FACULDADE DE ODONTOLOGIA

ANÁLISE DA MICROBIOTA DE FERIDAS CIRÚRGICAS EM MAXILAS DE RATOS SOB TERAPIA COM BISFOSFONATOS

RENATA CHIAPINOTTO BOFF

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ANALYSIS OF MICROBIOTA OF SURGICAL WOUNDS IN MAXILLAE OF RATS SUBJECTED TO BISPHOSPHONATE THERAPY

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Orientadora: Profª. Drª. Karen Cherubini

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De tudo ficaram três coisas:

A certeza de que estamos sempre começando...

A certeza de que precisamos continuar...

A certeza de que podemos ser interrompidos antes de terminar.

Façamos da interrupção um caminho novo...

Da queda, um passo de dança...

Do medo, uma escada...

Do sonho, uma ponte...

Da procura, um encontro.

Fernando Sabino (1923-2004)

Dedicatória

Aos meus pais, Marcos e Jaqueline, e à minha irmã,

Roberta, por todo amor e incentivo.

Ao meu padrinho Ricardo, por ter sido um exemplo de

garra e determinação.

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Resumo

RESUMO

Bisfosfonatos são fármacos amplamente empregados no tratamento de doenças caracterizadas por intensa reabsorção óssea e têm sido associados a quadros de osteonecrose maxilar (BRONJ). A BRONJ é uma condição de difícil tratamento, cuja etiologia, embora considerada multifatorial, não foi completamente esclarecida. Entre outros fatores etiológicos, a participação dos microrganismos como fator principal ou secundário tem sido discutida na literatura. O presente estudo teve por objetivo avaliar, por meio de histomorfometria nas técnicas de Gram, Gomori-Grocott e imunoistoquímica (IHQ), os microrganismos de feridas cirúrgicas induzidas em maxilas de ratos sob terapia com bisfosfonatos. Trinta e quatro ratos foram distribuídos em três grupos de acordo com o tratamento administrado: (1) grupo ácido zoledrônico (n=12); (2) grupo clodronato (n=12); e (3) grupo-controle (n=10, solução salina). Sessenta dias após iniciado o tratamento, os animais foram submetidos a exodontias e indução cirúrgica de lesão de tecido mole. Decorridos 42 dias dos procedimentos cirúrgicos, foi realizada a eutanásia, e os espécimes foram submetidos a processamento histológico. No sítio das exodontias, o grupo ácido zoledrônico teve maior prevalência de *Actinomyces* que o clodronato e o controle, enquanto no sítio da ferida de tecido mole, ambos os grupos-teste exibiram maiores valores que o controle. A ocorrência de *Candida* não diferiu significativamente entre os grupos. No sítio de exodontia, cocos Gram-positivos isolados foram mais prevalentes no grupo ácido zoledrônico, ao passo que, na região de ferida de tecido mole, eles foram mais prevalentes nos grupos-teste que no controle. A prevalência de cocos Gram-negativos isolados não exibiu diferença significativa entre os grupos no sítio das exodontias; entretanto, no sítio da ferida de tecido mole, foi maior no grupo ácido zoledrônico. Os bacilos não diferiram entre os grupos no sítio de exodontia, mas na área de ferida de tecido mole, foram mais prevalentes nos grupos-teste. A ocorrência de diplococos, estreptococos e estafilococos foi rara e não exibiu diferença significativa entre os grupos. Tanto em sítio de exodontia, quanto de ferida de tecido mole, o *Actinomyces* foi o microrganismo mais prevalente seguido pelos cocos isolados e bacilos. Estreptococos, estafilococos, *Candida* e diplococos não exercem papel significativo no desenvolvimento das lesões. O sítio anatômico parece ser fator determinante dos microrganismos envolvidos.

Palavras-chave: Bisfosfonatos; Etiopatogênese; Infecção; Biofilme; *Actinomyces* sp.

Summary

SUMMARY

Bisphosphonates are drugs widelly used to treat diseases characterized by intense bone resorption. Although very efficacious, they are also associated with cases of osteonecrosis in jaw bones called *bisphosphonate-related osteonecrosis of the jaws* (BRONJ). The disease is difficult to treat and its etiology is considered multifactorial but it has not yet been completely understood. Among other etiologic factors, the major or secondary role of microorganisms in BRONJ has been discussed. The aim of this work was to evaluate by means of histomorphometry in Gram, Gomori-Grocott and immunohistochemistry (IHC) the microorganisms found in surgically induced wounds in maxillae of rats subjected to bisphosphonate therapy. Thirty-four rats were allocated into three groups according to the treatment received: (1) zoledronic acid group (n=12); (2) clodronate group (n=12); and (3) control group (n=10, normal saline). Sixty days after started the treatment, tooth extractions and surgical wounds in soft tissue were performed. Forty-two days after surgeries, rats were euthanized, the specimens were processed and histological analysis performed. At the tooth extraction site, the zoledronic acid group had higher prevalence of *Actinomyces* than the clodronate and control, whereas at the soft tissue wound, both test groups showed higher values than control. *Candida* occurrence did not significantly differ between the groups. At the tooth extraction site, single Gram-positive cocci were more prevalent in the zoledronic acid, whereas at the soft tissue wound, they were more prevalent in both test groups than controls. Single Gram-negative cocci did not show any significant difference between the groups at the tooth extraction site; at the soft tissue wound, they were more prevalent in the zoledronic acid group. At the tooth extraction site, bacilli did not differ between the groups, whereas at the soft tissue wound site, they were significantly higher in the test groups. Diplococci, streptococci and staphylococci were rarely found, without significant difference between the groups. *Actinomyces* was the most prevalent microorganism, followed by single cocci and bacilli. Streptococci, staphylococci, *Candida* and diplococci do not seem to play a significant role in such lesions whereas the anatomical site seems to be a determinant of the microorganisms involved.

Key words: Bisphosphonates; Etiopathogenesis; Infection; Biofilm; *Actinomyces* sp.

Sumário

SUMÁRIO

Introdução

1 INTRODUÇÃO

Os bisfosfonatos são análogos químicos do ácido pirofosfórico que, no organismo humano, se encontra sob a forma de pirofosfato, um inibidor natural da reabsorção óssea (Fernandes *et al.*, 2005). Enquanto o pirofosfato exibe a estrutura P-O-P, os bisfosfonatos possuem um átomo de carbono substituindo o oxigênio (P-C-P). Essa estrutura P-C-P, além de permitir a ligação aos cristais de hidroxiapatita com alta afinidade, também aceita a presença de nitrogênio em uma das cadeias laterais, o que confere aumento significativo da potência da droga (Ruggiero; Woo, 2008). Em função de propriedades farmacológicas como potente inibição da atividade osteoclástica com supressão da remodelação óssea, acúmulo na matriz óssea mineralizada por muitos anos e forte aderência aos cristais de hidroxiapatita concentrando-se seletivamente no osso (Hansen *et al*., 2006; Neville *et al.*, 2009; Reid, 2009), esses medicamentos têm sido a primeira escolha para o tratamento de enfermidades ósseas como osteogênese imperfeita, osteoporose, doença de Paget e mieloma múltiplo, bem como metástases ósseas e complicações provenientes de neoplasias malignas (AAOMS, 2007; Diego *et al*., 2007; Gliklich; Wilson, 2009; Hansen *et al*., 2006; Kumar *et al*., 2010; Neville *et al.*, 2009).

Um importante efeito adverso do uso dos bisfosfonatos é a osteonecrose, lesão que pode ocorrer espontaneamente ou após trauma dentoalveolar e que se restringe ao complexo maxilomandibular. A osteonecrose maxilar associada aos bisfosfonatos é definida pela presença de osso exposto na cavidade oral, sem evidência de cicatrização por um prazo mínimo de oito semanas, em pacientes sob terapia ou já expostos a esses fármacos e que nunca foram submetidos à radioterapia de cabeça e pescoço (AAOMS, 2007; Ruggiero *et al*., 2009; Saia *et al*., 2010). Recentemente, casos de lesões sem exposição óssea, especialmente em estágios iniciais, também têm sido relatados. Nestes, sinais e sintomas como dor, aumento de volume nos ossos maxilares, inflamação gengival,

bolsa periodontal profunda, infecção, mobilidade dentária e fístula, que não estariam relacionados a nenhuma outra lesão odontológica ou desordem local ou sistêmica, além da osteonecrose, têm sido observados semanas ou meses antes da exposição óssea propriamente dita. Ao exame radiográfico, observa-se radiolucidez mal-definida que, geralmente, exibe área radiopaca de sequestro ósseo (Fedele *et al*., 2010; Junquera; Gallego, 2008; Lazarovici *et al.*, 2009; Mawardi *et al*., 2009). As teorias que tentam explicar a etiopatogênese da lesão baseiam-se no efeito de cessação do remodelamento ósseo por meio da inibição dos osteoclastos, bem como nas propriedades antiangiogênicas desses compostos (Agarwal; Rao, 2012). Além disso, a participação dos microrganismos no desenvolvimento da osteonecrose maxilar associada aos bisfosfonatos também tem sido apontada (Kaplan *et al*., 2009; Kumar *et al*., 2010).

A cavidade oral é colonizada por uma grande variedade de bactérias, e os ossos maxilares estão, frequentemente, envolvidos em processos sépticos de origem periodontal ou pulpar (AAOMS, 2007). Estudos têm mostrado a colonização do osso afetado por múltiplos morfotipos bacterianos como *Fusobacterium*, *Streptococcus*, *Selenomonas*, *Bacillus* (Sedghizadeh *et al.*, 2008*;* Sedghizadeh *et al.*, 2009*)*, *Staphylococcus* e espiroquetas (Sedghizadeh *et al*., 2009), além de *Actinomyces* e organismos fúngicos compatíveis com *Candida albicans* (Lee *et al*., 2011; Hansen *et al.*, 2006; Sedghizadeh *et al*., 2008; Sedghizadeh *et al*., 2009).

Embora em alguns casos não cause diretamente o início da necrose, o biofilme bacteriano parece ter papel essencial no problema clínico representado pela osteonecrose associada aos bisfosfonatos. Como a maioria dos casos dessa enfermidade envolve osso exposto e comorbidades que afetam a cicatrização da ferida ou a função imunológica dos pacientes, o osso impregnado de bisfosfonato apresenta-se menos resistente à infecção e à colonização bacteriana do que o osso normal (Kumar *et al.*, 2010). A identificação de

biofilmes em infecções persistentes é imprescindível para a escolha de terapias adequadas a fim de possibilitar a cicatrização do tecido no controle clínico da osteonecrose. A presente pesquisa teve por objetivo analisar, por meio de histomorfometria, os microrganismos presentes em feridas cirúrgicas em maxilas de ratos sob terapia com bisfosfonatos. O trabalho foi estruturado sob a forma de dois artigos científicos, sendo que o primeiro apresenta uma revisão da literatura sobre o tema, e o outro consiste na apresentação do experimento conduzido.

2 ARTIGO 1

O artigo a seguir intitula-se ***Important aspects regarding the role of microorganisms in bisphosphonate-related osteonecrosis of the jaws*** e foi formatado de acordo com as normas do periódico *Archives of Oral Biology* (Anexos A e B).

Important aspects regarding the role of microorganisms in bisphosphonate-related osteonecrosis of the jaws

Renata Chiapinotto Boff

Fernanda Gonçalves Salum

Maria Antonia Figueiredo

Karen Cherubini

Postgraduate Program, Dental College, Pontifical Catholic University of Rio Grande do Sul - PUCRS

Running title: *Microorganisms and BRONJ*

Keywords: bisphosphonate; osteonecrosis of the jaws; infection, *Actinomyces* sp.; *Candida* sp.

Corresponding author

Karen Cherubini
Serviço de Estomatologia, Hospital São Lucas, PUCRS
Av Ipiranga, 6690, sala 231
Porto Alegre RS Brazil
CEP 90610-000
Telephone/fax: 55(51)33203254
E-mail: karen.cherubini@pucrs.br

ABSTRACT

Bisphosphonate-related osteonecrosis of the jaws (BRONJ) is an important side effect of bisphosphonates, whose etiopathogenesis has not been completely elucidated. Theories pointing to bone turnover and angiogenesis inhibition, as well as effects on epithelial cells of oral mucosa and the role of microorganisms have been reported. Nevertheless, the true contribution of each one of these factors to BRONJ is unknown. We present here a literature review focusing on important aspects regarding the role of microorganisms in BRONJ development. Knowledge about specific microbiota and its role in the etiopathogenesis of this disease can help the optimization of preventive and therapeutic interventions in patients with or at-risk for BRONJ.

Introduction

Bisphosphonates are potent inhibitors of bone resorption widely used to treat osteogenesis imperfecta, osteoporosis and Paget's disease as well as adjuvant therapy in the management of multiple myeloma, bone metastases and malignancy complications such as hypercalcemia.[1-6] Concentrations of these drugs capable of inhibiting bone metabolism can also impair tissue healing after induced or physiological trauma, which has been associated with cases of necrotic bone exposure to oral environment.[7] Bisphosphonate-related osteonecrosis of the jaws (BRONJ), first reported in 2003,[8] is an important side effect of these drugs.[9] The condition is described as spontaneous bone exposure or non-healing wounds after tooth extraction, which may or may not involve infection and fistulization.[10] Clinical features can also include pain, erythema and pathological jaw fracture.[6,10] According to the American Association of Oral and Maxillofacial Surgeons (AAOMS),[2] three conditions are needed to define a case of BRONJ: (a) current or previous therapy with

bisphosphonates; (b) exposed necrotic bone in the maxilla or mandible lasting more than eight weeks; and (c) absence of head and neck radiation therapy.

BRONJ affects maxilla and mandible and has related-risk factors such as tooth extractions,[11,12] diabetes,[11,13] tobacco use,[11] trauma caused by prosthetics appliances, oral infection,[14] poor oral hygiene, subnutrition[15] and bone manipulation.[16] Many theories try to explain BRONJ etiopathogenesis.[11,17-21] One of them suggests that bone remodeling cessation related to osteoclast inhibition produced by bisphosphonates is the cause of the disease. These drugs have an affinity for the bone mineral matrix, and it is believed that they inhibit bone resorption by inducing osteoclast apoptosis and/or inhibiting osteoclast function. Bisphosphonates are not metabolized by bone, where they can stay in their original form for many years. During bone remodeling they are released from hydroxyapatite crystals and internalized by osteoclasts. Non-nitrogen-containing bisphosphonates are metabolized in the osteoclast cytoplasm into non-hydrolyzable ATP analogues, which are cytotoxic compounds that lead to cell death. Nitrogen-containing bisphosphonates, in turn, disrupt the mevalonate pathway, a biosynthetic pathway needed for cholesterol and isoprenoid lipid synthesis, leading to inhibition of protein prenylation, which determines osteoclast apoptosis. Bisphosphonates also stimulate osteoblasts to produce osteoprotegerin (OPG), an inhibitor of osteoclast differentiation.[22-24] Such effects impair the homeostatic cycle of bone remodeling and repair, especially in the jaws, which have more intense metabolic activity than other skeletal bones.[18,22] In inflammatory processes of the oral cavity, bisphosphonate-impregnated alveolar bone cannot be resorbed because of osteoclast inhibition, which leads to bone exposure to an environment rich in bacterial toxins, inflammatory cytokines and oxidative stress. This environment is highly toxic to bone cells, which can result in osteonecrosis.[20]

There is also evidence that bisphosphonates inhibit vascular endothelial functions, either *in vitro* or *in vivo*. Reduced endothelial cell proliferation, apoptosis induction and reduced formation of capillary tubes were observed *in vitro*. *In vivo*, zoledronic acid, ibandronate and, to a lesser extent, clodronate inhibited ventral prostate revascularization in castrated male rats under testosterone stimulation.[17] Therefore, another theory supports the idea that bisphosphonates inhibit endothelial cell proliferation in the jaws, leading to blood vessel loss and consequent avascular necrosis.[18] Landesberg *et al.*,[19] in turn, proposed that, after a trauma, oral epithelial cells are subjected to local increase in bisphosphonate levels, which inhibits normal regeneration of epithelium, thereby favoring the persistence of bone exposure and BRONJ development.

Still, there is strong evidence that infection is closely related to BRONJ etiopathogenesis,[21,25] especially regarding the constant findings of *Actinomyces* colonies in histological examinations of the lesions.[26] In bisphosphonate users, the site of tooth extraction favors infection because of (a) less inflammatory response and vascularization of the tissues, (b) increased bacterial adhesion to bisphosphonate-coated bone, and (c) persistence of exposed bone to oral cavity consequent to inhibition of both bone resorption and epithelial covering, which can provide a substrate for bacterial growth.[21]

The participation of microorganisms in the etiopathogenesis of these lesions was at first classified as secondary, but the possibility of a major role of microbial agents has been suggested.[6,11,16,26-28] We present here a literature review focusing on important aspects related to the role of microorganisms in BRONJ etiopathogenesis.

Colonization by oral microorganisms in BRONJ

In the oral cavity, bone can be easily exposed to the abundant bacterial and fungal microbiota, which has the potential of causing biofilm-mediated diseases.[29] Although routinely exposed to oral microorganisms that include over 750 recognized bacteria,[30] the

jaws are generally resistant to colonization. Therefore, for colonization to occur, it is necessary to have a combination of patient susceptibility and the presence of potentially pathogenic microorganisms, such as *Actinomyces*, which predominates either in BRONJ cases or in jaw osteomyelitis.[29]

Biofilms are complex microbial communities attached to surfaces, which can harbor single or multiple microbial populations or microcolonies. Microbial cells are incorporated in a matrix of extracellular polymeric substance they produce to be linked to and to communicate with each other and with the environment.[31] Such bacterial communities play an important role in BRONJ pathogenesis. It is probable that biofilm development in these lesions results from their chronic course and local environment, even though it could be favored by the presence of bisphosphonates on the bone surface.[26] The presence of microorganisms compatible with *Actinomyces* spp. and yeast colonies in contact with necrotic bone and within empty lacunae in BRONJ lesions[32,33] suggests an essential role of infectious agents in BRONJ pathogenesis.[6]

Saia *et al*.[25] evaluated 60 patients at high risk for osteonecrosis, who were being treated with nitrogen-containing bisphosphonates and who were subjected to surgical tooth extractions. The patients stopped bisphosphonate therapy for one month after tooth extraction and were evaluated at three, six and twelve months. Biopsies of alveolar bone were performed during the tooth extraction procedure, and 54 patients showed normal bone architecture and vascularization on histopathological examination, whereas six patients showed baseline osteomyelitis. No sign of bone necrosis was detected at this moment in any specimen. Nevertheless, three months after tooth extractions, four out of the six patients with baseline osteomyelitis developed osteonecrosis and, one more at six months follow-up. Because osteonecrosis developed only in patients who had osteomyelitis prior to surgery, the authors believed that it was unlikely that trauma from tooth extraction was responsible

for the lesion. Moreover, as bone necrosis was not detected in any specimen at the moment of tooth extraction, the hypothesis that BRONJ is an infectious disease and that the oral microbiota plays an important role in its pathogenesis was reinforced.

Sedghizadeh *et al.*[32] observed by means of scanning electron microscopy the presence of biofilm in bone specimens of four patients with BRONJ who had been subjected to surgical bone debridement and sequestrectomy. Bone specimens revealed large areas occluded by biofilm that comprised predominantly bacteria and occasionally some yeasts incorporated in the extracellular polymeric substance. Bacteria identified in all bone samples comprised Gram-positives and Gram-negatives and also included aerobes, even though the majority were anaerobes or facultative anaerobes. Species of *Fusobacterium*, *Bacillus*, *Actinomyces*, *Staphylococcus*, *Streptococcus*, *Selenomonas* and three different morphological variants of spirochetes were observed in the biofilms. Fungal morphotypes compatible with *Candida* spp. were also evident in all cases. The organisms identified were compatible with normal oral microbiota, particularly with that involved in periodontal, pulpal, periapical (bacterial) and mucosal (fungal) diseases. The authors observed that, when the specimens were cut in cross-section, bacteria were evident on all internal surfaces, indicating the presence of bacterial biofilm in deep bone cavities and not only on its surface exposed to the contaminated oral cavity. Moreover, all specimens showed bone regions where host eukaryotic cells were adjacent to or trapped within the biofilm. Nevertheless, no eukaryotic cell was observed in or near resorption pits. Therefore, considering that resorption was evident in all samples in the cases of either BRONJ or osteomyelitis reported by Sedghizadeh *et al.*,[29] it is probable that resorption had been caused by microbial biofilms. According to the authors, the fact that patients were taking bisphosphonates, which are antiresorptive drugs, supports this hypothesis. Also, all cases exhibited large surface areas occluded by well-developed biofilms, which comprised microbial organisms

embedded in the extracellular polymeric substance. Both osteomyelitis and osteonecrosis cases showed multispecies microbial biofilms on internal and external surfaces along the depth of the excised bone.

Like *Actinomyces israelii*, which can be seen in dental biofilm and calculus, advanced periodontitis, infected dental root canals and periapical infections,[34] many pathogenic bacteria of the oral cavity, which are associated with osteonecrosis, can invade jaw bones and cause destruction through direct and indirect mechanisms. Some of these are: (a) destruction of non-cellular components of bone caused by release of acids and proteases; (b) induction of cellular processes that stimulate bone degradation; and (c) inhibition of bone matrix synthesis.[35] Bacteria can invade osteoblasts, producing functional disturbances and apoptosis, which leads to dysregulation of bone remodeling[36] (Fig. 1).

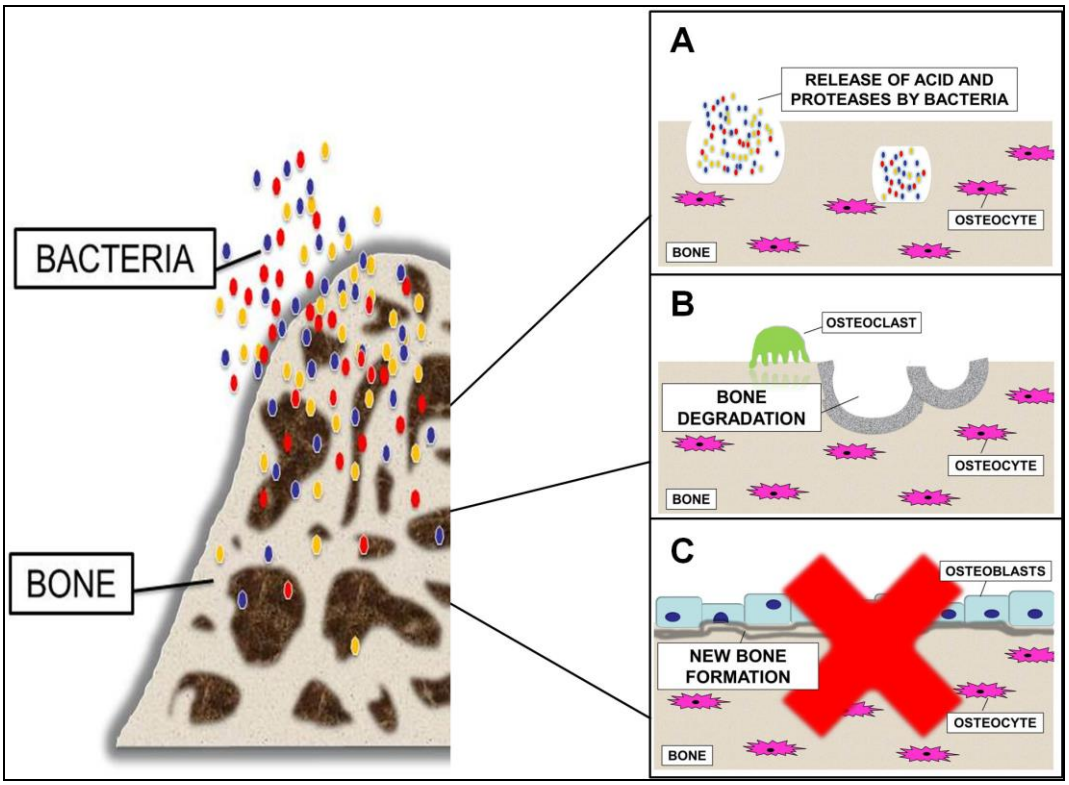

Fig. 1 – Mechanisms of jaw bones destruction by oral pathogenic bacteria. (A) Destruction of non-cellular components of bone caused by acids and proteases release; (B) induction of cellular processes that stimulate bone degradation; (C) inhibition of bone matrix synthesis

Chemical mediators of bone resorption produced by bacteria include proteins such as porins[35,36] and collagen-degrading enzymes such as collagenases. Through this process, amino acids required for bacterial growth are obtained and anaerobic niches are created in bone, which favors bacterial growth and spread.[37] Such proteins can interfere with host cell cycle, which represents an important mechanism of bacterial virulence.[35] Bacteria and their products cause inflammatory bone loss in many infections, which can determine significant morbidity. In most cases of infection-associated chronic inflammation, Gram-negative bacteria and their products have been implicated as etiologic factors. Under these conditions, there is a typically inflammatory infiltrate of mononuclear cells.[38] Lipopolysaccharides are inflammatory components of Gram-negative bacteria, and like some inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-alpha), these bacterial products can directly induce osteoclastogenesis. It is known that osteoblasts regulate osteoclastogenesis by receptor activator of NF-kB ligand (RANKL), also designated osteoprotegerin ligand (OPGL), which can be induced by bacteria and in this way contribute to chronic inflammation-associated bone loss.[38] Jiang *et al*.[38] demonstrated through incubation of leukocytes with *P. gingivalis*, that bacteria can directly induce osteoclast formation from leukocytes. It was also observed that either bacteria or lipopolysaccharides increased osteoclastogenesis in a dose-dependent manner.

Internalization protects bacteria from both host immune system and antibiotic therapy, which would explain the persistence of some bone infections.[36] Kos and Luczak[39] defend the hypothesis that bisphosphonates promote jaw osteonecrosis by facilitating bacterial colonization. According to these authors, bone necrosis and osteomyelitis that occur during bisphosphonate administration result from the more intense bacterial adhesion to bone coated with this drug. This promotes bone surface colonization predominantly by Gram-positive strains, including *Actinomyces*, which creates favorable conditions for the

development of chronic infection resistant to therapy. Such bacterial adhesion to bone surface would occur by direct electrostatic interaction with the amino-cationic group of nitrogen-containing bisphosphonates, through direct interaction between surface proteins or by providing an amino acid mimic on the surface of bone hydroxyapatite, which interacts with microbial surface components that recognize adhesive matrix molecules (MSCRAMM) mediating increased bacterial adhesion (Fig. 2).

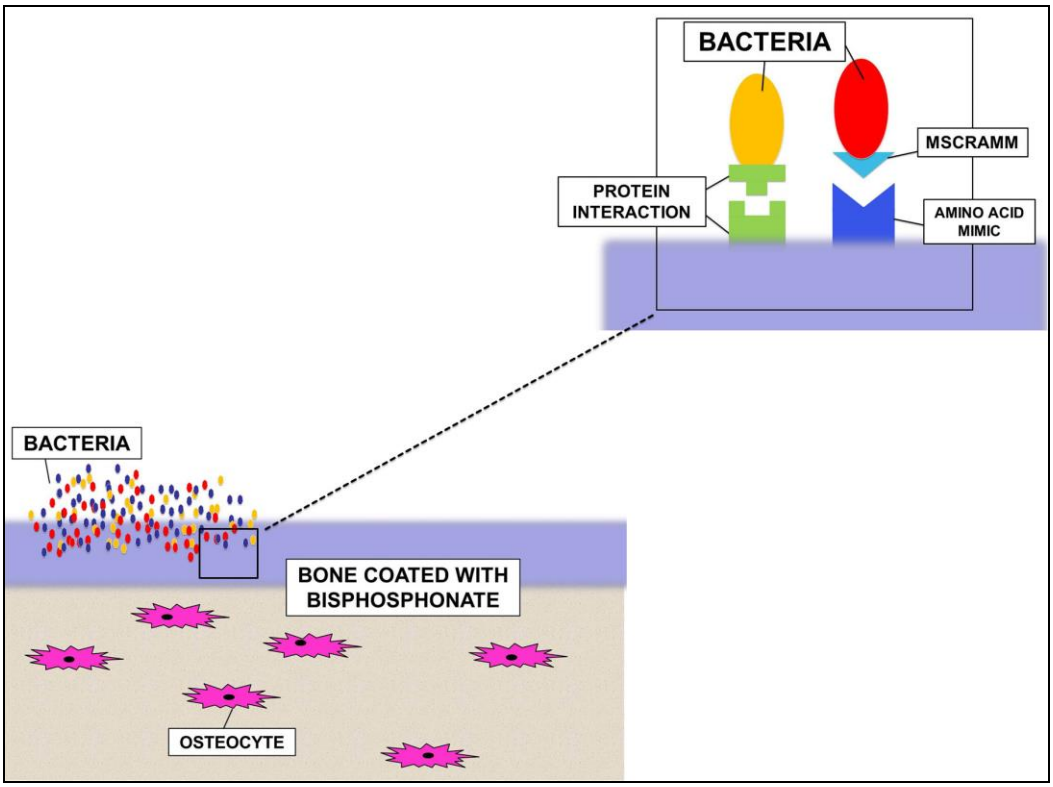

Fig. 2 – Interaction between bisphosphonate and bacteria according to Kos and Luczak[39]. The cationic amino group of nitrogen-containing bisphosphonate may attract bacteria by direct electrostatic interaction, through a direct surface protein interaction or by providing an amino acid mimic on the surface of the bone hydroxyapatite which interacts with MSCRAMM (microbial surface components that recognize adhesive matrix molecules)

Bone exposure during surgery or tooth extraction works as a triggering factor that opens the door for bacterial invasion. This would explain the strong correlation between BRONJ and dental surgeries. Also, the higher susceptibility of jaws to infection, when compared to

other bones, reinforces this hypothesis. Jaw bones easily come in direct contact with the external environment because of the thin layer of overlying mucosa, constant exposure to trauma and presence of teeth. According to Kos and Luczak,[39] the confirmation of this theory of infection could result in more rational antibiotic therapy, with drugs specifically directed to a hypovascular and hypocellular bone.

Actinomyces spp., *Candida* spp. and other microorganisms

BRONJ cases exhibit numerous microbial morphotypes, whereas bisphosphonate-non-related osteomyelitis cases show a higher prevalence of monospecies with a predominance of *Actinomyces* spp. In the former, bone becomes more susceptible to being colonized by various microorganisms that usually do not, such as superficial fungal organisms (*Candida albicans*) and some of the more benign bacterial morphotypes found in osteonecrosis.[29]

A microbial variety has been identified in BRONJ, where *Actinomyces* colonies in contact with non-vital bone are a consistent histological finding.[1,40,41] These colonies were observed in all BRONJ and osteoradionecrosis cases studied by Hansen *et al*.[1] The authors used histological samples subjected to hematoxylin and eosin (H&E), Grocott, Gram and periodic-acid Schiff (PAS) staining. Bacteria were observed in greater amounts at sites of necrotic bone, which differed from other bone regions by notable signs of erosion and numerous irregularly shaped contours. Also, bacterial filaments were interspersed with some inflammatory cells, especially neutrophilic granulocytes. Other microorganisms were not observed, except in one case of BRONJ that showed fungal spores superficially located, compatible with *Candida* spp.

The bacterial profile in soft tissues in BRONJ lesions has been analyzed by means of polymerase chain reaction (PCR) using 16S rRNA-based denaturing gradient gel electrophoresis (DGGE) and sequencing.[15] Samples were collected from five patients under antibiotic therapy and five patients under no treatment. The study showed no

significant difference in bacterial diversity (cultivable or non-cultivable) of BRONJ tissue samples. Nevertheless, the results indicated that species⁄phylotypes affiliated with the genera *Parvimonas* and *Peptostreptococcus* were more prevalent in the group treated with antibiotics, whereas *Fusobacterium*, *Atopobium*, and *Streptococcus* existed predominantly in the non-treated group indicating changes in biofilm composition. Both groups showed a high number of *Actinomyces*, which was attributed to the fastidious nature of the bacteria and not to its association with BRONJ. According to the study, the tissues affected by this disease are strongly colonized by oral bacteria, and systemic antibiotic therapy failed to restrict bacterial colonization, without efficient wound healing.[15]

Hansen *et al.*[42] evaluated archived material from 45 patients with actinomycosis of the jaws, and found that 42 had malignancy treated by head and neck radiation therapy or by bisphosphonate and that three had no malignancy. All patients, with or without malignancy, exhibited similar histological features: (a) *Actinomyces* colonies in direct association with bone; (b) mixed inflammatory infiltrate in bone marrow spaces with variable amount of osteoclasts; and (c) pseudoepitheliomatous hyperplasia in up to 60% of cases. *Actinomyces* colonies were principally adhered to bone without inflammatory cells. In three out of the 45 cases, fungi forming non-septated hyphae and spores were also observed, which probably corresponded to *Candida*. In contrast to *Actinomyces*, mycotic filaments were always broader showing a double-linear lining.

Merigo *et al.*[43] reported four cases of BRONJ that occurred without previous dental extraction, in patients using pamidronate and zoledronic acid. According to the authors, *Candida albicans* was observed in two cases, whereas one case showed *Actinomyces* within the bone lesion on microbiological and histopathological examinations. Diego *et al.*,[3] in turn, found necrotizing osteitis associated with bacterial colonies in all 10 cases of zoledronic acid-associated osteonecrosis evaluated by histopathological examination. Also,

histopathology was available in 30 out of 101 cases of BRONJ reported by Lazarovici *et al.*,[44] where 93% showed *Actinomyces* colonies identified by Gram and PAS staining. Senel *et al.*[45] reported one case of osteonecrosis related to the use of oral clodronate during five-year treatment of multiple myeloma. Histopathology of the infected bone exhibited dense infiltrate of plasma cells, polymorphonuclear leukocytes and lymphocytes as well as numerous *Actinomyces* colonies. Badros *et al.*[46] investigated BRONJ cases in multiple myeloma patients. On histological examination, they observed osteomyelitis and areas of acellular necrotic bone, whereas the microbiological examination showed filamentous microorganisms compatible with *Actinomyces* in seven out of 20 patients.

Maahs *et al.*[12] analyzed through H&E the tooth extraction sites in rats treated with bisphosphonates. They observed that 80% of the animals treated with zoledronic acid showed osteonecrosis with high prevalence of microorganisms, most of them compatible with *Actinomyces* sp. On the other hand, the alendronate group did not have any case of osteonecrosis and had low prevalence of microorganisms, with no significant difference from controls for this variable. According to the authors, the high prevalence of microorganisms in the zoledronic acid group resulted from the osteonecrotic lesion.

Jacobsen *et al.*[47] reported the clinical and histological features of 110 cases of jaw osteonecrosis associated with bone resorption-inhibiting drugs, such as bisphosphonates and RANKL inhibitors. Seventy-four percent of patients showed clinical bone exposure, and in 23% a fistula was observed with only radiographic evidence of affected bone. Pain was the major concern in 75% of patients, where 100% showed signs of infection at the site of the affected bone, with pus discharge, abscesses or inflammation of surrounding soft tissue. Histological analysis was performed in 64 specimens, where all of them showed necrotic bone (acellular bone, without osteocytes, osteoclasts or osteoblasts) with signs of

acute and chronic inflammation, besides bacterial colonization. Plaques of *Actinomyces* were specifically described in 72% of the cases.

Fourteen BRONJ patients who received intravenous bisphosphonate to treat bone metastases and hypercalcemia were diagnosed by Dannemann *et al.*[48] Clinically, the most prevalent finding was exposed necrotic bone, occurring in 13 patients. Eleven patients reported discomfort or strong acute pain. Six patients showed hypoesthesia of the inferior alveolar nerve. Inflammatory signs were observed in all cases and four out of them showed soft tissue abscess. Histopathological examination of necrotic bone fragments revealed acute and chronic inflammatory alterations with bone marrow fibrosis, plasma cell infiltration, and colonization by pathogens. Microbiological analysis was performed in six cases and showed fungal and bacterial colonization with pathogens of normal oral microbiota such as *Actinomyces*, *Lactobacillus*, *Candida glabrata* and other microorganisms determining aggressive infection of the bone and surrounding soft tissues. According to the authors, such results suggest that bisphosphonates are not an isolated cause of osteonecrosis. However, they play an important role in the pathogenesis of the lesion if associated with other synergistic factors such as oral microbiota. The authors believe that infection plays a major role in BRONJ pathogenesis. They found that even after a very successful surgery, some patients had bone dehiscence later on. Nevertheless, symptoms were completely alleviated after subjecting patients to antibiotic therapy, including antibiotics and antimicrobial rinsing.

Badros *et al.*[46] observed species of *Peptostreptococcus*, *Streptococcus*, *Eikenella*, *Prevotella*, *Porphyromonas* and *Fusobacterium* in nine out of 20 BRONJ patients evaluated. According to the authors, the contribution of these microorganisms to soft tissue infection and osteomyelitis is unknown. Wongchuensoontorn *et al.*[49] reported three cases of BRONJ that progressed to pathological fracture. Microbiological culture showed

Streptococcus intermedius, *Peptostreptococcus* spp. and *Bacteroides melaninogenicus* in the first patient, *Actinomyces israelii* and *Bacteroides fragilis* in the second, and *Enterococcus faecalis* and *Bacteroides fragilis* in the third.

O'Ryan and Lo[50] reported 30 cases of patients treated with oral bisphosphonate that developed BRONJ after tooth extraction or oral trauma or spontaneously. The cases subjected to histopathological examination showed *Actinomyces* spp. as a common feature. Most patients with oral trauma history also showed the presence of *Streptococcus* spp., *Prevotella* and *Klebsiella*, as well as *Pseudomonas* in those who had had tooth extraction.

Treatment implications

BRONJ management is a challenging problem, since up to now there is no efficacious therapy.[14,47,51] Depending on the clinical conditions and type of bisphosphonate used, surgical treatment can be recommended,[47] but there is no agreement about the adequacy of this therapeutic option.[51] In this context, biofilm organisms have been the clinical target for the prevention and treatment of the disease, aiming to reduce morbidity and costs associated with it.[6] Mouth rinses with antimicrobial substances such as 0.12% chlorhexidine and hydrogen peroxide, three to four times daily have been recommended as local therapy to reduce bacterial colonization.[14,43,52-54] Systemic antibiotics associated with mouth rinses are recommended for more advanced cases. Even though antibiotic regimen should be established according to the antibiogram,[48] some protocols are in current use. As most of the microorganisms isolated from BRONJ lesions are sensitive to penicillin, oral amoxicillin at 1.5 to 3 g daily has been the therapeutic choice.[44] Quinolones, metronidazole, clindamycin, doxycycline and erythromycin have been used in patients allergic to penicillin. These drugs must be administered over a long-term period, which can vary from several months to more than a year. Intravenous antibiotics should be used when

lesions do not respond to oral route.[2,44] Chlorhexidine has been beneficial in the control of surface bacteria, which can help the recovery of bone-exposed regions in BRONJ.[55] Anyway, it is important to recall that, besides bacteria, fungi have also been found in BRONJ lesions.[1,32,33,42,43,48]

Malhotra *et al.*[56] evaluated the efficacy of six different mouth rinses against *Streptococcus mutans*, *Lactobacillus* and *Candida albicans*. The rinses were (a) 0.12% chlorhexidine digluconate, (b) 0.2% sodium fluoride, (c) propolis mouth rinse and (d, e, f) combinations of these three substances. Among the mouth rinses, chlorhexidine was the most efficacious against *Streptococcus mutans* and *Lactobacillus.* In all test groups the smallest inhibition zone occurred for *Candida albicans,* even though chlorhexidine was the mouth rinse with the highest inhibition against it. At low concentrations, chlorhexidine is bacteriostatic, whereas at high concentrations it is bactericidal.[57] These effects are based on its ability to impair the integrity of bacterial membranes.[58] At low concentrations, it increases the permeability of bacterial cell membranes with leakage of intracellular components. At high concentrations, it induces the precipitation of the bacterial cytoplasm and consequently causes cell death.[56] Besides a broad spectrum of antimicrobial activity, chlorhexidine has an antifungal effect particularly efficient against *Candida albicans.*[59] The lower antifungal than antibacterial activity is related to the basic differences in the external cellular structure of bacteria and fungi, as the latter have a rigid external wall of chitin.[56] Despite showing lower antifungal than antibacterial activity, chlorhexidine has proved efficacy against *Candida albicans,*[59] which seems to be the major yeast found in BRONJ lesions.

Final considerations

BRONJ etiopathogenesis has not been completely elucidated, even though there are many theories trying to explain it. Some of them point to infection as the major and not just a

secondary event.[6,21,25,48] There are authors who defend the idea that bone impregnated with bisphosphonate is less resistant to bacterial infection and colonization than normal bone, serving as an ideal incubator for periapical and periodontal bacteria, which stimulate a chronic inflammatory immune response.[60] There are also speculations suggesting an electrostatic interaction between nitrogen-containing bisphosphonates and Gram-positive bacteria, which would favor bone infection by these microorganisms.[39] Moreover, although *Actinomyces* spp. is the most frequent microorganism found in BRONJ lesions, there are many other bacterial and fungal species of the oral microbiota that are also found in the affected bone (Table 1).

In fact, even if bacterial biofilm does not directly cause the development of BRONJ, it indeed plays an essential role in the clinical repercussion of the disease.[6] In this way, regardless of having a major or secondary role in BRONJ etiopathogenesis, microorganisms are up to now important determinants of the therapeutic measures in this disease. Therefore, BRONJ treatment cannot ignore the different species found in the lesions, including fungal ones. Further studies aiming to classify and quantify all these diverse microorganisms are needed. The strict relationship between microorganisms and the type of bisphosphonate used, as well as the possible electrostatic interactions between nitrogen-containing bisphosphonates and bacteria must be investigated.

Table 1- Reports on microorganisms involved in bisphosphonate-related osteonecrosis of the jaws (BRONJ)

Microorganisms	**positive cases / total (%)**	**Bisphosphonate**	**Method**	**Reference**
Actinomyces *Candida* spp.	8/8 (100%) 1/8 (12.5%)	Pamidronate, zoledronic acid, ibandronate	Clinical study, histopathology (H&E, PAS, Gram, Grocott)	Hansen *et al.*[1]
Actinomyces sp. *Actinomyces* sp.	10/10 (100%) 4/10 (36,4%)	Zoledronic acid Alendronate	*In vivo* study (rats), histopathology (H&E)	Maahs *et al.*[12]
Fusobacterium, Bacillus, Actinomyces, Candida spp., *Staphylococcus, Streptococcus, Selenomonas, Treponemas*	4*	Pamidronate (1), zoledronic acid (1), alendronate (2)	Clinical study Histopathology (H&E), SEM	Sedghizadeh *et al.*[32]
Actinomyces Yeast colonies	13/13 (100%) *Common finding**	Zoledronic acid, alendronate, risedronate, ibandronate	Retrospective study, histopathology, SEM	Lee *et al.*[33]
Actinomyces *Actinomyces israelii* *Candida*	26/26 (100%) 7/7 (100%) 3/26 (11,54%)	Zoledronic acid, pamidronate	Clinical study, histopathology (H&E, Grocott, Gram, PAS, Goldner or Elastica-van Gieson) PCR, SEM	Hansen *et al.*[42]
Actinomyces *Candida albicans*	1/4 (25%) 2/4 (50%)	Pamidronate and zoledronic acid (n=2) Zoledronic acid (n=2)	Clinical study, histopathology (H&E, PAS) and microbiology	Merigo *et al.* [43]
Actinomyces	28/30 (93%)	Pamidronate, zoledronic acid, alendronate, risedronate, clodronate	Clinical study, histopathology (Gram and PAS)	Lazarovici *et al.*[44]
Actinomyces	1/1 (100%)	Clodronate	Clinical study, histopathology (H&E)	Senel *et al.*[45]
Actinomyces *Peptostreptococcus, Streptococcus, Eikenella, Prevotella, Porphyromonas, Fusobacterium*	7/20 (35%) 9/20 (45%)	Zoledronic acid, pamidronate	Clinical study, histopathology Culture	Badros *et al.*[46]
Actinomyces	46/64 (72%)	Zoledronic acid, pamidronate, alendronate, ibandronate, risedronate	Clinical study, histopathology	Jacobsen *et al.*[47]
Actinomyces, Lactobacillus, Candida glabrata and other microorganisms	6*	Zoledronic acid, pamidronate	Clinical study, microbiology	Dannemann *et al.*[48]

Streptococcus intermedius, Peptostreptococcus spp., *Bacteroides melaninogenicus*,	1/3 (33.3%)	Zoledronic acid, alendronate	Clinical study, culture	Wongchuensoontorn *et al.*[49]
Actinomyces israelii, Bacteroides fragilis	1/3 (33.3%)			
Enterococcus faecalis, Bacteroides fragilis	1/3 (33.3%)			
Actinomyces spp., *Streptococcus* spp., *Prevotella*, *Klebsiella* e *Pseudomonas*	12*	Alendronate, ibandronate, etidronate	Retrospective study, histopathology	O'Ryan & Lo[50]

H&E= hematoxylin and eosin; PAS=periodic acid-Schiff; SEM= scanning electron microscopy
*Without other specification

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3 ARTIGO 2

O artigo a seguir intitula-se ***Microorganisms related to surgical wounds in maxilla of rats subjected to bisphosphonate therapy: a histomorphometric analysis*** e foi formatado de acordo com as normas do periódico *International Journal of Oral and Maxillofacial Surgery* (Anexos C e D).

Microorganisms related to surgical wounds in maxilla of rats subjected to bisphosphonate therapy: a histomorphometric analysis

Renata Chiapinotto Boff

Ana Carolina Uchoa Vasconcelos

Fernanda Gonçalves Salum

Maria Antonia Figueiredo

Tiago Giuliani Lopes

Vinicius Duval da Silva

Karen Cherubini

Postgraduate Program of Dental College, Pontifical Catholic University of Rio Grande do Sul – PUCRS, Porto Alegre, RS, Brazil

Department of Pathology, Hospital São Lucas, Pontifical Catholic University of Rio Grande do Sul – PUCRS, Porto Alegre, RS, Brazil

Running title: *Microorganisms and bisphosphonates*

Keywords: osteonecrosis; bisphosphonates; microorganisms; *Actinomyces* sp.; *Candida* sp.

Corresponding author

Karen Cherubini

Serviço de Estomatologia, Hospital São Lucas - PUCRS

Av. Ipiranga, 6690/231

Porto Alegre, RS, Brazil 90610-000

Telephone/Fax: 55 51 33203254

Email: karen.cherubini@pucrs.br

ABSTRACT

This study determined the microorganisms in surgical wounds in the maxillae of rats subjected to bisphosphonate therapy. Rats were allocated into three groups: zoledronic acid, clodronate and control. Tooth extractions and soft tissue wounds were performed and histologically analyzed (Gram, Gomori-Grocott and immunohistochemical staining). At the tooth extraction site, *Actinomyces* occurred most in the zoledronic acid group, but more in both test groups in soft tissue wound. *Candida* did not significantly differ between the groups. At the tooth extraction site, Gram-positive single cocci were more prevalent in the zoledronic acid group, whereas more prevalent in both test groups in soft tissue wound. Gram-negative single cocci did not show any significant difference between the groups at the tooth extraction site; in soft tissue wound, they were more prevalent in the zoledronic acid group. At the tooth extraction site, bacilli did not differ between the groups, whereas they showed significantly higher counts in the test groups in soft tissue wound. Diplococci, streptococci and staphylococci were rarely found, without differences between the groups. *Actinomyces* was the most prevalent microorganism, followed by single cocci and bacilli. Streptococci, staphylococci, *Candida* and diplococci did not seem to play a significant role in these lesions.

INTRODUCTION

Bisphosphonates have been widely used to treat diseases characterized by intense bone resorption such as postmenopausal osteoporosis, Paget's disease and bone metastases of cancer.[1] However, these drugs have also been associated with an important side effect, bisphosphonate-related osteonecrosis of the jaws (BRONJ). This condition is seen on clinical examination as spontaneous bone exposure or non-healing tooth extraction wounds, which may or may not involve infection, fistulization and fracture.[2]

Bisphosphonate potency, mechanism of action and potential to cause BRONJ vary according to the presence or absence of nitrogen in the side chain chemical structure. After being internalized by osteoclasts, non-nitrogen containing bisphosphonates are metabolized into non-hydrolyzable ATP analogues, whose intracellular accumulation determines osteoclast function loss or apoptosis. Nitrogen-containing bisphosphonates, on the other hand, inhibit farnesyl pyrophosphate synthase, a key enzyme of the mevalonate pathway, which is responsible for cholesterol and isoprenoid lipid production. This inhibition reduces cellular activity and promotes apoptosis in osteoclasts.[3-5] Nitrogen-containing bisphosphonates are more potent and far most associated with BRONJ than non-nitrogen containing ones.[4,6,7] Zoledronic acid is a potent nitrogen-containing bisphosphonate most used to treat bone metastases, whereas clodronate, also used for this purpose, belongs to the non-nitrogen-containing group.[8]

Although BRONJ etiopathogenesis has not yet been completely elucidated, it has been classified as multifactorial. Studies have reported the following factors: (a) the severe suppression of bone remodeling,[9,10] (b) bisphosphonate toxicity to bone and soft tissues,[11] and (c) the hypovascularization caused by angiogenesis suppression.[12,13] Recently, infectious processes, which were first classified as a secondary factor, have been pointed as having a major role in the disease development.[14,15]

Sedghizadeh *et al.*[16] observed multiple bacterial morphotypes in BRONJ lesions, especially *Fusobacterium*, *Streptococcus*, *Actinomyces*, *Selenomonas* and *Bacillus*. Such microorganisms comprise Gram-positive and Gram-negative ones, as well as aerobes and anaerobes, normally found in the oral cavity and many times associated with dental infection. Moreover, the authors also found fungal organisms compatible with *Candida albicans* colonizing bone in all cases studied and also coaggregating bacterial organisms of biofilm. *Actinomyces* colonies in contact with non-vital bone have been a consistent histological finding in BRONJ cases,[12,17,18] but the importance of other microorganisms such as *Candida albicans* has not yet been determined. According to Kos and Luczak,[19] bone coated with bisphosphonate is more susceptible to colonization, because of a more intense bacterial adhesion to bone, probably by direct electrostatic interaction between the amino-cationic group of nitrogen-containing bisphosphonates and Gram-positive bacteria.

BRONJ is a serious complication of bisphosphonate use, whose resolution is difficult to achieve, and a specific etiologic factor is unknown.[15,20,21] Up to now, microorganisms have been the major target in clinical approach of patients suffering from this disease.[15] Accordingly, knowledge of the microbiota involved can improve the rational use of drugs to prevent or to treat BRONJ. The present study evaluated, by means of histomorphometry in Gram, Gomori-Grocott and immunohistochemical staining, the microorganisms found in surgically induced wounds in the maxillae of rats subjected to nitrogen-containing and non-nitrogen-containing bisphosphonate therapy.

Material and methods

This study was approved by the Ethics Committee for Animal Use of Pontifical Catholic University of Rio Grande do Sul. The sample comprised 34 female Wistar rats (*Rattus norvegicus*), 120 days old and mean weight of 230 g. The animals were maintained in appropriate cages, at 22ºC and light/dark cycle of 12 hours (lights turned on at 7:00 a.m.

and turned off at 7:00 p.m.). Nuvilab-Cr1 chow (Nuvital, Colombo, PR, Brazil) and filtered water were given *ad libitum*. The rats were allocated into 3 groups according to the treatment received: (1) zoledronic acid group: 12 rats treated with zoledronic acid (Novartis Pharma AG, Basileia, Switzerland), (2) clodronate group: 12 rats treated with clodronate (Jenahexal Pharma GmbH, Turingia, Germany) and (3) control group: 10 rats treated with normal saline. Bisphosphonates were administered until the end of the experiment by the intraperitoneal (IP) route, every 28 days, at doses of 0.6 mg/kg for zoledronic acid[22,23] and 20 mg/kg for clodronate.[23] The control group received 1 mL of normal saline, IP, also every 28 days. The animals were subjected to tooth extractions and surgically induced wound of soft tissue as follows.

Tooth extractions were performed at 60 days after starting the treatment, under IP anesthesia with 5% ketamine hydrochloride (Vetbrands, Jacareí, SP, Brazil) at 100 mg/kg and 2% xylazine hydrochloride (Vetbrands) at 10 mg/kg. The 3 upper right molars were extracted using a Hollenback carver #3S (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) and pediatric forceps (Edlo, Canoas, RS, Brazil), both previously adapted for the tooth size. Right after the tooth extractions, an elliptical, 3 mm long and 1 mm deep surgical wound was made in the soft tissue of the left side of the hard palate, considering the left second upper molar as a reference and using a Bard-Parker scalpel handle with a #15 blade (Solidor, São Paulo, SP, Brazil). In the postoperative period, 50 mg/kg paracetamol (Medley S/A, Campinas, SP, Brazil) was administered IP. The animals were euthanized by means of deep anesthesia with isoflurane (Cristália, Porto Alegre, RS, Brazil), at 102 days of bisphosphonate therapy. The maxillae were dissected, and the specimens fixed in 10% buffered formalin (TopGlass, Porto Alegre, RS, Brazil) for 24 hours. Next, specimens were decalcified with ethylenediaminetetraacetic acid (EDTA, Biodinâmica, Ibiporã, PR, Brazil) for 30 days and embedded in paraffin.

Histological processing

Histological sections were obtained and processed for hematoxylin and eosin (H&E) and Gomori-Grocott staining following the standard-protocol, as well as for Brown-Hopps modified Gram[24] and immunohistochemical staining as described below. H&E technique was used to evaluate the adequacy of the samples.

Brown-Hopps modified Gram

After being deparaffinized, the 4 µm-thick histological sections were stained with 1% crystal violet solution for 1 minute and the slides rinsed in running water. Gram's iodine was applied for 1 minute and the slides rinsed. The slides were destained with acetone immediately followed by rinsing in running water. Afterwards, 1% basic fuchsin was applied for 5 minutes, and the slides rinsed in running water and dried. Differentiation was performed with galego solution twice, 1 minute each. The slides were rinsed again, and acetone was applied for 30 seconds followed by picric acid-acetone solution for 3 minutes and two changes of acetone-xylene. Finally, xylene was applied for dehydration, and coverslips were mounted with Entellan (Merck, Darmstadt, Germany). Gram-positive bacteria stained blue, and Gram-negative, red; the background stained yellow.

Immunohistochemical processing

Three micrometer-thick sections were deparaffinized and rehydrated. Antigen retrieval was performed at 98°C with Envision Flex *(*Dako Corporation, Santa Barbara, CA, USA*)* in pTLINK (Dako), and endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol. The immunohistochemical staining method based on capillary action was used (Shandon Sequenza Immunostaining, Thermo Scientific Shandon, Fremont, CA, USA), where the sections were incubated with polyclonal anti-*Candida albicans* antibody (Abnova, Jhongli, Taoyuan County, Taiwan) diluted in antibody diluent with background

reduction (1:5000, Dako). After rinsing in PBS, the Advance HRP system (Dako) was applied, and the sections were incubated with diaminobenzidine solution (Liquid Substrate Chromogen System, Dako). After being washed and counterstained with Harris hematoxylin and incubation with 37 mM ammonia, the sections were dehydrated in ethanol and xylene and coverslipped with Entellan (Merck).

Capture and analysis of the images

Images were captured by means of a Zeiss Axioskop 40 (Carl Zeiss, Oberkochen, Germany) light microscope equipped with a CoolSnap Pro videocamera (Media Cybernetics, Bethesda, MD, USA) connected to a microcomputer with a plaque Image Pro Capture Kit. Images were captured in a standardized manner using the 100x objective for Gram, and the 20x objective for Grocott and immunohistochemical staining. In the Gram staining, 20 fields were captured for each slide (10 for tooth extraction site and 10 for soft tissue wound); in Grocott staining, 12 fields per slide (6 for tooth extraction site and 6 for soft tissue wound); and in immunohistochemistry, 8 fields per slide (4 for tooth extraction site and 4 for soft tissue wound). Images were stored in TIFF (Tagged Image File Format) format and analyzed by one blinded and calibrated observer. The analyses were performed in Image Pro Plus 4.5.1 software (Media Cybernetics) using the manual point counting technique for Grocott staining (Fig. 1) and the semiautomated segmentation technique for immunohistochemistry[25] (Fig. 2). Blindness consisted in not knowing the group to which each image belonged. Intraobserver calibration was performed by analyzing 24 images in each technique, which were analyzed twice, at different moments. The results of these two analyses were tested by the intraclass correlation coefficient, which showed $r>0.8$. Images in Gram staining were analyzed semiquantitatively using the following scores: (-) = absent; (+) = slight (≤ 25% of the field); (++) = moderate (>25% and ≤ 50% of the field);

(+++) = intense (>50%). Analyses were performed according to the criteria reported in the literature.[26-28]

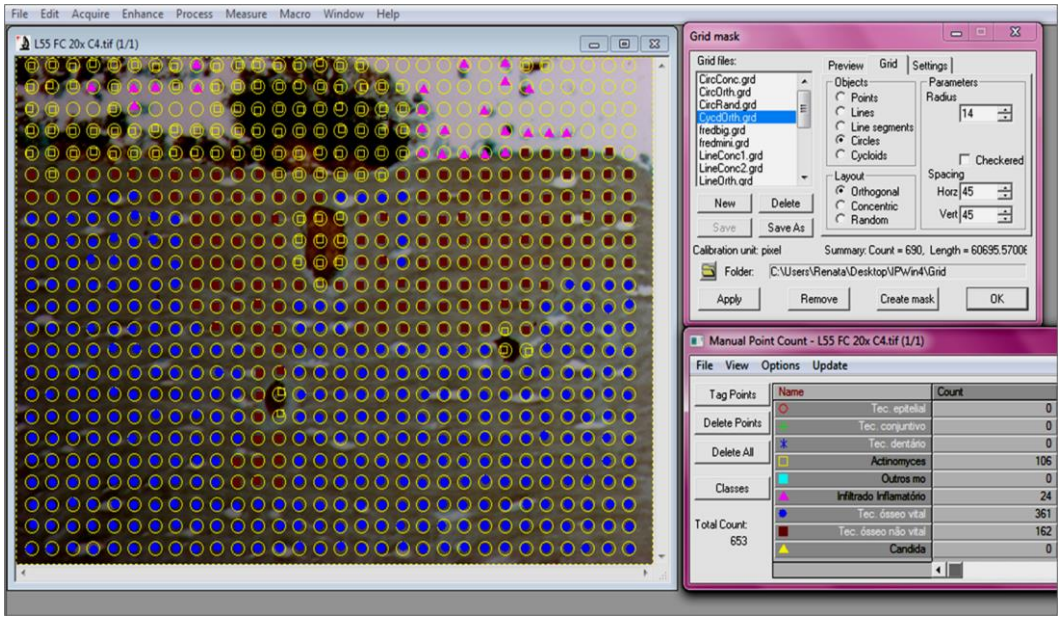

Figure 1: Analysis of Gomori-Grocott image by means of manual point counting technique in Image ProPlus 4.5.1 software (Media Cybernetics, Bethesda, MD, USA)

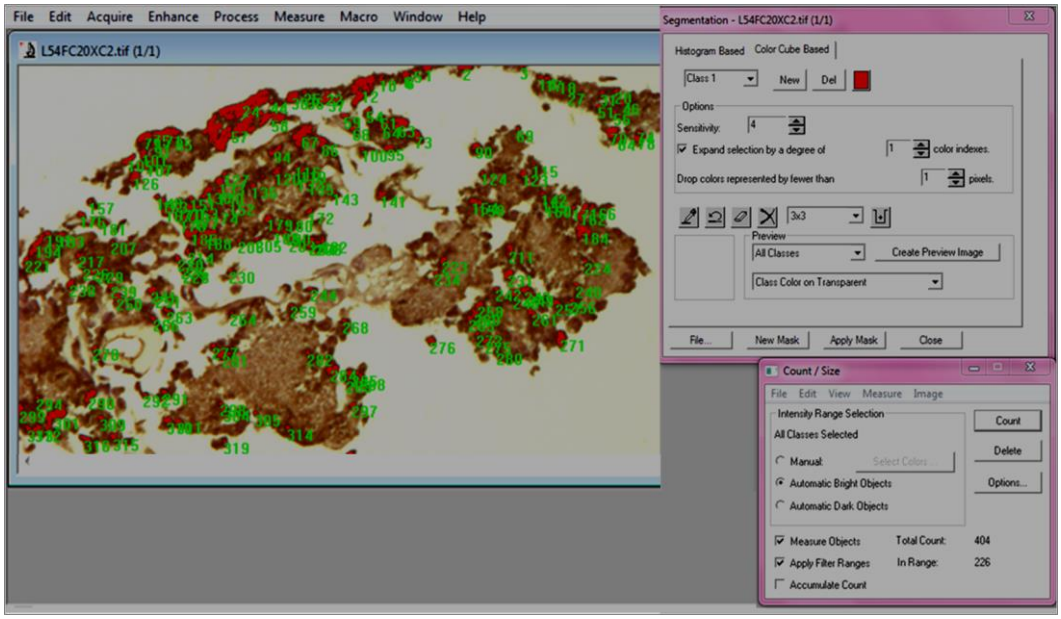

Figure 2: Analysis of immunohistochemistry image by means of semiautomated segmentation technique in Image ProPlus 4.5.1 software (Media Cybernetics, Bethesda, MD, USA

Statistical analysis

The results were analyzed by means of descriptive (mean, standard deviation, median, 25th percentile and 75th percentile) and inferential statistics. Kruskal-Wallis and chi-square tests were applied and complemented, respectively, by the Student-Newman-Keul test and analysis of adjusted residuals. Data were processed in SPSS 18.0 software (Statistical Package for the Social Sciences, Chicago, IL, USA) and evaluated at the 5% level of significance.

RESULTS

Gram staining (Fig. 3)

The Gram staining results are presented in Tables 1 and 2 and were tested by the Kruskal-Wallis test complemented by the Student-Newman-Keuls test and also by the chi-square test complemented by adjusted residual analysis (α=0.05).

Actinomyces sp.

At the tooth extraction site, the zoledronic acid group showed significantly higher *Actinomyces* sp. counts than did the clodronate and control groups, which did not significantly differ from each other. At the soft tissue wound site, the three groups differed significantly regarding this variable, with the highest values occurring in the zoledronic acid group followed by clodronate and control groups (Kruskal-Wallis complemented by Student-Newman-Keuls test, α=0.05).

Single cocci

At the tooth extraction site, Gram-positive single cocci were significantly more prevalent in the zoledronic acid group than in the control group, but this variable did not differ between the zoledronic acid and clodronate groups, or between the clodronate and control groups. At the soft tissue wound site, zoledronic acid and clodronate did not display

significantly different results, but both showed significantly higher values than the control group. Gram-negative single cocci did not show any significant difference between the groups at the tooth extraction site. At the soft tissue wound site, they were more prevalent in the zoledronic acid group compared to clodronate and control, without any significant difference between these two groups (Kruskal-Wallis complemented by Student-Newman-Keuls test, α=0.05).

Diplococci

Both Gram-positive and Gram-negative diplococci did not differ between the groups at either the tooth extraction site or the soft tissue wound site (Kruskal-Wallis complemented by Student-Newman-Keuls test, α=0.05).

Streptococci and staphylococci

Streptococci and staphylococci were rarely found and their counts did not significantly differ between the groups at either the tooth extraction site or the soft tissue wound site (Kruskal-Wallis complemented by Student-Newman-Keuls test, α=0.05).

Bacilli

At tooth extraction site, both Gram-positive and Gram-negative bacilli did not significantly differ between the groups. At the soft tissue wound site, both Gram-positive and Gram-negative bacilli differed significantly between the test groups and control, where the zoledronic acid and the clodronate groups did not differ from each other, but both showed higher values than the control group (Kruskal-Wallis complemented by Student-Newman-Keuls test, α=0.05).

Gomori-Grocott staining (Fig. 3)

The results of Grocott staining are presented in Tables 3 and 4 and were evaluated by the Kruskal-Wallis test complemented by the Student-Newman-Keuls test (α=0.05).

At the tooth extraction site, the proportion of *Actinomyces* sp. was significantly greater in the zoledronic acid group compared to the clodronate and control groups, but the latter two did not differ significantly from each other. At the soft tissue wound site, the proportion of *Actinomyces* sp. did not differ significantly between the zoledronic acid and clodronate groups, but these two groups showed higher values than the control group. *Candida* sp. was rarely found and there was no significant difference in its proportions between the three groups analyzed at either the tooth extraction site or the soft tissue wound site.

At the tooth extraction site, the proportion of inflammatory infiltrate was greater in the zoledronic acid group than in the clodronate and control groups, without significant difference between these two groups. At the soft tissue wound site, this variable did not significantly differ between the zoledronic acid and the control groups, but these two groups showed lower values than the clodronate group. Non-vital bone was significantly greater in the test groups than in the control, although this difference was significant for the soft tissue wound site ($P<0.001$) but not for tooth extraction site ($P=0.068$).

Immunohistochemical analysis

Immunohistochemical quantification for C*andida albicans* at both tooth extraction site and soft tissue wound site did not differ significantly between the three groups analyzed (Kruskal-Wallis test, $\alpha = 0.05$, Table 5).

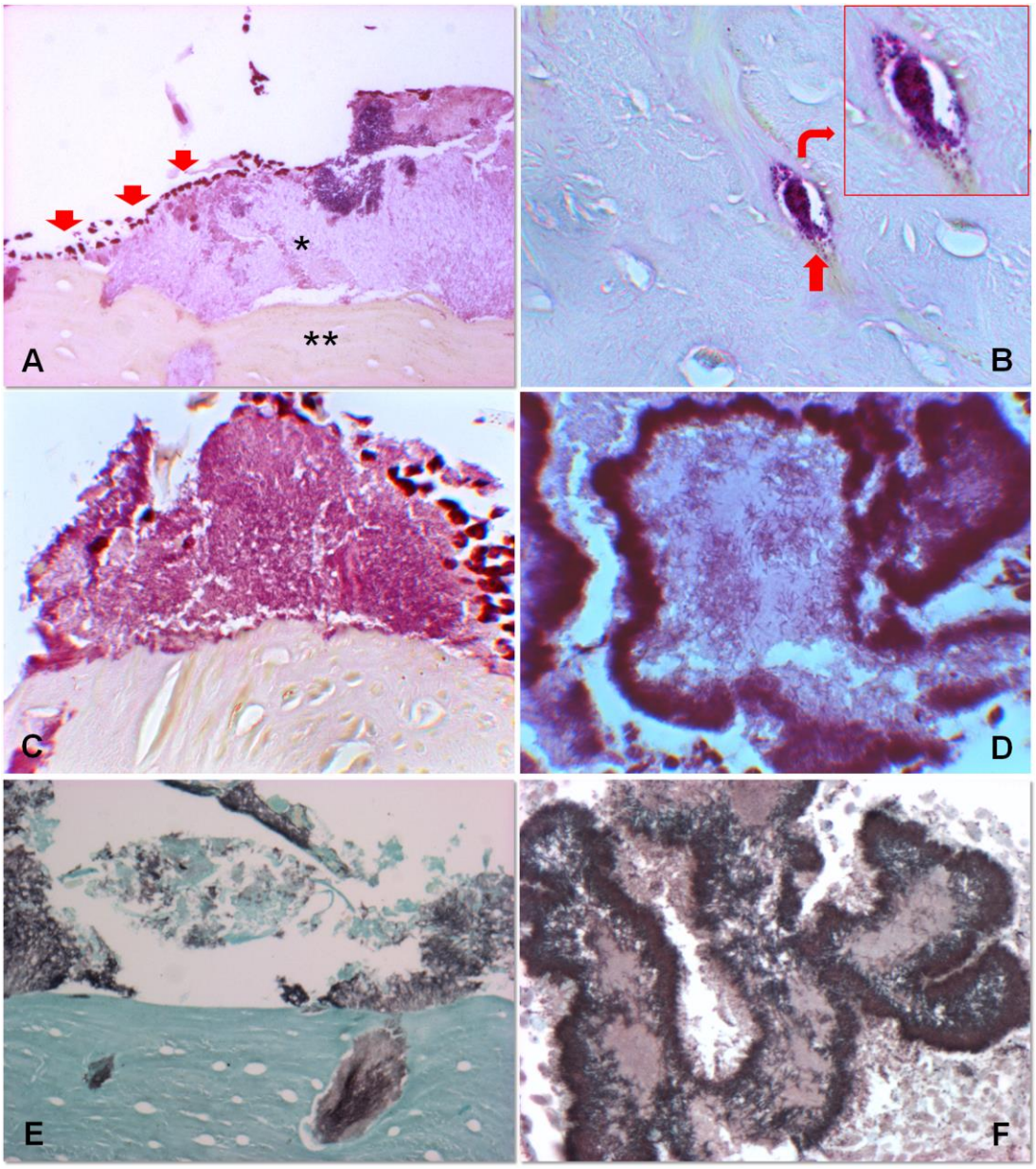

Figure 3: Histopathological examination of biofilm in surgical wound sites. **Gram staining**: Relation of biofilm (*) with inflammatory cells (arrows) and non-vital bone tissue (**) (A, 400x); Biofilm within bone lacuna showing cocci [arrows, (B, 1000x)]; Biofilm on the surface and within bone lacunae (C, 400x); *Actinomyces* colonies (D, 1000x). **Gomori-Grocott**: *Actinomyces* colonies (E, 400x and F, 1000x)

Table 1 - Gram stain analysis at the tooth extraction site in the zoledronic acid, clodronate and control groups

Microorganisms	**Score**	**Groups**						
		Zoledronic acid		Clodronate		Control		
		n	%	n	%	n	%	*P*
Actinomyces sp.	-	79	65.8	**110***	91.7	75	83.3	<0.01*
	+	1	0.8	0	0.0	0	0.0	
	++	**33***	27.5	7	5.8	9	10.0	
	+++	7	5.8	3	2.5	6	6.7	
	Mean rank	**188^{A}**		**146.44 ^{B}**		**160.92 ^{B}**		<0.01**
Gram + single cocci	-	29	24.2	39	32.5	**48***	53.3	<0.01*
	+	62	51.7	**64***	53.3	23	25.6	
	++	28	23.3	15	12.5	17	18.9	
	+++	1	0.8	2	1.7	2	2.2	
	Mean rank	**184.50^{A}**		**163.28^{AB}**		**143.12^{B}**		<0.01**
Gram – single cocci	-	17	14.2	22	18.3	14	15.6	0.231
	+	80	66.7	87	72.5	65	72.2	
	++	**23***	19.2	11	9.2	11	12.2	
	+++	0	0.0	0	0.0	0	0.0	
	Mean rank	175.86		156.07		164.26		0.133**
Gram + diplococci	-	115	95.8	116	96.7	83	92.2	0.302*
	+	5	4.2	4	3.3	7	7.8	
	++	0	0.0	0	0.0	0	0.0	
	+++	0	0.0	0	0.0	0	0.0	
	Mean rank	164.38		163.00		170.33		0.303**
Gram – diplococci	-	95	79.2	104	86.7	76	84.4	0.426*
	+	24	20.0	16	13.3	14	15.6	
	++	1	0.8	0	0.0	0	0.0	
	+++	0	0.0	0	0.0	0	0.0	
	Mean rank	172.50		159.93		163.59		0.270**
Streptococci	-	119	99.2	117	97.5	87	96.7	0.432*
	+	1	0.8	3	2.5	3	3.3	
	++	0	0.0	0	0.0	0	0.0	
	+++	0	0.0	0	0.0	0	0.0	
	Mean rank	163.38		166.13		167.50		0.433**
Staphylococci	-	119	99.2	120	100.0	90	100.0	0.416*
	+	1	0.8	0	0.0	0	0.0	
	++	0	0.0	0	0.0	0	0.0	
	+++	0	0.0	0	0.0	0	0.0	
	Mean rank	166.38		165.00		165.00		0.417**
Gram + bacilli	-	54	45.0	54	45.0	42	46.7	0.491*
	+	39	32.5	49	40.8	30	33.3	
	++	27	22.5	16	13.3	17	18.9	
	+++	0	0.0	1	0.8	1	1.1	
	Mean rank	169.23		162.07		165.11		0.820**

Gram – bacilli	-	31	25.8	31	25.8	21	23.3	0.489*
	+	68	56.7	77	64.2	54	60.0	
	++	21	17.5	12	10.0	15	16.7	
	+++	0	0.0	0	0.0	0	0.0	
	Mean rank	168.19		158.93		170.68		0.543**

*Chi-square test complemented by adjusted residual analysis; α=0.05. Bold values indicate significant difference.

**Kruskal-Wallis complemented by the Student-Newman-Keuls test, $P \leq 0.05$; different letters indicate significant difference between groups. Score: (-) absent; (+) slight; (++) moderate; (+++) intense

Table 2 - Gram stain analysis at the soft tissue wound site in the zoledronic acid, clodronate and control groups

Microorganisms	**Score**	**Groups**						
		Zoledronic acid		Clodronate		Control		
		n	%	n	%	n	%	*P*
Actinomyces sp.	-	53	44.2	69	57.5	**80***	80.0	<0.01*
	+	7	5.8	8	6.7	0	0.0	
	++	**35***	29.2	27	22.5	13	13.0	
	+++	**25***	20.8	16	13.3	7	7.0	
	Mean rank	**197.39^{A}**		**171.94^{B}**		**136.51^{C}**		<0.01**
Gram + Single cocci	-	16	13.3	26	21.7	**40***	40.0	<0.01*
	+	55	45.8	57	47.5	39	39.0	
	++	**49***	40.8	37	30.8	21	21.0	
	+++	0	0.0	0	0.0	0	0.0	
	Mean rank	**195.14^{A}**		**172.53^{A}**		**138.49^{B}**		<0.01**
Gram - Single cocci	-	9	7.5	15	12.5	**21***	21.0	<0.01*
	+	80	66.7	89	74.2	69	69.0	
	++	**28***	23.3	16	13.3	10	10.0	
	+++	**3***	2.5	0	0.0	0	0.0	
	Mean rank	**192.32^{A}**		**166.28^{B}**		**149.39^{B}**		<0.01**
Gram + diplococci	-	117	97.5	117	97.5	97	97.0	0.966*
	+	3	2.5	3	2.5	3	3.0	
	++	0	0.0	0	0.0	0	0.0	
	+++	0	0.0	0	0.0	0	0.0	
	Mean rank	170.25		170.25		171.10		0.966**
Gram – diplococci	-	94	78.3	96	80.0	81	81.0	0.113*
	+	22	18.3	24	20.0	19	19.0	
	++	**4***	3.3	0	0.0	0	0.0	
	+++	0	0.0	0	0.0	0	0.0	
	Mean rank	173.55		169.60		167.92		0.819**
Streptococci	-	119	99.2	114	95.0	99	99.0	0.059*
	+	1	0.8	**6***	5.0	1	1.0	
	++	0	0.0	0	0.0	0	0.0	
	+++	0	0.0	0	0.0	0	0.0	
	Mean rank	167.92		175.00		168.20		0.059**
Staphylococci	-	120	100.0	119	99.2	100	100.0	0.399*
	+	0	0.0	1	0.8	0	0.0	
	++	0	0.0	0	0.0	0	0.0	
	+++	0	0.0	0	0.0	0	0.0	
	Mean rank	170.00		171.42		170.00		0.400**
Gram + bacilli	-	45	37.5	27	22.5	**54***	54.0	<0.01*
	+	36	30.0	**51***	42.5	26	26.0	
	++	38	31.7	40	33.3	20	20.0	
	+++	1	0.8	2	1.7	0	0.0	
	Mean rank	**172.90^{A}**		**193.88^{A}**		**139.57^{B}**		<0.01**

Gram – bacilli	-	29	24.2	21	17.5	**40***	40.0	<0.01*
	+	63	52.5	**85***	70.8	49	49.0	
	++	**28***	23.3	14	11.7	11	11.0	
	+++	0	0.0	0	0.0	0	0.0	
	Mean rank	**183.49^{A}**		**178.47^{A}**		**145.35^{B}**		<0.01**

*Chi-square test complemented by adjusted residual analysis; α=0.05. Bold values indicate significant difference. **Kruskal-Wallis complemented by the Student-Newman-Keuls test, $P \leq 0.05$; different letters indicate significant difference between groups. Score: (-) absent; (+) slight; (++) moderate; (+++) intense

Table 3 - Gomori-Grocott staining analysis at the tooth extraction site in the zoledronic acid, clodronate and control groups

Feature	Groups															
	Zoledronic acid (%)					Clodronate (%)					Control (%)					
	MD	P25	P75	Mean	SD	MD	P25	P75	Mean	SD	MD	P25	P75	Mean	SD	*P**
Actinomyces sp.	**6.59^{A}**	3.94	20.76	12.58	11.73	**2.85^{B}**	0.46	5.17	3.43	3.55	**0.27^{B}**	0.00	3.46	2.91	5.95	0.004
Candida sp.	0.00	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.03	0.10	0.00	0.00	0.00	0.00	0.00	0.424
Inflammatory infiltrate	**0.58^{A}**	0.00	4.08	2.98	5.12	**0.00^{B}**	0.00	0.35	1.55	5.03	**0.00^{B}**	0.00	0.22	0.29	0.67	0.042
Non-vital bone	14.40	3.83	23.96	14.99	11.90	8.85	0.30	14.22	11.65	14.24	0.71	0.00	8.96	4.27	6.23	0.068
Vital bone	10.03	0.63	19.14	11.57	11.30	10.71	1.40	23.00	13.86	14.85	18.49	0.00	27.01	15.88	14.55	0.864
Epithelial tissue	8.97	3.03	24.10	12.43	10.54	15.59	6.42	21.71	16.95	13.25	14.63	10.70	23.45	16.24	7.17	0.465
Connective tissue	42.00	31.89	52.78	41.56	17.86	51.05	37.77	70.66	51.55	18.31	61.19	53.99	68.59	59.46	10.00	0.054
Dental tissue	0.00	0.00	0.00	0.36	1.22	0.00	0.00	0.00	0.54	1.89	0.00	0.00	0.27	0.85	2.34	0.743

* Bold medians followed by different letters indicate features that differed significantly between groups; Kruskal-Wallis test complemented by the Student-Newman-Keuls test, $\alpha = 0.05$

MD=median; P25 = 25th percentile; P75 =75th percentile; SD = standard deviation

Table 4 - Gomori-Grocott staining analysis at the soft tissue wound site in the zoledronic acid, clodronate and control groups

Feature	Groups															
	Zoledronic acid (%)					Clodronate (%)					Control (%)					
	MD	P25	P75	Mean	SD	MD	P25	P75	Mean	SD	MD	P25	P75	Mean	SD	*P**
Actinomyces sp.	**14.60**^{A}	9.67	19.13	14.90	7.97	**15.46**^{A}	2.84	30.16	16.57	13.80	**2.29**^{B}	0.58	3.46	2.20	1.64	0.003
Candida sp.	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.400
Inflammatory infiltrate	**1.12**^{A}	0.00	2.33	1.29	1.41	**5.76**^{B}	2.09	12.41	6.79	5.95	**0.00**^{A}	0.00	1.10	1.97	4.89	0.006
Non-vital bone	**23.43**^{A}	14.35	38.32	26.46	12.31	**21.23**^{A}	14.33	33.21	23.28	12.58	**4.03**^{B}	0.00	6.22	3.87	3.40	0.000
Vital bone	**5.74**^{AB}	0.88	31.49	13.04	15.36	**0.72**^{A}	0.00	5.21	3.71	6.42	**14.14**^{B}	4.18	32.06	18.02	16.04	0.030
Epithelial tissue	9,07	3.16	11.77	7.83	5.12	8,39	5.57	13.07	10.66	8.16	10,57	4.16	27.12	14.36	11.31	0.446
Connective tissue	**29.04**^{A}	23.89	50.91	33.53	16.13	**36.41**^{A}	22.15	55.25	37.55	16.45	**53.83**^{B}	49.75	69.07	57.87	15.84	0.013
Dental tissue	0.00	0.00	1.61	2.35	6.07	0.00	0.00	1.65	1.40	2.85	0.00	0.00	0.76	1.64	4.47	0.875

*Bold medians followed by different letters indicate features that differed significantly between groups; Kruskal-Wallis test complemented by the Student-Newman-Keuls test, α=0.05

MD=median; P25 = 25th percentile; P75 =75th percentile; SD = standard deviation

Table 5. Quantification (μm^2) of anti-*Candida albicans* antibody immunostaining at tooth extraction and soft tissue wound sites in the zoledronic acid, clodronate and control groups

			Tooth extraction	**Soft tissue wound**
Groups	Zoledronic acid	Median	4,658.73	4,464.29
		P25	122.63	2,935.10
		P75	16,358.48	15,266.80
		Mean	8,239.61	9,861.53
		SD	9,207.16	11,781.50
	Clodronate	Median	3,769.79	7,799.06
		P25	1,446.73	3,363.48
		P75	10,430.22	15,977.39
		Mean	6,648.98	10,654.86
		SD	7,267.71	9,000.56
	Control	Median	3,706.74	3,432.66
		P25	0.00	584.33
		P75	10,483.64	9,147.74
		Mean	6,292.87	5,751.61
		SD	7,793.52	6,552.54
		*P**	0.850	0.283

*Kruskal-Wallis test; $\alpha = 0.05$

P25 = 25th percentile; P75 =75th percentile; SD= standard deviation

DISCUSSION

Actinomyces sp. was the most frequently found microorganism at the surgical wound sites, with counts being significantly higher in the zoledronic acid and clodronate groups than control. This happened with both Gram and Grocott staining, and also the bisphosphonate groups showed higher amounts of non-vital bone, which corroborates reports in the literature about the predominance of *Actinomyces* spp. in BRONJ lesions, even using the H&E technique.[22,29]

Candida spp. has been reported as an important microorganism in BRONJ lesions,[16,17,30] but our results do not agree with this finding. We observed insignificant rates for *Candida* sp. in Grocott staining, and even though immunohistochemistry (IHC) showed positive staining, it did not show a significant difference between the test groups and control. This result suggests that *Candida* does not play an important role in the lesions associated with bisphosphonates. *Candida albicans* is a facultative anaerobe whose primary habitats are aerobic sites such as the dorsum of the tongue[31] and other epithelial surfaces of the oral mucosa.[31] Moreover, it is known that *Candida albicans* grows slowly in the anaerobic environment not supporting biofilm formation,[32] which suggests that BRONJ sites would not favor its colonization.

Anyway, considering that the immunostaining with anti-*Candida albicans* antibody occurred mainly on the periphery of *Actinomyces* colonies, some points should be taken into account here. First, it is known that *Actinomyces* and *Candida albicans* can coaggregate, where a protein on the *Candida* surface may interact with carbohydrates or carbohydrate-containing molecules on the surface of the *Actinomyces*. This intergeneric coaggregation between these oral microorganisms is considered an important factor in oral colonization by the yeast.[33] From this point of view, it is possible that such coaggregation was not observed in our sample on Grocott staining because of intrinsic limitations of the

technique, whereas immunostaining with anti-*Candida albicans* antibody was capable of disclosing it. On the other hand, another point to state is the possibility that the positive immunostaining resulted from a cross-reaction through some epitopes shared between *Actinomyces* and *Candida*.[34] This would be corroborated by the low frequency of *Candida* in the oral cavity of rats[35] and constitutes another plausible theory to understand the discrepancies between our Grocott and IHC results. Actually, to clarify this issue, the samples should be analyzed using other methods such as quantitative PCR and scanning electron microscopy.

After *Actinomyces* sp., single cocci and bacilli were the bacteria most observed in the wound sites. The significant differences of occurrence of these microorganisms between the groups varied according to their Gram positivity or negativity, bisphosphonate type and also the wound site. In the soft tissue wound, both test groups had higher amounts of *Actinomyces* sp., single cocci (Gram-positive) and bacilli (Gram-positive and Gram-negative) than controls; at the tooth extraction site differences in relation to controls occurred just in the zoledronic acid group for *Actinomyces* sp. and Gram-positive cocci. Considering that soft tissue wounds were adjacent to periodontal structures and sometimes developed affecting them, this finding suggests that the anatomic site, favoring colonization by anaerobes or microaerophiles, can also favor the colonization of the wounds by the resident microorganisms.[36]

Kos and Luczak[19] defended the theory that nitrogen containing bisphosphonates would increase bacterial adhesion to bone through electrostatic interactions between the amino-cationic group and Gram-positive bacteria. Our findings disagree with this possibility as we observed that at the surgical wound sites the occurrence of Gram-positive single cocci did not vary significantly between the zoledronic acid (nitrogen containing) and the clodronate (non-nitrogen containing) groups, whereas Gram-positive

diplococci, streptococci and staphylococci bacteria did not vary significantly between the three groups analyzed. Also, Gram-positive bacilli did not vary significantly between the three groups at the tooth extraction site, while at the soft tissue wound site the test groups did not differ significantly from each other. Therefore, only *Actinomyces* sp. behavior could corroborate the theory of Kos and Luczak,[19] as it was more prevalent in the zoledronic acid than clodronate group at the tooth extraction site. However, at the soft tissue wound site, the three groups differed significantly from each other. Accordingly, although without statistical significance, the zoledronic acid group had higher prevalence of non-vital bone at the tooth extraction site, which could be the cause of its highest prevalence of *Actinomyces* sp. In this context, it seems that the type of bacteria (Gram-positive or Gram-negative) colonizing the wound sites depends more on the resident bacteria of the specific anatomic site than on the nitrogen presence in the bisphosphonate side chain.

BRONJ lesions tend to provide an anaerobic environment, which favors colonization by microaerophiles and anaerobes, such as *Actinomyces israelii*, *Staphylococcus aureus*, *Parvimonas micra*, *Fusobacterium nucleatum*, *Streptococcus oralis*, *Eikenella corrodens* and *Enterococcus faecalis*.[36] *Actinomyces* are filamentous Gram-positive bacteria, which can be strict or facultative anaerobes. To proliferate and determine disease, they need a polymicrobial system and tissue injury. All this microbiota works synergistically to create this environment through the destruction of the vascularized aerobic tissues creating poorly oxygenated granulation tissue, which supports the growth and multiplication of *Actinomyces*. Severity and chronicity of the tissue injury are factors that can increase anaerobe growth.[37] Streptococci, in turn, are prominent members of resident oral microbiota. Most species of streptococci are not virulent, especially in their natural habitat. Thus, although many orofacial streptococcal infections occur, they are

relatively non-specific, resulting from the mixed microbiota.[38] In turn, even though *Staphylococcus aureus* has a remarkable capacity to adapt to different infection niches[39] and is by far the most common pathogenic microorganism involved in the development of osteomyelitis,[40] it was not a remarkable organism in our sample.

In conclusion, *Actinomyces* sp. was the most prevalent microorganism in bisphosphonate-related wounds, followed by single cocci and bacilli. Moreover, streptococci, staphylococci, *Candida* sp. and diplococci did not seem to play a significant role in these lesions, whereas the anatomical site seemed to be a determinant of the microbiota involved. This study provided a general panoramic view of the microorganisms in surgical wounds of rats subjected to bisphosphonate therapy. Further studies applying more specific techniques need to be conducted to classify in a more specific way the microorganisms found here as related to BRONJ lesions.

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Discussão Geral

4 DISCUSSÃO GERAL

Os bisfosfonatos são fármacos amplamente empregados no tratamento de doenças do metabolismo ósseo e metástases ósseas do câncer. Entretanto, seu principal efeito adverso, a osteonecrose maxilar (*bisphosphonate-related osteonecrosis of the jaws*, BRONJ), tem despertado a atenção de diversos pesquisadores (Janovska *et al*., 2013; Kumar *et al*., 2010; Lesclous *et al.*, 2009; Marx *et al*., 2005). Apesar de ainda ser considerada uma condição de baixa prevalência, o crescente número de casos e a morbidade significativa exigem o completo esclarecimento da etiopatogênese da BRONJ (Saia *et al*., 2010).

O mecanismo de ação dos bisfosfonatos baseia-se na inibição da remodelação óssea, com importante efeito sobre osteoclastos e osteoblastos, sendo que a maior potência e, consequentemente, o maior risco de desenvolvimento de BRONJ estão associados à presença de nitrogênio em sua estrutura química (Badel *et al.*, 2013). O ácido zoledrônico, bisfosfonato mais potente para uso clínico, exibe átomos de nitrogênio em uma cadeia heterocíclica, o que lhe confere tal propriedade. O clodronato, por sua vez, pertence à primeira geração de bisfosfonatos e não possui nitrogênio em sua formulação (Russell, 2007).

A presença de microrganismos e trauma constante no meio bucal, associada à alta taxa de remodelação dos ossos maxilares são justificativas apresentadas para a ocorrência de BRONJ exclusivamente nesses ossos (Kumar *et al.*, 2010; Marx *et al*., 2005; Sedghizadeh *et al*., 2008; Sedghizadeh *et al*., 2009). A cavidade oral é habitada por centenas de espécies de bactérias e fungos que, na maioria dos ambientes naturais, existem predominantemente como biofilme em vez de células planctônicas ou *free-floating* (Douglas, 2003). A possibilidade de que o osso impregnado por bisfosfonato

facilite a adesão microbiana por meio de interações eletrostáticas entre microrganismos Gram-positivos e o grupo amino dos bisfosfonatos nitrogenados também já foi apontada (Kos; Luczak, 2009). Considerando que a formação de biofilme, incluindo organismos bacterianos e fúngicos, tem sido discutida por muitos estudos clínicos como um importante agente na etiopatogênese da BRONJ (Ji *et al*., 2012; Kumar *et al*., 2010; Sedghizadeh *et al*., 2008; Sedghizadeh *et al*., 2009), a presente pesquisa teve por objetivo analisar, por meio de histomorfometria nas técnicas de Gram, Grocott e imunoistoquímica, a relação entre a microbiota de feridas cirúrgicas em maxila de ratos e o uso de bisfosfonato nitrogenado e não-nitrogenado.

A prevalência dos diferentes grupos e arranjos microbianos variou em relação à gram positividade ou negatividade, ao tipo de bisfosfonato e sítio anatômico da lesão. A maior prevalência de *Actinomyces* sp. entre os microrganismos corroborou os achados de estudos prévios que relatam ser este o principal componente da microbiota oral associado à osteonecrose (Hansen *et al*., 2006; Kaplan *et al*., 2009; Lee *et al*., 2011; Maahs *et al*., 2011). É importante salientar que esses resultados foram congruentes nas colorações de Gram e Grocott. Os outros microrganismos que exibiram associação com o uso dos fármacos classificaram-se como cocos (Gram-positivos) e bacilos (Gram-positivos e Gram-negativos), sem, no entanto, diferir significativamente entre ácido zoledrônico e clodronato.

Os resultados do presente estudo não corroboram os relatos da literatura sobre a presença de organismos fúngicos compatíveis com *Candida* spp. em casos de BRONJ (Hansen *et al*., 2006; Merigo *et al*., 2005; Sedghizadeh *et al*., 2008; Sedghizadeh *et al*., 2009). O achado de que a prevalência desse fungo não diferiu entre os grupos-teste e o grupo-controle sugere que a *Candida* não tenha papel significativo no desenvolvimento das lesões associadas a bisfosfonatos. Por outro lado, o padrão de marcação do anticorpo

anti-*Candida albicans* foi compatível com a ocorrência de coagregação desta com os *Actinomyces*, ou mesmo de ocorrência de reação cruzada entre esses organismos (Grimaudo *et al.*, 1996; Reiss *et al*., 1985). Também os arranjos de estreptococos e estafilococos constituíram achados raros e sua prevalência não diferiu entre os grupos avaliados. Embora participem da etiopatogênese de diversas enfermidades orais (Krzyściak *et al*., 2013; Liljemark; Bloomquist, 1996; Sklavounos *et al.*, 1986), esses microrganismos não exibiram ocorrência significativa nos grupos-teste (bisfosfonatos), o que sugere que não exerçam papel importante na patogênese da BRONJ.

Ainda, a comparação dos resultados verificados para a ocorrência dos microrganismos entre os grupos ácido zoledrônico e clodronato não está de acordo com a teoria de Kos e Luczak (2009), que aponta a possibilidade de interações eletrostáticas entre microrganismos Gram-positivos e bisfosfonatos nitrogenados. Por outro lado, microrganismos anaeróbios ou anaeróbios facultativos parecem possuir relação direta com os casos de BRONJ, provavelmente beneficiados pela topografia das lesões, que favorece seu crescimento e sobrevida em detrimento de microrganismos aeróbios (Harrington, 1996; Sedghizadeh *et al.*, 2008). Assim, a composição do biofilme parece estar mais relacionada com o sítio das lesões e sua microbiota residente, do que propriamente à presença ou ausência de nitrogênio na estrutura química do fármaco.

Mediante os achados do presente estudo, é possível inferir que os microrganismos desempenham importante papel nas lesões associadas aos bisfosfonatos, uma vez que sua presença é fator constante. Entre as populações microbianas que participam do biofilme nessas lesões destacam-se os *Actinomyces*, os cocos e os bacilos, especialmente os Gram-negativos. Por outro lado, *Candida*, *Streptococcus* e *Staphylococcus* não constituíram achados importantes. Isso sugere que os protocolos de terapia antimicrobiana em pacientes com risco de desenvolver BRONJ ou com lesão já

instalada devam ser voltados para esses microrganismos, estando, de modo geral, dispensadas terapias antifúngicas específicas. A conduta ideal é proceder-se à cultura com antibiograma para determinação da droga antimicrobiana mais adequada a cada caso. Entretanto, para situações clínicas em que a profilaxia faz-se necessária ou mesmo em que a cultura tenha resultado falso-negativo, fato frequente com anaeróbios e anaeróbios facultativos (Schmidt *et al.*, 2011), protocolos de penicilina associados ou não a metronidazol ainda parecem efetivos. Já a terapia antifúngica específica não se faz mandatória, constituindo a clorexidina uma boa opção para a terapia tópica isolada ou complementar à terapia antibacteriana sistêmica, uma vez que seu espectro de ação inclui bactérias Gram-positivas e negativas, bem como organismos fúngicos (Mohammadi; Abbott, 2009; Puig Silla *et al.*, 2008)

O presente estudo estabeleceu um desenho panorâmico dos grupos de microrganismos observados em lesões associadas a procedimentos cirúrgicos e uso de bisfosfonato nitrogenado e não-nitrogenado. Entretanto, novas pesquisas devem ser desenvolvidas para identificar, dentro de tais grupos e de forma mais específica, os agentes efetivamente envolvidos nessas lesões. O conhecimento objetivo e específico dos microrganismos associados à BRONJ, além de nortear condutas preventivas e terapêuticas para pacientes em risco de desenvolver ou já acometidos pela enfermidade, poderá auxiliar no esclarecimento de sua etiopatogênese.

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Anexos

ANEXO A

Normas para submissão de manuscritos ao periódico *Archives of Oral Biology*

ANEXO B

Comprovante de submissão do manuscrito ao periódico *Archives of Oral Biology*

Submission Confirmation for Important aspects regarding the role of microorganisms in bisphosphonate-related osteonecrosis of the jaws

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ANEXO C

Normas para submissão de manuscritos ao periódico *International Journal of Oral and Maxillofacial Surgery*

ANEXO D

Comprovante de submissão de manuscritos ao periódico *International Journal of Oral and Maxillofacial Surgery*

Submission Confirmation for Microorganisms related to surgical wounds in maxilla of rats subjected to bisphosphonate therapy: a histomorphometric analysis

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