FACULDADE DE ODONTOLOGIA

AVALIAÇÃO MACRO E MICROSCÓPICA DE LESÕES ORAIS INDUZIDAS POR PROCEDIMENTOS CIRÚRGICOS EM RATOS SOB TERAPIA COM BISFOSFONATOS

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MACRO AND MICROSCOPIC EVALUATION OF LESIONS INDUCED BY ORAL SURGICAL PROCEDURES IN RATS UNDER THERAPY WITH BISPHOSPHONATES

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PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

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Tese apresentada como requisito para obtenção do título de Doutor pelo Programa de Pós-Graduação da Faculdade de Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul. Área de Concentração, Estomatologia Clínica

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Porto Alegre

2012

DEDICATÓRIA

Dedico este trabalho aos meus pais, Fátima e Paulo, e às minhas irmãs Fabíola e Paula,
pelo amor generoso e desprendido.
Amo-os profunda e infinitamente.

AGRADECIMENTOS

À minha orientadora, Dra. Karen Cherubini, exemplo de dedicação, competência e responsabilidade pelo exercício acadêmico. Sou extremamente grata pelo ensinamento generoso e, sobretudo, pela constante (e incansável) disponibilidade a mim dedicada durante todo o período em que convivemos. Por sua grande contribuição ao meu amadurecimento profissional, muito obrigada!

À minha avó Urçula, aos meus tios e cunhado, pela ternura, confiança e carinho constantes.

Às minhas sobrinha e afilhada, lindas e cheias de vida, Beatriz e Clara, por não me deixarem esquecer que a vida, quando colorida de *pink* com brilhos, pode ser muito mais divertida.

Ao meu sobrinho Pedro, por entender que, "por uns tempos", a tia Carol precisou morar no computador.

Às minhas grandes e especiais amigas Cláudia, Carla, Tatiana, Flávia, Sílvia, Mara e Emmanuela. Este trabalho jamais teria acontecido sem o apoio de vocês. Obrigada por fazerem a minha vida valer muito a pena! Orgulho imenso em tê-las presentes comigo. Amo, de uma forma especial, cada uma de vocês!

Às professoras do Doutorado em Estomatologia Clínica, Dra. Liliane Soares Yurgel, Dra. Maria Antonia Zancanaro de Figueiredo e Dra. Fernanda Gonçalves Salum, pelo exemplo de competência e profissionalismo.

Ao Dr. Alan Arrieira Azambuja, pelo apoio atencioso e dedicado durante a pesquisa.

Ao professor Dr. Vinícius Duval da Silva, pelo apoio técnico fornecido durante a etapa de captura e análise das imagens histológicas.

Ao Técnico do Laboratório de Anatomia Patológica e Citopatologia do Hospital São Lucas da PUC-RS, Tiago Giulliani Lopes, por toda a disponibilidade.

Aos colegas do Programa de Pós-graduação, em especial a Maria Noel, Márcia Fava, Wâneza Borges, Soraya Berti, Jonas Dantas e Luiz Arteche (*in memoriam*), pelos bons momentos compartilhados nesses anos de PUC-RS.

A Luciana Giacometti, por todo apoio.

Aos funcionários do Programa de Pós-Graduação em Odontologia, Ana Lúcia, Paulo, Marcos e Davenir, pela atenção e competência.

À Faculdade de Farmácia da PUCRS, por disponibilizar o suporte técnico necessário à realização do experimento.

Ao CNPq, pelo apoio financeiro.

EPÍGRAFE

Não existem fatos, apenas interpretações.

Friedrich Nietzsche

(1844-1900)

RESUMO

RESUMO

A presente pesquisa teve por objetivo comparar a influência dos bisfosfonatos ácido zoledrônico e clodronato no reparo de feridas cirúrgicas induzidas por meio de exodontia e lesão de tecido mole oral. Trinta e quatro ratos (*Rattus novergicus*, linhagem Wistar) foram distribuídos em três grupos: (1) 12 animais tratados com ácido zoledrônico; (2) 12 animais tratados com clodronato; e (3) 10 animais que receberam solução salina. Decorridos 90 dias do início do tratamento, os animais foram submetidos a exodontias e procedimentos cirúrgicos em tecido mole, ambos na maxila. Aos 180 dias de administração dos fármacos, foi realizada a eutanásia. Após avaliação macroscópica, as maxilas foram processadas, e os cortes histológicos corados por hematoxilina e eosina (H&E), bem como submetidos a processamento imunoistoquímico com os marcadores RANKL, OPG, von Willebrand e caspase-3. Nas lâminas coradas por H&E, foram quantificadas as variáveis osso não-vital, osso vital, infiltrado inflamatório, colônias microbianas, tecido epitelial e tecido conjuntivo, sendo que, no sítio das exodontias, os restos radiculares também foram avaliados. A análise histológica foi realizada por meio do programa *Image Pro Plus*. Na avaliação macroscópica, no sítio das exodontias o grupo ácido zoledrônico exibiu associação com solução de continuidade da mucosa (qui-quadrado, análise de resíduos ajustados, $p<0,001$), enquanto na área de lesão de tecido mole, nenhum grupo exibiu essa associação (qui-quadrado, $p=0,151$). No sítio das exodontias, o grupo ácido zoledrônico exibiu proporção significativamente maior de osso não-vital e colônias microbianas do que os demais grupos. Na lesão de tecido mole, a proporção de osso não-vital e colônias microbianas foi significativamente maior nos grupos ácido zoledrônico e clodronato do que no controle. Não houve diferença significativa das variáveis tecido epitelial, infiltrado inflamatório e resto radicular entre os grupos (Kruskal-Wallis teste de comparações múltiplas, $p>0,05$). A expressão imunoistoquímica de RANKL, OPG, von Willebrand e caspase-3 também não diferiu significativamente entre os grupos (Kruskal-Wallis, $p>0,05$). Os resultados permitem concluir que (1) ambos, ácido zoledrônico e clodronato, são capazes de induzir osteonecrose; (2) de acordo com a análise imunoistoquímica, é pouco provável que eventos intrínsecos à mucosa oral sejam os iniciadores da osteonecrose.

Palavras-chave: bisfosfonatos, osteonecrose, marcadores biológicos, ratos

SUMMARY

SUMMARY

The aim of this work was to compare clodronate and zoledronic acid effect on the repair of surgical wounds induced by tooth extraction and oral soft tissue lesion. Thirty-four rats (*Rattus novergicus*, Wistar) were allocated into 3 groups: (1) 12 animals treated with zoledronic acid; (2) 12 animals treated with clodronate; and (3) 10 animals that were given saline solution. Elapsed 90 days from the beginning of the treatment, the animals were subjected to tooth extractions and surgical-induced soft tissue injury in maxillae. At 180 days of drug administration, they were euthanized. After macroscopic evaluation, maxillae were processed and histological cuts were stained with hematoxylin and eosin (H&E). Immunohistochemical expression of RANKL, OPG, von Willebrand and caspase-3 was also evaluated. Non-vital bone, inflammatory infiltrate, microbial colonies, epithelial tissue, connective tissue and vital bone were quantified at the tissue wound sites. At the tooth extraction site, root fragments were also evaluated. The variables were quantified with Image Pro Plus software. Macroscopic analysis at the tooth extraction site showed that zoledronic acid group was associated with loss of mucosal integrity (chi-square, residual adjusted analysis, $p<0.001$), whereas at the soft tissue wound site, no group showed this association (chi-square, $p=0.151$). At the tooth extraction site, the zoledronic acid group showed greater proportion of non-vital bone and microbial colonies in comparison with the other groups. At the soft tissue wound site, the proportions of non-vital bone and microbial colonies were greater in the zoledronic acid and clodronate groups than in the control group. There was no significant difference for epithelial tissue, inflammatory infiltrate and root fragments between the groups (Kruskal-Wallis test complemented by its multiple comparisons test, $p>0.05$). Immunohistochemical expression of RANKL, OPG, von Willebrand and caspase-3 at tooth extraction and soft tissue wound sites did not differ significantly between the three groups analyzed (Kruskal-Wallis test, $p>0.05$). According to the results, (1) both bisphosphonates zoledronic acid and clodronate are capable of inducing osteonecrosis; (2) the immunohistochemical analysis suggests that the involvement of soft tissues as the initiator of osteonecrosis development is less probable than has been pointed out.

Key words: bisphosphonates, osteonecrosis, biological markers, rats

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BMP	Bone morphogenetic protein
CD 31	Cluster of differentiation 31
CD 34	Cluster of differentiation 34
CTX	Carboxy terminal collagen cross links
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
H&E	Hematoxylin-eosin
IL-1	Interleukin-1
JPEG	Joint Photographic Expert Group
M-CSF	Macrophage colony-stimulating factor
MMP	Matrix metalloproteinase
NTX	Aminoterminal telopeptide
OCIF	Osteoclastogenesis inhibitory factor
OCN	Osteocalcin
OPG	Osteoprotegerin
OPGL	OPG ligand
OPN	Osteopontin
PCR	Polymerase chain reaction
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor-kappa B
RANKL	Receptor activator of nuclear factor- kappa-B ligand
RT-PCR	Reverse transcription-polymerase chain reaction
RUNX2	Runt-related transcription factor 2
TGF-β1	Transforming growth factor beta 1
TNF	Tumor necrosis factor
TRANCE	TNF-related activation-induced cytokine
TRAP	Tartrate resistant acid phosphatase
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick endlabeling
VEGF	Vascular endothelial growth factor
vWF	Willebrand factor

SUMÁRIO

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INTRODUÇÃO

1 INTRODUÇÃO

Bisfosfonatos são análogos sintéticos do pirofosfato inorgânico, um regulador endógeno do metabolismo ósseo, e são empregados na prevenção e no tratamento de doenças caracterizadas por excessiva reabsorção óssea. Esses fármacos podem ser classificados, de acordo com a presença ou ausência de nitrogênio em sua composição, respectivamente, em nitrogenados e não-nitrogenados. Ácido zoledrônico, alendronato, risedronato, olpadronato e icandronato pertencem ao grupo dos nitrogenados, enquanto clodronato, tiludronato e etidronato classificam-se como não-nitrogenados (MIGLIORATI et al., 2005; RUGGIERO et al., 2009).

A osteonecrose por bisfosfonato é uma condição que se caracteriza por exposição óssea persistente no complexo maxilomandibular de pacientes submetidos ao tratamento com o fármaco, mas sem histórico de radioterapia da região de cabeça e pescoço. O achado clínico habitual consiste em área de mucosa ulcerada com exposição de osso desvitalizado localizada, mais frequentemente, na região póstero-lingual da mandíbula (RUGGIERO; WOO, 2008; RUGGIERO et al., 2009). Os achados radiográficos são inespecíficos e podem assemelhar-se a lesão periapical, osteomielite e neoplasia maligna primária ou metastática. Ao exame histológico, podem-se observar áreas compatíveis com necrose óssea evidenciada por regiões de infiltrado celular inflamatório e osso avascular e acelular (KHOSLA et al., 2007). A condição é de difícil tratamento e este, em muitos casos, visa apenas a preservar a qualidade de vida do paciente por meio do controle da dor e da infecção, bem como prevenir a ocorrência de novas áreas de necrose (RUGGIERO; WOO, 2008).

A hipótese de que a etiopatogenia da osteonecrose por bisfosfonatos tem origem multifatorial é suportada por estudos que apontam os efeitos do fármaco sobre o metabolismo ósseo (KELLINSALMI et al., 2005; SENEL et al., 2010; SONIS et al., 2009)

e sobre a vascularização sanguínea (BI et al., 2010; NAIDU et al., 2008; WALTER et al., 2010; YAMADA et al., 2009; WOOD et al., 2002), bem como pela participação de agentes infecciosos (KOBAYASHI et al., 2010). Pesquisas revelam, ainda, que os bisfosfonatos são tóxicos ao epitélio oral, o que suscita dúvidas quanto à origem da lesão ser no tecido ósseo ou na mucosa de revestimento (LANDESBERG et al., 2008). Além disso, a maior parte das pesquisas que avaliam os efeitos do fármaco sobre os tecidos e sua relação com a osteonecrose são realizadas com bisfosfonatos nitrogenados, sendo poucas as investigações sobre o comportamento tecidual mediante o emprego dos não-nitrogenados.

A presente tese é composta por dois trabalhos apresentados sob a forma de artigos científicos. O primeiro teve por objetivo apresentar uma revisão da literatura concernente a métodos laboratoriais e biomarcadores disponíveis para avaliação dos efeitos biológicos dos bisfosfonatos. O segundo descreve o experimento, cujo objetivo foi comparar a influência dos bisfosfonatos nitrogenados (ácido zoledrônico) e não nitrogenados (clodronato) no reparo de feridas cirúrgicas induzidas em osso e em tecido mole da maxila.

ARTIGO 1

2 ARTIGO 1

O artigo a seguir intitula-se **Laboratory methods and biomarkers in the evaluation of bisphosphonate effects on body tissues - A literature review** e foi formatado de acordo com as normas do periódico *Oral Oncology* (Anexo A).

Laboratory methods and biomarkers in the evaluation of bisphosphonate effects on body tissues - A literature review

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Running title: *Biomarkers and bisphosphonates*

ABSTRACT

Bisphosphonates are extensively used to treat bone metabolism disorders, but these drugs have an important side effect - jaw osteonecrosis. Therefore, studies have been conducted aimed at better understand their mechanism of action and to determine a course to neutralize this important side effect. We present here a literature review focusing on the laboratory methods available for investigating bisphosphonate effects on body tissues. There are many different methods available for this purpose, but the correct indication of these techniques and the knowledge of their limitations are of crucial importance for the understanding of bisphosphonate effects on body tissues.

Key words: bisphosphonates; biomarkers; tissues

Introduction

Bisphosphonates are drugs used in the prevention and treatment of bone metabolism diseases with intense resorption activity.[1,2] This is because of their ability to inhibit both bone resorption[3] and angiogenesis.[4] However, some effects of these drugs on non-mineralized tissues have also been reported.[5,6]

These compounds are able to interfere with remodeling of bone tissue by acting on different cells.[4,7-9] Their effect on osteoclasts occurs by inhibiting recruitment and differentiation, decreasing life span[10] and promoting apoptosis.[7,11,12] The mechanism through which bisphosphonates affect osteoblasts is still unclear, but some *in vitro* studies have demonstrated that high concentrations of nitrogen-containing bisphosphonates inhibit osteoblast proliferation, adhesion, and migration[13,14] as well as inducing osteoblasts to produce osteoprotegerin (OPG).[15] Studies have shown that zoledronic acid and, to a lesser extent, clodronate have antiangiogenic effects, tending to inhibit proliferation, migration,

and adhesion of endothelial cells.[16,17] Zoledronic acid inhibits the proliferation and migration of these cells *in vitro* in a dose-dependent way.[18] Studies have also postulated that bisphosphonates, especially the most potent ones, are able to modulate the secretion of specific growth factors, such as transforming growth factor β1 (TGF-β1).[13]

Adverse effects related to therapy with these drugs are sporadic and depend on the class of the drug, as well as the route and frequency of administration. Bisphosphonate-related ostonecrosis of the jaws was first described in 2003 and is characterized by an area of exposed bone in the maxillofacial region, which persists for at least eight weeks in patients undergoing therapy with these drugs and with no history of head and neck radiation therapy.[19-21] Several theories have been proposed to explain the pathogenesis of this lesion, whose etiology is multifactorial.[22] It is believed that the impact of the drug on different cell types is capable of contributing to the difficulty of tissue healing after the damage, resulting in clinical manifestation of the disease. The challenge of treating osteonecrosis has demanded research on the mechanism of action of bisphosphonate and its effects on biological tissues,[23] aiming to establish criteria to guide the management of patients using bisphosphonate or with osteonecrosis.[24] The present study reviewed the scientific literature with regard to laboratory methods and biomarkers available for the evaluation of biological effects of bisphosphonates, and we discuss here their indication, limitations, advantages and disadvantages.

Evaluation of bisphosphonate effects on bone cells

Hematoxylin-eosin evaluation (H&E)

On H&E examination, bisphosphonate-related osteonecrosis shows non-vital bone tissue infected by microorganisms, as well as inflammatory areas.[25,26] In bisphosphonate users without osteonecrosis, bone tissue shows some morphologic changes, with

osteoclasts being the focus of attention. In this examination, they appear as acidophilic multinucleated giant-cells (Fig. 1A). The active osteoclasts are found intimately associated with the bone surface through their active pole. At this site, the ruffled border and the clear zone are noted, which is a small area demarking the borders of the region to be reabsorbed. The osteoclast-bone interface is poorly stained and is not easily seen. The inactive osteoclasts, in turn, do not stay in contact with bone structure and tend to assume a rounded shape. The H&E technique is easily performed, has low cost and also permits the visualization of active and inactive osteoclasts, an important feature regarding bisphosphonates. The major limitation lies in the identification of osteoclasts with just one or no nucleus. In these cases, they could be wrongly classified as pre-osteoclasts or not even be identified.[27]

There are some reports about the decrease in osteoclasts in bone under aminobisphosphonate treatment,[28,29] and histomorphometric evaluations in H&E have found increased trabecular density in alveolar bone,[30] tibia and femur.[31,32] Still, there are also reports of the absence of either changes in osteoclast number or significant increase in trabecular density in alveolar bone of rats treated with alendronate.[33]

Tartrate-resistant acid phosphatase (TRAP)

TRAP is a glycoprotein synthesized by differentiated cells of the monohistiocytic system that can be found in plasma, placenta, macrophages and leukocytes, and is more significantly expressed in osteoclasts.[34] It is one of the proteins secreted in the bone-osteoclast interface, and its increased expression is directly related to bone resorption.[35] TRAP is used as a resorption marker and its activity is inhibited by calcitonin.[36]

TRAP can also be used as a serum marker of osteoclast activity[34] and is identified by a histochemical method, where it shows purple staining, because of the reduction of

iron ions located at its active site.[27] The technique has been applied in investigations of bisphosphonate effects using cell cultures[37,38] and animal models,[39,40] showing a decreased number of osteoclasts after bisphosphonate use.

As TRAP staining only detects osteoclast cells (Fig. 1B), it is more specific than H&E. However, the specimen decalcifying process with strong acids such as nitric, formic and trichloroacetic impairs the TRAP technique. These acids cause enzymatic denaturation, which leads to failure in cell staining.[41] Moreover, as it is an enzymatic method, it is highly sensitive to environmental factors such as pH and temperature variation.[35]

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)

TUNEL detects apoptotic cells *in situ* by means of specific staining of fragmented DNA. It has already been used to investigate bisphosphonate effects on keratinocytes,[5,42] endothelial cells, fibroblasts, osteoblasts[43,44] and osteoclasts.[45] However, the specificity of this technique has been discussed regarding the fact that the increase in nuclear condensation during apoptosis impairs the access of TUNEL reagents to the target structures (apoptotic nucleus). Also, the method can show false positive results in cases of necrotic or mitotic cells staining.[46]

Combined TRAP and TUNEL technique

When stained exclusively by TRAP, osteoclasts exhibit notable cytoplasmic activity, which is revealed by a red color. If these cells are subjected to TUNEL, the positivity is identified by vacuoles containing yellowish-brown bodies. Therefore, TUNEL

and TRAP combination shows apoptotic osteoclasts as red cells containing yellowish-brown vacuoles.[47,48]

When compared with caspases, especially with the effector ones, the combined TRAP/TUNEL technique can show lower specificity because of the possible staining of necrotic and mitotic osteoclasts.[47] Still, this combined technique allows the nucleus of apoptotic osteoclasts to be distinguished from that of osteoblasts or osteocytes, which have casually been internalized by osteoclasts during bone resorption.[47,48]

Caspases

Apoptosis involves the activation of caspases, which make up a family of cysteine proteases that play a fundamental role in the cleavage of intracellular substrates. These proteins are synthesized as inactive precursors (pro-caspases), where they are activated after receiving a cell death signal.[49,50] Until now, 14 types of caspases have been identified, and they are classified according to their activity as inflammatory (1,4,5,11,12,13,14), initiators (2,8,9,10) and effectors (3,6,7). The activation of apoptosis can occur through two pathways. The extrinsic route is started by external signals such as free-radicals and physical or chemical agents, which stimulate cell surface receptors. The intrinsic route can occur as a result of cellular stress that causes morphofunctional changes in mitochondria. In both pathways, there is triggering of the caspase cascade, which culminates with caspase-3 activation.[51]

Caspases have been used in investigations into the effects of bisphosphonate on various cells, including bone and endothelial cells, fibroblasts and keratinocytes.[6,18] Studies have demonstrated pro- and antiapoptotic effects of bisphosphonates on osteoclasts and osteoblasts/osteocytes, respectively. Benford et al. (2001)[49] observed increased activity of caspase-3 in osteoclasts treated with nitrogen-containing bisphosphonates (alendronate,

zoledronic acid, risedronate, pamidronate) and non-nitrogen containing ones (clodronate, tiludronate, etidronate). According to these authors, high concentrations of clodronate, alendronate and zoledronic acid produce a similar proportion of apoptotic osteoclasts. This suggests that the greater antiresorptive effect of nitrogen-containing bisphosphonates is related not only to apoptosis, but also to effects on osteoclast migration and differentiation. Similarly, using caspase-3, Plotkin et al. (2006)[52] found that nitrogen- and non nitrogen-containing bisphosphonates exert apoptotic effect on osteoclasts. On the other hand, both groups of drugs inhibited osteoblast and osteocyte apoptosis at doses three times lower than that used for osteoclasts.

Receptor activator of nuclear factor-kappa B (RANK), receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG)

Bone remodeling is characterized by the balance between activation and apoptosis of osteoblasts and osteoclasts. Different stages of development of this process are regulated by diverse growth factors, cytokines and hormones, including macrophage colony-stimulating factor (M-CSF), interleukin-1 (IL-1), transforming growth factor β (TGF-β), tumor necrosis factor (TNF), D3-vitamin, calcitonin and parathyroid hormone (PTH).[53]

The discovery of a triad of proteins belonging to the TNF family and capable of regulating maturation, function and survival of osteoclasts has improved the understanding of bone metabolism.[54] Such proteins are RANKL also called TNF-related activation induced cytokine (TRANCE) or OPG ligand (OPGL), RANK, and OPG, also called osteoclastogenesis inhibitory factor (OCIF).[55-57]

Osteoclastogenesis requires osteoclast activation by two molecules characterized by complementary activities, M-CSF and RANKL. M-CSF increases osteoclast precursors, whereas RANKL, which is expressed on the osteoblast surface, exerts its effects when

bound to RANK receptor expressed on the osteoclast precursor cell surface, inducing the signaling of the cascade that leads to osteoclast differentiation and maturation.[55,58,59]

RANK is a transmembrane receptor found in osteoclast precursor cells, dendritic cells, fibroblasts and T cells.[60] RANKL, on the other hand, is a protein produced by osteoblasts and T cells, which can be expressed as a cell membrane cytokine or be released as a soluble factor. The two forms of RANKL have different functional properties, where the membrane receptor is known as a more effective osteoclastogenesis mediator. These two proteins (RANK and RANKL) can be quantified by many methods. Nevertheless, immunosorbent assay (ELISA) has the limitation of detecting just the soluble form of RANKL, which is less effective in expressing osteoclast formation.[56]

OPG is a protein expressed by a variety of cell types, including immune cells, osteoblasts and endothelial cells. It is considered an antagonist receptor of RANKL, as it blocks the RANK/RANKL interaction and inhibits the final stage of osteoclast differentiation. All these proteins show cytoplasmic staining (Fig.1 C and D). *In vitro* studies report that OPG effects include inhibition of both differentiation and activation of osteoclasts, therefore reducing bone resorption.[61]

Investigations into the relationship between serum levels of RANKL and OPG in patients undergoing bisphosphonate therapy have shown controversial results. Martini et al. (2006)[56] observed serum OPG levels significantly higher in patients with Paget disease treated with nitrogen-containing bisphosphonates. However, Alvarez et al. (2003)[62] reported a decrease in serum OPG in Paget patients after treatment with tiludronate. This same study, however, did not observe changes in serum levels of RANKL. Mountzios et al. (2010),[63] in turn, did not find any significant change in serum levels of OPG and RANKL in patients with bone metastases treated with zoledronic acid.

In vitro studies report that nitrogen-containing bisphosphonates increase the expression of OPG and decrease the expression of RANKL in different cell types. Pan et al. (2004)[64] investigated the effect of zoledronic acid on OPG and RANKL expression in osteoblast cultures using flow cytometry, Western blotting and ELISA. According to this study, bisphosphonates inhibit osteoclast differentiation through decreased expression of RANKL and increased OPG secretion in osteoblasts. This finding corroborates the report of Viereck et al. (2002),[15] who demonstrated by means of reverse transcription-polymerase chain reaction (RT-PCR) and ELISA, that zoledronic acid and sodium pamidronate increase the expression and secretion of OPG in osteoblasts in a dose-dependent manner. According to Tipton et al. (2011),[57] pamidronate and alendronate increased OPG production and decreased RANKL in gingival fibroblasts, changing the OPG/RANKL ratio, which suggests that these cells, located next to resorption sites, can be directly involved in alveolar bone metabolism regulation.

The RANK/RANKL/OPG triad shows a strong potential to be used in diagnosis and follow-up of patients with bone metabolism diseases. However, these markers still have restricted indication in clinical practice, because of their technical limitations, which include interference by physiological factors. Thus, they cannot be indicated without considering the clinical criteria usually applied to the diagnosis of the disease.[56]

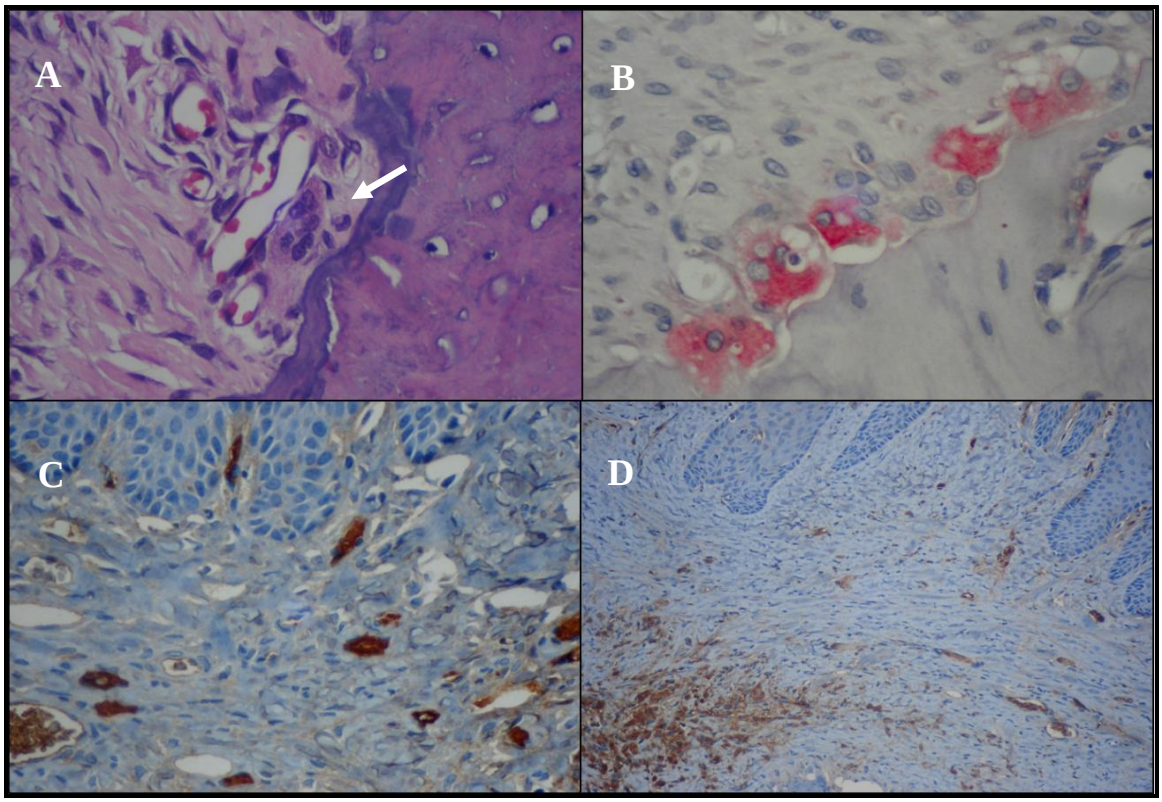

Figure 1. Osteoclast (white arrow) in H&E stain (original magnification x 400) (A); Osteoclasts stained by TRAP (original magnification x 400) (B); Immunostaining of RANKL (original magnification x 400) (C); Immunostaining of OPG (original magnification x 200) (D).

Osteocalcin (OCN)

OCN is a peptide secreted by mature osteoblasts, hypertrophic chondrocytes and odontoblasts, classified as the most abundant noncollagenous protein of bone matrix.[65,66] The affinity to hydroxyapatite seems to be sufficient for this protein to play an important role in the regulation and maturation of crystal and preservation of bone matrix.[67] OCN is considered a marker of mature osteoblasts, as it increases with the start of the mineralization process.[68] The lack of specificity is a limitation to the use of this marker because both osteolysis and osteogenesis release this protein into the serum.[66]

Studies on bisphosphonate interference with OCN levels suggest that these compounds cause metabolic changes in osteoblasts.[69,70] Koch et al. (2010)[71] evaluated gene expression of OCN by means of RT-PCR. These authors found that zoledronic acid and

ibandronate increase, in a dose-dependent manner, the expression of OCN in osteoblasts, whereas clodronate does not significantly change its expression, even when administered at high doses. Therefore, it is probable that nitrogen-containing bisphosphonates increase bone density by stimulating osteoblast differentiation.

Osteopontin (OPN)

OPN is a glycoprotein involved in many physiological and pathological events, including processes of immune response, tumorigenesis, and cell proliferation, migration and differentiation. It exists either as a membrane-bound molecule of the extracellular matrix in mineralized tissues, or as a cytokine in body fluids. In bone tissue, it is found in pre-osteoblasts, osteocytes and osteoclasts.[72] It is expressed not only in bone tissue, but also in macrophages, lymphocytes, dentin, cementum, kidneys and brain, which reflects its multiplicity of functions.[73]

OPN is upregulated by hormones and cytokines, such as calcitonin and fibroblast growth factors, and suppressed by bisphosphonates.[53] Downregulation by bisphosphonates in bone is compatible with osteoclast activity suppression.[73] Increased OPN was observed in cementoblasts treated with zoledronic acid.[74] Also, serum levels of OPN were increased in patients with bone metastases after treatment with zoledronic acid,[63] and higher immunohistochemical expression of this protein in periodontal ligament cells of rats under etidronate therapy has been reported.[75] These reports support the idea of bisphosphonates having the ability to increase bone mass. Nevertheless, the association of OPN with a variety of diseases and physiological situations in response to stress makes it a low-specificity method to evaluate bisphosphonate effects on bone tissue.[72]

Runt-related transcription factor 2 (RUNX2)

The RUNX family comprises a group of three transcription factor proteins: Runx1, Runx2 and Runx3. These proteins are expressed in bone and hematopoietic system and are involved in a series of neoplasms either as a tumor promoter or suppressor.[76] RUNX2 exerts known effects on bone development through the regulation of osteoblast differentiation.[76,77]

Few studies have focused on bisphosphonate effects on RUNX2 levels. Koch et al. (2010)[71] observed by means of RT- PCR that zoledronic acid and ibandronate increase the expression of the RUNX2 gene in osteoblasts, whereas clodronate does not cause significant changes in this expression, even when administered at high concentrations.

Alkaline phosphatase

Alkaline phosphatase is an ectoenzyme expressed in three isoforms, namely placental, intestinal and liver/bone/kidney forms, the latter being a nonspecific one which can be found in almost all tissues. The bone isoform, located on the surface of osteoblasts and chondrocytes, represents a marker of the middle stage of osteogenesis, appearing during the matrix maturation phase.[68]

A study analyzing the effect of zoledronic acid on metabolic activity of osteoblasts showed that the increase in drug concentration matched with a decrease in alkaline phosphatase production.[78] Other studies, on the other hand, found neither an increase in gene expression of alkaline phosphatase in osteoblasts treated with alendronate[79] nor significant changes in serum levels of alkaline phosphatase in patients treated with this drug.[80]

Carboxy terminal collagen cross links (CTX)/amino-terminal telopeptide (NTX)

CTX and NTX are products from the degradation of type I collagen, the major organic component of extracellular matrix. During collagen degradation, CTX and NTX fragments are released into the blood circulation. As both segments are small enough to be eliminated by the renal route, they can be measured by serum and urine examination. These are not specific markers as they are found in tissues other than bone, and their detection should be interpreted in the clinical context.[68,81]

Urine levels have as a disadvantage the necessity of correcting results for creatinine excretion to compensate variations in urine dilution. This requires a second evaluation, which generates some complications inherent to the technique. According to some authors, as serum measurement does not have this limitation, it is the most reliable method to evaluate CTX and NTX.[81] Moreover, studies comparing urinary and serum results with these markers report that there is still no consensus about their correlation.[82] Regardless of the evaluation method or the marker used, the technique shows great variability according to the sampling time and processing and storage of specimens, as well as the age and sex of the patients. Therefore, it is crucial to consider these limitations to minimize interpretation errors.[81]

Studies report significant reduction of CTX and NTX in patients with bone metastases after 6 months of clodronate therapy[83] and in patients with osteoporosis treated with zoledronic acid (yearly) and alendronate (weekly).[84] These reports reinforce the idea that nitrogen- and non-nitrogen-containing bisphosphonates inhibit osteoclast function.[81]

Bone morphogenetic proteins (BMPs) and matrix metalloproteinase proteins (MMPs)

BMPs comprise a series of proteins from TGF-β family, which are involved in the morphogenesis and organogenesis of different tissues and organs. Until now, nine types of

BMPs have been described, and with exception of BMP-1, all of them seem to be capable of inducing bone formation. MMPs, in turn, are proteins capable of degrading the components of extracellular matrix and are involved in a variety of physiological and pathological events of bone remodeling and inflammatory response regulation. These proteins can be distributed into five subfamilies: collagenases (MMP-1, MMP-8, MMP-13), stromelysins (MMP-3, MMP-10), gelatinases (MMP-2, MMP-9), matrilysines (MMP-7, MMP-26) and membrane-type metalloproteinases (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25), according to their structure and the type of substrate to which they bind.[85]

Studies report that bisphosphonate therapy is related to decreased expression of MMPs in periodontal tissue of rats,[86] as well as to a reduction in their serum levels in humans.[87] There are also reports about the association of these drugs with increased expression of BMPs in human bone marrow and periodontal tissue.[88,89]

Evaluation of bisphosphonate effects on angiogenesis

Vascular endothelial growth factor (VEGF)

VEGF is the major mitogenic factor for endothelial cell growth, either in physiological or pathological conditions. VEGF is produced by keratinocytes, macrophages, fibroblasts and mastocytes, and it is capable of potentiating vascular permeability and is directly involved in vascular differentiation and formation, also working as an antiapoptotic factor in endothelial cells of recently formed vessels.[90,91]

It was reported that the immunohistochemical expression of VEGF in tooth extraction area did not significantly differ between rats treated with zoledronic acid and alendronate and controls.[26] Also, a prospective study did not show a significant reduction in serum VEGF levels in cancer patients treated with nitrogen- and non nitrogen-

containing bisphosphonates.[92,93] Nevertheless, there are reports of decreased levels of circulating VEGF in cancer patients after zoledronic acid administration,[94,95] as well as significantly reduced expression of VEGF caused by zoledronic acid *in vivo* and *in vitro*.[96]

Cluster of differentiation 34 (CD34)

CD34 is a glycoprotein expressed on the surface of progenitor or primitive cells of the hematopoietic system. Although highly sensitive, it shows poor specificity, a reason for which the morphological identification of the stained structures is an important auxiliary tool for the immunohistochemical method. The technique also has the disadvantage of staining lymphatic vessels, which can cause false-positive results.[97]

Animal models with prostate cancer under alendronate therapy showed significant reduction of CD34 immunohistochemical expression in capillary vessels.[98] Also in flow cytometry, serum levels of CD34 were significantly higher in patients with multiple myeloma treated with nitrogen-containing bisphosphonate who developed osteonecrosis than in those who did not have the lesion. This result suggests an association between osteonecrosis and reduced vascular proliferation.[99]

Cluster of differentiation 31 (CD31)

CD31 is a protein expressed by hematopoietic and endothelial cells. An *in vivo* study showed no significant change in gene expression of CD31 in the gingival tissue of rats undergoing zoledronic acid therapy and subjected to oral lesion induction.[100] On the other hand, an *in vitro* study using CD31 immunohistochemistry observed reduced vascular density associated with either zoledronic acid or clodronate.[101]

von Willebrand factor (vWF)

vWF or factor VIII is a glycoprotein exclusively produced by endothelial cells and megakaryocytes. It is routinely used to identify blood vessels in histological samples.[102] When compared to other vascular markers, this is a highly specific protein for the detection of endothelial cells. Also, as it is released in situations of damage to these cells, vWF has been indicated as a marker of endothelial dysfunction.[103]

Freitas et al. (2005)[104] evaluated angiogenic activity in hemangiomas and pyogenic granulomas by immunostaining with anti-CD31, anti-VEGF and anti-vWF. The samples stained with anti-vWF showed a more uniform and more easily interpreted immunostaining pattern. However, CD 31 and vWF are not capable of differentiating new vessels from the preexisting ones, whereas VEGF is capable of overcoming this limitation (SCHOR et al., 1998).[105]

Final considerations

Bisphosphonates are relatively recent drugs whose effects on body tissues are partially known. Jaw osteonecrosis associated with these drugs has demanded new studies in this field. In this review, we list some markers that can serve this purpose. Actually, there are many other methods available, each one with its specificities, advantages and disadvantages. Most proteins presented here have been assessed through biochemical,[67] immunohistochemical[86] and genetic methods.[88] Moreover, studies using them in the investigation of bisphosphonate effects show controversial results, which seem to be supported by the lack of standardization of methods, type of bone structure evaluated, length of use, type and dose of the drug, as well as presence or absence of previous metabolically induced disease.[33] The correct indication of these techniques and the

understanding of their limitations are of crucial importance for understanding the effects of bisphosphonate on body tissues.

Acknowledgements

We thank Dr. A. Leyva (U.S.A) for English editing the manuscript.

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ARTIGO 2

3 ARTIGO 2

O artigo a seguir intitula-se **Comparison of effects of clodronate and zoledronic acid on the repair of maxilla surgical wounds - Histomorphometric, RANKL, OPG, von Willebrand factor and caspase-3 evaluation** e foi formatado de acordo com as normas do periódico *Journal of Oral Pathology & Medicine* (Anexo B).

Comparison of effects of clodronate and zoledronic acid on the repair of maxilla surgical wounds - Histomorphometric, RANKL, OPG, von Willebrand factor and caspase-3 evaluation

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Running title: *Bisphosphonates and surgical wounds*

ABSTRACT

BACKGROUND. The aim of this study was to compare clodronate and zoledronic acid regarding their influence on the repair of surgical wounds in maxillae (soft tissue wound and tooth extraction) and their relation to osteonecrosis.

MATERIAL AND METHODS. Thirty-four Wistar rats were allocated into 3 groups according to the treatment received: (1) 12 animals treated with zoledronic acid; (2) 12 animals treated with clodronate; and (3) 10 animals that were given saline solution. All animals were subjected to tooth extractions and surgically induced soft tissue injury. Histological analysis of the wound sites was performed by means of hematoxylin-eosin (H&E) staining and immunohistochemical staining for RANKL, OPG, von Willebrand factor and caspase-3.

RESULTS. The zoledronic acid group showed higher incidence of non-vital bone than did the clodronate group at the tooth extraction site. At the soft tissue wound site, there were no significant differences in non-vital bone between the test groups. RANKL, OPG, von Willebrand factor and caspase-3 did not show significant differences between the groups for both sites of surgical procedures.

CONCLUSION. Both of the bisphosphonates zoledronic acid and clodronate are capable of inducing maxillary osteonecrosis. Immunohistochemical analysis suggests that the involvement of soft tissues as the initiator of osteonecrosis development is less probable than has been pointed out.

Key words: bisphosphonates; zoledronic acid; clodronate; osteonecrosis

Introduction

Bisphosphonates are classified as nitrogen- and non-nitrogen according, respectively, to the presence or absence of nitrogen in their chemical structure. These drugs are associated with osteonecrosis of the jaws, an important adverse effect first described in 2003. The disease is characterized by exposure of bone to the oral cavity that does not heal within eight weeks, in patients who have received bisphosphonates without history of radiotherapy in the head and neck region (1, 2). In some cases, the condition is refractory to treatment, where it is only possible to preserve the patient's quality of life by controlling pain and infection, as well as preventing the occurrence of new areas of necrosis (3).

Bisphosphonates inhibit bone resorption through direct and indirect effects on osteoclasts, which undergo apoptosis or become unable to differentiate from hematopoietic stem cells (4-6). Other effects of these drugs, such as impairment of both angiogenesis (7-11) and epithelial cell proliferation (12), support the hypothesis that osteonecrosis of the jaws has a multifactorial etiology.

The pathway of receptor activator of nuclear factor-kB ligand (RANKL) and osteoprotegerin (OPG) is one of the main regulators of the molecular mechanisms involved in the development and function of osteoclasts. Studies on bisphosphonates, *in vitro* and *in vivo*, have shown controversial results when these proteins are used as immunohistochemical, genetic and serum markers (13, 14). It was also demonstrated that nitrogen-containing bisphosphonates are able to cause toxicity to the cells of oral epithelium, raising concerns whether disease onset could occur in bone tissue or in oral mucosa (15, 16). Moreover, most studies evaluating the effects of the drug on body tissues have been performed with nitrogen-containing bisphosphonates, which generate many concerns about tissue behavior when non-nitrogen-containing ones are used (15, 17).

There are many case reports and some animal model studies in which the use of zoledronic acid is associated with jaw osteonecrosis. Nevertheless, even though clodronate is the most prescribed non-nitrogen-containing bisphosphonate, there are a few case-reports in the literature on osteonecrosis of the jaws induced by this drug (18). In some of them, patients had used nitrogen-containing before the treatment with non-nitrogen ones. Nor has there been a study about clodronate effects using animal models. The aim of this work was to compare the effects of clodronate and zoledronic acid on the repair of surgical wounds of maxillae. Microscopic features of tooth extraction and soft tissue injury areas were evaluated by means of hematoxylin-eosin (H&E) staining and immunohistochemical detection of RANKL, OPG, von Willebrand factor (vWF) and caspase-3.

Material and methods

Animals

The present study was approved by the Ethics Committee for Animal Use 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 comprised 34 female rats (*Rattus norvegicus*, Wistar strain) from the animal facility of the Federal University of Pelotas, which had a mean age of 120 days and a mean weight of 230 g. Animals were individually identified on the tails and housed in plastic cages (5 per cage) placed in ventilated racks (Alesco, Monte Mor, SP, Brazil) at a temperature of 22°C with a 12-h light/dark cycle (lights on at 7:00 am and off at 7:00 pm). During the experiments, a standard diet of rat chow (Nuvilab, Colombo, PR, Brazil) and filtered water were provided *ad libitum*. The cleaning and changing of the cages were done three times a week. No experimental procedures were carried out in the place where the animals were kept in order to avoid any type of stress behavior. The animals were randomly allocated into 3 groups,

according to the bisphosphonate used: (1) zoledronic acid group: 12 animals that were treated with the nitrogen-containing bisphosphonate zoledronic acid (Novartis Pharma AG, Basel, Switzerland) intraperitoneally (0.6 mg/kg, every 28 days); (2) clodronate group: 12 animals that were treated with the non-nitrogen-containing bisphosphonate clodronate (Jenahexal Pharma GmbH, Thuringia, Germany), intraperitoneally (20 mg/kg, every 28 days); and (3) control group: 10 animals that were given saline solution (0.9% sodium chloride), intraperitoneally every 28 days.

Surgical procedures

All animals were subjected to tooth extractions and surgical-induced soft tissue injury as described below. Oroscopy was performed after the anesthesia and before the surgical procedures to certify that there were no previous oral lesions. The animals were given intraperitoneal paracetamol at a dose of 50 mg/kg (Medley S/A, Campinas, SP, Brazil) after the surgery for postoperative analgesia.

Tooth extractions

Tooth extractions were performed 60 days after the beginning of the experiment. Animals were anesthetized with a single intraperitoneal injection of a mixture of ketamine hydrochloride 5% (100 mg/kg; Vetbrands, Jacareí, SP, Brazil) and xylazine hydrochloride 2% (10 mg/kg; Vetbrands, Jacareí, SP, Brazil). The three upper right molars were extracted using an adapted 3s spatula (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) for luxation and a pediatric forceps (Edlo, Canoas, RS, Brazil) whose functional portion was adapted to the size of the tooth.

Surgically induced soft tissue wound

Immediately after the tooth extractions, a surgical wound was made on the mucosa of the hard palate at the opposite side (left side) of the maxilla, with reference to the second left upper molar, and using a surgical scalpel with no. 3 Bard-Parker handle (Solidor, São Paulo, SP, Brazil) and a no. 15 blade (Solidor, São Paulo, SP, Brazil). The incision was elliptical, 3 mm long and 1 mm deep. The size of the lesion was monitored by a millimeter ruler guide and a periodontal probe.

Euthanasia of the animals, macroscopic evaluation and dissection of the maxillae

After completing 102 days of drug administration, the animals were euthanized by deep anesthesia with isoflurane (Cristalia, Porto Alegre, RS, Brazil) in an appropriate anesthesia chamber. The specimens were then examined by means of a no.5 clinical probe (SS White, Duflex, Rio de Janeiro, RJ, Brazil) to determine the presence/absence of oral mucosal lesion. Afterwards, the maxillae were dissected and fixed for 24 h in 10% buffered formalin (TopGlass, Porto Alegre, RS, Brazil).

Histological processing

The specimen was cut into two fragments in the coronal direction, using a steel-sanding disc at low speed and subjected to decalcification in ethylenediaminetetraacetic acid (EDTA, Biodinâmica, Ibiporã, PR, Brazil) solution for 30 days. Next, they were paraffin-embedded, cut into 4-µm sections and stained with hematoxylin and eosin (H&E) as well as immunohistochemically processed.

Antigen retrieval was carried out in a 100°C water bath for 40 min, using Tris/EDTA buffer, pH 9 (20 mM Tris/0.65 mM EDTA). Endogenous peroxidase was

blocked with a 3% solution of hydrogen peroxide in methanol for 30 min, followed by three cycles of washing with PBS. The blocking of nonspecific binding was done with the commercial solution Protein Block Serum-Free (Dako, Carpinteria, CA, USA) for 30 min at room temperature. The immunohistochemical staining method based on capillary action was used (Thermo, Shandon, CA, USA), where the sections were incubated overnight at 2°C with dilutions of the following antibodies: anti-RANKL (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:500; anti-OPG goat polyclonal antibody (SC8468 - Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100; anti-vWF (Biocare, Concord, Massachusetts, USA) and anti-caspase-3 (Novocastra, Newcastle, UK) diluted at 1:500. After incubation with the primary antibody, sections were washed with 3 passages in PBS. For amplification of the antigen-antibody reaction, the Picture Max system (Invitrogen, Carlsbad, CA, USA) was used according to manufacturer's recommendations. The slides were then washed with PBS and incubated with diaminobenzidine solution for 5 min. The detection system used was Dako LSAB Kit (Dako, Carpinteria, CA, USA). Color development was carried out with the chromogen 3'-diaminoabenzidine and phosphate buffer solution containing 0.002% hydrogen peroxide. All markers had external and internal controls.

Histological analysis

The histological sections were digitized using a Zeiss Axioskop 40 light microscope (Zeiss, Göettingen, Germany), connected to a Roper Scientific video camera (Media Cybernetics, Bethesda, MD, USA) and a Pentium IV 2.2 GHZ computer with 512 MB RAM, 160 GB hard drive, and Image Pro Capture Kit Platform (Media Cybernetics). The images were captured using 5x (H&E stain) and 10x (immunohistochemistry) objectives and stored in Joint Photographic Expert Group (JPEG) format. For H&E analysis, 5 fields

were selected in a standardized manner in each slide to include the whole area of tooth extractions and soft tissue wound. For immunohistochemistry, 2 fields were selected in a standardized manner to include the epithelial and connective tissue at both sites evaluated.

The images were analyzed by a calibrated and blinded examiner. The calibration consisted of evaluating a series of 20 histological images for each technique (H&E and immunohistochemistry) twice, at two different moments. The results of these two evaluations were subjected to a paired *t* test and Pearson's correlation coefficient, showing the absence of a significant difference ($p > 0.05$) and a strong correlation ($r > 0.8$).

H&E analysis

The H&E images were analyzed using the manual counting technique in the Image Pro Plus 4.5.1 software (Media Cybernetics, Bethesda, MD, USA). A quantitative analysis was made for the variables epithelial tissue, connective tissue, root fragments, microbial colonies, inflammatory infiltrate, non-vital bone, and vital bone. A point-grid of 532 points was superimposed to each image and each point was counted according to the matching morphological structure (Fig. 1).

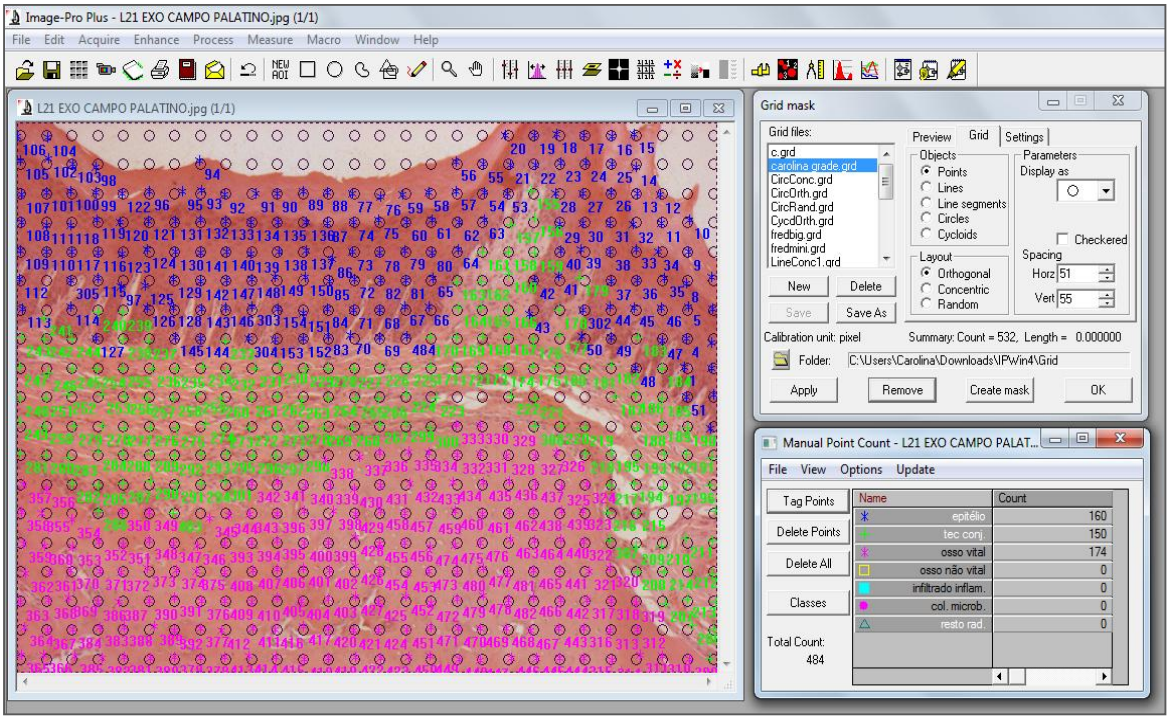

Figure 1. Quantification of histological features in 1 of the 3 fields examined at the site of tooth extraction using Image Pro Plus 4.5.1 software (Media Cybernetics, Bethesda, MD, USA; H&E stain, x 5 objective).

Immunohistochemical analysis

The immunohistochemical expression of RANKL, OPG, vWF and caspase-3 was quantified, according to Amenábar et al. (19) by means of semi-automated segmentation technique in the Image Pro Plus 4.5.1 software (Media Cybernetics, Bethesda, MD, USA; Fig. 2).

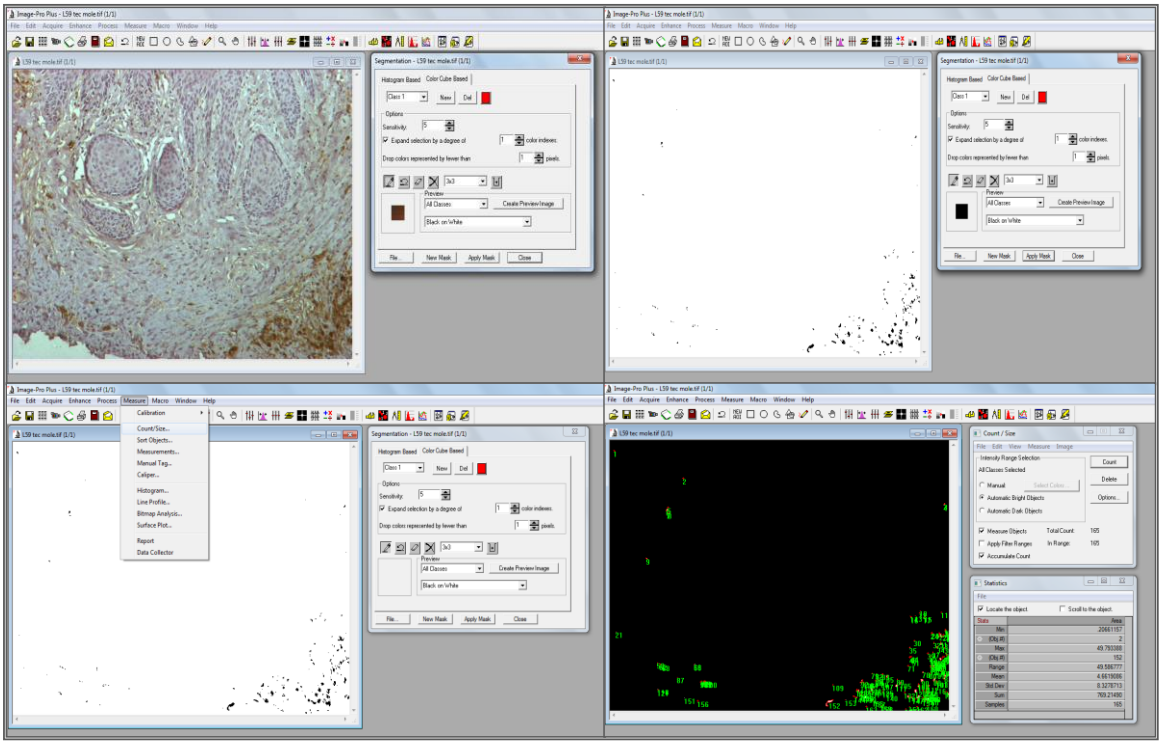

Figure 2. Quantification of immunohistochemical expression of OPG by means of semi-automated segmentation technique using Image Pro Plus 4.5.1 software (Media Cybernetics, Bethesda, MD, USA; x10 objective)

Statistical analysis

The data were analyzed by descriptive statistics, chi-squared test complemented by analysis of adjusted residuals, and Kruskal-Wallis test complemented by its multiple comparisons test, setting the level of significance at 5%. The statistics were processed by the SPSS 17.0 software (Statistical Package for the Social Sciences, Chicago, IL, USA).

Results

Clinical and macroscopic evaluation

On oral examination before tooth extractions, no animal exhibited oral mucosal lesions. Table 1 presents the results of macroscopic evaluation (after euthanasia) for tooth extraction and soft tissue wound sites. Zoledronic acid was associated with the loss of mucosal integrity in the tooth extraction site (chi-squared test complemented by analysis of

adjusted residuals, $p<0.001$). Neither zoledronic acid nor clodronate was associated with loss of mucosal integrity at the soft tissue wound site (chi-squared test, $p=0.151$).

Table 1. Sample distribution according to presence/absence of loss of mucosal integrity on macroscopic examination of the tooth extraction site and soft tissue wound site

Group	Loss of mucosal integrity							
	Tooth extraction site				Soft tissue wound site			
	Present		Absent		Present		Absent	
	n	%	n	%	n	%	n	%
Zoledronic acid (n=12)	**12***	**100***	0	0	9	75	3	25
Clodronate (n=12)	10	83.3	2	16.7	9	75	3	25
Control (n=10)	1	10	9	90	4	40	6	60
Total	23	67.6	11	32.3	22	64.7	12	35.2

n = number of animals
*Significant difference; chi-square test; analysis of adjusted residuals; p<0.001

H&E Analysis

Table 2 shows the results for the frequency (presence/absence) of non-vital bone in the groups evaluated. By means of the chi-squared test, complemented by analysis of adjusted residuals, it was observed that (1) zoledronic acid was associated with non-vital bone at the tooth extraction site ($p<0.001$); (2) at the soft tissue wound site, both zoledronic acid and clodronate were associated with non-vital bone ($p<0.001$).

Table 2. Sample distribution according to presence/absence of non-vital bone at the tooth extraction and soft tissue wound sites on microscopic examination

Group	Non-vital bone							
	Tooth extraction site				Soft tissue wound site			
	Present		Absent		Present		Absent	
	n	%	n	%	n	%	n	%
Zoledronic acid (n=12)	**12***	**100***	0	0	**12***	**100***	0	0
Clodronate (n=12)	5	41.6	7	58.3	**12***	**100***	0	0
Control (n=10)	0	0	10	100	4	40	6	60

n = number of animals
*Significant difference; chi-square test; analysis of adjusted residuals; $p<0.001$

The results for the proportions of the histological variables are presented in Tables 3 and 4, respectively, for the tooth extractions and soft tissue wound sites. At the tooth extraction site, the proportion of non-vital bone and microbial colonies 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. There was no significant difference in vital bone and root fragments between the groups. The proportion of connective tissue was significantly greater in the clodronate compared to the zoledronic acid group, but they did not differ significantly from the control group (Table 3, Kruskal-Wallis test complemented by multiple comparisons test, $\alpha = 0.05$).

At the soft tissue wound site, the proportions of nonvital bone and microbial colonies were significantly greater in the zoledronic acid and clodronate groups than in the control. The proportion of connective tissue was significantly greater in the control group compared to the clodronate and zoledronic acid groups, whereas the proportion of vital bone was significantly greater in the control group compared to the clodronate group, but these two did not differ significantly from the zoledronic acid group. There was no significant difference in epithelial tissue and inflammatory infiltrate between the groups

(Table 4, Kruskal-Wallis test complemented by multiple comparisons test, α = 0.05, Fig.3-A-D).

Table 3. Quantification of histological features (H&E stain) at the tooth extraction site in the zoledronic acid, clodronate and control groups

Histological feature	Group								
	Zoledronic acid (%)			Clodronate (%)			Control (%)		
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
Non-vital bone	15.38	10.02	**13.78^{A}**	1.28	2.12	**0^{B}**	0	0	**0^{B}**
Epithelial tissue	18.74	5.90	18.37	16.20	3.46	16.73	18.47	4.70	18.82
Connective tissue	28.13	8.23	**30.44^{B}**	41.73	10.44	**42.42^{A}**	37.48	8.68	**34.49^{AB}**
Vital bone	16.39	13.84	13.42	24.97	15.74	20.58	31.13	14.63	31.33
Inflammatory infiltrate	8.04	6.36	5.74	7.97	7.57	5.37	8.41	7.88	6.17
Microbial colonies	3.46	2.77	**3.32^{A}**	0.49	0.60	**0.21^{B}**	0.38	0.72	**0.08^{B}**
Root fragments	9.83	7.89	8.37	7.33	5.04	7.97	3.40	4.79	0.79

* Bold printed medians, followed by different letters, indicate features that differed significantly between groups; Kruskal-Wallis test complemented by multiple comparisons test, $p \leq 0.05$
SD = Standard deviation

Table 4. Quantification of histological features (H&E stain) at the soft tissue wound site in the zoledronic acid, clodronate and control groups.

Histological feature	Group								
	Zoledronic acid (%)			Clodronate (%)			Control (%)		
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
Non-vital bone	31.35	8.89	**30.79^{A}**	21.99	6.55	**23.26^{A}**	3.65	5.61	**0^{B}**
Epithelial tissue	12.21	3.65	11.44	13.96	5.6	13.11	12.62	2.10	12.98
Connective tissue	31.22	5.95	**30.43^{C}**	41.03	9.10	**43.99^{B}**	52.11	9.33	**51.96^{A}**
Vital bone	11.49	8.73	**11.32^{AB}**	3.73	3.57	**3.51^{B}**	22.95	16.44	**27.77^{A}**
Inflammatory infiltrate	10.12	8.87	8.47	16.33	7.67	15.89	8.00	7.49	8.92
Microbial colonies	3.58	3.34	**2.35^{A}**	2.94	2.60	**2.29^{A}**	0.34	0.85	**0^{B}**

** Bold printed medians, followed by different letters, indicate features that differed significantly between groups; Kruskal-Wallis test complemented by multiple comparisons test, $p \leq 0.05$
SD = Standard deviation

Immunohistochemical analysis

Immunohistochemical quantification for RANKL, OPG, vWF and caspase-3 at tooth extraction and soft tissue wound sites did not differ significantly between the three groups analyzed (Tables 5 and 6, Kruskal-Wallis test, α=0.05, Fig.3-D, E).

Table 5. Immunohistochemical quantification of RANKL, OPG, von Willebrand factor (vWF) and caspase-3 at tooth extraction site in the zoledronic acid, clodronate and control groups

Marker	Group									
	Zoledronic acid (mm^2)			Clodronate (mm^2)			Control (mm^2)			
	Mean	SD	MD	Mean	SD	MD	Mean	SD	MD	*P**
RANKL	0.38	0.32	0.35	0.34	0.38	0.25	0.42	0.44	0.38	0.76
OPG	0.31	0.30	0.22	0.35	0.20	0.31	0.26	0.20	0.21	0.55
vWF	0.50	0.35	0.39	0.77	0.61	0.58	0.49	0.27	0.41	0.58
Caspase 3	1.72	2.11	1.10	1.03	0.97	0.89	1.33	1.15	1.11	0.74

*Kruskal-Wallis test, α=0.05
SD = Standard deviation; MD = Median

Table 6. Immunohistochemical quantification of OPG, RANKL, von Willebrand factor (vWF) and caspase-3 at soft tissue wound site in the zoledronic acid, clodronate and control groups

Marker	Group									
	Zoledronic acid (mm^2)			Clodronate (mm^2)			Control (mm^2)			
	Mean	SD	MD	Mean	SD	MD	Mean	SD	MD	*P**
RANKL	0.35	0.40	0.13	0.41	0.34	0.32	0.52	0.55	0.30	0.77
OPG	0.36	0.33	0.30	0.29	0.26	0.16	0.59	0.32	0.71	0.08
vWF	0.97	0.93	0.67	0.61	0.58	0.30	0.79	0.71	0.60	0.46
Caspase 3	1.41	1.18	1.27	1.52	2.20	0.82	1.30	0.92	1.14	0.75

*Kruskal-Wallis test, α=0.05
SD = Standard deviation; MD = Median

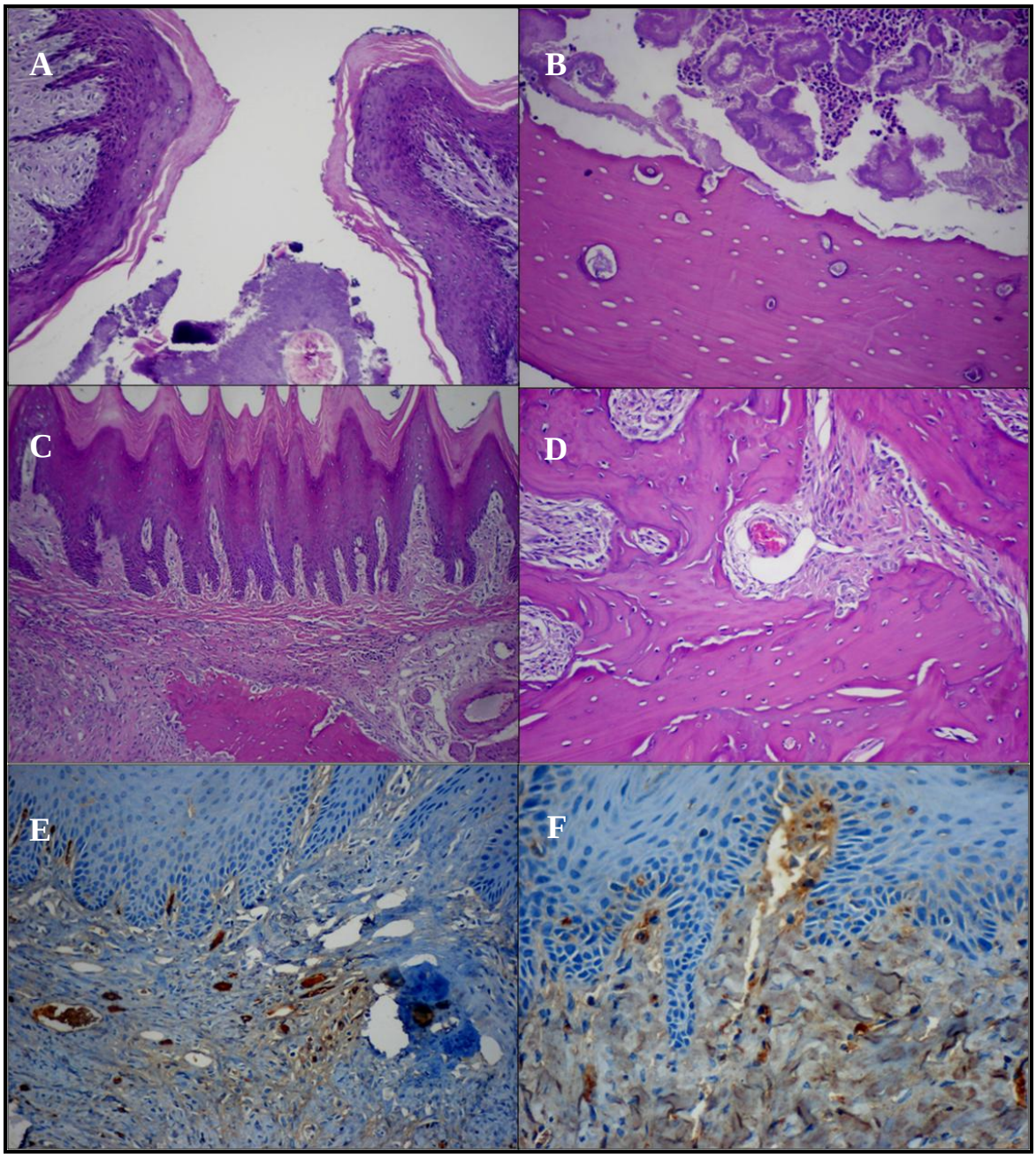

Figure 3. Persistent soft tissue defect showing the disruption of epithelium (H&E stain, original magnification x 100) (A); Non-vital bone, microbial colonies, and inflammatory infiltrate (H&E stain, original magnification x 200) (B); Complete tissue repair (H&E stain, original magnification x 100) (C); Vital bone (H&E stain, original magnification x 400) (D); Immunostaining of RANKL (original magnification x 200) (E); Immunostaining of OPG (original magnification x 400) (F).

Discussion

No animal in the three groups evaluated showed oral mucosal lesion prior to the surgical procedures, which indicates the lesions observed afterwards were associated with the

surgical procedure and bisphosphonate use. On macroscopic examination, an association was observed between loss of mucosal integrity at the tooth extraction site and zoledronic acid use. The occurrence of osteonecrosis and root fragments seems to explain this finding in both test groups and control. At the soft wound site, however, this association was not observed in any group, even with all animals in the test groups showing non-vital bone. This finding is in agreement with reports suggesting that bisphosphonate-associated jaw osteonecrosis can occur without bone exposure (20-22). Still, it disagrees with the hypothesis that soft tissues could occupy the first position in the pathophysiology of osteonecrosis (23-26).

The frequency of non-vital bone was significantly higher at the soft tissue wound site than at the tooth extraction site. The anatomical specificities of the sites subjected to the surgical procedures, as well as the type of lesion induced, could have contributed to this result. After a tooth extraction, the alveolar socket is first filled with the clot, which gives rise to a neoformed connective tissue, rich in fibroblasts and capillaries, which in turn promotes healing through the formation of well-organized bone trabeculae (27). The buccal and lingual bone walls remain covered by mucosa, healing occurs by secondary intention (28), and growing epithelial cells restore the epithelial continuity of the mucosa (29). In the case of bisphosphonate users, this process would be impaired by diminished vascularization (7) and reduced bone neoformation (16). Moreover, the hard palate has poor vascularization and its mucosa is firmly adhered to the periosteum (30). The wound at this site seems to have the potential of exposing a wider area of bone tissue, with less blood supply, if compared to the tooth extraction site, which could explain its higher prevalence of osteonecrosis. Therefore, the results of non-vital bone for the soft tissue wound site suggest that oral mucosal lesions, depending on their location, vascularization, submucosal thickness and relationship with the subjacent bone tissue, constitute a sufficient risk factor

for the occurrence of osteonecrosis associated with bisphosphonates, with no necessity of more invasive interventions such as tooth extractions. This is true not only for nitrogen-containing bisphosphonates, but also to non-nitrogen ones.

Nevertheless, it is important to recall that the maxilla, the bone subjected to tooth extractions in the present study, because of its higher vascularization (31) and turnover, is less prone to bisphosphonate osteonecrosis compared to the mandible is (32). Thus, it is plausible to infer that if we had done the tooth extractions in the mandible instead of maxilla, the prevalence of osteonecrosis could have been higher at this wound site than at the soft tissue wound site.

On microscopic examination, the tooth extraction site showed non-vital bone in 100% of the animals in the zoledronic acid group. This finding is in agreement with other studies, according to which the trauma constitutes a sufficient risk factor for osteonecrosis in zoledronic acid users (31, 33, 34). On the other hand, at the same site, the clodronate group did not show an association with non-vital bone, even though 5 (41.6%) out of 12 animals showed this feature. In humans, the lower prevalence of osteoncerosis associated with non-nitrogen bisphosphonates, especially clodronate, could be explained by the fact that these compounds have lower potency, and are less prescribed than nitrogen-containing ones (35). Besides that, the duration of non-nitrogen-containing bisphosphonates use needed for the occurrence of osteonecrosis is significantly longer (18). Nonetheless, considering that there was an association between non-vital bone and clodronate at the soft tissue wound site, it is also plausible to infer that a larger sample size would have given us a different result for the tooth extraction site in the clodronate group.

At the tooth extraction site, the zoledronic acid group had a higher proportion of microbial colonies than the clodronate and control. This is in accordance with the higher proportion of non-vital bone found in the zoledronic acid group, since osteonecrosis is

characteristically accompanied by microbial infection, especially by *Actinomyces* sp. (36). Nevertheless, there are reports on the antimicrobial effect of clodronate on *Staphylococcus aureus* and *Pseudomonas aeruginosa* (37). Zoledronic acid effects on microorganisms are also reported, although in an opposite direction, as this drug has been shown to improve, *in vitro,* the adhesion of *S. mutans* to the bone hydroxyapatite, promoting bacterial growth in culture dishes (12). Therefore, the greater occurrence of microbial colonies in the zoledronic acid group when compared to clodronate could be related to the specific interactions of each one of these two bisphosphonates with microorganisms.

At the site of soft tissue wound, the clodronate and zoledronic acid groups showed a lower proportion of fibrous connective tissue when compared to the control group. These findings are in agreement with studies showing that bisphosphonates are able to decrease fibroblast proliferation (38). Curiously, at this same site, vital bone proportion was significantly lower in the clodronate than in the control group, whereas the latter and the zoledronic acid group did not differ from each other. It is known that bisphosphonates promote nearly complete suppression of bone turnover (39, 40), but in this case we do not know why clodronate had a greater effect than did zoledronic acid.

The immunohistochemical quantification of RANKL, OPG, vWF and caspase-3 did not show significant differences between the groups evaluated, either at the soft tissue wound site or the tooth extraction site. The bisphosphonate effects on the epithelial cells of the oral mucosa are poorly understood, and until now, it has not been determined if osteonecrosis starts in bone tissue or in oral mucosa (15, 25, 38).

The present study quantified caspase-3 immunohistochemical expression in epithelial and connective tissues aiming to determine if higher rates of apoptosis in these tissues could favor osteonecrosis development. The results are in accordance with the reports of no increase in caspase-3 expression in oral keratinocytes and gingival fibroblasts

treated with nitrogen-containing bisphosphonates (15, 38). The lack of a difference in caspase-3 expression between the groups suggests that if the soft tissues are the target of the onset of osteonecrois (41), this involvement is not related to increased apoptosis of these cells. Moreover, studies report some bisphosphonate effects on keratinocytes and fibroblasts, such as loss of cell adhesion and decreased cell migration and proliferation (15, 38), relating them to the ability of promoting necrosis. Therefore, considering the hypothesis of bisphosphonate-related-osteonecrosis starting in soft tissue, it is possible that cell necrosis and/or impairment of critical events to oral wound healing such as cell proliferation, migration and differentiation would be the factor responsible for the lesion (15, 38).

The lack of significant differences in vWF expression between the groups evaluated in this study suggests that bisphosphonates are not capable of inhibiting vascularization in oral soft tissues. This agrees with the reports of Wehrhan et al. (42), who found that the immunohistochemical expression of CD31 in mucoperiosteal tissue did not differ between bisphosphonate users with and without osteonecrosis. However, studies investigating the bisphosphonate effects on angiogenesis show conflicting results.

According to some *in vitro* studies, zoledronic acid inhibits human endothelial cell differentiation (43), reduces their proliferation (44, 45), induces their apoptosis (11) and decreases the formation of capillary tubes (43). Similarly, other *in vitro* studies report that clodronate inhibits endothelial cell proliferation (45, 46). On the other hand, immunohistochemical expression of VEGF in bone tissue at the tooth extraction site did not differ significantly between rats treated with bisphosphonates and controls (33). There are also reports of a significant reduction in serum levels of VEGF in cancer patients treated with nitrogen-containing and non-nitrogen-containing bisphosphonates (47, 48).

The disagreements between the studies, either *in vitro* or *in vivo,* reflect the difficulties in comparing them because of the different methods applied.

vWF, whose expression was examined in the present work, is an important marker of vascularization, but it does not differentiate new vessels from preexisting ones (49). One could infer that by using a marker capable of indentifying new vessels, such as VEGF, we could have found a different result indicating an effect of bisphoshonate on angiogenesis. Nevertheless, we should consider the phase of healing in which the immunohistochemical analysis was performed here. In rats, the healing process at tooth extraction sites is completed by 40 days and neoformed vessels should be detected at 7 days (27), but not at the time at which we performed the evaluation. At this moment, the differentiation between new and preexisting vessels does not seem to influence the results anymore. Considering the conditions of the present study, as well as the conflicting results with VEGF and also the fact that vWF immunostaining pattern is more uniform if compared with VEGF and CD31 (50), we chose vWF as the marker of vascularization in our study.

The lack of significant differences in the immunohistochemical expression of OPG and RANKL between the zoledronic acid, clodronate and control groups would suggest that bisphosphonates are not capable of increasing the OPG/RANKL ratio. In our study, these proteins, which are also expressed in endothelial cells and fibroblasts, were quantified in connective tissue, 6 weeks after the surgical procedures, corresponding to the time of complete bone formation (51). This site of evaluation was chosen because we wanted to analyze the alterations in soft tissue that could be related to the onset of osteonecrosis and also because connective tissue is the matrix of bone neoformation. At this site, growth factors, cytokines and prostaglandins exert their effects on the recruitment, replication and differentiation of bone precursor cells (52). It is possible that an evaluation at the time corresponding to the highest metabolic activity could have shown different

results, that is, at one week after tooth extraction, when there are important changes in the connective tissue. Also, it is worth pointing out that at the soft tissue wound site, the statistical comparison of OPG results between groups showed p=0.08, which suggests that a larger sample size could have shown a significant result.

It is also important to consider that most of the *in vivo* studies evaluating the effects of bisphosphonates on the behavior of these proteins have measured their serum levels (53, 54). Maybe this analysis does not represent what actually occurs in the environment of bone cells, oral keratinocytes and fibroblasts (54). Moreover, we have to consider that the serum levels of RANKL reflect only its soluble form, excluding the major mediator of osteoclastogenesis, which is its form as a cytokine of cell membrane (55). Studies comparing the expression of these two forms of RANKL would be helpful.

It is worth recalling that the immunohistochemical analysis was conducted in epithelial and connective tissues, not in bone tissue. Considering this point, the absence of any significant result for the markers used (vWF, caspase-3, OPG, RANKL) in the test groups suggests that the major bisphosphonate effects occur in bone tissue. The high bone affinity of these drugs (40) corroborates this idea. According to the literature, 70% of the zoledronic acid administered binds to hydroxyapatite crystals, whereas the rest of the dose is eliminated unaltered by renal excretion (56). The situation with clodronate is similar except for the fact that the absence of the hydroxyl group in this compound reduces its ability to bind to hydroxyapatite crystals, compared to zoledronic acid (57).

In conclusion, both bisphosphonates zoledronic acid and clodronate are capable of inducing maxillary osteonecrosis. The findings of immunohistochemical analysis suggest that the involvement of soft tissues as the initiator of osteonecrosis development is less probable than has been pointed out. To elucidate this point, further studies should focus on

growth factors such as insulin-like growth factor and transforming growth factor-b, critical to oral wound healing, by means of molecular biology techniques such as PCR.

Acknowledgements

We thank the Novartis Laboratory for the donation of zoledronic acid and Dr. Fernanda Morrone, Head of Laboratório Farmacologia Aplicada, Faculdade de Farmácia da PUCRS for permitting the use of laboratory facilities. We are also grateful to Dr. A. Leyva (U.S.A.) for English editing the manuscript.

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DISCUSSÃO GERAL

4 DISCUSSÃO GERAL

A eficácia dos bisfosfonatos na prevenção e no tratamento de doenças caracterizadas por excessiva reabsorção óssea foi o fator responsável pela ampla difusão do uso desses fármacos a partir de sua introdução no mercado farmacêutico na década de 60 (RUSSELL, 2006). Entretanto, a ocorrência de casos de osteonecrose maxilar passou a constituir um importante efeito adverso, que trouxe algumas restrições ao uso desses medicamentos, bem como a necessidade de condutas preventivas e curativas para a condição (RUGGIERO, 2011).

A literatura comporta uma série de pesquisas que investigam os efeitos dos bisfosfonatos por meio da aplicação de diversos marcadores e métodos distintos. A associação com a osteonecrose é comprovada (MAAHS et al., 2011), existindo, entretanto, variações da intensidade do risco de acordo com as condições do paciente e especificidades do fármaco (RUGGIERO, 2011). Por outro lado, o mecanismo exato pelo qual a osteonecrose é induzida não está claramente definido. Algumas publicações levantam a possibilidade de a lesão iniciar na mucosa e, nesse contexto, têm explorado o efeito tóxico sobre células epiteliais incluindo as gastrointestinais, as renais e as da mucosa oral (LANDESBERG et al., 2008, PERAZELLA, 2003, TWISS et al., 1994). O desenvolvimento de úlceras e erosões gástricas é um reconhecido efeito colateral associado à administração oral de bisfosfonatos nitrogenados (WALLACE et al., 1999). No entanto, não está definido se o efeito que exercem sobre o epitélio oral seria capaz de iniciar a osteonecrose dos maxilares.

No presente estudo, verificou-se que, a despeito da ocorrência de osso não-vital, algumas lesões de tecido mole exibiram regeneração completa da mucosa, não tendo sido observada, nesse sítio, associação da variável solução de continuidade da mucosa com o uso de bisfosfonato. Tal achado, somado aos resultados observados para os marcadores

imunoistoquímicos caspase-3, von Willebrand, OPG, RANK e RANKL indica pouca repercussão dos efeitos dos bisfosfonatos sobre a mucosa oral. A ausência de associação entre a expressão de caspase-3 e os bisfosfonatos empregados sugere que não haja aumento das taxas de apoptose em epitélio e conjuntivo. Assim, o importante efeito que esses fármacos exercem no tecido ósseo ao interferirem na via do mevalonato ou transformarem-se em análogos de ATP e, consequentemente, levarem os osteoclastos à apoptose (LI et al., 2011; RUSSELL, 2006) parece não ser um evento significativo nesses outros tecidos. Isso, provavelmente, pela distribuição significativamente menor do fármaco em sítios extra-ósseos (RUSSELL, 2007). Ainda, é provável que seja a diferença de sua biodisponibilidade o fator responsável pelos resultados divergentes ao compararem-se pesquisas *in vitro* e *in vivo*. A maior parte das pesquisas que relatam efeitos significativos dos bisfosfonatos fora do tecido ósseo é composta por estudos com cultura de células, nos quais, por vezes, as concentrações do fármaco empregadas são superiores àquelas aplicadas *in vivo* e, mesmo quando em concentrações equivalentes às doses terapêuticas, sua biodisponibilidade é maior.

A análise realizada em apenas um período após as intervenções cirúrgicas, no entanto, não permitiu considerar o efeito dos fármacos de acordo com a cronologia do processo de reparo. É possível que os bisfosfonatos interfiram em fases iniciais desse processo, quando há intensa proliferação de fibroblastos e queratinócitos além da deposição de colágeno (RAVOSA et al., 2011). Assim, estudos que permitam avaliar a dinâmica do reparo tecidual, considerando a sequência de eventos que o constituem, podem ser úteis para determinar se alterações intrínsecas aos tecidos moles orais contribuem para a iniciação e/ou progressão da osteonecrose associada ao uso desses fármacos.

Os resultados são sugestivos, entretanto, de que os efeitos preponderantes ocorram, de fato, no tecido ósseo. Recentemente, estudos clínicos têm relatado casos de osteonecrose maxilar em pacientes usuários de denosumab, um anticorpo monoclonal empregado na prevenção e no tratamento de doenças caracterizadas por excessiva reabsorção óssea (HENRY et al., 2011). Essa droga, cuja farmacocinética difere da dos bisfosfonatos, exerce seus efeitos ao ligar-se ao RANKL, inibindo a atividade osteoclástica (STOPECK et al., 2010). Tais achados reforçam a hipótese de que o prejuízo da função osteoclástica constitui evento primário na patogênese da condição.

Ao compararem-se os efeitos de bisfosfonatos nitrogenados e não-nitrogenados, o clodronato, importante representante dos não-nitrogenados, foi capaz de induzir a ocorrência de osso não-vital, embora em menor prevalência. É preciso lembrar que esse medicamento foi administrado por via parenteral, o que resulta em doses cumulativas significativamente mais elevadas que as resultantes da administração oral. Assim, considerando-se a meia-vida dos bisfosfonatos, sua afinidade pelo tecido ósseo e o fato de não serem metabolizados neste sítio (LI et al., 2011), é plausível inferir que o risco de osteonecrose seja proporcional à dose cumulativa no tecido ósseo. Esta, por sua vez, estará na dependência da estrutura química do fármaco e da via de administração. A ausência do radical hidroxila confere ao clodronato menor afinidade à estrutura óssea, se comparado ao ácido zoledrônico (LI et al., 2011). Entretanto, o menor risco de osteonecrose que estaria associado à menor afinidade ao tecido ósseo poderá ser compensado pelo maior tempo de uso.

Os achados do presente estudo permitem inferir que tanto bisfosfonatos nitrogenados quanto não-nitrogenados são capazes de induzir a ocorrência de osteonecrose, e seu efeito sobre a mucosa oral não parece constituir fator iniciador da lesão. A alta afinidade desses compostos pela hidroxiapatita (RUSSEL, 2006) e os relatos de sua

potente atividade na supressão da remodelação óssea (ALLEN et al., 2010) corroboram a ideia de que seus efeitos ocorram, primordialmente, em estrutura óssea. Ainda, a maior parte dos estudos que avaliam os efeitos biológicos dos bisfosfonatos o faz por meio de ensaios *in vitro*, com dosagens e condições que podem não representar as da rotina clínica. Assim, muitos aspectos relacionados à etiopatogenia da doença permanecem pouco esclarecidos.

A presente pesquisa, ao testar o efeito do ácido zoledrônico e do clodronato em feridas induzidas em modelo animal, obteve alguns resultados divergentes dos relatados na literatura. Novas pesquisas que investiguem a distribuição desses fármacos nos diferentes tecidos, correlacionando-a aos possíveis efeitos farmacológicos, poderão corroborar tais achados. A melhor compreensão dos eventos biofarmacológicos que envolvem os bisfosfonatos no organismo humano poderá nortear condutas e fornecer critérios para tomada de decisão mediante pacientes usuários desses fármacos, sejam eles portadores ou não de osteonecrose maxilar.

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ANEXOS

ANEXO A

Karen Cherubini

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www.wma.net/e/policy/b3.htm) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used.

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HEINIC GS, GREENSPAN D, MACPHAIL LA, et al. Oral Histoplasma capsulatum infection in association with HIV infection: a case report. J Oral Pathol Med 1992; 21: 85-9.

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(6) Book
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(8) Published proceedings paper
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(9) Agency publication

MUIR C, WATERHOUSE J, MACK T, POWELL J, WHELAN S. Cancer incidence in five continents: Vol. 5. Lyon: International Agency for Research on Cancer, 1987; IARC Scientific Publications No. 88.

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ANEXO C

Comissão Científica e de Ética
Faculdade da Odontologia da PUCRS

Porto Alegre 08 de janeiro de 2009

O Projeto de: Tese

Protocolado sob nº:	0105/08
Intitulado:	Avaliação macro e microscópica de lesões orais induzidas por procedimentos cirúrgicos em ratos sob terapia com bisfosfonatos
Pesquisador Responsável:	Profa. Dra. Karen Cherubini
Pesquisadores Associados	Ana Carolina Uchoa Vasconcelos
Nível:	Doutorado

Foi ***aprovado*** pela Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS em *07 de janeiro de 2009.*

Este projeto deverá ser imediatamente encaminhado ao CEUA/PUCRS

Prof. Dr. Eraldo Luiz Batista Júnior
Presidente da Comissão Científica e de Ética da
Faculdade de Odontologia da PUCRS

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ANEXO D

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

Ofício 043/09 - CEUA

Porto Alegre, 16 de abril de 2009.

Senhora Pesquisadora:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 09/00083, intitulado: **"Avaliação macro e microscópica de lesões orais induzidas por procedimentos cirúrgicos em ratos sob terapia com bisfosfonatos".**

Sua investigação está autorizada a partir da presente data.

Relatórios do andamento do projeto devem ser entregues a este Comitê.

Atenciosamente,

Profa. Dr. Anamaria Feijó
Coordenadora do CEUA – PUCRS

Ilma. Sra.
Profa. Dra. Karen Cherubini
Faculdade de Odontologia
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