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BRUNA RATZKOWSKI

EFEITO DO INIBIDOR DE TIROSINA-QUINASE SUNITINIBE SOBRE A CICATRIZAÇÃO ALVEOLAR PÓS-EXODONTIA: ESTUDO HISTOMORFOMÉTRICO

Porto Alegre
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ESCOLA DE CIÊNCIAS DA SAÚDE
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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

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Gaste mais horas realizando que sonhando, fazendo que planejando, vivendo que esperando, porque, embora quem quase morre esteja vivo, quem quase vive já morreu.

Sarah Westphal (1983-)

Dedicatória

Especialmente à minha família, por estar ao meu lado

durante toda essa jornada.

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Resumo

RESUMO

A osteonecrose dos maxilares associada a medicamentos (MRONJ) é um importante efeito adverso que tem acometido pacientes em tratamento com diferentes drogas anticâncer, incluindo fármacos antiangiogênicos. O presente estudo teve por objetivo investigar o efeito do antiangiogênico inibidor de tirosina-quinase sunitinibe sobre o reparo ósseo alveolar em sítios de exodontias. Ratos Wistar (n=52) foram distribuídos em quatro grupos de acordo com o tratamento administrado: (1) sunitinibe (n=13); (2) sunitinibe/ácido zoledrônico (n=13); (3) ácido zoledrônico (n=13); (4) grupo-controle (n=13). Os animais foram submetidos a exodontias dos molares superiores do lado direito, e as maxilas dissecadas e macro e microscopicamente analisadas. Na avaliação macroscópica, o grupo ácido zoledrônico exibiu prevalência de lesão da mucosa oral significativamente maior que a dos demais grupos. O tamanho das lesões, entretanto, não diferiu significativamente entre os grupos. O grupo sunitinibe/ácido zoledrônico teve significativamente menos tecido epitelial que os grupos ácido zoledrônico e controle, mas não exibiu diferença significativa em comparação ao grupo sunitinibe. Os demais grupos não exibiram diferença significativa para essa variável. Os grupos sunitinibe/ácido zoledrônico e ácido zoledrônico não diferiram entre si, mas tiveram quantidade de tecido conjuntivo significativamente menor e de osso não-vital e colônias microbianas significativamente maior do que os grupos sunitinibe e controle, enquanto esses dois últimos grupos não diferiram significativamente entre si na avaliação dessas variáveis. Osso vital, infiltrado inflamatório e fragmento dentário não diferiram significativamente entre os grupos.

Conclusão: O antiangiogênico sunitinibe, quando administrado de forma isolada, não está associado à ocorrência de osso não-vital, enquanto a combinação sunitinibe/ácido zoledrônico ou o uso do ácido zoledrônico de forma isolada exibem associação com a ocorrência de osso não-vital.

Palavras-chave: drogas antiangiogênicas; sunitinibe; MRONJ; angiogênese; ácido zoledrônico

Summary

SUMMARY

Medication-related osteonecrosis of the jaw (MRONJ) is an important side effect that has been affecting patients undergoing different anticancer therapies, including antiangiogenic drugs. The aim of this study was to investigate the effect of tyrosine kinase inhibitor sunitinib on tissue repair at tooth extraction sites. Fifty-two Wistar rats were allocated into four groups according to the treatment received: (1) sunitinib (n=13); (2) sunitinib/zoledronic acid (n=13); (3) zoledronic acid (n=13); (4) control group (n=13). The animals were subjected to extractions of the right upper molars, and maxillae were dissected and macro- and microscopically analyzed. On macroscopic evaluation, the zoledronic acid group showed a significantly higher prevalence of oral mucosal lesion than the other groups; however, the size of this lesion did not significantly differ between groups. The sunitinib/zoledronic acid group had significantly less epithelium than the zoledronic acid and control group, but showed no significant difference compared to the sunitinib group. The other groups did not show any significant difference regarding this variable. The sunitinib/zoledronic acid and zoledronic acid groups did not differ from each other, but had significantly less connective tissue and more non-vital bone and microbial colonies than the sunitinib and control groups, whereas these latter two groups did not significantly differ from each other. Vital bone, inflammatory infiltrate and tooth fragment did not significantly differ between the groups.

Conclusion: Sunitinib alone is not associated with non-vital bone, whereas the sunitinib/zoledronic acid combination and zoledronic acid alone are.

Key words: antiangiogenic drugs; sunitinib; jaw osteonecrosis; angiogenesis; zoledronic acid

Sumário

SUMÁRIO

Introdução

1 INTRODUÇÃO

O termo *osteonecrose* denomina o resultado comum de uma série de condições que levam à morte do tecido ósseo, não se referindo a uma entidade clínica específica. É uma condição frequente, que pode tanto passar despercebida ao exame clínico, quanto determinar o colapso da estrutura óssea, levando a dor articular, destruição e perda de função do tecido ósseo (Fondi; Franchi, 2007). Ao acometer os ossos maxilares, com base em sua etiologia, a condição é classificada em osteorradionecrose, quando acomete pacientes que foram submetidos a radioterapia de cabeça e pescoço; e osteonecrose medicamentosa, quando associada ao uso de fármacos por parte de pacientes que não sofreram radioterapia prévia (Marx; Tursun, 2012).

Desde 2003, um número crescente de casos de osteonecrose maxilar em pacientes usuários de bisfosfonatos tem sido relatado na literatura, sendo a enfermidade, inicialmente, denominada *osteonecrose maxilar associada a bisfosfonatos* (*bisphosphonate-related osteonecrosis of the jaw,* BRONJ) (Marx, 2003; Ruggiero *et al*., 2007). Anos mais tarde, o fármaco antirreabsortivo denosumabe também se mostrou capaz de determinar o desenvolvimento da doença e, posteriormente, casos associados a antiangiogênicos passaram a ser relatados. Com isso, em 2014, foi proposta a nomenclatura *osteonecrose maxilar associada a medicamentos* (*medication-related osteonecrosis of the jaw*, MRONJ), estendendo sua associação aos três grupos de drogas: bisfosfonatos, denosumabe e antiangiogênicos (Ruggiero *et al*., 2014). A MRONJ é definida como área de osso exposto ou sondável na região maxilofacial, que não cicatriza no prazo de oito semanas, associada a sinais e sintomas como dor, edema, parestesia, infecção, ulceração dos tecidos moles e alterações radiográficas em pacientes que tenham sido tratados com os referidos fármacos e não tenham histórico de radioterapia de cabeça e pescoço (Weber *et al*., 2016). Fatores locais desempenham papel significativo para o

desenvolvimento da MRONJ, sendo a cirurgia dentoalveolar um importante fator de risco, com 52 a 61% dos pacientes relatando a exodontia como evento prévio ao desenvolvimento da lesão (Fehm *et al*., 2009; Vahtsevanos *et al*., 2009).

Considerando o papel crucial da angiogênese no desenvolvimento e na progressão de tumores malignos (Baka *et al.*, 2006), diversos medicamentos antiangiogênicos foram lançados no mercado e vêm sendo empregados como terapia anticâncer. De acordo com o mecanismo de ação, essas drogas são classificadas como anticorpos anti-VEGF (bevacizumabe, aflibercept, pegaptanibe, ranibizumabe); imunomoduladores (talidomida, lenalidomida) e inibidores de quinase (sunitinibe, sorafenibe, sirolimo, temsirolimo, everolimo, pazopanibe, vatalanibe, vandetanibe, regorafenibe, lenvatinibe, axitinibe) (Abel Mahedi Mohamed *et al*., 2018; Christodoulou *et al.,* 2009; Ramírez *et al.,* 2015). O sunitinibe, introduzido no mercado em 2006 pela empresa farmacêutica Pfizer (Pfizer, New York, NY, USA), é um antiangiogênico inibidor de tirosina-quinase, que tem sido associado ao desenvolvimento de MRONJ (Fleissig *et al.*, 2012). Também há relatos de que o risco dessa enfermidade aumenta significativamente em pacientes que usam sunitinibe e bisfosfonatos simultaneamente (Ramírez *et al.,* 2015).

Vários relatos de casos que associam o uso de antiangiogênicos à MRONJ são de pacientes que usaram bisfosfonatos ou denosumabe previamente ou concomitantemente ao uso da droga antiangiogênica (Beuselinck *et al*., 2012). A dificuldade de manejo e tratamento da MRONJ associada a bisfosfonatos resulta, em parte, da meia-vida extremamente prolongada desses fármacos, que pode ser superior a dez anos, o que leva a doses cumulativas elevadas no tecido ósseo e efeito residual persistente (Marx, 2014; Ruggiero *et al*., 2014). Os demais fármacos, mesmo que efetivamente associados à osteonecrose, geram algumas ressalvas e observações para suas especificidades. O

denosumabe e os antiangiogênicos não são incorporados pelo tecido ósseo e têm meia-vida variando entre 2,5 e 32 dias (Bodnar, 2014; Narayanan, 2013), o que reduz, potencialmente, a gravidade dos quadros de MRONJ e lhes confere melhor prognóstico. Por outro lado, o persistente efeito dos bisfosfonatos, além de comprometer a resposta ao tratamento dos quadros de MRONJ, também gera a suspeita de que alguns casos associados a antiangiogênicos possam ter sido, de fato, causados pelo uso de bisfosfonatos.

A etiopatogênese específica da MRONJ continua indefinida (Rosella *et al*., 2016), e os relatos de casos associados a antiangiogênicos ainda deixam alguma margem de dúvida, uma vez que esses fármacos são, frequentemente, administrados de forma conjunta ou subsequente ao uso de bisfosfosfonatos ou denosumabe (Ruggiero, 2015; Sivolella *et al*., 2013). Em função disso, o presente estudo teve por objetivo investigar a associação entre MRONJ e fármacos antiangiogênicos. O trabalho foi estruturado sob a forma de dois artigos: o primeiro artigo apresenta uma revisão da literatura sobre o tema em questão, enquanto o segundo artigo relata o experimento *in vivo* em que foi conduzida análise histomorfométrica do osso alveolar de ratos submetidos a exodontias durante terapia com sunitinibe.

2 ARTIGO 1

O artigo a seguir intitula-se **An overview of relationship between antiangiogenics and medication-related osteonecrosis of the jaw** e foi formatado de acordo com as normas do periódico *Gerodontology* (Anexo A).

An overview of relationship between antiangiogenics and medication-related osteonecrosis of the jaw

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Review Article

ABSTRACT

With the constant emergence of novel therapies and the longer life expectancy of cancer patients, adverse effects of anticancer treatment have drawn attention and demanded special care. Medication-related osteonecrosis of the jaw (MRONJ) is a potentially debilitating adverse effect, which has been reported in cancer patients undergoing therapy with antiresorptive and antiangiogenic drugs. We present here a literature review focusing on the relationship between this condition and antiangiogenics. The number of reported cases of MRONJ associated with these drugs has increased. It seems evident that when antiangiogenics are used in combination with bisphosphonates or denosumab, MRONJ occurrence is very likely. Anyway, some doubts remain concerning the capacity of antiangiogenics *per se,* with no other drug association, to determine MRONJ. Further studies using animal models and capable of isolating variables would be helpful in clarifying the relationship of antiangiogenics, as a single drug therapy, with the development of MRONJ.

INTRODUCTION

Osteonecrosis of the jaw is a potentially debilitating adverse effect, which has been reported in cancer patients subjected to different drug therapies.[1] In 2003, Marx[2] reported 36 cases of this disease related to the bisphosphonates zoledronate and pamidronate, which were described as painful bone exposures in the mandible, maxilla or both, non-responsive to either drugs or surgical treatment. Since then, numerous similar cases of jaw osteonecrosis associated with bisphosphonates have been reported.[3,4] At first, the condition was called bisphosphonate-related osteonecrosis of the jaw (BRONJ). However, in 2014, the term *medication-related osteonecrosis of the jaw* (MRONJ) was recommended by the American Association of Oral and Maxillofacial Surgeons (AAOMS), extending the cause of the disease to other drugs.[5]

MRONJ diagnosis should meet certain criteria: (1) current or previous use of bisphosphonate or other antiresorptive or antiangiogenic drug; (2) exposed or probing bone through intra- or extraoral fistula in the oral and maxillofacial region persisting for more than eight weeks; (3) absence of head and neck radiation therapy; and (4) absence of tumor/metastasis in the involved region.[5,6] The condition can be associated with pain, swelling, paresthesia, infection, soft tissue ulceration and radiographic alterations.[7] It is estimated that 65% of cases are located in the mandible, 28.4% in the maxilla and 6.5% in both maxilla and mandible.[8-10] Local factors play a significant role in the development of osteonecrosis, with dentoalveolar surgery being a major risk factor. Previous tooth extraction was reported in 52 to 61% of cases.[11,12] MRONJ etiopathogenesis is still controversial, and therefore, prevention becomes the focus of management.

Currently, there are three groups of drugs related to lesion development: bisphosphonates, denosumab and antiangiogenics.[8,13] We present here a literature review focusing on antiangiogenic anticancer drugs and their relationship with jaw osteonecrosis.

Angiogenesis and antiangiogenics

Angiogenesis is defined as the process of new blood vessel formation, where vascular endothelial growth factor family (VEGF) plays essential roles, in either physiological or pathological conditions. This family comprises five different members: VEGF-A (also known as VEGF), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PLGF), where VEGF is the one most involved in blood vessel formation.[14] Angiogenesis can be induced in an uncontrolled manner in many pathological conditions such as cancer and ischemic, inflammatory, infectious and immunological disturbances.[15] It is a complex biological process that supports the growth and metastatic potential of many tumors. Accordingly, tumor biology has become the basis for cancer therapy, and understanding how new blood vessels are formed during tumor growth has led to new therapies targeting such process.[16]

Based on their ability to block tumor growth by interfering with neoangiogenesis, antiangiogenics have been launched in the pharmaceutical market as antitumor agents.[16] The main action is inhibition of VEGF, which is expressed in most malignant tumors.[17] Some of these agents inhibit endothelial cells directly; others inhibit the angiogenesis signaling cascade or block the ability of endothelial cells to break down the extracellular matrix. The agents that directly target VEGF neutralize the protein, thereby blocking tumor expression of the angiogenic factor on endothelial cells.[18,19] According to their mechanism of action, antiangiogenics have been classified as anti-VEGF antibodies (bevacizumab, aflibercept, pegaptanib, ranibizumab), immunomodulators (thalidomide, lenalidomide) and kinase inhibitors (axitinib, everolimus, lenvatinib, pazopanib, regorafenib, sirolimus, sunitinib, sorafenib, temsirolimus, vandetanib, vatalanib).[5]

Receptor tyrosine kinases (RTKs) are proteins involved in various important signaling pathways and are directly related to proliferation, differentiation and cell migration processes. The family comprises receptors for growth factors and are involved in the development and progression of many types of malignant tumors. Some of these are VEGF receptors (VEGFRs), platelet-derived growth factor receptors (PDGFRs), fibroblast growth factor receptors (FGFRs), epidermal growth factor receptor (EGFR), RAF (rapidly accelerated fibrosarcoma) kinases and c-Kit (a receptor of the pluripotent cell growth factor, stem cell factor). Tyrosine kinase inhibitors (TKIs), in turn, are small molecules capable of interacting with RTKs inhibiting their activation and consequently various pro-angiogenic signaling pathways.[20,21]

Therapy with antiangiogenics involves high toxicity and adverse effects. The systemic disturbance caused in the signaling pathways that control angiogenic activity is associated with hemorrhagic complications and gastrointestinal perforations.[22] Also, these drugs have recently been related to MRONJ.[23-25]

Anti-VEGF antibodies

Bevacizumab

Bevacizumab was the first antiangiogenic drug approved for clinical use.[26] It is a humanized monoclonal antibody that recognizes and blocks VEGF, then inhibiting its interaction with VEGFR-1 and VEGFR-2 receptors located on the surface of endothelial cells.[27,28] As VEGF activity is neutralized, tumor vascularization is reduced and tumor growth inhibited.[14] Bevacizumab has been prescribed to treat some malignant tumors, such as metastatic colorectal cancer (CCRm), glioblastoma, lung cancer and neoplastic neurovascular diseases. Besides, it has been widely applied in ophthalmology to treat retinal lesions and neovascular diseases.[29]

With the VEGF signaling pathway as a target, bevacizumab would compromise the integrity of microvessels in the jaw and lead to subclinical compromise of the osteon.[30,31] In a meta-analysis where 3,560 patients received only bevacizumab, MRONJ prevalence was 0.2%, but it increased up to 0.9% when combined with bisphosphonates.[32] According to other reports, the risk among patients receiving combined therapy of bevacizumab and bisphosphonates increases to 2%.[23,33]

Aflibercept

Aflibercept is a recombinant human fusion protein that blocks the VEGF pathway through high-affinity binding to the VEGF-A and VEGF-B isoforms and the placental growth factor-1 and -2 isoforms, and it is indicated for the treatment of metastatic colorectal cancer.[34] Ponzetti *et al*.[35] reported a case of a 64-year-old female patient that was diagnosed with adenocarcinoma of the transverse colon with unresectable bilateral liver metastases. This patient had a history of unresolved chronic periodontitis and developed MRONJ after having received eleven cycles (six months) of chemotherapy with aflibercept.[35]

Kinase inhibitors

Sunitinib

Sunitinib was launched on the market in 2006 by Pfizer Pharmaceuticals (New York, NY, USA). It is a TKI that blocks some RTKs, including VEGFR and platelet-derived growth factor receptor (PDGFR). RTK inhibition prevents the cancer cell processes of proliferation, migration, differentiation, neoangiogenesis and invasion, becoming an important tool in the treatment of malignant tumors.[36,37] This drug is indicated for the treatment of gastrointestinal stromal tumor, metastatic renal cell cancer and pancreatic neuroendocrine tumor.[36]

In cellular and biochemical assays, sunitinib is a strong inhibitor of VEGFR-1, VEGFR-2, fetal liver tyrosine kinase 3 (FLT3), KIT (stem-cell factor [SCF] receptor), PDGFRα, and PDGFRβ.[38,39] *In vitro*, it was shown to induce apoptosis of umbilical vein endothelial cells.[39] Adverse effects such as diarrhea, mucositis, taste changes, skin lesions and hypertension were reported by patients undergoing sunitinib therapy. Most adverse effects are reversible and osteonecrosis has been reported.[23-25]

The incidence of complications increases when antiangiogenics are combined with chemotherapy.[40] Likewise, MRONJ risk increases in patients using sunitinib and bisphosphonate concomitantly, with a prevalence ranging from 0.9 to 2.4%.[41] This probably occurs because VEGFR inactivation impairs tissue healing, and sunitinib-related mucositis can contribute to MRONJ development.[42] The etiopathogenesis of bisphosphonate-related osteonecrosis has not yet been clarified. Nonetheless, in the case of antiangiogenics, it seems reasonable to associate the lesion with the interference of these drugs with major factors related to jaw bone remodeling and wound repair (VEGF and PDGF). The inhibition of these important factors for tissue healing could lead to osteonecrosis.[24] Considering the combined toxic effect of antiangiogenics in patients who have used bisphosphonates, it

seems that osteonecrosis results from impairment of both angiogenesis and bone remodeling.[33]

Fleissig *et al*.[24] reported a case of a 58-year-old patient on sunitinib to treat renal cancer, who had a history of neither bisphosphonate nor corticosteroid use and who developed mandibular osteonecrosis after a tooth extraction. Another study evaluating patients undergoing renal cancer treatment in nine Italian centers reported that 44 patients developed osteonecrosis, with zoledronic acid being the most frequently used nitrogen-containing bisphosphonate (93%), whereas the most commonly used antiangiogenic was sunitinib (80%). The major precipitating event was dental/periodontal infection (34%), followed by tooth extraction (30%).[25]

Everolimus

Everolimus is a drug that inhibits mTOR (mammalian target of rapamycin) activity, which is involved in cell growth and metabolism.[43] Yamamoto *et al.*[43] reported a case of a 67-year-old patient on everolimus for the treatment of breast cancer. The patient developed mandibular osteonecrosis despite not having any history of bisphosphonate use and no relevant past dental history, such as tooth extraction.

Sorafenib

Sorafenib is an oral multikinase inhibitor of VEGFR and PDGFR used for treatment of advanced hepatocellular carcinoma.[44] Garuti *et al*.[45] reported a case of a 74-year-old patient who was treated with sorafenib, did not receive bisphosphonates or other antiangiogenic drugs, and developed mandibular osteonecrosis at a site previously subjected to tooth extraction.

A retrospective study of patients undergoing bisphosphonate therapy combined or not with antiangiogenics found that out of 116 patients, only 25 used antiangiogenics. Among them, 22 received bevacizumab, 2 sunitinib and one sorafenib. Four (16%) out of

these 25 patients developed MRONJ when receiving antiangiogenic and bisphosphonate concomitantly. On the other hand, one (1.1%) out of 91 patients receiving only bisphosphonate developed MRONJ.[23] Cases of jaw osteonecrosis after tooth extraction in patients treated with sunitinib, imatinib and pazopanib were reported in a retrospective study.[46]

Immunomodulators

Therapies such as thalidomide and lenalidomide have provided benefits in the treatment of multiple myeloma (MM), but they have been associated with adverse events such as MRONJ.[47] Cetiner *et al*.[48] conducted a study in the Hematology Department of Gazi University Hospital with 32 patients (19 men, 13 women) who had been treated for multiple myeloma. Fifty percent (16/32) of patients had received post-transplant thalidomide maintenance and 31% (10/32) had received thalidomide with dexamethasone during induction treatment. In the total sample of 32 patients, MRONJ was detected clinically and radiographically in five patients,[48] four of them receiving thalidomide. Anyway, there was no significant difference in MRONJ frequency between patients treated or not with thalidomide.

FINAL CONSIDERATIONS

The number of case reports of MRONJ associated with antiangiogenic drugs has increased lately (Table 1). Considering that VEGF is crucial for tissue healing and that these drugs inhibit VEGF, some authors believe that antiangiogenics would impair tissue healing and would be associated with MRONJ.[23-25] Moreover, VEGF plays an important role in the regulation of osteoclast function, differentiation, and survival,[56,57] which would contribute to MRONJ onset.

Antiangiogenics are frequently prescribed in combination with some antiresorptive drugs such as bisphosphonates[58] and denosumab.[59] The capacity of these combined therapies in increasing MRONJ risk has been reported.[33,46] On the other hand, it is known that bisphosphonates and denosumab can *per se* cause MRONJ, whereas for antiangiogenics, such possibility still needs to be investigated. New research using animal models and capable of controlling biases and isolating variables are needed to clearly confirm the ability of antiangiogenics to contribute to the development of MRONJ when used as a single drug therapy.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1 – Literature reports on antiangiogenic-related osteonecrosis of the jaw

Type of study	Disease/sample size (n)	Drug/dose	Time of use (months)	Risk factor/comorbidities	Other drugs	Site	Reference
Case report	Breast cancer (n=1)	Bevacizumab 15 mg/kg every 3 weeks	6	None	Doxorubicin, cyclophosphamide, albumin-bound nanoparticle-paclitaxel, capecitabine	Mandible	Estilo *et al*. (2008)[30]
Case report	Breast cancer (n=1)	Bevacizumab Dose: NS	1	Tooth extraction	Liposomal doxorubicin	Maxilla	Greuter *et al*. (2008)[49]
Case report	Lung adenocarcinoma (n=1)	Bevacizumab 7.5 mg/kg,8gr.	0.5	Tooth extraction	Cisplatin and gemcitabine	Mandible	Serra *et al*. (2009)[50]
Case report	Renal cell carcinoma (n=1)	Sunitinib Dose: NS	14	Tooth extraction	Zoledronic acid	Mandible	Ayllon *et al.* (2009)[33]
Case report	Renal cell carcinoma (n=1)	Sunitinib 50 mg/day on a 4-week and 2-week-off schedule	5	Tooth extraction	Interferon, vinblastine, sorafenib	Mandible	Koch *et al*. (2011)[51]
Case report	Renal cell carcinoma (n=1)	Sunitinib 50mg/day on a 4 week and 2-week-off schedule	12	Tooth extraction	NS	Mandible	Fleissig *et al*. (2012) [24]
Case report	Colon carcinoma (n=1)	Bevacizumab 5 mg/kg every 2 weeks	3	None	Fluorouracil, leucovorin, oxaliplatin	Mandible	Dişel *et al*. (2012)[52]
Case report	Retinal vascular thrombosis (n=1)	Bevacizumab 2.5 mg/month (intravitreal)	24	None	NS	Mandible	Hopp *et al*. (2012) [29]
Case report	Pancreatic carcinoma (n=1)	Bevacizumab Dose: NS	4	Oral ulcer	Gemcitabine, erlotinib, folinic acid, 5-fluorouracil, oxaliplatin paclitaxel and sorafenib	Mandible	Pakosch *et al*. (2013)[53]
Retrospective	Renal cell carcinoma (n=6)	Sunitinib 50 mg/day on a 4-week and 2-week-off schedule	NS	Spontaneous MRONJ (n=2), dental procedure (n=2), denture-induced trauma (n=2)	Zoledronic acid	NS	Smidt-Hansen *et al.* (2013)[54]

Case report	Breast cancer (n=1)	Bevacizumab 15 mg/kg IV infusion	4	Tooth extraction	Carboplatin, docetaxel and cortisone, cyclophosphamide, epirubicin, 5-fluorouracil	Mandible	Nikitakis *et al.* (2016)[55]
Retrospective	Renal cell carcinoma (n=44)	Sunitinib, everolimus, bevacizumab, sorafenib Dose: NS	1 to 26	Periodontal infection (34%), tooth extraction (30%), ill-fitting dentures (9%), other oral surgical procedures (4.5%)	Zoledronic acid, pamidronate, ibandronate	Mandible (52%) Maxilla (36%)	Fusco *et al.* (2015)[25]
Case report	Hepatocellular carcinoma (n=1)	Sorafenib 400 mg/day	3	Tooth extraction	Furosemide, potassium canrenoate, bisoprolol, allopurinol, tamsulosin, hydroxychloroquine, vitamin D and sertraline	Mandible	Garuti *et al*. (2016)[45]
Case report	Adenocarcinoma of colon (n=1)	Aflibercept	6	Chronic periodontitis	Cetuximab plus capecitabine/oxaliplatin	Mandible	Ponzetti *et al.* (2016)[35]
Case report	Breast cancer (n=1)	Everolimus 10 mg/day	2	None	Exemestane, tamoxifen and fulvestrant	Mandible	Yamamoto *et al.* (2017)[43]
Retrospective	ALL (n=1) CLL (n=1) GM (n=1) MM (n=1) NSCLC (n=2) RCC (n=1)	Afatinib, bevacizumab, cetuximab, dasatinib, everolimus, erlotinib, imatinib, nilotinib, pazopanib, sunitinib, thalidomide Dose: NS	0.5 to 48	Tooth extraction	Alendronate, zolendronic acid pamidronate, denosumab, carboplatin, cisplatin, vinorelbine, alimeta, temozolomide, irinotecan, cyclophosphamide, alkeran, oxaliplatin, cytarabine, vincristine, blinatumomab, capecitabine, trastuzumab	Mandible Maxilla	Abel Mahedi Mohamed *et al*. (2018)[46]

NS=not specified; IV= intravenous
ALL=acute lymphocytic leukemia; CLL=chronic lymphocytic leukemia; GM=glioblastoma multiforme; MM=multiple myeloma; NSCLC=non–small-cell lung cancer; RCC=renal cell carcinoma

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Artigo 2

3 ARTIGO 2

O artigo a seguir intitula-se **Effect of tyrosine kinase inhibitor *sunitinib* on tissue repair at tooth extraction sites: a histomorphometric study in Wistar rats** e foi formatado de acordo com as normas do periódico ***Oral Oncology*** (Anexo B).

Effect of tyrosine kinase inhibitor *sunitinib* on tissue repair at tooth extraction sites: a histomorphometric study in Wistar rats

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Running Title: *Sunitinib and tooth extraction healing*

ABSTRACT

Objective: The aim of this study was to investigate the effect of sunitinib on tissue repair at tooth extraction sites.

Material and Methods: Fifty-two Wistar rats were allocated into four groups according to the treatment received: (1) sunitinib; (2) sunitinib/zoledronic acid; (3) zoledronic acid; (4) control group. The animals were subjected to extractions of the right upper molars, and maxillae were dissected and macro- and microscopically analyzed.

Results: On macroscopic evaluation, the zoledronic acid group showed a significantly higher prevalence of oral mucosal lesion than the other groups; however, the size of this lesion did not significantly differ between groups. The sunitinib/zoledronic acid group had significantly less epithelium than the zoledronic acid and control group, but showed no significant difference compared to the sunitinib group. The other groups did not show any significant difference regarding this variable. The sunitinib/zoledronic acid and zoledronic acid groups did not differ from each other, but had significantly less connective tissue and more non-vital bone and microbial colonies than the sunitinib and control groups, whereas these latter two groups did not significantly differ from each other. Vital bone, inflammatory infiltrate and tooth fragment did not significantly differ between the groups.

Conclusion: Sunitinib alone is not associated with non-vital bone, whereas the sunitinib/zoledronic acid combination and zoledronic acid alone are.

INTRODUCTION

Medication-related osteonecrosis of the jaw (MRONJ) is an adverse effect that has been reported in cancer patients subjected to different anticancer drug therapies [1]. The first cases of MRONJ were related to bisphosphonates in 2003 [2], and since then, other anticancer drugs have been associated with the disease [3,4]. Local factors play a significant role in MRONJ etiology, where tooth extraction is a major one, with 52 to 61% of patients reporting this intervention before lesion onset [5,6].

Currently, three groups of drugs are known to be MRONJ-related: bisphosphonates, denosumab and antiangiogenics [7,8]. The main action of antiangiogenics is the inhibition of vascular endothelial growth factor (VEGF), which is expressed in the majority of malignant tumors [9], and tumor neoangiogenesis is thereby suppressed. This group comprises bevacizumab, sunitinib, cabozantinib, everolimus, lenalidomide, pazopanib, ramucirumab, sorafenib, aflibercept, thalidomide and sirolimus. Sunitinib is a receptor tyrosine kinase (RTK) inhibitor launched on the market in 2006 (Pfizer, New York, NY, USA) [10]. RTK inhibition blocks multiple targets including VEGFR-1, VEGFR-2, fetal liver tyrosine kinase 3 (FLT3), PDGFRα and PDGFRβ in cellular and biochemical assays, which in turn, inhibits cell proliferation, migration and differentiation and neoangiogenesis and cancer cell invasion [11,12]. Sunitinib has been indicated in the treatment of stromal gastrointestinal carcinoma, metastatic renal cell cancer and pancreatic neuroendocrine tumor [11].

The risk of MRONJ increases for patients being treated with sunitinib combined with intravenous bisphosphonate, showing a prevalence of 0.9 to 2.4% [13]. This happens because VEGFR inactivation and consequent angiogenesis blockade impairs tissue healing [14], hampering bone healing and remodeling [15]. Several reports in the literature corroborate the notion of increased risk of MRONJ in such patients [7,14,16,17]. Koch *et al.* [18], in turn, reported a case of patient undergoing only sunitinib therapy, who developed MRONJ after a tooth extraction. These authors pointed to sunitinib as a possible causative factor of MRONJ even when used as single drug therapy.

MRONJ association with antiangiogenics still has some obscure points since most reported cases refer to patients having undergone or undergoing treatment with

both sunitinib and bisphosphonate. Therefore, the aim of this study was to investigate the effect of sunitinib on tissue repair at tooth extraction sites in animal models.

MATERIAL AND METHODS

The present study was approved by the Ethics Committee on Animal Use of Pontifical Catholic University of Rio Grande do Sul (CEUA-PUCRS) under protocol #8305. The sample was composed of 52 female rats (*Rattus novergicus*, Wistar strain) from the Central Facility (CEMBE/PUCRS), with a mean age of 70 days and mean weight of 250 g. The calculation of the sample size, with a margin of error of 1%, significance level of 5% and power of 80%, based on Maahs *et al*. [19], indicated the need for 11 rats per group (software WinPepi, version 11.28). This number was increased by 2 per group (20%) considering possible losses during the experiment period.

The animals were kept in microisolator cages with controlled temperature (23±1ºC) and 12-h light-dark cycle, with lighting of 300 lux in the center of the room and 60 lux inside the cages. The cages were cleaned and exchanged according to the facility center protocol, and feed (Nuvilab, Colombo, PR, Brazil) and filtered water were provided *ad libitum*. The animals were randomly allocated into 4 groups: (1) 13 animals that were given sunitinib (SU11248; sutent; Pfizer, Inc., New York, NY, USA); (2) 13 animals that were given sunitinib and zoledronic acid (Novartis Pharma, Basel, Switzerland); (3) 13 animals that were given zoledronic acid; and (4) control group: 13 animals with no drug. The first administration of both drugs was carried out at the beginning of the experiment, after labeling and weighing of the animals. Sunitinib was administered by gavage at a dose of 6 mg/kg/day for 35 days, and zoledronic acid was administered by the intraperitoneal route (IP) at a dose of 0.3 mg/kg/week for a total of 5 doses. In the control group, 6 rats received IP saline at the amount of 1 mL/kg/week,

and 7 rats received filtered water, 1 mL/kg/day by gavage. The animals were weighed every 7 days to adjust the doses.

Tooth extractions

Tooth extractions were performed 15 days after beginning the experiment, respecting a 3-day wash-out period (48 h before and 24 h after the tooth extractions) for sunitinib. The procedure was performed under deep anesthesia with mixture of ketamine (100 mg/kg; Syntec, Cotia, SP, Brazil) and xylazine (10 mg/kg; Syntec, Cotia, SP Brazil) administered IP, with the animal in dorsal decubitus [20]. The right upper molars were extracted using a lever movement with a #3s Hollenback carver (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) and pediatric forceps (Edlo, Canoas, RS, Brazil) whose functional portion was adapted to the size of the teeth. Right after the tooth extractions, the animals were returned to the cages where they remained on a sterile surgical pad and under controlled body temperature until the anesthetic effect subsided. During the postoperative period, the animals received dipyrone IP at a dose of 200 mg/kg every 24 h for three days, and mashed chow was provided. A total of 5 animals were lost due to complications during the surgical procedure: 2 animals from the sunitinib group, 2 animals from the sunitinib/zoledronic acid group and 1 animal from the zoledronic acid group. Six rats from the sunitinib group and 5 rats from the sunitinib/zoledronic acid group developed skin desquamation and necrosis, as well as edema of the extremities.

Euthanasia, macroscopic evaluation, and preparation of the specimens

The animals were sedated by IP administration of 5% ketamine hydrochloride at a dose of 70 mg/kg and 2% xylazine hydrochloride at a dose of 7 mg/kg and subjected to cardiac puncture and exsanguination. After exsanguination, an overdose of the ketamine and xylazine mixture was also administered. After euthanasia, the maxilla was dissected and subjected to macroscopic evaluation to determine the presence/absence and size of

oral mucosal lesion in the area of tooth extractions by using a #5 dental explorer and a periodontal probe (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil). The observer was blinded to the group examined and oral mucosal lesion was considered if there was loss of mucosal integrity. The specimens (maxillae) were then fixed for 24 h in 10% buffered formalin. After fixation, the osteotomized segment comprising the tooth extraction area was cut in the middle in a buccal–lingual direction into two pieces, both of them displaying the area of interest at the cut surface.

After decalcification in 10% nitric acid for 8 h, the specimens were embedded in paraffin, and 4 μm-thick sections were obtained, processed and stained with hematoxylin and eosin (H&E).

Capture of the images and histological analysis

Histological images were captured with an Olympus BX-43 light microscope (Olympus, Tokyo, Japan), connected to a computer with Olympus DP-73 digital camera (Olympus). Five fields of each slide were captured using a 10x objective, and the images were stored as uncompressed TIFF (tag image file format). The analysis was carried out by means of the manual point-counting technique (Image Pro Plus 5.1, Media Cybernetics, Bethesda, MD, USA) [21], where epithelium, connective tissue, vital bone, non-vital bone, inflammatory infiltrate, microbial colonies, and tooth fragment were quantified [19] (Fig.1). The observer was blinded (not knowing the group to which each image belonged) and calibrated. Calibration consisted of analyzing a series of 35 images, twice, at two different moments. The results of these analyses were tested by intraclass correlation coefficient, which showed $r = 0.9$.

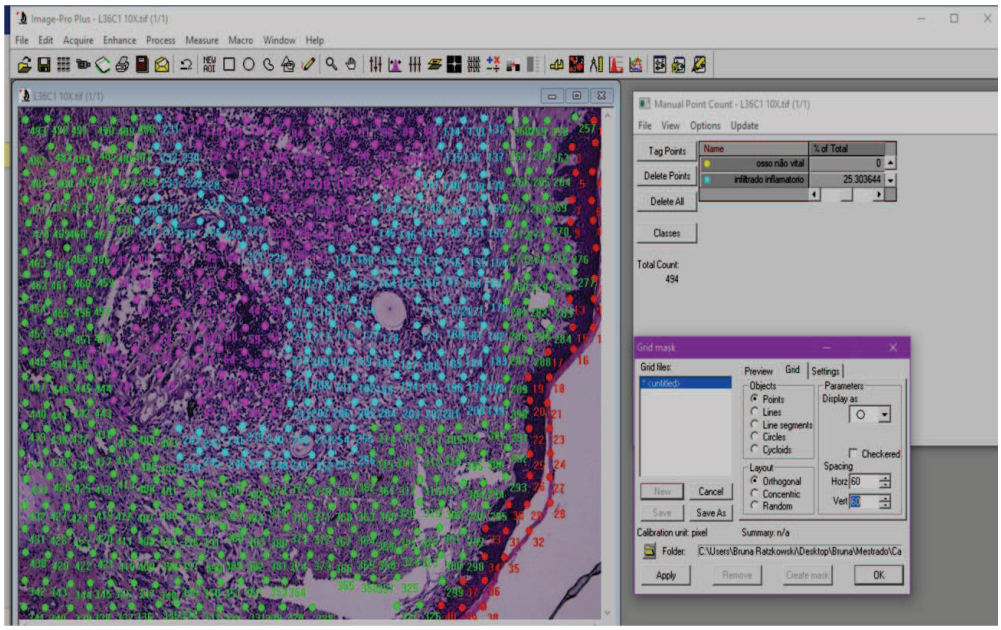

Figure 1 – Quantitative histological analysis by means of manual point-counting technique (Image Pro-plus software, Media Cybernetics, Bethesda, MD, USA)

Statistical analysis

Data were analyzed with descriptive and inferential statistics. The chi-square test was used to compare oral mucosal lesions and non-vital bone frequencies between the groups, and the Kruskal-Wallis test complemented by the Student-Newman-Keuls test was applied to compare the size of oral lesions and the measure of histological variables. The Spearman coefficient tested the relationship between the variables. Statistical analysis was performed in SPSS 17.0 (Statistical Product and Service Solutions, SPSS Inc, USA) at a significance level of 5%.

RESULTS

Macroscopic analysis

The zoledronic acid group showed a significantly higher frequency of oral mucosal lesion than the other groups (*P*=0.046). There was no difference in lesion occurrence between the sunitinib, sunitinib/zoledronic acid and control groups (chi-square, adjusted residual analysis, Table 1). With regard to lesion size, the groups did not show any significant difference (Kruskal-Wallis, *P*=0.670, Table 2).

Table 1 – Macroscopic analysis: sample distribution according to presence/absence of oral mucosal lesion (loss of mucosal integrity)

Group	Presence		Absence		Total		
	n	%	n	%	n	%	*P**
Sunitinib	9	81.82	2	18.18	11	100	
Sunitinib/zoledronic acid	7	63.64	4	36.36	11	100	**0.046**
Zoledronic acid	**12****	100	-	0	12	100	
Control	7	53.85	**6****	46.15	13	100	

n=number of animals; **P* value for chi-square test
**Statistically significant, chi-square test, adjusted residual analysis, α=0.05

Table 2 – Macroscopic analysis: size of the oral lesions (mm^2)

Group	**Size** (mm^2)				
	Mean	SD	Median	P25	P75
Sunitinib	2.66	3.22	1.00	0.25	5.00
Sunitinib/zoledronic acid	4.27	4.08	3.50	0.00	8.00
Zoledronic acid	2.58	2.65	1.75	1.00	2.88
Control	2.62	3.75	1.00	0.00	4.75
*P**			0.670		

**P* value for Kruskal-Wallis, α=0.05

Histological analysis

Presence/absence of non-vital bone in the sample

Table 3 displays the sample distribution in the groups according to the presence/absence of non-vital bone. The sunitinib and control groups were associated with absence of non-vital bone, whereas the sunitinib/zoledronic acid group showed an association with the presence of non-vital bone. Although the zoledronic acid group showed 66.7% of animals with non-vital bone, this was not statistically significant (chi-square, adjusted residual analysis, α=0.05).

Table 3 – Sample distribution according to presence/absence of non-vital bone

Group	**Non-vital bone**						*P**
	Presence		Absence		Total		
	n	%	n	%	n	%	
Sunitinib	2	18.2	**9****	81.8	11	23.4	
Sunitinib/zoledronic acid	**9****	81.8	2	18.2	11	23.4	
Zoledronic acid	8	66.7	4	33.3	12	25.5	**0.003**
Control	3	23.1	**10****	76.9	13	27.7	
Total	22	46.8	25	53.2	47	100	

n=number of animals; **P* value for chi-square test, adjusted residual analysis, α=0.05
**Statistically significant

Quantitative analysis of the histological variables

The sunitinib/zoledronic acid group had significantly less epithelium than the zoledronic acid group and the control, but showed no significant difference with regard to the sunitinib group. There was no significant difference in this variable between the other groups. The sunitinib/zoledronic acid and the zoledronic acid groups did not differ from each other, but had significantly less connective tissue and more non-vital bone and microbial colonies than the sunitinib and the control groups, where the latter two groups did not significantly differ from each other with regard to these variables. Vital

bone (P=0.328), inflammatory infiltrate (P=0.117) and tooth fragment (P=0.309) did not significantly differ between the groups evaluated (Kruskal-Wallis, Student-Newman-Keuls, α=0.05, Table 4). Figure 2 illustrates some of the histological variables analyzed.

Table 5 displays the values for "r" in correlation analysis between the variables using Spearman coefficient. Epithelium was negatively correlated with tooth fragment (r= -0.423); connective tissue was negatively correlated with vital bone (r= -0.407), non-vital bone (r= -0.537), inflammatory infiltrate (r= -0.417), and microbial colonies (r= -0.387); vital bone was negatively correlated with inflammatory infiltrate (r=-0.454). Non-vital bone was positively correlated with inflammatory infiltrate (r=0.523) and with microbial colonies (r=0.603). Inflammatory infiltrate was positively correlated with microbial colonies (r=0.401).

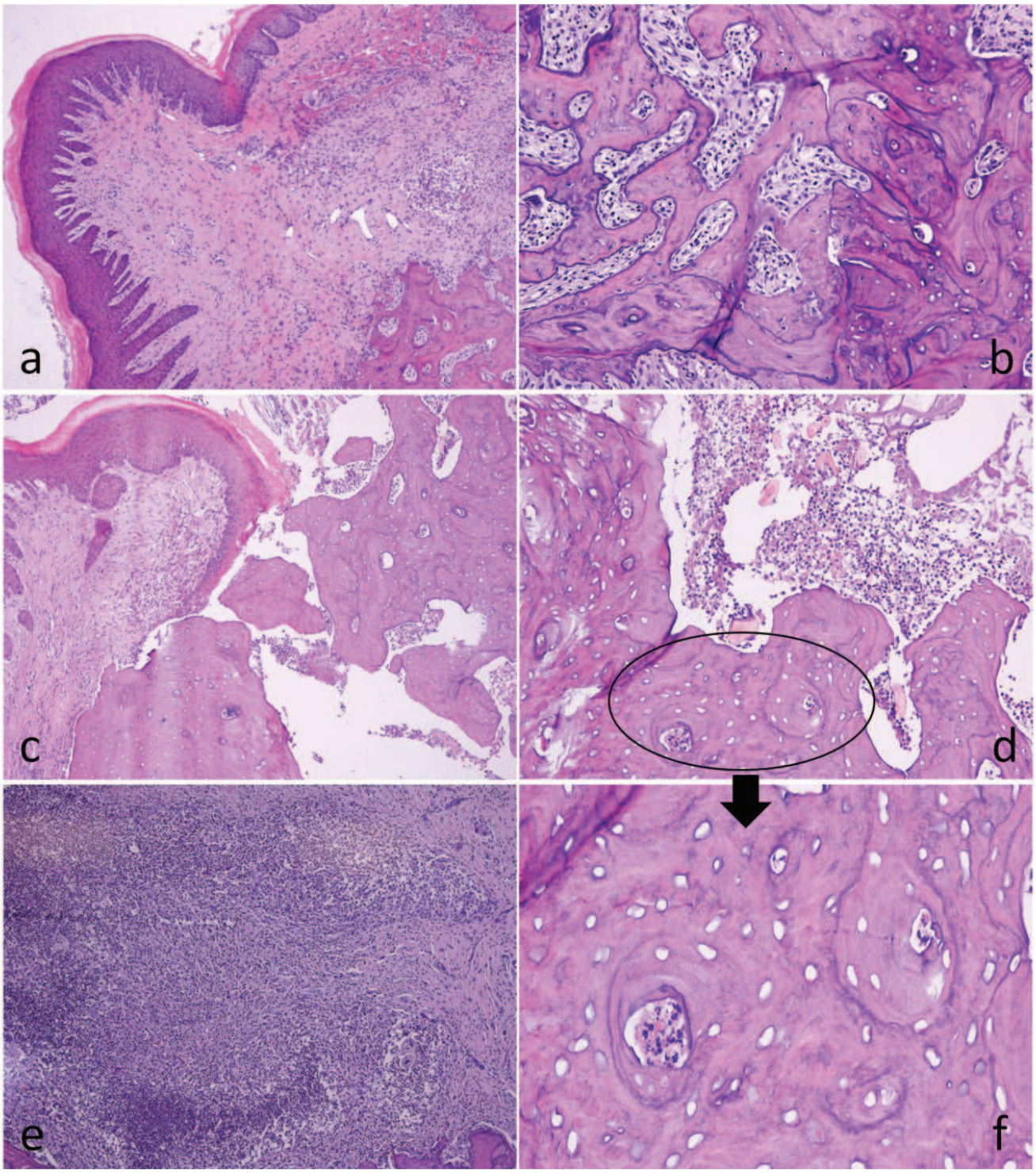

Figure 2 – Histological features on microscopic analysis: (a) complete healing of the surgical wound (H&E, 100X); (b) vital bone (H&E, 200X); (c) non-healing surgical wound showing loss of integrity of oral mucosa and non-vital bone (H&E, 100X); (d) non-vital bone area (H&E, 100X); (e) inflammatory infiltrate in the connective tissue (H&E, 200X); (f) close-up of the non-vital bone seen in the "d" image (H&E, 400X)

Table 4 – Histological analysis: quantification (% of the microscopic field) of the histological variables in the groups analyzed

Variable	Group												*P**
	Sunitinib			Sunitinib/zoledronic acid			Zoledronic acid			Control			
	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	
Epithelium	**6.57^{AB}**	8.25	5.22	**3.92^{B}**	5.27	2.97	**7.80^{A}**	8.41	4.27	**9.61^{A}**	10.29	3.90	**0.02**
Connective tissue	**49.37^{A}**	48.64	9.95	**25.54^{B}**	25.44	8.30	**26.15^{B}**	26.41	11.33	**44.04^{A}**	40.83	10.49	**0.0000**
Vital bone	30.33 ^{A}	29.35	11.16	41.19 ^{A}	36.71	16.71	36.29 ^{A}	39.08	21.92	33.03 ^{A}	32.08	7.86	0.3280
Non-vital bone	**0.00^{A}**	0.62	1.45	**7.61^{B}**	10.77	10.70	**3.03^{B}**	5.64	7.54	**0.00^{A}**	0.71	1.90	**0.0006**
Inflammatory infiltrate	1.55^{A}	6.78	10.59	7.41 ^{A}	10.93	10.65	12.27^{A}	13.28	9.97	6.27 ^{A}	7.49	5.78	0.1175
Microbial colonies	**0.00^{A}**	0.84	2.45	**1.44^{B}**	3.19	5.43	**1.08^{B}**	3.95	6.46	**0.00^{A}**	0.21	0.36	**0.0197**
Tooth fragment	1.91^{A}	5.50	8.19	5.19 ^{A}	7.70	8.76	1.66 ^{A}	3.24	4.28	10.25 ^{A}	8.38	7.13	0.3099

**P* value for Kruskal-Wallis, α=0.05
Medians followed by different letters in the row showed significant difference, Kruskal-Wallis complemented by Student-Newman-Keuls, α=0.05

Table 5 – "*r*" values in correlation analysis between the variables using Spearman coefficient

Variable	Epithelium	Connective tissue	Vital bone	Non-vital bone	Inflammatory infiltrate	Microbial colonies	Tooth fragment
Epithelium	1						
Connective tissue	0.231	1					
Vital bone	-0.213	**-0.407***	1				
Non-vital bone	-0.231	**-0.537***	-0.084	1			
Inflammatory infiltrate	0.030	**-0.417***	**-0.454***	**0.523***	1		
Microbial colonies	-0.214	**-0.387***	-0.198	**0.603***	**0.401***	1	
Tooth fragment	**-0.423***	-0.182	0.129	-0.155	-0.073	0.009	1

*Correlation is significant at the 0.01 level

DISCUSSION

The zoledronic acid group was the only one that showed an association with oral mucosal lesion on macroscopic analysis, whereas lesion frequency in the sunitinib groups did not significantly differ compared to control. The odd finding here was that the sunitinib/zoledronic acid group had no association with lesion, where it was expected to have at least the same rate as the zoledronic acid group. However, it is important to point out that since the control group had similar results as the experimental ones, it is more plausible that some of the macroscopic lesions could have resulted from the tooth fragments persisting at the extraction site and not as a consequence of the drug used, which agrees with the results for this variable in the histological analysis. These facts reinforce the great importance of microscopic evaluation.

Considering the frequency of animals having non-vital bone on microscopic examination, the groups treated with zoledronic acid whether or not in combination with sunitinib showed the highest prevalence, although only the sunitinib/zoledronic acid group showed a statistically significant difference. This finding indicated that sunitinib could potentiate the effect of zoledronic acid, whereas sunitinib alone would not be capable of causing the lesion. Another point to consider is that this was a dichotomous analysis in a relatively small sample, where non-vital bone criterion was bone tissue with empty lacunae (with no osteocytes) [22-24]. This analysis did not take into account the amount of this variable or the other features usually observed in MRONJ lesions, such as microbial biofilm and inflammatory infiltrate [25,26]. We know that empty lacunae can sometimes be an artifact resulting from the histological process [27]. These factors could impart a bias in this evaluation, and therefore, the quantitative analysis of the histological features must also be considered.

The sunitinib/zoledronic acid group had significantly less epithelium, which agrees with the results for non-vital bone in this group, since oral mucosa is incapable of re-epithelialization and of uniting the edges of the wound in areas of osteonecrosis [28,29]. Our findings are also in agreement with the literature, in that the initial damage induced by sunitinib in the oral cavity may affect not only vascular tissue but also keratinocytes [30]. Accordingly, it is believed that oral mucositis caused by sunitinib could progress to osteonecrosis [14]. Connective tissue levels, in turn, were significantly less in the sunitinib/zoledronic acid and zoledronic acid groups and also negatively correlated with non-vital bone, inflammatory infiltrate and microbial colonies, indicating that its lower levels in these groups were a result of the occurrence of osteonecrosis. These same groups (sunitinib/zoledronic acid and zoledronic acid) had significantly greater amounts of non-vital bone and microbial colonies than did the sunitinib and control groups, where these latter two groups did not significantly differ from each other. This is an important finding, where sunitinib seemed to have detrimental effects on bone repair only when combined with zoledronic acid. On the other hand, zoledronic acid combined or not with sunitinib was capable of impairing the healing of the surgical wound, as previously reported [8,19,31]. This would suggest that sunitinib causes non-vital bone only if combined with zoledronic acid, and considering that the sunitinib/zoledronic acid group did not show statistically greater levels of this variable than the zoledronic acid group, sunitinib did not potentiate the effect of zoledronic acid. This is corroborated by the finding that non-vital bone did not differ between the sunitinib group and control.

Our results suggest that the association of MRONJ with antiangiogenics still leaves some doubts, considering that these drugs are often administered in combination with bisphosphonates [16] and denosumab [32], either concomitantly or sequentially. Maybe the growing number of case reports of antiangiogenic-related MRONJ should be critically

considered, especially making sure that the patient has not undergone bisphosphonate therapy in preceding years, since this drug (bisphosphonate) has such a long half-life and long-lasting effects over the time elapsed [33-35].

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

The present study was approved by the Ethics Committee on Animal Use of Pontifical Catholic University of Rio Grande do Sul (CEUA #8305). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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HIGHLIGHTS

The number of antiangiogenic-related MRONJ cases has increased.

Antiangiogenics are often used in combination with bisphosphonates and denosumab.

Relationship of antiangiogenics with MRONJ needs to be investigated.

Discussão Geral

4 DISCUSSÃO GERAL

A osteonecrose maxilar associada a medicamentos (MRONJ) é uma enfermidade de etiopatogenia incerta e, muitas vezes, de difícil tratamento. Inicialmente, os casos foram associados a bisfosfonatos e, posteriormente, denosumabe e antiangiogênicos também passaram a compor o rol de medicamentos associados à condição (Marx, 2003; Ruggiero *et al*., 2004; Ruggiero *et al*., 2014). Os primeiros relatos de MRONJ relacionada ao uso de antiangiogênicos datam de 2008 e, até o momento, cerca de 35 casos já foram relatados na literatura (Estilo *et al*., 2008; Pimolbutr *et al*., 2018). Esses fármacos são empregados na terapia anticâncer de pacientes portadores de tumor gastrointestinal, carcinoma de células renais e tumor neuroendócrino, entre outros (Ruggiero *et al*., 2014). Muitos dos pacientes encontram-se em fase avançada da doença e usam ou já usaram uma ampla variedade de fármacos, incluindo quimioterápicos, denosumabe e bisfosfonatos (Beuselink *et al*., 2012; Sivolella *et al*., 2013). Considerando o acima exposto, bem como o fato de que os bisfosfonatos têm efeito persistente, mesmo após cessada sua administração, parece temerário afirmar que os antiangiogênicos tenham *per se* capacidade de determinar o desenvolvimento de MRONJ. Foi nesse contexto que se originou a ideia do presente estudo, cujo experimento *in vivo* conduziu análise histomorfométrica de sítios de exodontias em ratos submetidos à terapia com o antiangiogênico sunitinibe, em administração isolada ou em combinação com o bisfosfonato ácido zoledrônico.

De acordo com os resultados obtidos, o grupo ácido zoledrônico foi o único que apresentou associação com a ocorrência de lesão da mucosa oral durante a análise macroscópica. Na avaliação de presença/ausência de osso não-vital ao exame microscópico, os grupos tratados com ácido zoledrônico, associado ou não ao sunitinibe,

exibiram a maior prevalência. Esses achados corroboram relatos da literatura de que o risco de MRONJ com o uso de ácido zoledrônico, independentemente de comorbidades sistêmicas, é comprovado (Hoff *et al*., 2008; Marx *et al*., 2005).

O grupo sunitinibe, entretanto, não diferiu significativamente do grupo-controle para frequência de lesão ao exame macroscópico. Esse resultado se repetiu na microscopia, tanto pela avaliação dicotômica, quanto pela avaliação quantitativa de osso não-vital, em que o grupo sunitinibe exibiu valores similares aos do grupo-controle. Tais achados sugerem que o uso isolado de sunitinibe não seria capaz de determinar a ocorrência de MRONJ.

Considerando as demais variáveis histológicas, o grupo sunitinibe/ácido zoledrônico exibiu significativamente menos epitélio, o que pode ser justificado pela maior ocorrência de osso não-vital neste grupo, uma vez que a mucosa oral é incapaz de reepitelizar as áreas de osteonecrose (Landesberg *et al.,* 2008, Ravosa *et al*., 2011). Além disso, o efeito do sunitinibe na cavidade oral afeta também os queratinócitos (Mignogna *et al*., 2009), o que pode ter colaborado para a menor ocorrência de epitélio no grupo que combinou os dois fármacos. Corroborando esses achados estão os relatos da literatura de que pacientes submetidos a terapia com sunitinibe podem apresentar vários efeitos adversos que incluem mucosite, alterações gustativas e lesões cutâneas (Christodoulou *et al*., 2009; Fleissig *et al.*, 2012).

Os grupos sunitinibe/ácido zoledrônico e ácido zoledrônico tiveram quantidades significativamente maiores de osso não-vital e de colônias microbianas do que os grupos sunitinibe e controle. Esse é um resultado importante, em que o sunitinibe parece estar associado a efeitos deletérios no reparo ósseo somente se combinado ao ácido zoledrônico, enquanto o ácido zoledrônico, independentemente de estar combinado ou não ao sunitinibe, é capaz de comprometer a cicatrização da ferida cirúrgica, como já

relatado anteriormente (Maahs *et al*., 2011; Marx *et al*., 2005; Schwartz, 2005). Ainda, o achado de que o grupo sunitinibe/ácido zoledrônico não exibiu quantidade significativamente maior de osso não-vital do que o grupo ácido zoledrônico, indica que o sunitinibe sequer potencializou o efeito do bisfosfonato. Isso é confirmado pelo fato de que a quantidade de osso não-vital não diferiu entre os grupos sunitinibe e controle.

Os resultados do presente estudo colocam em questionamento a capacidade de os antiangiogênicos *per se* determinarem o desenvolvimento de MRONJ. Uma vez que essas drogas são, frequentemente, administradas em combinação com bisfosfonatos e denosumabe (Sivolella *et al.,* 2013), algumas de suas propriedades farmacológicas devem ser ponderadas. Os bisfosfonatos ligam-se ao cálcio da hidroxiapatita, incorporando-se ao tecido ósseo, e sua meia-vida pode ser superior a dez anos (Marx, 2014). Isto é, mesmo após suspensa a administração, o paciente tratado com bisfosfonato permanece sob efeito do fármaco (Marx *et al*., 2005). Já o denosumabe e os antiangiogênicos, têm meia-vida mais curta, que vai de 2,5 a 32 dias, sendo eliminados mais rapidamente (Narayanan, 2013; Bodnar, 2014). Isso deve ser levado em conta na avaliação dos pacientes que desenvolvem MRONJ durante o tratamento com antiangiogênicos. As características farmacológicas dos antiangiogênicos, principalmente sua meia-vida e a não incorporação ao tecido ósseo, fazem pensar que a MRONJ a eles associada seria mais fácil de controlar e teria melhor prognóstico. Assim, casos dessa enfermidade em pacientes usuários de antiangiogênicos, que se mostrem resistentes e não-responsivos ao tratamento, devem despertar a necessidade de certificação da ausência de uso de bisfosfonato na história médica atual ou pregressa do paciente.

Os resultados do presente estudo são preliminares e levantam uma possibilidade que deve ser mais profundamente investigada. É preciso lembrar que muitos dos

fármacos em questão são medicamentos lançados há pouco tempo no mercado. À medida que o contingente de pacientes usuários dessas terapias cresce, novos casos de efeitos adversos surgem e o conhecimento vai sendo sedimentado por meio de evidências e de novas pesquisas. Assim, estudos *in vivo* com os demais antiangiogênicos disponíveis no mercado para tratamento anticâncer, bem como estudos de casos com maior número de pacientes seriam úteis para elucidar a relação desses fármacos com a MRONJ.

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ANEXO A

Normas para submissão de artigos ao periódico *Gerodontology*

https://onlinelibrary.wiley.com/journal/17412358

ANEXO B

Normas para submissão de artigos ao periódico *Oral Oncology*

https://www.journals.elsevier.com/oral-oncology

ANEXO C

SIPESQ

Sistema de Pesquisas da PUCRS

Código SIPESQ: 8305

Porto Alegre, 4 de outubro de 2017.

Prezado(a) Pesquisador(a),

A Comissão Científica da FACULDADE DE ODONTOLOGIA da PUCRS apreciou e aprovou o Projeto de Pesquisa "Efeito do inibidor de quinase sunitinibe sobre a cicatrização alveolar pós-exodontia: estudo histomorfométrico e imunoistoquímico 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 D

SIPESQ

Sistema de Pesquisas da PUCRS

Código SIPESQ: 8305 Porto Alegre,25 de outubro de 2017

Prezado(a) Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou o Projeto de Pesquisa "Efeito do inibidor de quinase sunitinibe sobre a cicatrização alveolar pós-exodontia: estudo histomorfométrico e imunoistoquímico 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.

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: 25/10/2017 - 25/04/2018

Nº de Animais	Espécie
52	Rattus norvegicus
Total de Animais: 52	

Atenciosamente,

Comissão de Ética no Uso de Animais(CEUA)

ESCOLA DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

EFEITO DO INIBIDOR DE TIROSINA-QUINASE SUNITINIBE SOBRE A CICATRIZAÇÃO ALVEOLAR PÓS-EXODONTIA: ESTUDO HISTOMORFOMÉTRICO

BRUNA RATZKOWSKI

2018

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
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EFFECT OF TYROSINE KINASE INHIBITOR SUNITINIB ON POST-EXTRACTION HEALING OF THE ALVEOLAR BONE: A HISTOMORPHOMETRIC STUDY

Porto Alegre

2018

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BRUNA RATZKOWSKI

EFEITO DO INIBIDOR DE TIROSINA-QUINASE SUNITINIBE SOBRE A CICATRIZAÇÃO ALVEOLAR PÓS-EXODONTIA: ESTUDO HISTOMORFOMÉTRICO

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

2018

Gaste mais horas realizando que sonhando, fazendo que planejando, vivendo que esperando, porque, embora quem quase morre esteja vivo, quem quase vive já morreu.

Sarah Westphal (1983-)

Dedicatória

Especialmente à minha família, por estar ao meu lado

durante toda essa jornada.

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Aos meus pais, Benjamin Ratzkowski e Eneida Ratzkowski, por, ao longo da minha vida, terem me ensinado os valores mais importantes, terem me proporcionado toda estrutura necessária para que eu pudesse percorrer meu caminho e, acima de tudo, por terem me dado muito amor.

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Resumo

RESUMO

A osteonecrose dos maxilares associada a medicamentos (MRONJ) é um importante efeito adverso que tem acometido pacientes em tratamento com diferentes drogas anticâncer, incluindo fármacos antiangiogênicos. O presente estudo teve por objetivo investigar o efeito do antiangiogênico inibidor de tirosina-quinase sunitinibe sobre o reparo ósseo alveolar em sítios de exodontias. Ratos Wistar (n=52) foram distribuídos em quatro grupos de acordo com o tratamento administrado: (1) sunitinibe (n=13); (2) sunitinibe/ácido zoledrônico (n=13); (3) ácido zoledrônico (n=13); (4) grupo-controle (n=13). Os animais foram submetidos a exodontias dos molares superiores do lado direito, e as maxilas dissecadas e macro e microscopicamente analisadas. Na avaliação macroscópica, o grupo ácido zoledrônico exibiu prevalência de lesão da mucosa oral significativamente maior que a dos demais grupos. O tamanho das lesões, entretanto, não diferiu significativamente entre os grupos. O grupo sunitinibe/ácido zoledrônico teve significativamente menos tecido epitelial que os grupos ácido zoledrônico e controle, mas não exibiu diferença significativa em comparação ao grupo sunitinibe. Os demais grupos não exibiram diferença significativa para essa variável. Os grupos sunitinibe/ácido zoledrônico e ácido zoledrônico não diferiram entre si, mas tiveram quantidade de tecido conjuntivo significativamente menor e de osso não-vital e colônias microbianas significativamente maior do que os grupos sunitinibe e controle, enquanto esses dois últimos grupos não diferiram significativamente entre si na avaliação dessas variáveis. Osso vital, infiltrado inflamatório e fragmento dentário não diferiram significativamente entre os grupos.

Conclusão: O antiangiogênico sunitinibe, quando administrado de forma isolada, não está associado à ocorrência de osso não-vital, enquanto a combinação sunitinibe/ácido zoledrônico ou o uso do ácido zoledrônico de forma isolada exibem associação com a ocorrência de osso não-vital.

Palavras-chave: drogas antiangiogênicas; sunitinibe; MRONJ; angiogênese; ácido zoledrônico

Summary

SUMMARY

Medication-related osteonecrosis of the jaw (MRONJ) is an important side effect that has been affecting patients undergoing different anticancer therapies, including antiangiogenic drugs. The aim of this study was to investigate the effect of tyrosine kinase inhibitor sunitinib on tissue repair at tooth extraction sites. Fifty-two Wistar rats were allocated into four groups according to the treatment received: (1) sunitinib (n=13); (2) sunitinib/zoledronic acid (n=13); (3) zoledronic acid (n=13); (4) control group (n=13). The animals were subjected to extractions of the right upper molars, and maxillae were dissected and macro- and microscopically analyzed. On macroscopic evaluation, the zoledronic acid group showed a significantly higher prevalence of oral mucosal lesion than the other groups; however, the size of this lesion did not significantly differ between groups. The sunitinib/zoledronic acid group had significantly less epithelium than the zoledronic acid and control group, but showed no significant difference compared to the sunitinib group. The other groups did not show any significant difference regarding this variable. The sunitinib/zoledronic acid and zoledronic acid groups did not differ from each other, but had significantly less connective tissue and more non-vital bone and microbial colonies than the sunitinib and control groups, whereas these latter two groups did not significantly differ from each other. Vital bone, inflammatory infiltrate and tooth fragment did not significantly differ between the groups.

Conclusion: Sunitinib alone is not associated with non-vital bone, whereas the sunitinib/zoledronic acid combination and zoledronic acid alone are.

Key words: antiangiogenic drugs; sunitinib; jaw osteonecrosis; angiogenesis; zoledronic acid

Sumário

SUMÁRIO

Introdução

1 INTRODUÇÃO

O termo *osteonecrose* denomina o resultado comum de uma série de condições que levam à morte do tecido ósseo, não se referindo a uma entidade clínica específica. É uma condição frequente, que pode tanto passar despercebida ao exame clínico, quanto determinar o colapso da estrutura óssea, levando a dor articular, destruição e perda de função do tecido ósseo (Fondi; Franchi, 2007). Ao acometer os ossos maxilares, com base em sua etiologia, a condição é classificada em osteorradionecrose, quando acomete pacientes que foram submetidos a radioterapia de cabeça e pescoço; e osteonecrose medicamentosa, quando associada ao uso de fármacos por parte de pacientes que não sofreram radioterapia prévia (Marx; Tursun, 2012).

Desde 2003, um número crescente de casos de osteonecrose maxilar em pacientes usuários de bisfosfonatos tem sido relatado na literatura, sendo a enfermidade, inicialmente, denominada *osteonecrose maxilar associada a bisfosfonatos* (*bisphosphonate-related osteonecrosis of the jaw,* BRONJ) (Marx, 2003; Ruggiero *et al*., 2007). Anos mais tarde, o fármaco antirreabsortivo denosumabe também se mostrou capaz de determinar o desenvolvimento da doença e, posteriormente, casos associados a antiangiogênicos passaram a ser relatados. Com isso, em 2014, foi proposta a nomenclatura *osteonecrose maxilar associada a medicamentos* (*medication-related osteonecrosis of the jaw*, MRONJ), estendendo sua associação aos três grupos de drogas: bisfosfonatos, denosumabe e antiangiogênicos (Ruggiero *et al*., 2014). A MRONJ é definida como área de osso exposto ou sondável na região maxilofacial, que não cicatriza no prazo de oito semanas, associada a sinais e sintomas como dor, edema, parestesia, infecção, ulceração dos tecidos moles e alterações radiográficas em pacientes que tenham sido tratados com os referidos fármacos e não tenham histórico de radioterapia de cabeça e pescoço (Weber *et al*., 2016). Fatores locais desempenham papel significativo para o

desenvolvimento da MRONJ, sendo a cirurgia dentoalveolar um importante fator de risco, com 52 a 61% dos pacientes relatando a exodontia como evento prévio ao desenvolvimento da lesão (Fehm *et al*., 2009; Vahtsevanos *et al*., 2009).

Considerando o papel crucial da angiogênese no desenvolvimento e na progressão de tumores malignos (Baka *et al.*, 2006), diversos medicamentos antiangiogênicos foram lançados no mercado e vêm sendo empregados como terapia anticâncer. De acordo com o mecanismo de ação, essas drogas são classificadas como anticorpos anti-VEGF (bevacizumabe, aflibercept, pegaptanibe, ranibizumabe); imunomoduladores (talidomida, lenalidomida) e inibidores de quinase (sunitinibe, sorafenibe, sirolimo, temsirolimo, everolimo, pazopanibe, vatalanibe, vandetanibe, regorafenibe, lenvatinibe, axitinibe) (Abel Mahedi Mohamed *et al*., 2018; Christodoulou *et al.,* 2009; Ramírez *et al.,* 2015). O sunitinibe, introduzido no mercado em 2006 pela empresa farmacêutica Pfizer (Pfizer, New York, NY, USA), é um antiangiogênico inibidor de tirosina-quinase, que tem sido associado ao desenvolvimento de MRONJ (Fleissig *et al.*, 2012). Também há relatos de que o risco dessa enfermidade aumenta significativamente em pacientes que usam sunitinibe e bisfosfonatos simultaneamente (Ramírez *et al.,* 2015).

Vários relatos de casos que associam o uso de antiangiogênicos à MRONJ são de pacientes que usaram bisfosfonatos ou denosumabe previamente ou concomitantemente ao uso da droga antiangiogênica (Beuselinck *et al*., 2012). A dificuldade de manejo e tratamento da MRONJ associada a bisfosfonatos resulta, em parte, da meia-vida extremamente prolongada desses fármacos, que pode ser superior a dez anos, o que leva a doses cumulativas elevadas no tecido ósseo e efeito residual persistente (Marx, 2014; Ruggiero *et al*., 2014). Os demais fármacos, mesmo que efetivamente associados à osteonecrose, geram algumas ressalvas e observações para suas especificidades. O

denosumabe e os antiangiogênicos não são incorporados pelo tecido ósseo e têm meia-vida variando entre 2,5 e 32 dias (Bodnar, 2014; Narayanan, 2013), o que reduz, potencialmente, a gravidade dos quadros de MRONJ e lhes confere melhor prognóstico. Por outro lado, o persistente efeito dos bisfosfonatos, além de comprometer a resposta ao tratamento dos quadros de MRONJ, também gera a suspeita de que alguns casos associados a antiangiogênicos possam ter sido, de fato, causados pelo uso de bisfosfonatos.

A etiopatogênese específica da MRONJ continua indefinida (Rosella *et al*., 2016), e os relatos de casos associados a antiangiogênicos ainda deixam alguma margem de dúvida, uma vez que esses fármacos são, frequentemente, administrados de forma conjunta ou subsequente ao uso de bisfosfosfonatos ou denosumabe (Ruggiero, 2015; Sivolella *et al*., 2013). Em função disso, o presente estudo teve por objetivo investigar a associação entre MRONJ e fármacos antiangiogênicos. O trabalho foi estruturado sob a forma de dois artigos: o primeiro artigo apresenta uma revisão da literatura sobre o tema em questão, enquanto o segundo artigo relata o experimento *in vivo* em que foi conduzida análise histomorfométrica do osso alveolar de ratos submetidos a exodontias durante terapia com sunitinibe.

Artigo 1

2 ARTIGO 1

O artigo a seguir intitula-se **An overview of relationship between antiangiogenics and medication-related osteonecrosis of the jaw** e foi formatado de acordo com as normas do periódico *Gerodontology* (Anexo A).

An overview of relationship between antiangiogenics and medication-related osteonecrosis of the jaw

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Key words: sunitinib; bevacizumab; MRONJ; osteonecrosis; antiangiogenic drugs

Running title: Osteonecrosis and antiangiogenics

Review Article

ABSTRACT

With the constant emergence of novel therapies and the longer life expectancy of cancer patients, adverse effects of anticancer treatment have drawn attention and demanded special care. Medication-related osteonecrosis of the jaw (MRONJ) is a potentially debilitating adverse effect, which has been reported in cancer patients undergoing therapy with antiresorptive and antiangiogenic drugs. We present here a literature review focusing on the relationship between this condition and antiangiogenics. The number of reported cases of MRONJ associated with these drugs has increased. It seems evident that when antiangiogenics are used in combination with bisphosphonates or denosumab, MRONJ occurrence is very likely. Anyway, some doubts remain concerning the capacity of antiangiogenics *per se,* with no other drug association, to determine MRONJ. Further studies using animal models and capable of isolating variables would be helpful in clarifying the relationship of antiangiogenics, as a single drug therapy, with the development of MRONJ.

INTRODUCTION

Osteonecrosis of the jaw is a potentially debilitating adverse effect, which has been reported in cancer patients subjected to different drug therapies.[1] In 2003, Marx[2] reported 36 cases of this disease related to the bisphosphonates zoledronate and pamidronate, which were described as painful bone exposures in the mandible, maxilla or both, non-responsive to either drugs or surgical treatment. Since then, numerous similar cases of jaw osteonecrosis associated with bisphosphonates have been reported.[3,4] At first, the condition was called bisphosphonate-related osteonecrosis of the jaw (BRONJ). However, in 2014, the term *medication-related osteonecrosis of the jaw* (MRONJ) was recommended by the American Association of Oral and Maxillofacial Surgeons (AAOMS), extending the cause of the disease to other drugs.[5]

MRONJ diagnosis should meet certain criteria: (1) current or previous use of bisphosphonate or other antiresorptive or antiangiogenic drug; (2) exposed or probing bone through intra- or extraoral fistula in the oral and maxillofacial region persisting for more than eight weeks; (3) absence of head and neck radiation therapy; and (4) absence of tumor/metastasis in the involved region.[5,6] The condition can be associated with pain, swelling, paresthesia, infection, soft tissue ulceration and radiographic alterations.[7] It is estimated that 65% of cases are located in the mandible, 28.4% in the maxilla and 6.5% in both maxilla and mandible.[8-10] Local factors play a significant role in the development of osteonecrosis, with dentoalveolar surgery being a major risk factor. Previous tooth extraction was reported in 52 to 61% of cases.[11,12] MRONJ etiopathogenesis is still controversial, and therefore, prevention becomes the focus of management.

Currently, there are three groups of drugs related to lesion development: bisphosphonates, denosumab and antiangiogenics.[8,13] We present here a literature review focusing on antiangiogenic anticancer drugs and their relationship with jaw osteonecrosis.

Angiogenesis and antiangiogenics

Angiogenesis is defined as the process of new blood vessel formation, where vascular endothelial growth factor family (VEGF) plays essential roles, in either physiological or pathological conditions. This family comprises five different members: VEGF-A (also known as VEGF), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PLGF), where VEGF is the one most involved in blood vessel formation.[14] Angiogenesis can be induced in an uncontrolled manner in many pathological conditions such as cancer and ischemic, inflammatory, infectious and immunological disturbances.[15] It is a complex biological process that supports the growth and metastatic potential of many tumors. Accordingly, tumor biology has become the basis for cancer therapy, and understanding how new blood vessels are formed during tumor growth has led to new therapies targeting such process.[16]

Based on their ability to block tumor growth by interfering with neoangiogenesis, antiangiogenics have been launched in the pharmaceutical market as antitumor agents.[16] The main action is inhibition of VEGF, which is expressed in most malignant tumors.[17] Some of these agents inhibit endothelial cells directly; others inhibit the angiogenesis signaling cascade or block the ability of endothelial cells to break down the extracellular matrix. The agents that directly target VEGF neutralize the protein, thereby blocking tumor expression of the angiogenic factor on endothelial cells.[18,19] According to their mechanism of action, antiangiogenics have been classified as anti-VEGF antibodies (bevacizumab, aflibercept, pegaptanib, ranibizumab), immunomodulators (thalidomide, lenalidomide) and kinase inhibitors (axitinib, everolimus, lenvatinib, pazopanib, regorafenib, sirolimus, sunitinib, sorafenib, temsirolimus, vandetanib, vatalanib).[5]

Receptor tyrosine kinases (RTKs) are proteins involved in various important signaling pathways and are directly related to proliferation, differentiation and cell migration processes. The family comprises receptors for growth factors and are involved in the development and progression of many types of malignant tumors. Some of these are VEGF receptors (VEGFRs), platelet-derived growth factor receptors (PDGFRs), fibroblast growth factor receptors (FGFRs), epidermal growth factor receptor (EGFR), RAF (rapidly accelerated fibrosarcoma) kinases and c-Kit (a receptor of the pluripotent cell growth factor, stem cell factor). Tyrosine kinase inhibitors (TKIs), in turn, are small molecules capable of interacting with RTKs inhibiting their activation and consequently various pro-angiogenic signaling pathways.[20,21]

Therapy with antiangiogenics involves high toxicity and adverse effects. The systemic disturbance caused in the signaling pathways that control angiogenic activity is associated with hemorrhagic complications and gastrointestinal perforations.[22] Also, these drugs have recently been related to MRONJ.[23-25]

Anti-VEGF antibodies

Bevacizumab

Bevacizumab was the first antiangiogenic drug approved for clinical use.[26] It is a humanized monoclonal antibody that recognizes and blocks VEGF, then inhibiting its interaction with VEGFR-1 and VEGFR-2 receptors located on the surface of endothelial cells.[27,28] As VEGF activity is neutralized, tumor vascularization is reduced and tumor growth inhibited.[14] Bevacizumab has been prescribed to treat some malignant tumors, such as metastatic colorectal cancer (CCRm), glioblastoma, lung cancer and neoplastic neurovascular diseases. Besides, it has been widely applied in ophthalmology to treat retinal lesions and neovascular diseases.[29]

With the VEGF signaling pathway as a target, bevacizumab would compromise the integrity of microvessels in the jaw and lead to subclinical compromise of the osteon.[30,31] In a meta-analysis where 3,560 patients received only bevacizumab, MRONJ prevalence was 0.2%, but it increased up to 0.9% when combined with bisphosphonates.[32] According to other reports, the risk among patients receiving combined therapy of bevacizumab and bisphosphonates increases to 2%.[23,33]

Aflibercept

Aflibercept is a recombinant human fusion protein that blocks the VEGF pathway through high-affinity binding to the VEGF-A and VEGF-B isoforms and the placental growth factor-1 and -2 isoforms, and it is indicated for the treatment of metastatic colorectal cancer.[34] Ponzetti *et al*.[35] reported a case of a 64-year-old female patient that was diagnosed with adenocarcinoma of the transverse colon with unresectable bilateral liver metastases. This patient had a history of unresolved chronic periodontitis and developed MRONJ after having received eleven cycles (six months) of chemotherapy with aflibercept.[35]

Kinase inhibitors

Sunitinib

Sunitinib was launched on the market in 2006 by Pfizer Pharmaceuticals (New York, NY, USA). It is a TKI that blocks some RTKs, including VEGFR and platelet-derived growth factor receptor (PDGFR). RTK inhibition prevents the cancer cell processes of proliferation, migration, differentiation, neoangiogenesis and invasion, becoming an important tool in the treatment of malignant tumors.[36,37] This drug is indicated for the treatment of gastrointestinal stromal tumor, metastatic renal cell cancer and pancreatic neuroendocrine tumor.[36]

In cellular and biochemical assays, sunitinib is a strong inhibitor of VEGFR-1, VEGFR-2, fetal liver tyrosine kinase 3 (FLT3), KIT (stem-cell factor [SCF] receptor), PDGFRα, and PDGFRβ.[38,39] *In vitro*, it was shown to induce apoptosis of umbilical vein endothelial cells.[39] Adverse effects such as diarrhea, mucositis, taste changes, skin lesions and hypertension were reported by patients undergoing sunitinib therapy. Most adverse effects are reversible and osteonecrosis has been reported.[23-25]

The incidence of complications increases when antiangiogenics are combined with chemotherapy.[40] Likewise, MRONJ risk increases in patients using sunitinib and bisphosphonate concomitantly, with a prevalence ranging from 0.9 to 2.4%.[41] This probably occurs because VEGFR inactivation impairs tissue healing, and sunitinib-related mucositis can contribute to MRONJ development.[42] The etiopathogenesis of bisphosphonate-related osteonecrosis has not yet been clarified. Nonetheless, in the case of antiangiogenics, it seems reasonable to associate the lesion with the interference of these drugs with major factors related to jaw bone remodeling and wound repair (VEGF and PDGF). The inhibition of these important factors for tissue healing could lead to osteonecrosis.[24] Considering the combined toxic effect of antiangiogenics in patients who have used bisphosphonates, it

seems that osteonecrosis results from impairment of both angiogenesis and bone remodeling.[33]

Fleissig *et al*.[24] reported a case of a 58-year-old patient on sunitinib to treat renal cancer, who had a history of neither bisphosphonate nor corticosteroid use and who developed mandibular osteonecrosis after a tooth extraction. Another study evaluating patients undergoing renal cancer treatment in nine Italian centers reported that 44 patients developed osteonecrosis, with zoledronic acid being the most frequently used nitrogen-containing bisphosphonate (93%), whereas the most commonly used antiangiogenic was sunitinib (80%). The major precipitating event was dental/periodontal infection (34%), followed by tooth extraction (30%).[25]

Everolimus

Everolimus is a drug that inhibits mTOR (mammalian target of rapamycin) activity, which is involved in cell growth and metabolism.[43] Yamamoto *et al.*[43] reported a case of a 67-year-old patient on everolimus for the treatment of breast cancer. The patient developed mandibular osteonecrosis despite not having any history of bisphosphonate use and no relevant past dental history, such as tooth extraction.

Sorafenib

Sorafenib is an oral multikinase inhibitor of VEGFR and PDGFR used for treatment of advanced hepatocellular carcinoma.[44] Garuti *et al*.[45] reported a case of a 74-year-old patient who was treated with sorafenib, did not receive bisphosphonates or other antiangiogenic drugs, and developed mandibular osteonecrosis at a site previously subjected to tooth extraction.

A retrospective study of patients undergoing bisphosphonate therapy combined or not with antiangiogenics found that out of 116 patients, only 25 used antiangiogenics. Among them, 22 received bevacizumab, 2 sunitinib and one sorafenib. Four (16%) out of

these 25 patients developed MRONJ when receiving antiangiogenic and bisphosphonate concomitantly. On the other hand, one (1.1%) out of 91 patients receiving only bisphosphonate developed MRONJ.[23] Cases of jaw osteonecrosis after tooth extraction in patients treated with sunitinib, imatinib and pazopanib were reported in a retrospective study.[46]

Immunomodulators

Therapies such as thalidomide and lenalidomide have provided benefits in the treatment of multiple myeloma (MM), but they have been associated with adverse events such as MRONJ.[47] Cetiner *et al*.[48] conducted a study in the Hematology Department of Gazi University Hospital with 32 patients (19 men, 13 women) who had been treated for multiple myeloma. Fifty percent (16/32) of patients had received post-transplant thalidomide maintenance and 31% (10/32) had received thalidomide with dexamethasone during induction treatment. In the total sample of 32 patients, MRONJ was detected clinically and radiographically in five patients,[48] four of them receiving thalidomide. Anyway, there was no significant difference in MRONJ frequency between patients treated or not with thalidomide.

FINAL CONSIDERATIONS

The number of case reports of MRONJ associated with antiangiogenic drugs has increased lately (Table 1). Considering that VEGF is crucial for tissue healing and that these drugs inhibit VEGF, some authors believe that antiangiogenics would impair tissue healing and would be associated with MRONJ.[23-25] Moreover, VEGF plays an important role in the regulation of osteoclast function, differentiation, and survival,[56,57] which would contribute to MRONJ onset.

Antiangiogenics are frequently prescribed in combination with some antiresorptive drugs such as bisphosphonates[58] and denosumab.[59] The capacity of these combined therapies in increasing MRONJ risk has been reported.[33,46] On the other hand, it is known that bisphosphonates and denosumab can *per se* cause MRONJ, whereas for antiangiogenics, such possibility still needs to be investigated. New research using animal models and capable of controlling biases and isolating variables are needed to clearly confirm the ability of antiangiogenics to contribute to the development of MRONJ when used as a single drug therapy.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1 – Literature reports on antiangiogenic-related osteonecrosis of the jaw

Type of study	Disease/sample size (n)	Drug/dose	Time of use (months)	Risk factor/comorbidities	Other drugs	Site	Reference
Case report	Breast cancer (n=1)	Bevacizumab 15 mg/kg every 3 weeks	6	None	Doxorubicin, cyclophosphamide, albumin-bound nanoparticle-paclitaxel, capecitabine	Mandible	Estilo *et al*. (2008)[30]
Case report	Breast cancer (n=1)	Bevacizumab Dose: NS	1	Tooth extraction	Liposomal doxorubicin	Maxilla	Greuter *et al*. (2008)[49]
Case report	Lung adenocarcinoma (n=1)	Bevacizumab 7.5 mg/kg,8gr.	0.5	Tooth extraction	Cisplatin and gemcitabine	Mandible	Serra *et al*. (2009)[50]
Case report	Renal cell carcinoma (n=1)	Sunitinib Dose: NS	14	Tooth extraction	Zoledronic acid	Mandible	Ayllon *et al.* (2009)[33]
Case report	Renal cell carcinoma (n=1)	Sunitinib 50 mg/day on a 4-week and 2-week-off schedule	5	Tooth extraction	Interferon, vinblastine, sorafenib	Mandible	Koch *et al*. (2011)[51]
Case report	Renal cell carcinoma (n=1)	Sunitinib 50mg/day on a 4 week and 2-week-off schedule	12	Tooth extraction	NS	Mandible	Fleissig *et al*. (2012) [24]
Case report	Colon carcinoma (n=1)	Bevacizumab 5 mg/kg every 2 weeks	3	None	Fluorouracil, leucovorin, oxaliplatin	Mandible	Dişel *et al*. (2012)[52]
Case report	Retinal vascular thrombosis (n=1)	Bevacizumab 2.5 mg/month (intravitreal)	24	None	NS	Mandible	Hopp *et al*. (2012) [29]
Case report	Pancreatic carcinoma (n=1)	Bevacizumab Dose: NS	4	Oral ulcer	Gemcitabine, erlotinib, folinic acid, 5-fluorouracil, oxaliplatin paclitaxel and sorafenib	Mandible	Pakosch *et al*. (2013)[53]
Retrospective	Renal cell carcinoma (n=6)	Sunitinib 50 mg/day on a 4-week and 2-week-off schedule	NS	Spontaneous MRONJ (n=2), dental procedure (n=2), denture-induced trauma (n=2)	Zoledronic acid	NS	Smidt-Hansen *et al.* (2013)[54]

Case report	Breast cancer (n=1)	Bevacizumab 15 mg/kg IV infusion	4	Tooth extraction	Carboplatin, docetaxel and cortisone, cyclophosphamide, epirubicin, 5-fluorouracil	Mandible	Nikitakis *et al.* (2016)[55]
Retrospective	Renal cell carcinoma (n=44)	Sunitinib, everolimus, bevacizumab, sorafenib Dose: NS	1 to 26	Periodontal infection (34%), tooth extraction (30%), ill-fitting dentures (9%), other oral surgical procedures (4.5%)	Zoledronic acid, pamidronate, ibandronate	Mandible (52%) Maxilla (36%)	Fusco *et al.* (2015)[25]
Case report	Hepatocellular carcinoma (n=1)	Sorafenib 400 mg/day	3	Tooth extraction	Furosemide, potassium canrenoate, bisoprolol, allopurinol, tamsulosin, hydroxychloroquine, vitamin D and sertraline	Mandible	Garuti *et al*. (2016)[45]
Case report	Adenocarcinoma of colon (n=1)	Aflibercept	6	Chronic periodontitis	Cetuximab plus capecitabine/oxaliplatin	Mandible	Ponzetti *et al.* (2016)[35]
Case report	Breast cancer (n=1)	Everolimus 10 mg/day	2	None	Exemestane, tamoxifen and fulvestrant	Mandible	Yamamoto *et al.* (2017)[43]
Retrospective	ALL (n=1) CLL (n=1) GM (n=1) MM (n=1) NSCLC (n=2) RCC (n=1)	Afatinib, bevacizumab, cetuximab, dasatinib, everolimus, erlotinib, imatinib, nilotinib, pazopanib, sunitinib, thalidomide Dose: NS	0.5 to 48	Tooth extraction	Alendronate, zolendronic acid pamidronate, denosumab, carboplatin, cisplatin, vinorelbine, alimeta, temozolomide, irinotecan, cyclophosphamide, alkeran, oxaliplatin, cytarabine, vincristine, blinatumomab, capecitabine, trastuzumab	Mandible Maxilla	Abel Mahedi Mohamed *et al*. (2018)[46]

NS=not specified; IV= intravenous
ALL=acute lymphocytic leukemia; CLL=chronic lymphocytic leukemia; GM=glioblastoma multiforme; MM=multiple myeloma; NSCLC=non–small-cell lung cancer; RCC=renal cell carcinoma

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Artigo 2

3 ARTIGO 2

O artigo a seguir intitula-se **Effect of tyrosine kinase inhibitor *sunitinib* on tissue repair at tooth extraction sites: a histomorphometric study in Wistar rats** e foi formatado de acordo com as normas do periódico ***Oral Oncology*** (Anexo B).

Effect of tyrosine kinase inhibitor *sunitinib* on tissue repair at tooth extraction sites: a histomorphometric study in Wistar rats

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Running Title: *Sunitinib and tooth extraction healing*

ABSTRACT

Objective: The aim of this study was to investigate the effect of sunitinib on tissue repair at tooth extraction sites.

Material and Methods: Fifty-two Wistar rats were allocated into four groups according to the treatment received: (1) sunitinib; (2) sunitinib/zoledronic acid; (3) zoledronic acid; (4) control group. The animals were subjected to extractions of the right upper molars, and maxillae were dissected and macro- and microscopically analyzed.

Results: On macroscopic evaluation, the zoledronic acid group showed a significantly higher prevalence of oral mucosal lesion than the other groups; however, the size of this lesion did not significantly differ between groups. The sunitinib/zoledronic acid group had significantly less epithelium than the zoledronic acid and control group, but showed no significant difference compared to the sunitinib group. The other groups did not show any significant difference regarding this variable. The sunitinib/zoledronic acid and zoledronic acid groups did not differ from each other, but had significantly less connective tissue and more non-vital bone and microbial colonies than the sunitinib and control groups, whereas these latter two groups did not significantly differ from each other. Vital bone, inflammatory infiltrate and tooth fragment did not significantly differ between the groups.

Conclusion: Sunitinib alone is not associated with non-vital bone, whereas the sunitinib/zoledronic acid combination and zoledronic acid alone are.

INTRODUCTION

Medication-related osteonecrosis of the jaw (MRONJ) is an adverse effect that has been reported in cancer patients subjected to different anticancer drug therapies [1]. The first cases of MRONJ were related to bisphosphonates in 2003 [2], and since then, other anticancer drugs have been associated with the disease [3,4]. Local factors play a significant role in MRONJ etiology, where tooth extraction is a major one, with 52 to 61% of patients reporting this intervention before lesion onset [5,6].

Currently, three groups of drugs are known to be MRONJ-related: bisphosphonates, denosumab and antiangiogenics [7,8]. The main action of antiangiogenics is the inhibition of vascular endothelial growth factor (VEGF), which is expressed in the majority of malignant tumors [9], and tumor neoangiogenesis is thereby suppressed. This group comprises bevacizumab, sunitinib, cabozantinib, everolimus, lenalidomide, pazopanib, ramucirumab, sorafenib, aflibercept, thalidomide and sirolimus. Sunitinib is a receptor tyrosine kinase (RTK) inhibitor launched on the market in 2006 (Pfizer, New York, NY, USA) [10]. RTK inhibition blocks multiple targets including VEGFR-1, VEGFR-2, fetal liver tyrosine kinase 3 (FLT3), PDGFRα and PDGFRβ in cellular and biochemical assays, which in turn, inhibits cell proliferation, migration and differentiation and neoangiogenesis and cancer cell invasion [11,12]. Sunitinib has been indicated in the treatment of stromal gastrointestinal carcinoma, metastatic renal cell cancer and pancreatic neuroendocrine tumor [11].

The risk of MRONJ increases for patients being treated with sunitinib combined with intravenous bisphosphonate, showing a prevalence of 0.9 to 2.4% [13]. This happens because VEGFR inactivation and consequent angiogenesis blockade impairs tissue healing [14], hampering bone healing and remodeling [15]. Several reports in the literature corroborate the notion of increased risk of MRONJ in such patients [7,14,16,17]. Koch *et al.* [18], in turn, reported a case of patient undergoing only sunitinib therapy, who developed MRONJ after a tooth extraction. These authors pointed to sunitinib as a possible causative factor of MRONJ even when used as single drug therapy.

MRONJ association with antiangiogenics still has some obscure points since most reported cases refer to patients having undergone or undergoing treatment with

both sunitinib and bisphosphonate. Therefore, the aim of this study was to investigate the effect of sunitinib on tissue repair at tooth extraction sites in animal models.

MATERIAL AND METHODS

The present study was approved by the Ethics Committee on Animal Use of Pontifical Catholic University of Rio Grande do Sul (CEUA-PUCRS) under protocol #8305. The sample was composed of 52 female rats (*Rattus novergicus*, Wistar strain) from the Central Facility (CEMBE/PUCRS), with a mean age of 70 days and mean weight of 250 g. The calculation of the sample size, with a margin of error of 1%, significance level of 5% and power of 80%, based on Maahs *et al*. [19], indicated the need for 11 rats per group (software WinPepi, version 11.28). This number was increased by 2 per group (20%) considering possible losses during the experiment period.

The animals were kept in microisolator cages with controlled temperature (23±1ºC) and 12-h light-dark cycle, with lighting of 300 lux in the center of the room and 60 lux inside the cages. The cages were cleaned and exchanged according to the facility center protocol, and feed (Nuvilab, Colombo, PR, Brazil) and filtered water were provided *ad libitum*. The animals were randomly allocated into 4 groups: (1) 13 animals that were given sunitinib (SU11248; sutent; Pfizer, Inc., New York, NY, USA); (2) 13 animals that were given sunitinib and zoledronic acid (Novartis Pharma, Basel, Switzerland); (3) 13 animals that were given zoledronic acid; and (4) control group: 13 animals with no drug. The first administration of both drugs was carried out at the beginning of the experiment, after labeling and weighing of the animals. Sunitinib was administered by gavage at a dose of 6 mg/kg/day for 35 days, and zoledronic acid was administered by the intraperitoneal route (IP) at a dose of 0.3 mg/kg/week for a total of 5 doses. In the control group, 6 rats received IP saline at the amount of 1 mL/kg/week,

and 7 rats received filtered water, 1 mL/kg/day by gavage. The animals were weighed every 7 days to adjust the doses.

Tooth extractions

Tooth extractions were performed 15 days after beginning the experiment, respecting a 3-day wash-out period (48 h before and 24 h after the tooth extractions) for sunitinib. The procedure was performed under deep anesthesia with mixture of ketamine (100 mg/kg; Syntec, Cotia, SP, Brazil) and xylazine (10 mg/kg; Syntec, Cotia, SP Brazil) administered IP, with the animal in dorsal decubitus [20]. The right upper molars were extracted using a lever movement with a #3s Hollenback carver (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) and pediatric forceps (Edlo, Canoas, RS, Brazil) whose functional portion was adapted to the size of the teeth. Right after the tooth extractions, the animals were returned to the cages where they remained on a sterile surgical pad and under controlled body temperature until the anesthetic effect subsided. During the postoperative period, the animals received dipyrone IP at a dose of 200 mg/kg every 24 h for three days, and mashed chow was provided. A total of 5 animals were lost due to complications during the surgical procedure: 2 animals from the sunitinib group, 2 animals from the sunitinib/zoledronic acid group and 1 animal from the zoledronic acid group. Six rats from the sunitinib group and 5 rats from the sunitinib/zoledronic acid group developed skin desquamation and necrosis, as well as edema of the extremities.

Euthanasia, macroscopic evaluation, and preparation of the specimens

The animals were sedated by IP administration of 5% ketamine hydrochloride at a dose of 70 mg/kg and 2% xylazine hydrochloride at a dose of 7 mg/kg and subjected to cardiac puncture and exsanguination. After exsanguination, an overdose of the ketamine and xylazine mixture was also administered. After euthanasia, the maxilla was dissected and subjected to macroscopic evaluation to determine the presence/absence and size of

oral mucosal lesion in the area of tooth extractions by using a #5 dental explorer and a periodontal probe (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil). The observer was blinded to the group examined and oral mucosal lesion was considered if there was loss of mucosal integrity. The specimens (maxillae) were then fixed for 24 h in 10% buffered formalin. After fixation, the osteotomized segment comprising the tooth extraction area was cut in the middle in a buccal–lingual direction into two pieces, both of them displaying the area of interest at the cut surface.

After decalcification in 10% nitric acid for 8 h, the specimens were embedded in paraffin, and 4 μm-thick sections were obtained, processed and stained with hematoxylin and eosin (H&E).

Capture of the images and histological analysis

Histological images were captured with an Olympus BX-43 light microscope (Olympus, Tokyo, Japan), connected to a computer with Olympus DP-73 digital camera (Olympus). Five fields of each slide were captured using a 10x objective, and the images were stored as uncompressed TIFF (tag image file format). The analysis was carried out by means of the manual point-counting technique (Image Pro Plus 5.1, Media Cybernetics, Bethesda, MD, USA) [21], where epithelium, connective tissue, vital bone, non-vital bone, inflammatory infiltrate, microbial colonies, and tooth fragment were quantified [19] (Fig.1). The observer was blinded (not knowing the group to which each image belonged) and calibrated. Calibration consisted of analyzing a series of 35 images, twice, at two different moments. The results of these analyses were tested by intraclass correlation coefficient, which showed $r = 0.9$.

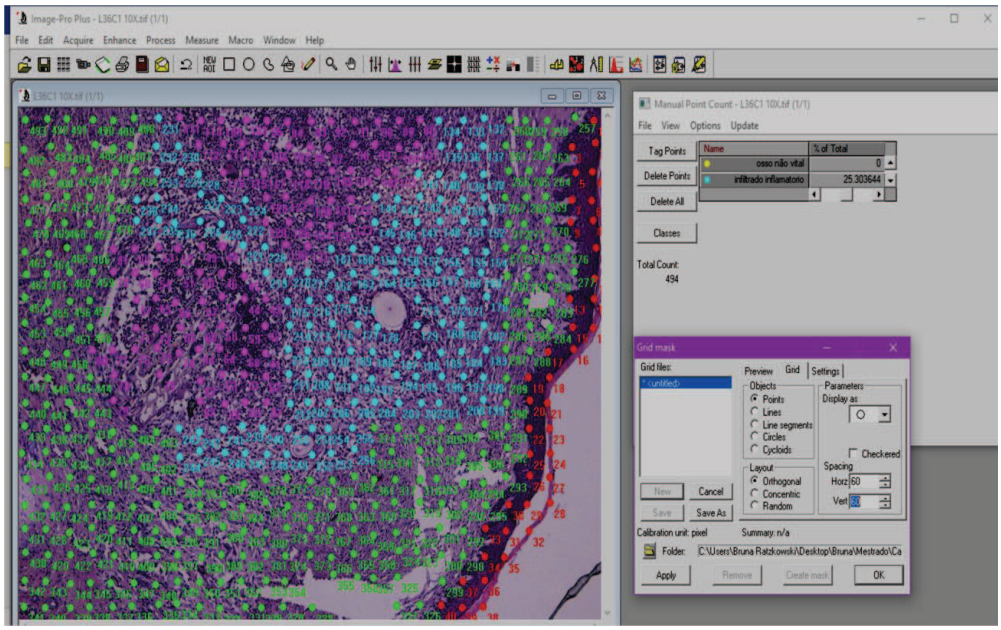

Figure 1 – Quantitative histological analysis by means of manual point-counting technique (Image Pro-plus software, Media Cybernetics, Bethesda, MD, USA)

Statistical analysis

Data were analyzed with descriptive and inferential statistics. The chi-square test was used to compare oral mucosal lesions and non-vital bone frequencies between the groups, and the Kruskal-Wallis test complemented by the Student-Newman-Keuls test was applied to compare the size of oral lesions and the measure of histological variables. The Spearman coefficient tested the relationship between the variables. Statistical analysis was performed in SPSS 17.0 (Statistical Product and Service Solutions, SPSS Inc, USA) at a significance level of 5%.

RESULTS

Macroscopic analysis

The zoledronic acid group showed a significantly higher frequency of oral mucosal lesion than the other groups (*P*=0.046). There was no difference in lesion occurrence between the sunitinib, sunitinib/zoledronic acid and control groups (chi-square, adjusted residual analysis, Table 1). With regard to lesion size, the groups did not show any significant difference (Kruskal-Wallis, *P*=0.670, Table 2).

Table 1 – Macroscopic analysis: sample distribution according to presence/absence of oral mucosal lesion (loss of mucosal integrity)

Group	Presence		Absence		Total		
	n	%	n	%	n	%	*P**
Sunitinib	9	81.82	2	18.18	11	100	
Sunitinib/zoledronic acid	7	63.64	4	36.36	11	100	**0.046**
Zoledronic acid	**12****	100	-	0	12	100	
Control	7	53.85	**6****	46.15	13	100	

n=number of animals; **P* value for chi-square test
**Statistically significant, chi-square test, adjusted residual analysis, α=0.05

Table 2 – Macroscopic analysis: size of the oral lesions (mm^2)

Group	**Size** (mm^2)				
	Mean	SD	Median	P25	P75
Sunitinib	2.66	3.22	1.00	0.25	5.00
Sunitinib/zoledronic acid	4.27	4.08	3.50	0.00	8.00
Zoledronic acid	2.58	2.65	1.75	1.00	2.88
Control	2.62	3.75	1.00	0.00	4.75
*P**			0.670		

**P* value for Kruskal-Wallis, α=0.05

Histological analysis

Presence/absence of non-vital bone in the sample

Table 3 displays the sample distribution in the groups according to the presence/absence of non-vital bone. The sunitinib and control groups were associated with absence of non-vital bone, whereas the sunitinib/zoledronic acid group showed an association with the presence of non-vital bone. Although the zoledronic acid group showed 66.7% of animals with non-vital bone, this was not statistically significant (chi-square, adjusted residual analysis, α=0.05).

Table 3 – Sample distribution according to presence/absence of non-vital bone

Group	**Non-vital bone**						*P**
	Presence		Absence		Total		
	n	%	n	%	n	%	
Sunitinib	2	18.2	**9****	81.8	11	23.4	
Sunitinib/zoledronic acid	**9****	81.8	2	18.2	11	23.4	
Zoledronic acid	8	66.7	4	33.3	12	25.5	**0.003**
Control	3	23.1	**10****	76.9	13	27.7	
Total	22	46.8	25	53.2	47	100	

n=number of animals; **P* value for chi-square test, adjusted residual analysis, α=0.05
**Statistically significant

Quantitative analysis of the histological variables

The sunitinib/zoledronic acid group had significantly less epithelium than the zoledronic acid group and the control, but showed no significant difference with regard to the sunitinib group. There was no significant difference in this variable between the other groups. The sunitinib/zoledronic acid and the zoledronic acid groups did not differ from each other, but had significantly less connective tissue and more non-vital bone and microbial colonies than the sunitinib and the control groups, where the latter two groups did not significantly differ from each other with regard to these variables. Vital

bone (P=0.328), inflammatory infiltrate (P=0.117) and tooth fragment (P=0.309) did not significantly differ between the groups evaluated (Kruskal-Wallis, Student-Newman-Keuls, α=0.05, Table 4). Figure 2 illustrates some of the histological variables analyzed.

Table 5 displays the values for "r" in correlation analysis between the variables using Spearman coefficient. Epithelium was negatively correlated with tooth fragment (r= -0.423); connective tissue was negatively correlated with vital bone (r= -0.407), non-vital bone (r= -0.537), inflammatory infiltrate (r= -0.417), and microbial colonies (r= -0.387); vital bone was negatively correlated with inflammatory infiltrate (r=-0.454). Non-vital bone was positively correlated with inflammatory infiltrate (r=0.523) and with microbial colonies (r=0.603). Inflammatory infiltrate was positively correlated with microbial colonies (r=0.401).

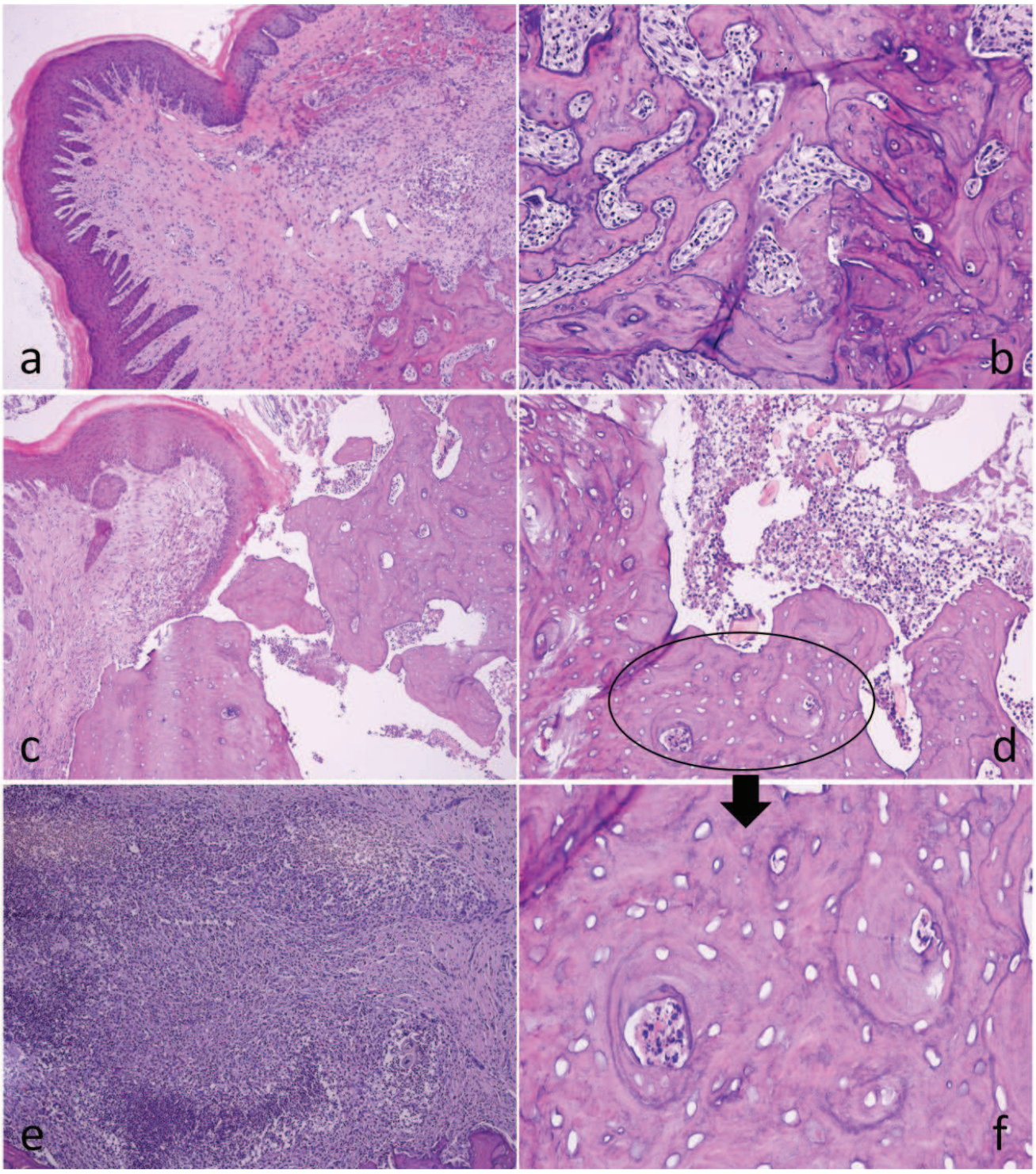

Figure 2 – Histological features on microscopic analysis: (a) complete healing of the surgical wound (H&E, 100X); (b) vital bone (H&E, 200X); (c) non-healing surgical wound showing loss of integrity of oral mucosa and non-vital bone (H&E, 100X); (d) non-vital bone area (H&E, 100X); (e) inflammatory infiltrate in the connective tissue (H&E, 200X); (f) close-up of the non-vital bone seen in the "d" image (H&E, 400X)

Table 4 – Histological analysis: quantification (% of the microscopic field) of the histological variables in the groups analyzed

Variable	Group												*P**
	Sunitinib			Sunitinib/zoledronic acid			Zoledronic acid			Control			
	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	
Epithelium	**6.57^{AB}**	8.25	5.22	**3.92^{B}**	5.27	2.97	**7.80^{A}**	8.41	4.27	**9.61^{A}**	10.29	3.90	**0.02**
Connective tissue	**49.37^{A}**	48.64	9.95	**25.54^{B}**	25.44	8.30	**26.15^{B}**	26.41	11.33	**44.04^{A}**	40.83	10.49	**0.0000**
Vital bone	30.33 ^{A}	29.35	11.16	41.19 ^{A}	36.71	16.71	36.29 ^{A}	39.08	21.92	33.03 ^{A}	32.08	7.86	0.3280
Non-vital bone	**0.00^{A}**	0.62	1.45	**7.61^{B}**	10.77	10.70	**3.03^{B}**	5.64	7.54	**0.00^{A}**	0.71	1.90	**0.0006**
Inflammatory infiltrate	1.55^{A}	6.78	10.59	7.41 ^{A}	10.93	10.65	12.27^{A}	13.28	9.97	6.27 ^{A}	7.49	5.78	0.1175
Microbial colonies	**0.00^{A}**	0.84	2.45	**1.44^{B}**	3.19	5.43	**1.08^{B}**	3.95	6.46	**0.00^{A}**	0.21	0.36	**0.0197**
Tooth fragment	1.91^{A}	5.50	8.19	5.19 ^{A}	7.70	8.76	1.66 ^{A}	3.24	4.28	10.25 ^{A}	8.38	7.13	0.3099

**P* value for Kruskal-Wallis, α=0.05
Medians followed by different letters in the row showed significant difference, Kruskal-Wallis complemented by Student-Newman-Keuls, α=0.05

Table 5 – "*r*" values in correlation analysis between the variables using Spearman coefficient

Variable	Epithelium	Connective tissue	Vital bone	Non-vital bone	Inflammatory infiltrate	Microbial colonies	Tooth fragment
Epithelium	1						
Connective tissue	0.231	1					
Vital bone	-0.213	**-0.407***	1				
Non-vital bone	-0.231	**-0.537***	-0.084	1			
Inflammatory infiltrate	0.030	**-0.417***	**-0.454***	**0.523***	1		
Microbial colonies	-0.214	**-0.387***	-0.198	**0.603***	**0.401***	1	
Tooth fragment	**-0.423***	-0.182	0.129	-0.155	-0.073	0.009	1

*Correlation is significant at the 0.01 level

DISCUSSION

The zoledronic acid group was the only one that showed an association with oral mucosal lesion on macroscopic analysis, whereas lesion frequency in the sunitinib groups did not significantly differ compared to control. The odd finding here was that the sunitinib/zoledronic acid group had no association with lesion, where it was expected to have at least the same rate as the zoledronic acid group. However, it is important to point out that since the control group had similar results as the experimental ones, it is more plausible that some of the macroscopic lesions could have resulted from the tooth fragments persisting at the extraction site and not as a consequence of the drug used, which agrees with the results for this variable in the histological analysis. These facts reinforce the great importance of microscopic evaluation.

Considering the frequency of animals having non-vital bone on microscopic examination, the groups treated with zoledronic acid whether or not in combination with sunitinib showed the highest prevalence, although only the sunitinib/zoledronic acid group showed a statistically significant difference. This finding indicated that sunitinib could potentiate the effect of zoledronic acid, whereas sunitinib alone would not be capable of causing the lesion. Another point to consider is that this was a dichotomous analysis in a relatively small sample, where non-vital bone criterion was bone tissue with empty lacunae (with no osteocytes) [22-24]. This analysis did not take into account the amount of this variable or the other features usually observed in MRONJ lesions, such as microbial biofilm and inflammatory infiltrate [25,26]. We know that empty lacunae can sometimes be an artifact resulting from the histological process [27]. These factors could impart a bias in this evaluation, and therefore, the quantitative analysis of the histological features must also be considered.

The sunitinib/zoledronic acid group had significantly less epithelium, which agrees with the results for non-vital bone in this group, since oral mucosa is incapable of re-epithelialization and of uniting the edges of the wound in areas of osteonecrosis [28,29]. Our findings are also in agreement with the literature, in that the initial damage induced by sunitinib in the oral cavity may affect not only vascular tissue but also keratinocytes [30]. Accordingly, it is believed that oral mucositis caused by sunitinib could progress to osteonecrosis [14]. Connective tissue levels, in turn, were significantly less in the sunitinib/zoledronic acid and zoledronic acid groups and also negatively correlated with non-vital bone, inflammatory infiltrate and microbial colonies, indicating that its lower levels in these groups were a result of the occurrence of osteonecrosis. These same groups (sunitinib/zoledronic acid and zoledronic acid) had significantly greater amounts of non-vital bone and microbial colonies than did the sunitinib and control groups, where these latter two groups did not significantly differ from each other. This is an important finding, where sunitinib seemed to have detrimental effects on bone repair only when combined with zoledronic acid. On the other hand, zoledronic acid combined or not with sunitinib was capable of impairing the healing of the surgical wound, as previously reported [8,19,31]. This would suggest that sunitinib causes non-vital bone only if combined with zoledronic acid, and considering that the sunitinib/zoledronic acid group did not show statistically greater levels of this variable than the zoledronic acid group, sunitinib did not potentiate the effect of zoledronic acid. This is corroborated by the finding that non-vital bone did not differ between the sunitinib group and control.

Our results suggest that the association of MRONJ with antiangiogenics still leaves some doubts, considering that these drugs are often administered in combination with bisphosphonates [16] and denosumab [32], either concomitantly or sequentially. Maybe the growing number of case reports of antiangiogenic-related MRONJ should be critically

considered, especially making sure that the patient has not undergone bisphosphonate therapy in preceding years, since this drug (bisphosphonate) has such a long half-life and long-lasting effects over the time elapsed [33-35].

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

The present study was approved by the Ethics Committee on Animal Use of Pontifical Catholic University of Rio Grande do Sul (CEUA #8305). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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HIGHLIGHTS

The number of antiangiogenic-related MRONJ cases has increased.

Antiangiogenics are often used in combination with bisphosphonates and denosumab.

Relationship of antiangiogenics with MRONJ needs to be investigated.

Discussão Geral

4 DISCUSSÃO GERAL

A osteonecrose maxilar associada a medicamentos (MRONJ) é uma enfermidade de etiopatogenia incerta e, muitas vezes, de difícil tratamento. Inicialmente, os casos foram associados a bisfosfonatos e, posteriormente, denosumabe e antiangiogênicos também passaram a compor o rol de medicamentos associados à condição (Marx, 2003; Ruggiero *et al*., 2004; Ruggiero *et al*., 2014). Os primeiros relatos de MRONJ relacionada ao uso de antiangiogênicos datam de 2008 e, até o momento, cerca de 35 casos já foram relatados na literatura (Estilo *et al*., 2008; Pimolbutr *et al*., 2018). Esses fármacos são empregados na terapia anticâncer de pacientes portadores de tumor gastrointestinal, carcinoma de células renais e tumor neuroendócrino, entre outros (Ruggiero *et al*., 2014). Muitos dos pacientes encontram-se em fase avançada da doença e usam ou já usaram uma ampla variedade de fármacos, incluindo quimioterápicos, denosumabe e bisfosfonatos (Beuselink *et al*., 2012; Sivolella *et al*., 2013). Considerando o acima exposto, bem como o fato de que os bisfosfonatos têm efeito persistente, mesmo após cessada sua administração, parece temerário afirmar que os antiangiogênicos tenham *per se* capacidade de determinar o desenvolvimento de MRONJ. Foi nesse contexto que se originou a ideia do presente estudo, cujo experimento *in vivo* conduziu análise histomorfométrica de sítios de exodontias em ratos submetidos à terapia com o antiangiogênico sunitinibe, em administração isolada ou em combinação com o bisfosfonato ácido zoledrônico.

De acordo com os resultados obtidos, o grupo ácido zoledrônico foi o único que apresentou associação com a ocorrência de lesão da mucosa oral durante a análise macroscópica. Na avaliação de presença/ausência de osso não-vital ao exame microscópico, os grupos tratados com ácido zoledrônico, associado ou não ao sunitinibe,

exibiram a maior prevalência. Esses achados corroboram relatos da literatura de que o risco de MRONJ com o uso de ácido zoledrônico, independentemente de comorbidades sistêmicas, é comprovado (Hoff *et al*., 2008; Marx *et al*., 2005).

O grupo sunitinibe, entretanto, não diferiu significativamente do grupo-controle para frequência de lesão ao exame macroscópico. Esse resultado se repetiu na microscopia, tanto pela avaliação dicotômica, quanto pela avaliação quantitativa de osso não-vital, em que o grupo sunitinibe exibiu valores similares aos do grupo-controle. Tais achados sugerem que o uso isolado de sunitinibe não seria capaz de determinar a ocorrência de MRONJ.

Considerando as demais variáveis histológicas, o grupo sunitinibe/ácido zoledrônico exibiu significativamente menos epitélio, o que pode ser justificado pela maior ocorrência de osso não-vital neste grupo, uma vez que a mucosa oral é incapaz de reepitelizar as áreas de osteonecrose (Landesberg *et al.,* 2008, Ravosa *et al*., 2011). Além disso, o efeito do sunitinibe na cavidade oral afeta também os queratinócitos (Mignogna *et al*., 2009), o que pode ter colaborado para a menor ocorrência de epitélio no grupo que combinou os dois fármacos. Corroborando esses achados estão os relatos da literatura de que pacientes submetidos a terapia com sunitinibe podem apresentar vários efeitos adversos que incluem mucosite, alterações gustativas e lesões cutâneas (Christodoulou *et al*., 2009; Fleissig *et al.*, 2012).

Os grupos sunitinibe/ácido zoledrônico e ácido zoledrônico tiveram quantidades significativamente maiores de osso não-vital e de colônias microbianas do que os grupos sunitinibe e controle. Esse é um resultado importante, em que o sunitinibe parece estar associado a efeitos deletérios no reparo ósseo somente se combinado ao ácido zoledrônico, enquanto o ácido zoledrônico, independentemente de estar combinado ou não ao sunitinibe, é capaz de comprometer a cicatrização da ferida cirúrgica, como já

relatado anteriormente (Maahs *et al*., 2011; Marx *et al*., 2005; Schwartz, 2005). Ainda, o achado de que o grupo sunitinibe/ácido zoledrônico não exibiu quantidade significativamente maior de osso não-vital do que o grupo ácido zoledrônico, indica que o sunitinibe sequer potencializou o efeito do bisfosfonato. Isso é confirmado pelo fato de que a quantidade de osso não-vital não diferiu entre os grupos sunitinibe e controle.

Os resultados do presente estudo colocam em questionamento a capacidade de os antiangiogênicos *per se* determinarem o desenvolvimento de MRONJ. Uma vez que essas drogas são, frequentemente, administradas em combinação com bisfosfonatos e denosumabe (Sivolella *et al.,* 2013), algumas de suas propriedades farmacológicas devem ser ponderadas. Os bisfosfonatos ligam-se ao cálcio da hidroxiapatita, incorporando-se ao tecido ósseo, e sua meia-vida pode ser superior a dez anos (Marx, 2014). Isto é, mesmo após suspensa a administração, o paciente tratado com bisfosfonato permanece sob efeito do fármaco (Marx *et al*., 2005). Já o denosumabe e os antiangiogênicos, têm meia-vida mais curta, que vai de 2,5 a 32 dias, sendo eliminados mais rapidamente (Narayanan, 2013; Bodnar, 2014). Isso deve ser levado em conta na avaliação dos pacientes que desenvolvem MRONJ durante o tratamento com antiangiogênicos. As características farmacológicas dos antiangiogênicos, principalmente sua meia-vida e a não incorporação ao tecido ósseo, fazem pensar que a MRONJ a eles associada seria mais fácil de controlar e teria melhor prognóstico. Assim, casos dessa enfermidade em pacientes usuários de antiangiogênicos, que se mostrem resistentes e não-responsivos ao tratamento, devem despertar a necessidade de certificação da ausência de uso de bisfosfonato na história médica atual ou pregressa do paciente.

Os resultados do presente estudo são preliminares e levantam uma possibilidade que deve ser mais profundamente investigada. É preciso lembrar que muitos dos

fármacos em questão são medicamentos lançados há pouco tempo no mercado. À medida que o contingente de pacientes usuários dessas terapias cresce, novos casos de efeitos adversos surgem e o conhecimento vai sendo sedimentado por meio de evidências e de novas pesquisas. Assim, estudos *in vivo* com os demais antiangiogênicos disponíveis no mercado para tratamento anticâncer, bem como estudos de casos com maior número de pacientes seriam úteis para elucidar a relação desses fármacos com a MRONJ.

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ANEXO A

Normas para submissão de artigos ao periódico *Gerodontology*

https://onlinelibrary.wiley.com/journal/17412358

ANEXO B

Normas para submissão de artigos ao periódico *Oral Oncology*

https://www.journals.elsevier.com/oral-oncology

ANEXO C

SIPESQ

Sistema de Pesquisas da PUCRS

Código SIPESQ: 8305

Porto Alegre, 4 de outubro de 2017.

Prezado(a) Pesquisador(a),

A Comissão Científica da FACULDADE DE ODONTOLOGIA da PUCRS apreciou e aprovou o Projeto de Pesquisa "Efeito do inibidor de quinase sunitinibe sobre a cicatrização alveolar pós-exodontia: estudo histomorfométrico e imunoistoquímico 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 D

SIPESQ

Sistema de Pesquisas da PUCRS

Código SIPESQ: 8305 Porto Alegre,25 de outubro de 2017

Prezado(a) Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou o Projeto de Pesquisa "Efeito do inibidor de quinase sunitinibe sobre a cicatrização alveolar pós-exodontia: estudo histomorfométrico e imunoistoquímico 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.

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: 25/10/2017 - 25/04/2018

Nº de Animais	Espécie
52	Rattus norvegicus
Total de Animais: 52	

Atenciosamente,

Comissão de Ética no Uso de Animais(CEUA)