1) + HTN Drug
Park et al. [19] CS				
Patient 20	F	68	OST	STE + AL (5)
Patient 21	F	69	OST + PLE + TB	STE + AL (10)
Patient 22	F	70	OST + HTN	STE + AL (6)
Manfredi et al. [20] CS				
Patient 23	F	68	OST + DM + HTN	AL (4.25)
Patient 24	F	65	OST + HTN	AL (5)
Patient 25	F	79	OST	AL (4)
Patient 26	F	57	OST + DM + HTN	AL (> 3)
Conte-Neto et al. [21] CR				
Patient 27	F	58	NO	AL (5)
Patient 28	F	68	OST	STE + METX + AL (7)

CS Case series, RS Retrospective study, CR Case report, NA Not available

OST Osteoporosis, HTN Hypertension, DM, Diabetes Mellitus, GU gastric ulcer, PLE Pleuritis, TB Tuberculosis, STE Steroids, MTX Methotrexate; AL Alendronate, RISE Risedronate, IBAN Ibandronate, OH oral hypoglycemicant

is unknown; therefore, the determination of the precise prevalence of BRONJ is extremely difficult but is possible to be estimated based on epidemiological studies.

Two reviews published recently, reporting patients that presented BRONJ induced by the use of oral BPs,

observed a very low prevalence of these lesions in patients with rheumatic diseases [22,23]. Other studies reported that the BRONJ prevalence in patients treated with oral BPs, ranges from 0.001% to 0.4% [17,24-27]. These numbers give the idea that the prevalence of



**Table 2 Clinical features of BRONJ lesions in patients with RA**

Authors	Trigger event	Clinical features	Stage	Site
Marunick et al [4]				
Patient 1	Extraction	BE + pain + sequestration	II	Md posterior lingual
Yarom et al [6]				
Patient 2	Implants	BE + pain + edema + PD + fistula + pathological fracture	III	Md posterior
Patient 3	Extraction	BE + NHS + pain + edema + PD + fistula	III	Md posterior
Malden & Pai [7]				
Patient 4	Extraction	BE + pain + NHS + edema	III	Mx posterior
Khamaisi et al [8]				
Patient 5	Dental surgery	NA	II	Md
Marx et al [9]				
Patient 6	Spontaneous	BE	I	Md lingual
Patient 7	Palatal CT graft	BE + erythema	II	Mx posterior palate
Patient 8	Spontaneous	BE	II	Md posterior lingual
Hamada [10]				
Patient 9	Spontaneous	Pain + edema + sequestration	II	Md
Barrow [11]				
Patient 10	Denture trauma	BE + pain + PD + erythema	II	Mx palatine
Junquera et al [12]				
Patient 12a	Extraction	BE + NHS + pain + hypoesthesia + erythema	II	Md anterior
Patient 12b	Spontaneous	BE + edema + trismus + pain + swelling	II	Md posterior lingual
Mehanna et al. [13]				
Patient 13	Spontaneous	BE + PD + trismus + abscess + pain	II	Md posterior
Favia et al. [14]				
Patient 14a	Extraction	BE + pain + PD	III	Mx posterior
Patient 14b	Extraction	BE + pain + PD	III	Md posterior
Kwon et al. [15]				
Patient 15	Spontaneous	BE + PD + edema	II	Mx and Md posterior
Sedghizadeh et al [16]				
Patient 16	Denture trauma	BE + pain + infection	II/III	Mx
Lo et al [17]				
Patient 17	Extraction	BE + PD edema	II	Md posterior lingual
Patient 18	Extraction	BE + pain + erythema	II	Mx posterior
Shin et al. [18]				
Patient 19	Implants	BE + pain + erythema + edema + increased probing depth	II	Mx posterior
Park et al [19]				
Patient 20	Implants	Pain + gingival bleeding + PI Pain	II	Md posterior
Patient 21	Extraction	+ PD + periodontitis	II	Mx posterior
Patient 22	Spontaneous	Pain + itching sensation + edema	III	Md posterior and anterior
Manfredi et al. [20]				
Patient 23	Spontaneous	BE + pain + infection	III	Md
Patient 24	Spontaneous	BE + pain + infection	II	Md
Patient 25	Spontaneous	BE + pain + infection	II	Md
Patient 26	Extraction	BE + pain + infection	II	Md
Conte-Neto et al. [21]				
Patient 27	Spontaneous	Pain + infection + increased probing depth + erythema	II	Md posterior
Patient 28	Spontaneous	BE + pain + infection + edema + erythema + increased probing depth + PD	II	Md posterior

BE Bone exposure, PD purulent discharge, NHS non-healing socket, CT connective tissue, PI periimplantitis, Md mandible, Mx maxillae

**Table 3 Radiographic and Histologic Features of BRONJ Lesions in Patients with RA**

Authors	Radiographic features	Histologic features
Marunick et al. [4]		
<i>Patient 1</i>	Osteolysis + sequestration	Dense nonvital bone + subacutely inflamed granulation tissue + bacterial colonies
Yarom et al. [6]		
<i>Patient 2</i>	ID	CLP inflammatory infiltrate + increased vascularity + necrotic bone + bacterial colonies ( <i>S. milleri</i> )
<i>Patient 3</i>	ID	CLP inflammatory infiltrate + increased vascularity + necrotic bone + bacterial colonies ( <i>S. viridans</i> )
Malden & Pai [7]		
<i>Patient 4</i>	Thickening of the left sinus floor and bone density alteration (suggestion of a retained root in the upper left second PM)	Sclerotic and necrotic bone
Khamaisi et al. [8]		
<i>Patient 5</i>	NA	NA
Marx et al. [9]		
<i>Patient 6</i>	NA	NA
<i>Patient 7</i>	NA	NA
<i>Patient 8</i>	NA	NA
Hamada [10]		
<i>Patient 9</i>	NA	NA
Barrow [11]		
<i>Patient 10</i>	Widening of the PLS	NA
Junquera et al. [12]		
<i>Patient 12a</i>	Generalized lytic pattern of bone destruction with superimposed sclerosis of the mandibular ramus	Necrotic osteitis + mixed infiltrate of lymphocytes and granulocytes + medullary fibrosis + numerous <i>Actinomyces</i> colonies
<i>Patient 12b</i>		
Mehanna et al. [13]		
<i>Patient 13</i>	Marked right neck collection with free gas and midline shift	Normal skin flora with scanty diphtheroids
Favia et al. [14]		
<i>Patient 14a</i>	ID	NA
<i>Patient 14b</i>	ID	NA
Kwon et al. [15]		
<i>Patient 15</i>	Osteomyelitis characteristics	NA
Sedghizadeh et al. [16]		
<i>Patient 16</i>	Ill-defined lytic lesion	NA
Lo et al. [17]		
<i>Patient 17</i>	Sequestration osteosclerosis, focal osteolysis with cortical disruption	<i>Actinomyces</i>
<i>Patient 18</i>	Irregular area of bony sclerosis	NA
Shin et al. [18]		
<i>Patient 19</i>	Alveolar bone resorption with internal scattered residual bone fragments, widening of LPS, and radiolucent lesion	NA
Park et al. [19]		
<i>Patient 20</i>	Ill-defined lytic lesion	Necrotic bone + acute and chronic non-specific inflammation + granulation formation
<i>Patient 21</i>	Ill-defined lytic lesion	Necrotic bone
<i>Patient 22</i>	Ill-defined lytic lesion, mixed radiolucent and radiopaque lesions	NONE
Manfredi et al. [20]		
<i>Patient 23</i>		

**Table 3 Radiographic and Histologic Features of BRONJ Lesions in Patients with RA (Continued)**

Patient 24	NA	NA
Patient 25		
Patient 26		
Conte-Neto et al. [21]		
Patient 27	Radiolucent lesions and sequestration	Necrotic lamellar bone + chronic and acute inflammatory cells + bacterial colonies
Patient 28	Osteosclerosis and osteolysis	NA

ID Impossible to determine, CLP chronic lympho-plasmacytic, NA not available, PLS periodontal ligament space, PM premolar

**Table 4 Management and Outcomes of BRONJ Lesions in Patients with RA**

Authors	Management	Outcomes
Marunick et al. [4]		
Patient 1	ATB + Oral Rinses and sequestrectomy	No healing
Yarom et al. [6]		
Patient 2	ATB and curettage + debridement + resection	No healing
Patient 3	ATB and curettage + debridement + resection	Partial healing
Malden & Pai [7]		
Patient 4	ATB + CLX + DD and debridement	Progressive improvement
Khamaisi et al. [8]		
Patient 5	NA	NA
Marx et al. [9]		
Patient 6	CLX + DD*	Complete healing
Patient 7	CLX + DD*	Complete healing
Patient 8	CLX + DD*	Complete healing
Hamada [10]		
Patient 9	CT	Partial Improvement
Barrow [11]		
Patient 10	NA	NA
Junquera et al. [12]		
Patient 12a	ATB + CLX	Complete healing
Patient 12b	DD and sequestrectomy	Remission of symptoms
Mehanna et al. [13]		
Patient 13	ATB + DD + drainage	Complete healing
Favia et al. [14]		
Patient 14a	ATB + CLX + DD	Partial Improvement
Patient 14b	ATB + CLX + DD	Partial Improvement
Kwon et al. [15]		
Patient 15	Sequestrectomy and CT and resection and debridement + DD	Sequestrectomy: no healing. CT: complete healing in mx and no healing in Md. Resection and debridement: healing after 6 months
Sedghizadeh et al. [16]		
Patient 16	ATB + CLX + DD + sequestrectomy + debridement	NA
Lo et al. [17]		

**Table 4 Management and Outcomes of BRONJ Lesions in Patients with RA (Continued)**

Patient 17	ATB + CLX + DD and sequestrectomy + debridement	No healing
Patient 18	ATB + CLX + DD	No healing
Shin et al. [18]		
Patient 19	ATB + DD + CLX + debridement	Satisfactory healing
Park et al. [19]		
Patient 20	ATB + DD + CLX + curettage	Satisfactory healing
Patient 21	ATB + DD + CLX + curettage	NA
Patient 22	ATB + DD + CLX	Satisfactory healing
Manfredi et al. [20]		
Patient 23	DD	NA
Patient 24	DD + ATB + Laser	Partial healing
Patient 25	ATB + Laser	Partial healing
Patient 26	ATB	Partial healing
Conte-Neto et al. [21]		
Patient 27	ATB + DD + CLX + sequestrectomy + debridement	Complete healing
Patient 28	ATB + DD + CLX + curettage + debridement	Complete healing

\*It was not possible to determine which ATB was prescribed to these patients  
 ATB antibiotic therapy, CLX chlorhexidine, DD drug discontinuation, CT conservative treatment, NA not available

BRONJ lesions in patients with RA is expected to be quite low, however it should be considered that the proportion of patients with rheumatic disease among all the population sampled in these studies is probably reduced as well.

At the same time, it is reasonable to expect an increased tendency in the number of BRONJ lesions in RA patients considering that there is a lack of knowledge about this disease among rheumatologists in many countries and that BPs are among the most frequent prescribed drugs in rheumatologic practice [28]. This is due to the high efficiency of BPs in the prevention and treatment of osteoporosis, which is a common feature in RA [29].

There is a considerable discussion in the literature whether aging plays a significant role in BRONJ development. Some studies found no statistically significant correlation between aging and BRONJ [30,31]. Therefore, the advanced age of the patients with BRONJ observed in the studies [6,8,10-12,14-16,18-21] may reflect nothing less than the increased BPs prescription to older patients compared with younger ones, since osteoporosis and RA are commonly seen in the elderly [29,32].

On the other hand, other authors include advanced age as a BRONJ co-factor [19,33,34], which could be related to the physiological effects of aging, including inflammatory issues [35], immune dysfunction [36], reduction of the blood flow and the remodeling ability [37,38], and increased oxidative stress [39]. In fact, these

features are all implicated with BRONJ pathogenesis and could explain why this disease is not reported in young patients, even with other risk factors associated [40]. Paradoxically, BPs have been prescribed for the treatment of steroid-induced osteonecrosis of the joints in pediatric populations [41].

Controversial aspects have also been discussed regarding gender as a BRONJ co-factor. Some studies found no statistically significant correlation between gender and BRONJ [33,34]. Therefore, we observed that the large proportion of female patients from the studies [4,6-11,13-16,18-21] can represent only a coincidence, since women take oral BPs more frequently than males, especially because RA and osteoporosis are more common in women [42].

In spite of that, other authors reported a positive correlation between gender and BRONJ [19]. It has been speculated that estrogen therapy may play a role in this correlation, since hormonal reposition has been associated with an increased risk of BRONJ [43]. The concern is that hormonal replacement therapy is likely to occur in RA, since this disease is often worsened after estrogen delivery [44]. In fact, the association of BPs and estrogen reposition is especially possible in patients with no satisfactory outcomes associated with a single drug therapy or who have very low bone density with multiple risks [45]. Moreover, treatment with estrogen/bisphosphonate conjugate drugs has also been described [46].

It is not surprising that osteoporosis was the most prevalent co-morbidity observed in the studies [4,9-11,14-16,18-21], since this is a common feature in RA patients for several reasons, including: (a) postmenopausal women represent part of the main risk group for RA and are at risk for accentuated bone loss; (b) steroid therapy is often prescribed for the treatment of RA; (c) physical inactivity is characteristic of RA due to disease activity; and (d) bone loss due to inflammatory disease mechanisms, such as elevated levels of systemic cytokines [29].

It has been shown that there is a direct relation between BRONJ occurrence and BPs potency [13,33], which is supported by the high incidence of BRONJ in patients receiving intravenous BPs [47], as well as by faster lesion onset in these patients [24]. This assumption corroborates with the higher incidence of BRONJ in patients using alendronate, as seen in the studies revised here [4,6-9,11,12,15,16,18-21], since it is the most potent drug among the BPs [9].

In contrast, Kos and Luczak [48] found no correlation between the type of BPs and BRONJ incidence, which reinforces the assumption that it can be only a coincidence. In fact, over the last decade, alendronate was among the most used drugs in the USA as first-line therapy for the prevention or treatment of osteoporosis [49], both in non-neoplastic BRONJ patients without RA [6,9,14,16] and in patients with RA [32]. Furthermore, it is reasonable to understand why ibandronate, a potent nitrogen-containing bisphosphonate (N-BPs) [7] that is much less prescribed compared with alendronate, is not associated with large-series cases of BRONJ [23].

It has been discussed that one of the most critical factors for BRONJ is the duration of oral BPs therapy [9,33]. It is believed that there is an increased incidence of oral BRONJ in patients treated with oral BPs for more than 3 years [5,9], which is in agreement with the findings of the studies revised here [4,6,9,12,15-17,19-21]. In fact, longer-term use of oral BPs may have a dose-equivalence effect, potentially approximating BPs levels in bone thought to be achieved only through high-dose intravenous delivery [16], and the total dose administered over a long period of time is important for the magnitude of the bone turnover reduction [50].

An interesting observation is that it has been suggested a trend of early-onset lesions due to steroids therapy [9]. However, this tendency was not confirmed in the patients of the studies revised in this paper, since the mean duration of BP therapy in steroid-treated patients was similar to patients that have not been treated with this drug. Furthermore, some authors reported early-onset lesions in patients with RA and no history of steroids [3,19].

The literature indicates a strong association of BRONJ with dental surgical procedures in all groups of patients

[3,5,9]. Although we also observed this tendency in the large number of lesions triggered by teeth extractions and dental implants in the studies that were retrieved [4,5,7-9,12,14,17-20], it is relevant to state that spontaneous occurrence of BRONJ lesions was observed in a significant proportion of individuals in several studies [9,10,12,13,15,19-21].

Most of the lesions in the studies were diagnosed in lower jaw at stage II or III [4,6-21] with the most common clinical findings being bone exposure, pain, edema, and purulent discharge. In fact, it also represents the same clinical aspects found in patients with no RA [6,9,14,16]. This evidence brings to light the lack of knowledge and attention about early clinical features that include non-specific signs and symptoms in the oral cavity, including no clinical evidence of bone exposure. The concern is that lesions can progress rapidly, and, in advanced stages, paresthesia, fistula formation, and pathologic fracture can also occur, even in RA patients [6].

The typical imaging finding in all groups of patients with BRONJ is the association of osteolysis and sclerosis aspects. Within this context, when it is considered that bone sclerosis is often detected in the initial stages of BRONJ [51], this sign can also be maintained in advanced stages; indeed, we observed in the studies that bone sclerosis was reported in six patients, all classified in advanced stages [7,12,17,19,21]. Therefore, a careful dental examination could allow an early diagnosis and intervention and assist the patients in responding more promptly to stage-specific treatment regimens for BRONJ.

Regarding the management and outcomes of BRONJ lesions in patients with RA, there is no widely accepted treatment protocol to BRONJ. Some authors believe that surgical procedures are not effective in patients with BRONJ and have led to further bone exposure, worsening of symptoms, and a greater risk of pathologic fracture, indicating conservative approaches as the best choice, including antibiotics, oral rinses with chlorhexidine, and 'drug holidays'[47]. In contrast, other authors believe that surgical procedures may achieve better outcomes in non-neoplastic patients [14].

In the papers that we revised, the lesions were treated either by conservative therapy (48%) or its association with surgical procedures (52%). Overall, this literature review showed that most of the lesions were treated with conservative approach [9,10,12,14,17,19,20] and were associated with the most positive outcomes including the complete healing of the lesions (33.3%) and no healing in only one patient (8.33%) [17] (Table 4). However, in other studies [4,6,7,12,13,15-19,21] the association of conservative and surgical therapy showed more diverse outcomes, with complete healing in only three lesions (25%) [13,21].

In fact, the management of BRONJ lesions is a great challenge, which reinforces the necessity of an adequate

oral care through routine dental examinations, education and motivation of the patients to adopt preventive measures in order to maintain a good oral hygiene and [52].

## Conclusion

The main characteristics of BRONJ patients with RA are generally similar to those with no RA. This critical review highlights a serious concern regarding the delayed diagnosis of BRONJ lesions, since most of these patients were diagnosed in stage II/III. It is clear that the rheumatologist needs to be aware of the potential risk of their patients developing BRONJ and must work together with the dentist to prevent and detect the lesions as soon as possible.

## Authors' contributions

NCN analyzed the records, reviewed all patients' data. ASB drafted the manuscript and helped in writing the text. RACM and EMJ drafted the manuscript and reviewed it critically. All authors read and approved the final manuscript.

## Authors' information

NCN is a PhD student from Implantology program at Araraquara School of Dentistry and ASB is a PhD student from Periodontology program at Araraquara School of Dentistry. EMJ and RACM are professors and chairmen of the Department of Diagnosis and Surgery, Division of Periodontology at Araraquara School of Dentistry.

## Competing interests

The authors declare that they have no competing interests.

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## *3 Proposição*



### **3 Objetivo geral**

Desenvolvimento de um modelo experimental de osteonecrose dos maxilares induzidas por bisfosfonatos em ratos, por meio de uma combinação de fatores de risco, como o uso prolongado de altas doses de alendronato por via parenteral, procedimento odontológico cirúrgico e o estresse crônico.

#### **3.3 Objetivos específicos**

- **Estudo 1:** Avaliar se altas doses diárias de alendronato por tempo prolongado provocam OMAB quando associadas à exodontia.
  
- **Estudo 2:** Avaliar se altas doses semanais de alendronato por tempo prolongado provocam OMAB quando associadas à exodontia.
  
- **Estudo 3:** Avaliar se altas doses semanais de alendronato por tempo prolongado provocam lesões osteonecróticas quando associadas a implantes instalados na tíbia em animais submetidos à indução de estresse crônico.
  
- **Estudo 4:** Avaliar comparativamente a influência de altas doses semanais de alendronato por tempo prolongado sobre a osseointegração de implantes maxilares e tibiais e, neste contexto, se o estresse crônico pode representar um fator de risco para a OMAB.

## ***4 Resultados***

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# ESTUDO 1

**Artigo 4** - Long-term treatment with alendronate increases the surgical difficulty degree during simple exodontias – an in vivo observation.

*Artigo submetido para publicação no periódico Head & Face Medicine*

Conte-Neto N, Spolidorio LC, Chierici-Marcantonio RA, Bastos AS, Marcantonio Jr E

**Artigo 5** - Experimental development of Bisphosphonate-related osteonecrosis of the jaws in rodents

*Artigo enviado para publicação no International Journal of Experimental Pathology*

Conte-Neto N, Spolidorio LC, Andrade CR, Guimarães MR, Bastos AS, Marcantonio Jr E

**Long-term treatment with alendronate increases the surgical difficulty during simple exodontias – an *in vivo* observation.**

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## **Abstract**

**Background:** Atraumatic teeth extractions protocols are highly encouraged in patients taking bisphosphonates (Bps) to reduce surgical trauma and, consequently, the risk of jaws osteonecrosis development. In this way, this paper aims to report the findings of increased surgical difficulty during simple exodontias in animals treated with bisphosphonates.

**Methods:** Sixty male Holtzman rats were randomly distributed into three groups of 20 animals and received daily subcutaneous administration of 1mg/kg (AL1) or 3mg/kg (AL3) of alendronate or saline solution (CTL). After 60 days of drug therapy all animals were submitted to first lower molars extractions under general anesthesia. Operatory surgical time and the frequency of teeth fractures were measured as principal outcomes and indicators of surgical difficulty degree.

**Results:** Animals treated with alendronate (AL1 and AL3) were associated to higher operatory times and increased frequency of teeth fractures compared to match controls.

**Conclusions:** The bisphosphonate therapy may be associated with an increased surgical difficulty and trauma following simple exodontias protocols, which is considered a critical issue when it comes to osteonecrosis development.

**Key-words:** bisphosphonates; tooth extraction; osteonecrosis

## **Background**

Tooth Extraction is one of the most common procedures in oral surgery practice and the difficulty to perform this procedure varies according to a sort of risk factors. Among these factors, the increased bone density has been recognized as a relevant feature [1, 2] that can be an aging physiologic issue or be resulted of antiresorptive drugs, including bisphosphonates (Bps) [3].

Currently, Bps are very often prescript around the world which has been generating a great concern due to the increasing number of Bisphosphonate-related Osteonecrosis of the Jaws (BRONJ). Considering the strong correlation between the etiology of this bone disease with tooth extractions [4-6] many efforts, have been made to reduce the surgical trauma in these patients [7, 8]. In this context this paper aims to report the findings of increased surgical difficulty, based on the analysis of operatory time and teeth fractures frequency, associated with bisphosphonate therapy.

## **Material and methods**

### *Animals*

Sixty male Holtzman rats weighting 155 to 200 g were used and randomly distributed into three groups of 20 animals each. The rats were housed in polypropylene cages in groups of five animals per cage, at controlled room temperature ( $23\pm 2^{\circ}\text{C}$ ), humidity ( $55\pm 10\%$ ), and 12/12 h light/dark cycle beginning at 7:00 a.m. Standard chow and tap water were available *ad libitum*. All the protocols described here were approved by local Ethics Committe of the School of Dentistry of Araraquara, São Paulo, Brazil (Protocol number 18/2009)

### *Treatments*

The animals received daily subcutaneous doses of alendronate (Ale, 1 or 3 mg/kg; ALCON, São Paulo, Brazil), or saline solution (0.9% NaCl; control group). After 60 days of alendronate or saline solution treatment, all animals were submitted to lower first molars extractions under general anesthesia using an intraperitoneal injection of ketamine (0.1 ml/100g body weight)

### *Teeth extraction*

Teeth extractions were performed by the same operator with the same technique in all animals. Initially, the rats were placed in a dorsal position and fixed in a special device. After that, the surrounding gingival was carefully detached from the lower first molars with a dental explorer and teeth luxation were made using a Hollenback Carver followed by the tooth removal with a forceps, adapted around the cervical line of the tooth.

### *Difficulty factors risk indicators*

The assessment of extraction difficulty degree was based on teeth fractures frequency and on the time spent to perform the extraction. This last parameter was defined as the interval between the utilization of the first instrument required to the tooth extraction until the use of the last instrument. Extraction time was measured using a digital timepiece for each case included in the study. The same individual (A.S.B.) measured all extraction times [9] to reduce possible bias.

Teeth fractures frequency were defined as a complete loose in tooth continuity involving crown and/or roots [10].

### *Statistical Analysis*

The data were evaluated using the GraphPad Prism 5.0 software package (GraphPad Inc., San Diego, CA USA). The normality of the data was assessed by the Kolmogorov-Smirnov test. Comparisons among groups were performed using the chi-squared test to teeth fractures frequency analysis and Kruskal–Wallis followed by Dunns post test to surgery duration evaluation (non-parametric data). Results are presented as frequency of teeth fractures. Statistical significance was set at 5% with 95% confidence intervals.

### **Results**

During lower first molars extractions of experimental animals, surgical difficulty was markedly increased in animals treated with alendronate 1mg (AL<sub>1</sub>) and 3 mg (AL<sub>3</sub>) when compared to control group (CTL). Group AL<sub>1</sub> and AL<sub>3</sub> animals presented a higher operatory time when compared to animals in control group (Figure 1).

Moreover, in the teeth fractures frequency analysis it was observed a significant difference between the groups. While animals in CTL group presented 8% of teeth fractures, animals in alendronate group presented 39.4% (AL<sub>1</sub>) ( $p < 0.01$ ) and 62.5% (AL<sub>3</sub>) ( $p < 0.001$ ). Thus, comparing AL<sub>1</sub> with AL<sub>3</sub> groups, it was observed that AL<sub>3</sub> animals presented teeth fractures frequency significantly increased compared with AL<sub>1</sub> group ( $p < 0.05$ ) (Figure 2).

### **Discussion**

In fact, the assessment of surgical difficulty is a relevant issue in the field of oral surgery since allows health professionals to plan operations more accurately helping to minimize surgical trauma, and risks of accidents and complications. This concern is highly relevant to patients treated with bisphosphonates due to the jaws osteonecrosis risk.



The assessment of extraction difficulty has been measured via a wide range of variables [9] and, among of them, the extraction time [11, 12] and complications frequency [13, 14] are well recognized indicators. For this reason, we selected these two variables as the outcome measures to evaluate the relationship of bisphosphonate therapy with surgical difficulty.

Our findings demonstrated that teeth extractions in animals treated with bisphosphonate require more surgical time when compared to control animals following simple extraction technique. In our opinion, the main reason to justify this surgical time difference is related to the presence of an increased bone density and consequently decrease bone elasticity, that are well recognized difficult factors to teeth extractions [1, 2] and are a result of Bps treatment [3].

Even with the absence of bone density evaluation and measurement methods, it is reasonably to believe that Bps treated animals presented a high bone density that is supported by the following points:

- 1- It is known that the bone effect of Bps is cumulative and assumes a bone absorption linear aspect until 5 mg/kg endovenously dosages [15]. Therefore, the long-term treatment of Bps in high dosages (1 and 3 mg/kg) used in this study resulted in an expressive Bps bone effect.
- 2- Bps were administered by subcutaneous route that are as effective as endovenously route regarding to drug bioavailability [15]. By this route it is estimated that more of 50% of the drug is available for bone matrix incorporation [16, 17].
- 3- Due to the high bone turnover in cortical alveolar bone is believed that, although controversial, the Bps bone absorption is higher when compared to other skeletal

sites [18], which can be justify by the alveolar lamina dura sclerosis seen in Bps treated patients with BRONJ in initial stage [19]. Besides, the mandibular bone has by itself a higher tissue degree of mineralization when compared to maxillae, been more prone to Bps effects and naturally increases surgical difficulty [9].

In this way, when it is opted to a simple exodontias technique, there is a highly dependence of the tooth to expand the bone tooth socket walls to allow its avulsion and in situations of an alveolar bone increased density there is a lack of sufficient socket expansion which obviously limits the teeth avulsion axis. Consequently, as it happened in this study, requires more surgical manipulation, thereby prolonging operating time [9], as well as the surgical trauma and increases the risk of accidents and complications.

Teeth fractures have been considerate the most frequent accidents during exodontias, in oral surgery practice [20]. They are usually related to inadequate instrumental use and excessive force use, which was one of the reasons that could justify the high frequency of teeth fractures in animals treated with Bps observed in this paper. The concern about this issue is that many efforts have been made to reduce the surgical trauma during teeth extractions by using atraumatic protocols in Bps treated patients [7, 8] since exodontias have been considerate as one the most frequent trigger factor to BRONJ [4-6].

In this context, as stated previously, in situations of an increased surgical difficulty degree there is a tendency to prolong surgical length and increase tissue trauma which in field of ONJ can lead to relevant implications:

- 1- Increase the inflammation of the alveolar bone [21], which could act in favor of the BRONJ lesions development according to the inflammatory theory [22].
- 2- Result in delayed extraction wound healing due to the compression of bone lining the socket impairing vascular penetration and results in thrombosis of the vessels

[21], which could act in favor of the BRONJ lesions development according to the angiogenic theory [23].

- 3- Increase the risk of dento-alveolar fractures, since when bone tissue becomes too highly mineralized, it also becomes brittle [24]. Moreover, it also makes the tissue more prone to microcrack initiation [25], which act in favor to the BRONJ lesions development according to the bone suppression theory [26].

Another concern that can be discussed regarding to the teeth fractures is about the approaches after these accidents:

- 1- If opted to extract the residual fragment, the surgical time can be prolonged and increases the tissue trauma, being sometimes necessary to perform bone removal techniques, which can contribute to BRONJ lesions as stated previously.
- 2- If opted to keep the residual fragment and follow the patient, the surgical trauma will be obviously lower; however, eventually tooth or bone fragments/remnants can lead to an increase in the risk of socket infection [21], which could also increase the risk of osteonecrosis according to the infectious theory [26].

Considering that the more atraumatic is the teeth extraction the better is for the healing process, with special mention in Bps treated patients, we highlight the strategies that can reduce the force intensity and the risk of teeth fracture during the exodontias, such as odontotomy techniques. Extractions without tooth sectioning might be responsible for a more traumatic and difficult surgery, especially in light of difficulty factors, such as increased bone density that can lead to several complications related to BRONJ lesions.

## **Conclusions**

The bisphosphonate therapy may be associated with an increased surgical difficulty and trauma following simple exodontias protocols, which is considered a critical issue when it comes to osteonecrosis development.

## **Consent**

Written informed consent was obtained from the patients for publication of these case reports and any accompanying images. A copy of the written consent form is available for review by the Editor-in-Chief of this journal.

## **Competing interests**

The authors declare that they have no competing interests.

## **Authors' contributions**

NCN performed one surgery under the supervision of the corresponding author, analyzed the records, reviewed all patients' data and designed the case report. ASB drafted the manuscript and helped in writing the text. LCS and RACM drafted the manuscript and reviewed it critically. EMJ performed one of the surgical procedures and reviewed the manuscript. All authors read and approved the final manuscript.

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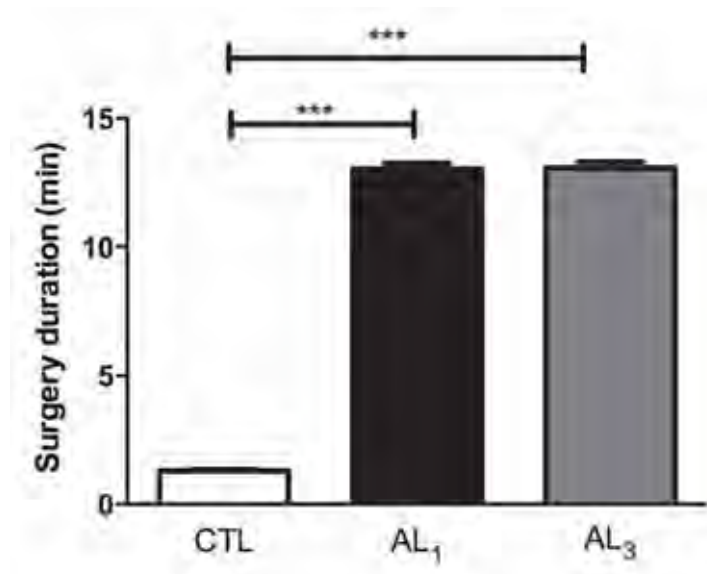
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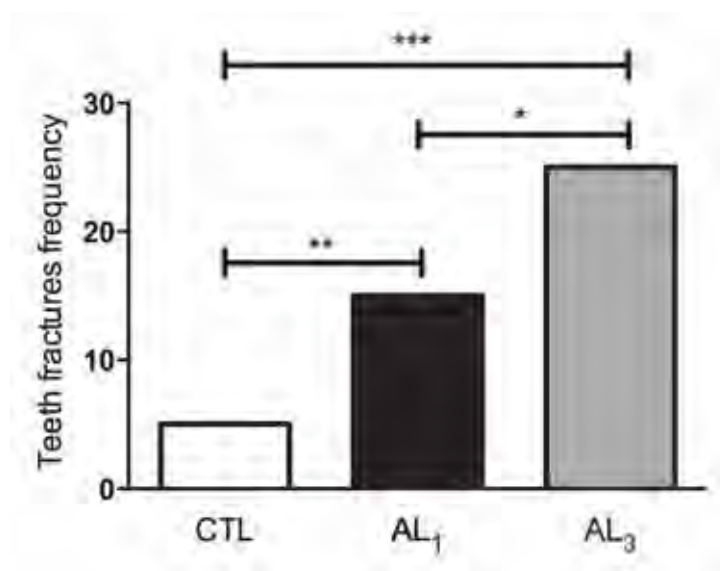
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## FIGURE LEGENDS



**Figure 1** – Surgery duration in experimental groups (\*\* $p < 0.001$ ; Kruskal–Wallis followed by Dunns post test).



**Figure 2** – Teeth fractures frequency in experimental groups. A total of 40 teeth extractions were performed for each group (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; chi-squared test).



## **Experimental development of bisphosphonate-related osteonecrosis of the jaws in rodents**

### **BRONJ-like lesions in rodents**

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**Abstract**

Bisphosphonate-related osteonecrosis of the jaws (BRONJ) has become of increased interest in the scientific community, due in particular to its as-yet-unsolved pathogenesis. An experimental model of BRONJ was induced in normal male rats (Alendronate; 1 mg/Kg/wk; n = 10) and matched controls (saline solution; n = 10). After 60 days of drug treatment, all animals were subjected to extractions of the left first lower molars and were euthanized at 3 and 28 days post-surgery. The following analyses were performed: (i) descriptive and quantitative (scores) histological evaluation; (ii) stereometry of distal sockets; and (iii) biochemical measurement of C-telopeptide cross-linked collagen type I (CTX) and bone-specific alkaline phosphatase (BALP). The results showed that, 28 days post-surgery, the animals treated with alendronate presented areas of exposed and necrotic bone, associated with significant infection, especially at the inter-alveolar septum area and crestal regions, compared with controls. The levels of CTX, BALP, and bone volume, as well as the degrees of inflammation and vascularization, were significantly reduced in these animals. Therefore, analysis of the data presented suggests that alendronate therapy is associated with the development of osteonecrosis in the jaws of rodents after tooth extraction.

**Keywords:** Alendronate; osteonecrosis; tooth extraction

## Introduction

Bisphosphonates (Bps) are synthetic analogs of inorganic pyrophosphate that have strong properties for the suppression of osteoclast-mediated bone resorption. They have been widely used to stabilize the bone loss occasioned by bone disorders, such as osteoporosis and Paget's disease (Rogers *et al.* 2000). However, since 2003, increasing reports of bone necrosis induced by Bps (BRONJ) have been described in the literature.

Several hypotheses concerning the pathogenesis of BRONJ have been proposed, but none has been accepted completely. However, analysis of data retrieved from clinical studies suggests that some co-factors may play a relevant role in the development of these lesions, even in the presence of BRONJ with no obvious co-morbidity factors (Marx *et al.* 2005; Ruggiero *et al.* 2004).

Within this context, many investigators have attempted to reproduce this pathological condition in experimental animals. Indeed, models of BRONJ-like lesions have been published in the literature, being developed by the association of Bps therapy, tooth extraction, and other co-factors, such as concomitant steroid use (Bi *et al.* 2010; Lopez-Jornet *et al.* 2010; Sonis *et al.* 2009), vitamin D deficiency (Hokugo *et al.* 2010), or increased socket damage (Biasotto *et al.* 2010). However, the great number of variables could reduce the reliability of these models.

The purpose of this study was to develop a suitable experimental model of BRONJ-like lesions by the association of only Bps therapy and tooth extraction, without other variable factors. Furthermore, we aimed to discuss critically relevant features that could justify the strong relationship between BRONJ and tooth extraction.

## Materials and methods

### *Animals and reagents*

The study was approved by the Ethics in Animal Research Committee of the School of Dentistry of Araraquara (UNESP, Brazil) (protocol number 18/2009). It included 20 male rats (*Rattus norvegicus*, albinus, Holtzmann), each weighing around 200 g. These animals were kept in a special room at São Paulo State University – UNESP, School of Dentistry of Araraquara and maintained on a 12:12-hour light/dark cycle (lights on at 7:00 a.m.) at  $23 \pm 2^\circ\text{C}$  with *ad libitum* access to a standard laboratory diet and water.

The alendronate (ALN) was purchased from ALCON Laboratory (São Paulo, SP, Brazil). The drug was dissolved in sterile physiological saline (0.9% NaCl) and diluted to the given concentrations.

### *Experimental design and surgical procedures*

After a 3-day acclimatization period, animals were randomly assigned in two experimental groups: AL (n = 10), including animals treated with daily subcutaneous doses of ALN (1 mg/kg); or CTL (n = 10), comprised of animals subjected to sterile physiological saline, following the same schedule as AL group.

After 60 days of the pharmacologic therapy, all animals were subjected to left lower first molar (M1) extraction under general anesthesia by a combination of ketamine chloridrate (Ketamina Agener, Agener União Ltda, São Paulo, SP, Brazil; 0.08 mL/100 g body weight) and Xylazine 2% (Rompum, Bayer S.A., São Paulo, SP, Brazil; 0.04 mL/100 g body weight).

The same professional performed the tooth extractions with the same technique in all animals. Initially, the rats were placed in a dorsal position and fixed in a special device. The surrounding gingivae were carefully detached from the lower first molars with a dental explorer. Then, with a Hollenback Carver, the tooth was luxated and separated into 2 segments (mesial and distal) that were removed with a forceps adapted around the cervical line of the segments.

After the surgical procedure, all animals received an intramuscular dose of antibiotic (Pentabiótico<sup>®</sup>, Wyeth-Whitehall Ltda, São Paulo, Brazil – 0.1 mg/Kg) and anti-inflammatory Ketoflex (Ketoprofen 1.0%, 0.03 mL/rat). Animals were euthanized by anesthesia overdose at 3 and 28 days after tooth extractions, and the ALN or sterile physiological saline administration protocol was maintained until the animals' death (Figure 1).

#### *Specimen processing*

All tissue blocks were immersed directly in 10% buffered formalin fixative solution for 48 h. After that, 5 specimens of each group/period were subjected to routine histological processing for descriptive and stereometric evaluation. All specimens were decalcified in tetrasodium-EDTA aqueous solution (0.5 M, pH 7.4) for 2–3 months, under agitation at room temperature. All specimens were then processed and included in paraffin blocks. Serial 4- $\mu$ m sections were obtained in the bucco-lingual direction, stained with hematoxylin and eosin, and referred for evaluation by light microscopy (Leica DM1200M; Leica Microsystems, Wetzlar, Hessen, Germany).

### *Histological and stereometric analyses*

One Board-certified oral pathologist blinded to the group assignments performed these analyses in three distinct moments to minimize discrepancy in the scores (kappa index=0,76). The histological endpoints were evaluated at 4 fields in each section (2 superior and 2 inferior) using magnifications of 50x, 100x and 200x. It included the degree of bone necrosis and infection, the quantity and quality of inflammation (acute, chronic, or mixed), as well as the vascularization degree (vessels number). These parameters were scored on a four-point scale 0 (absent; 0%), 1(mild;  $\geq 10\%$ ), 2 (moderate;  $>10$  and  $\leq 50\%$ ) and 3 (increased;  $> 50\%$ ).

The stereometric analysis was performed with Leica Application software Suite 3.8.0 (Leica Microsystems LTD, Heerburgg, Germany). The measurements were performed at the distal root of the left M1 at the regions of interest (ROIs) that included 3 different areas inside the sockets. Initially, a standard ROI was determined by identification of a quadrangular area extending from the level of the cemento-enamel junction (CEJ) of the left second mandibular (M2) to the apical end level of the M2 and between the mesial and distal alveolar bone surfaces. This quadrangular area was divided into 3 equal ROIs (1, 2, and 3) (Figure 2).

The variables analyzed included the percentage of the root socket filled with bone tissue (BV) in each ROI, following the nomenclature and abbreviations recommended by the American Society for Bone and Mineral Research (Parfitt, 1988). Bone volume (%) represented bone volume ( $\text{mm}^3$ ) per total tissue volume ( $\text{mm}^3$ ). All analyses were performed at a magnification of 100x, and 3 measurements were taken for each specimen, to complete the stereometric analysis. The distance between the selected sections was 50  $\mu\text{m}$ .

### *Assessment of bone turnover biochemical markers*

Blood samples were collected on the day of sacrifice by cardiac puncture and centrifuged for plasma separation. The levels of serum collagen type 1 cross-linked C-telopeptide (CTX) and bone-specific alkaline phosphatase (BALP) were determined by enzyme-linked immunosorbent assay (ELISA) kits (CUSABIO BIOTECH CO., Ltd, Wuhan, P.R. China).

### *Statistical analysis*

Data were initially subjected to the Kolmogorov-Smirnov normality test. We then performed comparisons among groups and periods for non-parametric data, using the Kruskal-Wallis test (non-parametric data), followed by Dunn's multiple-comparison test or analysis of variance (ANOVA), then Tukey tests for parametric data. The data were evaluated by means of GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA), and statistical significance was set at  $p < 0.05$  with 95% confidence intervals.

## **Results**

### *Clinical features*

Twenty-eight days after tooth extraction, all animals of the AL group presented with partial epithelial coverage, exhibiting areas of bone exposure. In contrast, control animals showed total epithelial coverage, without signs of inflammation (Figure 3). Furthermore, some of the animals in the AL group exhibited areas of exposed bone around the upper incisors, which were associated with coronal fractures (Figure 4).

### *Histological features*

Three days after tooth extraction, all groups exhibited areas of necrotic bone and infection, especially at the inter-alveolar septum area (Figure 5). However, animals treated with ALN presented significantly increased necrotic bone at 28 days ( $p < 0.01$ ), while control animals displayed a statistically significant decrease in the same period ( $p < 0.01$ ) (Graphic 1a and Figure 6). Furthermore, the CTL animals also exhibited statistically significantly decreased infection at 28 days ( $p < 0.001$ ), while the AL group did not (Graphic 1b).

Regarding the inflammatory condition, animals in the control group exhibited scores significantly higher at 3 days compared with those in the AL group ( $p < 0.01$ ), and the scores decreased markedly at 28 days only in control animals ( $p < 0.001$ ), while no statistical changes were observed in the AL group (Graphic 2a). Furthermore, while inflammation was predominantly acute at 3 days in control animals, it was mainly chronic in the AL group in the same time. At 28 days, both groups presented characteristic chronic inflammation (Graphic 2b).

No important differences in vascularization were found between the groups at 3 days. However, at 28 days, animals treated with ALN presented significantly less vascularization compared with the control animals ( $p < 0.01$ ). Furthermore, while this process increased from 3 to 28 days in control animals ( $p < 0.05$ ), no differences were observed in the AL group in the same periods (Graphic 3).

Regarding the BV count, we observed that animals of the AL group presented rates significantly lower than those in the control group at ROI 2 ( $p < 0.001$ ), while no statistically significant differences were found in ROIs 1 and 3 (Graphic 4).



### *Bone metabolism markers*

Animals treated with ALN presented the lowest values of BALP at both 3 ( $p < 0.05$ ) and 28 days ( $p < 0.001$ ) when compared with control animals. This same tendency was observed for CTX levels ( $p < 0.05$ ) (Graphic 5).

## **Discussion**

The development of animal models of pathological conditions has several advantages for the study of disease. As can be seen with the increasing reports of BRONJ from around the world, many efforts have been made to obtain experimental models of these lesions. However, most of them are associated with different variables, including Bps therapy, tooth extraction, concomitant steroid use (Bi *et al.* 2010), vitamin D deficiency (Hokugo *et al.*, 2010), or increased damage to the alveolar socket (Biasotto *et al.* 2010). Thus, in an attempt to increase the model's reliability, we reduced the number of variables and demonstrated the development of experimental BRONJ-like lesions using only the combination of Bps therapy and tooth extraction.

With the purpose of creating a favorable environment for the development of BRONJ, we first established a model of bone turnover suppression, based on the theory that, with reduced turnover, we would observe an increase in the numbers and sizes of matrix necrosis areas in jaws (Allen and Burr 2008). Thus, we prescribed long-term therapy with high non-toxic dosages of ALN (Lin 1996), based on the following assumptions: a) The magnitude of the reduction of bone turnover is closely related to the total Bps dose administered over a long period of time (Chapurlat and Delmas 2006); and b) the bone absorption of Bps follows a linear pattern until the

administration of intravenous doses of 5 mg/kg (Lin 1996), with no evidence of bone saturation or pharmacological interference with subsequent doses (Porras *et al.* 1999).

Indeed, our findings of the lowest levels of CTX and BALP, which are markers of bone metabolism (Pagani *et al.* 2005), in the AL group confirm the suppression of bone metabolism. Furthermore, the relevance of the Bps treatment schedule used in our study is supported by the findings of transient impairment (Aguirre *et al.* 2010; Hikita *et al.* 2009) or even the promotion of healing action of Bps (Jee *et al.* 2010) when lower cumulative dosages were associated with tooth extraction, in contrast to the prolonged bone lesions found in this study. Therefore, the Bps schedule seems to be closely related to BRONJ pathogenesis.

Although BRONJ lesions can occur spontaneously (Conte-Neto *et al.* 2011), most of the reports have been associated with surgical procedures (Ruggiero *et al.* 2009). In this study, we opted to use tooth extraction as the trigger agent, based on the fact that analysis of data retrieved from clinical and *in vivo* studies (Bi *et al.* 2010; Lopez-Jornet *et al.* 2010; Thumbigere-Math *et al.* 2009; Treister *et al.* 2009) suggested a strong relationship between tooth extraction and BRONJ. Some authors have reported that this kind of bone necrosis may be related to the impairment of bone capacity to accommodate the increasing demand for healing that is required in situations of tissue trauma such as surgical procedures and infection (Woo *et al.* 2005).

Indeed, our study confirmed that tooth extraction appears to be relevant to the development of BRONJ-like disease. Of note, we also demonstrated some bone lesions around upper incisors of ALN-treated animals that, curiously, were present only in the animals with coronal tooth fractures. Considering that these animals

constantly chewed their cages, our hypothesis is that this habit could generate an overload on periodontal tissue, thus facilitating lesion development and supporting the role of trauma as a BRONJ trigger factor (Woo *et al.* 2005).

In contrast, considering the environment and dynamics of alveolar socket healing, we believed that, besides trauma, other factors, such as infection and ischemic conditions, might also explain the strong relationship between tooth extraction and BRONJ. This is based on the potential of Bps to impair several physiologic events that occur after tooth extraction, including inflammation (Breuil and Euller-Ziegler 2006), angiogenesis, resorption activity, and epithelial migration (Kobayashi *et al.* 2010). Therefore, Bps not only impairs tissue scaffold re-establishment, but also leaves the organism more vulnerable to occasional harmful effects of micro-organisms and their by-products.

In this study, we demonstrated that Bps therapy is associated with an important impairment of vascularization in the later stages of alveolar healing. These findings can be reasonably explained by the inhibitory effects of Bps on angiogenesis (Fournier *et al.* 2002; Kobayashi *et al.* 2010), which is of particular interest considering that BRONJ lesions could be a result of ischemic changes to tissues (El-Salem *et al.* 2011).

In contrast, Aguirre *et al.* (2010), using low doses of ALN, observed only a transient impairment of angiogenesis after tooth extraction. These differences indicate that Bps could exert a dose-dependent effect on vascularization, and no anti-antigenic effect is observed with clinical dosing regimens (Biver *et al.* 2010). Furthermore, considering that, at 3 days, no differences in the degree of vascularization were found

in either group, the eight-week pre-treatment with ALN did not seem to interfere significantly with angiogenesis, at least in this phase.

It is well-known that inflammation is a natural step in the wound-healing process, being important for the removal of contaminating micro-organisms (Guo and Dipietro 2010). Indeed, after tooth extraction, we showed that the exposed bone, especially at the inter-radicular region, naturally became infected, regardless of the administration of Bps. However, in the AL group, this infection was present even in later periods.

Possible explanations for these features may be related to the changes in the inflammatory response induced by Bps. Some authors have stated that Bps may exert anti-inflammatory effects, decreasing the release of pro-inflammatory cytokines (Breuil and Euller-Ziegler 2006) and depressing the innate immune system for a prolonged time due to an impairment of neutrophils (Kuiper *et al.* 2011). Indeed, our findings demonstrated that the inflammatory response at 3 days was chronic instead of acute and significantly lower in animals treated with ALN. This is of special concern, since neutrophils are the first line of defense against micro-organisms, and a critical concentration of these cells is required for the effective killing of bacteria (Li *et al.* 2002).

In the context of infection, the dynamics of osteoclast activity during alveolar socket healing seems to be relevant. Soon after tooth extraction, a high degree of resorption takes place within the sockets to remove necrotic bone and bone debris (Lin *et al.* 1994). In this way, the inhibitory effects of Bps on osteoclasts, especially at the inter-dental alveolar area, allow for bone exposure to an environment rich in bacterial toxins, inflammatory cytokines, and oxidative stress (Aghaloo *et al.* 2011).

Indeed, we observed a high degree of bone necrosis and infection at the inter-dental alveolar area. Of note, necrotic bone fragments themselves can act as avascular foci for further bacterial adherence (Rodner *et al.* 2003), which could also be facilitated by Bps, since it has been demonstrated that BRONJ could result from increased bacterial adhesion to bone coated with these drugs (Kos 2011).

Another condition that could act in favor of infection is the soft-tissue impairment observed in animals treated with ALN, given the essential role of epithelial coverage protection against oral bacterial infection (Kawahara *et al.* 1998). After tooth extraction, there is naturally a high demand for oral epithelial cell migration, since healing occurs by secondary intention. This aspect is of special concern in the field of Bps, since these drugs inhibit the migration of oral epithelial cells (Kobayashi *et al.* 2010; Landesberg *et al.* 2008). In contrast, considering that the exposed bone areas were mainly located close to the infection, the potential impairment of bacteria and their by-products and their effects on soft-tissue healing should not be taken for granted, since infection can also impair epithelial cell growth (Pollanen *et al.* 1997).

Within this context, a relevant point to be discussed is the diagnostic criteria for BRONJ. The persistence of bone exposure in the oral cavity for at least 2 months has been recognized as an established diagnostic criterion (Ruggiero *et al.* 2009), and this concept has been extended to animal models of BRONJ-like lesions (Bi *et al.* 2010; Biasotto *et al.* 2010; El-Salem *et al.* 2011). However, given the inherent metabolic differences between species, we believe that this clinical human concept might not be applicable to animals.

Although the alveolar healing phases are similar in both humans and rats, they occur more rapidly in rodents than in humans (Bodner *et al.* 1993), lasting about one-third of the time required for human healing (Okamoto and de Russo 1973). Therefore, the four-week bone exposure in rats observed in this study could be considered a clinical concept of BRONJ in rats. To support this assumption, studies have shown transient impairment of Bps in alveolar socket healing, and these effects have not been extended beyond 14 days (Aguirre *et al.* 2010).

The effects of Bps on osteogenesis are not yet well-established in the literature. Our findings demonstrated that, 28 days after tooth extraction, the ALN-treated animals showed the lowest levels of bone volume at the ROI2. These features are in accordance with our outcomes of lower levels of BALP in the ALN group, which is in agreement with authors who found inhibitory effects of ALN over osteogenesis (Kobayashi *et al.* 2010).

In contrast, other authors did not find evidence of the direct effects of Bps on the ability of osteoblasts to produce bone matrix *in vivo* (Allen *et al.* 2006; Feher *et al.* 2010), which supports our results of similar bone volume levels at apical regions in both groups. Therefore, it is also possible that Bps indirectly reduced bone volume, delaying the initial steps of wound healing, since osteogenesis begins at the apical region, where statistically significant differences were not observed between groups, as previously stated.

In conclusion, we believe that infection may be closely related to lesion development. Indeed, the environment of tooth extraction in the field of Bps treatment is favorable to the development of infection, for several reasons: 1) lower inflammation response and degree of vascularization; 2) increased bacterial adhesion

to bone coated with Bps; and 3) maintenance of exposed bone to the oral cavity due to resorption and epithelial coverage impairment, which could act as a substrate for bacterial growth.

Within the limitations of this study, our results clearly demonstrated that the association of alendronate therapy and tooth extraction was able to induce BRONJ-like lesions in rodents. Further studies are necessary to investigate whether lower dosages of Bps, compatible with therapeutic doses, could also be related to the development of bone lesions.

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GRAPHICS AND FIGURES

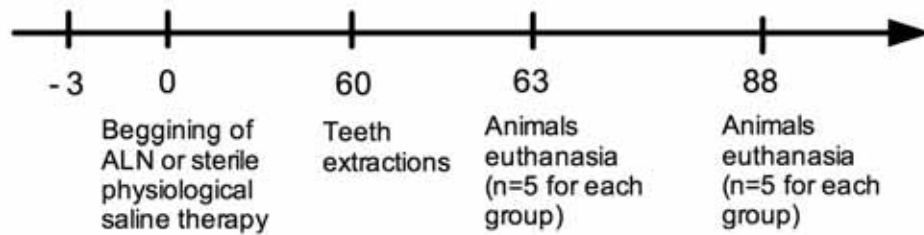


Figure 1 – Experimental design. After a 3-day acclimatization period, animals were randomly assigned to one of two experimental groups: AL (n = 10), which included animals receiving daily subcutaneous doses of alendronate (ALN) (1 mg/kg); or CTL (n = 10), composed of animals receiving sterile physiological saline, following the same schedule as for ALN. After 60 days of the drug therapy, all animals were subjected to left lower first molar (M1) extraction under general anesthesia. Animals were euthanized by anesthesia overdose at 3 and 28 days after tooth extraction, and the ALN or sterile physiological saline administration was maintained until sacrifice.

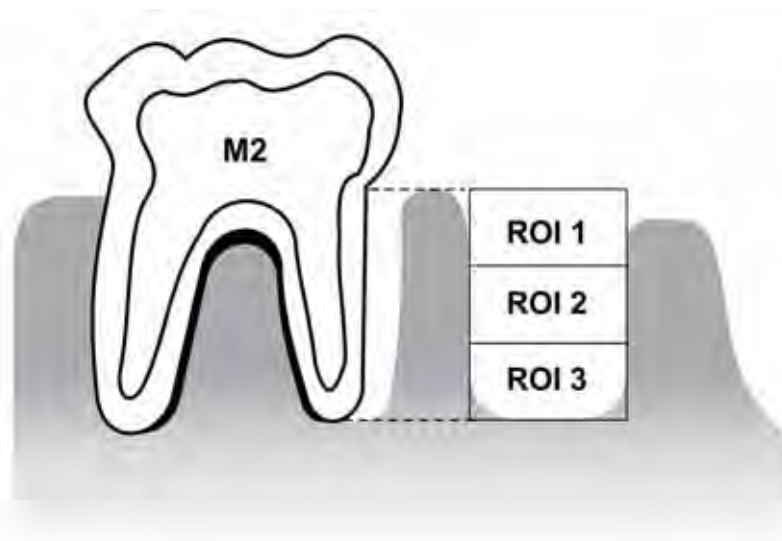


Figure 2 – Schematic view of the regions of interest (ROIs) for stereometric analysis. The measurements were done within the distal root of the left M1 at the ROIs that included three different areas inside the sockets. Initially, a standard ROI was determined by a quadrangular area extending from the level of the cemento-enamel junction (CEJ) of the left second mandibular (M2) to the apical end level of the M2 and between the mesial and distal alveolar bone surfaces. This quadrangular area was subsequently divided into three equal ROIs (1, 2, and 3).

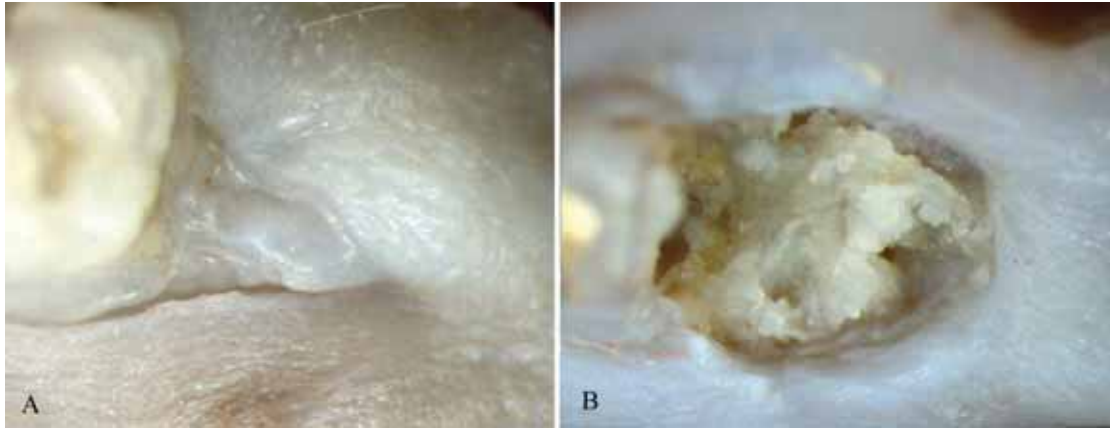


Figure 3 – Clinical aspects of a lower first molar (M1) alveolar socket 28 days after tooth extraction. Animals treated with sterile physiological saline (A) presented a full epithelial lining on the alveolar socket, with no signs of inflammation or infection. In contrast, animals treated with alendronate (B) exhibited significant areas of exposed bone with a marked impairment of alveolar socket re-epithelialization.



Figure 4 –Clinical aspects of bone lesions around upper incisors of animals treated with alendronate. Some animals of the AL group presented, at euthanasia, areas of exposed bone surrounding the upper incisors. Of note, these lesion areas were associated, in all animals, with coronal fractures.

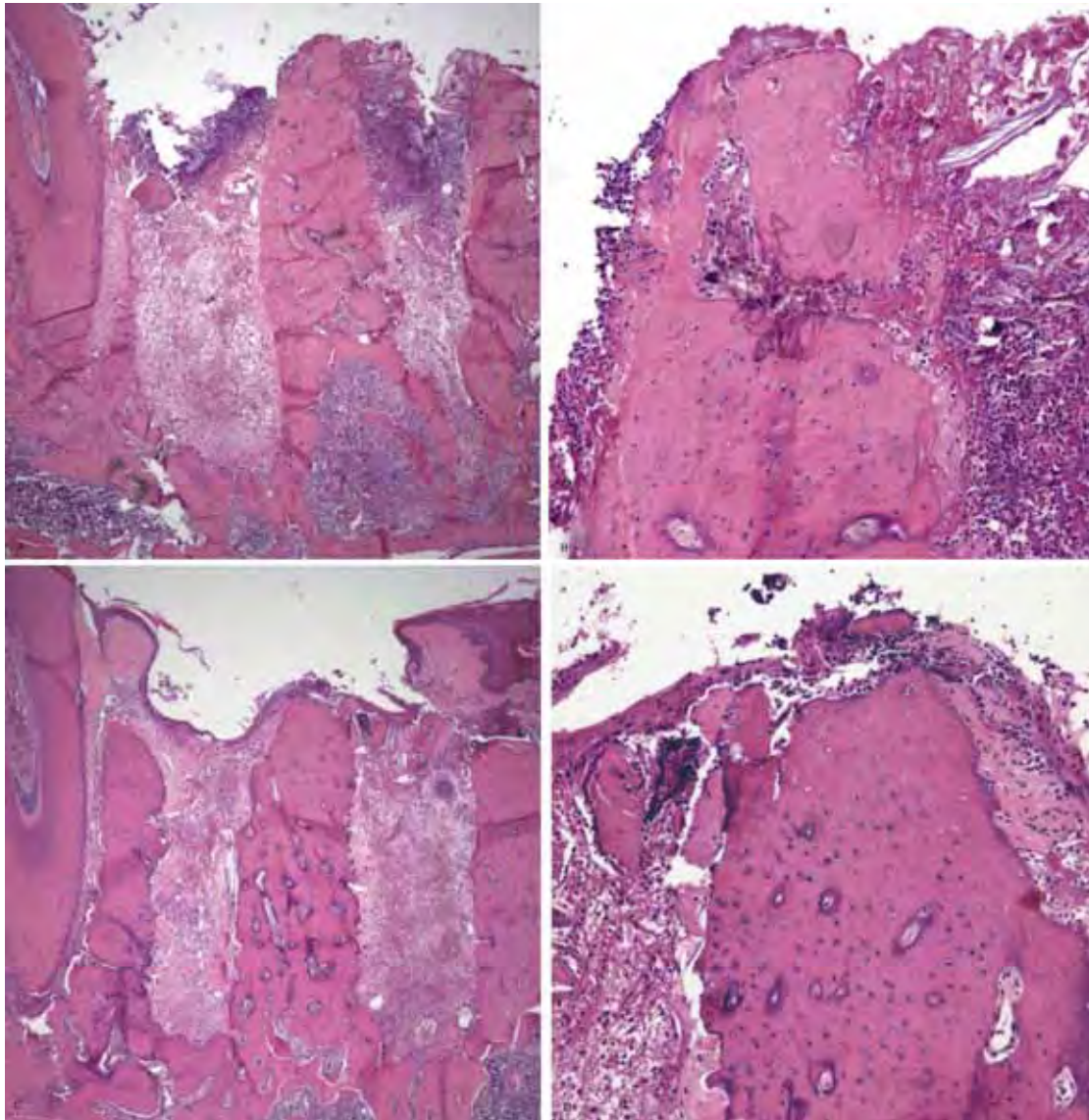


Figure 5 – Histologic sections of tooth sockets 3 days after left lower first molar extraction and stained with hematoxylin and eosin. Both the CTL (A) and AL (C) groups presented granulation tissue formation at this time (original magnification x25), as well as empty osteocyte lacunae at superior areas of the inter-radicular septum (B, CTL; D, AL; original magnification x100).

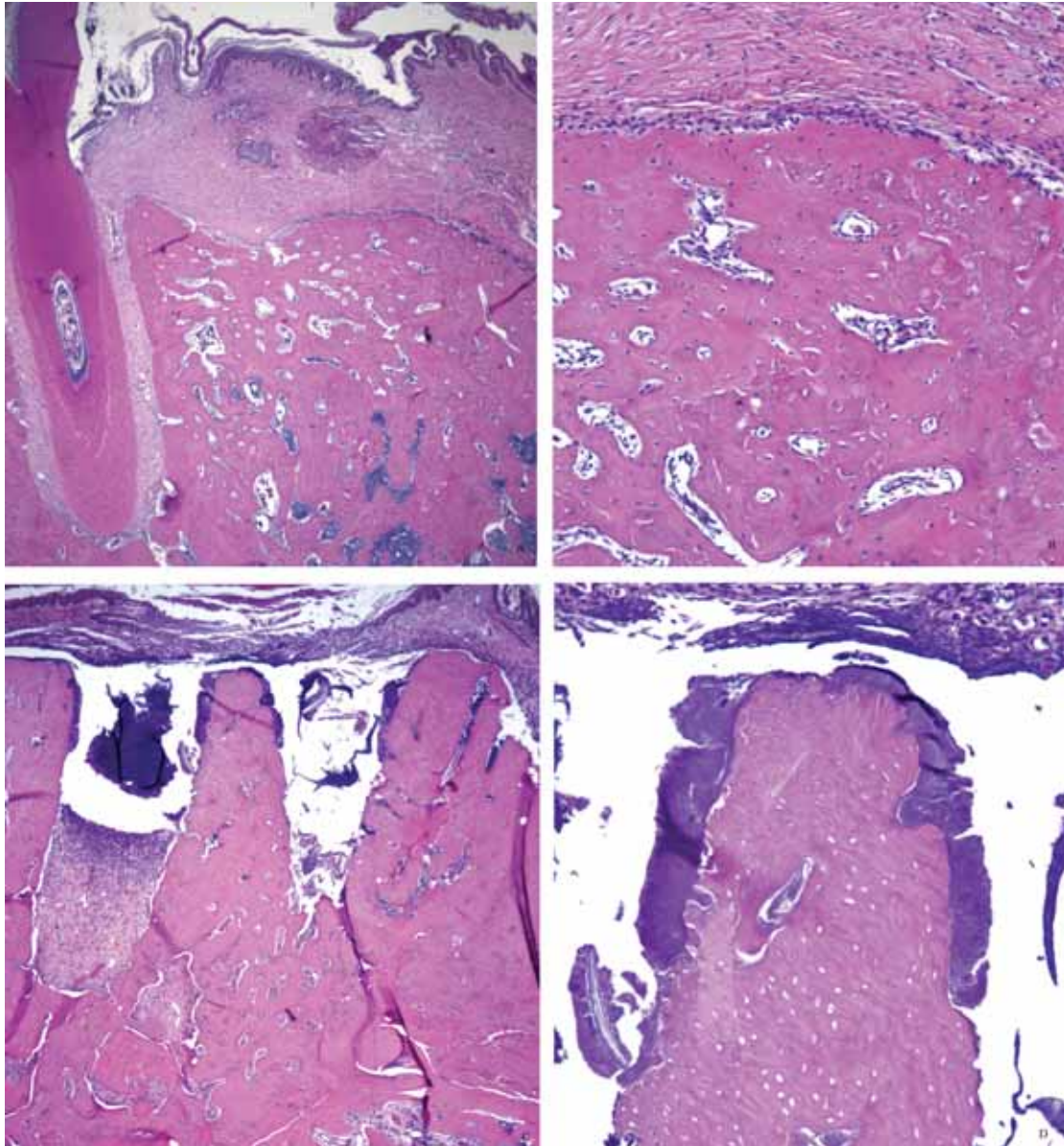
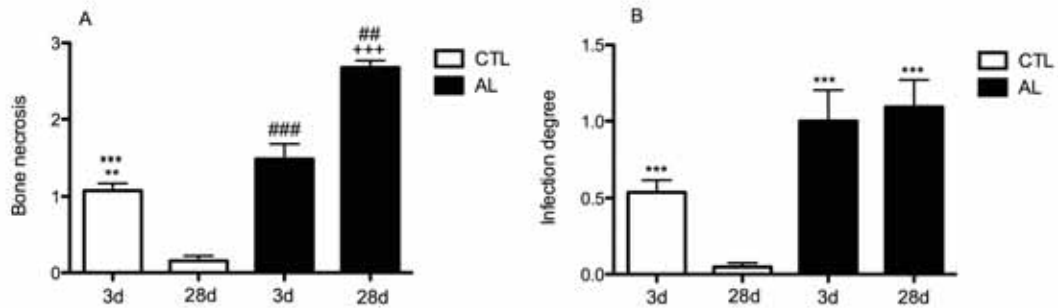
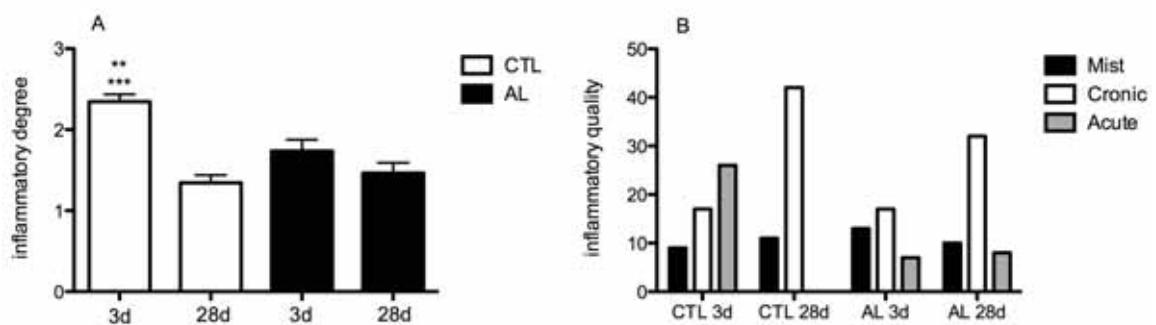


Figure 6 – Histologic sections of tooth sockets 28 days after left lower first molar extraction and stained with hematoxylin and eosin. A) Animals in the CTL group presented complete alveolar bone regeneration, while animals in the AL group (C) exhibited only slight bone formation, restricted to the apical socket area (original magnification x25). The animals treated with alendronate (D) demonstrated retention of the inter-radicular septum, which was associated with bone necrosis and infection, while in the CTL (B) group this area was completely remodeled, with signs of bone necrosis (original magnification x100).

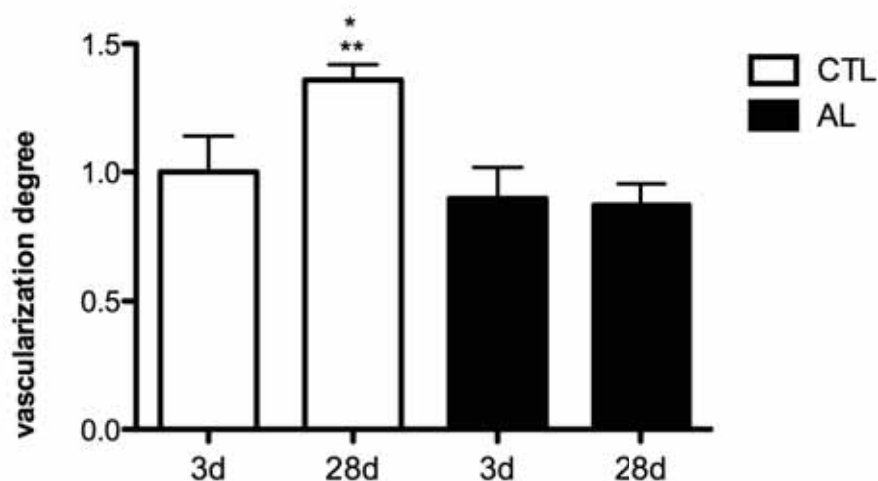




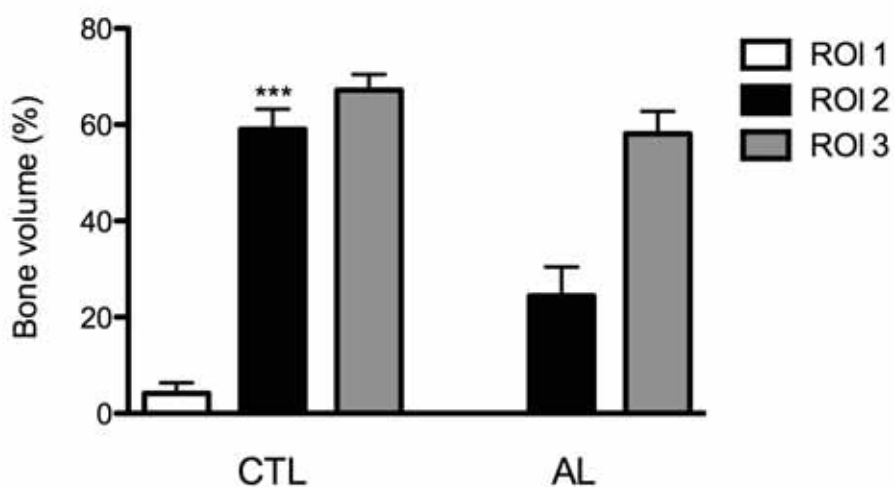
Graphic 1 - The effects of alendronate therapy on bone necrotic levels (A) and degrees of infection (B) at 3 and 28 days after tooth extraction. A) Animals treated with ALN developed a higher proportion of bone necrosis compared with matched controls (\*\* $p < 0.01$  in relation to CTL 28d; ## in relation to AL 3d; \*\*\* $p < 0.001$  in relation to AL 28d; ### $p < 0.001$  in relation to CTL 28d and AL 28d; +++ $p < 0.001$  in relation to CTL 28d). B) Animals treated with ALN presented an increased degree of infection compared with matched controls (\*\*\* $p < 0.001$  in relation to CTL 28d).



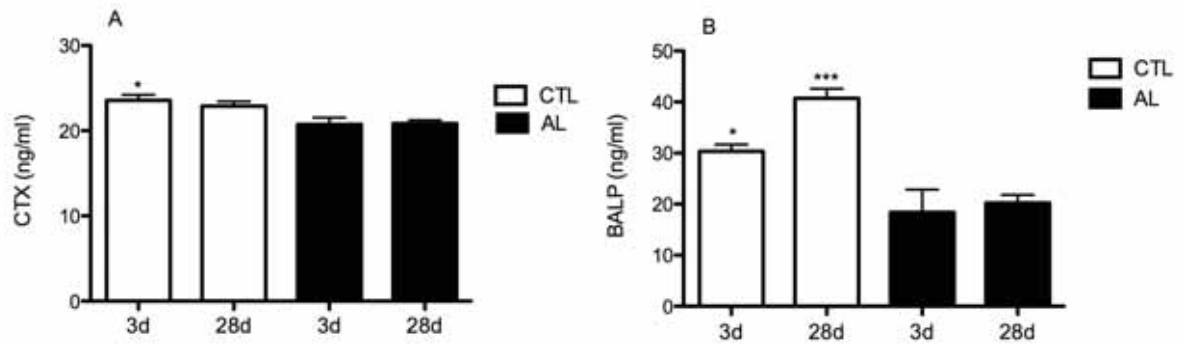
Graphic 2- The effects of alendronate therapy on the degree (A) and quality (B) of inflammation at 3 and 28 days after tooth extraction. At 3 days after tooth extraction, animals in the CTL group presented a higher degree of inflammatory response compared with all groups and periods (\*\*\* $p < 0.001$  in relation to CTL 28d and AL 3d; \*\* $p < 0.01$  in relation to AL 28d).



Graphic 3 – Degree of vascularization in experimental groups. At 28 days after tooth extraction, animals in the CTL group presented the highest levels of vascularization compared with all groups and periods (\* $p < 0.05$  in relation to CTL 3d and AL 3d; \*\* $p < 0.01$  in relation to AL 28d).



Graphic 4 – Bone volume (%) in experimental groups 28 days after tooth extractions. The region of interest 2 (ROI 2) of CTL animals exhibited a statistically significant increase in bone volume compared with the ROI 2 of animals treated with alendronate (\*\* $p < 0.001$  in relation to ROI 2 AL). Furthermore, no significant differences were found in other ROIs.



Graphic 5 – Levels of CTX (A) and BALP (B) in experimental groups at 3 and 28 days after tooth extractions. In the CTX analysis, animals in the CTL group presented levels statistically significantly higher at 3 days compared with all periods for group AL (\* $p < 0.05$  in relation to 3d and 28d AL). At 28 days, no significant differences were found between groups and periods. In the BALP analysis, at 3 days, significant differences were found between the CTL and AL groups (\* $p < 0.05$  in relation to 3d AL), and at 28 days, animals in CTL exhibited increased levels of BALP compared with all periods for the AL group (\*\* $p < 0.001$  in relation to AL periods).

# ESTUDO 2

**Artigo 6** - Bisphosphonate-related osteonecrosis of the jaws in rodents – an experimental study

Conte-Neto N, Spolidorio LC, Andrade CR, Bastos AS, Esteves JC, Marcantonio Jr E.

## **Bisphosphonate-related osteonecrosis of the jaws in rodents – an experimental study**

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**Abstract**

**Background:** The aim of this study is to develop an experimental model of Bisphosphonate-related osteonecrosis of the jaws in rodents

**Materials and Methods:** Adult male Holtzmann rats were assigned in two experimental groups to receive alendronate (AL; 1mg/kg/week; n=6) or saline solution (CTL; n=6). After 60 days of drug therapy, all animals were submitted to first lower molar extraction (M1) and, 28 days after the surgical procedure, animals were euthanized.

**Results:** animals treated with alendronate presented areas of exposed and necrotic bone, associated to a significant infection process, especially at inter-alveolar septum area and crestal regions. The vascularization degree and the levels of C-telopeptide cross-linked collagen type I and bone-specific alkaline phosphatase and bone volume were significantly reduced in these animals. Furthermore, on the radiographic analysis animals treated with alendronate presented an evident sclerosis of the M1 lamina dura associated to a decreased radiographic density at M1 sockets.

**CONCLUSIONS:** the data presented suggest that the alendronate therapy is associated to the development of osteonecrosis in the jaws in rodents.

**Key-words:** Osteonecrosis; alendronate; infection

## INTRODUCTION

During the last years, the Public Health statistics have been included the bisphosphonates (Bps) among the most common drugs prescribed in the world, especially due to the high effectiveness of these drugs in the treatment of several bone diseases[1]. However, since 2003, a great concern has been generated regarding the side-effects of Bps through increasing.

In this context, the Bps-related osteonecrosis of the jaws (BRONJ) represent a great challenge in scientific field and even with many efforts to develop experimental models of this disease, none of the BRONJ pathogenesis hypotheses is completely accepted yet. On the other hand, strong correlations have been made between this bone necrosis and possible co-factors, including Bps type and treatment length[2], as well trigger agents, highlighting to surgical procedures with bone manipulation, such as dental implants and teeth extractions[3].

Data retrieved from clinical studies indicate a strong association between teeth extractions and BRONJ[4-6]. In this way, the effects of Bps therapy on the alveolar sockets healing have been focus of several *in vivo* studies [7-10] [11]. Recently, we demonstrated the development of BRONJ-like lesions in rodents by the association of teeth extractions with daily high dosages of alendronate (ALN) in a long-term way (unpublished data). However, studies using lower dosages of Bps showed only a transient impairment on the alveolar socket healing after teeth extractions [12, 13].

Within this context, the aim of this paper is to evaluate if lower doses of ALN, which were correspondent in rats to the cumulative dosages for the management of rheumatic diseases[14], are able to induce BRONJ-like lesions in rodents.

Furthermore, we aimed to perform a critical discussion over the reasons behind the strong association between teeth extractions and this disease.

## **MATERIALS AND METHODS**

### ***Animals and reagents***

The study protocol was approved by the Ethics in Animal Research Committee of the School of Dentistry of Araraquara (UNESP, SP, Brazil) under protocol number 18 / 2009. It included twelve male rats (*Rattus norvegicus*, albinus, Holtzmann) weighing around 200g. These animals were kept at a special facility room at São Paulo State University – UNESP, School of Dentistry of Araraquara and maintained in a 12:12 hour light/dark cycle (lights on at 7:00 a.m.) at  $23\pm 2^{\circ}\text{C}$  with ad libitum access to a standard laboratory diet and water.

The Alendronate (ALN) was purchased from ALCON laboratory (São Paulo, SP, Brazil). The drug was dissolved in sterile physiological saline (0.9% NaCl) and diluted to the given concentrations.

### ***Experimental Design***

After a 3-day acclimatization period, animals were randomly assigned in two experimental groups: AL (n=6) that included animals receiving weekly subcutaneous doses of ALN (1mg/kg) and group CTL (n=6) composed by animals receiving sterile physiological saline, following the same schedule of ALN.

After 60 days of the pharmacologic therapy, all animals were submitted to the left lower first molars (M1) extractions under general anesthesia by a combination of ketamine chloridrate (Ketamina Agener, Agener União Ltda, Sao Paulo, SP, Brazil;



0.08ml/100g) and Xylazine 2% (Rompum, Bayer S.A., Sao Paulo, SP, Brazil; 0.04ml/100g).

The same professional performed the teeth extractions with the same technique in all animals. Initially, the rats were placed in a dorsal position and fixed in a special device. After that, the surrounding gingival was carefully detached from the lower first molars with a dental explorer. After that, with a Hollenback Carver tooth was luxated and separated in two segments (mesial and distal) that were removed with a forceps adapted around the cervical line of the segments.

After surgical procedure, all animals received an intramuscular dose of antibiotic (Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, Brazil – 0.1mg/Kg) and anti-inflammatory Ketoflex (Ketoprofen 1%, 0.03 ml/rat). Animals were euthanized by anesthesia overdose at 3 and 28 days after teeth extractions and the ALN or sterile physiological saline administrations were maintained until the sacrifice (Figure 1).

### ***Specimens processing***

Initially, all tissues blocks were immersed directly in 10% buffered formalin fixative solution for 48 h. After that, 6 specimens of each group were submitted to routine histological processing for descriptive and stequiometric evaluation. All specimens were decalcified in tetrasodium-EDTA aqueous solution (0.5 M, pH 7.4) for 2–3 mo, under agitation at room temperature. After that, all specimens were processed and included in paraffin blocks. Serial 4µm sections were obtained in the bucco-lingual direction, stained with hematoxylin and eosin and referred to light microscopic evaluation (Leica DM1200M; Leica Microsystems, Wetzlar, Hessen, Germany).

### ***Histological and stereometric analysis***

One board-certified oral pathologist who was blinded to this study effects performed these analysis. The histological endpoints were evaluated at four fields in each section (two superior and two inferior) and included changes in soft tissues, degree of bone necrosis and infection, quantity and quality of inflammation (acute, chronic or mist), as well the vascularization degree (vessels number). These parameters were scored on a four-point scale 0 (absent; 0%), 1(mild;  $\geq 10\%$ ), 2 (moderate;  $>10$  and  $\leq 50\%$ ) and 3 (increased;  $> 50\%$ ).

The stereometric analysis was performed using the Leica Application Suite 3.8.0 (Leica Microsystems LTD, Heerburg, Germany). The measures were done within the distal root of the left M1 at the regions of interest (ROIs) that included three different areas inside the sockets. Initially, it was determined a standard ROI by a quadrangular area extending from the level of the cementoenamel junction (CEJ) of the left second mandibular (M2) to the apical end level of the M2 and between the mesial and distal alveolar bone surface. After that, this quadrangular area was divided into three equal ROIs (1, 2 and 3) (Figure 2).

The variable analyzed included the percentage of the root socket filled with bone tissue (BV) in each ROI, following the nomenclature and abbreviations of the recommendation of the American Society for Bone and Mineral Research[15]. Bone volume (%) represents bone volume (mm<sup>3</sup>) per total tissue volume (mm<sup>3</sup>). All analyses were performed at magnification of 100x and a total of 3 measurements were taken for each specimen to complete the stereometric analysis. The distance between the selected sections was 50  $\mu\text{m}$ .

### ***Image acquisition***

For the radiographic evaluation, a digital radiograph of the left alveolar socket was taken immediately after surgery. The left mandible was fixed in a holding device with the vertical long axis of alveolar socket perpendicularly to the central X-ray beam and parallel to the sensor at 40-cm focus-object distance. The X-ray unit was operated at 70 KVp, 10 mA, and 0.2 s (Expectro 70x, Dabi Atlante, Ribeirao Preto, SP, Brazil).

### ***Radiographic bone density***

A single blinded calibrated examiner evaluated mandible radiographies. The radiographic bone density in the mandible was determined by the analysis of the gray scale in an area of 15 x 15 pixels at the ROIs included three different regions within the distal root of the left M1, as described previously. Furthermore, we also evaluated one region immediately after the mesial socket of left M1, which corresponded the body mandibular bone density.

This analysis was done using the image-analysis software Image Tool 2.03 (UTHSCA, San Antonio, Texas, EUA), which provided the gray scale average and standard deviation in these pre-determined regions by means of a histogram graph. The bone density calculations were performed by the average gray levels of the evaluated ROIs, which were divided by the gray level of the implant, to compensate minimal differences among radiographs, since the density of the metallic standard was the same in all specimens[16, 17]

### ***Assessment of bone turnover biochemical markers***

Blood samples were collected at the day of sacrifice by cardiac puncture in order to assess the levels of serum collagen type 1 cross- linked C-telopeptide (CTX) and Bone-specific alkaline phosphatase (bone ALP) (CUSABIO BIOTECH CO., Ltd, Wuhan, P.R. China) by enzyme-linked immunosorbent assay (ELISA) kits.

### ***Statistical Analysis***

Data were submitted initially to Kolmogorov-Smirnov normality test. Then, comparisons among groups were performed using the test T (parametric data) or Mann–Whitney U test (non-parametric data). Data were analyzed statistically using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA) and statistical significance was set at  $p < 0.05$  with 95% confidence intervals.

## **RESULTS**

### **Clinical and histological features**

All animals of group AL presented a degree of epithelial coverage statistically lower when compared to control animals ( $p < 0.001$ ) (Graphic 1). Consequently, animals treated with alendronate exhibited different degrees of bone exposure, while control animals presented with a complete epithelial healing without signals of inflammation (Figure 3).

A markedly bone necrosis associated with an infection process was observed in animals treated with alendronate, especially at inter-alveolar septum area, while control animals didn't displayed anyone of these features ( $p < 0.0001$ ) (Graphic 2 and Figure 4). Furthermore, animals in AL group presented a lower degree of vascularization ( $p < 0.01$ ) when compared to control group (Graphic 3), while no

statistical differences were found concerning the inflammation quality and degree between these animals (Graphic 4).

Regarding to BV count, we observed that at ROI 1 and ROI 2 animals of group AL presented rates significantly lesser than CTL animals ( $p < 0.01$ ), while no statistical differences were found in ROI 3 (Graphic 5).

### **Radiographic aspects**

On the radiographic analysis, it could be observed that animals treated with ALN showed a degree of bone radiographic density (BRD) significantly lesser at ROI 1 ( $p < 0.01$ ) and ROI 2 ( $p < 0.001$ ) when compared to control animals (Graphic 6). Furthermore, in AL group the linings of the alveolar socket were more evident when compared to control animals and also presented a sclerosis on the M1 alveolar lamina dura (Figure 5), as well the highest values of BRD at ROI 4 area ( $p < 0.001$ ) (Graphic 6).

### **Bone metabolism markers**

Animals treated with alendronate presented the lowest values of BALP ( $p < 0.01$ ) and CTX ( $p < 0.001$ ) when compared to control animals (Graphic 7).

## **DISCUSSION**

Several experimental models of BRONJ-like lesions have been published in the literature, but most of them were developed by the association of Bps, teeth extractions and other co-factors, such as concomitant steroids[7-9], vitamin D deficiency[10] or increased damage to socket[11], which are confounding issues to the evaluate the natural relation between Bps and osteonecrosis. From this point of

view, in this paper, we demonstrated the development of BRONJ-like lesions in rodents only by the association of Bps and teeth extractions.

The first step in this paper was to establish a chronic bone remodeling suppression model, since it is a relevant risk factor to BRONJ[18, 19]. To this aim we adopted chronic ALN therapy with doses correspondent in rats to the cumulative dosages for the management of rheumatic diseases[14].

The prerogative to this schedule was made basing on the observations that the total Bps dose administered over a long period of time is important for the magnitude of the reduction in bone turnover[20], especially involving high potency Bps in a high bioavailability route[21]. Indeed, animals in AL group presented the lowest levels of CTX and BALP, which are markers of the bone metabolism[22], supporting the effectiveness of our Bps treatment schedule to suppress the bone turnover.

The next step was to increase, through teeth extractions, the jaws demand of bone remodeling in a favorable environment to the development of bone lesions. This issue was based firstly on the strong association between teeth extractions and BRONJ as indicated by clinical studies [4-6]. Furthermore, we also considered the assumption that BRONJ could be related to the compromised bone incapacity to meet the increasing healing demand that is required in situations of tissue trauma and infection[23].

Indeed, 28 days after teeth extractions all animals treated with alendronate presented with areas of bone exposition and non-vital tissue, which were absent in control animals. In our opinion, the reasons that could explain this important correlation between teeth extractions and osteonecrosis may be related to

considerations in the environment and dynamics of the alveolar socket healing with the BRONJ theories.

After teeth extractions, there is, naturally, a high requirement for wound healing, especially at initial phases, and, in the field of Bps, this is worsened since these drugs impair the angiogenesis and resorption process after teeth extractions[13, 24]. Indeed, these features are in agreement with our findings of lower CTX levels and vascularization degree in animals treated with alendronate. Therefore, the association of a high demand requirement in an already compromised bone-remodeling environment could leave to the collapse of the wound repair, considering the BRONJ suppression theory [23].

Within this context of wound healing impairment, the role of the osteoclast activity in teeth extractions healing could represent a relevant issue. In these wounds, a high resorption activity is required to remove bone debris and necrotic bone, especially in the inter-dental alveolar septum that is more exposed to the environment rich in bacterial toxins, inflammatory cytokines, or oxidative stress[25].

This is of special concern in fields of Bps, since some authors believe that BRONJ may be associated with changes in oral bacterial behaviors, due to an increasing of bacteria activity[24]. Indeed, as in this paper, other studies have been demonstrated areas of active infection[9] and necrotic bone at inter-alveolar bone region that have been attributed to the prolonged retention of this area[12, 13].

In this discussion, it is important to state that even in the presence of a relevant infection process in animals treated with alendronate, no differences in the inflammatory response were found between the groups. This is particularly interesting since a microbial infection typically trigger host immune responses [26] and therefore

enhance the degree of inflammatory process. A possible explanation to this finding could be related to the dose dependent anti-inflammatory effects of Bps [27] that could, in a long-term way, leave to immunosuppressive conditions, acting in favor to the infection establishment.

Still discussing infection issues, we also demonstrated a varied degree of impairment in soft tissue coverage in animals treated with alendronate, which is a relevant issue due to the essential role of epithelial coverage protection from oral bacterial infection[28]. Indeed, it was show that Bps inhibits migration activity of oral epithelial cells [24, 29]. This aspect could be a concern to teeth sockets healing, since it requires a high demand of oral epithelial cells migration, considering that the degree of reepithelialization depends of the depth and width of the wound[30].

On the other hand, it is relevant to state that an infection process is also able to impair the epithelial cell growing[31]. Furthermore, the areas of epithelial absence in our paper were mainly located in the vicinity of the infection. Therefore, we can't discard the role of bacteria and it products on the impairment of the epithelial coverage

In the field of bone exposition, according to AAMOS[18], one of the BRONJ diagnosis criteria is the persistence of bone exposure in oral cavity for at least 8 weeks. In our opinion, this concept can't be strictly extended to animal experimental models, due to inherent metabolic differences between these species. Although both rodents and humans share basically similar sequences of alveolar healing, in rats they occur more rapid than in human[32], at about one third of the time of human healing process[33]. Furthermore, when transient, the negative effects of Bps on socket healing have not been extended lather than 14 days[12, 13].



Therefore, it is reasonable to believe that a bone exposition until 28 days in rodents could be considered a BRONJ-like lesion, especially in the presence of the histological features. This assumption is also supported by our radiographic findings of osseous sclerosis around the alveolar margin and lamina dura, as well by the non-healing sockets, demonstrated by the lower level of radiographic density. Taken together, these features are commonly presentations of BRONJ lesions [34-36].

In this paper, we found lesser BV values at medium and apical regions in AL group, which is coherent with the lowest levels of BALP in this group. These features are in agreement with authors that found inhibitory effects of alendronate on the osteogenesis[24]. On the other hand, we also observed no statistical differences on the bone volume at the sockets apical area between groups, which reinforces, at least in part, other authors who found no direct effects of Bps on the ability of osteoblasts to produce bone matrix in vivo[37, 38].

In fact, the effects of Bps over the bone formation process are not yet clearly established in the literature, but it seems to be dose-dependent[39]. Therefore, it is possible that in this study dosage, alendronate could exert a partial impairment of bone formation directly or indirectly by delaying the previous steps of sockets healing, since the osteogenesis begin at apical areas of the sockets[40]. On the other hand, it is relevant to state that bacterium and its products could be involved in this process, since they are also able to impair the osteogenesis process[41].

Within the limitation of this study, our outcomes clearly demonstrated that the association of alendronate therapy and teeth extractions was able to induce BRONJ-like lesions in rodents. This experimental could be useful to further studies addressing other issues related to the BRONJ pathways, including other trigger agents, such as

osseointegrated implants, as well as to develop preventive and therapeutic approaches to this disease.

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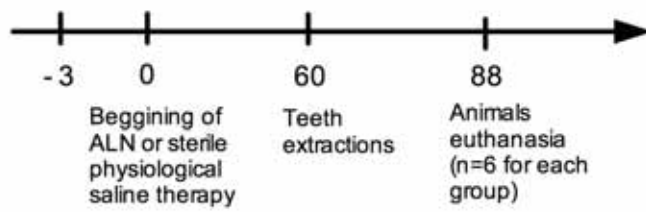
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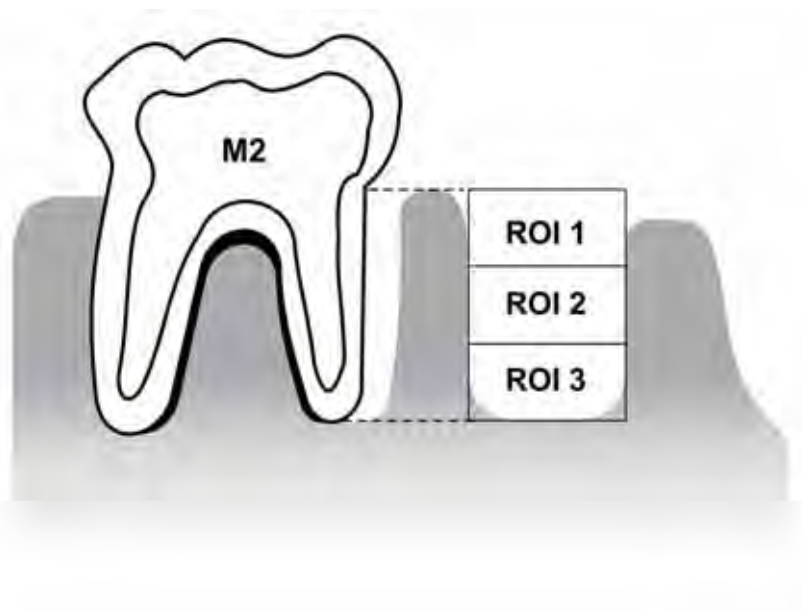
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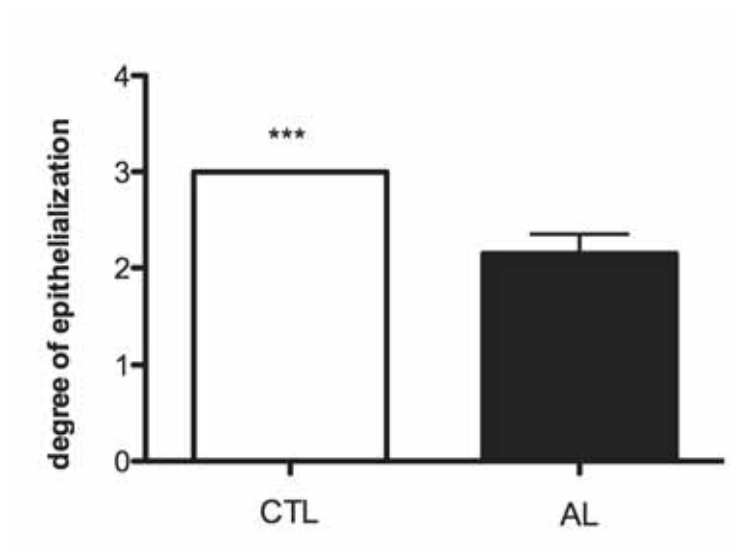
## GRAPHIC AND FIGURES



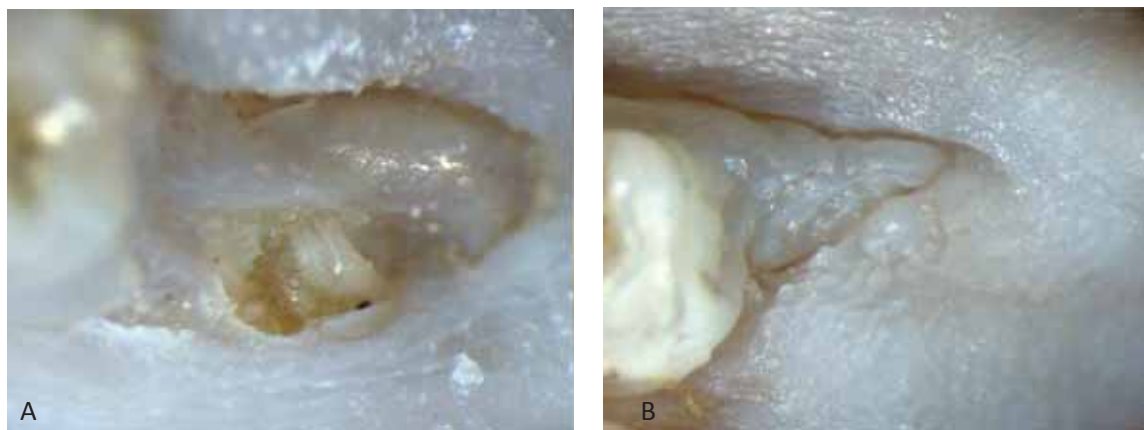
**Figure 1** – Experimental Design



**Figure 2** – Schematic view of the regions of interest (ROIs) for estereometric analysis.

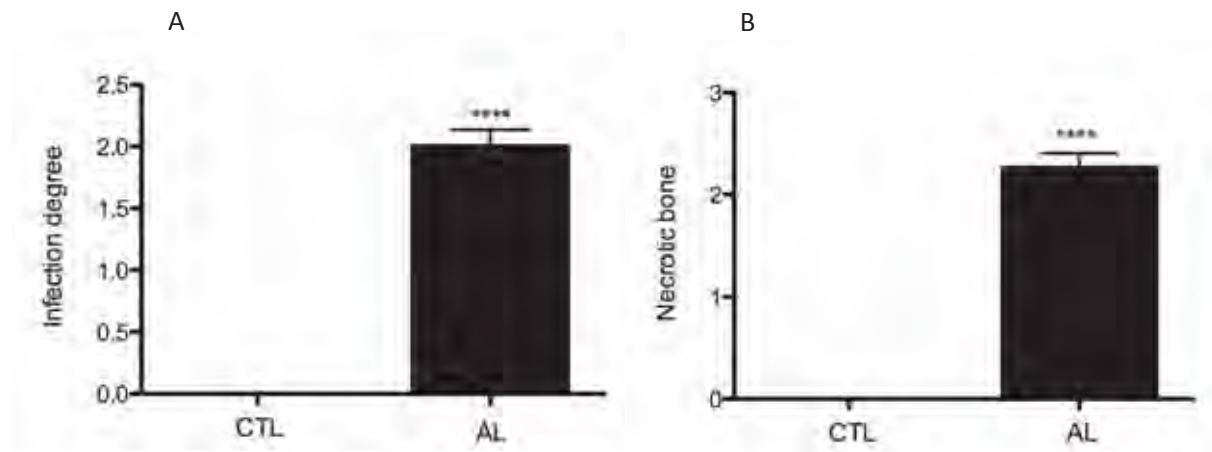


**Graphic 1** – Degree of Epithelialization between experimental groups (\*\* $p < 0.001$ )

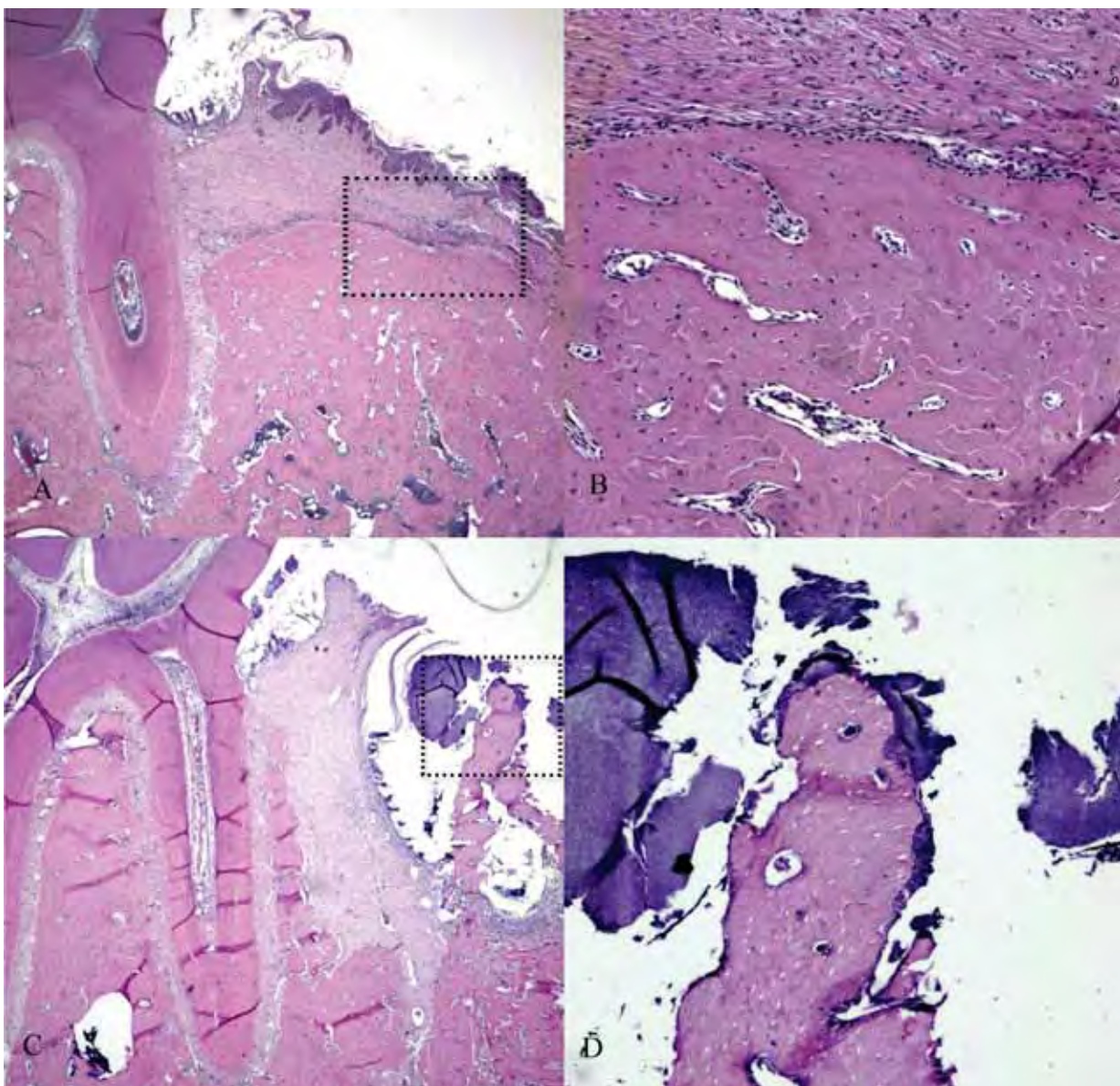


**Figure 3-** Clinical aspect of alveolar socket healing after 28 days of teeth extraction (25x) in animals treated with ALN (A), and sterile physiological saline(B).

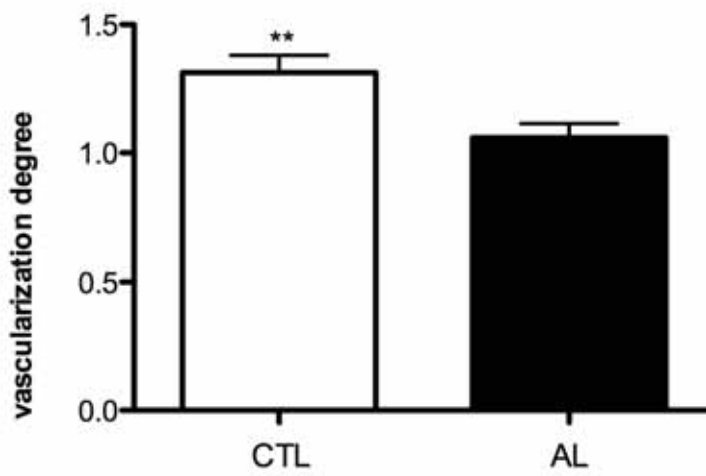




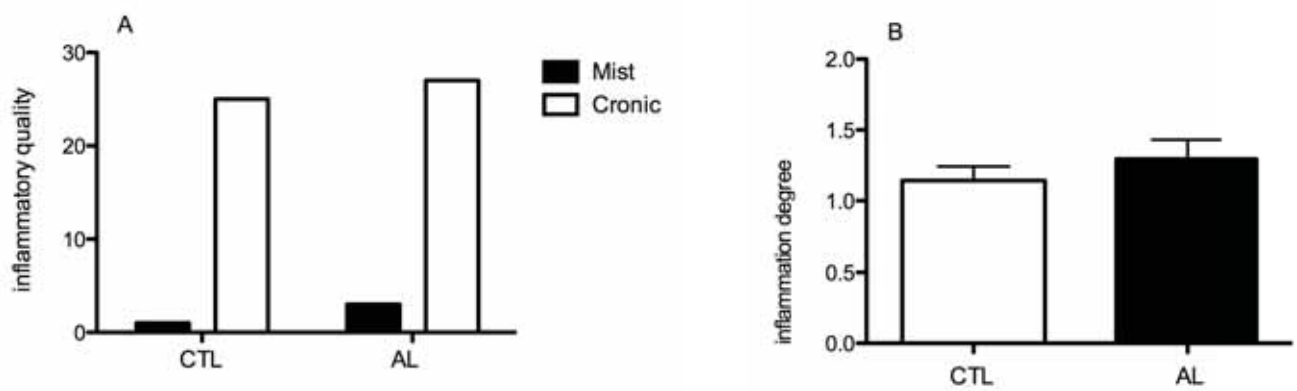
**Graphic 2** – Degree of infection (A) and necrotic bone (B) in experimental groups (\*\*\*\* $p < 0.0001$ )



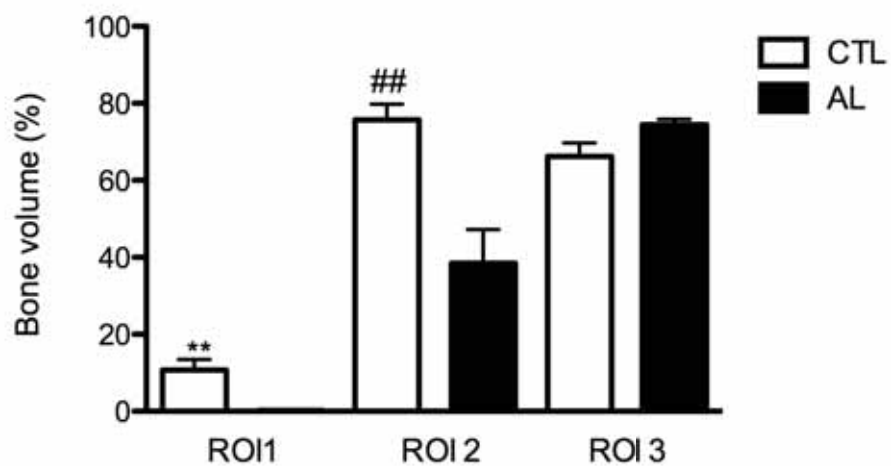
**Figure 4** – Histological features of the alveolar socket healing after 28 days of teeth extraction in animals treated with sterile physiological saline (A and B), and alendronate (C and D)



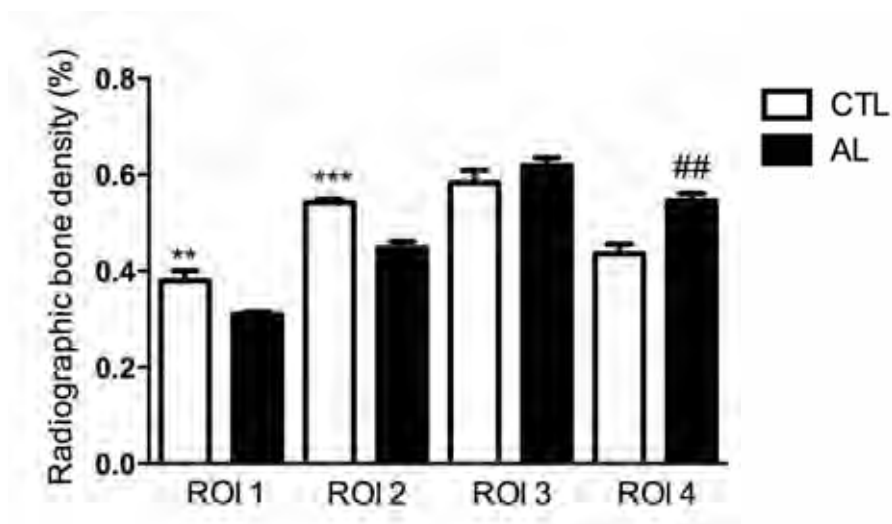
**Graphic 3** – Degree of vascularization in experimental groups (\*\*p<0.01)



**Graphic 4** – Quality (A) and degree (B) of the inflammatory response in experimental groups



**Graphic 5-** Percent of BV present at ROIs 1, 2 and 3 in experimental groups (\*\* $p < 0.01$  in relation to ROI 1 of AL; ## $p < 0.01$  in relation to ROI 2 of AL)



**Graphic 6 -** Percent of radiographic bone density present at ROIs 1, 2, 3 and 4 in experimental groups (\*\* $p < 0.01$  in relation to ROI 1 of AL; \*\*\* $p < 0.001$  in relation to ROI 2 of AL; ## $p < 0.01$  in relation to ROI 4 of AL)

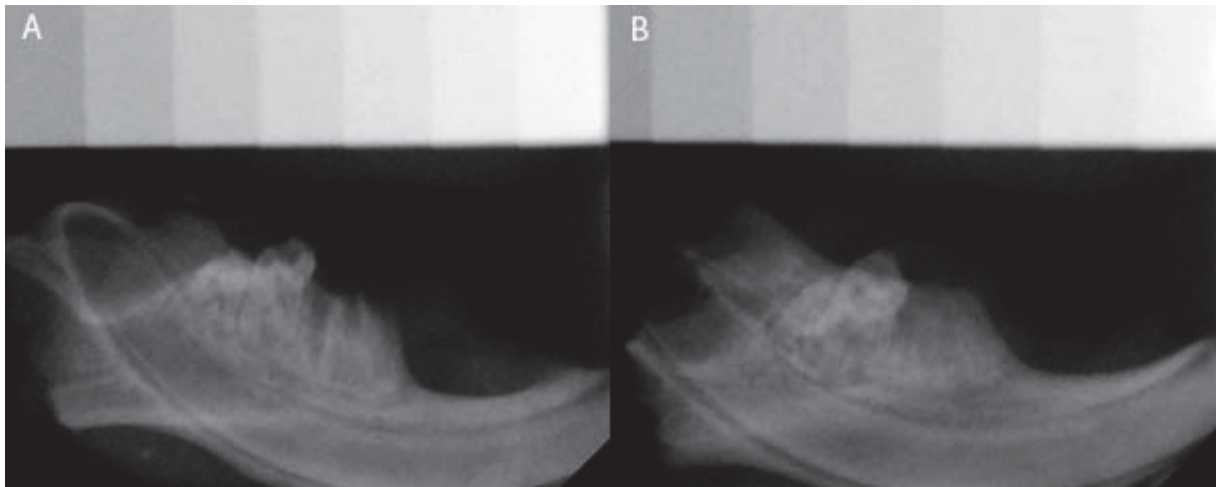
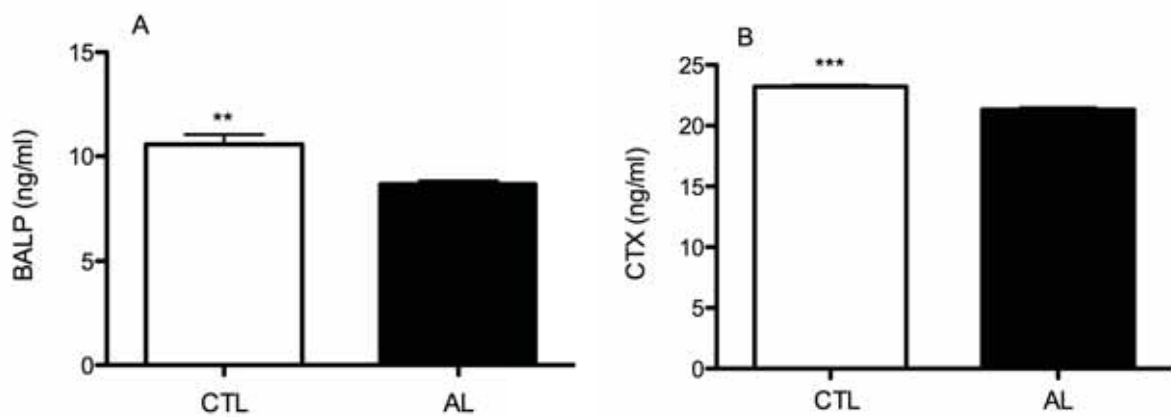


Figure 5 – Radiographic features of the alveolar socket healing after 28 days of teeth extraction in animals treated with alendronate (A), and sterile physiological saline (B)



Graphic 7- Levels of BALP (A) and CTX (B) in experimental groups (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ )

# ESTUDO 3

**Artigo 7** - Effects of chronic stress and alendronate therapy on the osseointegration of titanium implants.

*Artigo enviado para publicação no periódico BONE*

Conte-Neto N, Spolidorio LC, Planeta SP, Cruz FC, Andrade CR, Bastos AS, Marcantonio Jr E

## **Effects of chronic stress and alendronate therapy on the osseointegration of titanium implants**

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**Abstract**

The purpose of this study was to evaluate the influence of chronic stress (CS) on implant osseointegration parameters and also to analyze whether alendronate (ALN) therapy could prevent these eventual stress-negative effects. Adult male Holtzman rats were assigned to one of four experimental groups: AL (ALN; 1 mg/kg/wk; n=12), ALS (ALN + chronic stress; 1 mg/kg/wk; n=12), CTL (sterile physiological saline; n=12), or CTLS (sterile physiological saline + chronic stress; n=12). After 58 days of drug therapy, the ALS and CTLS groups were exposed to chronic stress, and two days later all animals underwent tibial implant installation. The animals were euthanized 28 days following the operative surgical procedure, and it was observed that the CTLS group presented an impairment of bone metabolism represented by lowest levels of bone specific alkaline phosphatase (BALP) and bone area fraction occupied (BAFO) values. Furthermore, these animals presented a higher proportion of bone necrotic matrix and infection. In contrast, the alendronate therapy showed increased osseointegration and torque value parameters, regardless of the exposure to stress. The highest level of bone necrotic matrix was observed in the animals from this group. Analysis of the data presented suggests that chronic stress partially impairs the osseointegration of tibial implants, and that alendronate therapy is able to prevent these negative effects.

**Keywords:** alendronate; osseointegration; bone; operative surgical procedure; infection

## **1-Introduction**

The relationship between stress and health has been the focus of many studies over the years. Indeed, a substantial body of evidence confirms that stress may play a relevant role in the pathogenesis of several diseases [1-4] and may also have been associated with the impairment of soft-tissue wound healing [5-7].

Stressful events activate the hypothalamic-pituitary-adrenal axis (HPA) and increase the release of corticotrophin-releasing hormone (CRH) from the hypothalamic paraventricular nucleus, causing the secretion of adrenocorticotropin (ACTH) from the anterior pituitary, which stimulates the secretion of corticosterone from the adrenal cortex [8, 9].

Although the mechanisms responsible for stress-induced impairment of wound healing have not yet been totally elucidated, evidence suggests that the most prominent pathway is through activation of the HPA axis [10]. Indeed, *in vivo* studies showed that the administration of glucocorticoids (GC) delays soft-tissue wound healing [11] and impairs implant osseointegration [12].

In the field of bone metabolism, many efforts have been made to develop strategies to stabilize the bone loss occasioned by diseases and drugs. In this context, bisphosphonates (BPs) have demonstrated positive effects on bone tissue, improving the osseointegration of implants [13-15] and preventing bone loss induced by the administration of GC [12, 16].

Although there is substantial evidence that exposure to chronic stress (CS) might lead to wound-healing impairment, the effects of stress on the osseointegration process have not yet been investigated. Therefore, the purpose of this study was to evaluate the influence of CS on osseointegration parameters and to analyze whether alendronate (ALN) therapy can prevent these eventual stress-negative effects.

## **2-Materials and Methods**

### *2.1- Animals*

The study was approved by the Ethics in Animal Research Committee of the School of Dentistry of Araraquara (UNESP, Brazil) (protocol number 18/2009) and



included a total of 48 male Holtzmann Albino rats, each weighing around 220 g. These animals were housed individually in single cages and kept in a special facility room at São Paulo State University–UNESP, School of Dentistry of Araraquara. Rats were maintained on a 12:12-hour light/dark cycle (lights on at 7:00 a.m.) at  $23 \pm 2^\circ\text{C}$  with *ad libitum* access to a standard laboratory diet and water. Body weight was monitored regularly.

## 2.2 - Drugs

The alendronate (ALN) was purchased from ALCON Laboratory (São Paulo, SP, Brazil). The drug was dissolved in sterile physiological saline (0.9% NaCl) and diluted to the given concentration.

## 2.3 - Experimental Protocol (Figure 1)

After a 3-day acclimatization period, animals were randomly assigned to one of four experimental groups: AL (n=12) and CTL (n=12) groups, including animals treated with subcutaneous administration of 1 mg/Kg of ALN or sterile physiological saline once a week, respectively; or ALS (n=12) and CTLS (n=12) groups, comprised of animals subjected to chronic stress (CS) and treated with ALN or sterile physiological saline, respectively, following the same posology of non-stressed groups.

### 2.3.1 - Implant surgical procedure

After 60 days of ALN or sterile physiological saline treatment, all animals were anesthetized by a combination of ketamine chloridrate (Ketamina Agener, Agener União Ltda, São Paulo, SP, Brazil; 0.08 mL/100 g body weight) and Xylazine 2% (Rompum, Bayer S.A., São Paulo, SP, Brazil; 0.04 mL/100 g body weight) and underwent trichotomy on the inner region of the leg and asepsis with povidone iodine solution. After that, an incision was made in the layers of the tibial metaphysis. The underlying bone was subjected to osteotomy with a starting drill of 1.8mm to accommodate the machined titanium implant with 4-mm length and 2.2-mm thickness (Neodente, Curitiba – Brazil) under abundant irrigation. The tissue was sutured with 4-0 silk thread (Ethicon, Division of Johnson & Johnson Medical Limited, São Jose dos Campos, São Paulo, Brazil).

Postoperatively, all animals received an intramuscular dose of antibiotic (Pentabiótico<sup>®</sup>, Wyeth-Whitehall Ltda, São Paulo, Brazil – 0.1 mg/Kg) and anti-inflammatory Ketoflex (Ketoprofen 1.0%, 0.03 mL/rat). The ALN or saline solution administration was maintained until 28 days after surgical procedures, when all animals were euthanized by anesthesia overdose.

### 2.3.2 - *Stress paradigm*

The chronic stress (CS) protocol was adapted from Marin et al. [17], beginning two days before the surgical procedures. The protocol consisted of exposure to three different cycles of stressors (10 consecutive days, each cycle) once a day for 30 days. In the first cycle, rats were maintained in a cold camera (4°C) for 15 min. In the second cycle, the rats were restrained in plastic cylinders [20.0 cm (length) x 5.5 cm (internal diameter)] for 1 hr/day. In the last cycle, they were subjected to forced swimming for 4 min.

All the stress sessions were performed in a room adjacent to the animal facility, and the non-stress group was left undisturbed except when their cages were cleaned.

## 2.4 – *Histological Analysis*

Initially, all tissue blocks were immersed directly in 10% buffered formalin fixative solution for 48 h. After that, 6 specimens of each group underwent routine histological processing, while 6 other specimens were prepared for hard tissue histology.

### 2.4.1 – *Soft-tissue histology*

In this analysis, all specimens were decalcified in tetrasodium-EDTA aqueous solution (0.5 M, pH 7.4) for 2-3 mo, under agitation at room temperature. After that, the tibial implants were carefully removed, processed, and included in paraffin blocks. Serial 4- $\mu$ m sections were obtained in the bucco-lingual direction, stained with hematoxylin and eosin, and referred for light microscopy (Leica DM1200M; Leica Microsystems, Wetzlar, Hesse, Germany) for descriptive and quantitative evaluation, which included the degree of bone necrosis, infection, and inflammation. These parameters were scored on a four-point scale 0 (absent; 0%), 1(mild;  $\geq 10\%$ ), 2 (moderate;  $>10$  and  $\leq 50\%$ ) and 3 (increased;  $> 50\%$ ).

#### *2.4.2 – Hard-tissue histology*

In this analysis, the specimens containing implants were prepared after dehydration by a series of ethanol solutions and embedded in methacrylate-based resin (Technovit 7200; Heraeus Kulzer, Wehrheim, Hesse, Germany). The blocks were initially sectioned at about 150  $\mu\text{m}$  using a specific system (EXAKT Apparatebau GmbH & Co., Norderstedt, Germany) [18] and subjected to grinding and polishing (EXAKT Apparatebau GmbH & Co.) to achieve a final thickness of approximately 30  $\mu\text{m}$ . After that, the sections were stained with Stevenel's blue/ acid fuchsin (1%) and referred for light microscopic evaluation.

Measurements of the percentages of bone-implant contact (BIC) and bone area fraction occupancy (BAFO) were performed at 100x magnification (Leica DM1200M; Leica Microsystems, Wetzlar, Hesse, Germany) using ImageJ 1.41o (National Institutes of Health, Bethesda, MD, USA).

#### *2.5 -Torque Removal Analysis*

Immediately after the animals were sacrificed, torque removal analysis was undertaken in 6 specimens from each group. The tibial implant was attached to a torque meter with a scale range of 0.1 to 10 Ncm and divisions of 0.05 Ncm (Tohnichi, Shanghai, China). A wrench was attached to the implant head to apply torque in the reverse direction of implant placement, until complete rupture of the bone/implant interface occurred, signaled by the rotation of the implant.

#### *2.6 -Assessment of Bone Turnover Biochemical Markers*

Blood samples were collected on the day of sacrifice by cardiac puncture, and the plasma was obtained after blood centrifugation at 3000 g for 10 min at 4°C and stored at -80°C until the analysis. The levels of serum collagen type 1 cross-linked C-telopeptide (CTX) and bone-specific alkaline phosphatase (BALP) (CUSABIO BIOTECH CO., Ltda, Wuhan, P.R. China) were determined by enzyme-linked immunosorbent assay (ELISA) kits.

#### *2.7 - Radioimmunoassay*

Blood samples were collected four times during the experiment: after the first stress session (baseline) and 24 hours after each cycle of stressors. The blood was

collected from the rat caudal artery and immediately centrifuged at 3000 g for 10 min at 4°C and stored at -80°C until the analysis. The radioimmunoassay for corticosterone was conducted with antibody obtained from Sigma (St. Louis, MO, USA) and (3H)-corticosterone from New England Nuclear (Boston, MA, USA). The method was adapted from that described by Sarnyai et al. [19].

### *2.8- Statistical Analysis*

The data were evaluated by means of the GraphPad Prism 5.0 software package (GraphPad Inc., San Diego, CA, USA). The normality of the data was assessed by the Kolmogorov-Smirnov test. The difference between the groups for parametric data was evaluated by analysis of variance (ANOVA) followed by Tukey tests or the F test. Statistical significance was set at 5%.

## **3-Results**

### *3.1- Body Weight*

The mean body weight of all rats was ~160 g at the beginning of the study, and there were no statistically significant differences among the groups ( $p < 0.05$ ). During the period of restraint stress, while control groups exhibited an increase of body weight, the stressed animals presented a significant weight loss ( $p < 0.001$ ) (Figure 2). In other cycles of stress, no statistical differences were found.

### *3.2 – Soft-tissue Histology*

Observations of histological sections showed that the bone tissue surrounding tibial implants in ALN groups was markedly compact, while a high volume of spongy and marrow bone was observed in control animals (Figure 3). Although matrix areas with necrosis were observed in all the groups, animals in the ALS group presented the highest values, which were statistically different from those of the CTL animals ( $p < 0.01$ ) (Figure 4a).

Furthermore, in animals with no ALN treatment, the exposure to stressors was associated with a significant increase in bone necrotic regions compared with that in the CTL group ( $p < 0.05$ ) (Figure 4a) and was associated with a relevant degree of infection in relation to all the groups ( $p < 0.001$ ) (Figure 4b). In contrast, animals in the

ALS groups presented a higher degree of inflammation compared with those in the AL group ( $p < 0.05$ ) (Figure 4c).

### 3.3 – *Hard-tissue Histology*

At 28 days following implantation, all groups presented osseointegrated implants (Figure 3), and BAFO measurements revealed that the CTLS group presented the lowest values when compared with CTL- ( $p < 0.05$ ) and ALN-treated animals ( $p < 0.001$ ). Furthermore, animals of the AL group showed no statistical differences in relation to ALS and CTL (Figure 5a).

Regarding the BIC analysis, it was observed that animals treated with ALN presented the highest values compared with those in the control groups ( $p < 0.001$ ). No significant differences were observed between groups with and without stress in the BIC parameter, although control animals subjected to CS presented lower values when compared with CTL animals (Figure 5b).

### 3.4 – *Torque Removal Analysis*

ALN treatment showed a significant increase in the torque removal values when compared with that in animals receiving sterile physiological saline, regardless of CS exposure ( $p < 0.001$ ). However, animals in the CTLS group showed trends toward lower values of torque removal when compared with those in the CTL group (Figure 5c).

### 3.5 - *Bone Metabolism Markers*

In general, the non-stressed groups presented the highest values of BALP when compared with stressed animals. The AL group presented higher values when compared with ALS ( $p < 0.001$ ) and CTLS ( $p < 0.05$ ). The CTL group presented higher values in relation to CTLS ( $p < 0.01$ ) and ALS ( $p < 0.001$ ). No statistical differences were found between ALN and sterile physiological saline-treated animals not subjected to CS (Figure 6a).

Regarding CTX analysis, animals treated with ALN presented the lowest values when compared with control animals, independent of CS exposure ( $p < 0.001$ ) (Figure 6b).

### 3.6 – Radioimmunoassay

Corticosterone levels were altered within weeks [ $F(1,63) = 9.63$ ;  $p < 0.001$ ], only in the animals exposed to chronic stress. These values were significantly higher in the third and fourth weeks when compared with baseline and with values in the first stress cycle ( $p < 0.05$ ). Furthermore, these rates were higher in relation to baseline and the first stress cycle of control animals ( $p < 0.05$ ) (Figure 7).

## 4 - Discussion

In spite of the absence of studies investigating the relationship between stress and bone healing, the harmful effects of stress on soft-tissue wound healing have been well-established in the medical literature [5-7]. To the best of our knowledge, this is the first study to address and demonstrate the negative effects of chronic stress (CS) on the osseointegration of titanium implants.

Corticosterone is the most abundant glucocorticoid in rats [20], and it has been considered a useful serum marker of the stress state in rodents [17, 21]. Our findings of higher levels of plasma corticosterone in animals exposed to stressors confirm the efficiency of the stress paradigm. Furthermore, these outcomes also indicate that the animals did not adapt to the stress protocols during the osseointegration period, which is a relevant issue in CS models.

It is believed that the physiologic pathways of stress-induced wound-healing impairment could be related to enhanced glucocorticoids (GC) [22, 23]. In this sense, in bone tissue, studies have shown that the administration of GC, especially long-term, stimulates osteoclast-mediated bone resorption [24], reduces osteoblast-mediated bone formation [25] and mineral bone density [26], and is associated with the development of osteonecrosis [27].

However, it is relevant to state that the effects of experimental stress should not be compared with those from the administration of exogenous steroids, for two main reasons. First, stress is associated with changes in behavioral responses and other physiological processes, including catecholamine release, that are also related to wound-healing delay [28]. Second, the mean potency of synthetic steroids is significantly higher compared with that of endogenous steroids [29], which means

that a strong difference in the intensity of the physiologic effects of both hormone categories can be expected.

Indeed, in spite of histometric and biomechanical measures, only the BAFO values were significantly decreased in stressed animals with no ALN therapy. In our opinion, these effects can be related to an impairment of bone metabolism, since the CS groups presented statistically lower values of BALP, even in ALN stressed animals. It is known that BALP is a product of osteoblast activity and, therefore, a marker of bone formation [30]. In contrast, the CTX levels, which are products of osteoclast activity [30], were not significantly different.

Although these findings suggest a partial impairment of bone healing around implants, it is relevant to state that the CTLS group was associated with an increased degree of infection and matrix areas with bone necrosis compared with the CTL group. Of note, the inflammatory response was not different between CTL and CTLS, which is particularly interesting since a microbial infection typically triggers host immune responses [31] and therefore enhances the degree of the inflammatory process.

Taken together, these findings could be explained by the suppressive effects of chronic stress on the immune response [32], which increase the vulnerability to infection [33] and might have an adverse effect on wound healing [34]. Although these features could be correlated with the increased necrotic bone areas, GC may induce osteonecrosis through different pathways, including effects on lipid metabolism and coagulation changes [35, 36].

In contrast, in animals treated with ALN, none of the osseointegration parameters was different between non-stressed and stressed animals, even considering that these latter animals had the lowest levels of BALP, as described previously. Furthermore, ALN therapy was associated with the highest values of histometric and biomechanical measurements. These findings are in agreement with those from other studies that confirm the potential of BPs to improve the osseointegration of implants [13-15], including causing reversal of the bone loss induced by the administration of steroids to titanium implants [12] and by periodontal disease [16].

However, the reasons for these positive effects have not yet been clearly established in the literature. While some authors justify these trends based on the suppression of the resorption process [37], others showed that BPs stimulate new bone formation around implants [38, 39]. Considering these studies and our findings, it is reasonable to believe that the inhibition of the resorption process may be a relevant factor, since the highest levels of CTX associated with similar levels of bone ALP were observed in ALN-treated animals, regardless of CS exposure.

Torque removal analysis is a test commonly performed to evaluate the strength of the bone-implant interface [40-42]. It has been demonstrated that ALN therapy significantly increases the values of implant torque removal [14, 43]. This was also confirmed in our study, even in animals subjected to CS. In fact, the close relation between this biomechanical test and the degree of bone in contact with the implant [44, 45] could support our findings of highest BIC values found in the ALN groups, as was also demonstrated by other authors [14, 43].

Nevertheless, it is relevant to state that bone mineral density (BMD) and bone architecture are key determinants of bone strength [46], and thus able to affect biomechanical implant tests. These observations can also explain the highest torque values in ALN-treated animals, since we showed a high degree of compact bone close to the implants in these animals, which is also favored by the markedly increased BMD that is expected to occur during BPs treatment [47]. In this way, associating these features with the higher BIC values found in ALN-treated animals, it could be speculated that BAFO measurement may not be a primary factor in torque removal analysis in the BP context, since we found similar levels of BAFO between non-stressed control and AL groups.

Although our study clearly demonstrated positive effects of ALN on osseointegration, it is relevant to state that animals treated with these drugs presented the highest values of necrotic bone matrix, especially in the ALS group. The reasons that could explain these findings can be related to the potential pathways of both BPs and GC to induce osteonecrosis [35, 36, 48]. In fact, this association is particularly dangerous to the jaws, since it has been shown to increase the severity of jaw osteonecrosis [49].



Curiously, no suppressive changes were observed in the degree of inflammation in the ALS group. Considering that necrotic cell death can induce an inflammatory response, especially through innate immune cells [50], we believe that the activation of this pathway could explain the higher degree of inflammation observed in the ALS group, since these animals presented the highest level of bone matrix necrosis.

## 5 - Conclusions

Within the limitations outlined above, the present study showed, for the first time, the negative role of chronic stress on the osseointegration of titanium implants in animals with no ALN treatment. Furthermore, the therapy with these drugs seems to improve osseointegration parameters and prevent the deleterious effects of stress. However, further studies, especially using jaw implants, are necessary to investigate the role of chronic BP therapy in implant survival and long-term implant osseointegration.

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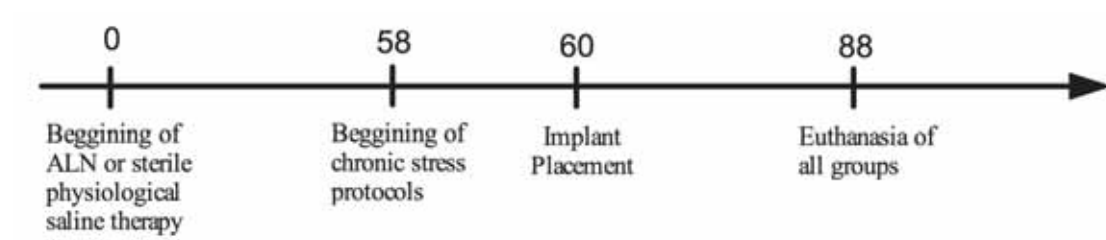
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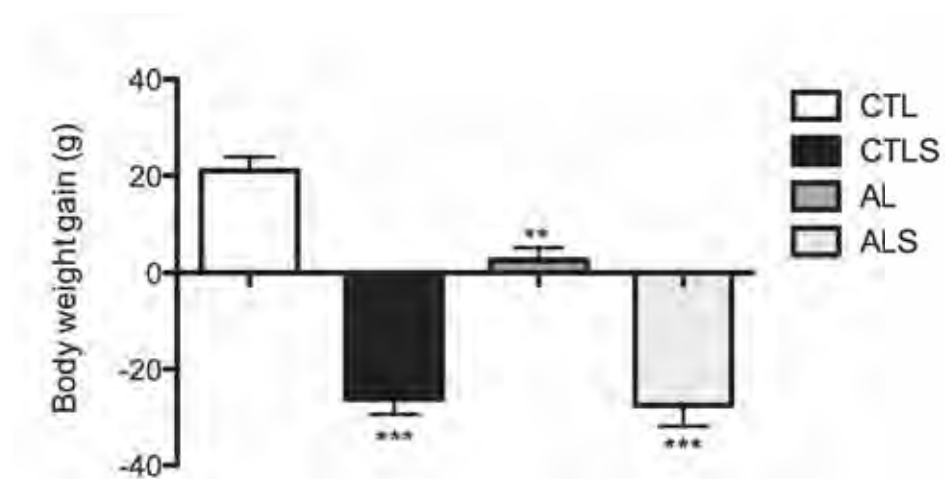
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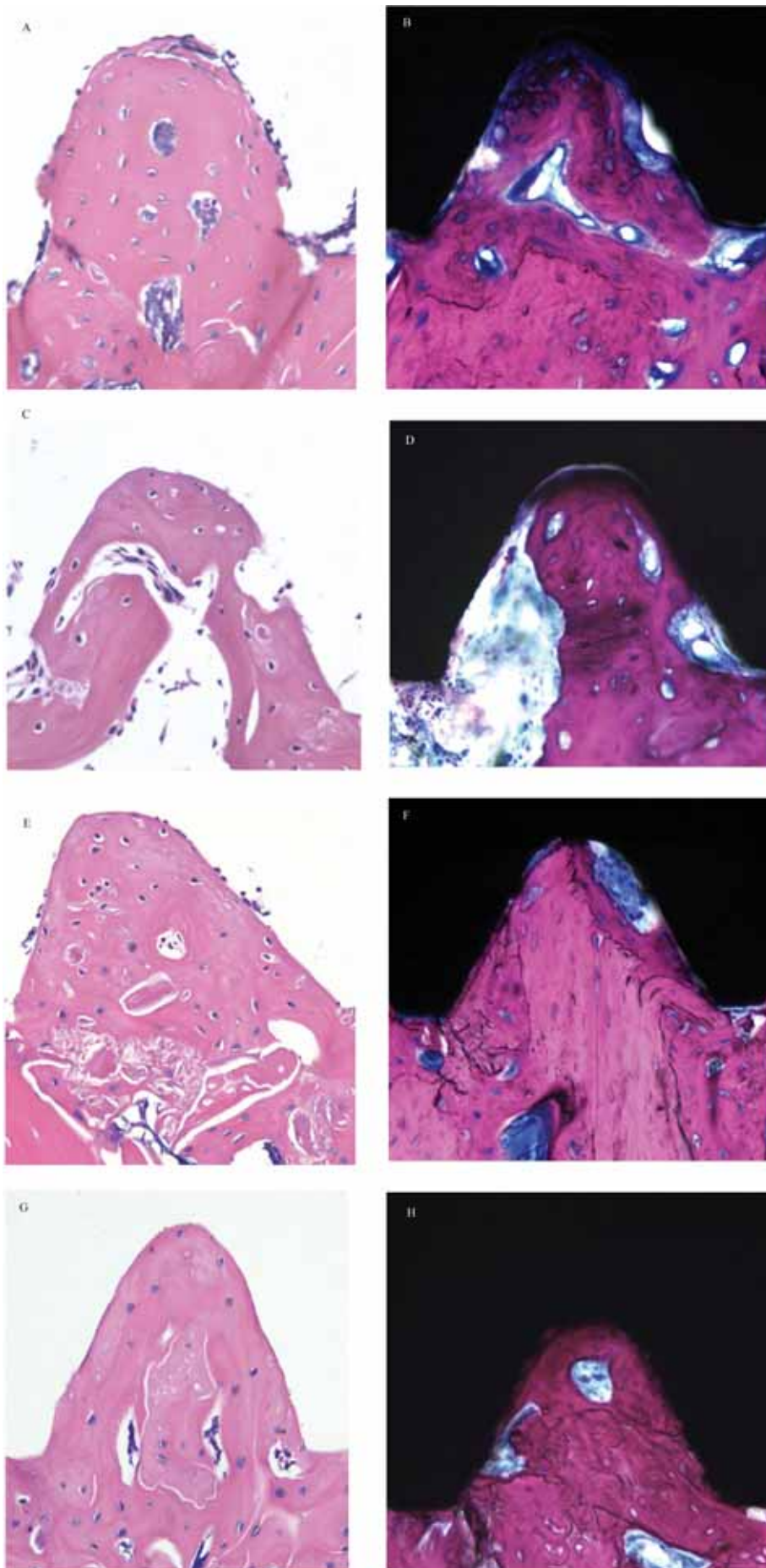
## FIGURES



**Figure 1** - Experimental design

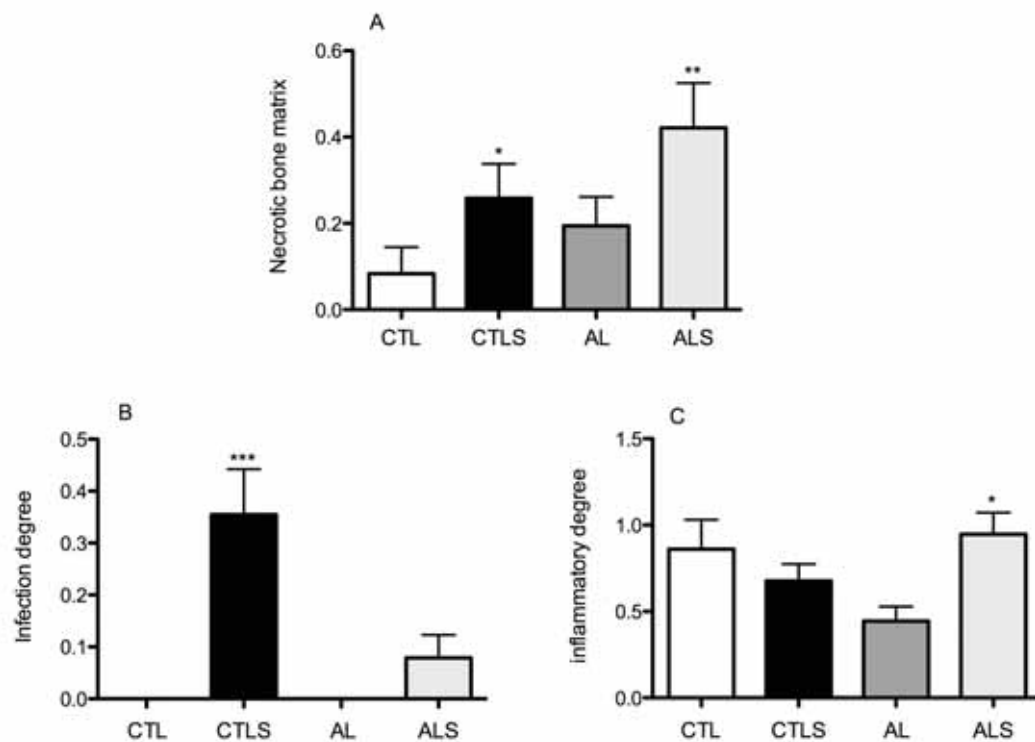


**Figure 2** - Body weight gain of rats subjected to repeated restraint stress (\*\*\* $p < 0.001$  in relation to CTL and AL; \*\* $p < 0.01$  in relation to CTL).

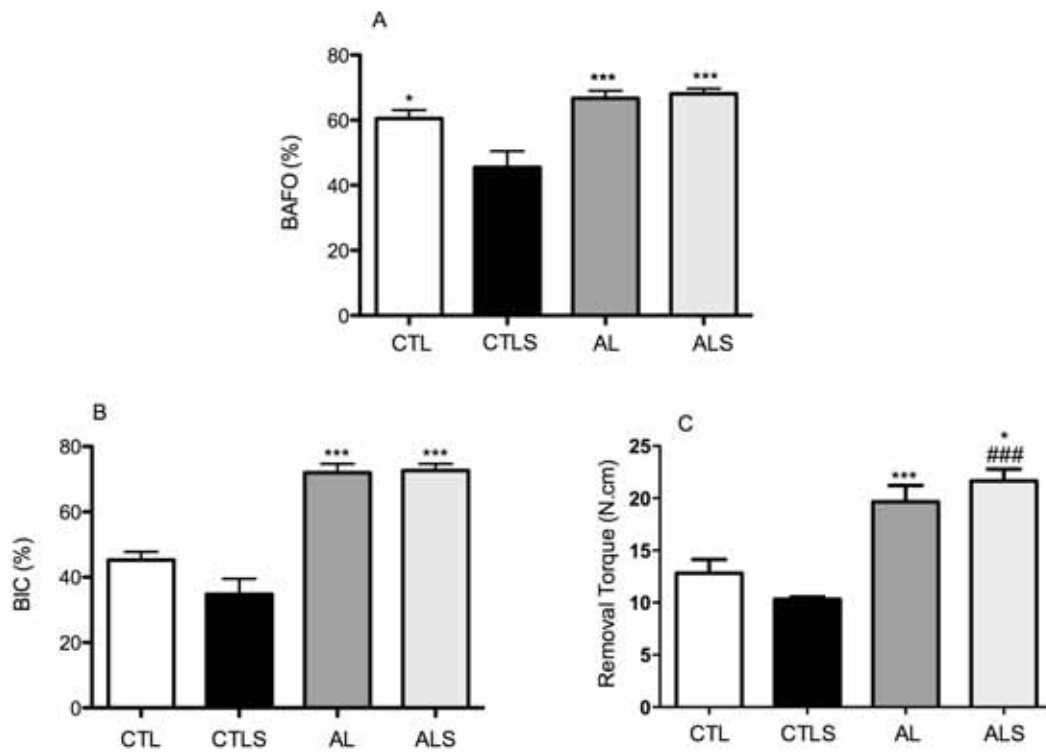




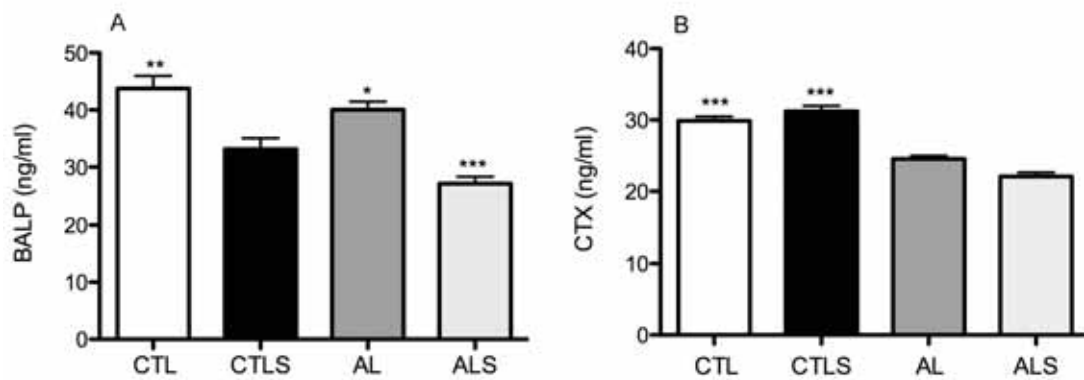
**Figure 3** –Histology aspects of implant cavity illustrated by longitudinal sections stained with hematoxylin and eosin (soft-tissue histology) and Stevenel’s blue and Acid fuchsin (hard-tissue histology) 4 weeks after implantation. Animals of groups CTL (A, B) and CTLS (C, D) presented a high volume of spongy and marrow bone was observed in control animals (x200). A high degree of compact bone was observed in groups AL (E, F) and ALS (G, H) (x200).



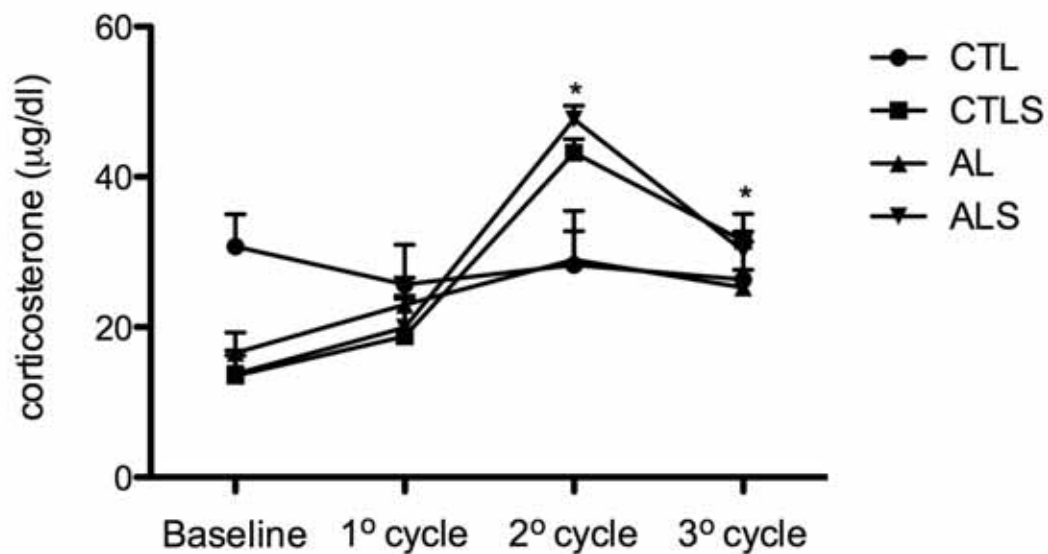
**Figure 4** – Histological features 28 days after tibial implant insertion. (A) Necrotic bone matrix proportion (\* $p < 0.05$  and \*\* $p < 0.01$  in relation to the CTL group). (B) Infection degree (\*\*\*) $p < 0.001$  in relation to all groups). (C) Inflammation degree (\* $p < 0.05$  in relation to the AL group).



**Figure 5** - Histomorphometric measures 28 days after tibial implant insertion. (A) BAFO parameter (\* $p < 0.05$  in relation to CTLS; \*\*\* $p < 0.001$  in relation to CTLS). (B) BIC parameter (\*\*\* $p < 0.001$  in relation to CTL and CTLS). (C) Torque removal values (\*\*\* $p < 0.001$  in relation to CTL and CTLS; ### $p < 0.001$  in relation to CTLS; \* $p < 0.05$  in relation to CTL).



**Figure 6** – Concentrations of bone metabolism markers 28 days after tibial implant insertion. (A) BALP (\*\* $p < 0.01$  in relation to CTL and CTLS; \*\*\* $p < 0.001$  in relation to CTLS; \* $p < 0.05$  in relation to CTLS). (B) CTX (\*\* $p < 0.001$  in relation to AL and ALS).



**Figure 7** – Plasma corticosterone levels in rats exposed to chronic stress during the experiment (\* $p < 0.05$  in relation to baseline and 1° cycle of the CTL and AL groups).

# Capítulo 4

**Artigo 8** – Comparative effects of alendronate therapy on the osseointegration of maxillary and tibial implants - an in vivo observation.

*Artigo em fase de redação*

Conte-Neto N, Spolidorio LC, Andrade CR, Bastos AS, Marcantonio Jr E

**Comparative effects of alendronate therapy on the osseointegration of maxillary and tibial implants - an in vivo observation.**

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## 1- Introduction

During the last years, the Public Health statistics have been included the bisphosphonates (Bps) among the most common drugs prescribed in the world[1], especially due to the high effectively of these drugs in the treatment of several bone diseases[2]. However, since 2003, a great concern has been generated regarding the increasing numbers of bisphosphonate-related osteonecrosis of the jaws (BRONJ)

BRONJ is a great challenge in scientific field and even with many efforts to develop experimental models of this bone necrosis, none of the BRONJ pathogenesis hypotheses is completely accepted yet. On the other hand, strong correlations have been made between BRONJ and possible co-factors, including Bps type and treatment length[3], as well trigger agents, highlighting to surgical procedures with bone manipulation, such as dental implants and teeth extractions[4].

Recently, our research group has established an experimental model to achieve BRONJ-like lesions in rodents by the association of a long-term alendronate (ALN) therapy and teeth extractions (data sent to publication). However, while data from clinical and *in vivo* studies indicate a strong evidence of the teeth extractions as BRONJ trigger agents[5-10], an additional risk of implant failure has not been demonstrated in patients who take oral Bps[11-13], on the contrary, animals treated with these drugs have an increase in osseointegration parameters of implants [14-16].

However, the great majority of animal studies investigating the relationship between Bps and osseointegration have been studying this issue in implants installed in other skeletal sites instead of the jaws[16-18]. Considering that the osteonecrosis related to Bps occurs exclusively at the jaws, the aim of this paper is to compare, in the same experimental model, the osseointegration process of tibial and maxillary implants, investigating if dental implants may be considerate a trigger factor to BRONJ.

## **2- Materials and Methods**

### *2.1- Animals*

The study protocol was approved by the Ethics in Animal Research Committee of the School of Dentistry of Araraquara (UNESP, Brazil) under number protocol 18/2009 and included a total of sixteen male Holtzmann Albinus rats, weighing around 200g. These animals were kept at a special facility room at São Paulo State University – UNESP, School of Dentistry of Araraquara and maintained in a 12:12 hour light/dark cycle (lights on at 7:00 a.m.) at  $23\pm 2^{\circ}\text{C}$  with ad libitum access to a standard laboratory diet and water.

### *2.2- Experimental Design*

After a 3-day acclimatization period, animals were randomly assigned in two experimental groups AL (n=8) and CTL (n=8) to receive weekly subcutaneous doses of ALN (ALCON laboratory, Sao Paulo, SP, Brazil) or sterile physiological saline (0.9% NaCl), respectively. After that the animals were

submitted to first upper maxillary extractions under general anesthesia by a combination of ketamine chloridrate (Ketamina Agener, Agener União Ltda, São Paulo, SP, Brazil; 0.08ml/100g body weight) and Xylazine 2% (Rompum, Bayer S.A., São Paulo, SP, Brazil; 0.04ml/100g body weight).

The therapy with ALN or sterile physiological saline started 30 days after upper teeth extractions, which correspond the healing period of alveolar socket healing [19]. After 60 days of the drugs treatment all animals were submitted to two maxillary implants in the healed sockets and two tibial implants under general anesthesia, following the same protocol of anesthesia describe previously. The ALN or sterile physiological saline administrations were maintained until 28 days after surgical procedures when all animals were euthanized by anesthesia overdose (Figure 1).

### *2.3- Surgical procedures*

The same operator performed all surgical procedures with the same technique in all animals.

#### *2.3.1- Teeth extractions*

Initially, the rats were placed in a dorsal position and fixed in a special device. After that, the surrounding gingival was carefully detached from the lower first molars with a dental explorer. After that, with a Hollenback Carver tooth was luxated and separated in two segments (mesial and distal) that were removed with a forceps adapted around the cervical line of the segments.

#### *2.3.2- Maxillary implants*



Initially, the rats were placed in a supine position and fixed in a special device. After that a small and linear incision was made in the mucosa followed by mucoperiosteal detachment and bone exposition. Then, an osteotomy was prepared bilaterally at the edentulous sites under copious saline irrigation to receive machined titanium implants (1.5 mm in diameter by 2.5 mm in length; Neodente, Curitiba, SC, Brazil). The flaps were sutured with vycril 6-0.

### *2.3.3- Tibial implants*

The animals were submitted to trichotomy on the inner region of the leg and asepsis with povidone iodine solution. After that, an incision was made in the layers on the tibial metaphysis. The underlying bone was subjected to osteotomy with a start drill of 1.8mm for the accommodation of the machined titanium implant with 4mm long and 2.2mm thickness (Neodente, Curitiba, PR, Brazil) under abundant irrigation. The tissue was sutured with silk thread 4-0 (Ethicon, Division of Johnson & Johnson Medical Limited, São Jose dos Campos, São Paulo, Brazil).

### *2.3.4 Post-operative procedures*

After surgical procedure, all animals received an intramuscular dose of antibiotic (Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, Brazil – 0.1mg/Kg) and anti-inflammatory Ketoflex (Ketoprofen 1%, 0.03 ml/rat).

### *2.4- Specimens processing*

Initially, all tissues blocks were immersed directly in 10% buffered formalin fixative solution for 48 h. After that, the specimens were submitted to

routine histological processing for descriptive evaluation or were prepared for hard tissue histology.

#### *2.4.1- Soft tissue histology*

To these analyses, all specimens were decalcified in tetrasodium-EDTA aqueous solution (0.5 M, pH 7.4) for 2–3 mo, under agitation at room temperature. After that, the implants were carefully removed and all specimens were processed and included in paraffin blocks. Serial 4 $\mu$ m sections were obtained in the bucco-lingual direction, stained with hematoxylin and eosin and referred to light microscopic evaluation to descriptive and quantitative analysis (Leica DM1200M; Leica Microsystems, Wetzlar, Hessen, Germany).

One board-certified oral pathologist who was blinded to this study effects performed theses analysis. The histological endpoints evaluated for quantitative analysis included the degree of bone necrosis and infection, as well the quantity and quality of inflammation (acute, chronic or mist). These parameters were scored on a four-point scale 0 (absent; 0%), 1(mild;  $\geq 10\%$ ), 2 (moderate;  $>10$  and  $\leq 50\%$ ) and 3 (increased;  $> 50\%$ ).

#### *2.4.2- Hard tissue histology*

To this analysis, the specimens containing implants were prepared after dehydration by a series of ethanol solutions and embedded in methacrylate-based resin (Technovit 7200; Heraeus Kulzer, Wehrheim, Hesse, Germany). The blocks were initially sectioned at about 150  $\mu$ m using a specific system (EXAKT Apparatebau GmbH & Co., Norderstedt, Germany)[20] and submitted to grinding

and polishing (EXAKT Apparatebau GmbH & Co.) to achieve a final thickness of approximately 30  $\mu\text{m}$ . After that, the sections were stained with Stevenel's blue/acid fuchsin (1%) and referred to light microscopic evaluation.

Measurements of the percentages of bone-implant contact (BIC) and bone area fraction occupancy (BAFO) were performed at 100x magnification (Leica DM1200M; Leica Microsystems, Wetzlar, Hessen, Germany) using ImageJ 1.41o (National Institutes of Health, Bethesda, MD).

### *2.5 -Torque removal analysis*

Analysis of the removal torque was made immediately after the animals sacrifice. The maxillary implants were attached to torque meter with a scale range of 0.1 and 10 Ncm and divisions of 0.05 Ncm (Tohnichi, Shanghai, China). The tibial implants were attached to torque meter with a scale range of 3 and 30 Ncm and divisions of 0.05 Ncm (Tohnichi, Shanghai, China). A wrench was attached to the implant head to apply torque in the reverse direction of implant placement, until complete rupture of the bone/implant interface, signaled by the rotation of the implant.

### *2.6- Statistical Analysis*

Data were submitted initially to Kolmogorov-Smirnov normality test. Then, comparisons among groups were performed using the Kruskal–Wallis followed by Dunn's Multiple Comparison Test (non-parametric data) or ANOVA followed by Tukey test (parametric data). Data were analyzed statistically using

GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA) and statistical significance was set at  $p < 0.05$  with 95% confidence intervals.

### **3- Results**

#### *3.1 – Soft tissue histology*

All animals of the AL and CTL groups presented with a completely epithelial closure over the region correspondent to the maxillary implant installation without signals of inflammation (Figure 1). However, the maxillary implant of AL group presented the highest proportion of necrotic bone matrix and infection degree compared to all groups ( $p < 0.001$ ) (Graphic 1a/b and Figure 2c). Furthermore, the inflammatory degree was statistically higher in maxillary implants of AL group compared to match control ( $p < 0.01$ ) and tibial implants ( $p < 0.001$ ) (Graphic 2c).

Regarding to descriptive histology, an evident difference in the bone architecture was observed, since animals in AL group in both maxillae and tibia presented a high degree of compact bone in contact to the implants, while animals in CTL group exhibited a high volume of spongius and marrow bone (Figures 2 and 3).

#### *3.2 - Hard tissue histology*

After 28 days of implant placement surgery, all groups presented osseointegrated fixations both in maxillae and tibial areas (Figures 2 and 3). However, tibial implants in AL group presented the highest levels of BIC when

compared to all groups and implants ( $p < 0.001$ ). The same tendency was observed comparing AL and CTL groups of maxillary implants ( $p < 0.01$ ) (Graphic 2a)

Regarding to the BAFO analysis of maxillary implants, animals in AL group presented statically higher values compared to CTL animals ( $p < 0.05$ ). On the other hand, in tibial implants no significant differences were found between groups (Graphic 2b).

### 3.3 – *Torque removal analysis*

Concerning to this biomechanical measure, tibial implants in AL group presented the highest levels when compared to all groups and implants ( $p < 0.001$ ). The same tendency was observed comparing AL and CTL groups of maxillary implants ( $p < 0.001$ ) (Graphic 2c).

## **4- Discussion**

Until now the role of dental implants as BRONJ trigger agents still remains unclear in the scientific field. In fact, an additional risk of implant failure has not been demonstrated in patients taking Bps[11-13], on the contrary, animals studies showed positive actions of Bps on the osseointegration process [14-16]. However, these animal models are not suitable to address the relation between BRONJ and implants, since they were carried out in other skeletal sites instead jaws. In this context, this paper showed that ALN therapy was associated to antagonistic effects on the osseointegration process according to the skeletal site,

demonstrating, at the same time, positive changes to tibial implants and deleterious actions to maxillae.

The hard tissue histology and biomechanical tests undoubtedly represent commonly parameters used to assess the bone healing around implants[16, 17, 21]. In this paper, considering only these parameters, we are prone to state that the therapy with ALN was associated to an increase of osseointegration parameters both in tibia and maxillae implants. However, in this last region the soft tissue histology revealed an extensive proportion of necrotic bone matrix and infection, which weren't observed in tibial implants. In this way, these findings bring to light the relevance of soft tissue histology to the real meaning of the hard tissue histology and biomechanical test, at least in the Bps context.

The reasons that justify the increase of histometric measures are not completely clear. In fact, the effects of Bps on the osteogenesis process are not yet well established in the literature. The positive changes observed in tibial region could be justified based on the suppression of the resorption process[15], or even by a new bone formation stimulus around implants[22, 23]. On the other hand, other authors didn't find evidences of Bps direct effects on the ability of osteoblasts to produce bone matrix in vivo[24, 25] or even inhibitory effects of ALN over osteogenesis[26], which could support our findings in maxillae area.

Regarding to the torque removal analysis, a particular consideration is relevant to be done. Although this biomechanical test is closely related to the degree of bone in contact with the implant[27, 28], which would reasonably

justify our findings, it has been shown that the mineral bone density (BMD) and the bone architecture are some of bone strength determinants [29] and, thus, may affect the removal torque test. These assumptions are especially relevant in the field of Bps, since these drugs are related to a significant increase of BMD[30], which naturally tend to result in a more compact bone tissue, as demonstrated in animals of AL group. Therefore, considering especially the maxillae parameters, it could be speculated that increase of BMD may be more significant to torque removal measure than histometric analysis.

This context of increase BMD bring to light our discussion of bone necrosis observed after maxillary implants. It has been showed that BRONJ occurs especially in the vicinity of areas with increased BMD, probably due to the compromised circulation and physicochemical overload [31]. This last aspect, in our opinion, seems to be highly pertinent to BRONJ meaning after implants.

Evidences retrieved from *in vivo* studies demonstrate that the surgical preparation to implant placement naturally result in an area of empty osteocytic lacunae at lateral areas of the bone cavity[32]. It occurs especially due to the heating generated by bone drilling[33, 34], however, in physiological conditions, the bone remodeling replace gradually the damaged bone to a compact one. Therefore, the bone regeneration associated with implants seems to be related to the biological activities of the recipient bone[35].

In the Bps context, considering that these drugs markedly suppress osteoclasts activity[2], the necrotic bone occasioned by tissue drilling can't be

replaced by vital bone, which result in the accumulation of non-vital tissue areas. This assumption explains our findings of extensive lesions at lateral edges of the bone cavity, which is the region where drilling effects are more intense. Furthermore, it has been shown that the drilling temperature increases as the bone mineral density (BMD) increases[33]. Therefore, it is reasonable to expect a higher trauma in bone tissue of Bps-treated animals, since these drugs increase the BMD[30].

These observations support the theory that BRONJ lesions are probably related to the compromised bone incapacity to meet the increasing healing demand that is required in situations of tissue trauma[36]. However, it couldn't explain the non-occurrence of these lesions in tibial region, since the effects of Bps are systemic and, in the both areas, the remodeling demand was increased. In this context, it was speculated that the jaws could be particularly affected, since the functional demand at these areas is increased due to the high rates of remodeling[37] and overload occasioned by masticatory forces[38].

Furthermore, some authors believe that the Bps uptake in alveolar bone is higher than other skeletal sites, which could allow a direct toxicity to the oral mucosa, resulting in exposed bone and secondary infection[39]. However, in our study we did not observe ulceration areas in oral mucosa, which highlight that differences on the environment between tibia and jaws seem to be relevant, especially concerning the oral environment that is rich in bacterial toxins, inflammatory cytokines, or oxidative stress[40].



In this paper we found that maxillary implants were associated to a high degree of infection, which weren't observed in tibial regions. Indeed, some authors believe that BRONJ could result from an increased bacterial adhesion to bone coated with these drugs[41]. Of note, necrotic bone fragments themselves, as demonstrated in our paper, can act as avascular foci for further bacterial adherence[42].

Concerning to inflammatory changes, we found that animals in AL group of maxillae implants presented the highest degree of inflammation. This aspect was probably due to the high infection degree and increased necrotic bone, since both aspects typically trigger host immune responses [26][43] and therefore enhance the degree of inflammatory process. In fact, it also explains the absence of significant inflammation in tibial implants.

These findings are particularly relevant since some authors found that Bps therapy was related to inflammatory changes in bone and soft tissues[44]. Furthermore, it has been suggested that the entire process of BRONJ appears to be associated with an inflammatory reaction[45], which may be mediated by the Gamma-delta T-cells (GDTC)[6]. Indeed, it has been demonstrated that BPs can activate these cells[46], and, moreover, the BP-induced mevalonate pathway inhibition[2] results in the accumulation of metabolic intermediates, including isopentenyl pyrophosphate (IPP)[47], which is a powerful activator of GDTC [46].

Regarding to the clinical pattern of the bone lesions induced by maxillary implants, it was not observed areas of bone exposition, which is in contrast to the diagnosis criteria's stated by BRONJ[48]. However, considering that this osteonecrosis presents different stages and clinical patterns we shouldn't disregard our findings as BRONJ-like lesions. Indeed, the necessity of bone exposed in the oral cavity have been questioned in the literature[10, 49, 50], since in initial stages this clinical feature is not already present[48] and, even in advanced stages, some authors observed that 30 to 45% of reports develops with no bone exposition [13, 49, 50].

Within this discussion, timing of the development of BRONJ classical patterns in reference to the placement of dental implants could be a relevant point[51]. Clinical studies have show that BRONJ reports related to dental implants occurs usually as a late complication[51-53], i.e. after prosthetic rehabilitation. Therefore, it could be speculated that to the classical clinical aspects of BRONJ is necessary the environment of constant loads being transmitted by the prosthesis system, which increases the demand of marginal bone remodeling of a compromised bone tissue, as stated previously[36].

Furthermore, the presence of periimplantar disease could play a role to the BRONJ development, considering that this disease is resulted from infection and/or overloading conditions that are factors related to BRONJ pathogenesis[36]. These assumptions are supported by the important relation of periodontal disease and BRONJ patogenesis[53, 54]. In this way, it could be hypothesized that periods longer that 28 days could be critical to BRONJ related to dental implants.

## 5- Conclusion

The outcomes of this study clearly demonstrated that Bps was related to bone necrosis development after maxillary implant, however, at the same time, increased osseointegration parameters of tibia implants that were associated to normal bone histology. Furthermore, more studies are necessary using animals models with prosthetic rehabilitation to investigate the role of chronic Bps therapy in implant survival and long-term implant osseointegration.

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## Graphics and Figures



Figure 1 – Clinical features of CTL (A) and AL (B) 28 days of maxillary implants placement, showing normal soft tissue coverage in both groups.

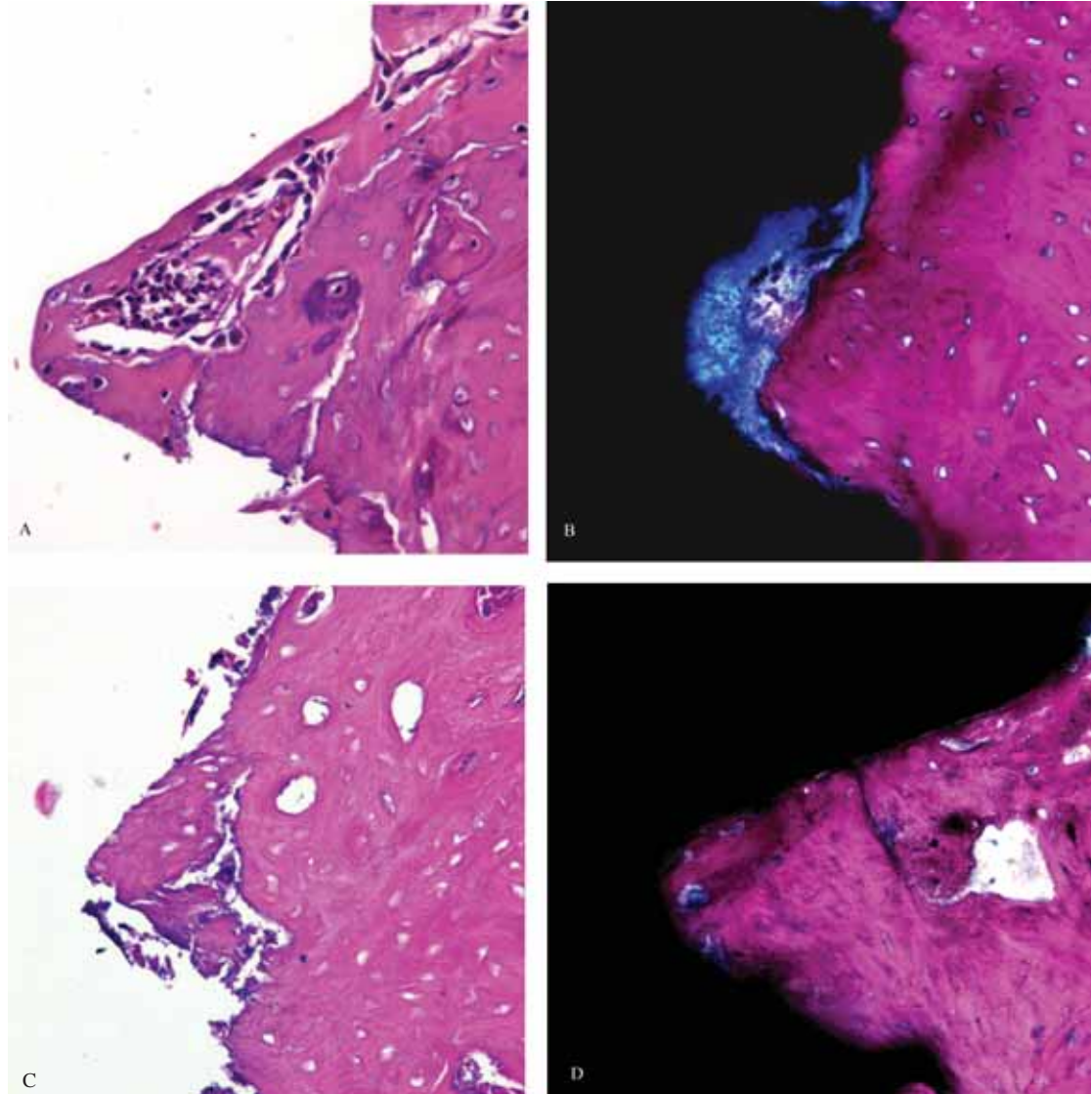


Figure 2 – Hard and soft tissue histology of maxillary implants after 28 days of surgery. Both AL (B) and CTL (D) groups presented bone in contact to implant, however animals treated with alendronate exhibited a high degree of bone necrosis (C), while CTL animals displayed a normal bone tissue (A).

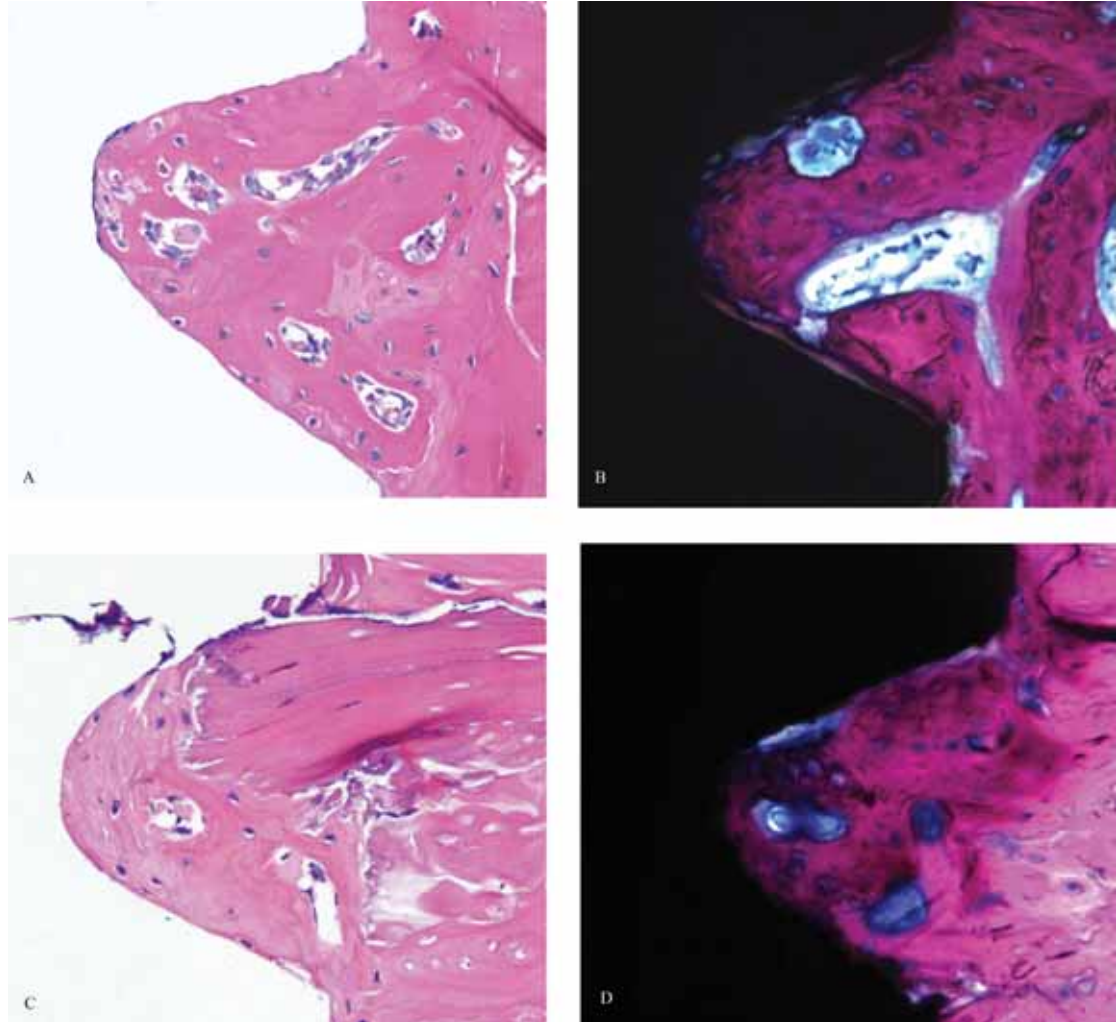
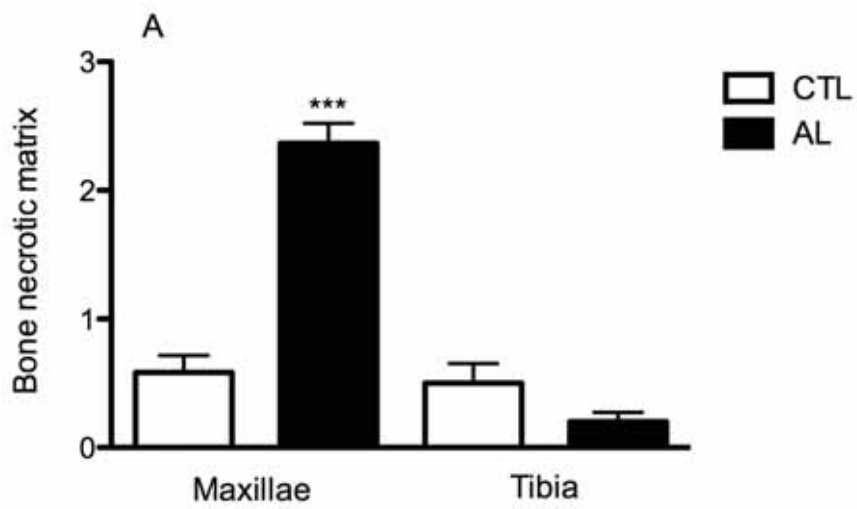
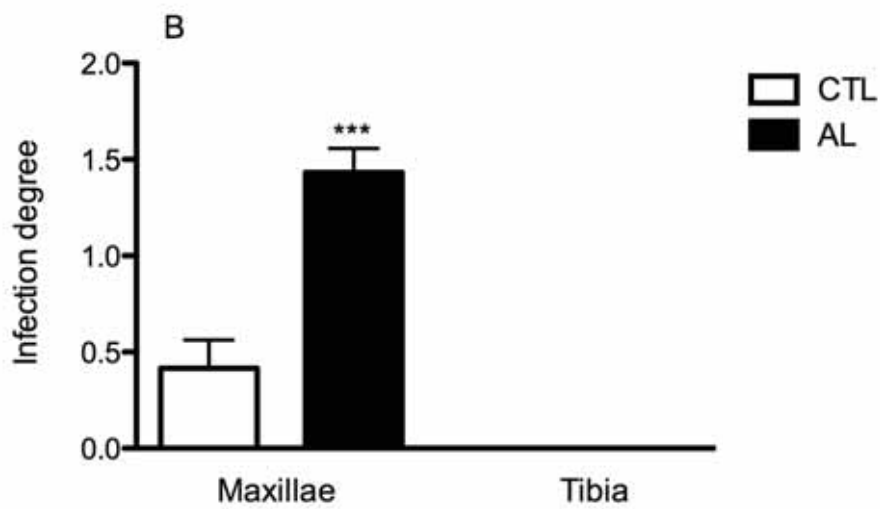


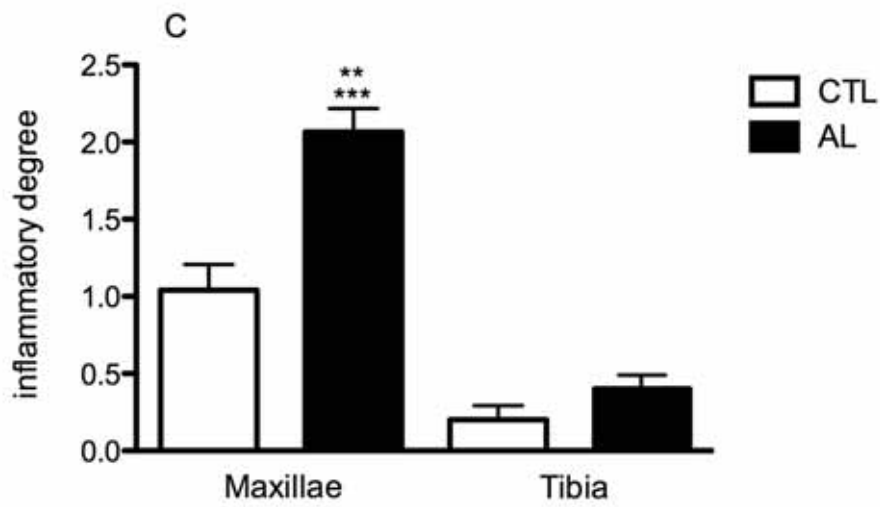
Figure 3 – Hard and soft tissue histology of tibial implants after 28 days of surgery. Both AL (A/B) and CTL (C/D) groups presented normal bone in contact to implant. Of note, animals treated with ALN exhibited an increase of empty osteocyte lacunae and a characteristic compact bone(C).



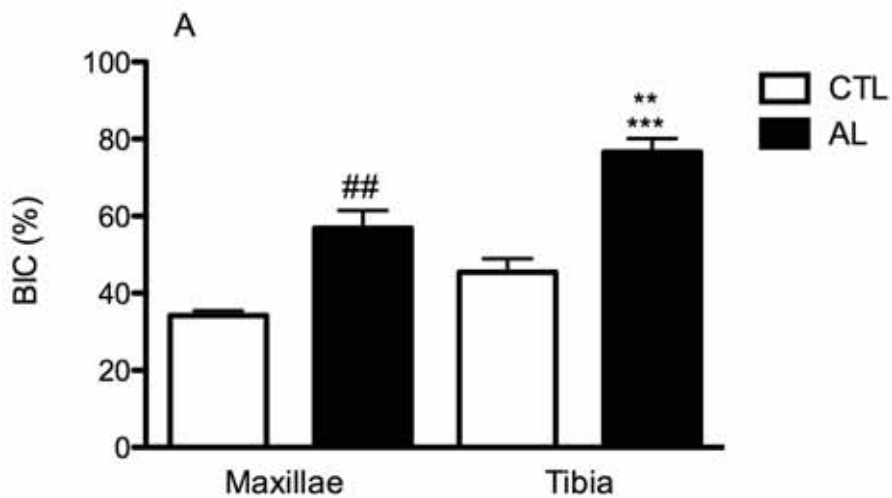
Graphic 1A – Bone necrotic matrix in maxillary and tibial implants (\*\*\*) $p < 0.001$  in relation to all groups).



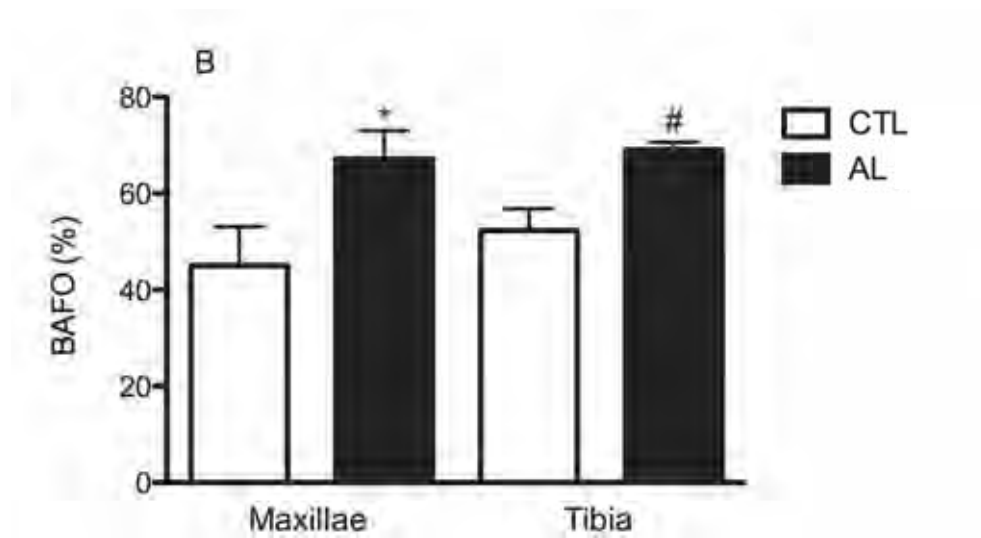
Graphic 1B – Infection degree in maxillary and tibial implants (\*\*\*) $p < 0.001$  in relation to all groups).



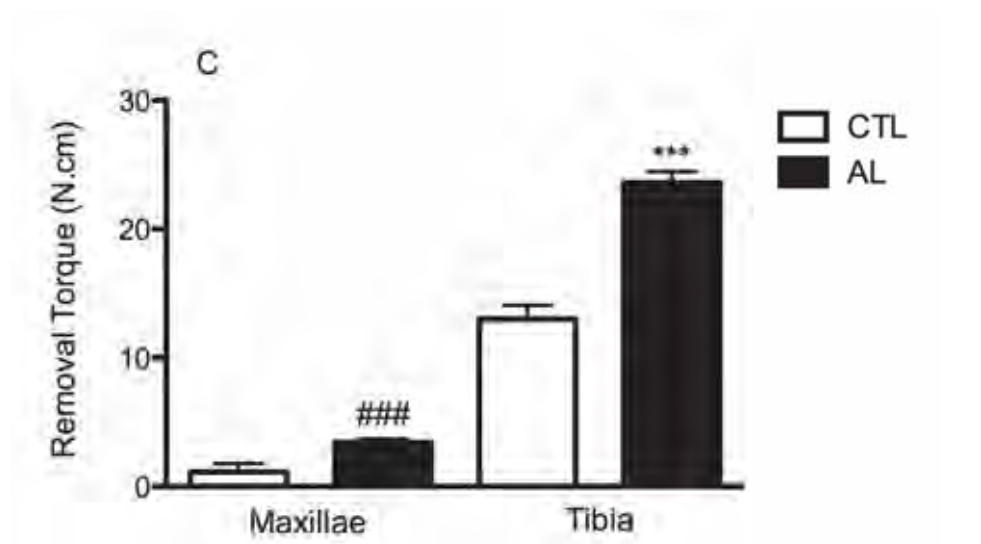
Graphic 1C – Inflammatory degree in maxillary and tibial implants ( \*\*p<0.01 in relation to maxillae CTL; \*\*\*p<0.001 in relation to Tibia CTL and AL).



Graphic 2A – BIC (%) of maxillary and tibial implants ( \*\*\*p<0.001 in relation to CTL maxillae and Tibia; \*\*p<0.01 in relation to AL maxillae; ##p<0.01 in relation to CTL maxillae)



Graphic 2B – BAFO (%) of maxillary and tibial implants (\*p<0.05 in relation to CTL maxillae; #p<0.05 in relation to CTL maxillae).



Graphic 2C – Torque removal values (N.cm) of maxillary and tibial implants (\*\*\*)p<0.001 in relation to all the groups; ###p<0.001 in relation to CTL maxillae).

## ***5 Discussão***

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A alta eficiência dos bisfosfonatos (BFs) na estabilização da perda óssea induzida por processos patológicos elevaram estas drogas à categoria dos fármacos que figuram entre os mais prescritos mundialmente. Entretanto, a partir de 2003 uma série crescente de relatos na literatura vem associando estas drogas ao desenvolvimento de áreas de necrose óssea nos maxilares<sup>20, 56, 68, 96</sup>

Muitas teorias têm sido propostas, porém a etiologia e patogênese da osteonecrose dos maxilares associadas aos bisfosfonatos (OMAB) ainda não foram elucidadas. Por estes motivos, diversos estudos têm sido dedicados ao desenvolvimento de modelos experimentais desta doença. No entanto, a grande maioria dos trabalhos vem descrevendo modelos de lesões por meio da associação de um grande número de variáveis, incluindo a terapia com BFs, extrações dentárias, uso concomitante de esteróides<sup>11, 62, 91</sup>, deficiência de vitamina D<sup>42</sup> ou trauma adicional em alvéolos pós-extração<sup>12</sup>. Neste contexto, com o objetivo de aumentar a confiabilidade dos modelos experimentais, nós reduzimos os números de variáveis e demonstramos o desenvolvimento de padrões diferentes de lesões osteonecróticas somente pela associação da terapia com BFs e procedimentos cirúrgicos bucais.

Para este fim, a primeira etapa deste projeto foi estabelecer um protocolo farmacológico com alendronato (ALE) favorável ao desenvolvimento da OMAB. Neste sentido, foram consideradas as seguintes observações:

- 1- Aspectos relacionados com os BFs, como tipo, dosagem, via de administração e tempo de tratamento representam fatores de risco bem estabelecidos para a OMAB em humanos<sup>83</sup>.



2- Alguns autores utilizando doses baixas de BFs observaram efeitos negativos transitórios<sup>4, 41</sup> ou até mesmo positivos ao reparo de alvéolos pós-extração<sup>43</sup> e à osseointegração de implantes<sup>34, 99</sup>.

3- Foi observado que a supressão do metabolismo ósseo esteve diretamente associada a um aumento do número e tamanho de matrizes de necrose óssea nos maxilares<sup>5</sup>.

4- Acredita-se que a magnitude da supressão da remodelação óssea está intimamente associada com a dose cumulativa total de BFs administrada sob um período longo de tempo<sup>19</sup>.

Diante destas considerações, nós entendemos que a dose cumulativa obtida antes da realização dos procedimentos cirúrgicos poderia representar um ponto crucial para o desenvolvimentos das lesões. Por este motivo, nós estabelecemos um protocolo de terapia prolongada com alendronato (ALE) em altas doses, não compatíveis (estudo 1) ou compatíveis às dosagens terapêuticas em animais para o tratamento de doenças reumáticas<sup>48</sup> (estudos 2, 3 e 4). De fato, utilizando os dois protocolos nós observamos uma supressão dos níveis de C-telopeptídeo de ligação cruzada do colágeno tipo I (CTX) (estudos 1, 2 e 3) e fosfatase alcalina específica óssea (FAO) (estudos 1 e 2), os quais representam marcadores bioquímicos da reabsorção e neoformação óssea, respectivamente<sup>73</sup>.

Embora a OMAB possa ocorrer espontaneamente<sup>20, 56, 67</sup>, a grande maioria das lesões está associada a procedimentos cirúrgicos bucais<sup>39, 83</sup>. Entretanto, enquanto os dados obtidos de trabalhos clínicos e *in vivo* indicam uma forte associação entre

exodontias e OMAB<sup>11, 12, 62, 66, 68, 70, 96, 98</sup>, o papel dos implantes dentais como fatores causais desta doença ainda não está claro na literatura. Esta controvérsia fundamenta-se em alguns aspectos, como o pequeno número de casos relatados<sup>20, 103</sup>, das altas taxas de sucesso de implantes em pacientes fazendo uso de BFs<sup>10, 31, 36, 44, 56</sup>, além da observação de efeitos positivos sobre os parâmetros de osseointegração em animais tratados com estas drogas<sup>34, 58, 71</sup>.

Neste contexto, nós observamos que tanto as extrações dentais quanto os implantes maxilares estiveram associados ao desenvolvimento de lesões osteonecróticas, entretanto, apresentaram-se com padrões clínicos diferentes. Por outro lado, nos implantes instalados na tíbia, a terapia com ALE se mostrou benéfica aos parâmetros de osseointegração estudados, inclusive evitando os efeitos deletérios induzidos pelo estresse crônico sobre o metabolismo ósseo.

A descrição destes resultados acentua a relevância sobre a discussão de alguns aspectos, incluindo: 1- a forte relação de causa e efeito entre a OMAB e os procedimentos cirúrgicos bucais; 2- os padrões diferenciados de lesões desenvolvidos após exodontias e implantes maxilares; 3- os efeitos positivos da terapia com ALE sobre os parâmetros de osseointegração em implantes tibiais.

Para alguns autores, a associação evidente entre o desenvolvimento da OMAB após procedimentos cirúrgicos na cavidade bucal pode ser justificada pelo fato destas lesões serem consequência da incapacidade de um tecido ósseo saturado com BFs cumprir com as demandas metabólicas aumentadas em situações de infecções e

trauma tecidual decorrente, por exemplo, de procedimentos cirúrgicos<sup>101</sup> ou de próteses totais mal-adaptadas<sup>90</sup>.

De fato, nós observamos que as lesões osteonecroticas desenvolveram-se eminentemente após as intervenções cirúrgicas bucais, mesmo tendo sido estabelecido um pré-tratamento de 8 semanas com altas doses de ALE. Por outro lado, nós também observamos em alguns animais tratados com ALE (estudo 1), a presença de áreas de exposição óssea em torno dos incisivos superiores, as quais não foram induzidas cirurgicamente. Entretanto, curiosamente, todas estas lesões estavam associadas a elementos dentais fraturados. Considerando que alguns animais tinham por costume roer as grades metálicas que recobriam as caixas nas quais estavam alocados, a nossa hipótese é que este hábito possa ter gerado uma sobrecarga aos tecidos periodontais. Conseqüentemente, houve um aumento pela demanda de remodelação, favorecendo o desenvolvimento das lesões de acordo com a teoria em questão<sup>101</sup>.

Esta observação reforça o reconhecimento da relevância do trauma na patogênese da OMAB, o que tem estimulado o desenvolvimento de protocolos atraumáticos de exodontias em pacientes tratados com BFs<sup>28, 86</sup>. Indubitavelmente, estes protocolos são extremamente relevantes para a prática Odonológica clínica, entretanto, nós entendemos que os procedimentos reconstrutivos, tais como implantes osseointegrados, também merecerem tais adaptações. A explicação para esta proposta baseia-se na relação técnica direta entre trauma tecidual e terapia com BFs,

estabelecida no momento em que o tratamento com estas drogas resulta no aumento importante da densidade óssea<sup>80</sup>.

Em se tratando de exodontias, a densidade óssea elevada é claramente reconhecida como um dos fatores que aumentam a dificuldade cirúrgica durante estes procedimentos<sup>17,63</sup>. Os nossos resultados estão de acordo com esta assertiva, pois observamos que as exodontias foram mais traumáticas nos animais tratados com alendronato (estudo 1), com base no maior tempo cirúrgico exigido e maior incidência de fraturas radiculares. Por estas razões, nós passamos a realizar extrações por seccionamento dental, visto que com esta técnica é possível reduzir o impedimento mecânico resultante do tecido ósseo, bem como o risco de fraturas dento-alveolares.

No âmbito da Implantodontia, estas observações também são pertinentes, visto que o trauma cirúrgico ocasionado pelo calor gerado na fresagem é diretamente proporcional a densidade óssea<sup>47</sup>. Desta forma, a preparação do leito receptor para os implantes tende a ser mais traumática em áreas de alta densidade óssea, o que explicaria, pelo menos em parte, os nossos achados de tecido ósseo não vital nas regiões laterais às fresagens. Estas observações fortalecem a relevância de se estabelecer protocolos atraumáticos de instalação de implantes nestas circunstâncias, especialmente em pacientes tratados com BFs.

No que se refere à patogênese da OMAB, nós entendemos que, além do trauma, aspectos ambientais e dinâmicos relacionados à cicatrização das feridas bucais também concorrem para o desenvolvimento destas lesões. Estas considerações

fundamentam-se no potencial dos BFs em comprometer diversos processos fisiológicos necessários para o processo cicatricial, incluindo a resposta inflamatória<sup>16, 59</sup>, a angiogênese, reabsorção óssea e migração epitelial<sup>51</sup>.

Em relação à dinâmica dos osteoclastos, está bem estabelecido que nas fases iniciais do processo cicatricial após exodontias e implantes dentais há uma intensa atividade reabsortiva com o intuito de remover o tecido ósseo necrótico e debris ósseos<sup>32, 61</sup>. Desta forma, a inibição dos osteoclastos induzida pelos BFs resulta na manutenção do tecido ósseo necrótico, o que particularmente explica as lesões osteonecroticas às margens da fresagem (estudo 4) e às localizadas na região de septo inter-radicular (estudos 1 e 2).

Além disso, permite que este tecido permaneça exposto à cavidade bucal rica em bactérias e seus produtos<sup>3</sup>, o que também resulta em necrose tecidual. Estes aspectos são particularmente relevantes no contexto da OMAB, visto que alguns autores demonstraram que esta doença pode ser resultado de uma adesão bacteriana aumentada ao tecido ósseo saturado com estas drogas, sendo mediada por uma proteína denominada de componente da superfície microbiana que reconhece moléculas de adesão da matriz e tem a capacidade de interagir com a hidroxiapatita do tecido ósseo<sup>52</sup>. De fato, nós observamos um alto grau de infecção associada às áreas de osteonecrose tanto nas exodontias quanto nos implantes maxilares.

Neste contexto de infecção e OMAB, sabe-se que a resposta inflamatória, dentre outras funções, atua ativamente na eliminação de microorganismos<sup>38</sup>, principalmente pelas ações dos neutrófilos que representam a primeira linha de defesa

contra a infecção<sup>59</sup>. Isto é particularmente relevante, considerando que nos períodos iniciais do reparo de alvéolos pós-extração (estudo 1), o infiltrado inflamatório foi eminentemente crônico e significativamente menor nos animais tratados com ALE. Enquanto que nos animais controle, a inflamação foi caracteristicamente aguda.

Além disso, é válido ressaltar que após 28 dias dos procedimentos cirúrgicos bucais não foram observadas diferenças significativas na intensidade da resposta inflamatória entre os grupos, mesmo na presença de um alto grau de infecção nos animais tratados com ALE. Este resultado é particularmente interessante pois sabe-se que uma infecção microbiana naturalmente induz a ativação da resposta imune do hospedeiro<sup>85</sup>, o que deveria aumentar a intensidade inflamatória nos animais infectados.

As razões que podem justificar estes achados podem estar relacionadas aos efeitos dos BFs sobre o processo inflamatório. Para alguns autores, os BFs possuem ações antiinflamatórias, diminuindo a liberação de citocinas pró-inflamatórias<sup>16</sup>, além da supressão prolongada do sistema imune inato devido a ações inibitórias sobre neutrófilos<sup>54</sup>. Dessa forma, pode ser especulado que a terapia prolongada com altas doses de BFs possa ter resultado em um estado constante de imunossupressão, além de inibir significativamente a resposta aguda, o que favoreceu a manutenção do processo infeccioso, pois uma concentração crítica dos neutrófilos é necessária para a eliminação efetiva das bactérias<sup>59</sup>.

Por outro lado, é válido destacar que após a instalação dos implantes na maxila foi observado um alto grau de resposta inflamatória, o que contradiz os

resultados dos outros estudos. Embora os BFs também estejam associados a respostas pró-inflamatórias<sup>35,40</sup>, estas usualmente ocorrem como reações de fase aguda, ou seja, após a primeira administração da droga<sup>40</sup>. Por este motivo, nós entendemos que estes resultados podem ter sido decorrentes da associação entre infecção e as extensas áreas de necrose óssea, visto que ambos estão envolvidos na ativação da resposta inflamatória<sup>46,69</sup>, o que também explicaria a ausência de inflamação significativa após a instalação dos implantes na Tíbia.

No que se refere às considerações vasculares, está bem estabelecido que o processo cicatricial decorrente das exodontias e implantes dentais é altamente dependente da revascularização<sup>32,61</sup>. No entanto, evidências substanciais demonstram que os BFs apresentam efeitos inibitórios importantes sobre a angiogênese<sup>30,51</sup>, o que explica, razoavelmente, os nossos resultados de menor grau de vascularização em períodos tardios de reparo após exodontias. Isto é particularmente relevante considerando que alguns autores acreditam que a OMAB possam ser resultado de condições isquêmicas, que acarretam em necrose avascular<sup>23</sup>. Além disso, é válido salientar que fragmentos ósseos necróticos podem atuar por si só como focos avasculares para aderência bacteriana<sup>81</sup>.

Outro aspecto relevante a ser discutido é a diferença nos padrões clínicos das lesões observadas após exodontias e implantes maxilares, o que é particularmente relevante no contexto infeccioso, considerando o papel relevante do tecido epitelial na proteção à infecção bacteriana bucal<sup>49</sup>. Baseado no potencial dos BFs em inibir a atividade migratória das células epiteliais normais<sup>51,55</sup>, entendemos que os alvéolos

foram particularmente afetados, pois houve uma alta demanda de migração de células epiteliais, conseqüente da cicatrização do tecido mole por segunda intenção, o que explica as áreas de exposição óssea observadas nestes alvéolos em animais tratados com ALE.

Por outro lado, nos implantes maxilares os efeitos inibitórios dos BFs sobre o tecido mole podem ter sido atenuados, devido a menor demanda pela migração de células epiteliais, pois a cicatrização foi estabelecida por primeira intenção, visto que o retalho muco-periosteal foi reposicionado e suturado. De fato, neste estudo não foram observadas ulcerações no tecido mole após as exodontias, mas sim uma inibição do fechamento da ferida.

Estas diferenças clínicas observadas reiteram a relevância da discussão sobre os critérios de diagnóstico comumente utilizados para a OMAB. Segundo a AAOMS (2009), a presença de exposição óssea na cavidade bucal por no mínimo 8 semanas é fundamental para a caracterização das lesões. Por este motivo, os modelos experimentais de OMAB *in vivo* tem frequentemente adotado este conceito<sup>11, 12, 23</sup>. Diante disso, nós entendemos que algumas considerações sobre este critério de diagnóstico devem ser pontuadas.

Em se tratando de tempo de exposição óssea, é válido ressaltar que existem diferenças metabólicas inerentes entre humanos e animais, de modo que, embora estas espécies compartilhem as mesmas fases de cicatrização alveolar, em ratos elas se desenvolvem mais rapidamente do que em humanos<sup>14</sup>, em uma proporção de aproximadamente 1/3 do tempo do reparo nos humanos<sup>72</sup>. Portanto, acreditamos que



o tempo de 8 semanas estabelecido para humanos não deve ser estritamente estendido aos animais, mas sim um período menor. Além disso, os estudos mostram que quando temporários, os efeitos inibitórios sobre a cicatrização alveolar não se estendem por mais de 14 dias<sup>4,41</sup>. Em nosso estudo, 4 semanas após as exodontias nós observamos aspectos clínicos, histológicos e radiográficos compatíveis com a OMAB.

No que se refere às lesões observadas após a instalação dos implantes maxilares, não observamos evidências de exposição óssea, o que contraria os critérios estabelecidos pela AAOMS (2009). Entretanto, entendemos que isto não impede a caracterização destas lesões como OMAB, visto que esta doença em humanos apresentam padrões clínicos distintos. A exigência de exposição óssea para diagnóstico desta doença vem sendo frequentemente contestada na literatura<sup>26, 45, 102</sup>, pois em estágios iniciais da doença esta característica clínica ainda não está presente<sup>83</sup> e, mesmo em estágios avançados, alguns autores observaram que cerca de 30 a 45% dos casos desenvolveram sem a presença de exposição óssea<sup>26, 45, 56</sup>.

Neste contexto, o tempo necessário para o desenvolvimento dos padrões clássicos da OMAB em relação a implantes dentais parece ser um ponto relevante. Estudos clínicos têm mostrado que os casos relatados desta doença após a instalação de implantes ocorrem geralmente como uma complicação tardia<sup>9, 20, 57, 65</sup>, ou seja, após a reabilitação protética. Dessa forma, pode ser especulado que para a ocorrência dos padrões característicos desta doença é importante o microambiente de transmissão constante das cargas pelo sistema protético, o que tende a aumentar a demanda pela remodelação óssea, em um tecido incapaz de cumprir com estas

exigências, conforme descrito anteriormente<sup>101</sup>. Além disso, é válido ressaltar que a presença da doença periimplantar pode concorrer para o desenvolvimento da OAMB, pelas considerações micro-ambientais nestes estados em relação às vertentes teórica da osteonecrose, além do fato de que a doença periodontal é considerada um importante fator de risco para a OMAB<sup>20,67</sup>. Neste sentido, períodos mais longos de avaliação e outros modelos animais com possibilidade de reabilitação protética podem ser necessários para o melhor entendimento do papel dos implantes dentais na gênese da OMAB.

Com relação aos efeitos dos BFs sobre a osteogênese, aspectos controversos tem sido descritos na literatura. Enquanto alguns autores observaram efeitos inibitórios do ALE sobre a osteogênese<sup>51</sup>, outros estudos não observaram evidências diretas de ações inibitórias destas drogas sobre a neoformação óssea in vivo<sup>7,27</sup>. De fato, os nossos resultados indicam que houve um comprometimento parcial da formação óssea nos animais tratados com ALE, especialmente nos alvéolos pós-extração. Entretanto, não se pode concluir se foi um efeito direto sobre a osteogênese ou se foi consequência secundária ao retardo das etapas iniciais do reparo alveolar, conforme descrito na literatura<sup>41</sup>.

Outro aspecto altamente relevante na discussão dos nossos resultados é a ausência de lesões osteonecróticas na região tibial. De fato, os efeitos in vivo dos BFs sobre a osseointegração usualmente tem sido avaliados por meio de implantes instalados na região tibial. Neste local, os resultados são realmente promissores indicando melhoras significativas nos parâmetros de osseointegração<sup>34,58,71</sup>, inclusive

evitando a perda óssea induzida pela administração de glicocorticóides<sup>18</sup>, que são hormônios liberados durante um estímulo estressor<sup>15</sup>.

Nesta tese nós mostramos pela primeira vez que a indução de estresse crônico foi associada a uma redução significativa dos níveis de FAO nos implantes tibiais. Entretanto, apenas os animais controle apresentaram redução no volume de tecido ósseo entre as espiras dos implantes. Dessa forma, nós observamos as mesmas tendências descritas pela literatura, de que a terapia com ALE evitou os efeitos deletérios do estresse sobre o tecido ósseo pela inibição do processo de reabsorção.

Estes achados são particularmente interessantes, pois mostram que a OMAB, ou pelo menos efeitos deletérios da terapia com BFs não desenvolvem-se em outros sítios esqueléticos, a não ser na cavidade bucal. As justificativas para estes efeitos controversos ainda não estão claramente estabelecidos na literatura, entretanto, algumas hipóteses tem sido elencadas, com base nas teorias que procuram explicar a gênese da OMAB.

Alguns autores baseiam-se na teoria de que a demanda funcional na cavidade bucal é particularmente aumentada devido à alta taxa de remodelação dos maxilares<sup>5</sup>, decorrentes do estímulo constante no ligamento periodontal<sup>67</sup>, da sobrecarga devido às forças mastigatórias<sup>95</sup> e como resultado de procedimentos odontológicos invasivos<sup>101</sup>. Entretanto, em nosso estudo tanto os implantes tibiais quando os maxilares foram instalados seguindo o mesmo protocolo, além de serem mantidos submersos, ou seja, livre de cargas funcionais.

Com base nesta observação e no fato de ambos implantes compartilharem as mesmas fases de osseointegração, especulamos que considerações ambientais possam ser relevantes. Esta hipótese reforça a teoria infecciosa sobre a patogênese da OMAB, onde os maxilares seriam especialmente sujeitos à infecção quando comparado a outras regiões do esqueleto humano, em virtude da espessura fina de mucosa que isola o tecido ósseo do meio bucal naturalmente contaminado, suscetibilidade ao trauma e presença dos dentes<sup>53</sup>.

É válido mencionar que na maior parte dos estudos os parâmetros de osseointegração frequentemente utilizados limitam-se a análise histológica de tecido calcificado e avaliação do torque de remoção<sup>33, 34, 71</sup>. Entretanto, os nossos resultados de maior BIC, BAFO e torque de remoção nos implantes maxilares em animais tratados com ALE evidenciam a importância da análise histológica de rotina, visto que nestes animais o tecido ósseo ao redor dos implantes era predominantemente não-vital.

Com relação aos efeitos do estresse crônico sobre os implantes instalados na maxila, as altas taxas destas fixações que não osseointegraram comprometeram o estabelecimento de um número adequado de espécimes para análise estatística. Em virtude do pequeno número de implantes instalados no estudo 5, não se pode concluir se este alto índice de insucesso foi decorrente de efeitos deletérios do estresse sobre o processo cicatricial.

## ***6 Conclusão***

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1- Exodontias em animais tratados com alendronato estiveram associadas à maior dificuldade cirúrgica e maior frequência de acidentes.

2- Altas doses diárias e/ou semanais de alendronato quando associadas a exodontias e implantes maxilares resultaram em padrões diferentes de OMAB.

3- Altas doses semanais de alendronato quando associadas a implantes tibiais resultaram em melhora dos parâmetros de osseointegração destes implantes.

4- O Estresse crônico quando associado a implantes tibiais em animais tratados com solução salina fisiológica resultaram em efeitos deletérios sobre a osseointegração destes implantes.

## ***7 Referências***

## Referências\*

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\* De acordo com a norma Vancouver. Disponível no site: [http://www.nlm.nih.gov/bsd/uniform\\_requirements.html](http://www.nlm.nih.gov/bsd/uniform_requirements.html)



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## ***8 Apêndice***

# *Apêndice 1*

## **Apêndice 1- MATERIAL E MÉTODO**

### **Animais**

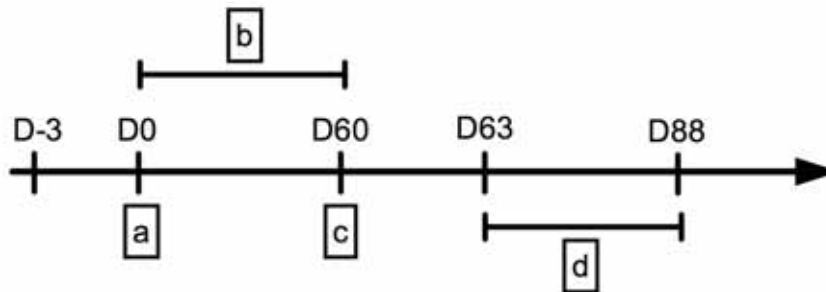
A manipulação animal seguiu o protocolo descrito pelo Comitê de Ética no Ensino e Pesquisa (CEEP) da faculdade de Odontologia de Araraquara (FOAr-UNESP) (protocolo número 18/2009).

Foram utilizados para esta pesquisa 152 ratos machos Holtzman, com peso variando entre 155 a 200g (6-8 semanas). Os animais foram numerados individualmente e alocados adequadamente em gaiolas de acordo com o grupo ao qual pertenceram, dispostas ao ambiente do Biotério da Faculdade de Odontologia de Araraquara – UNESP e ao Biotério veiculado à Disciplina de Farmacologia do Departamento de Princípios Ativos Naturais e Toxicologia (PANT) da Faculdade de Ciências Farmacêuticas do Campus de Araraquara – UNESP. Os animais foram aclimatizados antes do início do estudo, sendo mantidos em condições ambientais com temperatura, umidade e luz controlados. A alimentação foi feita com ração normal (pellets ou triturada) e água *ad libitum*.

### **Desenho dos estudos**

#### **ESTUDO 1**

##### ***Fluxograma***



**FIGURA 1-** Desenho experimental

**Item a:** após um período de três dias para adequação dos animais ao biotério, 60 animais foram distribuídos aleatoriamente em três grupos:

- a) Grupo AL<sub>1</sub> (n=20) recebeu doses de alendronato de 1 mg/kg/dia.
- b) Grupo AL<sub>3</sub> (n=20) recebeu doses de alendronato de 3 mg/kg/dia.
- c) Grupo CTL (n=20) recebeu tratamento com solução salina fisiológica diariamente, correspondendo ao grupo controle.

**Item b:** durante os primeiros 60 dias, os animais foram tratados por meio de injeção subcutânea de alendronato ou solução salina fisiológica, de acordo com o grupo.

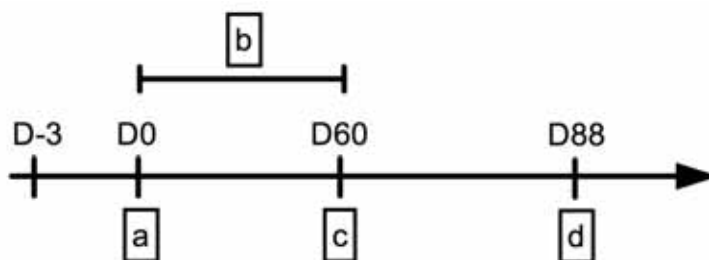
**Item c:** após este período de 60 dias, todos os animais foram anestesiados para a extração do primeiro molar inferior esquerdo e o mesmo protocolo farmacológico com alendronato ou soro fisiológico foi mantido até o sacrifício dos animais.

**Item d:** nos períodos de 3 e 28 dias após as extrações dentárias, os animais foram novamente anestesiados para coleta de uma amostra sanguínea por meio de punção

cardíaca para análise bioquímica, sendo em seguida sacrificados por meio de aprofundamento de anestesia para retirada dos espécimes. Em cada período foram sacrificados cinco animais de cada grupo.

## **ESTUDO 2**

### *Fluxograma*



**FIGURA 2-** Desenho experimental

**Item a:** após um período de três dias para adequação dos animais ao biotério, 12 animais foram distribuídos aleatoriamente em dois grupos:

- a) Grupo AL (n=6) recebeu doses de alendronato de 1 mg/kg/semana
- b) Grupo CTL (n=6) recebeu tratamento com solução salina fisiológica, 1x/semana, correspondendo ao grupo controle.

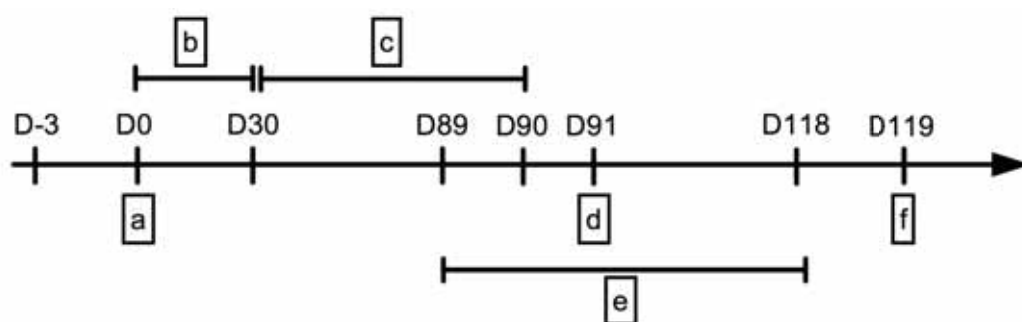
**Item b:** durante os primeiros 60 dias, os animais foram tratados por meio de injeção subcutânea de alendronato (grupo AL) ou solução salina fisiológica (grupo CTL).

**Item c:** após este período de 60 dias, todos os animais foram anestesiados para a extração do primeiro molar inferior esquerdo e o mesmo protocolo farmacológico com alendronato ou soro fisiológico foi mantido até o sacrifício dos animais.

**Item d:** no período de 28 dias após as extrações dentárias, os animais foram novamente anestesiados para coleta de uma amostra sanguínea por meio de punção cardíaca para análise bioquímica, sendo em seguida sacrificados por meio de aprofundamento de anestesia para retirada dos espécimes.

### ESTUDO 3

#### *Fluxograma*



**FIGURA 3-** Desenho experimental

**Item a:** após um período de três dias para adequação dos animais ao biotério, 48 animais foram distribuídos aleatoriamente em quatro grupos:



- a) Grupo AL (n=12) recebeu doses de 1mg/Kg/semana de alendronato.
- b) Grupo ALS (n=12) recebeu doses de 1mg/Kg/semana de alendronato associado à indução de estresse.
- c) Grupo CTL (n=12) recebeu tratamento com solução salina fisiológica uma vez por semana
- d) Grupo CTLS (n=12) recebeu tratamento com solução salina fisiológica uma vez por semana associado à indução de estresse.

Em seguida, os animais foram anestesiados para a extração dos primeiro molares superiores e inferiores (direito e esquerdo).

**Item b:** período de 30 dias (Dia 0 ao 30) para que ocorra reparação alveolar.

**Item c:** durante o período do dia 30 ao 90 (60 dias), os animais foram tratados por meio de injeção subcutânea de alendronato (AL e ALS) ou solução salina fisiológica (CTL e CTLS), uma vez por semana. No dia 80 foi coletada uma amostra sanguínea por meio de secção caudal para radioimunoensaio, objetivando a detecção dos níveis basais de corticosterona.

**Item d:** no dia 91, os animais foram anestesiados e submetidos a procedimento cirúrgico para instalação de implantes bilateralmente na região da metáfise tibial e nas maxilas.

**Item e:** o protocolo de indução de estresse (grupos ALS e CTLS) teve início no dia 89\* e permaneceu até o dia 118, para que compreenda o período necessário para

osseointegração em ratos que é de 28 dias, tanto para implantes maxilares quanto tibiais<sup>32, 67</sup>. Todos os animais foram estressados no mesmo período (tarde) em todos ciclos e 24 horas após cada ciclo de estresse (dia 99, dia 109 e dia 119) foram coletadas novas amostras sanguíneas por meio de secção caudal para radioimunoensaio, para confirmar que os animais permaneceram com os níveis elevados de corticosterona em todos os ciclos de estresse e, portanto, durante todas as fases da osseointegração.

\* O protocolo de estresse teve início dois dias antes da instalação dos implantes, visto que no momento dos procedimentos cirúrgicos os animais já deveriam estar sob efeito do estresse, uma vez que o processo de osseointegração tem início logo após a instalação dos implantes, envolvendo respostas vasculares e inflamatórias.

### **Metodologias e protocolo de exposição ao estresse**

#### **Cronologia dos protocolos de indução de estresse crônico**



**FIGURA 4-** Metodologias e cronologia de indução de estresse crônico

O modelo de estresse crônico (EC) consistiu de exposição diária ao agente estressor, de forma que a cada período de 10 dias consecutivos, os animais foram expostos a apenas um tipo de agente estressor uma vez ao dia. Após aleatorização, a cronologia de exposição ao EC ficou estabelecida da seguinte forma: exposição ao frio, restrição de movimento e natação forçada (Figura 4). Desnecessário dizer que o protocolo de estresse foi realizado da mesma forma tanto no grupo controle quanto tratado com alendronato.

A alternância entre os agentes estressores é justificada, pois toda mudança significativa gera uma necessidade de adaptação por parte do organismo e essa, por sua vez, exerce um papel determinante na patogênese do estresse. Neste sentido, conforme estabelecido pelo trabalho de Marin et al.<sup>64</sup>, em um período de 10 dias não há adaptação do animal ao agente estressor, garantindo, desta forma, a manutenção de níveis elevados de corticosterona, que é um dos biomarcadores para o estresse em ratos, ao longo de todo o experimento.

Todos os agentes estressores foram aplicados em uma sala adjacente aos animais para facilitar o transporte.

### **Descrição das metodologias de indução de estresse**

#### ***1) Exposição ao frio***

Os animais alocados em gaiolas individuais foram expostos a uma temperatura de 4 °C durante 15 minutos por 10 dias<sup>64</sup>

## **2) *Estresse por contenção***

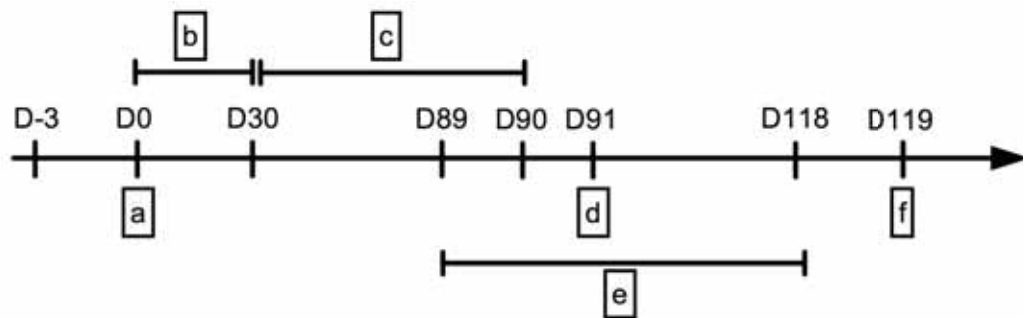
O estresse por contenção foi realizado alocando os animais em tubo de plásticos perfurados (5.5 cm em diâmetro x 18 cm em comprimento) durante 1 hora por 10 dias com a finalidade de restringir e isolar os animais, sem resultar em sintomatologia dolorosa desnecessária<sup>64</sup>.

## **3) *Estresse por natação***

Os ratos foram submetidos individualmente à natação forçada em um tanque cilíndrico de PVC, com diâmetro de 22 cm. A altura da coluna de água foi de 35-38 cm, suficiente para evitar que os animais encostassem as patas ou a cauda no fundo do tanque. Foi realizada uma sessão de natação por dia durante 10 dias consecutivos por 4 minutos. Em seguida, os ratos permaneceram em sala aquecida até estarem completamente secos e então retornaram às gaiolas-moradia<sup>64</sup>

**Item f:** após 29 dias da instalação dos implantes (um dia após o término da exposição aos agentes estressores), todos os animais foram sacrificados por meio de aprofundamento da anestesia. Antes do sacrifício, foram coletadas amostras de sangue por meio de secção caudal para radioimunoensaio e punção cardíaca para análise bioquímica.

## **ESTUDO 4**



**FIGURA 5-** Desenho experimental

Este estudo foi realizado seguindo o mesmo desenho experimental do estudo 3, com duas alterações metodológicas:

- 1- A utilização de 8 animais por grupo.
- 2- A exodontia dos primeiros molares inferiores antagonistas e a utilização de ração triturada. Estas modificações foram realizadas com o intuito de reduzir a sobrecarga sobre os implantes.

## **INTERVENÇÕES**

### **Administração dos Bisfosfonatos**

#### **Obtenção dos bisfosfonatos**

As soluções de alendronato foram preparadas por meio de dissolução do sal monossódico do ácido alendrônico (ALCON, São Paulo, SP, Brasil) em solução

salina fisiológica (NaCl a 0,9%) às concentrações apropriadas para obter as doses correspondentes.

### **Doses administradas**

Os bisfosfonatos têm demonstrado uma pequena toxicidade após administração aguda, subaguda ou crônica, em razão da sua rápida incorporação nos tecidos calcificados e a sua curta presença na corrente sanguínea<sup>29</sup>.

As dosagens que foram utilizadas no estudo 1 estão acima das doses terapêuticas empregadas, uma vez que altas concentrações da droga estão entre os fatores de risco para o desenvolvimento da osteonecrose<sup>83</sup>. A absorção do bisfosfonato pelo tecido ósseo é dose dependente, seguindo um padrão linear após doses endovenosas de até 5 mg/kg<sup>60</sup>, não havendo evidências de saturação ou influência de uma dose EV na farmacocinética de doses subseqüentes<sup>77</sup>. Além disso, em altas concentrações os bisfosfonatos têm mostrado efeitos citotóxicos diretos em outras células, incluindo fibroblastos<sup>5</sup>, o que pode estar associado com as lesões clínicas de osteonecrose.

A dose de alendronato de 1mg/kg de peso corporal administrada uma vez por semana utilizada nos estudos 2, 3 e 4 compreende uma dose eficaz e segura para se obter supressão da reabsorção óssea em ratos<sup>8</sup>, sendo compatível com dosagens correspondentes em ratos para tratamento de doenças reumáticas<sup>48</sup>.

### **Via de administração**

Os animais receberam a droga por administração subcutânea, uma vez que esta é tão efetiva quanto a endovenosa no que diz respeito à biodisponibilidade da droga<sup>60</sup>, eliminando a toxicidade gastrointestinal após administração oral e o maior risco de toxicidade sistêmica, especialmente renal, uma vez que foram utilizadas altas doses neste estudo.

### **Tempo de tratamento**

O uso de bisfosfonatos por um período de três anos tem sido considerado como um fator de risco para o desenvolvimento de osteonecrose<sup>83</sup>, o que corresponde a aproximadamente a um período de oito semanas em ratos<sup>79</sup>. Além disso, é possível que com exposições prolongadas as concentrações locais da droga atinjam níveis elevados<sup>6</sup>.

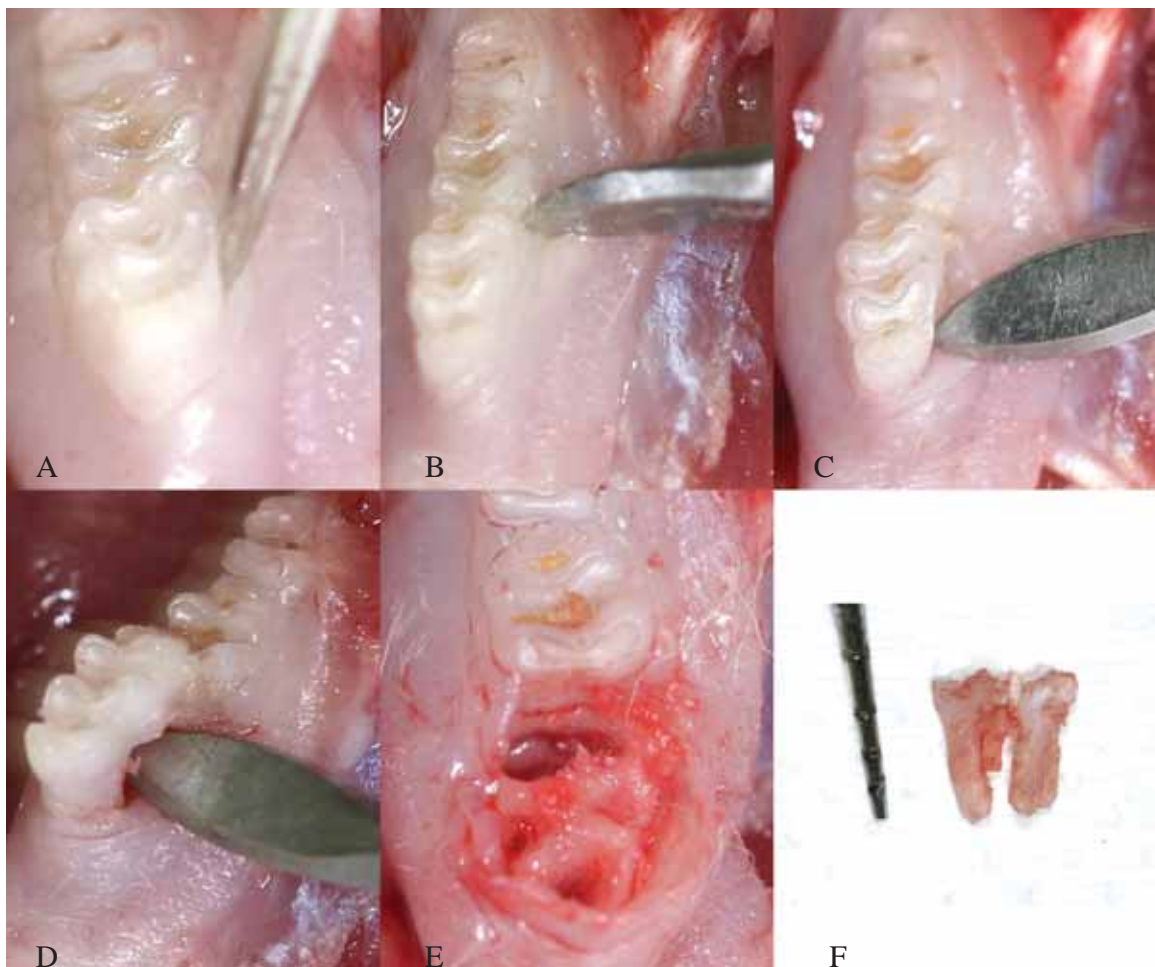
### **Procedimentos cirúrgicos**

Antes dos procedimentos cirúrgicos os animais foram anestesiados por meio de uma combinação de cloridrato de ketamina (Ketamina Agener®; Agener União Ltda, São Paulo, SP, Brasil) na concentração de 0,08 ml/100g de peso corpóreo e xilazina 2% (Rompum ® Bayer S.A. São Paulo, SP, Brazil) na concentração de 0,04 ml/100g de peso corpóreo, via intramuscular<sup>34</sup>.

### **Extração dentária**

As extrações dos primeiros molares superiores (estudos 3 e 4) e inferiores (todos os estudos) foram realizadas pelo mesmo operador utilizando a mesma técnica cirúrgica em todos os grupos. Inicialmente, os animais foram estabilizados em posição supina para exodontia dos primeiros molares superiores ou em posição dorsal para extração dos primeiros molares inferiores. Em seguida, com auxílio de uma sonda exploradora, foi realizado a sindesmotomia do tecido gengival, correndo a ponta do explorador em torno do dente em questão. Após este descolamento, os dentes foram luxados em sentido méso-distal e cérvico-apical, sendo, então, divididos em dois segmentos com auxílio de uma espátula Hollenback posicionada na região de furca. Os segmentos foram então extraídos utilizando uma pinça hemostática (Figura 6)

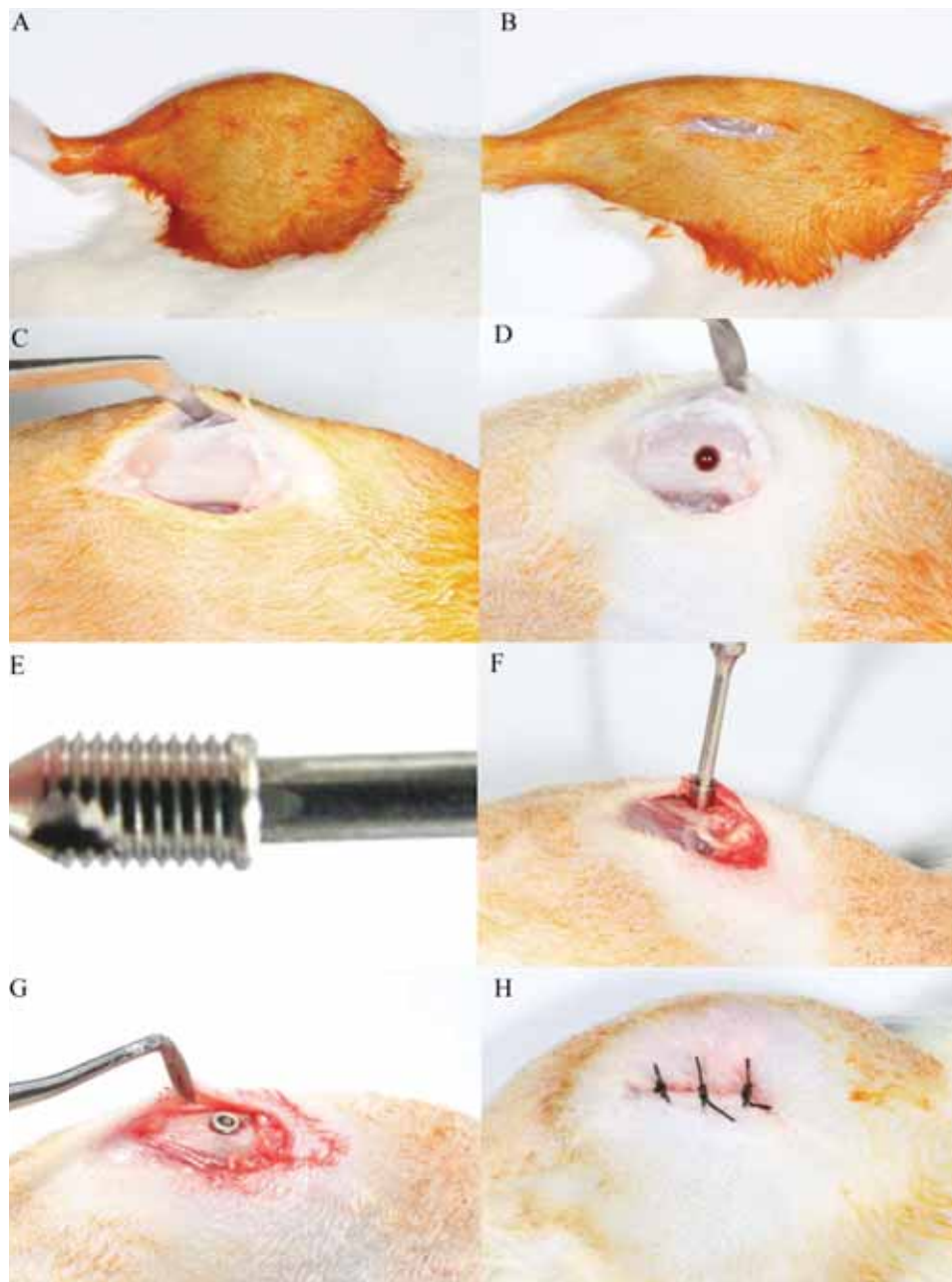




**FIGURA 6** – Protocolo cirúrgico para extração dentária nos animais. A) Descolamento da gengiva inserida com sonda exploradora; B) Luxação méso-distal utilizando uma espátula Hollenback; C e D) Luxação cérvico-apical e separação das raízes; E) Alvéolo pós-extração; F) Primeiro molar inferior segmentado.

### **Implantes tibiais**

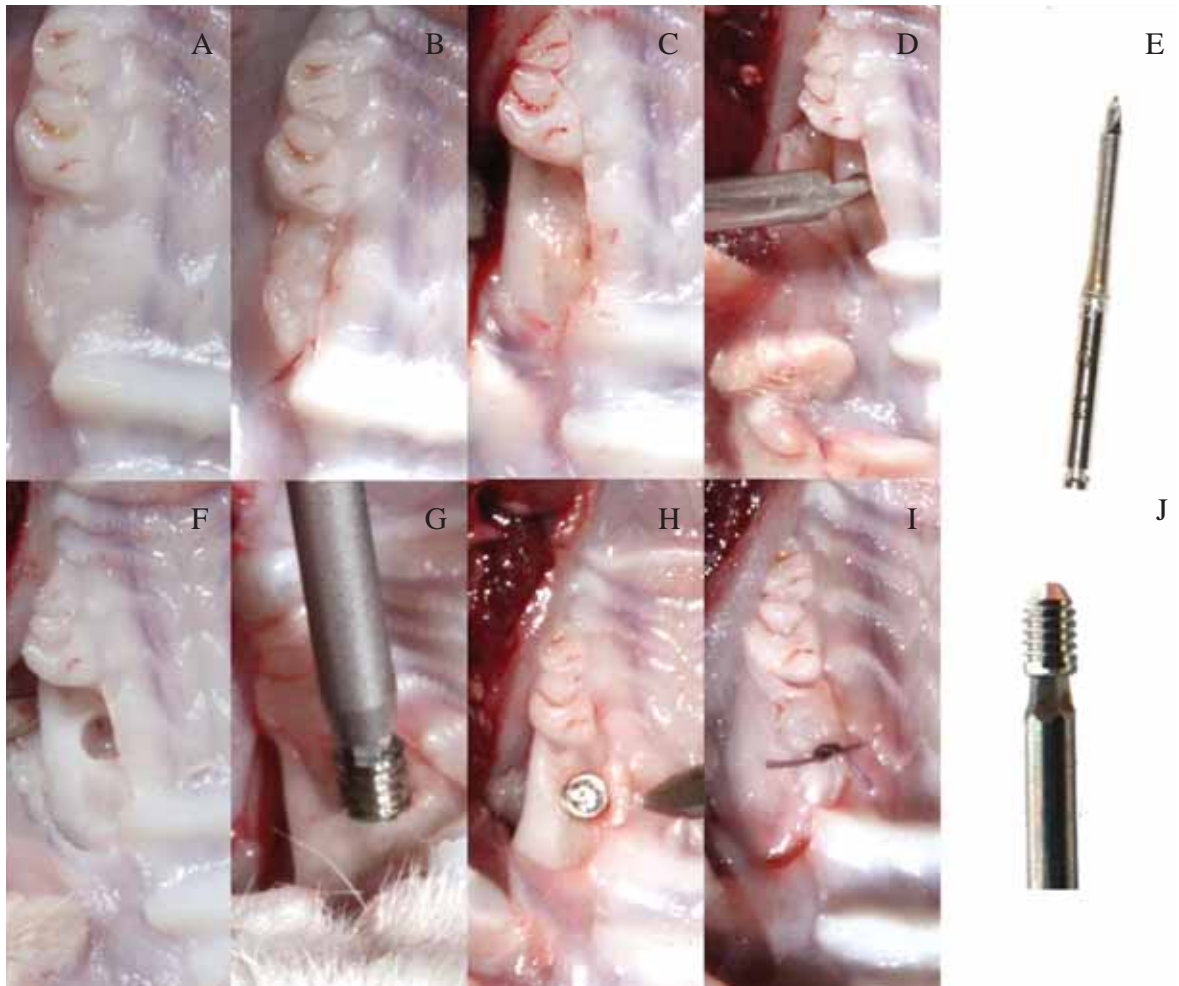
Inicialmente os animais foram submetidos à tricotomia da região interna da perna, sendo realizada a anti-sepsia com gaze estéril embebida em solução de iodopovidona. Uma incisão de aproximadamente 20 mm foi realizada e após uma dissecação delicada, o tecido ósseo foi submetido a uma osteotomia, realizada por meio de uma fresa lança de 1.8 mm de diâmetro para acomodar um implante de titânio de superfície lisa de 4 mm de comprimento por 2.2 mm de espessura (Neodente, Curitiba, PR, Brasil). Todas as perfurações foram realizadas com um motor elétrico ajustado a 1000 RPM, sob abundante irrigação com solução salina estéril, com contra-ângulo redutor de 20:1. O implante foi instalado com a ajuda de uma chave digital. Todo o ferimento foi suturado em planos, internamente com um fio reabsorvível e externamente com fio de seda (Figura 7).



**FIGURA 7** – Protocolo cirúrgico para instalação dos implantes tibiais. A) Antissepsia da face interna da tíbia; B e C) Incisão e descolamento para exposição do tecido ósseo receptor do implante; D) Cavidade óssea preparada; E) Implante de titânio com superfície lisa medindo 4.0 x 2.2 mm; F) Instalação do implante com chave digital; G) Posição final do implante na tíbia; H) Tecido suturado com fio de seda 4-0.

### **Implantes maxilares**

Os implantes maxilares foram instalados nos alvéolos regenerados correspondentes aos primeiros molares superiores extraídos. Para a instalação dos implantes os animais foram anestesiados como descritos anteriormente. Inicialmente, foram realizadas incisões seguido pelo descolamento mucoperiosteal do retalho. Em seguida, foram realizadas perfurações bilateralmente nas áreas edêntulas com fresas de 1.2 mm de diâmetro para a inserção dos implantes, cujo tamanho e formato foram especialmente desenhados para a instalação na maxila dos ratos, com as dimensões de 2.5 mm de comprimento por 1.5 mm de espessura (Neodente, Curitiba, PR, Brasil). Todas as perfurações foram realizadas com um motor elétrico ajustado a 1000 RPM, sob abundante irrigação com solução salina estéril, com contra-ângulo redutor de 20:1 (Figura 8).



**FIGURA 8** – Protocolo cirúrgico para instalação dos implantes maxilares. A) Área receptora para os implantes; B e C) Incisão e descolamento para exposição do tecido ósseo receptor do implante; D e E) Preparação da cavidade óssea utilizando uma fresa de 1.2mm; F) Leito do implante após a fresagem; G) Instalação do implante com chave digital; H) Posição final do implante na maxila; I) Tecido suturado com fio de vycril 6-0; J) implante de titânio com superfície lisa medindo 2.0 x 1.5 mm.

### **Cuidados pós-operatórios**

Os animais receberam uma dose única, por via intramuscular, de antibiótico (Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, Brasil - 0,1mg/Kg) e de Ketoflex (cetoprofeno 1%, 0,03ml/rato).

### **Procedimentos laboratoriais e metodologia aplicada**

#### **Indicadores do grau de dificuldade cirúrgica**

Esta avaliação foi realizada com base na frequência de fraturas dentárias e no tempo necessário para a realização das exodontias. Este último parâmetro foi definido como o intervalo de tempo entre a utilização do primeiro instrumento cirúrgico e a avulsão dentária, mensurado com auxílio de um cronômetro digital. Esta análise foi realizada pelo mesmo indivíduo em todos os procedimentos para redução de possíveis vieses<sup>93</sup>.

As fraturas dentárias foram definidas como a perda completa de continuidade dental podendo envolver a coroa e/ou raízes<sup>76</sup>.

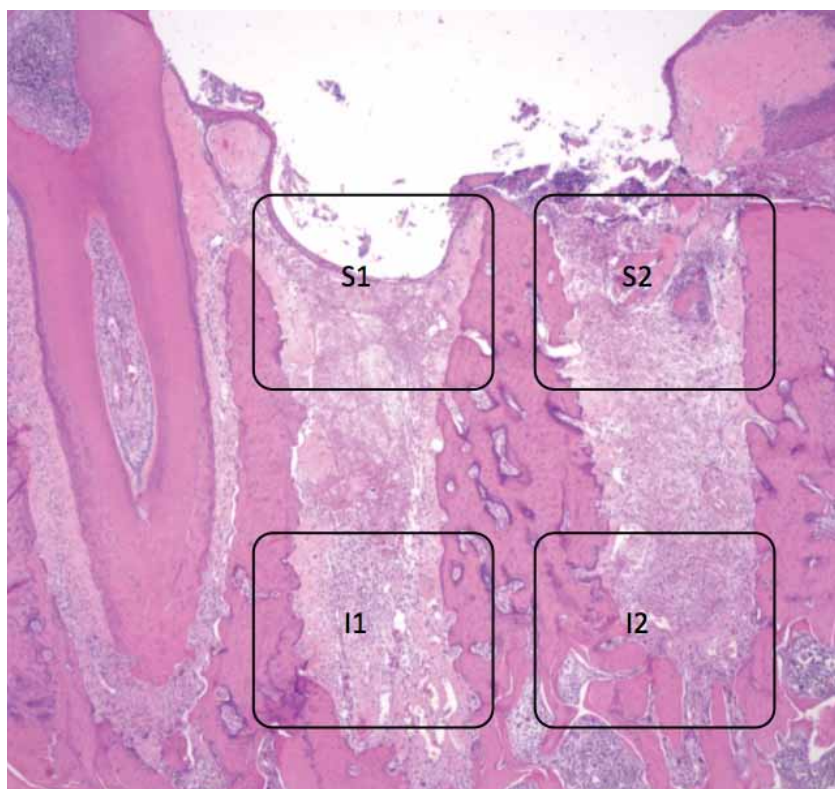
#### **Processamento das amostras para análises histológica**

Os espécimes foram obtidos no momento do sacrifício nos períodos correspondentes a cada estudo. Todos estes espécimes foram fixados em solução de paraformaldeído a 4% por 48 horas. Em seguida, estes espécimes foram então descalcificados por 60 dias com ácido etilenodiamino tetra-acético (EDTA) com pH 7.8. Após a descalcificação, os implantes foram cuidadosamente removidos (estudo 3

e 4) e todos os espécimes foram processados e embebidos em parafina. A partir de então, o bloco obtido foi cortado com o intuito de obter vários espécimes com 4  $\mu$ m em espessura, os quais foram corados com hematoxilina eosina (HE), sendo então destinados para a análise histológica descritiva por meio de microscopia de luz convencional (todos os estudos) e análise estereométrica (estudo 1 e 2). O número de cortes destinados para cada análise foi calculado de acordo com o número de cortes obtidos de cada alvéolo.

#### **Análise histológica (todos os estudos)**

A análise histológica foi realizada em três momentos distintos por um Patologista experiente e cego para os efeitos do estudo. Esta análise foi realizada em quatro regiões nas lâminas: 2 superiores – terço superior dos alvéolos mesial e distal; 2 inferiores – terço inferior dos alvéolos mesial e distal (Figura 9), utilizando aumentos de 100, 200, 400 e 1000x. Os parâmetros avaliados incluíram alterações no tecido ósseo e epitelial, proporção de tecido ósseo não-vital, grau de infecção e vascularização (número de vasos), além da intensidade e qualidade do processo inflamatório (agudo, crônico ou misto) (Tabela 1). Os parâmetros quantitativos foram estabelecidos a partir de escores, sendo 0 (ausente; 0%), 1 (brando;  $\geq 10\%$ ), 2 (moderado;  $>10$  e  $\leq 50\%$ ) e 3 (aumentada;  $> 50\%$ ).



**FIGURA 9-** Áreas selecionadas para análise histológica quantitativa por escores

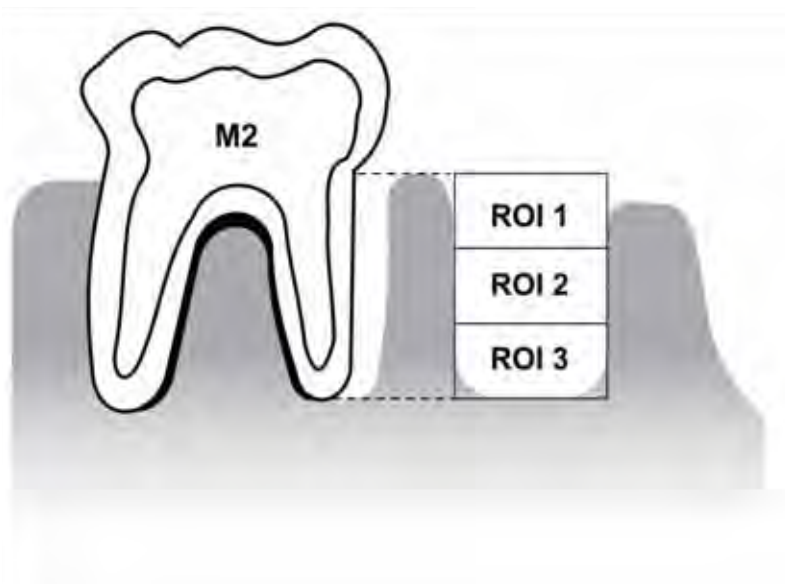
**Tabela 1-** Descrição dos parâmetros utilizados para análise histológica por escores

<b>Parâmetro</b>	<b>Descrição</b>
Neoformação óssea	Linhas de reversão
Reepitelização	Organização das células epiteliais
Tecido ósseo não-vital	Ausência de osteócitos; colonização bacteriana; ausência de vascularização
Infecção	Proporção de bactérias
Neovascularização	Proporção de vasos sanguíneos
Inflamação	Proporção de células inflamatórias



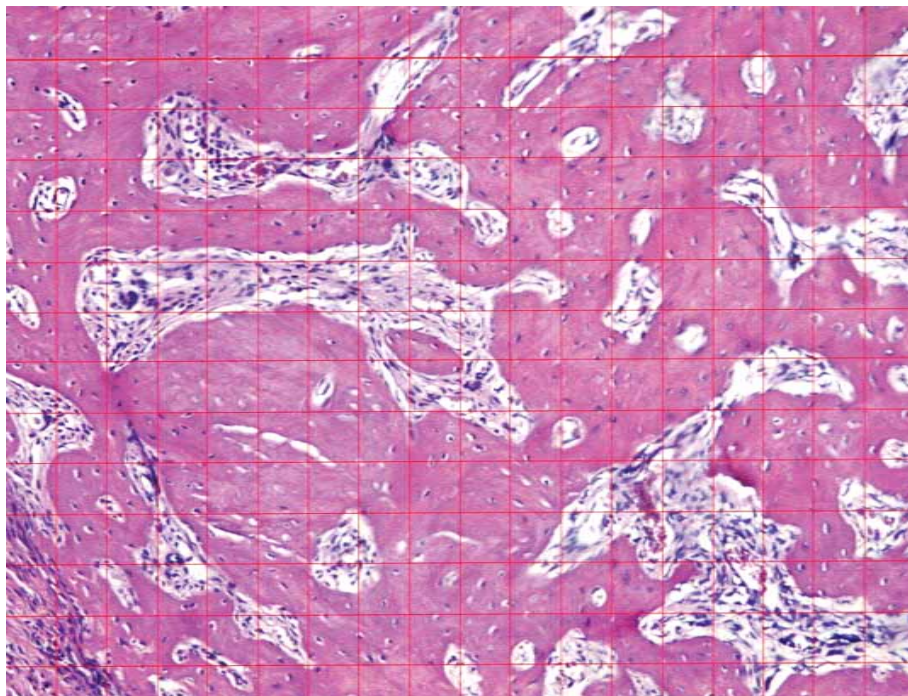
### **Análise estereométrica dos alvéolos dentais (estudo 1 e 2)**

Esta análise foi realizada com auxílio do software Leica Application Suite 3.8.0 (Leica Microsystems LTD, Heerburgg, Germany). As medidas foram realizadas no alvéolo distal do primeiro molar inferior esquerdo em três regiões diferentes no alvéolo (1-apical, 2-média, 3-superior). Para determinação destas áreas de interesse, inicialmente foi estabelecida uma área retangular padrão, estendendo-se do nível da junção cimento-esmalte (JCE) do segundo molar inferior esquerdo (M2) até a região apical do M2, e entre a parede óssea alveolar mesial e distal do alvéolo de interesse. Em seguida, esta área foi dividida em três partes iguais, as quais corresponderam às regiões de interesse (1, 2 e 3) (figura 10)



**FIGURA 10** - Regiões de interesse 1, 2 e 3 para realização da análise estereométrica.

Em seguida, para cada ROI foi realizada uma fotografia com um aumento de 100x, sobre a qual era desenhada uma grade cuja intersecção das linhas totalizavam 221 pontos (Figura 11). O parâmetro estereométrico mensurado incluiu a porcentagem do volume ósseo (VO%), que representa o volume de tecido ósseo (VO,  $\text{mm}^3$ ; número de pontos coincidentes sobre o tecido ósseo) pelo total de volume tecidual pelo volume total de tecido (VT,  $\text{mm}^3$ ; 221 pontos), seguindo a nomenclatura estabelecida pela Sociedade Americana para Pesquisa Mineral e Óssea<sup>74</sup>. As análises foram realizadas em um total de três lâminas diferentes para cada animal. A distância entre os cortes selecionados foi de 50  $\mu\text{m}$ .



**FIGURA 11-** Grade posicionada na região de interesse (100x).

### **Análise radiográfica dos alvéolos dentais (estudo 2)**

As imagens radiográficas foram obtidas imediatamente após o sacrifício dos animais, por meio de um sistema de imagem digital direta – CDR (*Computed Dental Radiography for Microsoft Windows*), o qual utiliza um sensor eletrônico em substituição ao filme radiográfico. As hemi-mandíbulas foram fixadas em um suporte de modo que estivessem paralelamente ao sensor e permitissem que o feixe de raios-X incidisse perpendicularmente ao longo eixo do alvéolo.

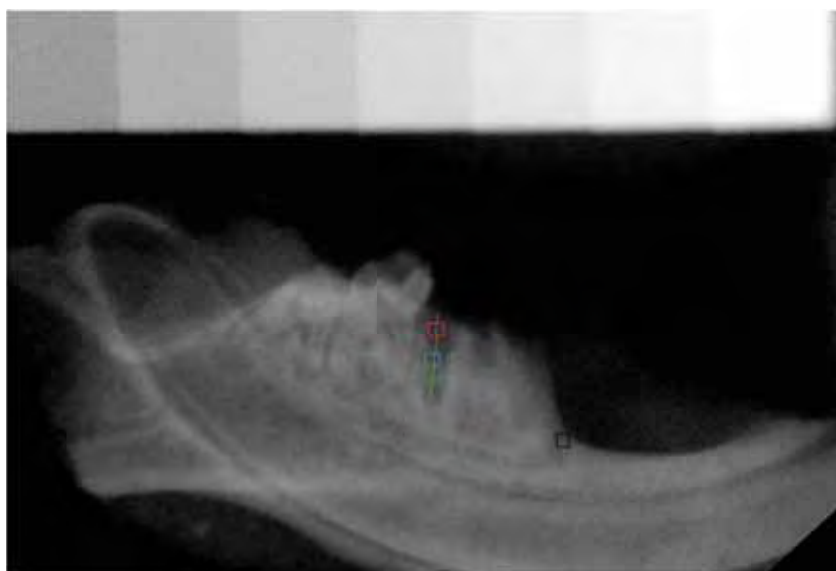
O Sensor foi exposto a tomada radiográfica a uma potência de 70 KVp e 10 mA, com tempo de exposição de 0,2 segundos (Expectro 70x, Dabi Atlante, Ribeirão Preto, SP, Brasil), com uma distância foco-sensor padronizada em 40 cm. Durante as tomadas radiográficas uma escala de alumínio, composta de 8 degraus, com 1 mm de diferença na altura entre os degraus, foi posicionada sobre o sensor e a avaliação da densidade foi comparada com a densidade da escala.

As imagens foram exportadas do programa Schick® em uma resolução de 640 ppi (pixel por polegada) com um tamanho de 900 x 641 dpi (pontos por polegada), sendo arquivadas em formato TIFF (Tagged Image File Format). Em seguida, estas imagens foram importadas para um software analisador de imagem denominado de Image Tool 2.03 (UTHSCA, San Antonio, Texas, EUA) o qual gera uma escala com os valores médio dos níveis de cinza baseado em um histograma.

A densidade óssea radiográfica foi avaliada em uma área de 15 x 15 pixel nas regiões de interesse no alvéolo distal do primeiro molar inferior esquerdo, utilizando-

se os valores médios dos níveis de cinza da radiografia divididos pela média dos níveis de cinza de um degrau selecionado na escala de alumínio, para compensar eventuais diferenças entre as radiografias, haja vista que foi utilizado a mesmo degrau da escala de Alumínio em todas as radiografias.

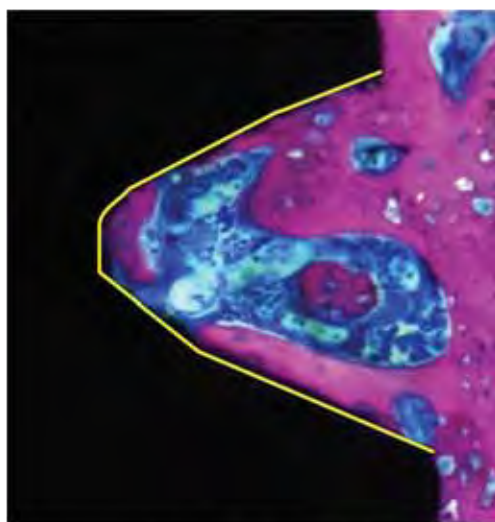
Para delimitação das regiões de interesse correspondentes ao alvéolo, inicialmente foi realizado uma medida linear da altura dos alvéolos mesial e distal. Em seguida, esta distância foi dividida por três para delimitação dos três terços do alvéolo (1-superior, 2-médio e 3-inferior), de modo que foram selecionadas três áreas diferentes correspondentes a cada terço do alvéolo para estimativa da densidade óssea radiográfica alveolar. Além disso, foi selecionada a região mesial ao alvéolo do primeiro molar inferior (região 4) para estimativa da densidade óssea radiográfica mandibular (Figura 12 ).



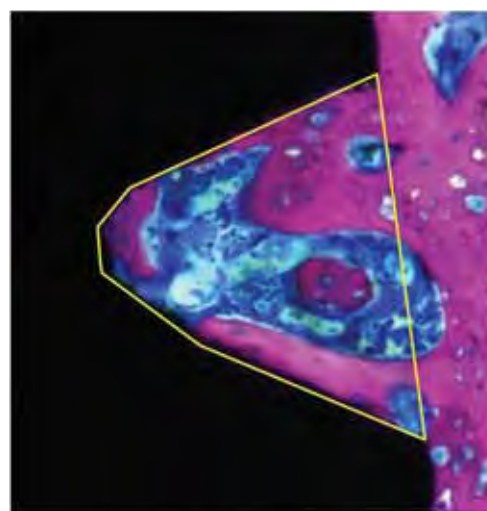
**FIGURA 12-** Regiões de interesse para análise da densidade óssea ( ■1 ■2 ■3 ■4 )

### **Análise histométrica dos implantes (estudos 3 e 4)**

Para esta análise, as amostras foram submetidas à infiltração plástica utilizando glicolmetacrilato (Technovit 7200 VLC) e álcool etílico. Após a infiltração plástica os espécimes foram incluídos em resina, polymerizados e montado em uma lâmina acrílica com o auxílio da resina Technovit 4000 (Kulzer, Wehrheim, Alemanha). Em seguida, foram cortados por meio de um sistema de corte (Exakt - Cutting. System,<sup>22</sup> e desgastados para resultar em um espécime de aproximadamente 30µm de espessura, quando foram então coradas com azul de Stevenel e fucsina ácida (1%) e os parâmetros histométricos analisados foram: extensão linear de tecido ósseo em contato direto com a superfície do implante (Figura 13); área entre as roscas ocupada por tecido ósseo (Figura 14). Estes parâmetros foram analisados nas faces mesial e distal dos implantes e expressos como a média das duas faces.

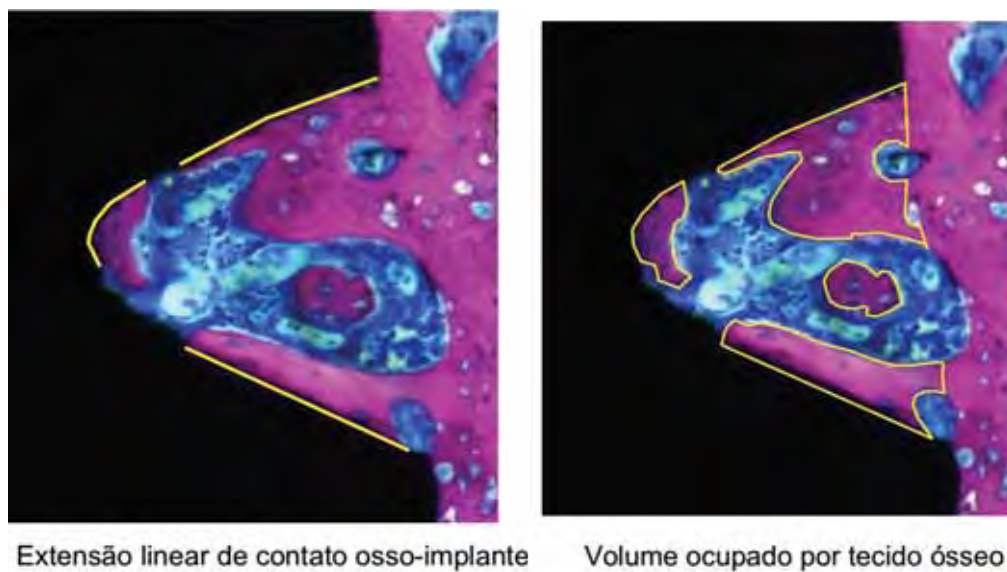


Extensão linear total



Volume total

**FIGURA 13-** Exemplo de mensuração do contato osso implante (BIC). O comprimento da linha amarela representa a área aonde o osso toca a superfície do implante (b), avaliada proporcionalmente ao comprimento total da rosca do implante (a).



**FIGURA 14-** Exemplo de mensuração do volume de osso entre as roscas do implante (BAFO). A linha amarela demarca a área total mensurada (a) e a área ocupada por tecido ósseo (b).

#### **Análise do torque de remoção (estudo 3 e 4)**

Imediatamente após o sacrifício dos animais, a tíbia e a maxila foram dissecadas e os implantes foram expostos e fixados em um dispositivo. Dois torquímetros analógicos (Tohnichi, modelo ATG24CN-S) foram utilizados para o teste, sendo um para a maxila (escala de 0 a 10 Ncm com intervalos regulares de 0.05 Ncm) e outro para tíbia (escala de 0 a 30 Ncm com intervalos regulares de 0.05

Ncm). Uma chave hexagonal foi adaptada à cabeça do implante e, em seguida, aplicada uma força reversa ao sentido de colocação do implante, até o rompimento completo da interface osso/implante, resultando em um giro do implante, sendo então registrado o valor apontado, assim considerado como a força necessária para rompimento da osseointegração.

### **Análise bioquímica (estudos 1, 2 e 3)**

As amostras de sangue total coletadas foram centrifugadas (1800 RPM durante 15min a 4°C) para separação do soro e foram congeladas em -80°C para posterior análise. Foram realizados testes específicos para avaliação da concentração de fosfatase alcalina específica para tecido ósseo (FAO) e do telopeptídeo C-terminal do colágeno tipo I (CTX) (CUSABIO BIOTECH CO., Ltd, Wuhan, P.R. China). Estas concentrações foram determinadas através de reação imunoenzimática de ELISA, por meio da utilização de kits comerciais, conforme a indicação do fabricante.

### **Radioimunoensaio para corticosterona (estudos 3 e 4)**

O radioimunoensaio para corticosterona foi realizado por meio da utilização de um anticorpo anti-corticosterone (Sigma, St. Louis, MO) e <sup>3</sup>H- corticosterona da New England Nuclear (Boston, MA). Esta metodologia foi adaptada a partir do método descrito por Sarnyai et al.<sup>84</sup>. Em suma, 100 µl de uma solução de anticorpo e <sup>3</sup>H-corticosterona (10,000 a 20,000 cpm/ml) foi adicionado a cada amostra de

plasma, misturado e incubado durante a noite a 4 °C. Foi utilizado carvão revestido com dextran para a adsorção dos esteróides livres após a incubação. Os tubos foram centrifugados a 2000×g por 15 min a 4 °C, o sobrenadante de cada tubo foi transferido para as vias de cintilação e a radioatividade será quantificada por espectrometria de cintilação líquida. Curvas padrão foram construídas utilizando 25, 50, 100, 250, 500, 750, 1000 e 2000 pg/100 µl de corticosterona.

### **Análise dos dados**

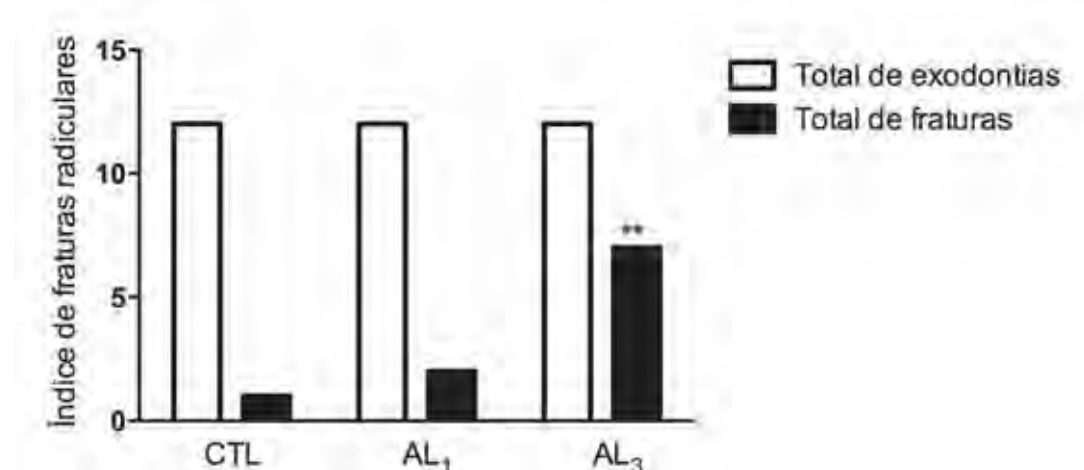
Os resultados referentes a frequência de fraturas radiculares foram submetidos ao teste chi-quadrado, enquanto que os outros resultados foram analisados pelo teste de D'Agostino ou Kolmogorov-Smirnov para avaliação da normalidade e, então, aplicados os testes paramétricos t de Student ou ANOVA seguido do teste de Tukey ou Teste para contraste entre os pares de médias (Teste F). No caso de dados não normais, foram utilizados testes não paramétricos Mann-Whitney ou Kruskal-Wallis seguido pelo pós-teste de Dunns, para a comparação das médias obtidas entre os grupos experimentais. O nível de significância estabelecido foi de 5%.



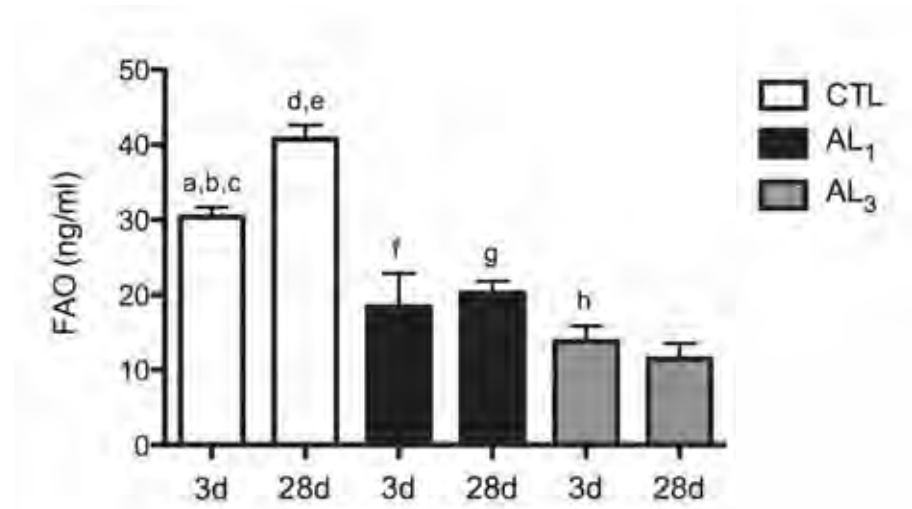
## *Apêndice 2*

## Apêndice 2 – Resultados adicionais

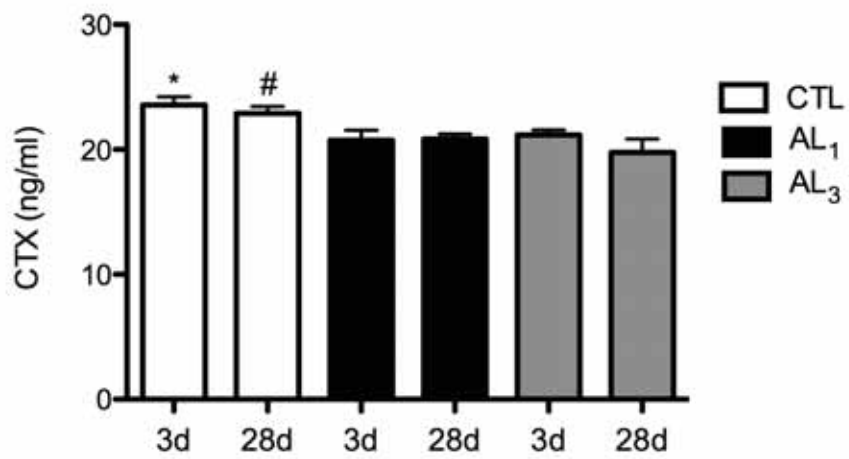
### Estudo 1



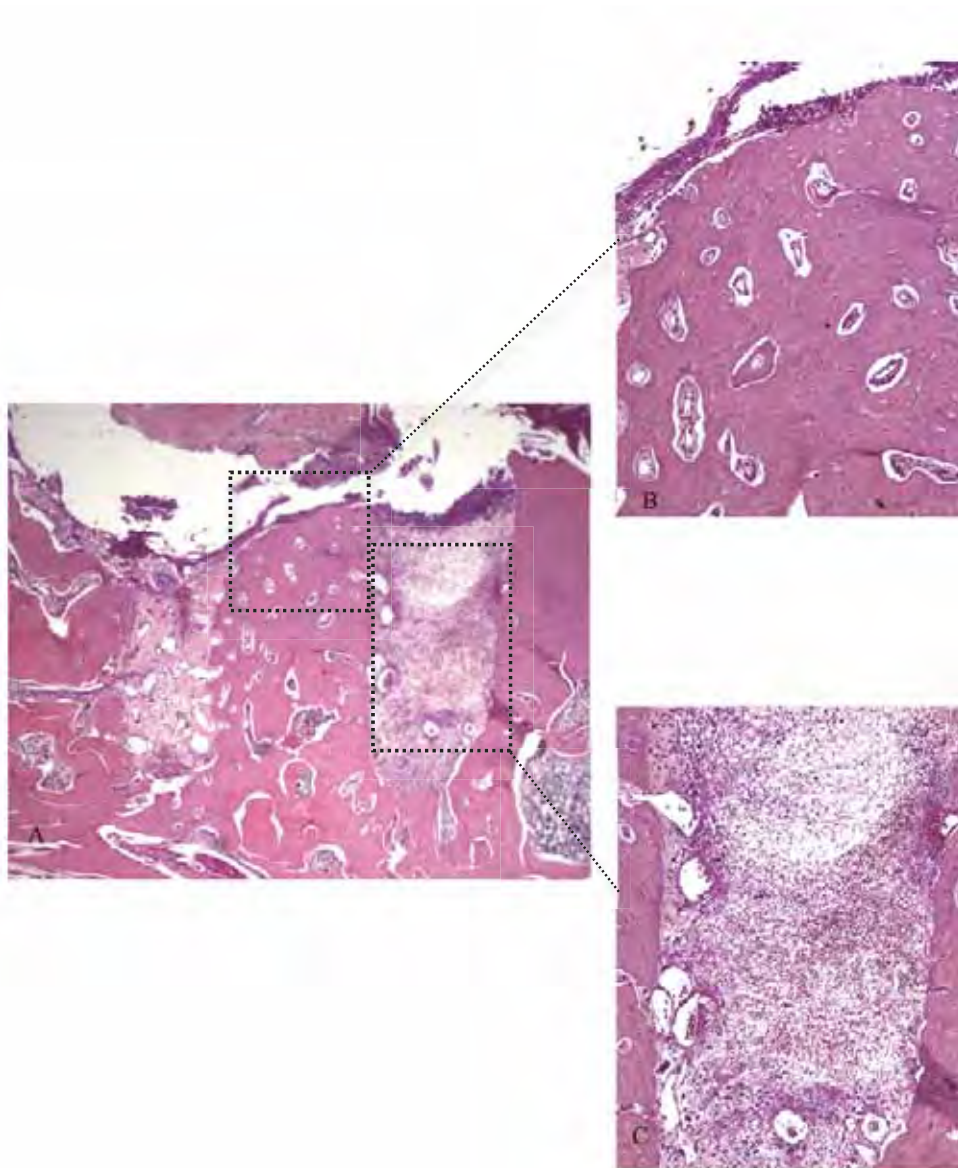
**FIGURA 1** – Índice de fraturas radiculares nos grupos experimentais utilizando a técnica de extração por secionamento dental (\*\* $p < 0.01$  em relação ao grupo CTL; Teste chi-quadrado)



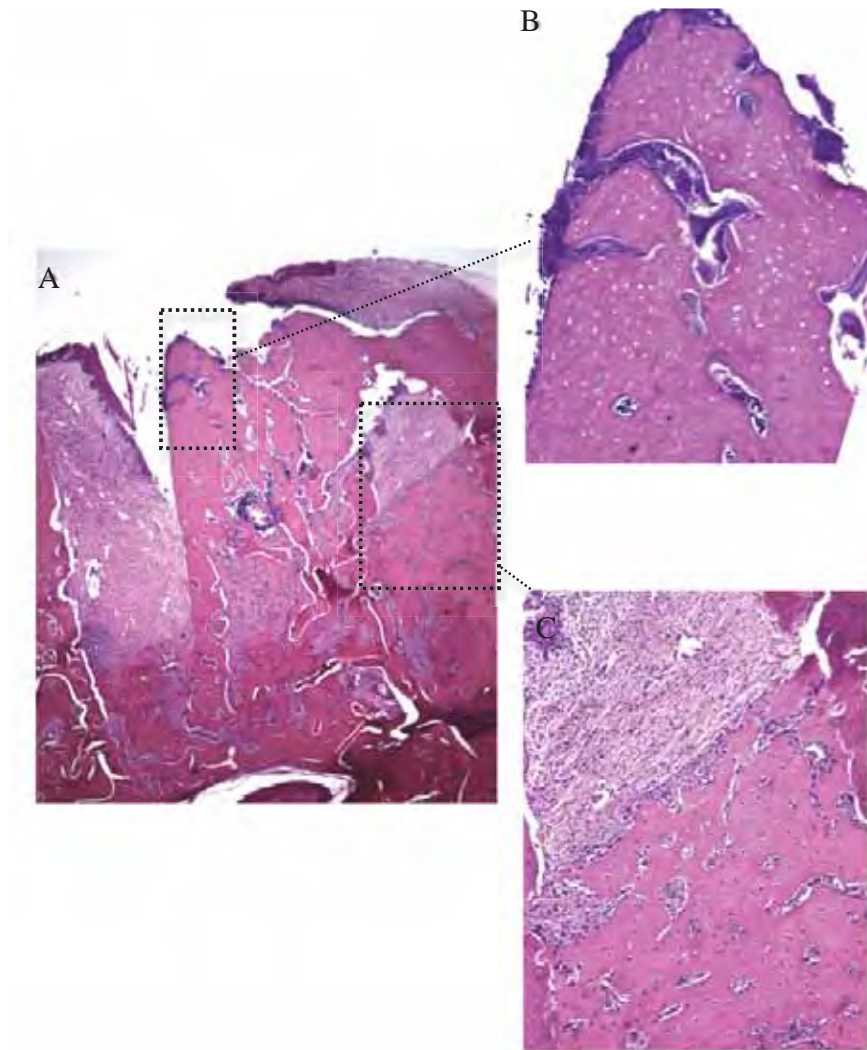
**FIGURA 2** – Níveis séricos de FAO nos períodos de 3 e 28 dias após as exodontias (<sup>a</sup> $p < 0.05$  em relação à CTL28d e AL<sub>1</sub>3d; <sup>d</sup> $p < 0.05$  em relação a AL<sub>1</sub>3d; <sup>b</sup> $p < 0.01$  em relação à AL<sub>3</sub> 28d; <sup>c,e,f,g,h</sup> $p < 0.001$  em relação à todos outros períodos e grupos; Teste ANOVA seguido do teste de Tukey).



**FIGURA 3** – Níveis séricos de CTX nos períodos de 3 e 28 dias após as exodontias (\* $p < 0.05$  – em relação ao período de 3 dias dos grupos AL<sub>1</sub> e AL<sub>3</sub>; # $p < 0,05$  – em relação aos períodos de 28 dias dos grupos AL<sub>1</sub> e AL<sub>3</sub>; Teste ANOVA seguido do teste de Tukey).

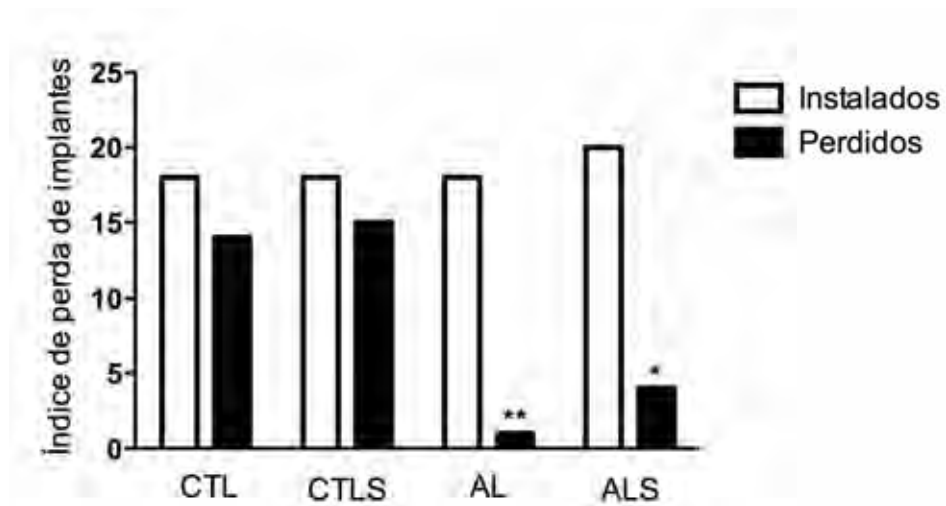


**FIGURA 4** – Corte histológico vestibulo-lingual correspondente ao alvéolo de um animal do grupo AL<sub>3</sub>, três dias após a exodontia do 1º molar inferior (HE). A) aumento de 25x; B) Aumento de 100x na região de septo inter-radicular evidenciando lacunas vazias de osteócitos; C) Aumento de 100x terço médio do alvéolo mesial, observando a formação de tecido de granulação.



**FIGURA 5** – Corte histológico vestibulo-lingual correspondente ao alvéolo de um animal do grupo AL<sub>3</sub>, 28 dias após a exodontia do 1º molar inferior (HE). A) aumento de 25x; B) Aumento de 100x na região de septo inter-radicular evidenciando áreas de osteonecrose associada à infecção; C) Aumento de 100x terço médio do alvéolo distal, observando a formação parcial de tecido ósseo associada a presença de granulação.

## Estudo 3



**FIGURA 6** – Índice de perda de implantes após 28 dias de instalação dos implantes maxilares (\* $p < 0.05$  e \*\* $p < 0.01$  em relação aos grupos CTL e CTLS; Teste chi-quadrado)

#### Estudo 4

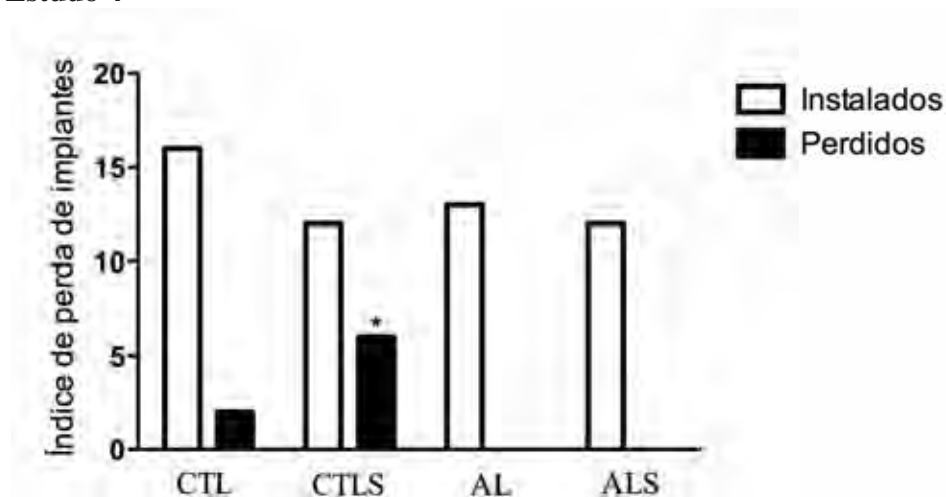


FIGURA 7 – Índice de perda de implantes após 28 dias de instalação dos implantes maxilares (\* $p < 0.05$  em relação aos grupos AL e ALS; Teste chi-quadrado)

**Tabela 1-** Distribuição dos espécimes maxilares para o estudo 4

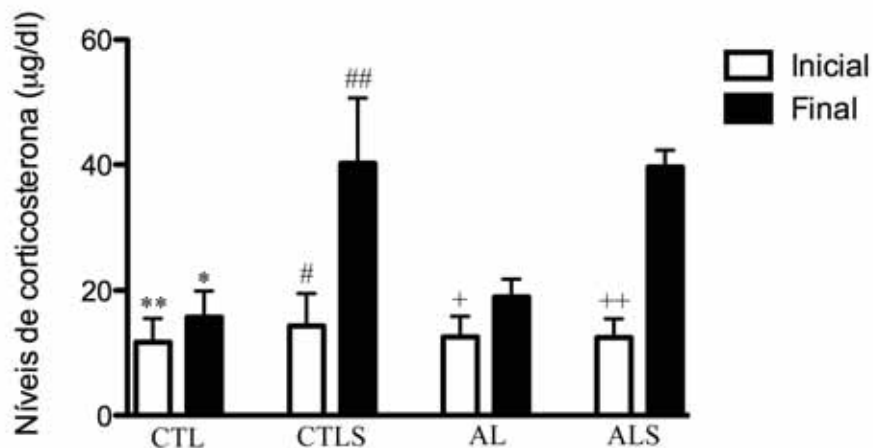
Grupo	Implantes instalados	Implantes perdidos	Histometria	Histologia	Torque de remoção
CTL	14	2	4	4	4
CTLS <sup>*†</sup>	12	6	2	2	2
AL <sup>#</sup>	15	0	5	5	5
ALS <sup>#</sup>	15	0	5	5	5

<sup>#</sup> 1 e <sup>\*</sup> 2 espécimes perdidos durante o processamento para análise histométrica/histológica

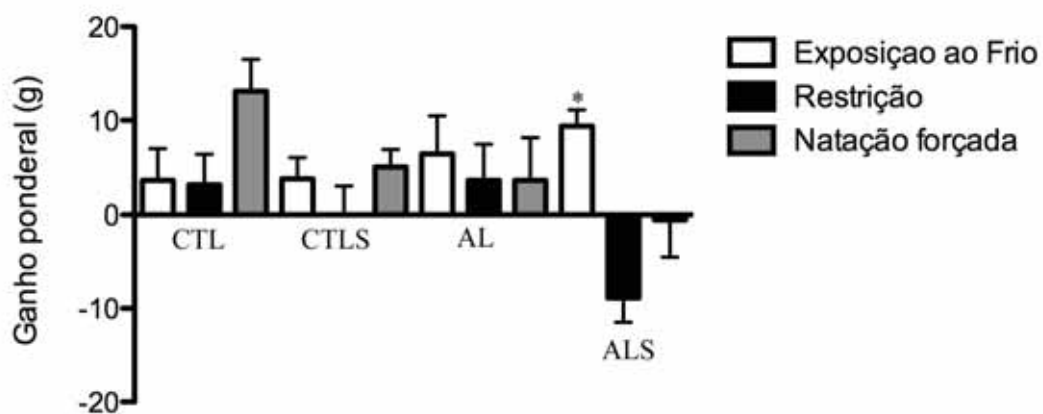
**Tabela 2-** Distribuição dos espécimes tibiais para o estudo 4

Grupo	Implantes instalados	Implantes perdidos	Histometria	Histologia	Torque de remoção
CTL	15	0	5	5	5
CTLS <sup>#†</sup>	13	0	4	4	5
AL	15	0	5	5	5
ALS <sup>*</sup>	15	0	4	4	5

<sup>#</sup>1 espécime e <sup>\*</sup>2 espécimes perdidos durante o processamento para análise histométrica/histológica

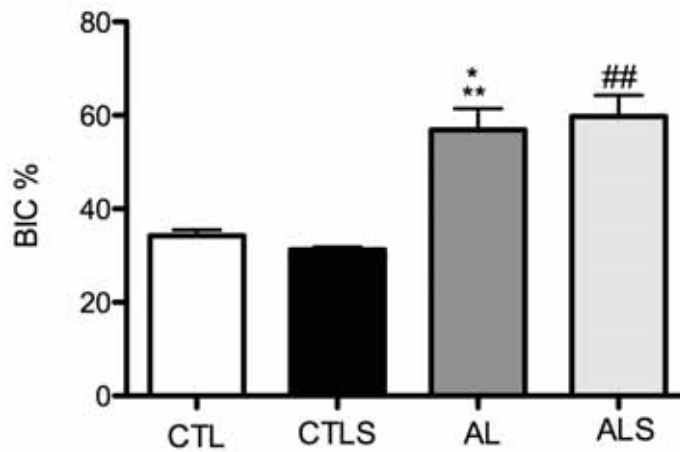


**FIGURA 8-** Níveis de corticosterona plasmática antes (inicial) e após (final) a exposição aos agentes estressores (\*# $p < 0.05$  em relação aos grupos CTLS e ALS finais; \*\* $p < 0.01$  em relação aos grupos CTLS e ALS finais; ## $p < 0.01$  em relação a AL e ALS iniciais; +/+ $p < 0.01$  em relação ao grupo ALS final; Teste ANOVA seguido do teste F).

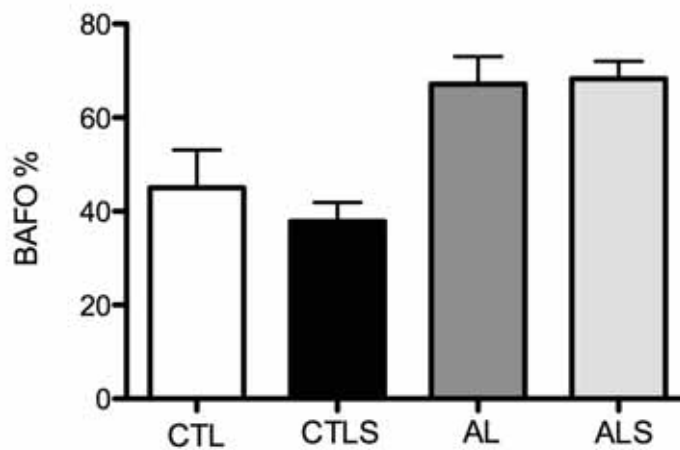


**FIGURA 9-** Ganho ponderal ao longo do período de exposição aos agentes estressores (\* $p < 0.05$  em relação ao período de Restrição no grupo ALS; Teste ANOVA seguido do teste de Tukey).

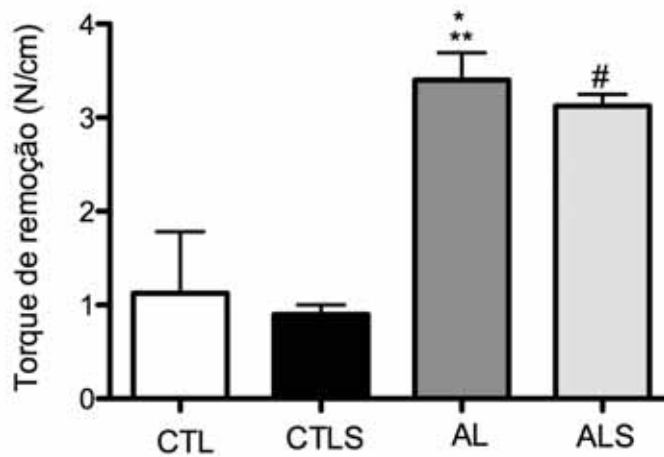




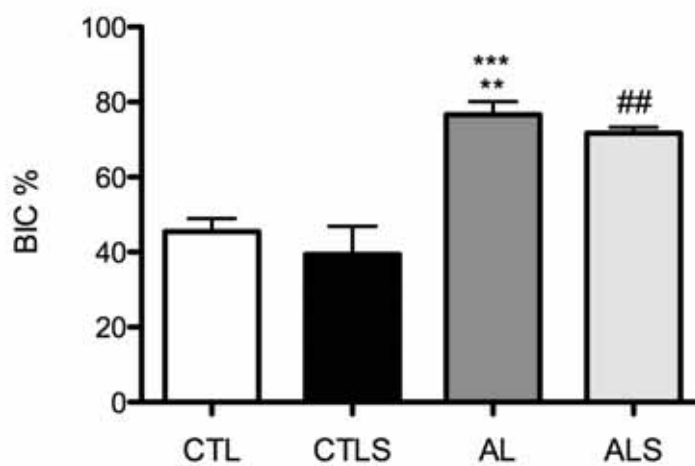
**FIGURA 10-** Contato osso-implante (BIC) após 28 dias da instalação dos implantes maxilares (\* $p < 0.05$  em relação ao grupo CTLS; \*\* $p < 0.01$  em relação ao grupo CTL; ## $p < 0.01$  em relação aos grupos CTL e CTLS; Teste ANOVA seguido do teste de Tukey).



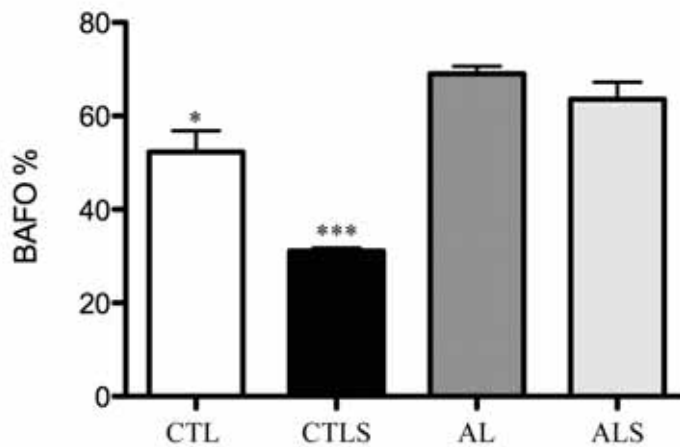
**FIGURA 11-** Volume ósseo entre as espiras (BAFO) dos implantes maxilares após 28 dias da instalação destas fixações (Teste ANOVA seguido do teste de Tukey).



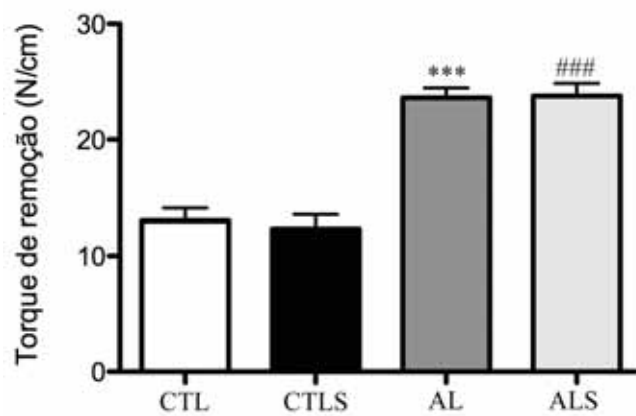
**FIGURA 12-** Torque de remoção dos implantes maxilares após 28 dias da instalação destas fixações (\* $p < 0.05$  em relação aos grupos CTLS; # $p < 0.05$  em relação aos grupos CTL e CTLS; \*\* $p < 0.01$  em relação ao grupos CTL; Teste ANOVA seguido do teste de Tukey).



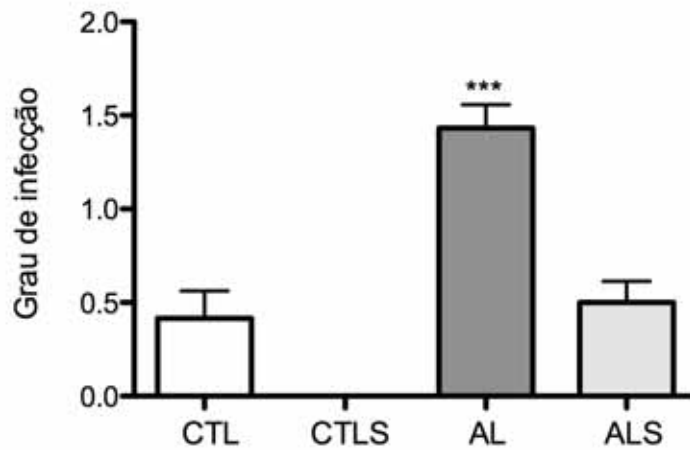
**FIGURA 13-** Contato osso-implante (BIC) após 28 dias da instalação dos implantes tibiais (\*\*/## $p < 0.01$  em relação aos grupos CTL e CTLS; \*\*\* $p < 0.001$  em relação ao grupo CTLS; Teste ANOVA seguido do teste de Tukey).



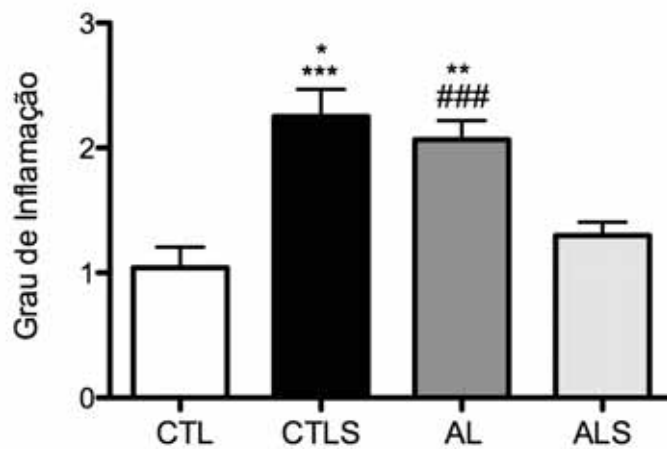
**FIGURA 14-** Volume ósseo entre as espiras (BAFO) dos implantes tibiais após 28 dias da instalação destas fixações ( $p^* < 0.05$  em relação aos grupos CTL e AL;  $***p < 0.001$  em relação ao grupo AL e ALS; Teste ANOVA seguido do teste de Tukey).



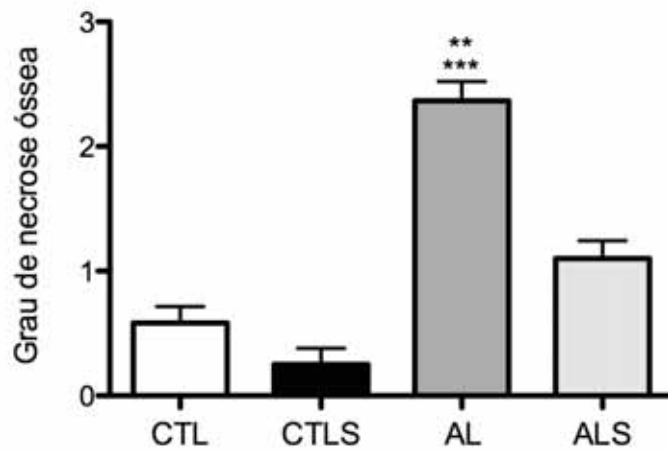
**FIGURA 15-** Torque de remoção dos implantes tibiais após 28 dias da instalação destas fixações ( $***p < 0.001$  e  $###p < 0.01$  em relação aos grupos CTL e CTLS; Teste ANOVA seguido do teste de Tukey).



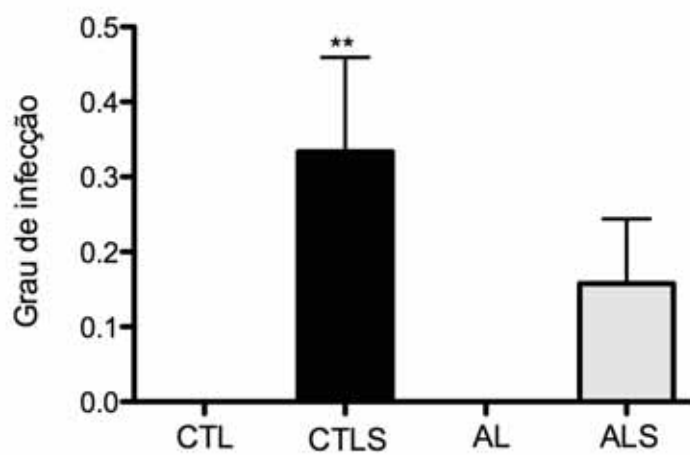
**FIGURA 16-** Grau de infecção observados nos implantes maxilares(\*\* $p < 0.001$  em relação aos grupos CTL, CTLS e ALS; Teste ANOVA seguido do teste de Tukey).



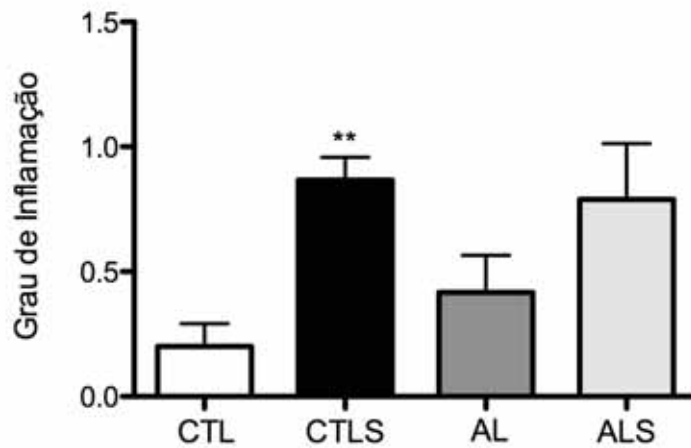
**FIGURA 17-** Grau de inflamação observado nos implantes maxilares(\*\* $p < 0.001$  e ### $p < 0.001$  em relação ao grupo CTL, \* $p < 0.05$  em relação ao grupo ALS; \*\* $p < 0.01$  em relação ao grupo ALS; Teste ANOVA seguido do teste de Tukey).



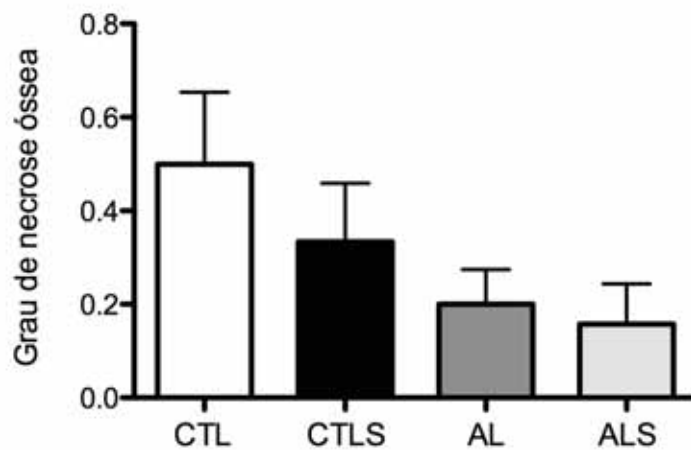
**FIGURA 18-** Grau de necrose óssea observado nos implantes maxilares(\*\*\* $p < 0.001$  em relação ao grupo CTL e CTLS; \*\* $p < 0.01$  em relação ao grupo ALS; Teste ANOVA seguido do teste de Tukey).



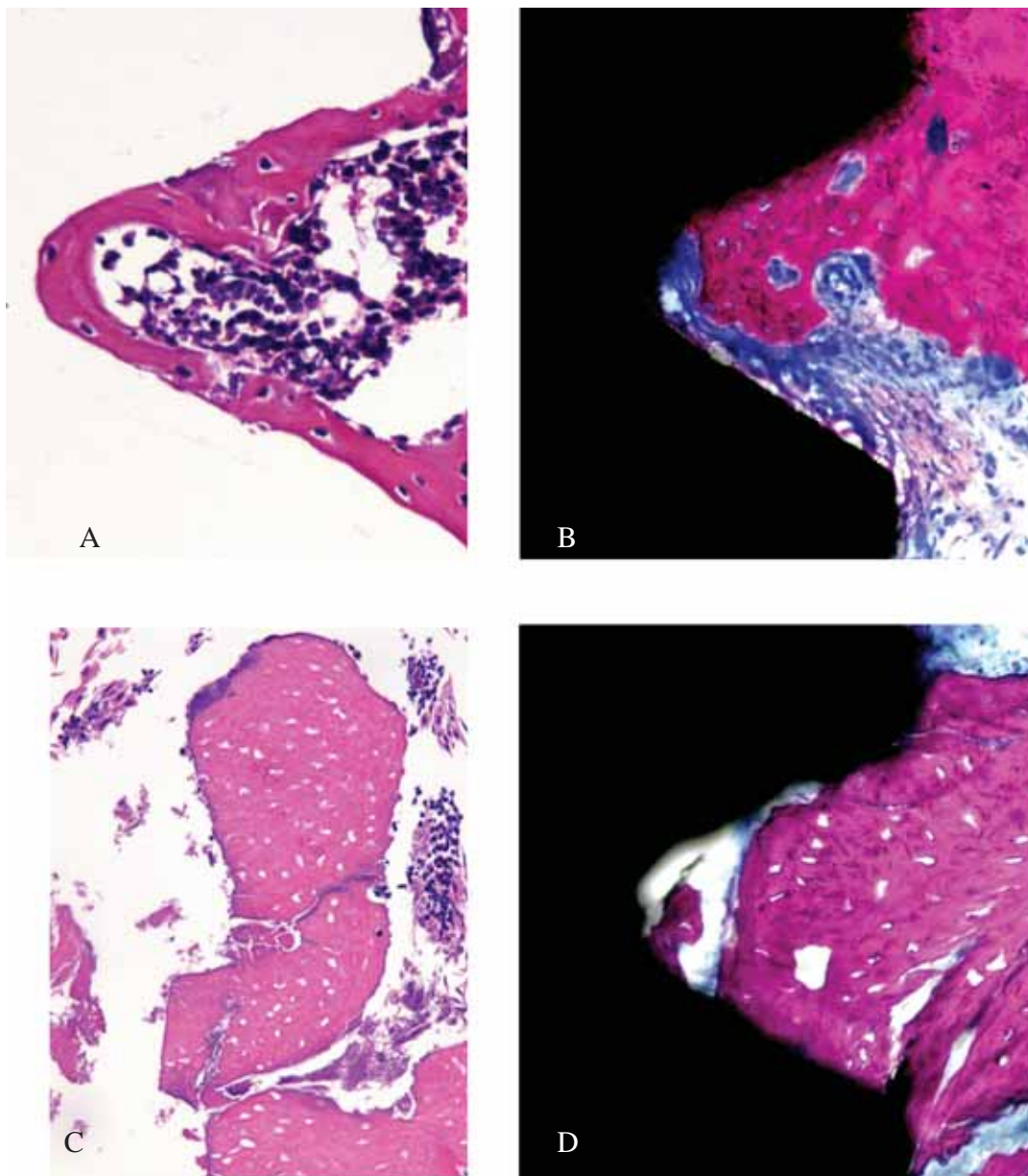
**FIGURA 19-** Grau de infecção observados nos implantes tibiais(\*\* $p < 0.01$  em relação ao grupo CTL e AL; Teste ANOVA seguido do teste de Tukey).



**FIGURA 20-** Grau de inflamação observado nos implantes tibiais(\*\*p<0.01 em relação ao grupo CTL; Teste ANOVA seguido do teste de Tukey).



**FIGURA 21-** Grau de necrose óssea observado nos implantes tibiais(\*\*\*p<0.001 em relação ao grupo CTL e CTLS; \*\*p<0.01 em relação ao grupo ALS; Teste ANOVA seguido do teste de Tukey).



**FIGURA 22-** Cortes referentes à histologia dos implantes maxilares para tecidos calcificados e descalcificados em animais submetidos aos protocolos de estresse crônico. Em ambos os grupos CTLS (B) e ALS (D) observa-se tecido ósseo em contato com o implante. No entanto, os animais tratados com alendronato (C) observam-se áreas extensas de necrose óssea e infecção, enquanto que no grupo CTLS o tecido ósseo encontrava-se vital.

***Anexos***

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# ***Anexo A***

## ANEXO A – Certificado do Comitê de Ética



UNIVERSIDADE ESTADUAL PAULISTA  
"JÚLIO DE MESQUITA FILHO"  
Câmpus de Araraquara



FACULDADE DE ODONTOLOGIA

Proc. CEEA nº 18/2009

Araraquara, 17 de novembro de 2009

Senhores Pesquisadores:

O Comitê de Ética em Experimentação Animal-CEEA desta Faculdade reunido em 16/10/2009, após a avaliação do projeto de sua responsabilidade intitulado "Uso do alendronato na indução de osteonecrose experimental: estudo em ratos" (Proc. CEEA nº 18/2009) AUTORIZA a realização da pesquisa, ficando a apresentação do RELATÓRIO FINAL para OUTUBRO/2010.

Atenciosamente.

  
Prof. Dr.<sup>a</sup> ELENY ZANELLA BALDUCCI  
Coordenadora do CEEA

Ao  
**Prof. Dr. ÉLCIO MARCANTONIO JUNIOR**  
DD. Pesquisador Responsável  
Departamento de Diagnóstico e Cirurgia

## ***Anexo B***

## Anexo B – Documento de autorização



Rua Prof. Carlos Liepin, 534 Jardim Bela Vista  
Nova Odessa / SP / 13460-000 - Brasil  
+ 55 19 3466 2083 www.editoranapoleao.com

Fazer Saber

### Autorização para uso de obras literárias

Pelo presente instrumento particular, a Editora Napoleão, estabelecida à Rua Prof. Carlos Liepin nº 534, Bela Vista, na cidade de Nova Odessa – SP – Brasil, CNPJ 06.228.693/0001-50, titular dos direitos morais e patrimoniais da obra intitulada *"Osteonecrose Maxilar Associada aos Bisfosfonatos"*, publicada no Livro *"Periodontologia e Implantodontia - Soluções estéticas e recursas clínicas"*, autoriza a utilização gratuita do mencionado capítulo pela Faculdade de Odontologia do Campus de Araraquara – Universidade Estadual Paulista "Júlio de Mesquita Filho", estabelecida à Rua Humaitá nº 1680, na cidade de Araraquara – São Paulo, CNPJ (48.031.918/0024-10), nos seguintes termos:

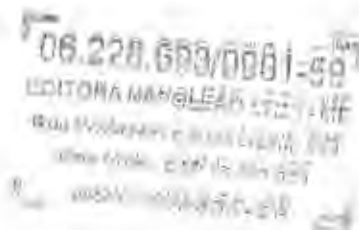
**Inclusão de uma cópia do referido capítulo na Tese de Doutorado intitulada *Uso de Alendronato para indução de osteonecrose experimental: estudo em ratos que está sendo desenvolvida pela FOAR – UNESP pelo doutorando Nicolau Conte Neto.***

Por esta ser a expressão da minha vontade, declaro que autorizo o uso acima descrito sem que nada haja a ser reclamado a título de direitos autorais e conexos.

Nova Odessa, 10 de Fevereiro de 2012.

  
LEONARDO NAPOLÉÃO

LEONARDO NAPOLÉÃO/SÓCIO ADMINISTRADOR



## ***Anexo C***

## **Anexo C – Documentos comprobatórios**

Article title: Long-term treatment with alendronate increases the surgical difficulty during simple exodontias - an in vivo observation.

MS ID : 2109051387712617

Authors : Nicolau Conte-Neto, Alliny S Bastos, Luis Carlos Spolidorio, Rosemary Adriana Chierici Marcantonio and Elcio Marcantonio Jr

Journal : Head & Face Medicine

Dear Dr Conte-Neto

Thank you for submitting your article. This acknowledgement and any queries below are for the contact author. This e-mail has also been copied to each author on the paper, as well as the person submitting. Please bear in mind that all queries regarding the paper should be made through the contact author.

A pdf file has been generated from your submitted manuscript and figures. We would be most grateful if you could check this file and let us know if any aspect is missing or incorrect. Any additional files you uploaded will also be sent in their original format for review.

[http://www.head-face-med.com/imedia/2109051387712617\\_article.pdf](http://www.head-face-med.com/imedia/2109051387712617_article.pdf) (210K)

For your records, please find below link(s) to the correspondence you uploaded with this submission. Please note there may be a short delay in creating this file.

[http://www.head-face-med.com/imedia/1679786472712621\\_comment.pdf](http://www.head-face-med.com/imedia/1679786472712621_comment.pdf)

**De:** "BONE (ELS)" <[bone@elsevier.com](mailto:bone@elsevier.com)>

**Data:** 9 de março de 2012 16:03:52 BRT

**Para:** [elciojr@foar.unesp.br](mailto:elciojr@foar.unesp.br)

**Assunto: Submission Confirmation**

Dear Prof. Elcio Marcantonio Jr,

Your submission entitled "Effects of chronic stress and alendronate therapy on the osseointegration of titanium implants" has been received by Bone

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NICOLAU CONTE NETO