FACULDADE DE ODONTOLOGIA

EFEITO DA OXIGENOTERAPIA HIPERBÁRICA SOBRE O REPARO ALVEOLAR PÓS-EXODONTIA EM RATOS WISTAR TRATADOS COM BISFOSFONATO - ANÁLISE HISTOMORFOMÉTRICA E IMUNOISTOQUÍMICA

MIGUEL LUCIANO SILVA

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EFFECT OF THE HYPERBARIC OXYGEN THERAPY ON POST-EXTRACTION ALVEOLAR HEALING IN WISTAR RATS TREATED WITH BISPHOSPHONATE - HISTOMORPHOMETRIC AND IMMUNOHISTOCHEMICAL ANALYSIS

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Epígrafe

A experiência nunca falha, apenas as nossas opiniões falham,

ao esperar da experiência aquilo que ela não é capaz de oferecer.

Leonardo da Vinci (1452-1519)

Dedicatória

Aos meus pais, Miguel e Helena, à minha esposa Gisela, e à minha irmã Priscila, por todo amor e incentivo.

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Resumo

RESUMO

A osteonecrose maxilar associada aos bisfosfonatos (*bisphosphonate-related osteonecrosis of the jaw*, BRONJ) é um importante efeito adverso desses fármacos. A condição exibe elevada morbidade e difícil tratamento, sendo que várias modalidades terapêuticas têm sido empregadas, entre as quais se destacam antibioticoterapia, intervenções cirúrgicas e terapias alternativas como a terapia a laser de baixa intensidade, plasma rico em plaquetas e oxigenoterapia hiperbárica (*hyperbaric oxygen therapy*, HBOT). Entretanto, não existe consenso sobre a efetividade da HBOT no tratamento da BRONJ. O presente estudo teve por objetivo investigar o efeito da HBOT em sítio de exodontias em ratos sob tratamento com bisfosfonatos. Trinta e cinco ratos Wistar foram tratados com ácido zoledrônico, submetidos a exodontias e distribuídos em grupos de acordo com o regime de HBOT recebido: (1) 7 dias de HBOT; (2) 14 dias de HBOT; (3) sem HBOT (controle de 7 dias); (4) sem HBOT (controle de 14 dias). O sítio das exodontias foi analisado por meio de histomorfometria e imunoistoquímica. O volume de exposição óssea não diferiu significativamente entre os períodos pré- e pós-HBOT, nem entre grupo-teste e controle. Aos 7 dias, o grupo HBOT exibiu proporção de epitélio e resto radicular significativamente menor que o controle. Aos 14 dias, a proporção de osso não-vital foi significativamente menor no grupo HBOT que no controle. Quando os grupos-teste foram comparados entre si, foi observada maior proporção de osso não-vital e menor proporção de resto radicular aos 7 dias. O grupo HBOT exibiu, aos 7 dias, expressão de VEGF, RANKL, BMP-2 e OPG significativamente menor que o controle, enquanto aos 14 dias essa diferença não foi significativa. Quando os grupos-teste foram comparados entre si, VEGF e OPG exibiram expressão significativamente maior aos 14 dias, enquanto RANKL e BMP-2 não exibiram diferença significativa.

Conclusão: A HBOT está associada a menor proporção de osso não-vital detectado microscopicamente em sítios de exodontias de ratos submetidos a terapia com bisfosfonato. O efeito parece ser dose-dependente e novos estudos são necessários para esclarecer os mecanismos responsáveis por esse efeito.

Palavras-chave: osteonecrose; bisfosfonatos; oxigenoterapia hiperbárica; ossos maxilares; ratos

Summary

SUMMARY

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is an important side-effect of bisphosphonates. The condition has high morbidity and its treatment is difficult, where many therapies have been tried including antibiotics, surgical interventions, and also some alternative therapies such as low-level laser therapy, platelet-rich plasma and hyperbaric oxygen therapy (HBOT). Nevertheless, there is no consensus about the effectiveness of HBOT in BRONJ. The aim of the present study was to investigate the effect of HBOT on tooth extraction site in rats treated with bisphosphonate. Thirty-five Wistar rats were treated with zoledronic acid, subjected to tooth extractions and allocated into groups according to HBOT regimen: (1) 7 days of HBOT; (2) 14 days of HBOT; (3) without HBOT (7-day control); (4) without HBOT (14-day control). The site of tooth extractions was analyzed by histomorphometry and immunohistochemistry. Bone exposure volume did not significantly differ between pre- and post-HBOT or between test groups and controls. At 7 days, the HBOT group showed amounts of epithelium and root fragment that were significantly less than the control. At 14 days, non-vital bone was significantly less in the HBOT group than in the control. HBOT groups compared to each other showed higher amounts of non-vital bone and less root fragment at 7 days. The HBOT group showed at 7 days lower expression of VEGF, RANKL, BMP-2 and OPG compared to the control, whereas at 14 days, there was no significant difference. Comparing HBOT groups at 7 and 14 days to each other, VEGF and OPG showed significantly higher expression at 14 days, whereas RANKL and BMP-2 did not show any significance.

Conclusion: HBOT can reduce the amounts of non-vital bone microscopically detected in tooth extraction sites of rats subjected to bisphosphonate therapy. The effect seems to occur in a dose-dependent mode. Further studies are required to clarify the mechanisms accounting for this effect.

Key words: osteonecrosis; bisphosphonates; hyperbaric oxygen therapy; jaws; rats

Sumário

SUMÁRIO

Introdução

1 INTRODUÇÃO

Bisfosfonatos são análogos sintéticos do pirofosfato em que o átomo central de oxigênio da estrutura P-O-P (fósforo-oxigênio-fósforo) é substituído por um átomo de carbono (P-C-P). Essa alteração faz com que tais compostos sejam resistentes à degradação enzimática e tenham meia-vida biológica longa o suficiente para interferir no metabolismo ósseo (Cremers *et al*., 2005; Licata, 1997). Diferentes substituintes nos radicais R_1 e R2, ligados ao carbono central da molécula, conferem características específicas para cada fármaco, sendo o radical R1 responsável pela afinidade aos cristais de hidroxiapatita, enquanto R2 confere potência e atividade farmacológica (Drake *et al*., 2008; Fleisch, 1998; Russel, 2006). Em R1, a presença de hidoxila resulta em maior fixação óssea, enquanto o cloro representa fixação reduzida (Fleisch, 1998). Um grupo nitrogenado em R2 aumenta a potência, que é mensurada comparativamente ao bisfosfonato não-nitrogenado etidronato (Drake *et al*., 2008; Fleisch, 1998; Russel, 2006). Assim, o ácido zoledrônico, que contém nitrogênio em um anel heterocíclico em sua estrutura química, tem potência 10.000 vezes superior à do etidronato (Russel, 2006).

Os bisfosfonatos de uso oral são empregados no tratamento da osteoporose e da osteopenia, bem como em condições menos prevalentes como a doença de Paget do osso e a osteogênese imperfeita (Allen; Burr, 2009; Gliklich; Wilson, 2009; Greenberg, 2004; Leite *et al*., 2006; Marx, 2003; Ruggiero *et al*., 2004; Sonis *et al*., 2009). Já os intravenosos são indicados para o tratamento de condições relacionadas ao câncer, como hipercalcemia, metástases ósseas de câncer de mama, próstata e pulmão, e nas lesões líticas do mieloma múltiplo (Bagan *et al*., 2005; Berenson, 2002; Cetiner *et al*., 2009).

A remodelação óssea é um processo fisiológico essencial à manutenção das características da estrutura óssea normal, que remove microdanos e repõe ossos danificados com tecido neoformado (Migliorati *et al*., 2005). Os bisfosfonatos interferem

nesse processo inibindo o *turnover* ósseo por meio da indução de apoptose e perda de função osteoclástica (Heras Rincón *et al*., 2007; Ruggiero *et al*., 2009). O fármaco incorpora-se à matriz óssea ligando-se ao cálcio e, durante a remodelação, é retomado pelos osteoclastos e internalizado no citoplasma (Migliorati *et al.*, 2005). Neste sítio, os bisfosfonatos nitrogenados são capazes de interromper a via do mevalonato, uma importante via metabólica que possui papel-chave no processo de diferenciação e crescimento celular. A via é precursora de hormônios esteroidais e isoprenoides não esteróis, provendo moléculas bioativas essenciais às células (Buhaescu; Izzedine, 2007). Os bisfosfonatos não-nitrogenados, por sua vez, após internalizados pelo osteoclasto são metabolizados em análogos de ATP citotóxicos, responsáveis pela inibição da função mitocondrial, o que resulta em apoptose (Drake *et al*., 2008; Rogers *et al.*, 1994; Russel, 2006). Também tem sido sugerido que o bisfosfonato acumulado no osso seja tóxico ao epitélio oral e iniba a cicatrização normal de lesões dos tecidos moles (Reid *et al.*, 2007; Reid; Cundy, 2009).

A ação dos bisfosfonatos sobre o metabolismo ósseo tem determinado um importante efeito adverso denominado *osteonecrose dos maxilares associada aos bisfosfonatos* (*bisphosphonate-related osteoecrosis of the jaw*, BRONJ). Marx (2003) foi um dos pioneiros a associar a enfermidade ao uso de pamidronato e ácido zoledrônico, e a condição foi inicialmente definida pela exposição de tecido ósseo na cavidade oral por um período igual ou superior a oito semanas em indivíduo usuário de bisfosfonato que não tivesse sido submetido a radioterapia de cabeça e pescoço (AAOMS, 2007; Reid; Cundy, 2009). Recentemente, casos sem exposição óssea evidente foram incorporados ao conceito da enfermidade (Fedele *et al.*, 2010; Fedele *et al.*, 2015; Junquera; Galego, 2008; Rugani *et al.*, 2014; Ruggiero *et al.*, 2014), bem como sua associação a outras drogas inibidoras da reabsorção óssea e antiangiogênicas, sendo proposta a denominação *medication-related*

osteonecrosis of the jaw (MRONJ). A MRONJ é definida como uma complicação em pacientes sob terapia medicamentosa com drogas inibidoras da reabsorção óssea e/ou antiangiogênicas que se caracteriza por exposição óssea ou osso passível de sondagem por meio de fístula intra ou extraoral em maxila e/ou mandíbula, persistente por mais de oito semanas em paciente sem história de radioterapia ou doença metastática na região (Ruggiero *et al.*, 2014).

A etiologia da BRONJ é multifatorial, acreditando-se que diversos fatores de risco contribuam para a manifestação e intensidade da lesão, entre eles o diabetes (Khamaisi *et al.*, 2007), o tabagismo (Wessel *et al.*, 2008), intervenções cirúrgicas orais e o uso frequente de medicamentos como corticosteroides (Allen; Burr, 2009; Ruggiero *et al.*, 2004). Entretanto, a ocorrência de casos espontâneos também tem sido relatada (Curi *et al.*, 2007; Gliklich; Wilson, 2009; Marx, 2003; Marx *et al.*, 2005; Marx *et al.*, 2007; Montebugnoli *et al.*, 2007; Ruggiero *et al.*, 2004; Wang *et al.*, 2003).

A BRONJ tem ocupado lugar de destaque na literatura científica em função de sua elevada morbidade e difícil tratamento. A abordagem tem incluído diversas modalidades terapêuticas, com alguns casos respondendo favoravelmente, enquanto outros são refratários (Curi *et al.*, 2007; Hinson *et al.*, 2015; Marx *et al.*, 2005; Ruggiero *et al.*, 2004; Yoneda *et al.*, 2010). Há relatos de tratamentos conservadores com o uso de antimicrobianos sistêmicos e tópicos, bem como de ressecção cirúrgica da área afetada (Heras Rincón *et al.*, 2007). Tratamentos alternativos tais como plasma rico em plaquetas, laserterapia de baixa intensidade e oxigenoterapia hiperbárica também têm sido empregados (Bocanegra-Pérez, 2012; Cetiner *et al.*, 2009; Curi *et al.*, 2007; Lam *et al.*, 2007; Martins *et al.*, 2012; Rugani *et al.*, 2014; Ruggiero *et al.*, 2014; Rupel *et al.*, 2014; Vescovi *et al.*, 2013; Vescovi *et al.*, 2014). A oxigenoterapia hiperbárica é indicada para o tratamento de diversas enfermidades (Gill; Bell, 2004; UHMS, 2015), em que se incluem

doenças do tecido ósseo (Levin *et al.*, 1999; Peskin *et al.*, 2001), como a osteomielite crônica e a osteorradionecrose (Wreford-Brown; Hampson, 2003). Mais recentemente, sua indicação passou a ser sugerida também para o tratamento da BRONJ, situação em que alguns estudos clínicos têm apresentado resultados estimulantes (Biasotto *et al.*, 2006; Fatema *et al.*, 2015; Freiberger *et al.*, 2007; Freiberger *et al.*, 2012; Shimura *et al.*, 2006), embora outros autores contestem essa indicação (Dimopoulos *et al.*, 2006; Heras Rincón *et al.*, 2007; Marx *et al.*, 2005; Migliorati *et al.*, 2005; Nastro *et al.*, 2007; Ruggiero *et al.*, 2014).

Considerando a premência de terapias resolutivas para a BRONJ, bem como o fato de que as pesquisas sobre a efetividade da oxigenoterapia hiperbárica nessa enfermidade consistem em estudos clínicos em que a padronização é difícil e variáveis intervenientes influenciam consideravelmente os resultados, a presente pesquisa teve por objetivo investigar o efeito da oxigenoterapia hiperbárica em sítio de exodontias em modelo animal sob tratamento com bisfosfonato. O trabalho está estruturado sob a forma de dois artigos científicos, sendo que o primeiro consiste em uma revisão da literatura que aborda efeitos, indicações e contraindicações da oxigenoterapia hiperbárica, enfocando sua aplicabilidade à BRONJ, enquanto o segundo apresenta o experimento desenvolvido.

Artigo 1

2 ARTIGO 1

O artigo a seguir intitula-se ***Important topics on hyperbaric oxygen therapy with focus on its application in bisphosphonate-related osteonecrosis of the jaw*** e foi formatado de acordo com as normas e submetido ao periódico *Archives of Oral Biology* (Anexos A e B).

Important topics on hyperbaric oxygen therapy with focus on its application in bisphosphonate-related osteonecrosis of the jaw

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Running title: *Hyperbaric oxygen therapy in BRONJ*

Key words: osteonecrosis; bisphosphonates; hyperbaric oxygen therapy

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ABSTRACT

Bisphosphonate-related osteonecrosis of the jaw (BRONJ), an important side effect of bisphosphonates, has high morbidity and is often refractory to treatment. Attempts to control the disease include antimicrobial therapy, surgical interventions and some alternative therapies such as low-level laser therapy, platelet-rich plasma and hyperbaric oxygen therapy (HBOT). HBOT has been successfully applied to treat osteoradionecrosis and osteomyelitis, but its indication in BRONJ is still controversial. We present here a literature review on some important aspects of HBOT and its indications with focus on BRONJ treatment. Considering the reported effects of angiogenesis inhibition and suppression of osteoclast activity by bisphosphonates, as well as the major role of microorganisms in the pathogenesis of the disease, it seems that HBOT could be an effective adjuvant therapy for BRONJ. Nevertheless, further clinical and experimental studies with standardized methods are required to better evaluate the suitability of this treatment.

INTRODUCTION

Bisphosphonates are drugs used to treat diseases associated with increased bone resorption, such as osteoporosis, multiple myeloma and bone metastases in cancer. In general, they are well tolerated by the human body, but in the last years, bisphosphonate-related osteonecrosis of the jaw (BRONJ) has been reported as an adverse effect of these drugs.[1-19] The condition is directly related to administration route, duration of use and cumulative dose of the drug. Poor oral hygiene, deficient oral health, diabetes, tooth extraction, and corticosteroid use are other relevant risk factors for the disease.[3,7,8,10,14,20-22] On clinical examination, it is classically characterized by persistent bone exposure in the maxilla and/or mandible in patients

subjected to bisphosphonate treatment, but without history of head and neck radiation therapy. Pain, suppuration and intra/extraoral fistulae can be present.[7,23-25] The treatment is complex, [3,7,9,10,13,23-26] and microorganisms play an important role in the disease etiopathogenesis.[27] More recently, cases of patients without exposure of the compromised bone to the oral environment have also been reported. In these situations, although oral mucosa seems normal, the subjacent bone shows radiographic signs of BRONJ and, in most cases, it becomes exposed after a while.[28-32]

BRONJ management includes, among other therapies, conservative clinical treatment with systemic and topical antibiotics, as well as surgical resection of the affected bone, combined or not, or with low-level laser therapy (LLLT), platelet-rich plasma (PRP) or hyperbaric oxygen therapy (HBOT).[3,4,26,30,31,33-43] Conservative clinical treatment with antibiotics, when not combined with other therapeutic options, can take more than two years for complete healing[44] or it can even be ineffective.[20,21,44] Cases of surgical resection, in turn, can result in either remission or appreciable aggravation of the disease.[24,26,30,31,33-35] LLLT[36-41] and PRP have been used as adjuvant agents,[3,4,42,43,45] with reports of both favorable[39,40,46] and unfavorable[41] results.

HBOT has shown some satisfactory results in the treatment of ostomyelitis and osteoradionecrosis. However, its indication for the treatment of BRONJ has been a controversial issue. Some authors have classified it as an effective adjuvant therapy in this disease,[23,47-50] whereas others question such effectiveness.[10,15,20,30,51,52] Considering the beneficial effects of HBOT in the treatment of various diseases including osteomyelitis and osteoradionecrosis, as well as the lack of agreement concerning its applicability in treating BRONJ, the present study aimed to review the

scientific literature on the effects, indications and contraindications of this therapy with focus on its applicability in BRONJ treatment.

Hyperbaric oxygen therapy (HBOT)

General aspects, effects, indications and contraindications (Fig. 1)

Although oxygen was not discovered until the XVIIIth century, it was in 1662 that Henshaw, in England, used hyperbaric medicine for the first time to treat lung diseases. He was inspired by the significant health improvement determined by climate changes and barometric variations observed at that time.[53-55] Anyway, HBOT was developed mainly in France, in the middle of the XIXth and beginning of the XXth century. In 1834, Junod described some beneficial effects of oxygenation under high pressure in humans, and he reported the construction of the first chamber for hyperbaric purposes.[53,55]

Basically, oxygen therapy consists in oxygen (O_2) supplementation to improve blood and tissue oxygenation of a patient with respiratory difficulties and hypoxia.[56] This therapy associated with increased atmospheric pressure provides an increase in oxygen saturation of the tissues, leading to profound transformations in the body.[54,57-59] The patient is placed in a chamber with atmospheric pressure of 1.4 absolute atmospheres (ATA) or more and, therefore, higher than the pressure at sea level (1 ATA). Treatment is conducted inside individual or collective chambers of high pressure, where the patient breathes 100% oxygen in an intermittent mode.[60,61]

In the last 20 years, there was a significant increase in investigations of HBOT applicability in the treatment of various diseases.[57] The major indications are: arterial gas embolism; carbon monoxide and cyanide poisoning; clostridial myositis and myonecrosis (gas gangrene); crush injury, compartment syndrome and other traumatic peripheral ischemia; decompression sickness; arterial insufficiency; severe anemia;

intracranial abscesses; necrotizing soft tissue infection; refractory osteomyelitis; compromised skin flaps and grafts; radiation tissue damage; thermal burns and idiopathic sudden sensorineural hearing loss.[60] The therapy is indicated as part of treatment to heal wounds, since it stimulates growth factor production, particularly vascular endothelial growth factor (VEGF).[62] Therefore, HBOT can be combined with surgical debridement, grafts, and antibiotic therapy. It is also an adjuvant in antithrombotic therapy as well as after stabilization of bone fragments, favoring fracture healing.[63]

The absolute contraindication for HBOT is restricted to cases of untreated pneumothorax, whereas relative contraindications include conditions of impaired pressure equalization and cardiac disease.[54] In the latter, HBOT can lead to acute pulmonary edema.[64]

Effects of HBOT

HBOT effects on the body are explained by the laws of physics that determine the behavior of an ideal gas. Boyle's, Dalton's, Henry's[54,65,66] and Fick's laws[65,66] are the most important ones, because they explain physiological and biochemical effects of hyperoxia on biological tissues.[66,67] According to Henry's law, the amount of gas dissolved in a liquid or tissue is proportional to the partial pressure of the gas in contact with that liquid or tissue. This is the basis for the increase in oxygen tension in the tissue subjected to HBOT.[54,65,66]

Most oxygen transported in the blood is carried by hemoglobin, which is saturated at 97% in normal atmospheric pressure. However, there is also some oxygen dissolved in the blood plasma. It is this oxygen that increases in a hyperbaric environment, maximizing tissue oxygenation. When we breathe normobaric air, the arterial oxygen tension is about 100 mmHg, whereas in tissues, the tension is

approximately 55 mmHg. In conditions of 100% oxygen at 3 ATA, arterial oxygen tension increases to 2000 mmHg and around 500 mmHg in tissues, which allows the transportation of 60 mL of oxygen per liter of blood, compared to 3 mL of oxygen per liter of blood at normal atmospheric pressure. Such situation is capable of maintaining tissue oxygenation without hemoglobin contribution.[54] The increase in oxygen pressure reduces the half-life of carboxyhemoglobin, responsible for carbon monoxide poisoning,[57,68] and it also reduces the volume of inert-gas bubbles inside blood vessels, which prevents air embolism and decompression sickness.[57]

High oxygen pressure can cause important hemodynamic and circulatory changes.[69] The most known are bradycardia and hyperoxic vasoconstriction, which were demonstrated in healthy individuals and tend to protect tissues from damage caused by hyperoxia.[70] Although the angiogenic effect of HBOT is known, its mechanism still requires elucidation.[57,71] Studies report that growth stimulating factors such as VEGF,[57,61,62,72] basic fibroblastic growth factor (bFGF),[61,73] hepatocyte growth factor (HGF)[73] and hypoxia-inducible factors 1 and 2 (HIF-1 and 2)[74] are increased with HBOT. Nevertheless, such aspects of these regulations are still controversial.[61,73,75] Zhang et al.,[75] for example, found HIF-1α reduction in spite of healing improvement with HBOT.

Circulating endothelial progenitor cells (EPC) are increased in HBOT[71] by means of mobilization of stem/progenitor cells (SPC) found inside bone marrow. Exposure to HBOT stimulates cell production and mobilization through nitric oxide synthesized in bone marrow via nitric oxide synthase activity, which is activated by hyperoxia.[71,74,76,77] These SPCs show receptors for growth factors such as VEGFR2 (VEGF receptor 2) and CXCR4 (CXC chemokine receptor 4). The latter is required to maintain stem cells in the ischemic or injured site, while the former is found in

EPCs.[71,74,76] Nitric oxide can be produced in various tissues and its potential beneficial or damaging effects depend on the tissue where it is generated.[78]

HBOT increases the production of reactive oxygen species (ROS),[49,54,57,78] which oxidize proteins and lipid membranes, causing damage to bacterial DNA and inhibiting bacterial metabolic function, being particularly effective against anaerobes. Bacterial killing by neutrophils require adequate tissue oxygen levels, with which effectiveness is directly correlated.[79,80] HBOT enhances the oxygen-dependent transport of some antibiotics through the bacterial cell wall, helping with body defense mechanisms and exerting synergistic effects when combined with antibiotic therapy. It is, therefore, an adjuvant therapy in the treatment of tissue infections involving either aerobic or anaerobic bacteria.[54] Moreover, vasoconstriction determined by HBOT reduces edema and favors injured tissue healing. This therapy is capable of promoting fibroblast activation, stimulating angiogenesis and regulating osteoclast activity.[57]

Regarding bone tissue, studies analyzing HBOT effects on autogenic grafts in animal models indicate acceleration of the union between the graft and receptor site. Also, the osteoinductive effect of this therapy on the activity of recombinant human bone morphogenetic protein-2 (rhBMP-2) has been reported, as well as higher activity of alkaline phosphatase in the groups treated with HBOT compared to controls. Such findings suggest that the effects of HBOT on bone tissue are similar to those obseverd in soft tissues.[81-83]

Side effects

The increase in oxygen pressure in the body not only exerts beneficial effects but can also produce undesirable effects related to oxygen toxicity to the tissue in question.[70] The majority of HBOT side effects are reversible, and their intensity depends on the

dose and, especially, on the duration of treatment.[84] The average number of sessions varies from 20 to 50, but there are reports of treatments lasting for many years. Hyperbaric protocols exceeding 100 sessions exhibit a significant increase in the incidence of side effects.[64]

The most frequent side effect is middle ear barotrauma, which is often associated with eustachian tube dysfunction, where pressure equalization is impaired. Another frequent effect is sinus squeeze in patients with upper respiratory tract infections or allergic rhinitis.[54,64] Eye disturbances such as myopia[54,64,85] and cataract[64,85] can occur in some cases. The mechanism of action is not clear, but it is related to oxygen toxicity, since oxygen passes through the cornea getting to eye fundus and its vessels. This effect is strictly related to the form of application of oxygen, where it is more significant when the helmet device is used, because in this case, the oxygen dose and its contact with eyes are greater. Myopia usually shows total remission after a resting period proportional to the time of treatment.[54,64]

Oxygen therapy modifies baseline levels of nitric oxide, which interacts with other ROS and also induces the production of superoxide dismutase (SOD), an enzyme with protective antioxidant effects. Nevertheless, there is a paradox here, as nitric oxide production may either exacerbate or mitigate the toxic effects of oxygen, depending on the particular nitric oxide synthase (NOS) isoform that produces it. Therefore oxygen therapies under high pressures or even normobaric can trigger damaging effects respectively to the central nervous system and lungs.[78]

An increase in free radicals was observed in the blood of patients subjected to HBOT.[54,86] When the body's antioxidant defenses are not completely effective, the increase in free radicals becomes harmful. This situation is called oxidative stress. Among cell targets, the genome is particularly vulnerable to this condition. Repair

mechanisms are involved in the removal of oxidative DNA damage and, if this does not happen in a proper way, initiation or progression of cancer can occur. However, the genotoxic effect of HBOT can be reduced by the alteration of the treatment protocol, reducing the time of exposure of the patient to the therapy.[84]

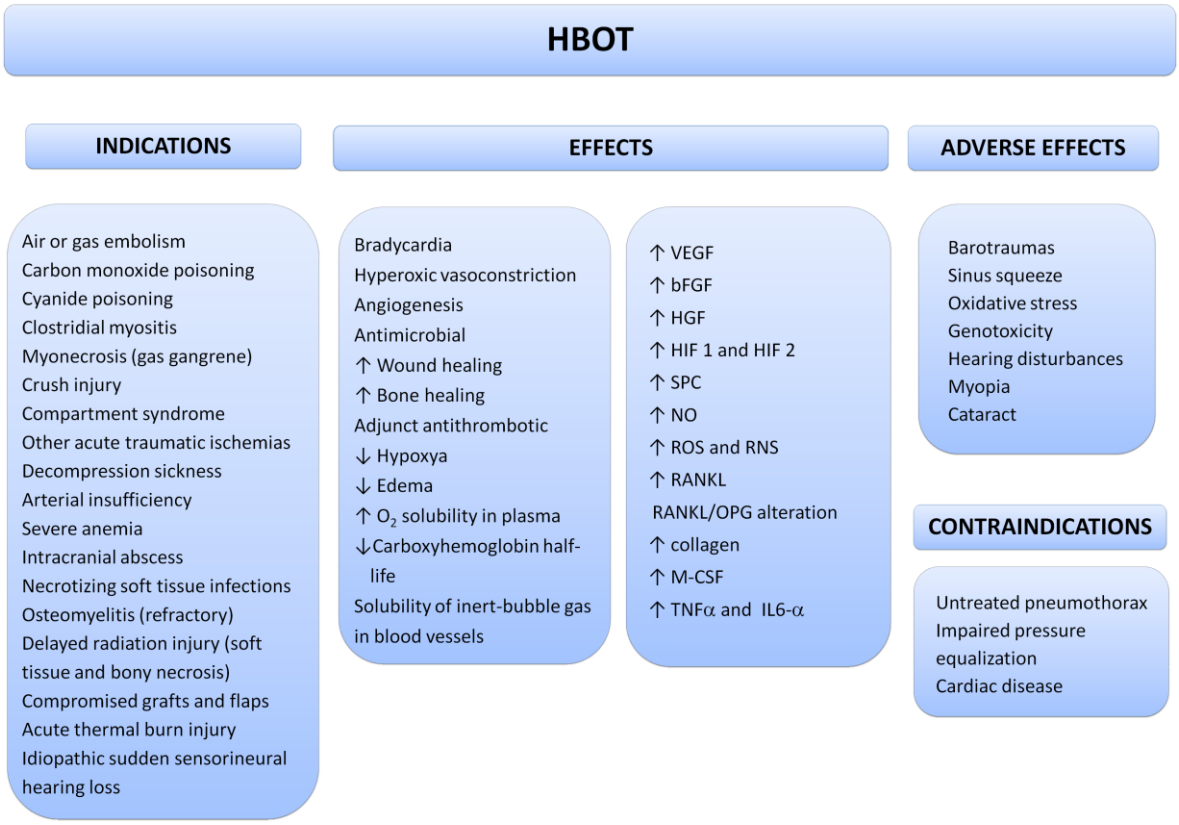

Figure 1 – Indications, contraindications, effects and adverse effects of hyperbaric oxygen therapy (HBOT). VEGF=vascular endothelial growth factor; bFGF=basic fibroblast growth factor; HGF=hepatocyte growth factor; HIF=hypoxia-inducible factor; SPC=stem/progenitor cell; NO=nitric oxide; ROS=reactive oxygen species; RNS=reactive nitrogen species; RANKL= receptor activator of nuclear factor kappa-B ligand; OPG=osteoprotegerin; M-CSF=macrophage colony-stimulating factor; TNF= tumor necrosis factor; IL=interleukin

HBOT in BRONJ

HBOT would be capable of neutralizing the antiangiogenic effect of bisphosphonates through ROS and reactive nitrogen species (RNS) production, which in turn would interfere with chemical mediators responsible for the regulation of osteoclast

production and activity.[87] Cultured cells subjected to hyperoxia can undergo changes in the concentration of certain chemical mediators such as early growth response protein 1 (Egr-1), basic fibroblast growth factor (bFGF), receptor activator of nuclear factor kappa-B ligand (RANKL), vascular endothelial growth factor receptor (VEGFR), and macrophage colony stimulating factor (M-CSF).[73,88] Oxidants in physiological concentrations stimulate collagen production by fibroblasts, which is proportional to the concentration of molecular oxygen (PO_2) in the range of 0 to 200 mmHg. The mechanism involves hydroxylation of procollagen, where participation of molecular oxygen is required.[72]

The highly reactive molecules ROS and RNS, increased by HBOT, exert some effects on osteoclast activity and differentiation and also regulate some critical aspects of bone metabolism. ROS stimulate RANKL expression, changing the RANKL/osteoprotegerin (OPG) ratio, which favors osteoclast differentiation, and interfere with RANK-associated ROS-sensitive transcription factor (NFkB). Oxygen-sensitive osteoclastogenic cytokines such as tumor necrosis factor (TNF), M-CSF, RANKL, and interleukin 6 (IL6) can suppress osteoclast apoptosis. Therefore, by stimulating the production of ROS and ROS-sensitive cytokines, HBOT would reverse bisphosphonate-induced osteoclast suppression.[87-89]

Reports on HBOT application in BRONJ

The literature reports studies evaluating HBOT in BRONJ treatment, and in most of them it is classified as a useful adjuvant therapy (Table 1). Nevertheless, Ruggiero et al.[11] reviewed 63 cases of BRONJ, where bisphosphonate had been given intravenously in 56 patients and orally in 7. All of them had lesions refractory to previous conservative treatment with debridement and antibiotic therapy. Most patients required surgical treatment to remove the necrotic bone (sequestrectomy,

maxillectomy). Two of them were subjected to 30 sessions of 1-hour HBOT prior to surgical marginal resection of the mandible, but without success in stopping disease progression.

Migliorati et al.[10] reported 18 cases of BRONJ (14 women and 4 men; mean age of 62 years), where 16 patients were subjected to tooth extractions or had trauma or infection of the jaws, while 2 had spontaneous lesions. Surgical intervention, antibiotic therapy, antiseptic rinses and HBOT were used. According to the authors, HBOT was performed in one patient without positive results, and discontinuation of bisphosphonate did not guarantee wound healing. Sequestrectomy and surgical debridement did not show good results either, leading to enlargement of the lesions.

Biasotto et al.[23] reported a series of 12 cases of BRONJ related to zoledronic acid, where antibiotic therapy and debridement were combined with 30 sessions of HBOT. According to the authors, the therapy was effective in obtaining symptomatic relief but did not improve the clinical aspect of the lesions.

Dimopoulos et al.[52] reported a case series of 202 patients with multiple myeloma under treatment with bisphosphonates, where 7.4% (9 men and 6 women) of them developed BRONJ. Treatment consisted in antibiotic therapy and minor debridement. One patient also received HBOT. According to the authors, HBOT did not improve the lesion, but they did not report the protocol used.

Magopoulos et al.[90] reviewed 60 cases of BRONJ (28 men and 32 women; mean age of 61 years), considering the site of the lesion (maxilla or mandible), associated trauma and chosen treatment. In 68.33% (n=41) of cases, BRONJ occurred after tooth extraction, in 15% (n=9) after chronic denture trauma, and spontaneously in 16.67% (n=10). They tested different treatment protocols, combined antibiotic therapy, surgery, discontinuation of bisphosphonate, HBOT and monitoring. Regardless of the

associated treatment, HBOT without withdrawal of bisphosphonate was not capable of inhibiting BRONJ recurrence in 6 patients. On the other hand, discontinuation of bisphosphonate combined with HBOT, surgery and antibiotic therapy led to complete healing of the lesions (n=4). All cases without bisphosphonate termination showed recurrence (n=16) or progression (n=11) of osteonecrosis, whereas all patients in whom bisphosphonate was stopped showed improvement with complete healing (n=9) or stabilization of necrosis (n=24).

Shimura et al.[50] reported a case of a 60-year-old patient with multiple myeloma who developed BRONJ after 13 months of oral minodronate and 4 weeks of intravenous incandronate. The patient was treated with antibiotic therapy followed by HBOT. According to the authors, early diagnosis of BRONJ is an important factor for the overcome, and HBOT combined with antibiotic therapy is effective.

Freiberger et al.[48] evaluated 16 patients with BRONJ, who were treated with HBOT, considering clinical response, period of remission, and stabilization or relapse of the lesion. The mean time of bisphosphonate treatment before the onset of the disease was 18 months, and the period between the onset and HBOT was 12 months. Right after therapy, 44% (n=7) of patients showed improvement. In 50% (n=8), lesions stabilized, where only 2 did not have remission, and 6% showed disease progression. According to the authors, HBOT can be beneficial; however, the outcome can be improved by the withdrawal of bisphosphonate.

Shirota et al.[91] reported a case of a 54-year-old female patient with BRONJ involving dental implants in maxilla, after 2 years of IV bisphosphonate use to treat bone metastases of breast cancer. Sequestrum and implant resection was followed by HBOT 1x/day for 60 min at 2 ATA for 10 days combined with antibiotic therapy. There was complete healing of the lesion 2 weeks after surgery. Bisphosphonate was

restarted 3 weeks after surgery, and the patient survived for 8 months without symptoms and then died of systemic complications.

Thumbigere-Math et al.[92] reported 26 cases of BRONJ, including 17 women and 9 men, with mean age of 64 years. Sixteen of them developed BRONJ after dental interventions, whereas 10 had spontaneous lesion. Most patients received systemic and topic antimicrobials, 18 (69.2%) received minor debridement, and 4 were given antibiotic therapy, subjected to debridement, and also received HBOT. Regarding the small sample size, it was not possible to establish an effective result with HBOT, even though it was possible to observe that spontaneous lesions had easier remission than did lesions caused by previous dental interventions.

Chiu et al.[93] reported a series of 12 cases of BRONJ classified at stages I (n=3), II (n=5) and III (n=4), with combined corticosteroid use for a long period. The development of lesions occurred after tooth extractions in 7 patients, in 1 after pre-prosthetic surgery, and in 2 with trauma by prosthetic appliance, and 2 did not have any related factor. All patients received HBOT combined with antibiotic therapy and some kind of debridement depending on the stage of the disease. Eight patients at stages I and II received flap surgery to cover the wound, and healing was by second intention in 3 stage III patients. Stage I patients (n=3) received 20 preoperative sessions, whereas the other patients (stages II and III, n=9) received 20 preoperative and 20 postoperative sessions of HBOT. All patients reported a significant reduction of symptoms and showed complete healing of the lesions between 3 (first intention healing) and 9 months (second intention healing) after the surgical procedure.

In a case report of a 72-year-old patient with breast cancer, Antonini et al.[94] described the successful treatment of BRONJ related to zoledronic acid. The lesion had spontaneous onset in the maxilla and one year of duration. Ten sessions of HBOT

were applied previous to surgery with debridement and curettage. After bone resection, guided bone regeneration with resorbable membrane, PRP, and closure with rotation flap were performed, along with antibiotic therapy and antiseptic rinses. Complete healing was achieved.

Freiberger et al.[49] tested HBOT as an adjunct to surgery and antibiotic therapy in BRONJ. By means of a randomized control trial, gingival healing, pain and quality of life were evaluated. One group of 25 BRONJ patients was subjected to conventional therapy combined with HBOT, and the other group was formed by 21 patients treated only with conventional therapy, without HBOT. At the first three-month follow-up period, 17 patients (68%) in the HBOT group showed improvement while only 8 (38.1%) in the control group. HBOT appeared to be a useful adjuvant therapy for BRONJ, especially in the most severe cases.

Hinson et al.[95] investigated whether discontinuation of bisphosphonate affected the outcome of BRONJ. Eighty-four patients were treated with debridement (n=42; 50%), major surgery (n=33; 39.3%), systemic antibiotic therapy (n=68; 80.9%) and alternative treatments (n=14; 16.7%), where 6 patients received HBOT. According to the authors, regardless of the therapeutic option used or disease stage, discontinuing bisphosphonate was the major factor, allowing faster resolution of the disease and reducing time of resolution by approximately 6 months.

FINAL CONSIDERATIONS

In general, the lack of standardization of methods employed to evaluate HBOT results makes it difficult to determine which factors are the most affected by the therapy and which of them can result in beneficial effects. Also, results differ between *in vitro* and *in vivo* studies, raising many questions about the indications.[96] Even though, the major

reason for HBOT indication is to increase oxygen in the circulating blood, whose influence on soft tissues is well known.[97,98]

Speculations about the effectiveness of HBOT in BRONJ are based on its angiogenic properties and effects on osteoclast differentiation through ROS and RNS,[87] as well as effectiveness against microorganisms, especially anaerobes.[54,79,80] Nevertheless, the reports evaluating such effectiveness consist of clinical studies with many intervening variables and lack of method standardization. According to them, it seems that regardless of the therapeutic option, BRONJ remission depends most on the withdrawal of bisphosphonate and time sufficient for bone metabolism recovery,[48,90,95] which can take months or even years depending on the type of bisphosphonate used and individual characteristics of the patient.[44] However, in the case of cancer patients, sometimes discontinuation of the drug may not be a viable choice.

Moreover, considering the obscure multifactorial etiopathogenesis of BRONJ, where infection, suppressed bone metabolism and suppressed angiogenesis seem to play a major role,[2,27] a multimodality treatment including antibiotic therapy, surgical interventions and HBOT should be evaluated in a specific way, considering the particularities of each patient, as well as risk-benefit and cost-benefit relationships. According to some authors, severe cases of BRONJ can be improved by the use of HBOT as part of a multimodal therapy.[48,49]

Although the literature reports some encouraging results about HBOT use in BRONJ, the issue needs to be clarified.[57] The effectiveness of this therapy in BRONJ treatment requires further studies including clinical, *in vitro* and *in vivo* ones, with rigorously designed methods investigating the repercussions of HBOT in tissues previously or currently treated with bisphosphonates.

Table 1 – Reports on the use of hyperbaric oxygen therapy (HBOT) in bisphosphonate-related osteonecrosis of the jaw (BRONJ)

Method	Total n / HBOT n	Oxygen therapy protocol	Results	Associations	Reference
Case series	63/2	30 sessions of 1-hour	Did not affect disease progression	ATB; antiseptic; debridement, surgical resection	Ruggiero et al.[11]
Case series	18/1	w/s	No improvement	ATB; antiseptic; debridement; sequestrectomy;	Migliorati et al.[10]
Case series	12/11	30 sessions	Symptomatology improvement	ATB; rifocin rinse; debridement; sequestrectomy	Biasotto et al.[23]
Case series	15/1	w/s	No improvement	ATB; minor debridement	Dimopoulos et al. [52]
Case report	1/1	w/s	HBOT associated with ATB is effective in BRONJ treatment	ATB	Shimura et al.[50]
Case series	60/10	w/s	HBOT without withdrawal of bisphosphonate=BRONJ relapse (n= 6); HBOT + bisphosphonate withdrawal=complete healing (n=4)	ATB; antiseptic; debridement; sequestrectomy	Magopoulos et al.[90]
Case report	2/1	2.4 ATA; 100% O_2/90min, 5X/week, 4 weeks.	Resolution	ATB; PRP	Lee et al. [45]
Case series	16/16	2 ATA; 100% O_2/120min; 2X/day, 37 sessions on average	14 of 16 improved by stage; 7 of 16 were in remission, 8 stabilized	ATB; debridement; BP discontinuation	Freiberger et al.[48]
Case report	1/1	2 ATA; 60 min/1x/day; 10 sessions	Complete healing	ATB; surgical resection	Shirota et al.[91]

Case series	26/4	w/s	Not possible to establish effectivenes of HBOT in BRONJ	ATB; antiseptic; debridement	Thumbigere-Math et al.[92]
Case report	1/1	2.4 ATA;100% O2/120min; 1x/day, 30 sessions	Complete healing	ATB; antiseptic; debridement; PRP; surgical resection;	Antonini et al.[94]
Case series	12/12	Stage I: 2.4 ATA, 90 min/20 sessions. Stage II/III: 2.4 ATA; 90 min/40 sessions	Improvement of symptoms; remission of lesion in all cases	ATB; debridement; sequestrectomy	Chiu et al.[93]
Randomized control trial	46/25	2 ATA; 100%O2/ 120 min; 2X/day, 40 sessions	17 of 25 patients showed improvement of symptomatology; HBOT is a useful adjuvant therapy	ATB; antiseptic; debridement	Freiberger et al.[49]
Case series	84/6	w/s	Regardless of the therapeutic option or stage of the disease, discontinuing of bisphosphonate is the major factor	ATB;debridement; surgical resection	Hinson et al.[95]

ATA=atmosphere absolute; ATB=antibiotic therapy; BP=bisphosphonate; BRONJ=bisphosphonate-related osteonecrosis of the jaw; HBOT=hyperbaric oxygen therapy;PRP=platelet-rich plasma; w/s=without any specification

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HIGHLIGHTS

- BRONJ has high morbidity and is often refractory to treatment

- Hyperbaric oxygen therapy has been used as an adjuvant therapy in BRONJ

- Further studies are needed to evaluate hyperbaric oxygen therapy in BRONJ

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Artigo 2

3 ARTIGO 2

O artigo a seguir intitula-se ***Effect of hyperbaric oxygen therapy on tooth extraction site in rats subjected to bisphosphonate therapy – Histomorphometric and immunohistochemical analysis*** e foi formatado de acordo com as normas e submetido ao periódico *Head & Neck* (Anexos C e D).

Effect of hyperbaric oxygen therapy on tooth extraction site in rats subjected to bisphosphonate therapy – Histomorphometric and immunohistochemical analysis

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Running title: *Hyperbaric oxygen therapy and bisphosphonates*

Key words: osteonecrosis; bisphosphonates; hyperbaric oxygen therapy; bone; jaws; rats

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ABSTRACT

Backgroound: This study aimed to investigate the effect of hyperbaric oxygen therapy (HBOT) on tooth extraction site in rats treated with bisphosphonate.

Methods: Rats were treated with zoledronic acid, subjected to tooth extractions and allocated into groups: (1) 7 days of HBOT; (2) 14 days of HBOT; (3) 7-day control; (4) 14-day control. The site of tooth extractions was analyzed by histomorphometry and immunohistochemistry.

Results: On macroscopic analysis, HBOT did not significantly affect bone exposure volume either at 7 or 14 days. On H&E analysis, the 14-day HBOT group showed less non-vital bone compared to both controls and 7-day HBOT group. HBOT significantly lowered expression of VEGF, RANKL, BMP-2 and OPG at 7 days, compared to control, whereas at 14 days, there was no significant difference for these variables.

Conclusion: HBOT can reduce the amounts of non-vital bone microscopically detected in tooth extraction sites of rats subjected to bisphosphonate therapy.

INTRODUCTION

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is one of the designations for a side-effect of bisphosphonates characterized on clinical examination as bone exposure in the maxilla and/or mandible, persistent for more than eight weeks, in patients treated with bisphosphonate who have not received head and neck radiation therapy.[1-7] More recently, the concept was extended to other antiresorptive and antiangiogenic drugs also capable of determining the lesion such as denosumab, bevacizumab and sunitinib, thus referred to as medication-related osteonecrosis of the jaw (MRONJ).[8] The definition was also expanded to include either exposed bone or bone that can be probed through an intra- or extraoral fistula in the maxilla and/or mandible, persistent for more than eight weeks, in a patient without history of head and neck radiation therapy and without metastasis in the jaw bones.[8] Intravenous administration of bisphosphonates, cancer and anticancer therapy,

tooth extraction, oral bone manipulating surgery, poor fitting dental appliances, intraoral trauma, duration of exposure to bisphosphonate treatment, glucocorticoids, co-morbid conditions, alcohol and/or tobacco abuse and pre-existing dental or periodontal disease are reported risk factors.[4]

Tissue response to treatment in BRONJ is poor, presumably because of osteoclast apoptosis and activity suppression induced by bisphosphonates,[2,4,9-15] and possibly because of its antiangiogenic effect.[2,16] A series of events involving chemical mediators, where vascular endothelial growth factor (VEGF)[16] is inhibited, and where the relation between receptor activator of nuclear factor kappa-B (RANK), its ligand (RANKL) and osteoprotegerin (OPG) is changed,[2,8,12,17-20] leads to bone turnover suppression. Therefore, bone remodeling, which depends on resorption and deposition processes, is impaired[21] and the bone healing process of wounds as well, including tooth extraction wounds, predisposing bone tissue to secondary infection.[2]

BRONJ treatment has been a challenging task. Discontinuation of bisphosphonate use and conservative antimicrobial therapy with systemic antibiotics and antiseptic rinses such as with chlorhexydine have been used,[18,22-25] combined or not with surgical interventions and alternative therapies. Surgical procedures such as necrotic bone removal and debridement of the margins have been defended,[7,11,26-33] and among the alternative therapies, low-level laser therapy (LLLT), platelet-rich plasma (PRP) and hyperbaric oxygen therapy (HBOT) have been used.[5,8,11,27-29,34-43] Meanwhile, the adequacy of some of these proposed therapies is still debated.[14,18,25,33]

HBOT has been indicated for a long time to treat chronic osteomyelitis of the jaw and osteoradionecrosis.[12,44-47] Its antimicrobial effects and wound healing capability are well recognized.[12,22,44,48-58] More recently, studies have determined that HBOT also generates reactive oxygen species (ROS) and reactive nitrogen species (RNS), which in

turn interfere with osteoclasts, RANKL and OPG, favoring osteoclast maturation and activity.[12]

On the basis of these concepts, it seems reasonable to indicate HBOT in BRONJ, where it has been referred to as an effective adjunct therapy. Nevertheless, these reported beneficial effects are mainly based on clinical studies,[5,12,22,59] most of them with inherent intervening variables and lacking standardization.[24,60-62] Moreover, some other reports do not support significant effectiveness of HBOT in BRONJ.[14,18,33,63,64] To determine this effectiveness, biological mechanisms and interactions involving bisphosphonates, BRONJ and HBOT need to be investigated, where the use of animal models could be helpful.[21,65] Accordingly, the aim of this work was to investigate by means of histomorphometric and immunohistochemical analysis the effect of HBOT on tooth extraction sites in rats subjected to bisphosphonate therapy.

MATERIAL AND METHODS

The present study was approved by the Ethics Committee on Animal Use of Pontifical Catholic University of Rio Grande do Sul and the Ethics Committee for Animal Use of the University of Caxias do Sul. The sample comprised 35 female Wistar rats (*Rattus norvegicus*), 140 days old and mean weight of 240 g, which were maintained in appropriate cages, at 22ºC and light/dark cycle of 12 h (lights turned on at 7:00 a.m. and turned off at 7:00 p.m.). Nuvilab-Cr1 chow (Nuvital, Colombo, PR, Brazil) and filtered water were given *ad libitum*. The animals were treated with zoledronic acid (Novartis Pharma AG, Basileia, Switzerland), subjected to tooth extractions and allocated into 4 groups according to the HBOT regimen: (1) 10 rats received HBOT for 7 days; (2) 11 rats received HBOT for 14 days; (3) 7-day control group of 7 rats not given HBOT; and (4) 14-day control group of 7 rats not given HBOT.

Zoledronic acid administration and tooth extractions

Zoledronic acid was administered by the intraperitoneal (IP) route, at a dose of 0.6 mg/kg[21,66] every 7 days[65] in a total of 5 doses. Forty-five days after starting zoledronic acid administration, tooth extractions were performed under IP anesthesia with 5% ketamine hydrochloride (Syntec, Cotia, SP, Brazil) at 100 mg/kg and 2% xylazine hydrochloride (Syntec) at 10 mg/kg.[67] The 3 upper right molars were extracted using a Hollenback carver #3S (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) and pediatric forceps (Edlo, Canoas, RS, Brazil), both previously adapted for the tooth size. In the postoperative period, 50 mg/kg paracetamol (Medley S/A, Campinas, SP, Brazil) was administered for 24 h.

Clinical evaluation and HBOT

Forty-five days after tooth extractions, clinical evaluation was performed and HBOT started. On clinical evaluation, the presence of bone exposure was investigated by using a #5 probe (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil). When present, lesions were measured with a digital caliper (resolution of 0.01 mm, series 500, Mytutoyo, Suzano, SP, Brazil) and a calibrated periodontal probe (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil). HBOT was performed by using an experimental hyperbaric chamber[68] (Janus and Pergher, Porto Alegre, RS, Brazil) at the Laboratory of Physiology, University of Caxias do Sul. Test group 1 (HBOT, 7 days) received daily sessions for 7 days, and test group 2 (HBOT, 14 days) received daily sessions for 14 days. Each session of HBOT lasted 90 min where in the first 30 to 35 min, the pressure inside the chamber was gradually increased with 100% humidified oxygen until 2.5 ATA (absolute atmosphere), and the temperature inside the chamber was kept at 24ºC. The recommended therapeutic period was 30 min with constant temperature and pressure monitoring, as well as visual observation of animals' behavior. At the end of the session, the chamber was gradually depressurized for 25 to 30

min to minimize possible barotrauma. Sessions were repeated daily at the same time. The control groups were not subjected to HBOT. The protocol was according to that established in the literature.[54,56,57,68-71]

Euthanasia of the animals and macroscopic evaluation

The animals were euthanized with isoflurane (Cristalia, Porto Alegre, RS, Brazil) inhalation in an appropriate chamber at 7 days of HBOT for test group 1 and 14 days for test group 2. Euthanasia in control groups 1 and 2 was as the same postoperative period as test groups 1 and 2, respectively. Right after euthanasia, a macroscopic evaluation was performed by using the same instruments and criteria used in the previous clinical examination.

Specimen processing

Maxillae were dissected, fixed in 10% buffered formalin for 24 h, and tooth extraction area was cut by using an extra fine diamond disc (American Burrs, Porto Alegre, RS, Brazil) at low speed. The osteotomized segment comprised the tooth extraction area with 2 mm safety margin and was cut in the midlle in a buccal-lingual direction into 2 pieces, both of them displaying the area of interest at the cut surface.

Histological processing

Specimens were decalcified in 17% (pH 7.0) ethylenediaminetetraacetic acid (EDTA, Biodinâmica, Ibiporã, PR, Brazil) at 44^{o}C for 30 days. The solution was renewed twice a week until complete decalcification. The specimens were then embedded in paraffin, resulting in 2 blocks per animal, and five 4 µm-thick sections were obtained from each block and mounted on microscope slides to be subjected to hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining.

Immunohistochemical processing

Tissue sections were deparaffinized, rehydrated and processed. Antigen retrieval was with enzymatic digestion using trypsin (Sigma, St. Louis, MO, USA), and endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol. Nonspecific bonds were inhibited with 2% bovine serum albumin (BSA). Sections were incubated overnight at 4°C with the primary antibodies for VEGF 1:200 [VEGF(C-1):sc-7269, Santa Cruz Biotechnology, Paso Robles, CA, USA], BMP-2 (bone morphogenetic protein-2) 1:100 [BMP-2(SS09):sc-73743, Abcam, Cambridge, UK], OPG 1:5000 [OPG(N-20):sc-8468, Santa Cruz Biotechnology] and RANKL 1:200 [RANKL(N-19):sc-7628), Santa Cruz Biotechnology]. Next, secondary antibody conjugated with biotin ligand and streptavidin-peroxidase complex (Dako, Carpinteria, CA, USA) was added at 30°C for 25 min. Staining was revealed with diaminobenzidine (DAB), and sections were counterstained with Harris hematoxylin. Slides were dehydrated in ethanol and xylene and coverslipped in Entellan (Merck Millipore, Darmstadt, Germany). Samples of placenta, breast tumor, muscle, and bone neoformation were used as positive controls respectively for VEGF, RANKL, BMP-2 and OPG. Samples processed without the primary antibodies served as the negative controls.

Capture and analysis of the images

Images were captured by means of a Zeiss Axioskop 40 (Carl Zeiss, Oberkochen, Germany) light microscope equipped with a CoolSnap Pro videocamera (Media Cybernetics, Bethesda, MD, USA) connected to a computer with a plaque Image Pro Capture Kit. Images were captured in a standardized manner using the 10x objective for H&E, and 20x objective for IHC. With H&E staining, 5 fields were captured for each slide; and in IHC, 4 fields per slide including the tooth extraction area. Images were stored

in uncompressed TIFF (Tagged Image File Format) and analyzed by one blinded and calibrated observer. The analyses were performed in Image Pro Plus 4.5.1 software (Media Cybernetics). In H&E images, quantitative analysis (proportion) was performed considering the variables epithelial tissue, vital bone, non-vital bone, inflammatory infiltrate, microbial colonies, root fragment and fibrous connective tissue. We used the manual point counting technique applying a grid with 705 points over each field image.[72] In IHC images, the stained area was quantified by using the semiautomated segmentation technique.[72] Blindness consisted in not knowing the group to which each image belonged. Intraobserver calibration was performed by using 40 images in each technique, which were analyzed twice at different moments. The results of these two analyses were tested by the intraclass correlation coefficient, which showed $r>0.7$.

Statistical analysis

Data was analyzed by means of descriptive (mean, median, standard deviation, 25th percentile, 75th percentile) and inferential statistics. Quantitative variables were compared between test and control groups using the Mann-Whitney test, whereas qualitative variables were tested with Fisher's exact test. The volume of bone exposure was compared within HBOT group, pre- and posttreatment, with the Wilcoxon test. Analysis was performed in SPSS 17.0, using a 5% level of significance.

RESULTS

Macroscopic analysis: volume of bone exposure

On macroscopic examination, the volume of bone exposure did not significantly differ between pre- and post-HBOT periods either for the 7-day (P=0.593) or 14-day (P=0.327) group (Table 1, Wilcoxon test, α=0.05). Considering the difference in bone exposure volume pre- and post-HBOT, the comparison between test groups and their respective

controls at 7 days (*P*=0.961) and at 14 days (*P*=0.172) did not show any significance (Table 1, Mann-Whitney test, α=0.05).

Table 1 – Macroscopic analysis of oral exposed bone in the hyperbaric oxygen therapy (HBOT) and control groups at 7 days and 14 days in the pre- and posttreatment periods

Time	Group	Exposed bone volume (mm^3)										
		Pre-HBOT			Post-HBOT				Difference (Post – Pre)			
		MD	P25	P75	MD	P25	P75	*P**	MD	P25	P75	*P***
7 days	HBOT	0.030	0.003	0.087	0.014	0.00	0.083	0.593	-0.004	-0.023	0.017	0.961
	Control	0.166	0.010	0.166	0.080	0.010	0.166	0.465	0.00	-0.091	0.009	
14 days	HBOT	0.050	0.002	0.133	0.050	0.000	0.106	0.327	0.00	-0.092	0.066	0.172
	Control	0.050	0.004	0.066	0.080	0.033	0.200	0.116	0.030	0.000	0.050	

HBOT=hyperbaric oxygen therapy; MD=median; P25=25th percentile; P75=75th percentile
**P*=P value for comparison of pre-HBOT and post-HBOT; Wilcoxon test, α=0.05
***P*=P value for comparison of HBOT and control; Mann-Whitney test, α=0.05

H&E analysis (Fig. 1)

At 7 days, the group treated with HBOT showed amounts of epithelium (*P*=0.01) and root fragment (*P*=0.002) significantly lower than in the control group. At 14 days, non-vital bone was significantly less in the HBOT group than in the control (*P*=0.049). Although vital bone was greater in the HBOT groups than in the controls, for 7-day (*P*=0.24) and 14-day (*P*=0.056) periods, this difference was not significant (Table 2, Mann-Whitney, α=0.05). When the HBOT groups were compared to each other, higher amounts of non-vital bone (*P*=0.012) and less root fragments (*P*=0.008) were found in the 7-day group than in the 14-day group (Table 3, Mann-Whitney test, α=0.05). No other significances were found in the proportion of the variables on H&E analysis.

Table 2 – Histological analysis in hematoxylin and eosin (H&E) staining in the hyperbaric oxygen therapy groups and the control groups at 7 days and 14 days

Variable	Group 7 Days							Group 14 Days						
	HBOT (%)			Control (%)				HBOT (%)			Control (%)			
	MD	P25	P75	MD	P25	P75	*P**	MD	P25	P75	MD	P25	P75	*P***
Epithelium	20.5	16.75	22	28	24	31	**0.01**	20	17	22	24	21	29	0.101
Vital bone	22.5	18.5	27.5	18	14	26	0.24	26	22	34	15	10	24	0.056
Non-vital bone	5	2	8.5	2	1	10	0.404	0	0	2	6	1	8	**0.049**
Inflammatory infiltrate	15	9	22.25	12	10	15	0.73	12	11	23	20	9	26	0.618
Microbial colonies	0	0	1	0	0	1	0.909	0	0	1	0	0	1	1
Root fragment	0	0	0.25	5	4	9	**0.002**	4	0	6	3	0	7	0.963
Connective tissue	34.5	25.75	47	28	24	31	0.142	33	20	37	28	23	32	0.683

HBOT=hyperbaric oxygen therapy; MD=median; P25=25th percentile P75=75th percentile
**P*=*P* value for comparison of HBOT and control at 7 days; Mann-Whitney test, α=0.05
***P*=*P* value for comparison of HBOT and control at 14 days; Mann-Whitney test, α=0.05
Bold values show a significant difference

Table 3 - Histological analysis in hematoxylin and eosin (H&E) staining in the hyperbaric oxygen therapy groups at 7 days and 14 days

Variable	HBOT (%)						
	7 days			14 days			
	MD	Mean	SD	MD	Mean	SD	*P**
Epithelium	20.5	19.6	2.459	20	21.27	5.236	0.721
Vital bone	22.5	22.9	5.109	26	27.18	9.579	0.259
Non-vital bone	5	6.2	5.75	0	1.64	2.73	**0.012**
Inflammatory infiltrate	15	15.4	6.415	12	17.27	10.974	1
Microbial colonies	0	0.4	0.516	0	0.45	0.82	0.799
Root fragment	0	0.4	0.966	4	3.64	3.443	**0.008**
Connective tissue	34.5	35.1	9.814	33	28.27	11.306	0.306

HBOT=hyperbaric oxygen therapy; MD=median
**P*=*P* value for Mann-Whitney test, α=0.05; bold values show a significant difference

Frequency of non-vital bone

When considering presence/absence of non-vital bone on H&E examination, there was no significant difference in frequency of this variable between the HBOT group and respective control either at 7 days (*P*=0.412) or 14 days (*P*=0.316). However, comparison of the HBOT groups, 7 versus 14 days, showed that the frequency of non-

vital bone was significantly higher at 7 days (P=0.035) (Table 4; Fisher's exact test, α=0.05).

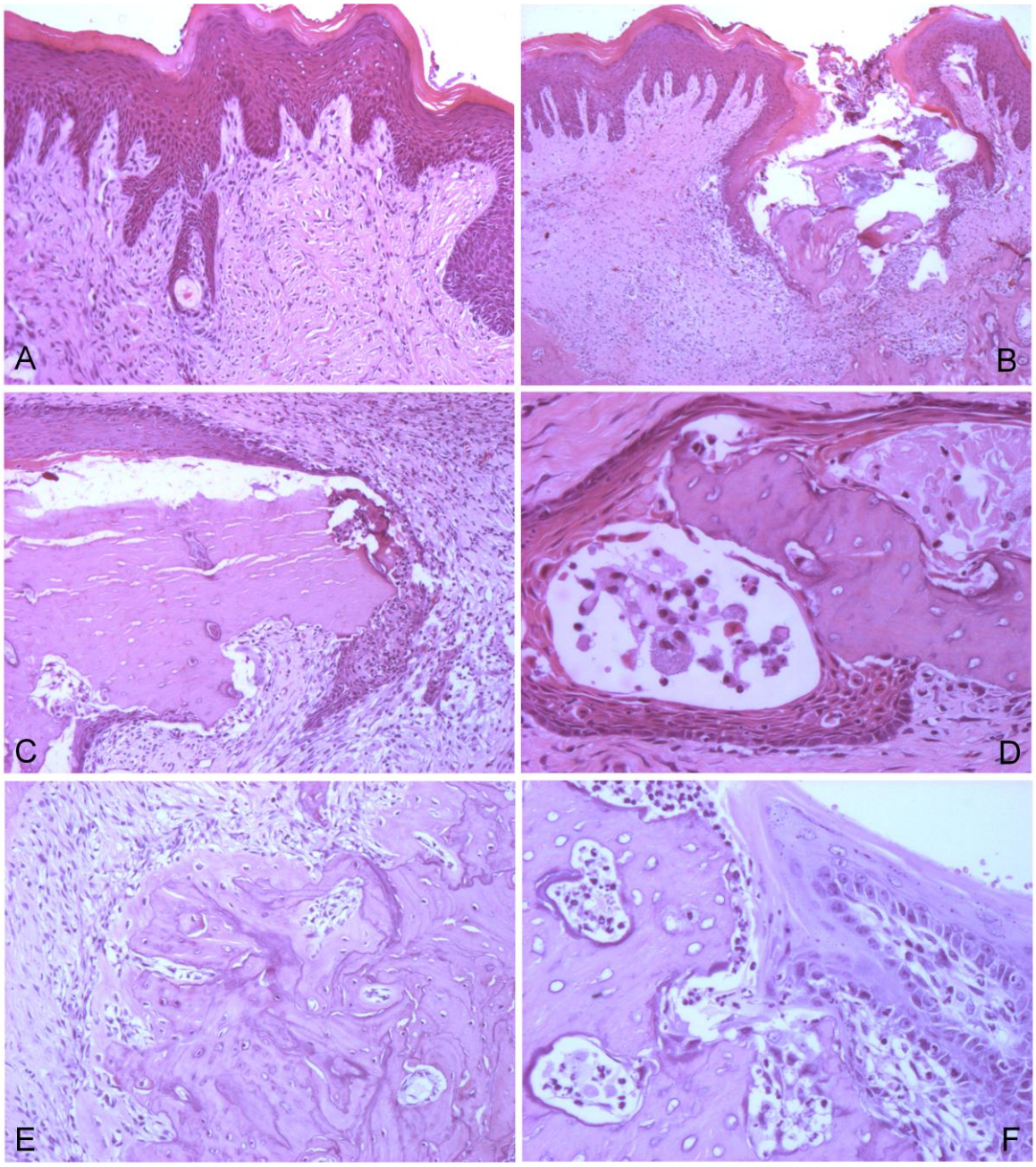

Figure 1 – Analysis of tooth extraction site in hematoxylin and eosin (H&E). Complete wound repair with epithelial and connective tissue (A, 100x); bone exposure (B, 100x) where non-vital bone is being circumscribed by soft tissue (C, 200x) with epithelial cell proliferation and inflammatory infiltrate (D, 400x). Vital bone showing neoformation (E, 400x) and non-vital bone with inflammatory infiltrate and adjacent epithelial cell proliferation (F, 400x).

Table 4 – Frequency of non-vital bone in the hyperbaric oxygen therapy and control groups at 7 days and 14days

		Non-vital bone						*P**
		Present		Absent		Total		
Time	**Group**	n	%	n	%	n	%	
7 days	HBOT	10	100	0	0	10	100	0.412
	Control	6	85.7	1	14.3	7	100	
14 days	HBOT	6	54.5	5	45.5	11	100	0.316
	Control	6	85.7	1	14.3	7	100	
7 days	HBOT	10	100	0	0	10	100	**0.035**
14 days	HBOT	6	54.5	5	45.5	11	100	

**P* value for Fisher's exact test, α=0.05; HBOT=hyperbaric oxygen therapy

Immunohistochemical analysis (Fig. 2)

At 7 days, the HBOT group showed lower expression than the control group for all the immune markers analyzed, VEGF (*P*=0.021), RANKL (*P*=0.005), BMP-2 (*P*=0.014) and OPG (*P*=0.042), whereas at 14 days of treatment, there was no significant difference between the groups for these variables (Table 5; Mann-Whitney test, α=0.05). Comparing the HBOT groups, 7 versus 14 days, VEGF (*P*=0.029) and OPG (*P*=0.043) showed significantly higher expression at 14 days of HBOT, whereas RANKL and BMP-2 did not show any significant difference (Table 6; Mann-Whitney test, α=0.05).

Table 5 – Immunostained area (μm^2) for VEGF, RANKL, BMP-2 and OPG in the hyperbaric oxygen therapy and the control groups at 7 days and 14 days

Marker	Group 7 Days							Group 14 Days						
	HBOT (μm^2)			Control (μm^2)				HBOT (μm^2)			Control (μm^2)			
	MD	P25	P75	MD	P25	P75	*P**	MD	P25	P75	MD	P25	P75	*P***
VEGF	94,235	64,441	141,909	123,194	76,475	196,887	**0.021**	125,894	76,475	196,887	119,359	73,229	198,615	0.879
RANKL	144,679	79,595	197,627	220,291	96,538	360,716	**0.005**	150,826	96,769	245,028	194,392	101,687	313,824	0.14
BMP-2	103,143	69,295	153,835	137,789	80,132	236,041	**0.014**	113,816	77,044	180,087	127,402	88,298	207,702	0.426
OPG	10,873	5142	27,383	18,620	10,546	38,419	**0.042**	18,264	9032	32,956	13,011	7860	38,340	0.594

HBOT=hyperbaric oxygen therapy; MD=median; P25=25th percentile; P75=75th percentile; VEGF=vascular endothelial growth factor; RANKL=receptor activator of nuclear factor kappa-B ligand; BMP-2=bone morphogenetic protein 2; OPG=osteoprotegerin
**P=P* value for comparison of HBOT and control at 7 days; Mann-Whitney test, α=0.05
***P=P* value for comparison of HBOT and control at 14 days; Mann-Whitney test, α=0.05
Bold values show significant difference

Table 6 – Immunostained area (μm^2) for VEGF, RANKL, BMP-2 and OPG in the hyperbaric oxygen therapy groups at 7 days and 14 days

Marker	HBOT						
	7 days (μm^2)			14 days (μm^2)			
	MD	P25	P75	MD	P25	P75	*P**
VEGF	94,235	64,441	141,909	125,894	76,475	196,887	**0.029**
RANKL	144,679	79,595	197,627	150,826	96,769	245,028	0.27
BMP-2	103,143	69,295	153,835	113,816	77,044	18,0087	0.225
OPG	10,873	5142	27,383	18,264	9032	32,956	**0.043**

HBOT=hyperbaric oxygen therapy; MD=median; P25=25th percentile; P75=75th percentile; VEGF=vascular endothelial growth factor; RANKL= receptor activator of nuclear factor kappa-B ligand; BMP-2=bone morphogenetic protein 2; OPG=osteoprotegerin
**P=P* value for Mann-Whitney test, α=0.05; Bold values show significant difference

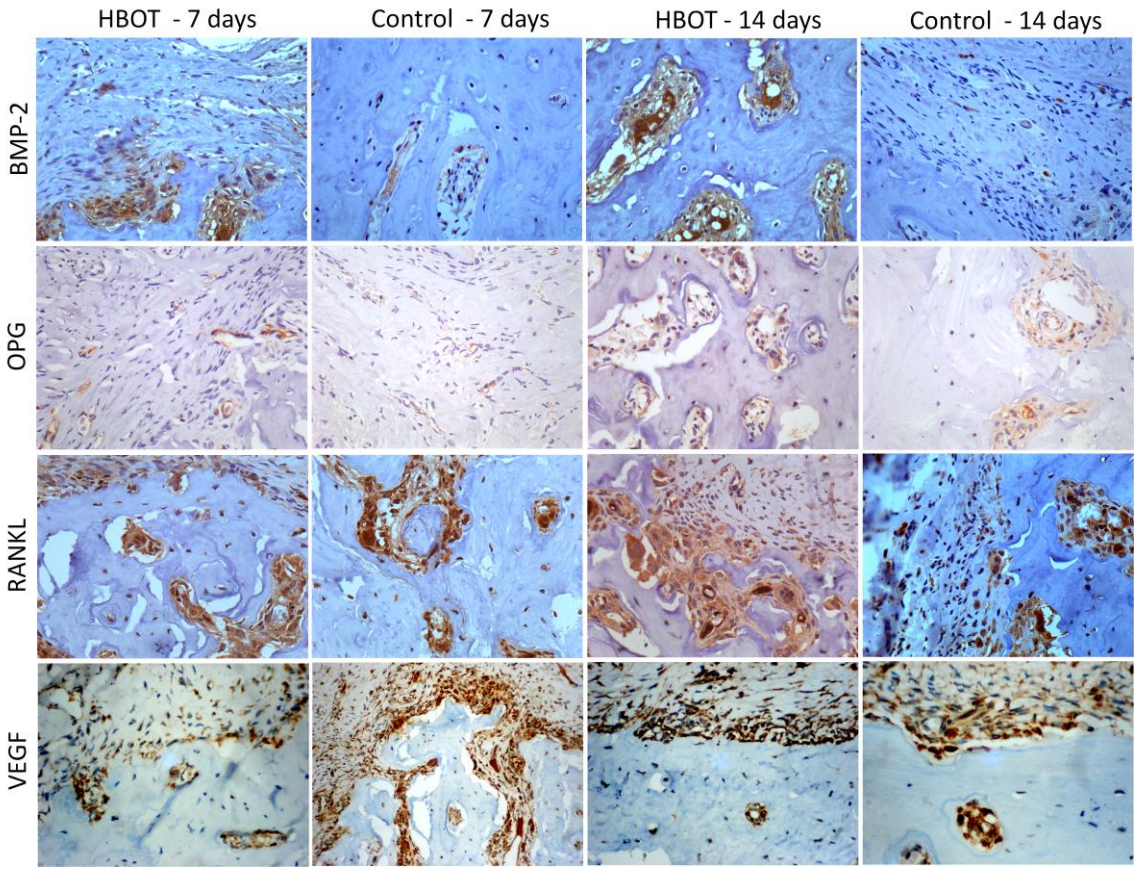

Figure 2 – Immunohistochemical analysis (400x) for BMP-2, OPG, RANKL and VEGF in oxygen therapy groups and controls at 7 and 14 days.

DISCUSSION

In the present study, the finding of no significant difference in volume of bone exposure between pre- and post-HBOT and also in the treatment effect on the size of the lesions, comparing the test groups and controls suggests that HBOT did not exert a significant effect on the clinical evolution of the lesions. This result agrees with some reports in the literature, which do not support the use of HBOT to treat lesions related to bisphosphonates.[14,18,32,33,63,64] Another possibility would be that the regimen of HBOT used in our study was not sufficient to determine macroscopically detectable improvement, since most protocols reported for BRONJ patients are longer.[22,26,33,61,73]

On the other hand, the lesions were very small making evaluation difficult, and some animals did not even develop detectable bone exposure after tooth extractions. However, it is important to recall here that according to recent updates, bone exposure should not be a *sine qua non* condition for BRONJ diagnosis.[8,74,75] Therefore, it seems crucial to consider not only the clinical evaluation but also the parameters microscopically analyzed.

On H&E examination, there was significantly less epithelium in the HBOT group than control at 7 days, whereas at 14 days this difference was no longer observed. Considering that the migration of oral epithelial cells is a major step for tooth socket closure and that it can be suppressed by zoledronic acid,[76] these results suggest impaired healing in animals subjected to HBOT at 7 days and some improvement at 14 days. Also, non-vital bone did not differ between HBOT group and control at 7 days but it was significantly less in the HBOT group than control at 14 days. Besides that, albeit not statistically significant, vital bone amount at 14 days was greater in HBOT than control. Considering that the *P* value for this comparison was 0.056, a larger sample size could have given a significant result for this variable. When the two HBOT groups were compared to each other, non-vital bone amounts were again significantly higher at 7 days. All these findings suggest that duration of treatment influences the results with HBOT, where it seems to become effective after 14 days. Moreover, the lower amounts of root fragments in the 7-day HBOT group reinforces that such results were not influenced by this iatrogenic local factor, which could impair wound healing. The significantly higher frequency of non-vital bone at 7 days when presence/absence of this variable was compared between HBOT groups also agrees with these findings. That is, longer treatment results in a greater probability of reducing non-vital bone and therefore healing improvement, which is corroborated by the reported cumulative effect of HBOT.

Since the atmospheric pressure used in our study was at the level to obtain the best therapeutic results, and recalling that in HBOT, oxygen is delivered at 100%, the way to increase the dose would be to extend the time of treatment, which can be adjusted according to the severity of the case.[77]

It has been reported that bisphosphonates inhibit angiogenesis[16,78] and promote OPG generation[79] and therefore inhibit osteoclastogenesis, besides inducing osteoclast apoptosis.[12,15] HBOT on the other hand would promote angiogenesis[51,80] and, by generating ROS and RNS, would interfere with bone metabolism, enhancing RANKL expression and consequently osteoclast differentiation.[12] Based on these assumptions it has been suggested that HBOT would counteract the effects of bisphosphonates.[12] Nevertheless, contrary to that expected, the HBOT group at 7 days showed significantly lower expression than the control for all the markers analyzed (VEGF, RANKL, BMP-2 and OPG). At first, it could mean that HBOT did not improve healing, as the process needs these mediators. However, at 14 days, there was no difference, and when comparing HBOT groups to each other (7 versus 14 days), VEGF and OPG were significantly higher at 14 days. Some points deserve to be considered here. Even though these mediators are necessary for wound repair, their expression can vary also in response to other stimuli, such as infection.[81-83] Therefore, when reducing infection, a well-known effect of HBOT,[80] the therapy could reduce local levels of VEGF, BMP-2 and RANKL. Another point to ponder is that hypoxia is considered a major factor in VEGF generation,[84] and that non-vital bone in BRONJ is colonized by anaerobes, especially *Actinomyces* sp.[85] In this context, it seems reasonable to infer that in this environment of infection and anaerobiosis, the sites not subjected to HBOT would need more VEGF than those subjected to HBOT, where presumably there would be less hypoxia. Still regarding this issue, Kalns et al.[86] reported that HBOT caused significant

downregulation of angiogenesis, which they attributed to the regimen of HBOT applied. According to the authors, the regimen was sufficient to maintain oxygen levels above the threshold required for VEGF expression and new vessel formation.

The expression of the markers evaluated in our study is not a dichotomous or an independent process. There are many factors, mediators and feedback mechanisms involved that can change gradients in the microenvironment and therefore the markers expression, including bisphosphonate type and dose, HBOT dose and the phase of the wound repair these therapies interfere with. VEGF inhibition or generation, e.g., can occur depending on the gradient of hypoxia or hyperoxia;[86] high doses of zoledronic acid can markedly enhance RANKL and, at lower intensity, OPG, whereas lower doses of this bisphosphonate can enhance OPG at higher rates than RANKL.[17] Different types of bisphosphonate are also related to distinct effects on these mediators.[17] The events are dynamic and the up- or downregulation of one mediator or the other at a specific moment can depend on the the site evaluated and the stimulus involved at that moment. Accordingly, because ROS and RNS are the basis for HBOT's signaling effects, its clinical consequences in any therapeutic situation will be dose-, tissue-, and time-specific.[12] More or less aggressive regimens of HBOT can produce different results, and thus the discrepancies between different studies.[86]

Eventually, according to the H&E results in our study, HBOT seems to produce beneficial effects on the repair process of tooth extraction sites in rats under bisphosphonate therapy. Anyway, further studies comparing the effects of this therapy with the other treatments currently used for BRONJ and also using different types and doses of bisphosphonate, as well as longer regimens of HBOT, could give us more precise information.

CONCLUSION

HBOT can reduce the amounts of non-vital bone microscopically detected in tooth extraction sites of rats subjected to bisphosphonate therapy. The effect seems to occur in a dose-dependent mode. Further studies are required to clarify the mechanisms accounting for this effect.

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Discussão Geral

4 DISCUSSÃO GERAL

Bisfosfonatos são potentes inibidores da reabsorção óssea que, em função de sua alta afinidade pelo cálcio da hidroxiapatita, fixam-se ao tecido ósseo onde podem ter meia-vida superior a 10 anos e exercem seus efeitos de inibição da função osteoclástica e indução dessas células à apoptose (Fleisch, 1998; Russel, 2006). Enfermidades como osteoporose (Cremers *et al.*, 2005), osteogênese imperfeita (Leite *et al.*, 2006), mieloma múltiplo (Berenson, 2002), hipercalcemia associada ao câncer, bem como metástases ósseas de tumores sólidos (Drake *et al.*, 2008) têm nesses agentes uma importante opção terapêutica. Entretanto, a despeito da alta eficácia, os bisfosfonatos exibem um significativo efeito adverso, a osteonecrose dos maxilares (*bisphosphonate-related osteonerosis of the jaw,* BRONJ), cujos relatos têm acontecido desde 2003 (Marx, 2003; Khosla *et al.*, 2007; Migliorati *et al.*, 2005; Ruggiero *et al.*, 2014; Yoneda *et al.*, 2010). A BRONJ é uma enfermidade de elevada morbidade e difícil tratamento, em que múltiplas modalidades terapêuticas têm sido indicadas (Curi *et al.*, 2007; Hinson *et al.*, 2015; Marx *et al.*, 2005; Ruggiero *et al.*, 2004; Yoneda *et al.*, 2010) e, mesmo assim, muitos casos mostram-se refratários. Terapia antimicrobiana, intervenções cirúrgicas conservadoras e radicais, bem como tratamentos alternativos têm sido aplicados sem que nenhum deles se mostre, de forma isolada ou mesmo combinada, realmente eficaz. A oxigenoterapia hiperbárica (*hyperbaric oxygen therapy*, HBOT) tem sido apontada como uma forma de tratamento passível de ser instituída nos casos de BRONJ (Biasotto *et al.*, 2006; Fatema *et al.*, 2015; Freiberger *et al.*, 2007; Freiberger *et al.*, 2012; Shimura *et al.*, 2006). Entretanto, os trabalhos que preconizam seu uso são estudos clínicos, muitos deles relatos de caso, havendo divergência sobre a efetividade da terapia (Dimopoulos *et al.*, 2006; Heras Rincón *et al.*, 2007; Marx *et al.*, 2005; Migliorati *et al.*, 2005; Nastro *et*

al., 2007; Ruggiero *et al*., 2014). A necessidade de um tratamento resolutivo e a falta de consenso a respeito da propriedade da indicação da HBOT para tratamento da BRONJ inspiraram a realização da presente pesquisa.

O experimento desenvolvido em modelo animal sob terapia com ácido zoledrônico e submetido a exodontias múltiplas permitiu controlar vieses inerentes aos estudos clínicos e avaliar de forma quantitativa o efeito da HBOT sobre o reparo tecidual no sítio das exodontias. Os resultados da análise macroscópica não exibiram diferença significativa das lesões antes e após o tratamento e nem entre grupos-teste e seus respectivos controles, o que poderia indicar ineficácia do tratamento. Por outro lado, a exposição óssea não parece mais ser condição *sine qua non* na definição dos casos de BRONJ (Fedele *et al.*, 2010; Fedele *et al.*, 2015; Ruggiero *et al.*, 2014), o que exige especial atenção aos resultados da avaliação microscópica.

A análise em hematoxilina e eosina (H&E) evidenciou proporção de osso não-vital significativamente menor no grupo HBOT aos 14 dias quando comparado ao controle. Por outro lado, aos sete dias, essa significância não foi observada, o que sugere que a terapia seja capaz de auxiliar no processo de reparo a partir de 14 dias de tratamento. Tal achado evidencia a influência do tempo de exposição e dose de oxigênio, já que a terapia exibe efeito cumulativo (D'Agostino *et al.*, 2008; Zang *et al.*, 2008). Talvez um período mais longo de exposição à HBOT tivesse repercutido em redução significativa das lesões também ao exame macroscópico. Uma vez que a pressão atmosférica aplicada estava na faixa capaz de determinar os melhores resultados terapêuticos (D'Agostini *et al.*, 2008; Zang *et al.*, 2008) e lembrando que o oxigênio é mantido sempre a 100%, a variável a ser ajustada seria o tempo de tratamento, que levaria a uma maior dose de HBOT.

Na análise imunoistoquímica, o grupo HBOT exibiu, aos sete dias, expressão significativamente menor dos marcadores avaliados (VEGF, RANKL, BMP-2 e OPG) que o controle. Já aos 14 dias, a diferença entre grupo-teste e controle não foi significativa para nenhum marcador. Na comparação dos grupos-teste entre si foi possível observar expressão significativamente maior de VEGF e OPG aos 14 dias, enquanto RANKL e BMP-2 não exibiram diferença significativa. Embora este achado pudesse sugerir uma tendência de aumento dose-dependente dos marcadores, o que concordaria com a literatura (Freiberger *et al.*, 2007; Freiberger., 2009; Mutlu *et al.*, 2012; Okubo *et al.*, 2001), é preciso considerar que também pode refletir o efeito do fator tempo independentemente do tratamento, daí a necessidade de comparação ao respectivo grupo-controle. Ainda considerando os resultados da análise imunoistoquímica, é preciso lembrar que vários fatores concorrem para o aumento ou para a diminuição da expressão dos marcadores avaliados, entre eles, dose e tipo de bisfosfonato, dose de HBOT e sítio avaliado (Kalns *et al.*, 2003; Koch *et al.*, 2012). Tais fatores contribuem para a divergência de resultados entre os estudos relatados na literatura (Freiberger, 2009; Kalns *et al.*, 2003), assim como podem ter influenciado os resultados da presente pesquisa.

A BRONJ é uma enfermidade de elevada morbidade, que compromete de forma significativa a qualidade de vida dos pacientes, sejam estes portadores de osteopenia, osteoporose ou câncer. O profissional assistente, por sua vez, depara-se com uma condição crônica, muitas vezes refratária ao tratamento, em que os recursos disponíveis ainda não são eficazes. Alguns autores consideram a suspensão do bisfosfonato fator crucial para o sucesso do tratamento da BRONJ (Freiberger *et al.*, 2007; Hinson *et al.*, 2015; Magopoulos *et al.*, 2007). Entretanto, mediante a condição clínica de alguns

pacientes, a suspensão da droga pode não constituir opção viável. Nesses casos, a HBOT concorreria como mais uma possibilidade de abordagem terapêutica.

Os resultados da presente pesquisa indicam que a HBOT determina efeitos favoráveis no reparo tecidual pós-exodontia em ratos tratados com bisfosfonato. Achado este que, respeitadas as limitações de estudos em modelo animal, pode ser extrapolado para a rotina clínica. Partindo-se da premissa de que a BRONJ é uma doença multifatorial e que nenhum dos tratamentos disponíveis se mostra eficaz isoladamente, a abordagem com várias modalidades terapêuticas, incluindo a HBOT, pode favorecer a evolução clínica dos pacientes. Ou seja, a HBOT pode ser considerada um método adjuvante no combate à BRONJ, ainda que cada caso deva ser avaliado de forma específica, sempre considerando as particularidades de cada paciente e ponderando tanto a relação risco/benefício quanto custo/benefício. A despeito disso, novas pesquisas com rigorosa padronização de método e emprego de distintos grupos de bisfosfonatos em diferentes doses, bem como outros regimes de HBOT, contemplando principalmente o quesito tempo de tratamento, fazem-se necessárias.

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Anexos

ANEXO A

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

www.elsevier.com/journals/archives-of-oral-biology/0003-9969?generatepdf=true

ANEXO B

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

Submission Confirmation for Important topics on hyperbaric oxygen therapy with focus on its application in bisphosphonate-related osteonecrosis of the jaw

ees.aob.0.31a2d6.406136b8@eesmail.elsevier.com em nome de Archives of Oral Biology [AOB@elsevier.com]
Enviado: segunda-feira, 1 de junho de 2015 9:35
Para: Karen Cherubini; karencherubibi66@gmail.com; Karen Cherubini

Archives of Oral Biology
Title: Important topics on hyperbaric oxygen therapy with focus on its application in bisphosphonate-related osteonecrosis of the jaw
Authors: Miguel L Silva, DDS; Leandro Tasso, Ph.D.; Maria A Figueiredo, Ph.D.; Fernanda G Salum, Ph.D.; Karen Cherubini, Ph.D.
Article Type: Review Article

Dear Karen,

Your submission entitled "Important topics on hyperbaric oxygen therapy with focus on its application in bisphosphonate-related osteonecrosis of the jaw" has been received by Archives of Oral Biology.

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Thank you for submitting your work to this journal. Please do not hesitate to contact me if you have any queries.

Kind regards,

(On behalf of the Editors)

Archives of Oral Biology

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ANEXO C

Normas para submissão de manuscritos ao periódico *Head & Neck*

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ANEXO D

Comprovante de submissão do manuscrito ao periódico *Head & Neck*

Manuscript submitted to Head & Neck - HED-15-0662, Authors Copy
onbehalfof+mcrapanz+mdanderson.org@manuscriptcentral.com em nome de mcrapanz@mdanderson.org
Enviado: sexta-feira, 5 de junho de 2015 14:38
Para: Karen Cherubini; kebini.ez@terra.com.br

05-Jun-2015

Manuscript number: HED-15-0662

Dear Prof. Cherubini:

We are pleased to receive your manuscript entitled Effect of hyperbaric oxygen therapy on tooth extraction sites in rats subjected to bisphosphonate therapy - Histomorphometric and immunohistochemical analysis by Silva, Miguel; Tasso, Leandro; Azambuja, Alan; Figueiredo, Maria Antonia; Salum, Fernanda; da Silva, Vinicius; Cherubini, Karen.

We will be sending it out for review shortly.

To track the progress of your manuscript through the editorial process using our new web-based system, simply point your browser to:

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Please remember in any future correspondence regarding this article to always include its manuscript ID number HED-15-0662.

If you experience problems associated with the submission web site, please contact the Wiley support staff directly at: mcrapanz@mdanderson.org

Many thanks for submitting your manuscript,

Dr. Ehab Hanna
Editor in Chief
Head & Neck

ANEXO E

Comissão Científica e de Ética
Faculdade da Odontologia da PUCRS

Porto Alegre 13 de Abril de 2011

O Projeto de: Tese

Protocolado sob nº:	0026/11
Intitulado:	Análise histomorfométrica e imunoistoquímica do efeito da oxigenoterapia hiperbárica sobre a osteonecrose dos maxilares induzida por bisfosfonatos
Pesquisador Responsável:	Profa. Dra. Karen Cherubini
Pesquisadores Associados	Miguel Luciano da Silva; Alan Arrieira Azambuja; Leandro Tasso
Nível:	Tese / Doutorado

Foi ***aprovado*** pela Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS em *13 de Abril de 2011.*

Este projeto deverá ser imediatamente encaminhado ao CEUA/PUCRS

Profa. Dra. Ana Maria Spohr
Presidente da Comissão Científica e de Ética da
Faculdade de Odontologia da PUCRS

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ANEXO F

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

Ofício 079/11 – CEUA Porto Alegre, 07 de julho de 2011.

Senhora Pesquisadora:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 11/00232, intitulado: **"Análise histomorfométrica e imunoistoquímica do efeito da oxigenoterapia hiperbárica sobre a osteonecrose dos maxililares induzida por bisfosfonato".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora do CEUA/PUCRS

Ilma. Sra.
Profa. Dra. Karen Cherubini
N/Universidade

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ANEXO G

COMISSÃO DE ÉTICA NO USO DE ANIMAIS
CEUA-UCS

Of.CEUA 008/2011

Caxias do Sul, 14 de Setembro de 2011.

Número: 006/11

Título: Análise histomorfométrica e imunoistoquímica do efeito da oxigenoterapia hiperbárica sobre a osteonecrose dos maxilares induzida por bisfosfonatos

Investigador principal: Leandro Tasso

A Comissão de Ética no Uso de Animais da Universidade de Caxias do Sul, em reunião ordinária do dia 13 de Setembro de 2011, analisou o projeto supracitado e o considerou aprovado em seus aspectos éticos e metodológicos de acordo com a Lei nº 11.794/08 que estabelece procedimentos para o uso científico de animais.

A CEUA deve ser informada de todos os eventos adversos ou fatos relevantes que alterem o curso normal do estudo.

Relatórios parciais devem ser apresentados a CEUA semestralmente e ao término do estudo.

Lembramos que o pesquisador responsável é o representante legal pela posse e guarda de todo material envolvido na pesquisa.

Salientamos que o membro da Comissão de Ética no Uso de Animais, não participou do processo de avaliação onde consta como investigador Principal.

Atenciosamente,

Prof. Ms. Luis Gonzaga de Moura Júnior
Coordenador CEUA/UCS