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MARCELO MATOS ROCHA

EFEITO DO USO COMBINADO DE BISFOSFONATO E ENXERTO XENÓGENO NA REPARAÇÃO ÓSSEA ALVEOLAR: ESTUDO HISTOMORFOMÉTRICO

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EFFECT OF COMBINED USE OF BISPHOSPHONATE AND XENOGENEIC BONE GRAFT IN ALVEOLAR BONE REPAIR: A HISTOMORPHOMETRIC STUDY

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Dissertação apresentada como requisito para obtenção do título de Mestre pelo Programa de Pós-Graduação em Odontologia, Área de Concentração: Estomatologia Clínica

Orientadora: Profª. Drª. Karen Cherubini

Porto Alegre

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O êxito da vida não se mede pelo caminho que você conquistou, mas sim pelas dificuldades que superou no caminho.

Abraham Lincoln (1809-1865)

Dedicatória

Especialmente à minha esposa, **Ana Paula**, pela paciência e compreensão em minhas ausências.

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Resumo

RESUMO

Os bisfosfonatos têm sido associados à ocorrência de osteonecrose maxilar (MRONJ, *medication-related osteonecrosis of the jaw*), condição que tem, entre os fatores de risco para sua ocorrência, as intervenções cirúrgicas dos ossos maxilares, principalmente as exodontias. Por outro lado, o uso de xenoenxerto para preenchimento alveolar após exodontia tornou-se procedimento frequente, com evidências clínicas de sua capacidade em melhorar as condições locais do sítio cirúrgico com vistas à reabilitação oral. O presente estudo teve por objetivo investigar o efeito do uso combinado de bisfosfonato e enxerto xenógeno (Bio-Oss®Collagen) no reparo ósseo alveolar após exodontia. Sessenta ratos foram distribuídos em cinco grupos de acordo com o tratamento recebido: ácido zoledrônico (grupo 1) e alendronato (grupo 2), ambos com xenoenxerto; ácido zoledrônico (grupo 3) e alendronato (grupo 4), ambos sem xenoenxerto; e grupo-controle (grupo 5). Todos os animais foram submetidos a exodontias, as maxilas foram dissecadas e analisadas por meio de exame macro- e microscópico. A frequência de lesão da mucosa oral não diferiu significativamente entre os grupos; entretanto essas lesões foram significativamente menores no grupo-controle. A prevalência de tecido conjuntivo fibroso foi maior nos grupos 2 (alendronato/Bio-Oss) e 5 (controle) do que nos grupos 3 (ácido zoledrônico) e 4 (alendronato). O grupo 4 (alendronato) teve maior quantidade de osso vital do que os grupos 1 (ácido zoledrônico/Bio-Oss), 3 (ácido zoledrônico) e controle. A quantidade de osso não-vital foi maior nos grupos ácido zoledrônico com e sem Bio-Oss (1 and 3), sendo que, ao comparar-se esses dois grupos entre si, essa variável foi maior no grupo 3. O grupo 3 exibiu mais infiltrado inflamatório do que os grupos 2, 4 e 5 e maior quantidade de colônias microbianas do que os demais grupos. Infiltrado inflamatório e colônias microbianas tiveram correlação negativa com osso vital e positiva com osso não-vital. Infiltrado inflamatório e colônias microbianas exibiram correlação positiva entre si.

Conclusão: O preenchimento do alvéolo pós-exodontia com Bio-Oss® Collagen não evitou a ocorrência de osso não-vital e infecção, mas foi capaz de diminuir a intensidade dessas variáveis nos ratos sob tratamento com ácido zoledrônico.

Palavras-chave: extração dentária; enxerto; osteonecrose; bisfosfonatos; osso alveolar

Summary

SUMMARY

Bisphosphonates have been associated with *medication-related osteonecrosis of the jaw* (MRONJ), a disorder that has some risk factors such as surgical interventions in the jaw bones, especially tooth extractions. On the other hand, xenogeneic grafts have been widely used to fill the alveolar socket after tooth extraction, with clinical evidences of improving local conditions of the surgical bed for oral rehabilitation. The aim of this study was to investigate the effect of combined bisphosphonate and the xenogeneic graft Bio-Oss® Collagen on alveolar bone repair after tooth extraction. Sixty rats were allocated into five groups according to the treatment received: zoledronic acid (group 1) and alendronate (group 2) both with xenogeneic graft; zoledronic acid (group 3) and alendronate (group 4) both without xenogeneic graft; and control (group 5). All animals were subjected to tooth extractions, and maxillae were dissected and macro- and microscopically analyzed. Frequency of oral mucosal wounds did not significantly differ between the groups; however, these lesions were significantly smaller in the control group. The amount of fibrous connective tissue was greater in groups 2 (alendronate/Bio-Oss) and 5 (control) than in 3 (zoledronic acid) and 4 (alendronate). Group 4 showed greater amounts of vital bone than did groups 1 (zoledronic acid/Bio-Oss), 3 and 5. The amounts of non-vital bone were greater in the zoledronic acid groups (1 and 3), where non-vital bone was less in group 1 than 3. Group 3 showed more inflammatory infiltrate than groups 2, 4 and 5. There were greater amounts of microbial colonies in group 3. Inflammatory infiltrate and microbial colonies were negatively correlated to vital bone and positively correlated to non-vital bone. Inflammatory infiltrate and microbial colonies were positively correlated to each other.

Conclusion: Post-extraction socket filling with Bio-Oss® Collagen did not prevent the occurrence of non-vital bone and infection, but it did lower the extent of these variables in rats under treatment with zoledronic acid.

Key words: tooth extraction; graft; osteonecrosis; bisphosphonates; alveolar bone

Sumário

SUMÁRIO

Introdução

1 INTRODUÇÃO

A osteonecrose dos maxilares associada a medicamentos (MRONJ-*medication-related osteonecrosis of the jaw*) é um efeito adverso que foi, primeiramente, associado ao uso de bisfosfonatos (Khosla *et al*., 2007; Marx, 2003; Ruggiero *et al.*, 2009) e, posteriormente, a outros fármacos como denosumabe e antiangiogênicos (Ruggiero *et al*., 2014). A enfermidade exibe alguns fatores de risco, entre os quais merecem destaque as intervenções cirúrgicas nos ossos maxilares, o que inclui exodontias, apicectomias, colocação de implantes dentários, entre outros (Migliorati *et al*., 2005; Ruggiero *et al*., 2006; Ruggiero *et al*., 2009). Os quadros envolvem significativa morbidade e, a despeito das inúmeras tentativas terapêuticas relatadas, não há, até o momento, um tratamento resolutivo (Khosla *et al.*, 2007).

Os bisfosfonatos estão indicados para o tratamento de doenças caracterizadas por quadros de intensa reabsorção óssea como osteoporose, metástases ósseas de tumores de mama, pulmão e próstata, bem como quadros de mieloma múltiplo (Greenberg, 2004; Ruggiero *et al.*, 2006). Esses fármacos são classificados em nitrogenados e não-nitrogenados e inibem a reabsorção osteoclástica por meio de distintos mecanismos de ação que resultam tanto em inibição da função osteoclástica, quanto em apoptose dessas células (Barni *et al*., 2006; Capelari *et al*., 2010). Como consequência, também o metabolismo ósseo é inibido, uma vez que depende dos processos de reabsorção e neoformação (Rodan; Fleisch, 1996). Segundo alguns autores, este seria o principal fator associado à ocorrência da osteonecrose (Khosla *et al*., 2007; Ruggiero *et al.*, 2009).

Os enxertos, por sua vez, têm sido amplamente empregados no intuito de promover a neoformação óssea, principalmente em áreas do osso alveolar que foram submetidas a exodontias. A incorporação de um enxerto ósseo pelo leito receptor depende do *turnover*

ósseo, e envolve osteogênese, reabsorção osteoclástica, osteoindução e osteocondução (Erdogan *et al*., 2007). No enxerto ósseo, a osteocondução atua como um suporte para o crescimento de capilares, tecido perivascular e células osteoprogenitoras do leito hospedeiro, permitindo a substituição gradual do enxerto por reabsorção das trabéculas ósseas antigas e formação de novo tecido ósseo (Garbuz *et al*., 1998). O uso de enxertos no aumento do volume ósseo alveolar visa a estimular a neoformação óssea em áreas de reabsorção grave. Nessa situação, o enxerto autógeno, a despeito de algumas limitações, como a necessidade de uma segunda cirurgia, é considerado padrão-ouro em função de suas propriedades de osteogenicidade, osteoindução e osteocondução (Fellah *et al*., 2008; Jemt; Lekholm, 2003; Jensen *et al*., 2006; Le Nihouannen *et al*., 2007; Mah *et al*., 2004). Xenoenxertos ou enxertos xenogênicos, são enxertos ósseos de outras espécies, diferentes da humana, tais como a bovina, que são usados como uma matriz calcificada (Kumar *et al*., 2013), e têm sido empregados com sucesso no intuito de preservar as dimensões alveolares em cavidades pós-exodontia, bem como no tratamento de defeitos estruturais do processo alveolar (Artzi *et al.,* 2000; Berghlund; Lindhe 1997; Carmagnola *et al.,* 2003; Froum *et al.*, 2002; Nevins *et al.,* 2006).

A literatura relata estudos com resultados conflitantes no que se refere ao uso combinado de bisfosfonatos e enxertos ósseos. Além disso, não há relatos a respeito do efeito desses fármacos especificamente sobre o osso alveolar receptor de enxerto. Em função disso, o presente estudo teve por objetivo investigar possíveis interações entre bisfosfonatos e enxertos ósseos. O trabalho foi estruturado sob a forma de dois artigos: o primeiro artigo apresenta uma revisão da literatura enfocando o tema em questão, enquanto o segundo artigo relata o experimento *in vivo* em que foi conduzida análise histomorfométrica do osso alveolar submetido a exodontias e uso combinado de bisfosfonato e enxerto xenógeno.

Artigo 1

2 ARTIGO 1

O artigo a seguir intitula-se ***Insights into interactions between bone grafts and bisphosphonates with focus on alveolar bone*** e foi formatado de acordo com as normas do periódico *Archives of Oral Biology* (Anexos A e B).

Insights into interactions between bone grafts and bisphosphonates with focus on alveolar bone

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Key words: tooth extraction; graft; osteonecrosis; bisphosphonates

Running title: *Bone grafts and bisphosphonates*

Review Article

ABSTRACT

Bisphosphonates are antiresorptive drugs that are widely used to treat osteoporosis, bone metastases of solid tumors and multiple myeloma as well. Since 2003, they have been associated with the adverse effect of osteonecrosis of the jaw (MRONJ), where surgical intervention in the alveolar bone is a major risk factor. Bone grafts, either autogenous, isogenous, allogeneic, xenogeneic, or alloplastic, in turn, have been indicated to improve bone formation, including alveolar bone augmentation. The target population of bisphosphonate therapy is most times also the one in need of bone grafts. Nevertheless, little is known about interactions between grafts and bisphosphonates. The authors reviewed the scientific literature with regard to important concepts concerning this issue. The literature seems a little controversial regarding the effects of bisphosphonate on bone grafting. Whereas some authors report improvement in graft incorporation when bisphosphonate is administered, others do not. The lack of standardization related to study design, dose, route of administration and type of bisphosphonate, as well as type of graft, may contribute to such disagreements and are important factors to consider in further studies.

Keywords: tooth extraction; bone graft; osteonecrosis; bisphosphonates

INTRODUCTION

Bisphosphonates increase bone density by inhibiting osteoclast activity. Since the remodeling process (repair) of the skeleton depends on osteoclastic resorption, these drugs also interfere with bone metabolism, impairing the normal repair process. They bind to calcium of hydroxyapatite on the bone surface, which explains the specific effects on mineralized bone tissue. During bone remodeling, bisphosphonates are released, becoming pharmacologically active, and continue to be released daily, even after ten years of

stopping medication, which evidences a lifelong drug behavior in humans (Rodan; Fleisch, 1996). Moreover, since 2003, these drugs have been associated with the adverse effect of osteonecrosis of the jaw (MRONJ), where surgical intervention in the alveolar bone is a major risk factor (Ruggiero *et al*., 2014).

Bone grafts have been used in oral and maxillofacial surgery to treat bone defects, especially in the case of dental implants, aimed at restoring an edentulous area (Kumar *et al*., 2013). Basically, grafts serve as a scaffold having functions such as osteogenesis, osteoinduction and osteoconduction in the host bed. Osteointegration is also a desirable property (Precheur, 2007). According to the literature, the gold-standard graft in the treatment of bone defects in humans is an autogenous graft. This kind of graft is the only one that provides immune-compatible living bone cells, essential for phase I of osteogenesis, which is responsible for bone cell proliferation (Silva Júnior *et al*., 2001). The other types of grafts are isogenous, allogenous and xenogenous, and several synthetic materials have been investigated and used as alloplastic grafts (Cancian *et al*., 1999; Davies, 2003; von Arx; Cochran, 2001). These biomaterials have been used to overcome the disadvantages of natural ones. Because of the inability of bone in some patients to repair bone defects, the need for investigations of biomaterials for bone regeneration has increased (Kim; Kim, 2013; Porter *et al*., 2009).

Considering that the age range of patients using bisphosphonates overlaps that of those in need of bone grafts, it is likely that, in many cases, these treatments will be concomitantly administered. Accordingly, the aim of the present study was to critically review the scientific literature with regard to biological concepts and interactions between bone grafts and bone tissue treated with bisphosphonates with special focus on alveolar bone.

Bone repair

Important processes such as osteogenesis, osteoclastic resorption and osteoinduction are involved in bone repair. Osteogenesis is a generic term referring to bone ability to regenerate and produce new bone; a function exerted by osteoblasts. Osteoclastic bone resorption releases bone minerals from bone matrix and is performed by osteoclasts. Osteoinduction is the stimulation of new bone formation through recruitment of pluripotential mesenchymal cells from the surrounding tissue. This function is exerted by soluble proteins derived from bone matrix especially the family of bone morphogenetic proteins (BMPs) (Garbuz *et al*., 1998).

Bone is a well-organized dynamic tissue, which can undergo remodeling according to environmental mechanical stress and hormonal activity (Erdogan *et al*., 2007). Bone remodeling is regulated by a combination of bone resorption and formation processes, where the cells primarily involved are osteoblasts and osteoclasts. These cells exert activity within a temporary anatomical structure called basic multicellular unit (BMU) (Sikavitsas *et al*., 2001). During bone deposition, osteoblasts synthesize a matrix which undergoes mineralization, whereas resorption performed by osteoclasts consists of bone mineral dissolution and catabolism of bone matrix components (Brats, 2013). Therefore, a mature BMU is formed by a leading front of osteoclasts followed by osteoblasts, some blood supply and associated connective tissue. Eventually, the resorbed bone is replaced by osteoblasts and bone neoformation, a process that takes approximately three months (Jilka, 2003).

Besides BMPs, other proteins and growth factors, such as transforming growth factor beta (TGF-beta), insulin-like growth factor I and II (IGF I and II), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) are responsible for regulating bone cell activity. They bind to receptors on the surface of cells and stimulate the

intracellular environment. Overall, this activity is translated by a protein kinase, which induces a series of events that, in turn, result in mRNA transcription and, finally, formation of proteins that will be used intra- or extracellularly.

BMPs are the most important growth factors in bone formation and healing. They are produced during fracture repair by mesenchymal cells, osteoblasts and chondrocytes. Different BMPs work independently or in combination with each other and with other members of the TGF family to trigger a cascade of events that support the development of bone and cartilage (Bessa *et al*., 2008; Dimitriou *et al*., 2005). Several types of cells, including osteoblasts and chondroblasts, participate in the process of bone morphogenesis and are functionally regulated by BMPs. BMPs are accommodated in the bone matrix, acting as pleiotropic regulators of cellular events such as chemotaxis, mitosis, differentiation, stimulation of extracellular matrix synthesis, phenotype maintenance and apoptosis (Kumar *et al*., 2013; Mulconrey *et al*., 2008; Valdes *et al*., 2009). These effects depend on the concentration, time of exposure and target cells (Chen *et al*., 2010; King *et al*., 1996; Yamashita *et al*., 1996).

Osteopontin (OPN), in turn, is a marker of osteogenic differentiation found in the periphery of the neoformed bone matrix and in the process of bone mineralization, preventing premature precipitation of calcium phosphate crystals in the collagen matrix (Hell *et al*., 2011). OPN has also been implicated in a variety of disease states, where it mediates various cellular functions such as adhesion, migration and survival of different cell types, including the regulation and propagation of inflammatory responses of macrophages, T cells and dendritic cells (Lund *et al*., 2009).

Osteoblasts and bone marrow stromal cells regulate osteoclastogenesis by means of monocyte-colony stimulating factor (M-CSF), receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) production. RANKL and M-CSF induce

progenitor cells that differentiate into osteoclasts through cell-to-cell contact between osteoblasts/stromal cells and osteoclasts (Kim *et al*., 2009; Lacey *et al*., 1998; Tay *et al*., 2004).

Alveolar bone regeneration and augmentation

Mature alveolar bone is a specialized part of the maxilla and mandible bones, which forms the primary scaffold to support the teeth. It is organized in layers oriented parallel to the coronal-apical tooth direction (Sodek; McKee, 2000). The success of alveolar repair is associated with this bone structure and its basic healing characteristics. According to Cardaropoli *et al*. (2003), after blood clot formation, five biological events are involved in post-extraction alveolar healing: (1) clot stabilization; (2) formation of a provisional connective tissue matrix (after 7 days); (3) woven bone (after 14-30 days); (4) lamellar bone (after 30-180 days); (5) resorption of lamellar bone and increase in bone marrow (after 60-180 days).

The term *alveolar bone augmentation* refers to any attempt to preserve or increase the height or the width of the residual alveolar ridge, or the repair of defects with grafts or biomaterials. The techniques include ridge preservation after tooth extraction, onlay autogenous bone block grafting, guided bone regeneration techniques, inlay or interpositional grafting, alveolar distraction osteogenesis, and ridge expansion/splitting techniques (Erdogan *et al*., 2007). The use of grafts in alveolar augmentation aims to improve new bone growth in areas of severe bone resorption. Several types of grafts have been used for this purpose. Among them, autogenous grafts are considered the gold-standard because of their properties of osteogenicity, osteoinduction and osteoconduction, in spite of some limitations such as the need of a second surgery. Another possibility is allografts, which are less osteogenic and more immunogenic and show resorption rates similar to autogenous ones, and also, they have the potential risk of disease transmission

(Fellah *et al*., 2008; Jemt; Lekholm, 2003; Jensen *et al*., 2006; Le Nihouannen *et al*., 2007; Mah *et al*., 2004). Xenografts, in turn, are obtained from nonhuman species, such as bovine, which are used as a calcified matrix (Kumar *et al*., 2013).

Alloplastic grafts or bone graft substitutes represented by biomaterials can also be used in alveolar bone regeneration and augmentation. A biomaterial is defined as any substance other than drug or the combination of substances, synthetic or natural in origin, which can be used for a time period, completely or partially, as part of a system, and capable of treating, stimulating or replacing any organ or tissue or function in the body (Williams, 2008). According to Carvalho *et al*. (2010), a biomaterial should have the following properties: does not induce thrombus formation; does not induce an adverse inflammatory response; is not toxic; is not carcinogenic; does not impair blood flow; and does not produce an acute or chronic inflammatory response that impairs the proper differentiation of adjacent tissues.

After an alveolar bone augmentation procedure, bone graft healing comprises a cascade of events involving osteogenesis, osteoclastic resorption, osteoinduction and osteoconduction (Erdogan *et al*., 2007). A bone graft shows osteoconduction, where it serves as a support for the ingrowth of capillaries, perivascular tissue and osteoprogenitor cells from the host bed. This scaffold allows the gradual replacement of the bone graft by resorption of old bone trabeculae and formation of new bone (Garbuz *et al*., 1998). Incorporation of bone graft in the receptor bone depends on bone turnover, which in turn is affected by the rates of osteoblast and osteoclast production, osteoclast apoptosis and BMU lifespan (Erdogan *et al*., 2007).

Effects of bisphosphonates on bone tissue

Bisphosphonate effects are exerted especially on osteoclasts, by either inhibiting their recruitment to bone surface and activity or shortening their lifespan by apoptosis induction

(Rodan; Fleish, 1996). Non-nitrogen-containing bisphosphonates, after being internalized by osteoclasts, are metabolized into adenosine triphosphate (ATP) analog molecules. The intracellular accumulation of these non-hydrolysable ATP analogs is cytotoxic to osteoclasts, since such compounds inhibit various ATP-dependent cellular processes, determining osteoclast apoptosis. Nitrogen-containing bisphosphonates, on the other hand, after being internalized by osteoclasts, inhibit farnesyl pyrophosphate synthase and disrupt the mevalonate pathway. Farnesyl pyrophosphate synthase is a key enzyme of this pathway, which is responsible for the production of cholesterol and other sterols and isoprenoid lipids. The isoprenylation of the proteins Rac, Rab and Rho, which play a central role in the regulation of osteoclast physiology, is inhibited, leading to cell loss of function and ultimately to apoptosis (Drake *et al*., 2008). Bisphosphonates affect bone remodeling, exerting direct effects on osteoclasts. However, an indirect mode of action occurs, where osteoclastogenesis is inhibited by osteoprotegerin (OPG), whose secretion by osteoblasts is induced during exposure to bisphosphonate (Kim *et al*., 2009; Lacey *et al*., 1998; Tay *et al*., 2004).

Bisphosphonates are released only when bone is resorbed, which accounts for its long half-life. After being phagocytized by osteoclasts, they can return to the circulation and be excreted by the kidneys or remain in the circulation until being reincorporated into bone tissue. This recycling process may explain why, after discontinuation of treatment, small amounts of bisphosphonates can be detected in the urine even after 10 years (Cartsos *et al*., 2008; Kwak *et al*., 2009; Rodan, 1998; Rodan; Fleisch, 1996; Toussaint *et al*., 2009). Although the primary action of bisphosphonates is osteoclast inhibition, there is increasing evidence that these drugs affect osteoblasts, stimulating their proliferation and maturation and inhibiting apoptosis, which would result in increased bone formation (von Knoch *et al*., 2005).

Bisphosphonates and bone grafts

Some studies have investigated the effects of bisphosphonate administration, either local or systemic, on different types of bone grafts. Either inhibition of graft resorption or improvement of bone neoformation has been reported. Improvement of bone formation was observed in free autogenic bone graft in rats after daily subcutaneous injection of alendronate for 12 weeks (Altundal *et al*., 2007). Local administration of this drug was also capable of improving bone formation when combined with bioactive glass (Srisubut *et al*., 2007). Accordingly, systemic zoledronic acid was used associated with bioactive glass and increased bone neoformation without blocking natural remodeling (Välimäki *et al*., 2006).

Bosemark *et al*. (2013) treated bone defects in rats using BMP and autograft bone, followed by a single subcutaneous injection of zoledronic acid (0.1 mg/kg) two weeks later. According to the authors, premature resorption was prevented during the initial formation of bone callus and an additional doubling of force was achieved. Combination of BMP and bisphosphonate provided a bony callus that was four times stronger than with the autogenous graft alone.

Kim *et al*. (2015) investigated whether the combination of alendronate with recombinant human bone morphogenetic protein-2 (rhBMP-2) would improve bone regeneration. They found that low concentrations of topical alendronate (1 mM) combined with rhBMP-2 had a synergistic effect on bone regeneration. The mechanism involved seemed to be a reduction in RANKL activity in osteoblasts. The authors concluded that alendronate with rhBMP-2 could be clinically applied in bony defect.

According to Mathijssen *et al*. (2014), systemically administered bisphosphonates could protect autogenous graft from an exceedingly fast resorption and stimulate bone neoformation. In grafts not treated with bisphosphonate, the new bone formed by osteoblasts is immediately resorbed by osteoclasts, whereas in grafts treated with

bisphosphonate, the newly formed bone is not resorbed. However, only revascularized bone can be reached by the drug, a limitation that could be overcome by using topical administration.

Toker *et al*. (2012), in turn, reported no improvement in bone neoformation after either local or systemic use of bisphosphonate combined with micro-macroporous biphasic calcium phosphate (MBCP) graft material in the calvaria of rats, which disagrees with other studies. Considering the risks of systemic treatment with alendronate, it was suggested that further investigations should be conducted, focusing on local administration of this drug at different doses and associated with different graft materials, which would possibly result in better effects on bone graft healing (Toker *et al*., 2012). Accordingly, topical alendronate did not influence the bone healing process when combined with calcium phosphate bone graft in rabbit tibia defects of a healthy bony environment (Schlickewei *et al*., 2015).

FINAL CONSIDERATIONS

Incorporation of bone grafts into the host bed depends on bone turnover, which in turn, is affected by osteoblast and osteoclast production, osteoclast apoptosis and BMU lifespan (Erdogan *et al*., 2007). Therefore, considering the known effects of bisphosphonates inhibiting bone metabolism, it could be inferred that bone treated with these drugs would impair graft incorporation. Nevertheless, studies investigating interactions between bone graft and host bed treated with bisphosphonate report conflicting results (Table 1). Some of them report improvement of graft healing with bisphosphonate use (Bosemark *et al*., 2013; Greiner *et al*., 2008; Jakobsen *et al*., 2006; Jakobsen *et al*., 2009; Srisubut *et al*., 2007; Välimäki *et al*., 2006), whereas others report no effect of these drugs on graft healing

(Aspenberg; Astrand, 2002; Kim *et al*., 2015; Myoung *et al*., 2001; Schlickewei *et al*., 2015; Toker *et al*., 2012).

Lack of standardization of studies may explain the disagreements between them and requires caution when comparing data. The methods applied including type of study, criteria for data analysis, type, dose and route of administration of bisphosphonate and type of graft used could have influenced the results (Toker *et al*., 2012). Regarding the studies with positive results with topical bisphosphonate immediately before the graft implant, the intriguing point would be how the effects are exerted, considering the short time for any of the known bisphosphonate mechanisms of action to occur (Consolaro; Consolaro, 2008). On the other hand, even if using systemic administration at a satisfactory dose and sufficient time for bisphosphonates to act, their known mechanisms of action seem to counteract those expected from bone grafts (Fig. 1). Moreover, most studies as presented in Table 1 have been performed in calvaria or tibia. A few of them were conducted in the mandible, and even these did not involve alveolar bone, which significantly compromises any extrapolation of the results to the jawbones.

Regarding the positive findings with the combined use of bisphosphonates and grafts, it is important to recall that many aspects of bisphosphonate mechanism of action remain obscure, and other novel properties such as induction of osteoblast proliferation have been described (von Knoch *et al*., 2005). According to some authors (Consolaro; Consolaro, 2008), bisphosphonates are not inhibitors but modulators of bone metabolism. Considering this point of view, the possibility of improved graft healing with their use seems reasonable. Therefore, further *in vivo* studies taking into account alternative effects of bisphosphonates and standardized methods, involving alveolar bone, are necessary for a better understanding of bone graft and bisphosphonate interactions. For now, clinicians

must critically evaluate patients in need of both therapies in such a way as to avoid undesirable effects such as MRONJ.

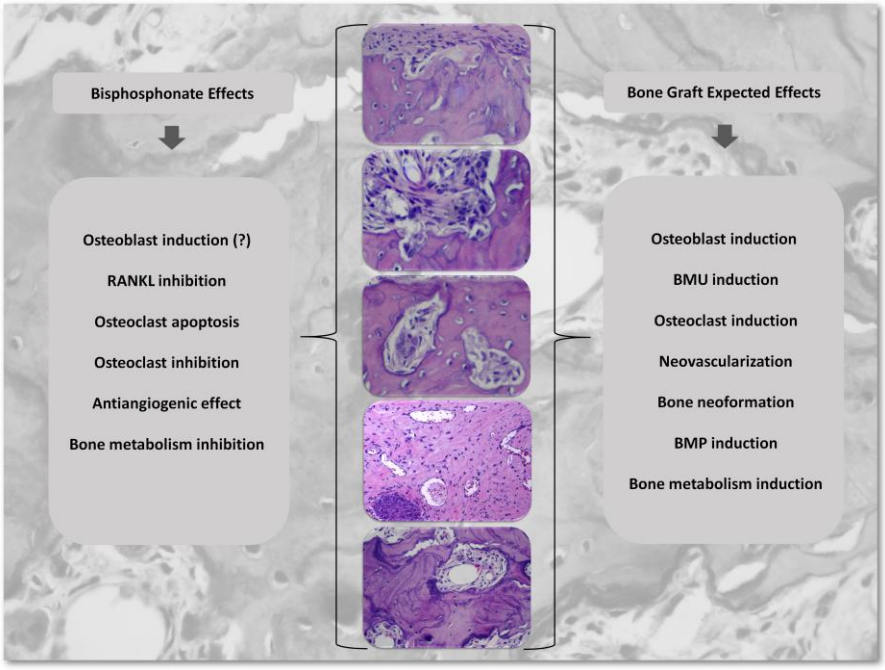

Figure 1 – Bisphosphonate effects and bone graft expected effects. BMU=bone morphofunctional unit; BMP=bone morphogenetic protein; RANKL= receptor activator of nuclear factor-kappa B ligand

Table 1 - Studies reported in the literature on combined use of bisphosphonates and bone grafts

Type of study, n, time	Bisphosphonate	Graft	Method/bone tested	Results	Reference
In vivo (rats) n= 60 4, 6 and 8 weeks	PAM IP 0.01 mg/kg/day	Autogenous free bone graft	Calvaria Evaluated the expression of CTR mRNA and OCIF	Decreased bone resorption Inhibition of CTR and up-regulation of OCIF	Myoung *et al*. (2001)
In vitro n=10 6 weeks	ALN Local 1 mg/mL	Allograft bone	Implanted frozen cancellous bone immersed in ALN for 10 min Histomorphometric analysis	Risk-free Prevent mechanical graft failure due to resorption	Aspenberg; Astrand (2002)
In vivo (dogs) n=8 12 weeks	ALN Topical	Titanium implant	Porous-coated titanium implants inserted with bone compaction into the knees.	Increase in total bone-to-implant contact and total bone density around the implants in the bisphosphonate-treated group	Jakobsen *et al*. (2006)
In vivo (rats) n=80 8 weeks	ZOL Subcutaneous 1.5 mg/kg	Bioactive glass	Tibia	Bioactive glass induced high local bone turnover. Bioactive glass incorporation seemed to benefit from ZOL therapy	Välimäki *et al*. (2006)
In vivo (rats) n= 26 4 weeks	ALN Topical	Bioactive glass	Surgical defect in the mandible (angle)	Improved bone formation on the histological examination	Srisubut *et al*. (2007)
In vivo (rats) n= 95 6 and 12 weeks	ZOL Topical	PDLLA	Midshaft fractures of tibia stabilized with either uncoated, PDLLA-coated, or ZOL-coated implants. Mechanically tested	Local application of ZOL from PDLLA coating appears to accelerate the achievement of mechanical stability in fractures	Greiner *et al*. (2008)
In vivo (dogs) n=10 12 weeks	ALN Local	Hydroxyapatite (HA)	HA-coated implants inserted into the proximal tibia; ALN applied prior to bone compaction. Histomorphometry and biomechanical push-out test	Improvement of the fixation of porous-coated implants that have also undergone HA-surface coating and peri-implant bone compaction	Jakobsen *et al*. (2009)

In vivo (rats) n=40 (10/group) 8 weeks	ALN Systemic & topical	MBCP	Bone defects in calvaria Histomorphometric analysis	Number of osteoblasts, osteoclasts and resorption lacunae significantly higher in the group MBCP with systemic ALN ALN, administered systemically or locally, did not increase bone regeneration with MBCP graft	Toker *et al*. (2012)
In vivo (rats) n=36 6 weeks	ZOL Systemic	Autograft and BMP	Bone defects (fracture) in tibia Mechanical test	Combination of BMP and bisphosphonate resulted in calluses 4 times stronger than autograft alone	Bosemark *et al*. (2013)
In vivo (rats) n=36 2 weeks	ZOL, topical 1 and 10 mM	rhBMP-2	Bone defects in calvaria	ZOL + rhBMP-2 showed synergistic effect on bone regeneration Reduced RANKL activity in osteoblasts	Kim *et al*. (2015)
In vivo (rabbits) n= 24 2, 4, 12 weeks	ALN Topical	Calcium phosphate	Bone defects in tibia	No increased callous formation after injection with calcium phosphate + alendronate	Schlickewei *et al*.(2015)
In vivo (rabbits) n=12 6 weeks	ALN systemic	Deproteinized bovine bone (xenograft) and autogenous graft	Maxillary sinus floor elevation	ALN stimulated bone formation and reduced fibrous tissue formation, especially in the xenograft group, following maxillary sinus augmentation grafts	Ayranci *et al*. (2015)

PAM=pamidronate; CTR=calcitonin receptor; OCIF=osteoclast inhibitory factor; ALN=sodium alendronate; ZOL=zoledronic acid; HA=hydroxyapatite; PDLLA=biodegradable poly (D,L-lactide); MBCP= micro-macroporous biphasic calcium phosphate; BMP=bone morphogenetic protein; rhBMP-2=recombinant human bone morphogenetic protein-2; RANKL= receptor activator of nuclear factor-kappa B ligand

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest related to this work.

HIGHLIGHTS

The target population of bisphosphonate therapy is most times also the one in need of bone grafts.

There is some controversy regarding the effects of bisphosphonate on bone grafting.

Lack of standardization contributes to disagreements of studies on bisphosphonates and grafts.

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3 ARTIGO 2

O artigo a seguir intitula-se ***Effect of combined use of bisphosphonate and xenogeneic graft on alveolar bone repair after tooth extraction: a histomorphometric study*** e foi formatado de acordo com as normas do periódico ***Clinical Oral Implants Research*** (Anexo C).

Effect of combined use of bisphosphonate and xenogeneic graft on alveolar bone repair after tooth extraction: a histomorphometric study

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Key words: tooth extraction; graft; osteonecrosis; bisphosphonates

Running title: *Bone graft and bisphosphonates*

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ABSTRACT

Objective: To investigate the effect of combined bisphosphonate and the xenogeneic graft Bio-Oss®Collagen on alveolar bone repair after tooth extraction.

Material and Methods: Sixty rats were allocated into five groups according to the treatment received: zoledronic acid (group 1) and alendronate (group 2) both with xenogeneic graft; zoledronic acid (group 3) and alendronate (group 4) both without xenogeneic graft; and control (group 5). All animals were subjected to tooth extractions, and maxillae were dissected and macro- and microscopically analyzed.

Results: Frequency of oral mucosal wounds did not significantly differ between the groups; however, these lesions were significantly smaller in the control group. The amount of fibrous connective tissue was greater in groups 2 (alendronate/Bio-Oss) and 5 (control) than in 3 (zoledronic acid) and 4 (alendronate). Group 4 showed greater amounts of vital bone than did groups 1 (zoledronic acid/Bio-Oss), 3 and 5. The amounts of non-vital bone were greater in the zoledronic acid groups (1 and 3), where non-vital bone was significantly less in group 1 than 3. Group 3 showed more inflammatory infiltrate than groups 2, 4 and 5. There were greater amounts of microbial colonies in group 3. Inflammatory infiltrate and microbial colonies were negatively correlated to vital bone and positively correlated to non-vital bone. Inflammatory infiltrate and microbial colonies were positively correlated to each other.

Conclusion: Post-extraction socket filling with Bio-Oss®Collagen did not prevent the occurrence of non-vital bone and infection, but it did lower the extent of these variables in rats under treatment with zoledronic acid.

INTRODUCTION

Bisphosphonates are a class of drugs capable of inhibiting osteoclast resorption (Michaelson; Smith, 2005), which are indicated to treat osteoporosis, Paget's disease, osteogenesis imperfecta, juvenile osteoporosis, multiple myeloma, and bone metastases of breast, lung and prostate cancer as well (Greenberg, 2004; Ruggiero *et al*., 2006). Jaw osteonecrosis, in turn, is an adverse effect of bisphosphonates named by the acronym MRONJ (medication-related osteonecrosis of the jaw). The condition is defined as

exposed or probing bone through intra- or extraoral fistula in the maxilla and/or mandible, persisting for more than eight weeks in a patient previously or currently under treatment with antiresorptive or antiangiogenic drugs and who has not received radiation therapy to the head and neck region and who has no metastasis in the maxillomandibular complex (Ruggiero *et al*., 2014). The condition is usually associated with pain, edema, paresthesia, infection and ulceration of soft tissues, and radiographic alterations (Khosla *et al.*, 2007).

MRONJ has high morbidity, with substantial impairment of the patient's quality of life, and is difficult to treat. Although several therapies have been attempted such as antimicrobials; surgical intervention with or without platelet-rich plasma, low-intensity laser therapy, hyperbaric oxygen therapy, piezo surgery, and ozone therapy, none of them is considered efficacious and definitive, which makes prevention still the best approach (Fliefel *et al*., 2015; Ruggiero *et al*., 2014). Even though its etiopathogenesis is not yet completely understood, MRONJ is considered a multifactorial disease; some risk factors have been reported, and surgical intervention in the jaws is the main one, with tooth extraction corresponding to 50% of cases (Kunchur *et al*., 2009). For patients using the oral bisphosphonate sodium alendronate, MRONJ risk is 0.03%, increasing to 0.5% in case of tooth extraction. On the other hand, for those under treatment with zoledronic acid, the estimated risk is 1.6% increasing to 14.8% if tooth extractions are performed (Ruggiero *et al*., 2014).

A particulate graft of deproteinized bovine bone mineral (Bio-Oss®, Geinstlish Pharma AG, Wolhusen Switzerland) has been successfully used in several studies either to preserve alveolar bone dimensions after tooth extraction or to treat structural defects of the alveolar process (Artzi *et al*., 2000; Carmagnola *et al*., 2003; Froum *et al*., 2002; Nevins *et al*., 2006). Animal-based experimental studies have repeatedly classified Bio-Oss® as a suitable bone substitute material (Berglundh; Lindhe, 1997; Klinge *et al*., 1992; Wetzel *et*

al., 1995). Such biomaterial works as a scaffold for neovascularization and mesenchymal cells for bone neoformation. More recently, Bio-Oss® was improved with 10% porcine collagen giving Geistlich Bio-Oss® Collagen, with improved handling properties and ability to efficiently induce bone formation.

While bisphosphonates have been used to increase bone density by inhibiting osteoclast activity (Rodan; Fleish, 1996) and are associated with MRONJ, especially after tooth extraction, biomaterials are applied to guide bone neoformation and to preserve alveolar bone after tooth extraction. Nevertheless, there are few reports about interactions between bisphosphonate, bone grafts and the alveolar bone subjected to tooth extraction. That is, the effect of a bone graft on alveolar socket repair in individuals systemically treated with bisphosphonate has not yet been adequately clarified. Therefore, the aim of this work was to investigate by means of histomorphometric analysis the effect of combined use of systemic bisphosphonate and a xenogeneic graft (Bio-Oss® Collagen) on alveolar bone repair after tooth extractions in animal models.

MATERIAL AND METHODS

The study protocol was approved by both the Ethics Committee on Animal Use of Pontifical Catholic University of Rio Grande do Sul (CEUA-PUCRS, protocol #7610) and the Ethics Committee on Animal Use of University of South Santa Catarina (UNISUL, protocol #16.029.4.02.IV). All procedures were in accordance with the guidelines of the *Guide for Care and Use of Laboratory Animals* (National Institute of Health publication No. 86–23, revised 1985). The sample comprised 60 female Wistar rats (*Rattus norvegicus*), 90 days old and with a body weight between 210 g and 290 g at the beginning of the experiment. The animals were maintained in appropriate cages, 4 rats each, placed on ventilated racks at 22 ± 1º C and light/dark cycle of 12 h (lights turned on at 7:00 a.m.

and turned off at 7:00 p.m.). Nuvilab-Cr1 chow (Nuvital, Colombo, PR, Brazil) and filtered water were provided *ad libitum*.

After 7 days of adaptation to the environmental conditions of the Central Animal Facility of UNISUL, the animals were allocated into 5 groups of 12 rats each according to the treatment: (1) zoledronic acid and xenogeneic graft; (2) sodium alendronate and xenogeneic graft; (3) zoledronic acid; (4) sodium alendronate; and (5) control group: saline. All groups, including the control, were subjected to tooth extractions. Groups 3 and 4 were respectively subjected to the same treatment as groups 1 and 2 but without alveolar socket filling with xenogeneic graft after tooth extractions, whereas the control group received neither bisphosphonate nor graft. This group received saline to simulate handling during drug administration. All animals were subjected to the same environmental and stress conditions in the laboratory. During drug administration and surgical procedures, we lost 6 animals in group 2, 2 animals in group 3, and 2 animals in group 5, ending with a final sample of 50 rats.

Drug administration

Zoledronic acid at 0.3 mg/kg/week was administered intraperitoneally (IP) to groups 1 and 3 for 60 days (Maahs *et al.*, 2011; Silva *et al*., 2017; Sonis *et al.*, 2009), totaling 8 doses. Sodium alendronate at 0.1 mg/kg/week was given by the subcutaneous (SC) route to groups 2 and 4 for 60 days (Berti-Couto *et al.*, 2014; Huang *et al*., 2005), also totaling 8 doses. The control group received saline at 0.1 mL/100 g of body weight, where half of the group received it IP and the other half by the SC route.

Surgical procedures

Tooth extractions were performed after 35 days of drug therapy under anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg) administered by the IP route (Gratton *et*

al., 1995). The three upper left molars were extracted by using a Hollenback #3s carver (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) and pediatric forceps (Quinelato, Schobell Industrial Ltda, Rio Claro, SP, Brazil), which were adapted to the size of the animal's teeth. Right after the tooth extractions, groups 1 and 2 received the biomaterial. A granulated inorganic bovine graft with 10% porcine collagen comprising a 100 mg block with standard size of 0.3-0.35 cm^3 [Bio-Oss® Collagen, Geistlich Pharma, Wolhusen, Switzerland (Bio-Oss)] was divided into 3 fragments to be inserted in the post-extraction sockets by using a Diethrich forceps and Lucas curette (Quinelato, Schobell Industrial Ltda, Rio Claro, SP, Brazil). One out of the 3 fragments was inserted into each maxilla (Fig.1). Post-operative analgesia was with IP paracetamol at 110 mg/kg every 12 h for two days.

Euthanasia and macroscopic evaluation

After 60 days of treatment, animals were euthanized with IP administration of thiopental sodium (40 mg/kg) associated with lidocaine (10 mg/kg). Maxillae were dissected and macroscopically examined with adequate lighting and a 12x magnifying glass, examining for oral mucosal wounds at the tooth extraction site (Fig.2).

Specimen processing

A segment of the maxilla was cut in a coronal orientation comprising the area of the tooth extractions by using a diamond disc (dbl sided w/o mandril, 0.30 mm thick, 7/8" diameter) (G&H Orthodontics, Franklin, IN, USA), at low speed rotation. Osteotomy comprised the whole area of tooth extractions, with a safety margin of 2 mm. This segment was then cut in the middle with coronal orientation to show the area of interest as the cut surface. The specimens were immersed in 10% buffered formalin for 24 h, decalcified with 10% nitric

acid for 36 h, and then paraffin embedded. Four-micrometer-thick tissue sections were obtained and processed by hematoxylin and eosin (H&E) staining.

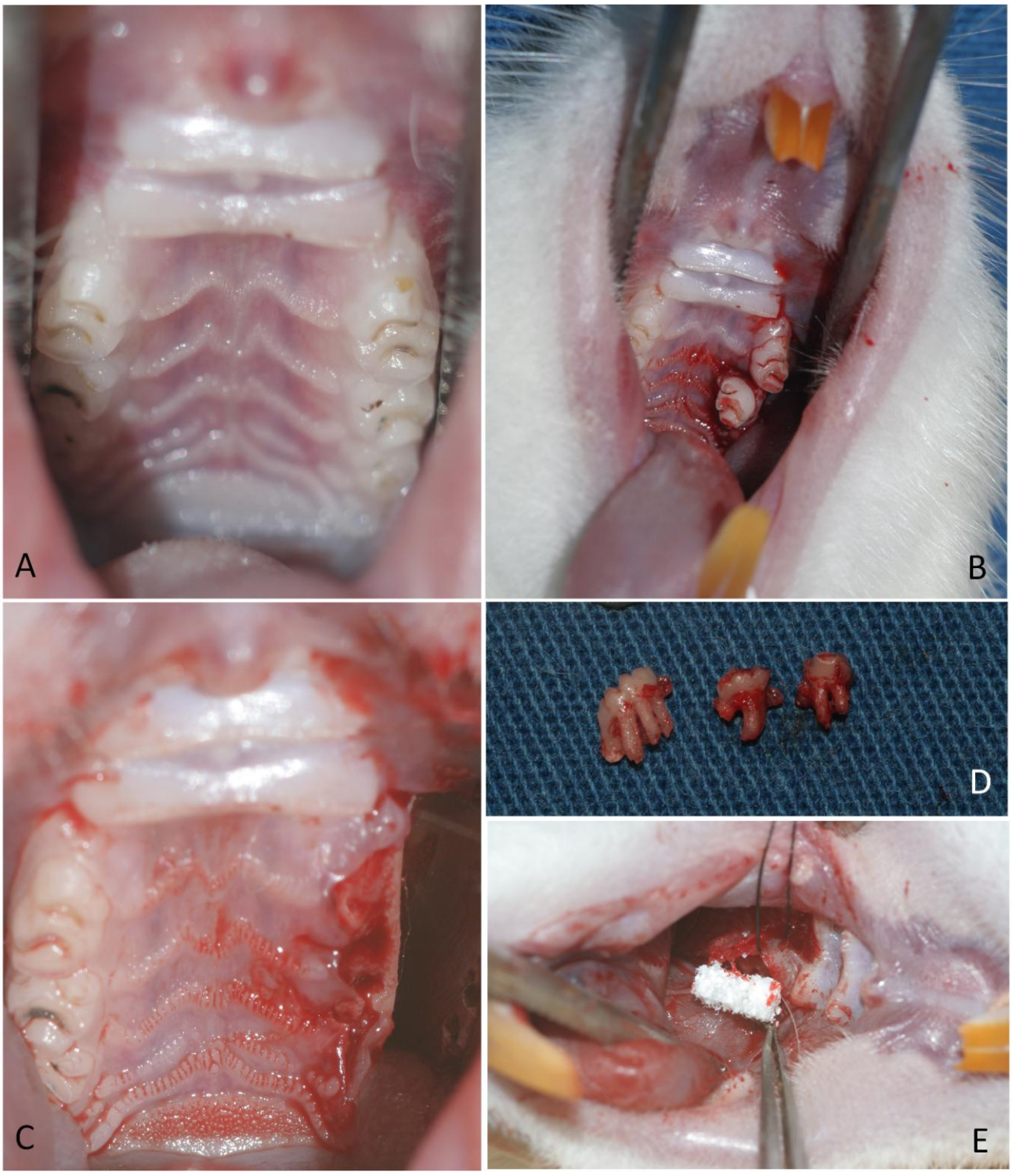

Figure 1 – Surgical procedure. (A) Clinical aspect of the maxilla, (B) upper second molar luxated, (C) clinical aspect of the maxilla immediately after tooth extraction, (D) upper molars extracted, (E) placement of Bio-Oss® Collagen in the alveolar sockets.

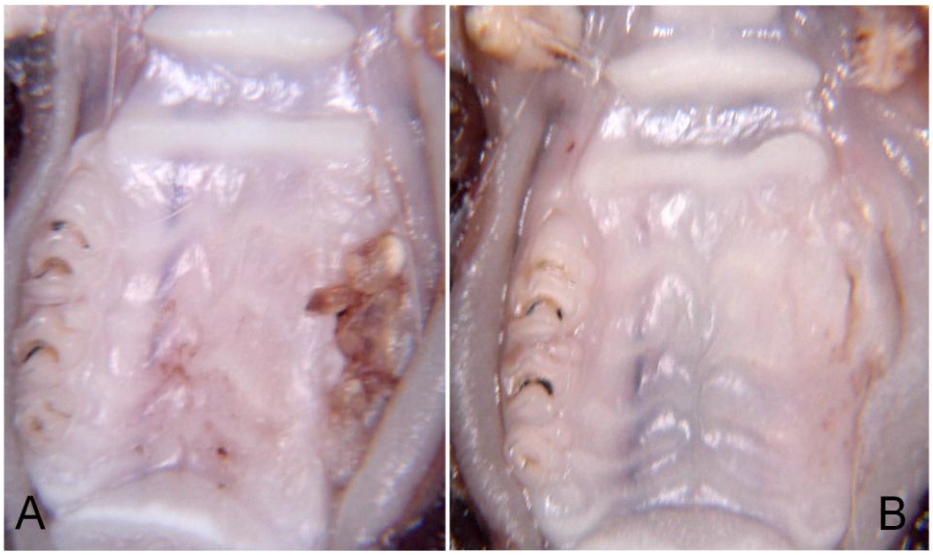

Figure 2 – Macroscopic examination. Site of tooth extractions showing (A) no wound healing and (B) wound healing.

Capture of the images and microscopic analysis

Histological images were captured by means of a digital system in an Olympus BX-43 light microscope (Olympus, Tokyo, Japan), connected to a computer with a Olympus DP-73 digital camera (Olympus). H&E images were captured with a 10 x objective and stored in TIFF format (True Image Format File) without compression. Five fields were captured for each H&E slide in a standardized manner, in a way to include the whole area of tooth extraction.

The images were analyzed in Image Pro Plus 5.1 software (Media Cybernetics, Bethesda, MD, USA), by a blinded (not knowing the group to which each image belonged) and calibrated observer. Intra-observer calibration consisted of analyzing a series of 30 images, twice, at two different moments. The results of these analyses were tested by intraclass correlation coefficient, which showed r=0.9. The following variables were analyzed: epithelial tissue, fibrous connective tissue, vital bone, non-vital bone, inflammatory infiltrate, microbial colonies, and tooth fragment. Analysis was performed overlaying a grid of 588 points over each captured image, where variables were quantified

by manual point-counting technique in the Image Pro-Plus software (Amenábar *et al*., 2006, Fig.3).

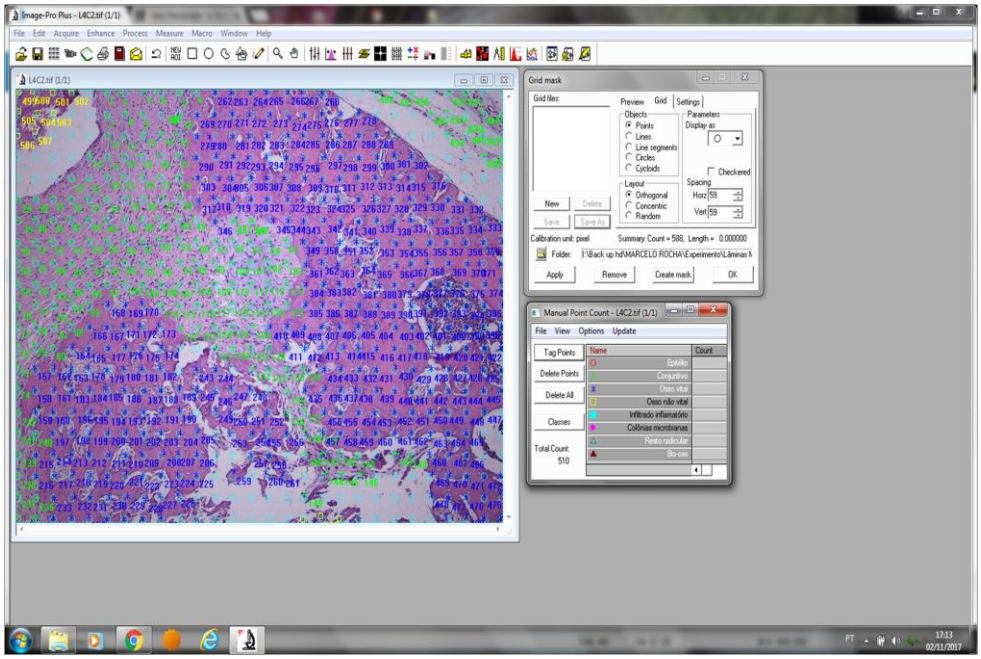

Figure 3 – Histological analysis of the hematoxylin and eosin (H&E) images in Image ProPlus software (Media Cybernetics, Bethesda, MD, USA) by using manual point-counting technique. A mask grid of 588 points was overlaid on the image and each variable was quantified

Statistical analysis

Data were analyzed with descriptive and inferential statistics. The frequency of oral mucosal wounds was compared between the groups by using the chi-square test complemented by adjusted residuals analysis. The quantitative variables were compared between the groups with ANOVA complemented by the Tukey multiple comparisons test, and the correlations were tested with Pearson's correlation coefficient. Analysis was performed in SPSS 17.0 (IBM Corp., Armonk, NY, USA) at a significance level of 5%.

RESULTS

Macroscopic analysis

The frequency of oral mucosal wounds did not significantly differ between the groups (chi-square test, adjusted residuals analysis, α=0.05, Table 1), whereas the control group showed association with absence of this variable (*P*=0.023). Oral mucosal lesions were significantly smaller in diameter in the control group than the other groups (ANOVA, Tukey test, *P*=0.003, Table 2).

Table 1 – Frequency of oral mucosal wounds

Group	Oral mucosal wounds						*P**
	Present		Absent		Total		
	n	%	n	%	n	%	
Zoledronic acid/Bio-Oss	12	24.0	-	-	12	24.0	0.023
Alendronate/ Bio-Oss	6	12.0	-	-	6	12.0	
Zoledronic acid	9	18.1	-	-	9	18.0	
Alendronate	12	24.0	-	-	12	24.0	
Control	8	16.0	**3****	**27.3****	11	22.0	
Total	47	94	3	6	50	100	

**P* value for chi-square test complemented by adjusted residuals analysis, α=0.05
***Bold printed value showed significant difference

Table 2 – Size of oral mucosal wounds

Group	Size of the lesion (greater diameter in cm)					*P**
	Mean	SD	Median	Minimum	Maximum	
Zoledronic acid/Bio-oss	0.255[A]	0.186	0.300	0.1	0.7	0.003
Alendronate/ Bio-oss	0.200 [A]	0.089	0.200	0.1	0.3	
Zoledronic acid	0.244 [A]	0.124	0.200	0.1	0.5	
Alendronate	0.204 [A]	0.099	0.200	0.0	0.4	
Control	0.057[B]	0.036	0.060	0.0	0.1	
Total	0.189	0.137	0.200	0.0	0.7	

**P* value for ANOVA complemented by Tukey multiple comparisons test, α=0.05
Means followed by different letters in the column showed significant difference; SD=standard deviation

Microscopic analysis

The amount of epithelial tissue and tooth fragment did not significantly differ between the groups. There was more fibrous connective tissue in the groups 2 (alendronate /Bio-Oss) and 5 (control) than in the groups 3 (zoledronic acid) and 4 (alendronate), whereas there was no significant difference for this variable between groups 1 (zoledronic acid/Bio-Oss), 2 (alendronate/Bio-Oss) and 5 (control). Group 4 showed greater amounts of vital bone than groups 1, 3 and 5, whereas groups 1, 2, 3 and 5 did not differ from each other. The amounts of non-vital bone were greater in groups 1 and 3, where group 1 had less non-vital bone than group 3. Groups 2, 4 and 5 did not differ from each other for this variable. Group 3 showed more inflammatory infiltrate than groups 2, 4 and 5; no differences occurred between group 1 and the other groups for this variable. There were greater amounts of microbial colonies in group 3 than in the other groups, whereas no differences were found between groups 1, 2, 4 and 5 for this variable (ANOVA, Tukey test, α=0.05, Table 3; Fig.4 and Fig.5).

Inflammatory infiltrate was negatively correlated to vital bone (r=-0.599) and positively correlated to non-vital bone (r=0.438). The same was seen for microbial colonies, with r=-0.425 and r=0.584, respectively, for vital and non-vital bone. Inflammatory infiltrate and microbial colonies were also positively correlated to each other (r= 0.602) (Pearson's coefficient, α=0.05, Table 4).

Table 3 – Microscopic analysis of tooth extraction site in the zoledronic and alendronate groups with and without Bio-Oss® and the control group

	Group															
	Zoledronic acid/Bio-oss®(1)			Alendronate/Bio-oss®(2)			Zoledronic acid (3)			Alendronate (4)			Control (5)			
Histological feature	Mean %	SD	SE	Mean %	SD	SE	Mean %	SD	SE	Mean %	SD	SE	Mean %	SD	SE	*P**
Epithelial tissue	11.49^{A}	1.32	1.70	10.51^{A}	1.17	2.14	8.78^{A}	1.07	1.60	9.30^{A}	1.12	1.45	9.20^{A}	1.19	1.61	0.755
Fibrous connective tissue	26.04^{AB}	1.4	1.80	31.55^{B}	1.31	2.39	21.66^{AC}	1.33	1.98	25.01^{AC}	1.289	1.66	32.43^{B}	1.53	2.06	**0.001**
Vital bone	44.67^{A}	3.36	4.33	47.89^{AB}	2.48	4.54	37.15^{A}	3.45	5.14	60.62^{B}	2.59	3.34	50.47^{A}	2.81	3.79	**0.002**
Non-vital bone	11.19^{A}	1.20	1.55	1.87^{B}	4.84	0.88	20.31^{C}	2.01	3.00	1.02^{B}	2.94	0.38	0.98^{B}	3.55	0.48	**0.000**
Inflammatory infiltrate	4.61^{AB}	5.81	0.75	2.66^{A}	4.60	0.840	7.34^{B}	7.67	1.14	2.51^{A}	5.09	0.66	3.40^{A}	6.85	0.92	**0.001**
Microbial colonies	0.55^{A}	0.790	0.10	0.22^{A}	0.53	0.097	1.91^{B}	2.50	0.37	0.3^{A}	0.89	0.11	0.19^{A}	0.67	0.09	**0.000**
Tooth fragment	1.44^{A}	4.48	0.58	5.29^{A}	12.43	2.27	3.04^{A}	9.26	1.38	1.48^{A}	5.45	0.70	3.31^{A}	9.23	1.24	0.179

SD=standard deviation; SE=standard error

**P*=ANOVA complemented by Tukey multiple comparisons test, α=0.05. Means followed by different letters in the row differed significantly

Table 4 –" *r*" value for Pearson's correlation coefficient

Variable	Epithelial tissue	Fibrous connective tissue	Vital bone	Non-vital bone	Inflammatory infiltrate	Microbial colonies	Tooth fragment
Epithelial tissue	1	-	-	-	-	-	-
Fibrous connective tissue	0.462**	1	-	-	-	-	-
Vital bone	-0.714**	-0.630**	1	-	-	-	-
Non-vital bone	0.138*	-0.095	-0.527**	1	-	-	-
Inflammatory infiltrate	0.210**	0.159*	-0.599**	0.438**	1	-	-
Microbial colonies	0.047	0.003	-0.425**	0.584**	0.602**	1	-
Tooth fragment	0.051	-0.008	-0.259**	-0.086	0.095	-0.040	1

*. Correlation is significant at the 0.05 level

**. Correlation is significant at the 0.01 level

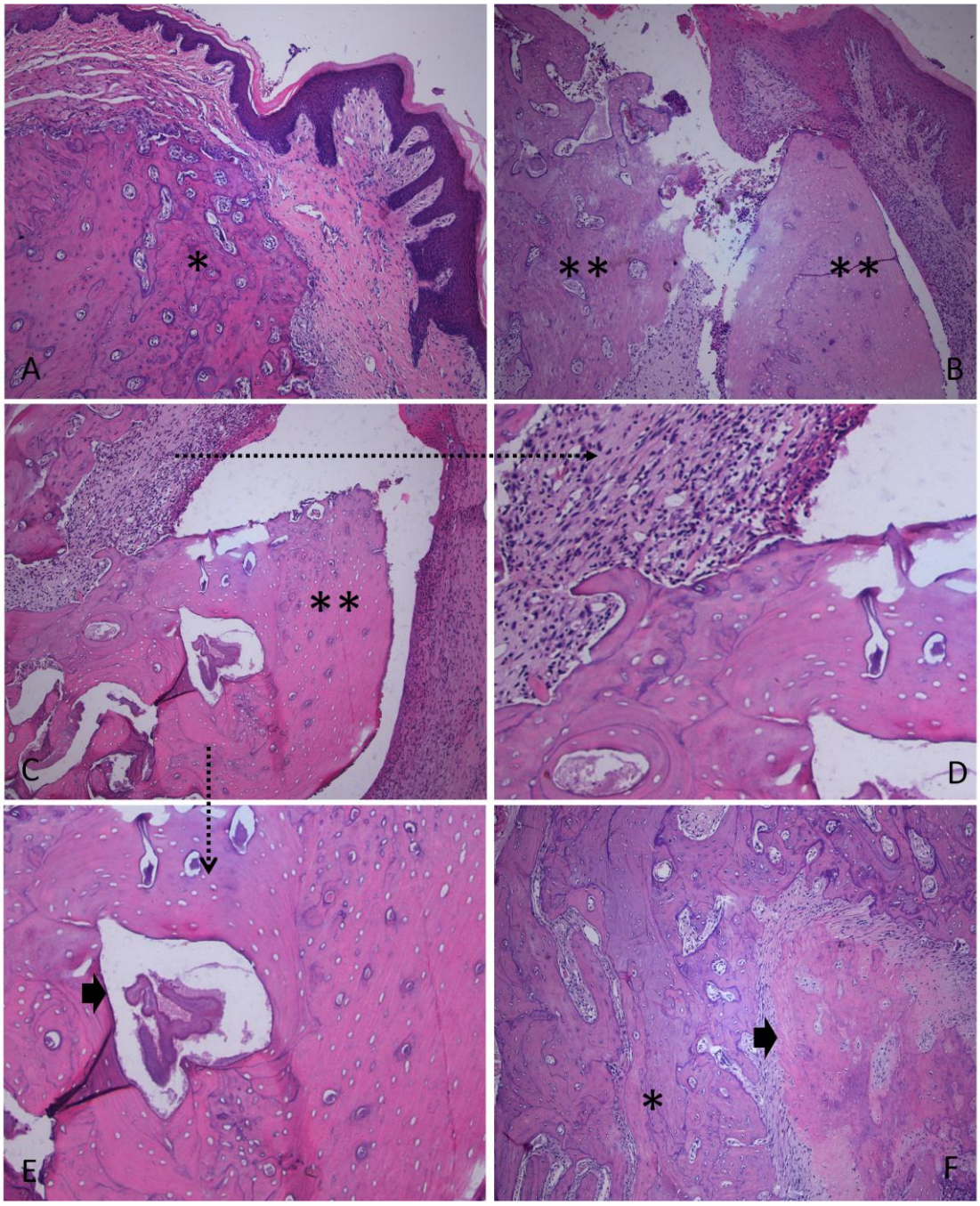

Figure 4 – Histological features with hematoxylin & eosin staining, 200x. (A) Complete healing of tooth extraction site with epithelial tissue, fibrous connective tissue and vital bone (*); (B) healing failure of the tooth extraction wound, with non-vital bone (**), microbial colonies, inflammatory infiltrate in connective tissue and loss of integrity of the epithelium; (C) non-vital bone showing microbial colonies replacing bone marrow and osteocytes (**), fibrous connective tissue with inflammatory infiltrate, which can be seen in close-up in images D and E; (F) Vital bone (*) and tooth fragment (arrow).

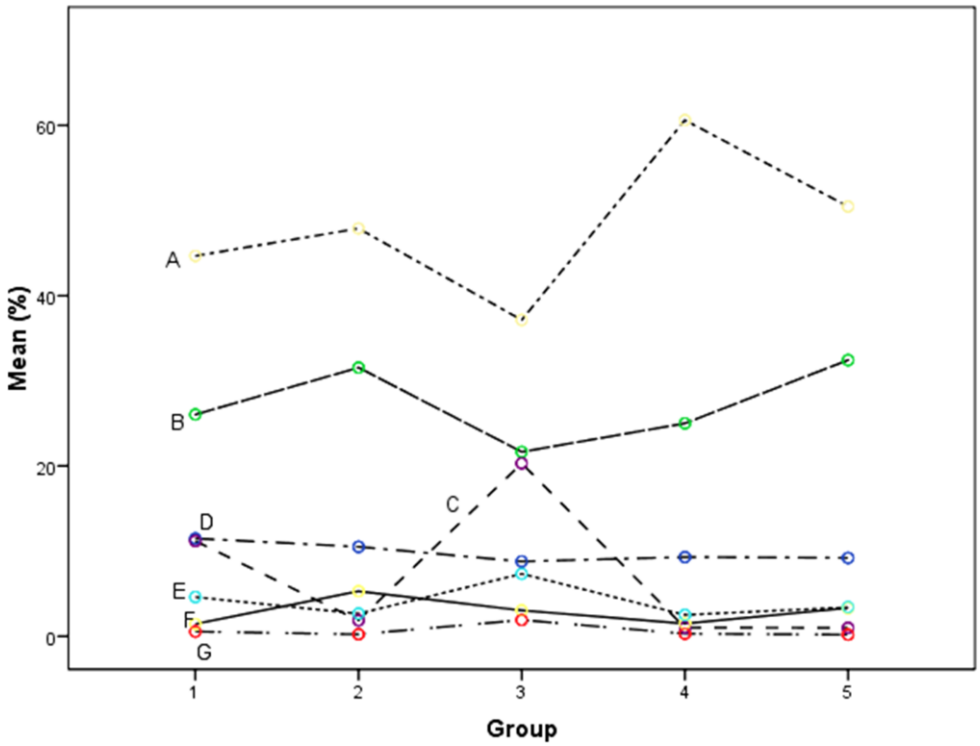

Figure 5 – Histological features in groups 1 (zoledronic acid/Bio-Oss), 2 (alendronate/Bio-Oss), 3 (zoledronic acid), 4 (alendronate) and 5 (control). A=vital bone; B=fibrous connective tissue; C=non-vital bone; D=epithelium; E=inflammatory infiltrate; F=tooth fragment; G=microbial colonies

DISCUSSION

As expected, macroscopic analysis showed significantly smaller oral mucosal wounds in the control-group. Nevertheless, this variable did not significantly differ between the test groups, suggesting Bio-Oss® Collagen (Bio-Oss) did not improve post-extraction healing in animal models under systemic bisphosphonate treatment. This finding is in agreement with the studies of Toker *et al*. (2012) and Schlickewei *et al*. (2015), who did not find improvement in bone regeneration using bone grafts associated with bisphosphonate, either topical or systemic.

Some interesting results were obtained with microscopic analysis. Both zoledronic acid groups (with and without Bio-Oss) showed greater amounts of non-vital bone. Even though, when comparing these two groups to each other, it seems that the group using Bio-

Oss had a better performance, with significantly less non-vital bone and fewer microbial colonies. Accordingly, there were greater amounts of microbial colonies in group 3 (zoledronic acid) than in the other groups. Considering the novel concept of non-exposed MRONJ (Fedele *et al*., 2010; Koth *et al*., 2016) and also the possibility that some macroscopic wounds have occurred because of the persistence of tooth fragments in the site of extraction, it seems that microscopic results would be more reliable than macroscopic ones.

According to the results, Bio-Oss did not prevent the occurrence of non-vital bone and microbial colonies; however, it seemed to reduce the prevalence of such variables. Although this biomaterial is known to be potentially osteoconductive and can induce bone neoformation (Thaller *et al*., 1994), in our study, the amount of vital bone was not greater in the groups that received Bio-Oss compared to those without Bio-Oss. Therefore, we could infer that Bio-Oss, in some way, was capable of directly inhibiting non-vital bone formation and infection. Regarding the lower prevalence of microorganisms in the Bio-Oss group when comparing the two zoledronic acid groups, this could have just been a consequence of the lower prevalence of non-vital bone, since both variables were positively correlated. On the other hand, taking into account the major role of microorganisms in the etiopathogenesis of MRONJ (Boff *et al*., 2014; Watters *et al*., 2013), we could infer that Bio-Oss has a direct effect in diminishing microbial populations and therefore leading to less non-vital bone. Considering this point of view, which component of Bio-Oss would be responsible for that? Since the formula of this product does not reveal any antimicrobial component, we could only infer that mechanical properties would exert such effect. That is, the scaffold formed by this biomaterial filling the socket (Araújo *et al*., 2010) would lead to less physical space for bacterial growth and also impair the access of microbial agents to the socket.

The alendronate groups with and without Bio-Oss, in turn, did not significantly differ from each other or from controls with regard to non-vital bone, microbial colonies and inflammatory infiltrate. These findings corroborate results of studies showing that zoledronic acid represents a higher risk for MRONJ than does alendronate (Marx *et al*., 2007; Ruggiero *et al*., 2009; Ruggiero *et al*., 2014), and therefore, if the biomaterial produced some improvement, it was not noted at all. Also, some antibacterial activity was earlier attributed to alendronate (Leon *et al*., 2006; Maahs *et al*., 2011), which could help to explain such findings. Moreover, the results for alendronate with greater amounts of vital bone in the alendronate group without Bio-Oss corroborate those for zoledronic acid, where Bio-Oss did not improve new bone formation in our sample. That is, the finding of less non-vital bone and fewer microorganisms might be related to other properties of Bio-Oss than improvement in new bone formation. Anyway, when alendronate groups with and without Bio-Oss were compared to each other, the former had significantly more fibrous connective tissue, which could indicate some repair improvement. Accordingly, the zoledronic acid/Bio-Oss group did not significantly differ from control-group for this variable, whereas the zoledronic acid and alendronate groups without Bio-Oss showed significantly less fibrous connective tissue than controls. Such findings suggest that the improvement associated with Bio-Oss in our study could be associated with fibrous connective tissue formation.

Pearson's correlation coefficient analysis reinforced our results, where fibrous connective tissue was negatively correlated to vital bone. Also there was a negative correlation of microbial colonies and inflammatory infiltrate with vital bone, but a positive correlation with non-vital bone. That is, these three variables comprised the picture of osteonecrosis in an interdependent relationship, supporting the role of microorganisms in MRONJ etiopathogenesis (Boff *et al*., 2014; Watters *et al*., 2013).

CONCLUSION

Post-extraction socket filling with Bio-Oss® Collagen did not prevent the occurrence of non-vital bone and infection, but it was capable of lowering the extent of these variables in rats treated with zoledronic acid.

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Discussão Geral

4 DISCUSSÃO GERAL

Desde 2003, os bisfosfonatos têm sido associados à ocorrência de osteonecrose maxilar (Marx, 2003; Ruggiero *et al*., 2004). Mais recentemente, outros fármacos como denosumabe e antiangiogênicos também foram associados à condição, que passou a ser denominada MRONJ (*medication-related osteonecrosis of the jaw*). A enfermidade envolve importante morbidade e dificuldades no manejo do paciente. Entre os fatores de risco à sua ocorrência, merecem destaque as intervenções cirúrgicas dos ossos maxilares, principalmente as exodontias (Kunchur *et al*., 2009; Ruggiero *et al*., 2014). A despeito das tentativas terapêuticas já relatadas, nenhum tratamento tem-se mostrado, de fato, eficaz (Fliefel *et al*., 2015; Ruggiero *et al*., 2014; Watters *et al*., 2013). Por outro lado, o uso de xenoenxerto para preenchimento alveolar pós-exodontia tornou-se procedimento frequente, com evidências clínicas de sua capacidade em melhorar as condições locais do sítio cirúrgico com vistas à reabilitação oral (Dahlin *et al*., 1988). Por vezes, a população em uso de bisfosfonatos é também aquela que necessitaria de enxertos ósseos. Foi esse o contexto que inspirou a realização da presente pesquisa, cujo objetivo foi investigar o efeito do uso de xenoenxerto em sítios de exodontia de modelo animal tratado com bisfosfonatos.

No presente estudo, os resultados da análise macroscópica não sugerem interferência do xenoenxerto na cicatrização pós-exodontia. Entretanto, a avaliação microscópica evidenciou maior prevalência de osso não-vital nos grupos tratados com ácido zoledrônico, enquanto os grupos alendronato não diferiram significativamente do controle para essa variável. À primeira vista, esse achado corrobora evidências já sedimentadas de que o risco de MRONJ é maior com o uso de ácido zoledrônico, se comparado ao alendronato (Hoff *et al*., 2008; Mercer *et al*., 2013). Entre os fatores para

tal são apontadas a maior potência e a maior dose cumulativa que caracterizam o ácido zoledrônico (Fernandes *et al*., 2005). Por outro lado, o grupo que associou ácido zoledrônico e xenoenxerto obteve melhores resultados, com menor prevalência das variáveis osso não-vital e colônias microbianas, se comparado ao grupo tratado apenas com ácido zoledrônico.

O material Bio-Oss® Collagen empregado no presente estudo é potencialmente osteocondutor e capaz de induzir neoformação óssea (Thaller *et al*., 1994), fatores que poderiam ter contribuído para os achados. Entretanto, a prevalência de osso vital não foi maior nos grupos que receberam Bio-Oss® Collagen, devendo existir outro fator associado a tal achado que não seja a neoformação óssea. Ainda, a menor prevalência de colônias microbianas pode ter sido mera consequência da menor prevalência de osso não-vital nesses grupos, já que as duas variáveis exibiram correlação positiva. Mas, se for considerado o ponto de vista de que os microrganismos têm papel fundamental no desenvolvimento da MRONJ (Boff *et al*., 2014), também é plausível inferir que, de alguma forma, o xenoenxerto seria capaz de diminuir a colonização microbiana no sítio cirúrgico. Partindo desse pressuposto, tal propriedade poderia ser consequente a algum componente desse biomaterial que tivesse atividade antimicrobiana. Entretanto, não há informações específicas disponíveis sobre outros componentes que façam parte da formulação do Bio-Oss® Collagen, além dos já citados: 90% de matriz de osso bovino mineralizada (carbapatita e hidroxiapatita) e 10% de colágeno porcino tipo I (Wang *et al*., 2016). Outra ilação plausível seria de que a propriedade mecânica do xenoenxerto de preencher o alvéolo, ocupando espaço físico ao formar um arcabouço, poderia ser um fator associado à menor colonização microbiana nesses animais. Por outro lado, nos grupos que empregaram Bio-Oss, a variável tecido conjuntivo fibroso não diferiu significativamente do grupo-controle, enquanto os grupos que não usaram o biomaterial

tiveram significativamente menos tecido conjuntivo fibroso que o grupo-controle. Talvez seja essa a explicação para os melhores resultados verificados no grupo ácido zoledrônico que empregou Bio-Oss.

A literatura evidencia resultados conflitantes entre estudos que investigam o uso associado de bisfosfonatos e enxerto ósseo (Aspenberg; Astrand, 2002; Ayranci *et al*., 2015; Bosemark *et al*., 2013; Greiner *et al*., 2008; Jakobsen *et al*., 2006; Jakobsen *et al*., 2009; Kim *et al*., 2015; Myoung *et al*., 2001; Schlickewei *et al*., 2015; Srisubut *et al*., 2007; Toker *et al*., 2012; Välimäk *et al*., 2006). Cabe ressaltar que a maior parte de tais estudos testa esses fatores em tíbia, fêmur e calvária, ossos cujo metabolismo difere daquele do osso alveolar e não sujeitos à ocorrência de MRONJ (Aspenberg; Astrand, 2002; Bosemark *et al*., 2013; Greiner *et al*., 2008; Jakobsen *et al*., 2006; Jakobsen *et al*., 2009; Kim *et al*., 2015; Myoung *et al*., 2001; Schlickewei *et al*., 2015; Toker *et al*., 2012; Välimäki *et al*., 2006). Procedimentos odontológicos invasivos como exodontias representam risco à ocorrência de MRONJ em usuários de bisfosfonato. Em se tratando do ácido zoledrônico, o risco é potencializado e, a princípio, haveria contraindicação para tais intervenções (Hoff *et al*., 2008; Ruggiero *et al*., 2014). Entretanto, em algumas situações clínicas, tratamentos conservadores podem não ser factíveis, e o procedimento cirúrgico, seja de exodontia ou outras intervenções em maxila e mandíbula torna-se inevitável. Nesses casos, de acordo com os achados do presente estudo, o Bio-Oss® Collagen, associado aos demais cuidados pré- trans- e pós-operatórios que devem ser rigorosamente respeitados nesse grupo de pacientes, poderia tornar-se uma alternativa com vistas a favorecer o prognóstico.

Os resultados do presente estudo são preliminares e apenas suscitam uma possibilidade que, a rigor, deve ser mais profundamente investigada. Novos estudos testando também outros enxertos, distintos procedimentos cirúrgicos, bem como

pesquisas *in vitro* e *in vivo* que investiguem a inter-relação entre enxerto, bisfosfonato e microrganismos poderão elucidar aspectos ainda obscuros. Nesse ínterim, a abordagem clínico-cirúrgica dos usuários de bisfosfonatos segue exigindo cautela e avaliação diferenciada, em que cada indivíduo deve ser abordado de acordo com suas especificidades, o que inclui condições clínicas do paciente, classe e tempo de uso do bisfosfonato e avaliação criteriosa de exames complementares.

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Anexos

ANEXO A

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

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ANEXO B

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

Submission Confirmation for Insights into interactions between bone grafts and bisphosphonates with focus on alveolar bone

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Hoje, 16:54

Karen Cherubini; karencherubibi66@gmail.com; Karen Cherubini

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ANEXO C

Normas para submissão de artigos ao periódico *Clinical Oral Implants Research*

http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1600-0501/homepage/ForAuthors.html

ANEXO D

S I P E S Q

Sistema de Pesquisas da PUCRS

Código SIPESQ: 7610 Porto Alegre, 21 de setembro de 2016.

Prezado(a) Pesquisador(a),

A Comissão Científica da FACULDADE DE ODONTOLOGIA da PUCRS apreciou e aprovou o Projeto de Pesquisa "EFEITO DO USO COMBINADO DE BISFOSFONATO E ENXERTO XENÓGENO NA REPARAÇÃO ÓSSEA ALVEOLAR: ESTUDO HISTOLÓGICO E NÍVEIS SÉRICOS DE CTX EM RATOS". Este projeto necessita da apreciação da Comissão de Ética no Uso de Animais (CEUA). Toda a documentação anexa deve ser idêntica à documentação enviada ao CEUA, juntamente com o Documento Unificado gerado pelo SIPESQ.

Atenciosamente,

Comissão Científica da FACULDADE DE ODONTOLOGIA

ANEXO E

S I P E S Q

Sistema de Pesquisas da PUCRS

Código SIPESQ: 7610 Porto Alegre,7 de dezembro de 2016

Prezado(a) Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou o Projeto de Pesquisa "EFEITO DO USO COMBINADO DE BISFOSFONATO E ENXERTO XENÓGENO NA REPARAÇÃO ÓSSEA ALVEOLAR: ESTUDO HISTOLÓGICO E NÍVEIS SÉRICOS DE CTX EM RATOS" coordenado por KAREN CHERUBINI.

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está autorizada a partir da presente data, conforme recomendações abaixo:

O projeto foi avaliado e aprovado pela CEUA/PUCRS. Entretanto, sua execução está condicionada à prévia aprovação pela CEUA da Universidade do Sul de Santa Catarina (UNISUL).

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Duração do Projeto: 07/12/2016 - 07/09/2017

Nº de Animais	Espécie
60	Rattus norvegicus
Total de Animais: 60	

ANEXO F

UNIVERSIDADE DO SUL DE SANTA CATARINA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS – CEUA/UNISUL

Palhoça, 13 de dezembro de 2016
Registro na CEUA (código): 16.029.4.02.IV

Ao Pesquisador/Professor(a): Karen Cherubini

Prezado(a),

Viemos por meio deste, certificar que a proposta de estudo e/ou projeto de pesquisa intitulada "Efeito do uso combinado de bisfosfanato e enxerto xenógeno na reparação óssea alveolar: estudo histológico e niveis séricos de CTX em ratos", registrada com o nº16.029.4.02.IV.IV, sob a responsabilidade de Karen Cherubini - que envolve a manutenção ou utilização de modelos animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei Federal nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi **aprovado** pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) desta Instituição, em reunião de 13 de dezembro de 2016.

A CEUA/UNISUL tem por finalidade cumprir e fazer cumprir, no âmbito da UNISUL e nos limites de suas atribuições, os dispostos na legislação Federal aplicável à criação, manutenção e a utilização de animais em atividades de ensino e de pesquisa, realizadas pelos corpos docente, discente e técnico-administrativo da UNISUL e pesquisadores de outras instituições, caracterizando-se a sua atuação como educativa, consultiva, de assessoria e fiscalização nas questões relativas à matéria, sob os aspectos: I - Ético; II - Legal: enquadramento na legislação vigente.

Gostaríamos de salientar que, embora aprovado, qualquer alteração dos procedimentos e metodologias que houver durante a realização do projeto em questão, deverá ser informada imediatamente à Comissão.

Atenciosamente,

Prof. Sandro Melim Sgrott
Coordenador da Comissão

Campus Florianópolis - Unidade Pedra Branca - Avenida Pedra Branca, 25 - Cidade Universitária Pedra Branca - CEP 88137-270 - Palhoça - SC - Fone 55-48-3279-1036 - ceua@unisul.br