FACULDADE DE ODONTOLOGIA

EFEITOS DOS BISFOSFONATOS NITROGENADOS SOBRE O OSSO ALVEOLAR

NICOLE DE MELLO RAHDE

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Uma janela aberta ao conhecimento científico é caminho árduo,
que se percorre com amor à verdade, sabedoria, coragem e
liberdade, com o qual se conquista a paz interior e
o progresso do mundo.

ÉLIDA GOMES TAVARES

(1936 -)

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RESUMO

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Os bisfosfonatos são drogas inibidoras da reabsorção óssea e têm sido associados a uma forma peculiar de osteonecrose dos maxilares. O efeito dessas drogas é investigado principalmente em tíbia e fêmur, sendo poucos os estudos conduzidos em maxila e mandíbula. A presente pesquisa teve por objetivo investigar, ao exame microscópico, o efeito dos bisfosfonatos nitrogenados alendronato e ácido zoledrônico sobre o osso alveolar. Trinta e um ratos fêmeos (*Rattus norvegicus,* Wistar) foram distribuídos em 3 grupos: (1) 11 animais tratados com alendronato (gavagem oral); (2) 10 animais tratados com ácido zoledrônico (intraperitoneal) e (3) 10 animais que não receberam bisfosfonato. Completado o período de 150 dias do início da terapia, os animais foram submetidos à eutanásia. As maxilas foram processadas e cortes histológicos foram corados por hematoxilina e eosina (HE) e picrossírius. Nos cortes corados por HE, foram realizadas a contagem de osteoclastos e a avaliação da densidade trabecular óssea. Nos cortes corados por picrossírius, a densidade de fibras colágenas dos espaços medulares foi determinada. Também foi realizado processamento imunoistoquímico para avaliação da expressão de osteoprotegerina (OPG). As variáveis foram quantificadas com o auxílio dos programas Adobe Photoshop CS3 e Image Pro Plus 4.5.1. O grupo ácido zoledrônico apresentou densidade trabecular significativamente maior que o grupo-controle (ANOVA, teste de Tukey, $P<0,001$), e o grupo alendronato não apresentou diferença significativa quando comparado aos demais grupos ($P>0,05$). Não houve diferença significativa para contagem de osteoclastos, densidade de fibras colágenas dos espaços medulares e expressão de OPG entre os grupos (ANOVA, $P>0,05$). Os resultados permitem concluir que (1) o ácido zoledrônico promove aumento da densidade trabecular do osso alveolar, enquanto o alendronato não produz esse efeito; (2) alendronato e ácido zoledrônico não estão associados à fibrose dos espaços medulares do osso alveolar. Os efeitos do alendronato e do ácido zoledrônico sobre o número de osteoclastos, bem como sobre a expressão imunoistoquímica de OPG, necessitam ser avaliados por novas pesquisas.

Palavras-chave: Bisfosfonatos, Alendronato, Ácido zoledrônico, Osteonec dos maxilares.

SUMMARY

SUMMARY

Bisphosphonates inhibit bone resorption and have been related to a peculiar form of osteonecrosis of the jaws. Nevertheless, bisphosphonate effects have been investigated mainly in bones like tibia and femur, with just few studies conducted on maxilla and mandible. The present research aimed to investigate bisphosphonates microscopic effects on alveolar bone. Thirty one female rats (*Rattus norvegicus*, Wistar) were allocated into 3 groups: (1) 11 animals treated with oral alendronate; (2) 10 animals treated with intraperitoneal zoledronic acid; and (3) 10 animals without bisphosphonate treatment. One hundred and fifty days after the beginning of the treatment, the animals were euthanized. Maxillae were processed and histological sections were stained with hematoxilin and eosin (H&E) to evaluate bone trabecular density and osteoclast count; and with picrosirius to evaluate collagen fiber density in medullary spaces. Immunohistochemical expression of osteoprotegerin (OPG) was also evaluated. The variables were quantified with Adobe photoshop CS3 and Image Pro Plus 4.5.1 softwares. Zoledronic acid group showed higher trabecular density than control group (ANOVA, Tukey's test, $P<0.001$), and alendronate group did not show significant difference when compared to the other groups ($P>0.05$). There was no significant difference in osteoclast count, collagen fiber density in medullary spaces and OPG expression among the groups (ANOVA, $P>0.05$). According to the results, (1) zoledronic acid promotes trabecular density increase while alendronate does not; (2) alendronate and zoledronic acid use is not associated to alveolar bone marrow fibrosis; (3) alendronate and zoledronic acid effects on osteoclast number and immunohistochemical expression of OPG need to be evaluated by other investigations.

Key-words: Bisphosphonates, Alendronate, Zoledronic acid, Osteonecrosis of the jaws.

SUMÁRIO

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INTRODUÇÃO

1. INTRODUÇÃO

Os bisfosfonatos são a mais potente classe de drogas inibidoras da atividade osteoclástica (RUSSELL et al., 2008) e têm como principal indicação o tratamento de enfermidades do metabolismo ósseo, tais como a osteoporose pós-menopausa e induzida por corticoterapia. Também são empregados no manejo de complicações relacionadas a neoplasias, como hipercalcemia maligna, lesões osteolíticas do mieloma múltiplo e metástases ósseas associadas ao câncer de mama, próstata, pulmão e a outros tumores de tecidos moles. Condições menos prevalentes, como a doença de Paget e a osteogênese imperfeita, podem ser igualmente tratadas por essas drogas (MARX, 2007; LANDESBERG et al., 2008; RUGGIERO et al., 2009).

Por serem análogos sintéticos do pirofosfato inorgânico, os bisfosfonatos apresentam alta afinidade por cristais de fosfato de cálcio (hidroxiapatita). Têm como alvo, portanto, a porção mineral do tecido ósseo, sítio em que permanecem por longo período de tempo, e seus efeitos podem persistir por, aproximadamente, dez anos (RUSSELL et al., 2008; MARX; CILLO; ULLOA, 2007). O mecanismo de ação baseia-se na inibição da reabsorção óssea por meio de efeitos diretos e indiretos sobre os osteoclastos. Após a administração, acumulam-se na superfície óssea, em locais de intensa reabsorção, sendo englobados diretamente pelos osteoclastos durante o processo normal de remodelamento. Uma vez no citoplasma da célula, promovem perda de função ou apoptose da mesma, por inibição de sistemas enzimáticos ou produção de metabólitos citotóxicos (SATO et al., 1991; LIN, 1996; ROGERS et al., 2000; THOMPSON et al., 2006; SARIN; de ROSSI; AKINTOYE, 2008). Também inibem a diferenciação das células da linhagem monócito-macrófago em osteoclastos (SAHNI et al., 1993), por meio de alterações da via celular do receptor ativador do fator nuclear κB

(RANK), seu ligante (RANKL) e receptor *decoy* osteoprotegerina (OPG) (VIERECK et al., 2002; PAN et al., 2004; ZHOU et al., 2005). Outros efeitos como inibição da angiogênese (SANTINI et al., 2002; ZERVAS et al., 2006), da proliferação e do reparo tecidual de células epiteliais orais *in vitro* (LANDESBERG et al., 2008), comprometimento da cicatrização da mucosa oral *in vivo* (MAAHS, 2008) e fibrose dos espaços medulares (HANSEN et al., 2006; BEDOGNI et al., 2008) foram relatados.

A osteonecrose maxilar associada ao uso de bisfosfonatos é um efeito adverso dessas drogas e constitui causa de significativa morbidade entre os pacientes acometidos. Para a confirmação diagnóstica da enfermidade, o paciente deve apresentar as seguintes características: tratamento atual ou prévio com bisfosfonatos, exposição do tecido ósseo do complexo maxilo-mandibular ao meio bucal persistente por mais de oito semanas e ausência de história de radioterapia na região de cabeça e pescoço (RUGGIERO et al., 2009). As lesões, geralmente, ocorrem após procedimentos cirúrgicos invasivos nos ossos maxilares, tais como exodontias, colocação de implantes, cirurgias periodontais e periapicais. Casos de exposição óssea espontânea e após trauma causado por próteses parciais removíveis também foram relatados e são atribuídos a características anatômicas e fisiológicas, pois são mais frequentes na região posterior da mandíbula, que apresenta mucosa de espessura fina (MARX et al., 2005; MIGLIORATI; SIEGEL; ELTING, 2006). Os principais sinais e sintomas incluem eritema, edema e ulceração da mucosa, supuração, sequestros ósseos, suscetibilidade à fratura patológica, dor e parestesia. A condição é refratária ao tratamento, e tentativas de debridamento local levam à piora do quadro (RUGGIERO et al., 2004; BAGAN et al., 2005; BAMIAS et al., 2005; MARX et al., 2005; MIGLIORATI; SIEGEL; ELTING, 2006; DUNSTAN; FELSENBERG; SEIBEL, 2007).

Apesar de a osteonecrose maxilar associada ao uso de bisfosfonatos resultar de efeitos recíprocos entre metabolismo ósseo, trauma local, aumento da demanda de reparo ósseo, infecção e hipovascularização (MIGLIORATI et al., 2005), sua patogênese ainda não é completamente conhecida. Além disso, os fatores responsáveis pelo acometimento exclusivo dos ossos maxilares são ignorados (MARX, 2007). A maioria das pesquisas *in vivo* que avaliam os efeitos dos bisfosfonatos são conduzidas em ossos como tíbia e fêmur. A investigação das repercussões microscópicas do uso de bisfosfonatos, especificamente sobre o osso alveolar, se faz necessária para o esclarecimento dessas questões.

O presente estudo compreende dois trabalhos apresentados sob a forma de artigos científicos. O primeiro teve como objetivo fundamentar o experimento por meio de uma revisão da literatura a respeito do mecanismo de ação dos bisfosfonatos e sua relação com a osteonecrose maxilar. O segundo artigo descreve o experimento, cujo objetivo foi investigar, ao exame microscópico, os efeitos dos bisfosfonatos nitrogenados sobre o osso alveolar.

ARTIGO 1

2. ARTIGO 1

O artigo a seguir intitula-se **“Mechanism of action of bisphosphonates and its relation to osteonecrosis of the jaws: A review of the literature”** e foi formatado de acordo com as normas do periódico *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* (Anexo A).

MECHANISM OF ACTION OF BISPHOSPHONATES AND ITS RELATION TO OSTEONECROSIS OF THE JAWS: A REVIEW OF THE LITERATURE

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Abstract

In the last years, many reports have been published on the occurrence of osteonecrosis of the jaws in patients using nitrogen-containing bisphosphonates who had not had radiotherapy in the craniofacial region. The disease is characterized by non-healing exposure of bone to the oral cavity, associated with pain, paresthesia, suppuration, bone sequestration and susceptibility to pathological fracture. This article addresses chemical features of bisphosphonates, indications and mechanism of action and also reviews one of their major side effects: osteonecrosis of the jaws.

INTRODUCTION

Bisphosphonates are chemical compounds with high affinity for calcium phosphate crystals, exerting their effects on bone tissue. This group of drugs has been widely used in the treatment of diseases of bone metabolism.[1] Their mechanism of action is based on the inhibition of bone resorption and thus of bone remodeling. There are direct and indirect effects on osteoclasts, which undergo apoptosis [2] or become unable to differentiate from hematopoietic stem cells.[3] Impairment of angiogenesis[4] and damage to epithelial cells have also been reported.[5] After administration, bisphosphonates accumulate on the bone surface, at sites of intense resorption, being engulfed by osteoclasts during normal remodeling. They exert intracellular effects, such as loss of function and apoptosis, mediated by the inhibition of enzymatic systems.[1]

Jaws are considered the exclusive site for the occurrence of bisphosphonate-related osteonecrosis.[6] The presence of teeth, which makes the jaws susceptible to bone exposure, along with the high rate of bone turnover, and the need for adequate bone metabolism and blood supply are pointed out as factors responsible for osteonecrosis of

the jaws.[7, 8] However, the factors responsible for the exclusive involvement of the jaws are still unknown.

The purpose of this work was to review the mechanism of action of bisphosphonates and its relation to osteonecrosis of the jaws.

CHEMICAL FEATURES AND INDICATIONS OF BISPHOSPHONATES

Bisphosphonates are considered the most potent class of drugs responsible for the inhibition of osteoclast activity. They have a chemical structure (Fig. 1) similar to that of inorganic pyrophosphate, an endogenous regulator of bone mineralization. The chemical structure is based on the presence of two phosphonate groups linked by phosphoether bonds to a central carbon (a P-C-P structure). The P-C-P structure is resistant to pyrophosphatases and acid hydrolysis. The phosphonate groups provide anchoring to divalent cations such as calcium. They form a three-dimensional structure in a bidentate manner, by coordination of one oxygen from each phosphonate group with the divalent cation. Two additional covalent bonds to the central carbon atom of bisphosphonates form two side chains, R1 and R2. The affinity for the hydroxyapatite can be increased further if one of the side chains (R1) is a hydroxyl or primary amino group, because this allows the formation of a tridentate conformation that is able to bind calcium more effectively.[1, 9] This property explains the long half-life of these drugs in bone tissue, being as long as approximately ten years.[6] R2 components, on the other hand, are responsible for antiresorptive potency, which is magnified in the presence of nitrogen. Thus, nitrogen-containing bisphosphonates (e.g., alendronate, ibandronate, pamidronate, risedronate and zoledronic acid) are more potent than non nitrogen-containing ones (e.g., etidronate, chlodronate and tiludronate).[1, 2, 6, 9, 10]

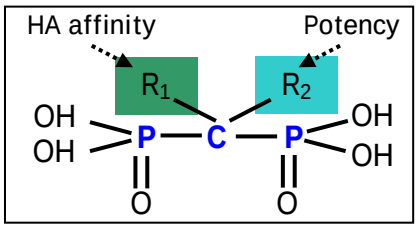

Fig.1. Bisphosphonate chemical structure (HA= hydroxyapatite).

Bisphosphonates are mainly indicated to treat postmenopausal and corticosteroid-induced osteoporosis, as well as to manage complications related to malignancies, such as hypercalcemia, osteolytic lesions of multiple myeloma, and breast, prostate and lung cancer bone metastases. Off-label use of bisphosphonates has also been reported in Paget's disease and osteogenesis imperfecta.[5, 6, 11] Oral bisphosphonates are generally indicated for the treatment of osteoporosis, where alendronate is the most common followed by risedronate and ibandronate.[6, 12, 13,14] Parenteral bisphosphonate formulations such as zoledronic acid, used once yearly and ibandronate, administered every three months can also be employed for this purpose.[11] The bisphosphonates indicated for the control of hypercalcemia and osteolytic lesions associated with malignancies are administered intravenously. The drug most used in these situations is zoledronic acid followed by pamidronate, both as monthly infusions.[6, 14]

MECHANISM OF ACTION OF BISPHOSPHONATES

The main mechanism of action of bisphosphonates is based on the inhibition of bone resorption and thus of bone remodeling through effects on osteoclasts which undergo apoptosis.[1] An increase in bone mineral density has been demonstrated, [6, 13, 15, 16] since some bisphosphonates such as etidronate, alendronate, pamidronate and olpadronate exert an anti-apoptotic effect on osteoblasts and osteocytes.[1, 17-19]

Direct effects on osteoclasts

After administration, part of the bisphosphonate is available for incorporation into the bone matrix at sites of intense osteoclastic activity.[2] The bisphosphonate remaining is excreted unmetabolized in the urine. Once incorporated into bone tissue, the drug is removed slowly and can remain in place for up to ten years.[20] During resorption, in the acidic environment of the Howship lacuna, bisphosphonate bound to bone is released, the drug reaches high concentrations in solution or in the form of calcium salts, and enters the osteoclast by endocytosis.[1]

Specific intracellular effects occur depending on the presence or absence of nitrogen in the side chain of the molecule. The non nitrogen-containing bisphosphonates are metabolized into cytotoxic analogues of adenosine triphosphate (ATP) and accumulate intracellularly interfering with osteoclast function through inhibition of ATP-dependent enzymes.[6] On the other hand, nitrogen-containing bisphosphonates are considered more potent, and impair the intracellular mevalonate pathway, which is associated with cholesterol biosynthesis. This effect by the latter bisphosphonates occurs through the inhibition of farnesylpyrophosphate synthase, which catalyzes the synthesis of isoprenoid lipids such as farnesylpyrophosphate and geranylgeranyl farnesylpyrophosphate. These molecules modulate prenylation, a structural change of small guanosine triphosphate-binding proteins (GTPases), such as Rhas and Rho. The lack of prenylated protein prevents the anchoring of other molecules of the signaling cascade, causing different changes in cellular function, such as loss of the ruffled border, cytoskeleton breakage, impairment of adhesion proteins and proton pump and inhibition of lysosomal enzymes, as well as apoptosis (Fig.2).[2, 9, 21-23]

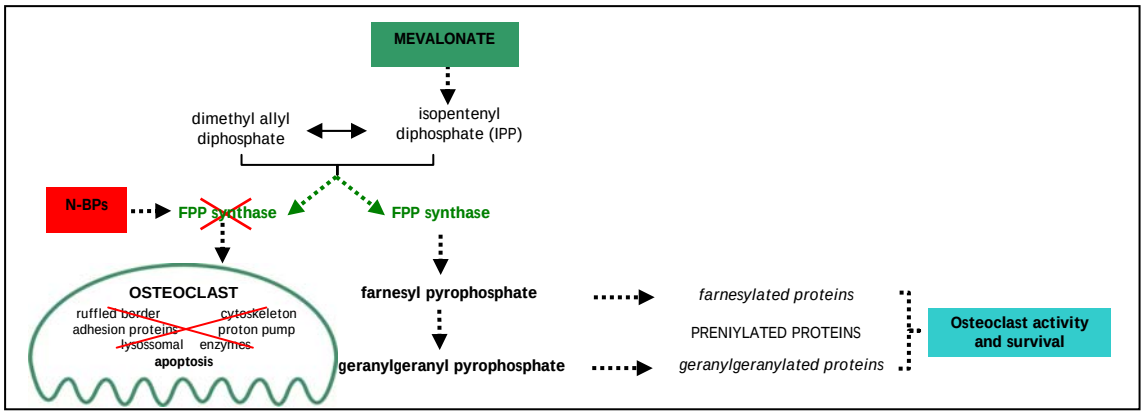

Fig. 2. Effects of bisphosphonates on mevalonate pathway, schematic representation. N-BPs inhibit FPP synthase, resulting in lack of prenylated proteins, which are essential to osteoclast activity and survival (N-BPs = nitrogen-containing bisphosphonates; FPP = farnesyl pyrophosphate).

Indirect effects

The mechanism of action of bisphosphonates is not only related to direct actions on osteoclastic activity. Indirect effects, mediated by osteoblasts, have also been observed. Sahni et al.[24] conducted a study in which cells of osteoblast lineage, previously treated with non nitrogen- and nitrogen-containing bisphosphonate for five minutes, were incubated in coculture with untreated osteoclasts attached to a mineral surface (ivory) for 24 hours. There was a decrease in osteoclastic activity resulting in a reduction in the number and diameter of resorption pits. This study was the first to launch the hypothesis that the effects of bisphosphonates on bone resorption also depend on osteoblasts, since they secrete proteins which either stimulate or impair osteoclastogenesis.[3]

RANK/RANKL/OPG pathway

One of the main regulators of the molecular mechanisms involved in the development and function of osteoclasts is the pathway of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) secreted by osteoblast lineage

cells. OPG is a member of the tumor necrosis factor receptor (TNFR) superfamily and acts as a decoy receptor for RANKL, the osteoclast differentiation-stimulating protein. The binding of RANKL to receptor activator of nuclear factor-κB (RANK) present on the surface membrane of monocyte-macrophage lineage cells, which are osteoclast precursors, produces the signal that leads to their differentiation into osteoclasts. This signal may be interrupted by the secretion of OPG, which binds to RANKL and thereby blocks the interaction with RANK.[6, 25-28]

Some cell culture studies demonstrated an increase in OPG expression in osteoblasts treated with bisphosphonates.[3, 27] Similar results were obtained by Zhou et al.[29] in an *in vivo* study. The authors evaluated OPG immunohistochemical expression in the tibia of mice bearing osteolytic tumors, treated with zoledronic acid. They found increased OPG expression in animals treated with the bisphosphonate in comparison to those that did not receive treatment.

Anti-angiogenic effect

Besides suppressing bone remodeling, bisphosphonates are reported to exert an anti-tumor effect by interfering with angiogenesis. The effect occurs when more potent drugs, such as zoledronic acid, are used. These compounds presumably modulate the secretion of specific growth factors, such as vascular endothelial growth factor (VEGF), and inhibit the proliferation, migration and adhesion of endothelial cells and the consequent formation of capillary tubes.[30, 31] Nevertheless, different results were found by Pampu et al.[32] in rabbits given a single dose of zoledronic acid and submitted 5 days later to mandibular distraction osteogenesis for 5 more days. After 32 days of a consolidation period, a histomorphometric evaluation was performed in the regenerated bone region. Even though it was not statistically significant, there was an increase in the

number of blood vessels. Moreover, Maahs[33] reported that the administration of neither alendronate nor zoledronic acid was associated with lower VEGF immunohistochemical expression in rat maxillae.

Effects on epithelial cells and collagen fibers

Other actions of bisphosphonates have been reported. Deleterious effects on epithelial cells *in vitro*,[5] as well as on oral mucosa healing *in vivo*[33] and increase in fibrous connective tissue (collagen fibers) in medullary spaces of bone tissue[15, 34] were observed.

Landesberg et al.[5] evaluated the effect of pamidronate on oral keratinocytes of mice *in vitro* and found inhibition of cell proliferation and tissue repair when these cells were exposed to therapeutic concentrations of the drug. The effects, however, did not occur at the expense of apoptosis. It is believed that pamidronate promotes necrosis.

BISPHOSPHONATE-RELATED OSTEONECROSIS OF THE JAWS

There are many cases of bisphosphonate-related osteonecrosis of the jaws (BRONJ) reported in the literature. The condition is characterized by exposed bone in the maxillofacial region that persists for more than eight weeks in patients treated with bisphosphonates who do not have a history of radiotherapy in the head and neck region. The lesions develop spontaneously or after trauma on bone areas covered with a thin mucosal layer or after dento-alveolar surgery.[6, 7, 35] Although a cause and effect relationship has not yet been established, several epidemiological and some experimental studies provide evidence to support a strong correlation between monthly intravenous bisphosphonate therapy associated with tooth extractions, and the development of osteonecrosis.[33, 36] Based on case series, case-control and cohort studies,

the estimated cumulative incidence ranges from 0.8% to 12%. Lower risk is observed with the use of oral bisphosphonates for the treatment of osteoporosis in comparison to intravenous bisphosphonates for the treatment of osteolytic tumors. However, the association of oral bisphosphonate use with co-factors could play a key role in the development of osteonecrosis.[11]

BRONJ is limited to the jaws,[6] since it has not so far been reported in other bones of the skeleton. The factors identified as possible reasons for the uniqueness of the maxilla and mandible in hosting the injury are: (1) the presence of teeth, which exposes bone to the external environment; (2) periodontal disease, abscesses, endodontic treatment as well as the occurrence of injuries[7] requiring bone metabolism and blood supply to maintain the appropriate balance;[37] and (3) the high bone turnover rate at these sites, which causes a higher drug uptake.[8]

Etiopathogenesis

BRONJ is the result of reciprocal complex effects between bone metabolism, local trauma, increased demand for bone repair, infection and hypovascularity.[8] Bone homeostasis depends on the balance between osteoblasts and osteoclasts. When bone resorption is impaired by inhibition of osteoclastic activity, the bone matrix is degraded and non-vital bone, which is characterized by the absence of osteocytes, accumulates.[15] In the jaws, the constant need for repair due to masticatory forces, coupled with the presence of infection when the bone is exposed to the oral environment, increases the demand for resorption to such a level that exceeds the response capacity of the tissue whose metabolism has been modified by bisphosphonates. The result is osteonecrosis.[4,8, 35]

Hansen et al.,[34] using H&E, Grocott and periodic acid-Schiff (PAS), found areas of bone erosion covered with *Actinomyces* sp. in all samples of osteonecrosis from patients receiving bisphosphonates. One sample showed fungi consistent with *Candida* sp. Osteoclasts were observed within resorption lacunae in five out of eight specimens evaluated.

The damage on epithelial cells caused by bisphosphonates, as demonstrated *in vitro* by Landesberg et al.[5], could contribute to the persistent exposure of underlying bone and the development of osteonecrosis.

Clinical features

Clinically, BRONJ occurs as one or multiple areas of alveolar bone exposed to the oral cavity (Fig. 3), which can display sequestration, purulent discharge, ulceration and/or swelling of the adjacent mucosa, fistula or pathological fracture. The patient may have pain or paresthesia; the mandible is more affected than the maxilla, and although less frequently, both bones can be affected simultaneously. The mandibular posterior region is the site of greatest prevalence of osteonecrosis due to compressive forces induced by the occlusion in that area.[6, 7, 11, 20, 37-44]

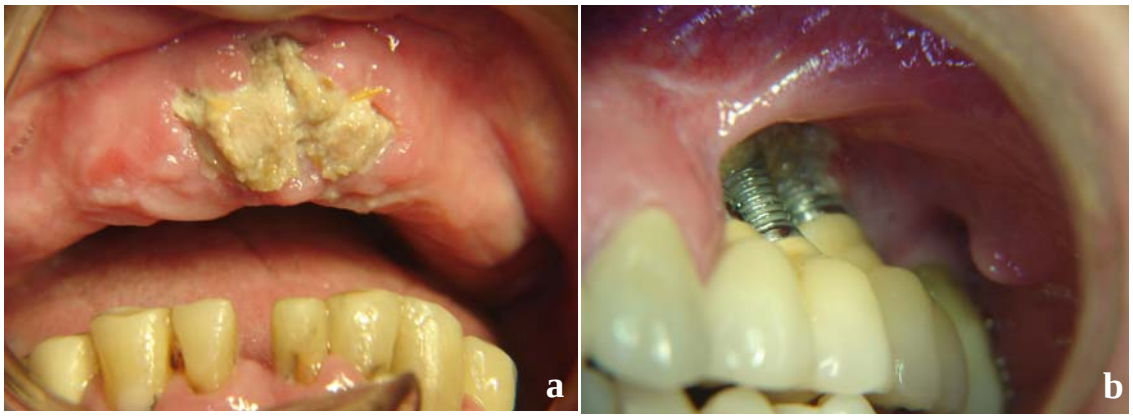

Fig.3. BRONJ clinical features. (a) 82-year-old male patient under zoledronic acid therapy for multiple myeloma, who developed osteonecrosis at the tooth extraction site; (b) 58-year-old

female patient treated for breast cancer bone metastasis with zoledronic acid, who developed osteonecrosis at implant placement sites.

Imaginological features

The radiographic examination shows ill-defined radiolucent areas in alveolar bone which has a mottled appearance suggestive of osteolytic lesion (Fig. 4) with or without radiopaque areas consistent with bone sequestration. Occasionally, the radiographic signs may be absent.[37, 41, 44] Sclerosis and loss of integrity of the lamina dura (Fig. 4), as well as thickening of the periodontal ligament space could be radiographic signs of subclinical damage caused by bisphosphonates.[6, 7, 11, 45]

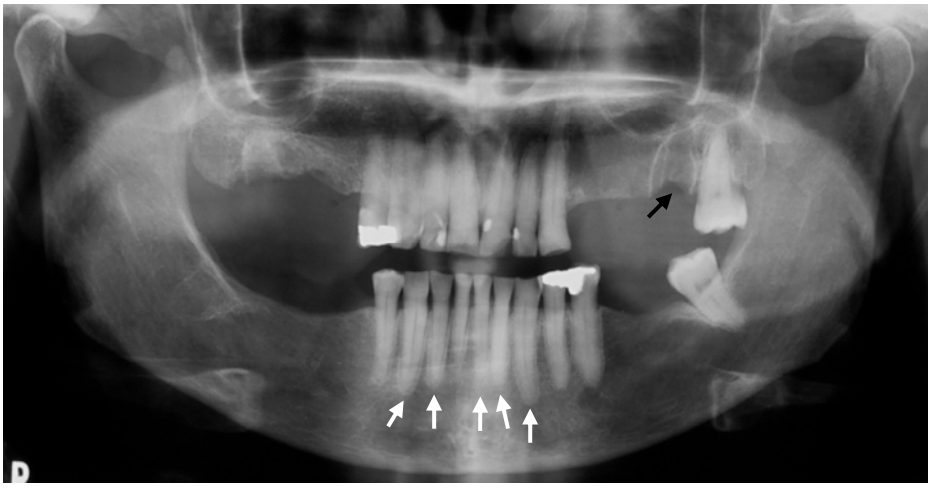

Fig. 4. Panoramic radiograph from a female patient under pamidronate therapy, who developed osteonecrosis after extraction of 2nd upper left molar. The black arrow shows osteolytic area in left maxilla; white arrows show sclerosis of lamina dura in lower teeth.

Computed tomography (CT) scans are used to identify alterations difficult to discern on radiographs. They provide three-dimensional information and better delineation of the lesion. Areas of clinically exposed bone in panoramic radiographic images tend to be smaller than the areas of injured bone shown by CT. CT also shows focal sclerosis in early disease with the presence of a disorganized trabecular pattern and poor corticomedullary differentiation.[46]

The findings of magnetic resonance imaging in six patients with a diagnosis of BRONJ showed, in early disease, loss of the normal hyperintensity of fatty marrow in the mandible and maxilla. More advanced BRONJ cases demonstrated bone destruction, soft tissue swelling, inferior alveolar nerve thickening, and pterygoid muscle swelling.[47]

O'Ryan et al.[48] evaluated bone scintigraphy imaging in patients receiving intravenous nitrogen-containing bisphosphonates. They identified positive tracer uptake in maxilla and mandible areas which subsequently developed BRONJ. According to this finding, bone scintigraphy could be useful in the early subclinical detection of BRONJ.

Histological features

On light microscopy, BRONJ appears as multiple areas of non-vital bone, partly confluent, interspersed with residual vital bone. Inflammatory infiltrate, *Actinomyces* sp. colonies and *Candida* sp. are frequent findings.[15, 33, 34, 49] Perinecrotic bone shows inflammatory reaction in the marrow spaces, with marrow fibrosis, inflammatory cell infiltration and blood vessels. Osteoclasts, in contact or not with the bone surface, are also observed.[15, 49]

Sonis et al.,[50] in an animal model of zoledronic acid-related osteonecrosis, found areas of acellular necrotic bone regularly associated with mucosal ulceration and a robust inflammatory response. Proliferation of small blood vessels and absence of *Actinomyces* sp. as determined by PAS staining were also observed.

Risk factors

Based on the latest findings available in the literature, the main risk factors for the development of osteonecrosis is the use of intravenous bisphosphonates and surgical interventions in bone tissue. Other factors related to bisphosphonates, as well as local,

demographic and systemic factors, should also be considered.[10, 11] Drug potency and duration of therapy are directly related to the risk of developing this injury. Zoledronic acid is more potent than pamidronate, which in turn is more potent than oral bisphosphonates.[11]

Local risk factors include dentoalveolar procedures such as tooth extractions, periodontal surgery involving bone, periapical surgery and implant placement. Specific anatomical sites, such as the mylohyoid ridge as well as the maxillary and mandibular *tori*, have a thin mucosa predisposing bisphosphonate users to the development of osteonecrosis. The concomitant presence of inflammatory dental disease such as periodontal and periapical abscesses also increases the risk.[11]

Age and race are demographic factors associated with the risk of developing osteonecrosis. The lesions occur most frequently in older Caucasian patients over 60 years of age. Systemic factors such as renal failure,[11, 51] diabetes,[8, 11] obesity, metastatic disease, low hemoglobin levels,[11] steroids, chemotherapy,[11, 37, 42, 52, 53] smoking[11, 13, 54] and alcohol ingestion[11, 45] can also predispose bisphosphonate users to osteonecrosis.

Treatment

Since the occurrence of the first reports of BRONJ until today, it has not been possible to determine a treatment strategy that promotes the cure of the disease. There is difficulty in obtaining adequate surgical margins, because the entire bone is exposed to bisphosphonate effects. Thus, surgical interventions can often exacerbate the injury. In mild cases, the lesion is controlled by the use of antimicrobial rinses. When there are signs of infection in soft tissues and associated symptoms, systemic antibiotics are used, often for extended periods. Mobile segments of bone sequestration can be removed gently, without exposing uninvolved bone. Elective bone surgery in the maxillofacial

region should be avoided. The other dental procedures that do not involve direct manipulation of bone tissue should be performed in order to prevent the need for tooth extractions.[6, 11]

In some situations, temporary discontinuation of bisphosphonate therapy under the guidance of the attending physician, along with monitoring serum biochemical markers of bone metabolism, may help therapeutic decisions regarding the management of the injury, especially in patients who have taken or are taking oral bisphosphonates.[6]

Hyperbaric oxygen therapy has been evaluated through randomized studies. Preliminary results indicate some improvement in symptoms and partial healing. However, it does not promote the complete repair of the lesions when used as single therapy.[55]

DISCUSSION

Bisphosphonate mechanism of action is based on impairment of bone resorption through direct and indirect effects on osteoclasts.[1] These effects are inhibition of osteoclastogenesis and osteoclastic function, as well as inducing apoptosis of mature cells, causing a reduction in osteoclast number.[2] The role of bone marrow stromal cells or their osteoblast progeny in the maturation of monocyte-macrophage lineage cells into osteoclasts is essential. These precursor cells secrete both RANKL, which stimulates osteoclastogenesis, and OPG, which inhibits it.[56] Bisphosphonates may stimulate OPG secretion and contribute, consequently, to impaired bone resorption, as indicated by some *in vitro*[3, 27] and *in vivo* studies.[29] Anti-angiogenic properties of bisphosphonates were also reported and are associated with anti-tumor activities.[31] However, the consequences of these effects in the jaws are not completely understood. Bisphosphonate actions on the jaws are likely more severe since the high bone turnover

rate in this skeletal region may result in higher drug uptake.[6] The complex microbial flora of the oral cavity associated with the low biological response of bisphosphonate-treated bone leads to several structural changes.[37] Osteonecrosis of the jaws is a serious side effect of bisphosphonates that impairs the quality of life of the patient. The major obstacle lies in the difficulties of developing an adequate therapeutic approach.[11] Furthermore, lack of information among physicians and dentists regarding bisphosphonate mechanism of action and osteonecrosis onset is disturbing, and prevention is still the best strategy available. Not only epidemiological studies, but also experimental investigations, using specific BRONJ animal models, are needed to elucidate aspects that are still obscure.

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ARTIGO 2

3. ARTIGO 2

O artigo a seguir intitula-se **“Microscopic and immunohistochemical evaluation of effects of nitrogen-containing bisphosphonates on the rat alveolar bone tissue”** e foi formatado de acordo com as normas do periódico *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology* (Anexo A).

MICROSCOPIC AND IMMUNOHISTOCHEMICAL EVALUATION OF EFFECTS OF NITROGEN-CONTAINING BISPHOSPHONATES ON THE RAT ALVEOLAR BONE TISSUE

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ABSTRACT

Objective. This study aimed to investigate effects of nitrogen-containing bisphosphonates on alveolar bone.

Study design. Thirty-one female Wistar rats were allocated into 3 groups: oral alendronate treatment (n=11); intraperitoneal zoledronic acid treatment (n=10); and control (n=10). After 150 days of treatment, the animals were euthanized, maxillae were processed, and histological sections of alveolar bone were stained with H&E (osteoclast count; trabecular density) and picrosirius (collagen fiber density in medullary spaces). Immunohistochemical expression of osteoprotegerin (OPG) was also evaluated.

Results. Osteoclast count, collagen fiber density and OPG expression were not significantly different between groups. Trabecular density was statistically higher in the zoledronic acid group than control (ANOVA; Tukey; p=0.038), but was not significantly different between alendronate and the other groups.

Conclusions. Zoledronic acid administration increased trabecular density of alveolar bone, while alendronate did not. Neither bisphosphonate tested caused marrow fibrosis; their effects on osteoclast number and OPG expression need further investigations.

INTRODUCTION

Bisphosphonates have been widely used in the treatment of bone metabolism disorders, such as osteoporosis and bone metastases.[1] These therapeutic agents inhibit bone resorption through direct and indirect effects on osteoclasts. After administration, circulating bisphosphonates bind to exposed hydroxyapatite crystals at resorption sites[21] and are engulfed by osteoclasts, exerting intracellular effects.[1] Non nitrogen-containing

bisphosphonates, such as chlodronate, etidronate and tiludronate are metabolized into cytotoxic analogues of ATP, which cause inhibition of osteoclastic activity and apoptosis.[9] Nitrogen-containing bisphosphonates, such as alendronate, risedronate, ibandronate, pamidronate and zoledronic acid, are considered more potent and inhibit the mevalonate pathway. These drugs prevent the formation of prenylated proteins, which regulate a variety of cellular processes involved in osteoclast function. Consequently, loss of osteoclast activity and apoptosis occur.[2, 9, 23]

Indirect effects have also been reported.[1, 24] *In vitro* studies showed that pamidronate[3] and zoledronic acid[3, 27] promoted osteoprotegerin (OPG) secretion by osteoblasts. Immunohistochemical expression of OPG was increased in bone tissue of mice bearing Ewing sarcoma, treated with zoledronic acid.[29] OPG acts as a soluble antagonist for receptor activator of nuclear factor-κB ligand (RANKL), the osteoclast differentiation stimulating protein. When RANKL binds to its receptor RANK on bone marrow mononuclear precursors, they differentiate into osteoclasts. However, when OPG interacts with RANKL, binding to RANK is prevented, and osteoclast differentiation does not occur.[28]

Direct and indirect actions of bisphosphonates reduce the number of osteoclasts available on the bone surface. As a consequence, bone mineral density and trabecular bone density increase.[6, 12, 16] Other effects, such as impairment of both angiogenesis[31] and epithelial cell proliferation,[5] as well as increase in the fibrous component (collagen fibers) of bone medullary space[15, 34] have also been reported.

The occurrence of osteonecrosis of the jaw in patients who have received nitrogen-containing bisphosphonates without history of radiotherapy in the head and neck region has brought concern. The disease is characterized by exposure of bone to the oral cavity, associated with pain, paresthesia, suppuration, bone sequestration and

susceptibility to pathological fracture. The condition is refractory to treatment, since debridement attempts may lead to injury exacerbation.[7, 20, 37, 38, 57]

It is important to consider that the majority of studies evaluating the effects of bisphosphonates on bone have been conducted on the tibia and femur.[58-63] The lack of knowledge about the effects of these drugs on alveolar bone, especially in regard to bone resorption and formation, as well as the restriction of osteonecrosis to the jaws reinforces the need for further investigations.

This study aimed to investigate effects of alendronate and zoledronic acid on the rat alveolar bone tissue, regarding osteoclast number, trabecular bone density, collagen fiber density in medullary spaces and immunohistochemical expression of OPG.

MATERIALS AND METHODS

Animals

The present study was approved by the Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul, and the procedures were carried out in accordance with institutional guidelines for animal care and use. The sample was composed of 31 female Wistar rats (*Rattus novergicus albinus*) aged 140 days and weighing 241 g on average; they were obtained from the animal facility of the Federal University of Pelotas (UFPEL, RS, Brazil). Animals were individually numbered on the tail and housed in plastic cages placed in ventilated racks (Alesco, Monte Mor, SP, Brazil) at a temperature of 22°C with a 12-h light/dark cycle. Animals were fed a standard diet of rat chow (Nuvilab, Colombo, PR, Brazil) and given water *ad libitum*.

No experimental procedures were carried out in the place where the animals were kept to avoid any type of behavioral stress.

Study design

The animals were randomly allocated into three groups, according to the bisphosphonate used: group 1 (n=11): alendronate (0.05 mg/kg, oral gavage, once a week); group 2 (n=10): zoledronic acid (0.6 mg/kg, intraperitoneally, every 28 days); and group 3 (n=10): control (no bisphosphonate used). After completing a period of 150 days of drug administration, the animals were euthanized by inhalation of isoflurane in an appropriate anesthesia chamber[64] (Cristalia, Porto Alegre, RS, Brazil), and the maxillae were removed and fixed in 10% buffered formalin for 24 h. Maxillae were transversally split in the region of the first and second molars, obtaining two fragments, called A and B.

Histological processing

Fragments A and B (n=62) were decalcified in formic acid solution, composed of 780 ml of 10% tribasic sodium citrate P.A. (Cromoline, Diadema, SP, Brazil) and 220 ml of 85% formic acid P.A. (Synth, Diadema, SP, Brazil), for 24 h. They were then paraffin-embedded, cut into 4-μm sections and stained with hematoxylin and eosin (H&E) and picrosirius, as well as submitted to immunohistochemical analysis for OPG detection.

Immunohistochemistry

Antigen retrieval was performed with Tris/EDTA buffer, pH 9 (20 mM Tris/0.65 mM EDTA) in a 99ºC water-bath for 30 min. Endogenous peroxidase was blocked with a 3% solution of hydrogen peroxide in methanol for 30 min. The sections were

incubated in anti-OPG goat polyclonal antibody (SC8468 – Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted at 1:100. The Dako LSAB kit was used as the detection system. Reaction products were visualized by immersing the sections in 0.03% diaminobenzidine solution containing 0.002% hydrogen peroxide. Hematoxylin was used for counterstaining. Negative control sections were treated identically, except that the primary antibody was substituted with phosphate-buffered saline.

Histological evaluation

The sections were digitized using a light microscope (Olympus BX-50; Olympus America Inc., Miami, FL, USA) coupled to a video camera (CCD-IRIS Sony DXc 107 A/107 AP; Sony, Park Ridge, NJ, USA), with Image Pro Plus 4.5.1 software (Media Cybernetics Inc., Bethesda, MD, USA). The images were analyzed by a calibrated and blinded examiner using Image Pro Plus 4.5.1 and Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA, USA) softwares. Intra-examiner agreement was determined with a paired *t* test ($P<0.05$), Pearson's correlation coefficient and intraclass correlation coefficient, according to the method tested. No significant difference and a strong correlation, as demonstrated by the Pearson correlation coefficient, were observed as follows: trabecular density - $P= 0.87$, R=0.90; collagen fiber density - $P=0.84$, R=0.90; and OPG expression - $P=0.47$; R=0.92. Intraclass correlation coefficient for osteoclast count was R=0.99.

Study endpoints

Osteoclast count

In the H&E stained sections, the number of osteoclasts was determined on the whole extent of alveolar bone surface, from buccal to palatal crest, with a x40 objective.

Large cells containing three or more nuclei located on the bone surface and displaying eosinophilic cytoplasm, were considered osteoclasts (Fig. 1).

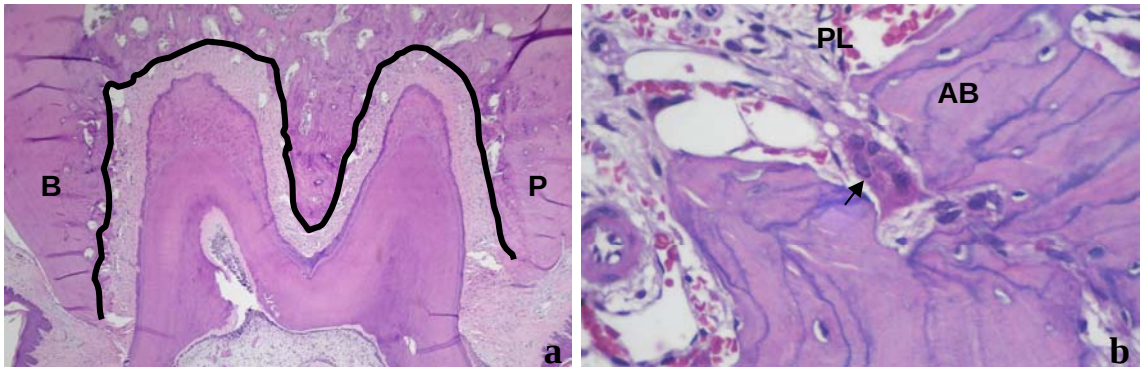

Fig.1. Osteoclast count. (a) H&E (40x); black line points out the surface, from buccal (B) to palatal crest (P), where osteoclasts were counted. (b) H&E (400x); osteoclast (arrow) attached to alveolar bone (AB) surface; (PL) periodontal ligament.

Trabecular bone density

Trabecular bone density was evaluated in the H&E sections in four alveolar bone fields (apical, interradicular, buccal and palatal). The histological images were digitized with a x10 objective and analyzed according to Mahl and Fontanella,[16] using Adobe Photoshop CS3 software (Adobe Systems Inc., San Jose, CA, USA). The *extract* filter discarded the structures not corresponding to alveolar bone, and the histogram function calculated total bone area in pixels. The *extract* filter was again used to select only the bone trabeculae, discarding the image of medullary spaces. The histogram function calculated trabecular area (Fig. 2). Trabecular bone density was considered the percentage of trabecular area in each field, calculated using the following formula: trabecular area x 100 / total bone area.

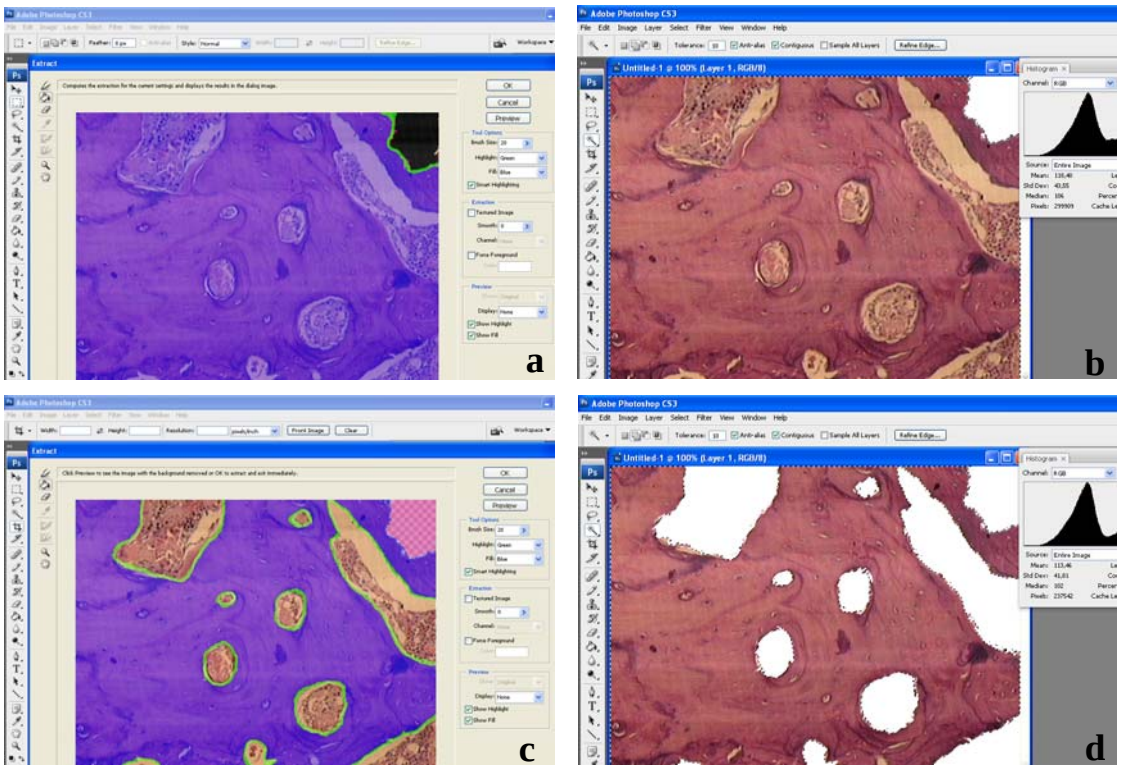

Fig.2. Trabecular density, H&E (x10 objective). (a), (b) Elimination of areas not corresponding to alveolar bone; (c) elimination of image of medullary spaces; (d) trabecular area quantification.

Collagen fiber density in the medullary spaces of alveolar bone

Collagen fiber density in the medullary spaces was evaluated in the picrosirius-stained sections in three bone fields (apical, buccal and palatal), using a x20 objective. Adobe Photoshop CS3 software was used to select only the medullary spaces in each field, with the *extract* filter. The semi-automated segmentation method[65, 66] was used with Image Pro Plus 4.5.1 software (Media Cybernetics Inc., Bethesda, MD, USA) for collagen fiber area quantification. As collagen fibers stained with picrosirius show a red color, the red areas were selected with the function *measure – count/size*. After that, a mask applied to the image converted it into black and white, and the white areas, corresponding to collagen fibers, were quantified in μ^2. The same procedure was performed to quantify total area (Fig. 3). Collagen fiber density was considered the

percentage of collagen area in each field, calculated using the following formula: collagen area x 100 / total area of medullary spaces.

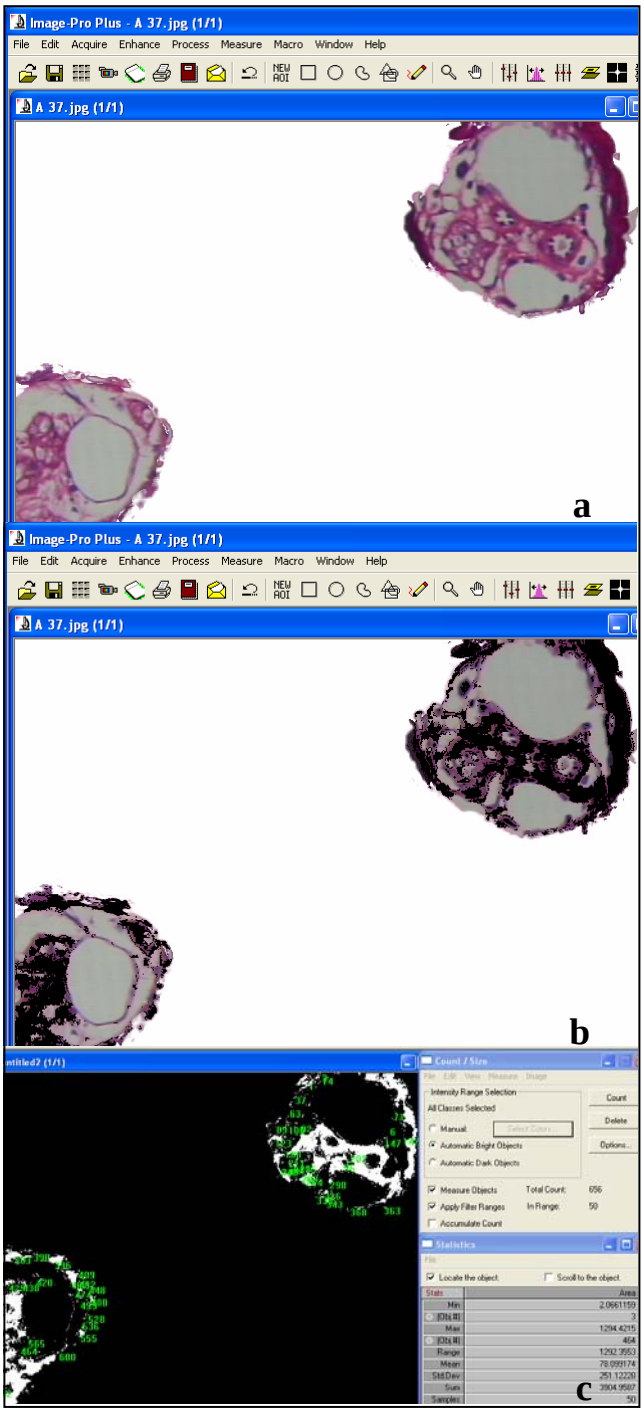

Fig.3. Collagen fiber density, picrosirius (x20 objective). (a) Medullary spaces previously selected; (b) selection of collagen-positive areas; (c) quantification of collagen-positive areas.

OPG expression

OPG expression was assessed in four alveolar bone fields (apical, interradicular, buccal and palatal), using a x20 objective, with the semi-automated method.[30] Alveolar bone areas expressing OPG showed an intense brown color. The brown areas were selected with the function *measure – count/size*. A mask applied to the image converted it into black and white. The white areas, corresponding to OPG-positive structures, were quantified in μ^2. The same procedure was performed to quantify total area (Fig. 3). Immunohistochemical expression of OPG in alveolar bone was calculated using the following formula: OPG-positive area x 100 / total bone area (Fig. 4).

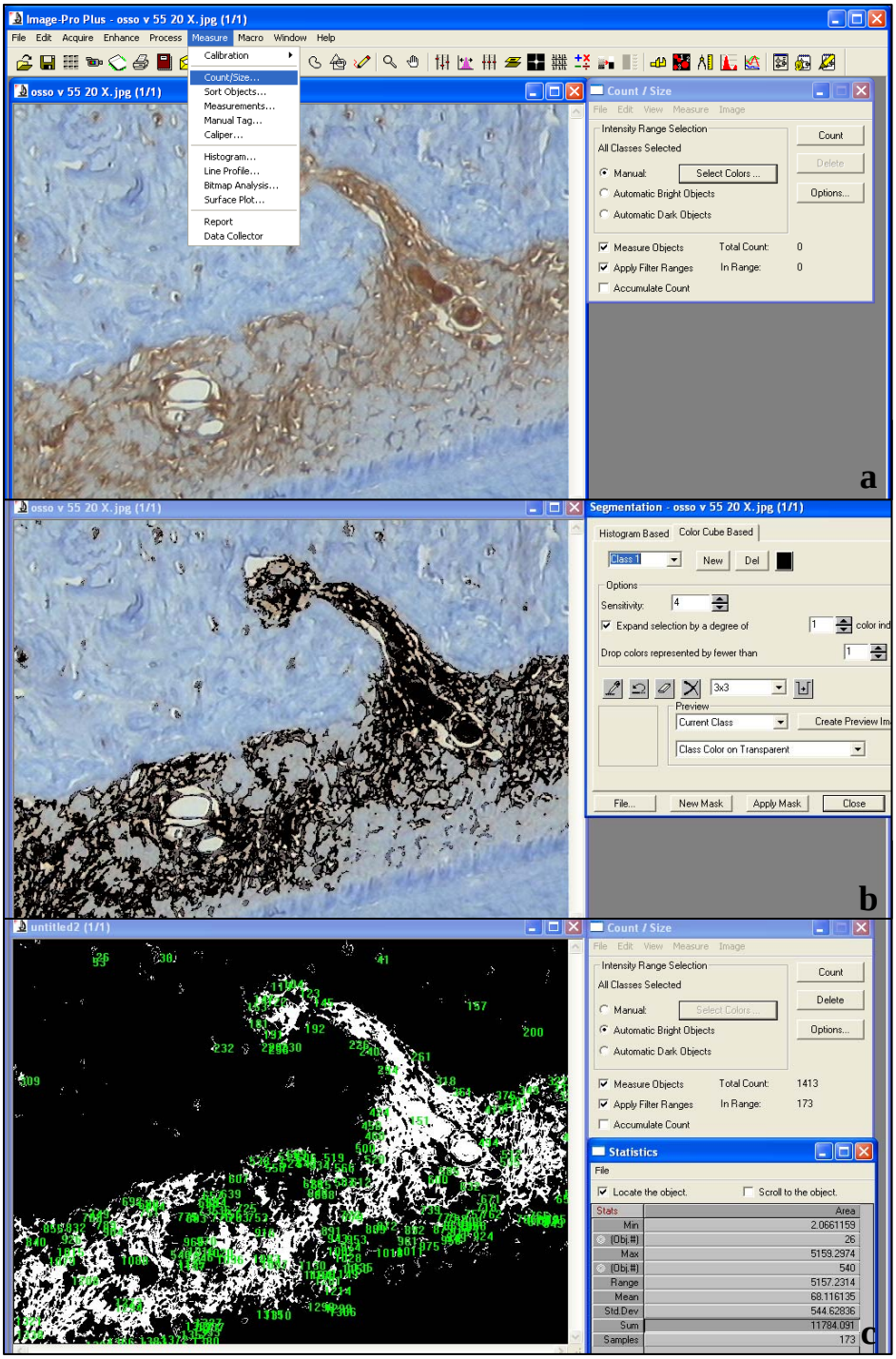

Fig.4. OPG immunohistochemical expression (x20 objective). (a) *Measure-count/size* function; (b) selection of OPG-positive areas; (c) quantification of OPG-positive areas.

Statistical Analysis

The data were analyzed by descriptive statistics, and the comparison of the variables tested between the three groups was performed using ANOVA and Tukey's test, at a 5% level of significance.

RESULTS

Osteoclast count

Table I displays the results of osteoclast counts in the H&E stained sections of alveolar bone surface. Although the number of osteoclasts was higher in the control group than in alendronate and zoledronic acid groups, there was no statistically significant difference between the three groups analyzed (ANOVA; *P*=0.208). Figure 5 illustrates osteoclast counting on alveolar bone.

Table I: Osteoclast count on alveolar bone surface

Group	Osteoclast number				
	n	Mean	SD	Minimum	Maximum
Alendronate	11	9.09	6.25	0.00	18.00
Zoledronic Acid	10	11.20	5.25	0.00	17.00
Control	10	14.0	9.87	1.00	19.00

SD=Standard deviation; n=Sample size
ANOVA (α=0.05); *P*=0.208

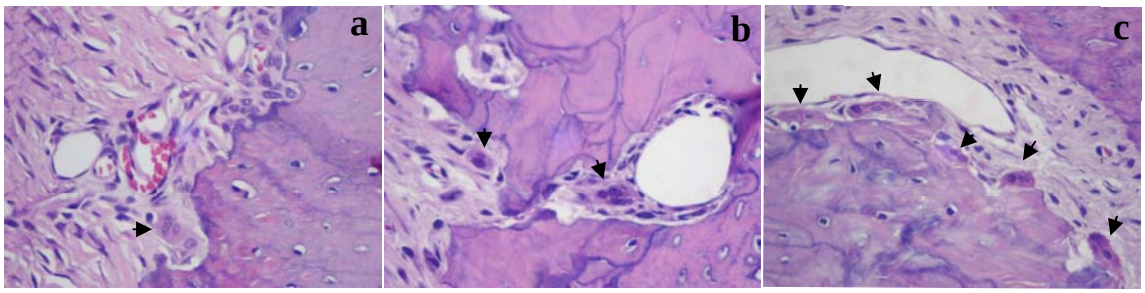

Fig.5. Histological sections showing osteoclast counting (arrows) on alveolar bone surface from (a) alendronate, (b) zoledronic acid and (c) control groups (H&E, 400x).

Trabecular bone density

Regardless of the bone fields evaluated, trabecular density was significantly higher in the zoledronic acid group, when compared to control (ANOVA and Tukey's test; *P*=0.038). There was no significant difference between the zoledronic acid and alendronate groups, nor between the alendronate and control groups. Regardless of the group evaluated, the apical field showed significantly lower trabecular density when compared to the interradicular, buccal and palatal ones (ANOVA; *P*<0.001) (Table II; Fig. 6).

Table II: Trabecular density of alveolar bone

Bone field	Group						Total	
	Alendronate		Zoledronic Acid		Control			
	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
Apical	91.20	5.13	91.94	3.01	87.67	5.86	90.39^{B}	4.97
Interradicular	94.70	5.80	98.07	1.48	95.07	3.22	95.74^{A}	4.25
Buccal	98.07	1.18	98.03	1.14	93.57	13.01	96.66^{A}	7.33
Palatal	98.85	0.55	98.08	1.40	98.36	0.64	98.46^{A}	0.95
Total	95.57^{ab}	4.91	96.45^{a}	3.30	93.67^{b}	8.03	95.27	5.73

SD=Standard deviation

ANOVA; Tukey's test (α=0.05) - Means followed by different upper case letters in the column are significantly different (*P*<0.001). Means followed by different lower case letters in the row are significantly different (*P*=0.038).

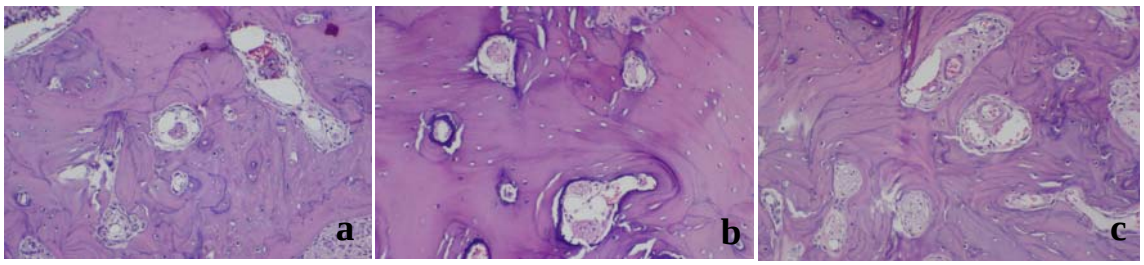

Fig.6. Histological sections showing alveolar bone trabecular density in (a) alendronate (b) zoledronic acid, and (c) control groups (H&E, 100x).

Collagen fiber density in medullary spaces of alveolar bone

There was no significant difference in collagen fiber density in medullary spaces between the alendronate, zoledronic acid and control groups, nor between the apical, buccal and palatal bone fields (ANOVA; *P*>0.05) (Table III; Fig. 7).

Table III: Collagen fiber density in medullary spaces of alveolar bone

Bone field	Group						Total	
	Alendronate		Zoledronic Acid		Control			
	Mean(%)	SD	Mean(%)	SD	Mean(%)	SD	Mean(%)	SD
Apical	42.05	10.91	39.88	13.08	37.45	9.11	39.87	10.85
Buccal	50.4	14.17	38.59	11.61	42.75	13.56	44.02	13.45
Palatal	46.72	17.75	45.84	23.84	37.38	8.90	42.96	16.49
Total	45.67	13.99	40.98	15.36	39.06	10.48	41.98	13.38

SD=Standard deviation
ANOVA (α=0.05); *P*>0.05

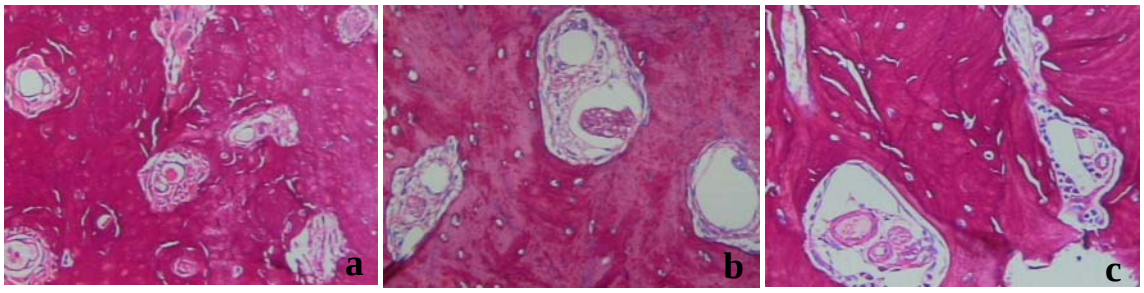

Fig.7. Histological sections showing collagen fiber in medullary spaces stained by picrosirius (red) in (a) alendronate, (b) zoledronic acid and (c) control groups (picrosirius, 200x).

OPG expression

There was no significant difference in immunohistochemical expression of OPG on alveolar bone between the alendronate, zoledronic acid and control groups (ANOVA; *P*>0.05). Regardless of the group evaluated, the interradicular field demonstrated significantly higher OPG expression than the other fields (ANOVA and Tukey's test; *P*=0.003) (Table IV; Fig. 8).

Table IV: OPG immunohistochemical expression in alveolar bone

Bone field	Group						Total	
	Alendronate		Zoledronic Acid		Control			
	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
Apical	11.72	3.90	17.69	9.88	14.64	6.81	14.48 ^{AB}	7.26
Interradicular	17.21	6.72	19.03	13.69	15.58	4.70	17.15 ^{A}	8.48
Buccal	8.73	6.10	11.50	6.52	12.12	7.92	10.72 ^{B}	6.80
Palatal	10.61	4.72	13.32	7.07	11.69	5.11	11.61 ^{B}	5.32
Total	12.07	6.17	15.34	9.77	13.51	6.25	13.48	7.44

SD=Standard deviation. ANOVA; Tukey's test (α=0.05). Means followed by different letters are significantly different (*P*=0.003).

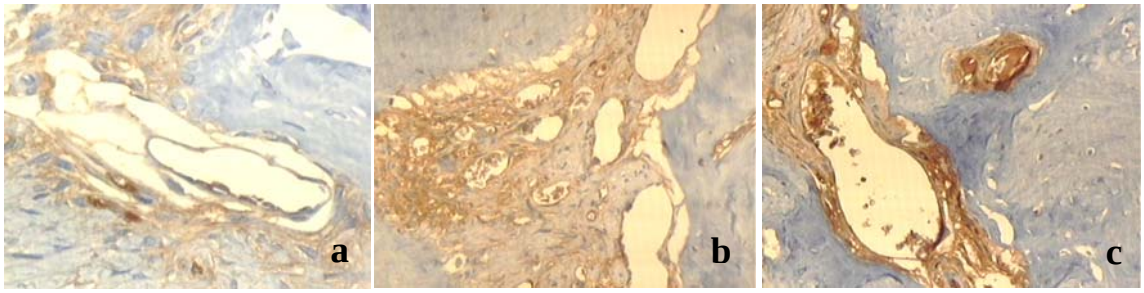

Fig.8. OPG expression on alveolar bone detected by immunohistochemistry: (a) alendronate, (b) zoledronic acid, and (c) control group (200x).

DISCUSSION

There was no significant difference in alveolar bone osteoclast count between the three groups analyzed – alendronate, zoledronic acid and control. Lower cell count would be expected in bisphosphonate-treated animals, since apoptosis of mature osteoclasts and suppression of osteoclastogenesis are reported effects of these drugs.[1] Some studies have shown lower osteoclast numbers in mouse bone tissue after bisphosphonate treatment.[58, 61, 63] However, conflicting results regarding this effect of bisphosphonates have been reported in the literature. Kimura et al.[67] and Spolidorio et al.[68] found no significant difference in osteoclast number in the tibia and alveolar bone

between alendronate-treated and untreated animals, which is consistent with the outcome of the present research.

Some points related to the different methods used in each study could explain such discrepancies. The majority of experiments evaluating the effect of bisphosphonates on bone, as in those performed by Ito et al.,[58] Tannehill-Gregg et al.[61] and Zheng et al.,[63] involved the induction of metabolic bone disease before therapy. The results of these studies showed lower osteoclast numbers when bisphosphonates were used. On the other hand, the present investigation and the studies performed by Kimura et al.[67] and Spolidorio et al.[68] evaluated the biological effect of drug administration in normal animals, without a induced disease model. In this situation, no difference in osteoclast number was observed between bisphosphonate and control group. Furthermore, factors such as dosage regimens, treatment duration, type of bisphosphonate and histological techniques are not standardized among experiments. Many studies employ tartrate-resistant acid phosphatase (TRAP), a specific histochemical marker for osteoclasts. When stained by this method, osteoclasts exhibit evident TRAP cytoplasmic activity identified by red staining.[69] The histochemical approach by specifically revealing cells bearing the marker appears to be more reliable than routine histology (H&E), since some osteoclasts, which often displays no nucleus or only one nucleus in histological sections, may go unrecognized during quantification procedures, as well as pre-osteoclasts.[70] However, the decalcification process may cause enzyme denaturation, resulting in a faint or absent stain by this method.[71] Thus, since typical multinucleated osteoclasts are generally easy to identify in routine histology and because this method demonstrates no significant difference in osteoclast number when compared to TRAP staining,[70] H&E was employed in the present study.

Although induction of osteoclast apoptosis would certainly inhibit bone resorption and decrease osteoclast numbers, bisphosphonates might exert other changes in osteoclasts that can also affect their ability to resorb bone. Some of these changes are disruption of osteoclast ruffled border and cytoskeleton, as well as prevention of both lysosomal enzyme release and ATP-dependent proton pump activity.[9] Actually, the initial effect of nitrogen-containing bisphosphonates seems to be impairment of osteoclast function. Eventually, these drugs may cause apoptosis and consequent reduction in osteoclast number, but this event does not appear to be a mandatory requirement for inhibition of resorption by bisphosphonates.[1]

Bisphosphonate action on bone tissue is dose-dependent. Studies have shown that the alendronate dose capable of inducing a decrease in osteoclast number is usually higher than that impairing cell activity.[1] In a previous *in vivo* study,[60] osteoclast number was evaluated after minodronic acid, a potent nitrogen-containing bisphosphonate, was administered to mice with collagen-induced arthritis. Reduction in osteoclast number was only seen in the high-dose minodronic acid group. When a 10-fold lower dose was used, there was no difference in osteoclast number between bisphosphonate and control group. In an *in vitro* study, alendronate inhibited bone resorption in pit assays at doses 10-fold lower than those reducing osteoclast number indicating that the oral bisphosphonate suppression of bone resorption was independent of its effects on apoptosis.[72] It should be noted that the alendronate dose used in the present study was based on the therapeutic dose prescribed for humans and adjusted according to rodents' metabolic rates[73] and to the longer treatment duration (150 days). Whether the calculated dose was only sufficient to promote inhibition of osteoclast function rather than inducing apoptosis requires further investigation.

Moreover, dysfunctional osteoclasts detached from bone surface were found in jaw bone biopsies obtained from areas of diseased but unexposed tissue in patients treated with zoledronic acid, who developed osteonecrosis.[15] This morphological feature indicates inhibition of osteoclast activity by impairment of ruffled border and sealing zone formation, caused by bisphosphonates.[9] Likewise, trans-iliac biopsies from alendronate-treated patients displayed higher osteoclast numbers, when compared to the placebo group. These cells, nevertheless, were detached from bone surface and exhibited pyknotic nuclei,[74, 75] a classic feature of cells undergoing apoptosis.[76] The evaluation of morphological features related to osteoclast activity or apoptosis in the H&E sections was not performed in the present study. Nevertheless, further studies applying this kind of analysis in the same *in vivo* model could disclose those features. Immunohistochemical staining of cellular structures related to osteoclast function, such as ATP-dependent proton pump[58] and methods for detecting cells undergoing apoptosis in histological sections, such as terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) or transmission electron microscopy[76] could address the questions presented here.

There was no significant difference in OPG expression between the alendronate, zoledronic acid and control groups in this research. This result suggests that the bisphosphonates tested did not stimulate OPG secretion in alveolar bone. As a consequence, the crucial signal to drive osteoclast development from hematopoietic progenitor cells would not be inhibited, which is consistent with the lack of effect on osteoclast number in the present study. A similar outcome was reported by Kim et al.[77] with alendronate use, which did not alter RANKL or OPG mRNA expression in mouse osteoblastic cells *in vitro*. Indeed, it has been reported that the mechanism of action of alendronate is not based on impairment of osteoclast recruitment, but on inhibition of

osteoclastic activity.[2] On the other hand, if statistical tests were not considered, the value obtained for OPG in zoledronic acid group would be higher than the other ones (*P*=0.14). It seems reasonable to consider the possibility of a larger sample size disclosing different results with statistical significance.

Zoledronic acid has been reported to promote an increase in OPG protein secretion by primary human osteoblast-like cells *in vitro*.[3, 27] However, peak plasma levels after intravenous infusion of zoledronic acid are reported to be far lower than that used in *in vitro* experiments. It is uncertain whether the high concentrations used in these studies accurately reflect what occurs in the bone microenvironment *in vivo*.[61] Although a significant increase in OPG expression was not observed at alveolar bone sites of bisphosphonate-treated animals in the present study, it was demonstrated by Zhou et al.[29] In a mouse model of Ewing sarcoma, tibia tissue in zoledronic acid-treated animals showed elevated OPG immunohistochemical expression when compared to untreated animals. Maybe alendronate and zoledronic acid behave differently in alveolar bone when compared to other skeletal sites. The present study showed not only a lack of OPG stimulation but also no changes in osteoclast numbers. Further investigations need to be conducted to resolve this question. In addition, the assessment of RANKL immunohistochemical expression could complement the results obtained. RANKL to OPG ratio is important in the regulation of bone resorption, since the balance between the expression of these two proteins dictates the quantity of bone resorbed.[29, 56]

The zoledronic acid group showed significantly higher trabecular bone density when compared to control group. The alendronate group, on the other hand, showed no statistical difference in trabecular density when compared to the zoledronic acid and control groups. The result concerning zoledronic acid was also reported by other studies in which intravenous bisphosphonate administration increased trabecular density in

bones bearing osteolytic tumors.[78-80] The result concerning alendronate was consistent with the study of Spolidorio et al.,[68] in which the effect of this bisphosphonate was evaluated in alveolar bone of rats without previous disease. In that experiment, no significant difference in trabecular density was found between alendronate and untreated animals, in three alveolar bone regions (buccal, interradicular and palatal). On the other hand, when compared to osteoporosis animal models, the results of the present study were conflicting. Those investigations reported increased trabecular density after oral bisphosphonate use.[12, 59, 81]

Such discrepancies could be explained by the notion that effects of bisphosphonate on bone volume may depend on the rate of bone remodeling before starting treatment. In studies using paired iliac crest biopsies, Borah et al.[82] showed that treatment with an oral nitrogen-containing bisphosphonate for 3 years was associated with an increase in bone volume in patients with high baseline bone turnover compared with no change in this parameter in patients with low baseline turnover. Bisphosphonate uptake in bone depends on hydroxyapatite exposure which occurs in resorption sites. Only the exposed hydroxyapatite is available and accessible to circulating bisphosphonates.[21] Since the animal model employed in the present study and also by Spolidorio et al.[68] did not have high bone turnover induced by any metabolic disease, probably a small amount of hydroxyapatite was exposed. Thus, bisphosphonate uptake and its consequent effects would likely be less prominent in normal animals than in models in which metabolic bone diseases such as osteoporosis were induced.[21] Maybe the lack of association between alendronate and trabecular enlargement was due to a lower uptake of bisphosphonate by the bone tissue because of its normal turnover. Zoledronic acid, on the other hand, promoted trabecular density increase despite the normal bone turnover. The divergent results between the bisphosphonates evaluated

could be explained by the higher mineral affinity of zoledronic acid, related to its three-dimensional molecular configuration and nitrogen disposition.[1]

There was no statistical difference in collagen fiber density in medullary spaces between the groups tested. This fact supports the idea that bisphosphonates *per se* do not promote bone marrow fibrosis. High amounts of fibrous connective tissue in medullary spaces was found in the jaws of patients with bisphosphonate-related osteonecrosis.[15, 34, 39, 49] Actually, marrow fibrosis is a histological feature associated with chronic inflammation in perinecrotic bone.[15, 34, 39, 49] Therefore, the cases of bone marrow fibrosis in patients under bisphosphonate therapy reported are more likely related to the osteonecrosis condition than the bisphosphonate effect.

Comparing the results obtained in each distinct bone field evaluated, there was no significant difference between groups for all variables tested. On the other hand, regardless of the group tested, trabecular density was higher in buccal, palatal and interradicular regions when compared to apical field. This difference, however, was probably due to anatomical and physiological factors related to the animal model and not to the bisphosphonate effects. It should be noted that buccal and palatal fields included the cervical area of the alveolar bone, as the respective crests were considered the initial reference point. Therefore, the results reported here are consistent with the concept that tissue alterations are more evident in cervical regions in maxillary rat molars. In apical sites, the effects are more subtle and show a direct relation to root morphology. Compared to humans, the roots of rats' upper molars are wider in the apical area providing the dissipation of masticatory forces toward the apical region.[83]

Some other aspects on the animal model employed in the present study should also be considered. For screening different therapeutic agents, the rat model has been generally accepted.[84] In fact, the leading preclinical model for osteoporosis is the

ovariectomized rat, in which bisphosphonate therapy is usually tested.[1] Rats' cancellous bone remodeling sites are very similar to those seen in humans, as well as skeleton anatomy.[84] Because of the unique physicochemical properties of bisphosphonates, data from animal studies, especially those related to absorption and deposition, can be reasonably extrapolated to humans.[21]

This study provided relevant evidence about the microscopic effects of oral and parenteral nitrogen-containing bisphosphonates on alveolar bone and showed trabecular enlargement with zoledronic acid use. On the other hand, collagen fiber density in medullary spaces, alveolar bone OPG expression and osteoclast count were not influenced by bisphosphonate administration. Additional studies exploring the balance between RANKL and OPG expression in alveolar bone, as well as the activity of osteoclasts, would contribute to resolving the questions still unanswered.

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DISCUSSÃO GERAL

4. DISCUSSÃO GERAL

A osteonecrose maxilar associada ao uso de bisfosfonatos constitui efeito adverso desses fármacos e caracteriza-se, ao exame físico, pela exposição de osso necrótico na cavidade oral. O controle da progressão da doença é a principal estratégia recomendada, uma vez que os procedimentos convencionais não se têm mostrado medidas terapêuticas eficazes (MIGLIORATI; SIEGEL; ELTING, 2006; RUGGIERO et al., 2009). Da mesma forma, a etiopatogênese da condição ainda não foi completamente esclarecida (RUSSELL et al., 2008). Talvez isso ocorra em função de a maioria das publicações basearem-se em observações pontuais de casos clínicos. A literatura prescinde de estudos epidemiológicos longitudinais bem como de investigações experimentais em modelo animal. Tais limitações colaboram para a falta de uma abordagem terapêutica adequada da doença (RUGGIERO et al., 2009).

As dificuldades no manejo dos pacientes que apresentam osteonecrose maxilar associada ao uso de bisfosfonatos inspiraram a concepção e estimularam a realização da presente pesquisa. Os bisfosfonatos mais empregados para o tratamento das complicações de neoplasias osteolíticas e da osteoporose são, respectivamente, o ácido zoledrônico e o alendronato (MARX et al., 2005). A maioria dos estudos analisa a ação dessas drogas em tíbia e fêmur, após a indução de doenças do metabolismo ósseo (TANNEHILL-GREGG et al., 2006; ZHENG et al., 2007). Poucas são as pesquisas que avaliam os efeitos biológicos da administração do ácido zoledrônico e do alendronato no osso alveolar sem a indução de doença óssea prévia. Partindo-se desse pressuposto e considerando-se os relatos da literatura acerca do mecanismo de ação dos bisfosfonatos, importantes variáveis associadas ao efeito desses fármacos sobre o osso alveolar foram investigadas. Ainda, a realização do experimento em modelo animal possibilitou isolar

os fatores a serem estudados e, assim, avaliar a ação dos bisfosfonatos independentemente das variáveis intervenientes que os pacientes, via de regra, apresentam (SONIS et al., 2009).

Os resultados acerca da densidade trabecular obtidos na presente pesquisa corroboram os resultados de outros estudos que relatam o efeito indutor do ácido zoledrônico sobre a formação óssea. O presente estudo demonstrou que, da mesma forma como ocorre em outros ossos do esqueleto (QUINN et al., 2005; DAUBINÉ et al., 2007; MORGAN et al., 2008; RUSSELL et al., 2008), a densidade trabecular aumenta no osso alveolar com o uso do ácido zoledrônico a despeito da ausência de doença metabólica óssea prévia. Em contrapartida, o emprego do alendronato não influenciou significativamente a densidade trabecular do osso alveolar, achado também relatado por Spolidorio et al. (2007). Entretanto, resultados controversos relativos a esse tópico podem ser observados na literatura. Em modelos animais de osteoporose, por exemplo, o emprego do alendronato promoveu aumento da densidade trabecular em tíbia e fêmur (DALLE CARBONARE et al., 2005; OGAWA et al., 2005; IWAMOTO et al., 2006).

Os bisfosfonatos avaliados também não influenciaram a densidade de fibras colágenas dos espaços medulares. Os resultados sugerem que esses fármacos, isoladamente, não sejam os responsáveis pela fibrose medular observada em casos de osteonecrose maxilar associada ao seu uso (BEDOGNI et al., 2008). A ocorrência da fibrose, portanto, parece resultar do processo inflamatório que acompanha a osteonecrose.

O osteoclasto é a célula-alvo dos bisfosfonatos (ROGERS et al., 2000). Esperava-se que essas drogas, por induzirem os osteoclastos à apoptose (ZHENG et al., 2007), viessem a determinar a diminuição da contagem dos mesmos na superfície

alveolar. Entretanto, não foi observado menor número de osteoclastos após o uso de alendronato ou de ácido zoledrônico. Reforçando esse contexto, a expressão imunoistoquímica de OPG também não foi modificada pelo emprego dos medicamentos. Como essa proteína participa do controle da diferenciação osteoclástica, inibindo-a, o aumento da sua expressão contribuiria para a diminuição da contagem dos osteoclastos (KIM et al., 2002) na superfície óssea. Esses resultados são complementares e sugerem que, nas condições da presente pesquisa, os bisfosfonatos não exerceram efeito significativo sobre a osteoclastogênese e sobre a apoptose dos osteoclastos.

O principal efeito dos bisfosfonatos é a inibição da reabsorção óssea (ROGERS et al., 2000). Entre os mecanismos de ação envolvidos nesse processo inibitório, estaria a indução dos osteoclastos à apoptose e consequente diminuição de seu número em cortes histológicos (ROGERS et al., 2000). O fato de, na presente pesquisa, não ter sido observada alteração da contagem de osteoclastos e da expressão de OPG nos grupos-teste reforça a ideia de que a inibição da reabsorção óssea mediada pelo fármaco envolva outros mecanismos além da apoptose dessas células. Talvez o efeito mais relevante da droga no osso alveolar seja a inibição da atividade dos osteoclastos e não a promoção de sua apoptose. Segundo algumas pesquisas realizadas com outros ossos do esqueleto, foi possível observar osteoclastos destacados da superfície óssea, que apresentaram encolhimento do citoplasma e núcleos hipercromáticos após a administração de bisfosfonatos. Tais alterações seriam indicativas da interrupção da atividade celular (ROGERS et al., 2000; BEDOGNI et al., 2008; JAIN; WEINSTEIN, 2009; WEINSTEIN; ROBERSON; MANOLAGAS, 2009). Futuras investigações que avaliem a morfologia e a atividade osteoclástica podem fornecer respostas às questões abordadas. O emprego de marcadores imunoistoquímicos

de estruturas celulares como a bomba de prótons, além de métodos para detectar células em processo de apoptose seriam procedimentos pertinentes.

No contexto atual da osteonecrose maxilar associada ao uso de bisfosfonatos, a prevenção constitui a melhor conduta. A abordagem terapêutica da enfermidade é assunto controverso e apresenta baixa resolutividade, a depender da gravidade do quadro e do fármaco em questão (RUGGIERO et al., 2009). A maioria das pesquisas são desenvolvidas em tíbia e fêmur de modelos animais (ITO et al., 2001; OGAWA et al., 2005; TANNEHILL-GREGG et al., 2006; YAO et al. , 2006; TANISHIMA et al., 2007; ZHENG et al., 2007). Entretanto, existem diferenças anatômicas e fisiológicas entre esses ossos e o complexo maxilomandibular. Esse fato torna-se evidente ao constatar-se a ausência de casos de osteonecrose associada a bisfosfonatos em outros ossos do esqueleto que não maxila e mandíbula (MARX, 2007). Sendo assim, muitos aspectos sobre o mecanismo de ação do fármaco e seus efeitos sobre o osso alveolar permanecem obscuros (RUGGIERO et al., 2009). Novas pesquisas que investiguem interações específicas entre osso alveolar e bisfosfonatos poderão identificar os agentes responsáveis pela exclusividade do complexo maxilo-mandibular em sediar a osteonecrose e, assim, contribuir tanto para a prevenção quanto para nortear uma abordagem terapêutica eficaz da doença.

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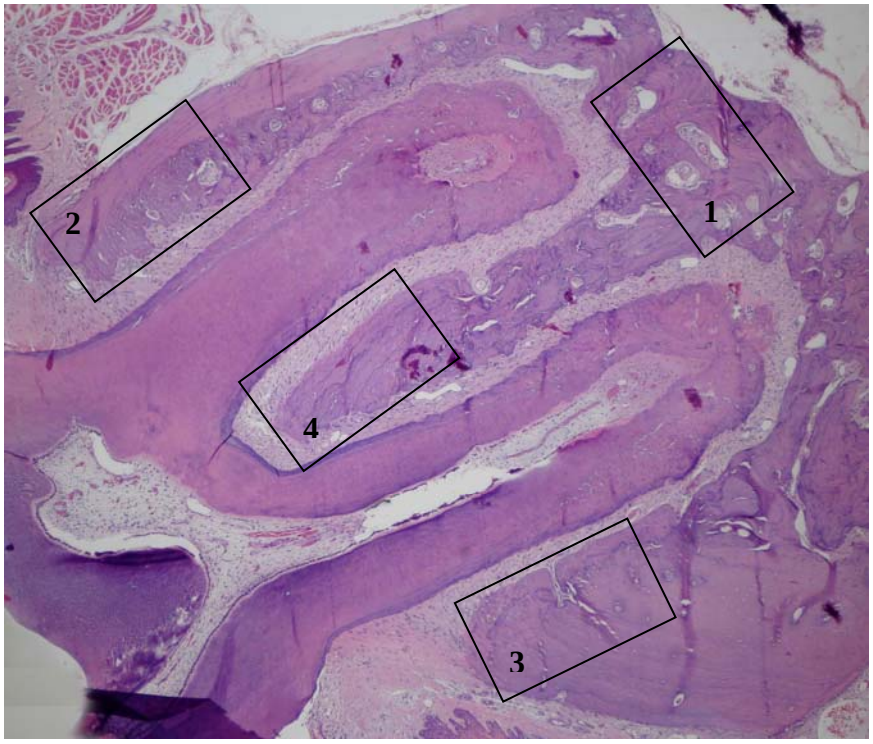

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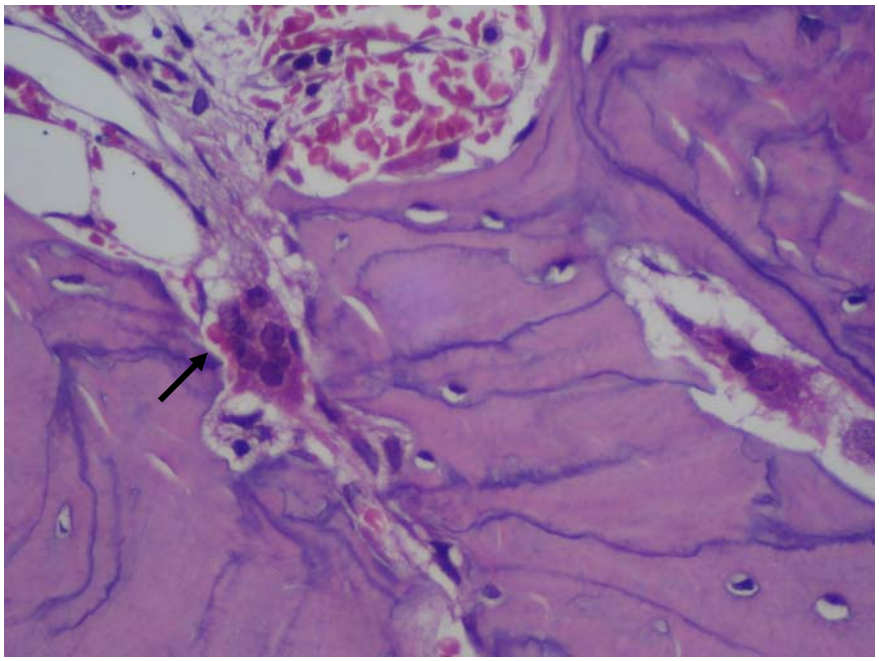

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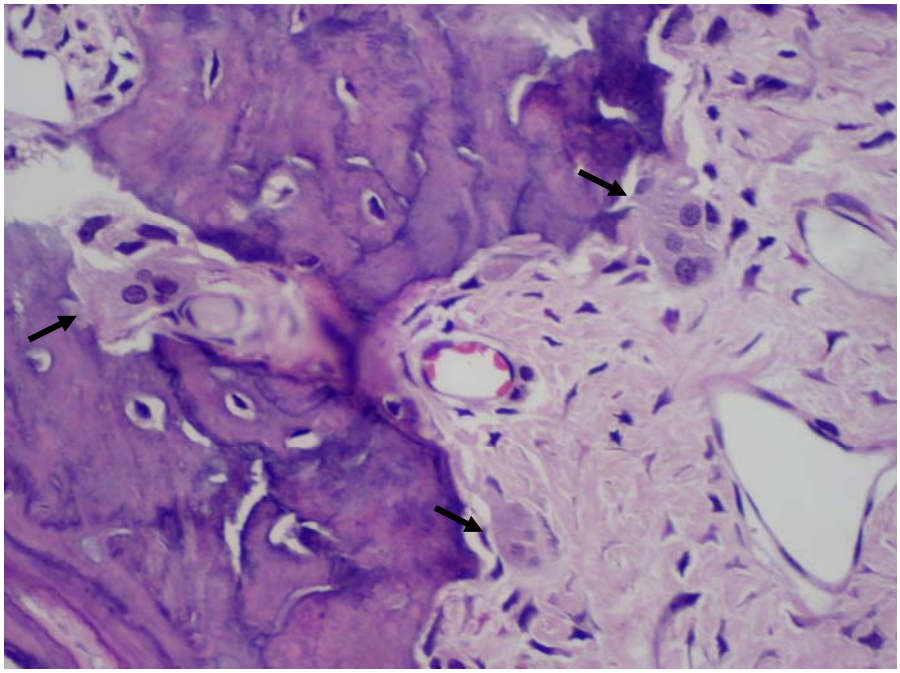
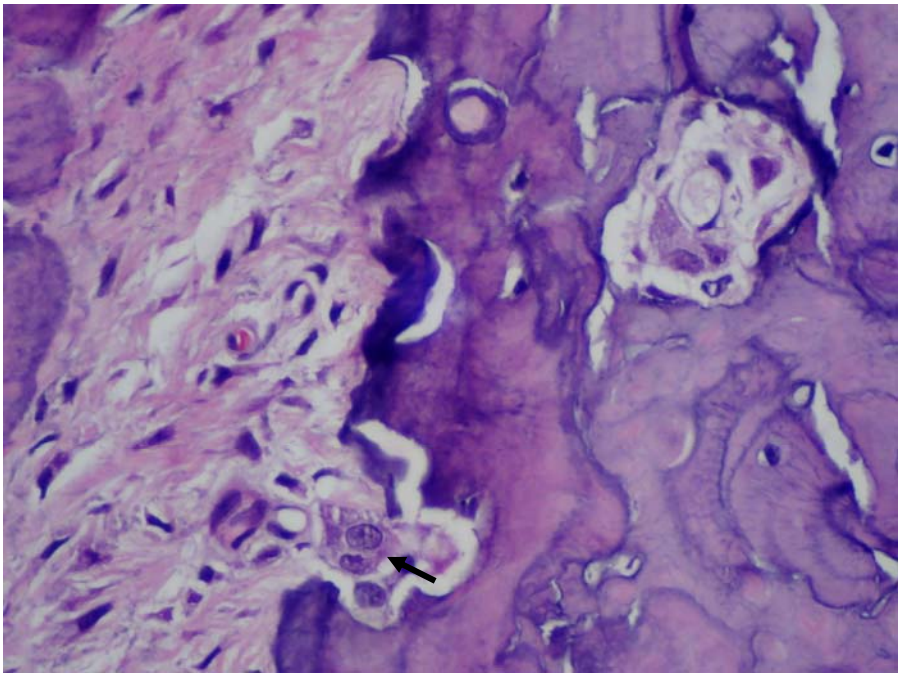


Osteoclastos (setas). Grupo alendronato, região apical (HE; aumento aproximado de 400x).



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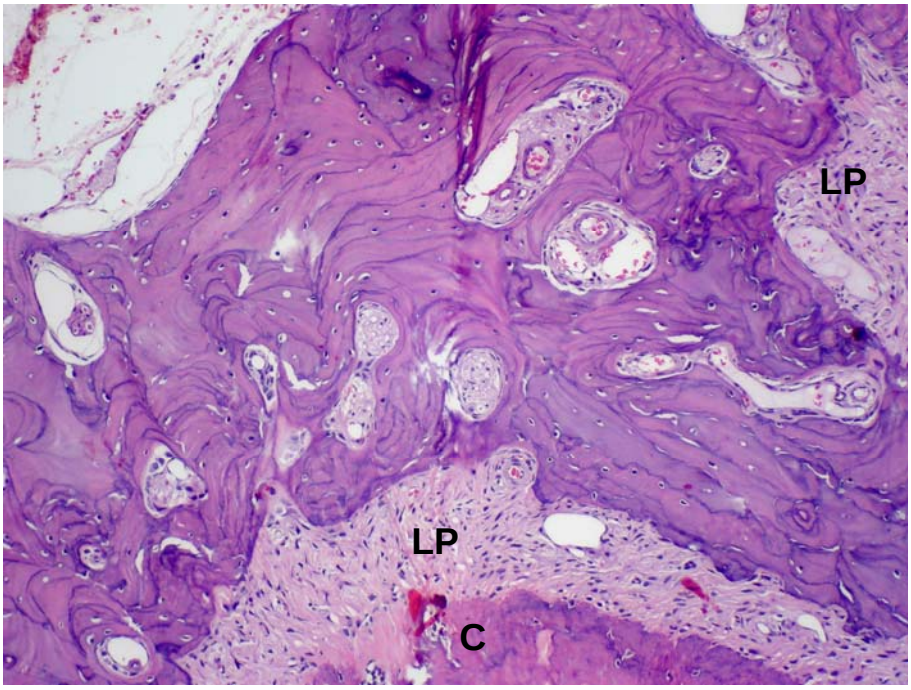

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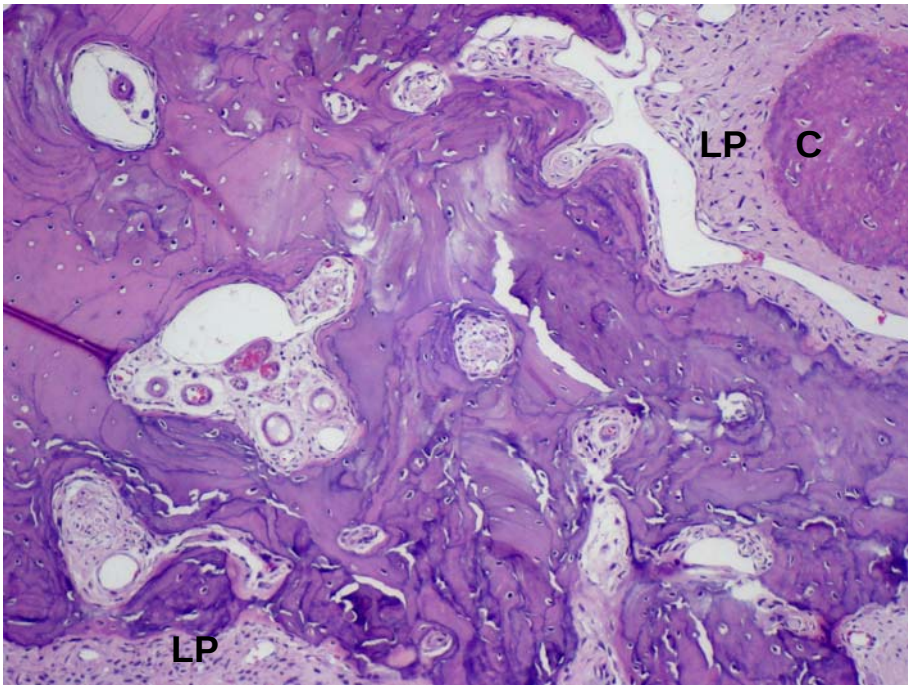

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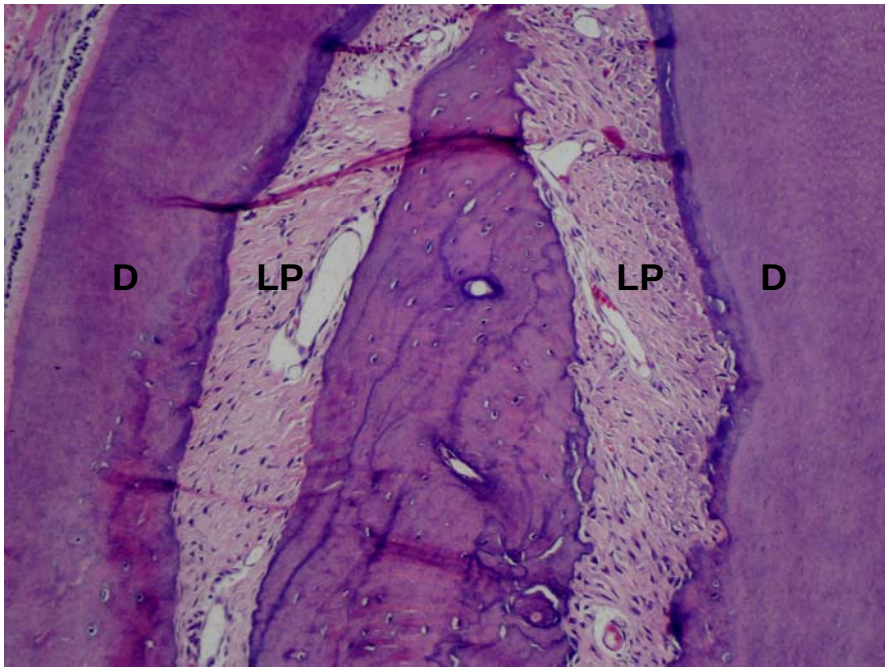

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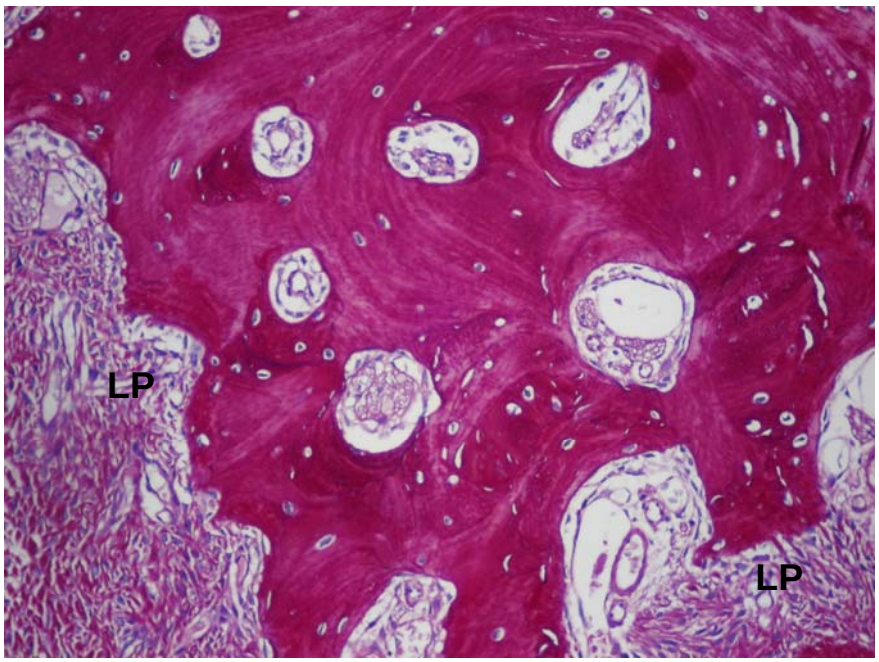

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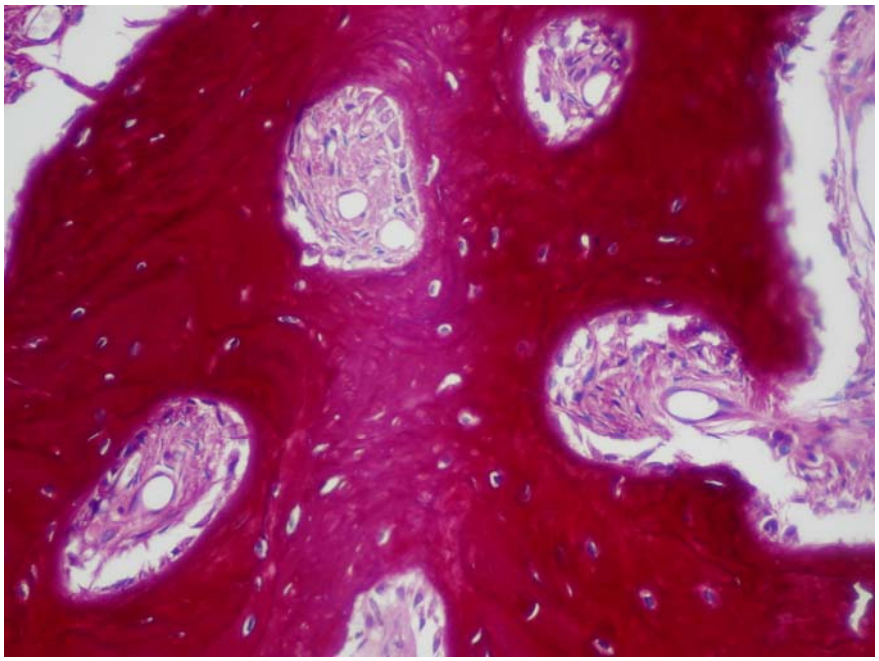
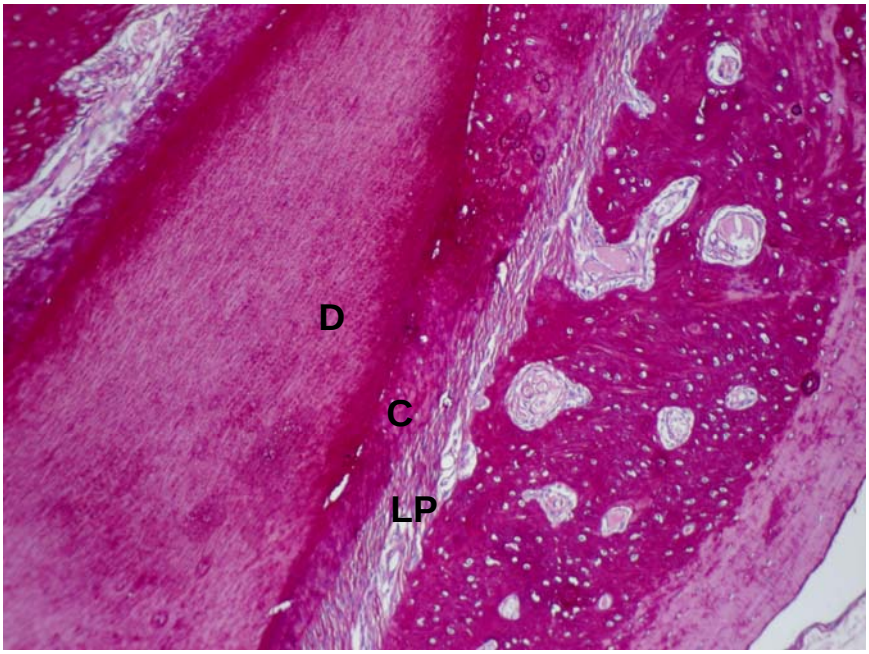
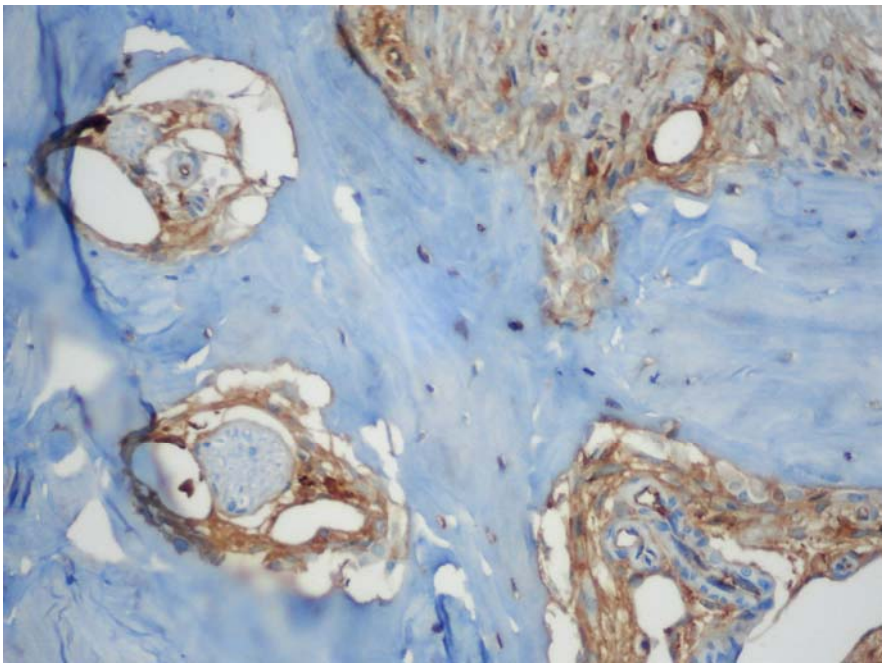


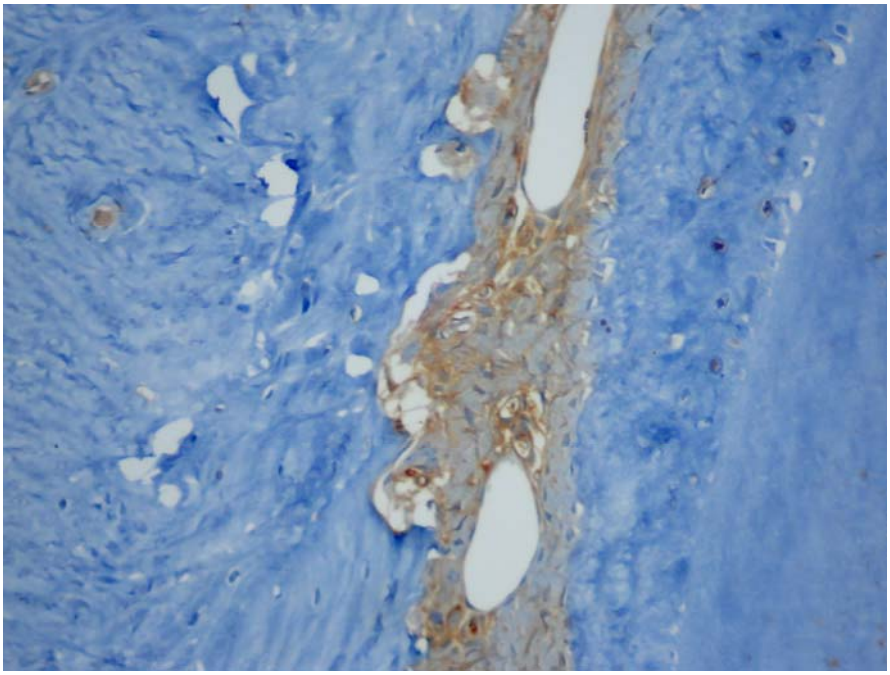
Fibras colágenas nos espaços medulares do osso alveolar. Grupo alendronato, região apical (picrossírius; aumento aproximado de 200x).



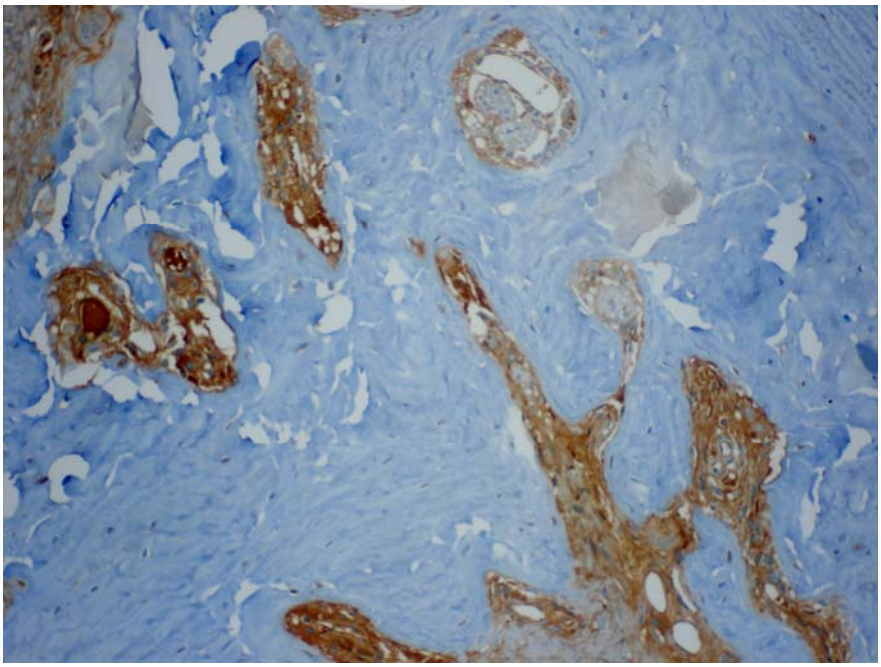
Fibras colágenas nos espaços medulares do osso alveolar. Grupo ácido zoledrônico, região vestibular (picrossírius; aumento aproximado de 100x). **LP**: ligamento periodontal; **C**: cemento; **D**: dentina.



Expressão de OPG no osso alveolar. Grupo-controle, região apical (imunoistoquímica; aumento aproximado de 200x).



Expressão de OPG no osso alveolar. Grupo alendronato, região palatina (imunoistoquímica; aumento aproximado de 200x).



Expressão de OPG no osso alveolar. Grupo ácido zoledrônico, região inter-radicular (imunoistoquímica; aumento aproximado de 200x).

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