UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de Pós-graduação em Biologia Celular

Juliano Douglas Silva Albergaria

ASSOCIAÇÃO DO RANELATO DE ESTRÔNCIO À ENXERTOS ÓSSEOS BOVINOS MINERALIZADOS E DESMINERALIZADOS: EFEITOS *IN VITRO* E *IN VIVO*.

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ATA DA DEFESA DE TESE DE DOUTORADO DE

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Às **nove horas** do dia **31 de agosto de 2021**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **"ASSOCIAÇÃO DO RANELATO DE ESTRÔNCIO À ENXERTOS ÓSSEOS BOVINOS MINERALIZADOS E DESMINERALIZADOS: EFEITOS IN VITRO E IN VIVO"**, requisito final para obtenção do grau de Doutor em Biologia Celular. Abrindo a sessão, a Presidente da Comissão, **Dra. Gerluza Aparecida Borges Silva**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	Indicação
Dra. Gerluza Aparecida Borges Silva	UFMG	Aprovado
Dra. Ivana Márcia Alves Diniz	UFMG	Aprovado
Dra. Michele Munk Pereira	UFJF	Aprovado
Dra. Erika Lorena Fonseca Costa de Alvarenga	UFSJ	Aprovado
Dr. Bruno Machado Bertassoli	UNIBH	Aprovado

Pelas indicações, o candidato foi considerado: Aprovado
O resultado final foi comunicado publicamente ao candidato pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 31 de agosto de 2021.**

Drª. Gerluza Aparecida Borges Silva (Orientadora)

Dra. Ivana Márcia Alves Diniz

Drª. Erika Lorena Fonseca Costa de Alvarenga

Dr. Bruno Machado Bertassoli

Drª. Michele Munk Pereira

Obs: Este documento não terá validade sem a assinatura do Coordenador

Dedico este trabalho aos meus pais que desde a infância me ensinaram os grandes valores das pequenas coisas, à minha noiva, família, amigos e mestres pela motivação e felicidade que me proporcionaram durante esta caminhada.

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RESUMO

Os defeitos ósseos constituem um sério problema para as clínicas odontológica e ortopédica e podem ser causados por diferentes motivos, como processos degenerativos, traumas, tumores e perdas dentárias**.** Várias estratégias são empregadas e estudos desenvolvidos para a reconstrução do tecido ósseo. Na atualidade, os enxertos ósseos bovinos mineralizados (OBM) constituem uma das alternativas mais utilizadas na clínica odontológica. Todavia, a utilização de matrizes desmineralizadas tem sido proposta por apresentar proteínas colagenosas e não colagenosas, melhorando a osteoindutividade e osteocondutividade do enxerto. O uso de terapias medicamentosas sistêmicas, como o ranelato de estrôncio (RE), associadas as enxertias ósseas para tratamento de fraturas, também têm sido investigadas. Assim sendo, num primeiro momento o nosso grupo de estudo avaliou a capacidade de xenoenxertos desmineralizados por ácido etilenodiaminotetracético (EDTA), em comparação com enxertos mineralizados, no processo de regeneração de defeitos ósseos intrabucais em ratos. Os efeitos das enxertias foram avaliados após 1, 7, 14, 21 e 49 dias sobre a epitelização do sítio cirúrgico e regeneração do tecido ósseo. As avaliações foram realizadas por macrofotografias padronizadas, radiografia e histologia. Os resultados mostraram que no período de 7 dias não houve diferença estatística na quantidade de tecido de granulação entre os grupos e aos 14 dias, todos os grupos apresentaram a região do sítio cirúrgico completamente epitelizada. Os resultados radiográficos e histológicos mostraram que o enxerto de osso bovino desmineralizado (OBD) induziu maior quantidade de neoformação óssea e osso maduro. Esses resultados revelaram que o OBD representa uma importante alternativa para terapias de reconstrução óssea na clínica. Em um segundo momento, avaliamos a reconstrução do modelo de defeito ósseo proposto e suas enxertias por histologia e ensaios radiográficos sobre dois métodos: radiopacidade por tons de cinza e análise fractal. Tanto a histologia quanto os dois métodos de avaliação radiográfica demonstraram maior quantidade de osso neoformado para o grupo desmineralizado. Todavia, a análise fractal não encontrou diferenças estatísticas no período final de avaliação. Dessa forma, a quantificação por escala de cinza pareceu ser mais efetiva quando comparada com a análise fractal. Porém, ressalta-se que na avaliação de enxertias mineralizadas, os dois métodos de avaliação apresentaram eficácia. Extrapolamos os testes de melhoria das propriedades osteocondutoras e osteoindutoras dos enxertos estudados, mineralizados e desmineralizados, por meio da adsorção de estrôncio (Sr), utilizando testes *in vitro* em culturas de MC3T3-E1, e *in vivo* por meio do modelo de defeito ósseo estudado nos períodos de 1, 7, 14, 21 e 60 dias. Inicialmente, os enxertos foram tratados com solução saturada de RE por 14 dias. A técnica de espectrometria de emissão atômica por plasma acoplado indutivamente (ICP-OES) foi empregada para avaliar a quantidade de Sr adsorvido nos biomateriais, sendo significativamente maior

no enxerto mineralizado. A técnica demonstrou também que o enxerto mineralizado liberou gradativamente o Sr adsorvido em meio aquoso ao longo de 14 dias. Foi realizado testes de citotoxicidade e atividade celular por MTT – períodos de 3 e 7 dias de cultura celular, e Fosfatase Alcalina (FA) – períodos de 7 e 10 dias de cultura celular, respectivamente. Os resultados mostraram que os enxertos associados ao Sr apresentaram maior viabilidade celular em todos os períodos avaliados. Quanto a atividade celular, os grupos tratados com enxertos sem adsorção de Sr demonstraram melhores resultados aos 7 dias de cultivo celular. Aos 10 dias, apenas o enxerto mineralizado sem Sr apresentou resultados superiores em atividade celular. Nas experimentações *in vivo*, as avaliações macroscópicas revelaram uma possível toxicidade do Sr ao epitélio nos períodos de 7 e 14 dias. Quanto a neoformação óssea, avaliada por radiografia e histomorfometria, os enxertos mineralizado e desmineralizado associados ao Sr apresentaram elevada quantificação de neoformação e maturação óssea no período final de avaliação. Esses resultados sugerem que o Sr associado aos xenoenxertos favorece a deposição óssea e não apresenta citotoxicidade aos osteoblastos, mas compromete a epitelização gengival nas concentrações estudadas.

Palavras-chave: Regeneração óssea; *scaffolds*; biomaterial orgânico; citotoxicidade.

ABSTRACT

Bone defects are a serious problem for dental and orthopedic clinics and can be caused by different reasons, such as degenerative processes, trauma, tumors and tooth loss. Several strategies are used and studies developed for bone tissue reconstruction. Currently, mineralized bovine bone grafts (MBB) constitute one of the most used alternatives in the dental clinic. However, the use of demineralized matrices has been proposed to present collagenous and non-collagenous proteins, improving graft osteoinductivity and osteoconductivity. The use of systemic drug therapies, such as strontium ranelate (SrRan), associated with bone grafts for the treatment of fractures, has also been investigated. Therefore, at first, this work evaluated the capacity of ethylenediaminetetraacetic acid (EDTA) demineralized xenografts compared to mineralized grafts in the process of regeneration of intrabuccal bone defects in rats. The effects of grafts were evaluated after 1, 7, 14, 21 and 49 days on surgical site epithelialization and bone tissue regeneration. Evaluations were performed by standardized macrophotographs, radiography and histology. The results showed that within 7 days there was no statistical difference in the amount of granulation tissue between the groups and at 14 days, all groups had the region of the surgical site completely epithelialized. The radiographic and histological results showed that demineralized bovine bone graft (DBB) induced a greater amount of bone neoformation and mature bone. These results revealed that DBB represents an important alternative for bone reconstruction therapies in the clinic. Secondly, we evaluated the reconstruction of the proposed bone defect and its grafts by histology and radiographic tests on two methods: grayscale radiopacity and fractal analysis. Both histology and the two methods of radiographic evaluation showed a larger amount of newly formed bone for the demineralized group. However, fractal analysis did not find statistical differences in the final evaluation period. Thus, gray scale quantification appeared to be more effective when compared to fractal analysis. It is noteworthy that in the evaluation of mineralized grafts, both evaluation methods were effective. We extrapolated the tests for improving the osteoconductive and osteoinductive properties of the studied, mineralized and demineralized grafts, by means of strontium (Sr) adsorption, using in vitro tests on MC3T3-E1 cultures, and in vivo using the bone defect model studied in the periods of 1, 7, 14, 21 and 60 days. Initially, the grafts were treated with saturated SrRan solution for 14 days. The Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-OES) technique was used to evaluate the amount of Sr adsorbed in biomaterials, being significantly higher in mineralized graft. The technique also demonstrated that the mineralized graft gradually released Sr adsorbed in aqueous medium over 14 days. Cytotoxicity and cell activity tests were performed by MTT - periods of 3 and 7 days of cell culture, and Alkaline Phosphatase (AP) - periods of 7 and 10 days of cell culture, respectively. The results showed that grafts associated with Sr

had greater cell viability in all periods evaluated. Regarding cellular activity, the groups treated with grafts without Sr adsorption showed better results at 7 days of cell culture. At 10 days, only the mineralized graft without Sr showed superior results in cellular activity. In *in vivo* experiments, macroscopic evaluations revealed a possible toxicity of Sr to the epithelium at 7 and 14 days. As for bone neoformation, evaluated by radiography and histomorphometry tests, the mineralized and demineralized grafts associated with the Sr showed high quantification of bone neoformation and maturation in the final evaluation period. These results suggest that Sr associated with xenografts favors bone deposition and does not present cytotoxicity to osteoblasts, but compromises gingival epithelialization at the studied concentrations.

Keywords: Bone Regeneration; scaffolds; organic biomaterial; cytotoxicity.

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LISTA DE SIGLAS E ABREVIATURAS

Capítulo I:

BC-control: blood clot

BO: Bio-Oss®

DBB: Demineralized bovine bone

EDTA: ethylenediamine tetraacetic acid

FA: fractal analysis

FD: fractal dimension

MGS: mean gray scale

ROIs: regions of interest

Capítulo II:

AP: Alkaline phosphatase

BC: Bone defects filled with blood clot

BMP: Bone Morphogenetic Proteins

CBFA1: Core-Binding Factor Alpha-1

D + Sr: Bone defects filled with strontium-adsorbed demineralized bovine bone graft.

D: Bone defects filled with strontium-free demineralized bovine bone graft

ICP-OES: Inductively Coupled Plasma - Atomic Emission Spectrometry

M + Sr: Bone defects filled with strontium-adsorbed mineralized bovine bone graft

M: Bone defects filled with strontium-free mineralized bonive bone graft

PBS: Phosphate buffered saline

RANKL: Receptor activator of nuclear factor kappa B ligand

RUNX2: Runt-Related Transcription Factor 2

Sr: Strontium

SrRan: Strontium ranelate

SUMÁRIO

INTRODUÇÃO

Os defeitos ósseos crâniomaxilofaciais constituem um problema para a clínica odontológica, pois comprometem não somente a estética dos pacientes, que por si já impactam em um prejuízo psicológico, mas especialmente as diversas funções do sistema estomatognático (Mittal *et al*., 2016). Esses defeitos podem ser consequentes de diferentes causas, como processos degenerativos, traumas, tumores e perdas dentárias (Watanabe *et al.*, 2016). Inúmeras pesquisas com variadas abordagens e estratégias têm sido desenvolvidas para a reconstrução do tecido ósseo (Shirmohammadi *et al.*, 2014; Jo *et al*., 2018; Yaghobee *et al.*, 2018; Naros *et al.*, 2019; Pichotano *et al.*, 2019).

Na atualidade, os enxertos ósseos bovinos mineralizados (OBM) constituem uma das alternativas mais utilizadas para a substituição óssea na clínica odontológica (Bahammam, 2016; de Assis Gonzaga *et al*., 2017). Por essa razão, a melhoria de suas propriedades osteocondutoras e osteoindutoras torna-se fator chave para terapias cada vez mais eficazes no restabelecimento do paciente (Drosos *et al*., 2015; Houdek *et al*., 2015).

A osteocondução consiste na migração de células osteoblásticas e osteoprogenitoras por meio do biomaterial transplantado, além do aporte para o crescimento e inserção de vasos sanguíneos durante o reparo ósseo. Já a osteoindução é a propriedade do biomaterial que induz o processo de diferenciação das células osteoprogenitoras em osteoblastos maduros (Elsalanty e Genecov, 2016). Os xenoenxertos são biomateriais conhecidos por apresentar uma boa osteocondução, mas baixa osteoindução (Nart *et al*., 2016; Yang *et al*., 2021).

Neste sentido alguns estudos têm sugerido a utilização de enxertos de matrizes ósseas desmineralizadas as quais apresentam proteínas colagenosas e não colagenosas, como as Proteínas Morfogenéticas Ósseas (BMPs) e osteopontina, capazes de melhorar a osteocondutividade e osteoindutividade (Fernandez de Grado *et al*., 2018). Essa melhoria favorece uma rápida revascularização permitindo o início do processo de osteogênese (Mahyudin *et al*., 2017).

Visando acelerar o reparo ósseo, indústrias voltadas para o mercado odontológico já disponibilizam produtos elaborados a partir da associação de proteínas derivadas da matriz óssea orgânica, como esponjas de colágeno com BMPs. A família das BMPs é considerada agente chave da indução osteoblástica por favorecer a diferenciação de células mesenquimais em células osteoprogenitoras (Sierra-Garcia *et al*., 2016) e seu uso tem aprovação do *Food and Drug Administration*.

A osteopontina é outra importante proteína exposta em enxertos ósseos desmineralizados que favorece a sua osteocondução. Essa é uma fosfoproteína da matriz extracelular que medeia diferentes funções biológicas (*Nam e Han*, 2016). A osteopontina estimula vias de sinalização celular por diferentes receptores na maioria das células, estimulando a migração das mesmas. Outro fator que favorece esse processo é a sua interação com integrinas, o que auxilia a adesão e sobrevivência das células (Yang *et al*., 2021). Além disso, essa proteína permite a formação de tecidos calcificados e a sua remodelação por possuir sítios de ligação ao cálcio (Ca). Essa ligação promove a formação de uma estrutura tridimensional que regula a biomineralização e também a atividade de osteoclastos (Lund *et al*., 2016)

Em um estudo realizado como minha dissertação de mestrado (Defesa em 16/03/2017), comparou-se os efeitos dos enxertos ósseos desmineralizados e mineralizados de origem nacional e importada na regeneração de defeito ósseo intrabucal em ratos. O defeito ósseo do experimento apresentou dimensões padronizadas, criados nos maxilares de ratos na região do primeiro molar superior. O enxerto desmineralizado proposto para teste possuía preservado em sua matriz orgânica colágeno tipo I, osteopontina e BMPs. Como controle negativo as cavidades foram preenchidas apenas com coágulo sanguíneo.

Após 1, 7, 14, 21 e 49 dias, os animais foram sacrificados para avaliação da neoformação óssea e dos efeitos dos biomateriais na epitelização da mucosa sobre o sítio cirúrgico em cada grupo estudado. A influência dos biomateriais no fechamento das feridas foi avaliada por meio de macrofotografias padronizadas e os efeitos dos biomateriais no reparo ósseo foram avaliados por análises radiográfica e histológica (tricrômico de masson e picrosirius red).

Os resultados revelaram que os biomateriais (mineralizados ou desmineralizados) não interferiram na cicatrização gengival. Todos os grupos apresentaram, aos 14 dias, a região do sítio cirúrgico completamente fechada, com a mucosa epitelizada, sem diferença estatística entre eles. Quanto ao reparo ósseo, os resultados radiográficos mostraram maior ganho de radiopacidade no período final de regeneração para os grupos tratados com o enxerto de osso bovino desmineralizado (OBD), sugerindo uma maior quantidade de neoformação óssea. Os resultados histológicos corroboraram com os achados radiográficos. Verificou-se maior quantidade de tecido ósseo neoformado, bem como maior nível de maturação óssea para o grupo OBD a partir de 14 dias pós cirurgia. Concluiu-se que os enxertos ósseos bovinos de natureza desmineralizada (OBD) merecem atenção dos pesquisadores. Isto por que podem evoluir para uma importante alternativa dentre as terapias de reconstrução óssea na clínica odontológica, especialmente quando a resistência mecânica inicial no sítio enxertado não é

condição prioritária. Esse estudo foi aceito para publicação na revista *Journal of the International Academy of Periodontology* e encontra-se no anexo I dessa tese. Ressalta-se que esse trabalho foi a base para o desenvolvimento da tese aqui apresentada.

Além das matrizes ósseas desmineralizadas, paralelamente, o uso de fármacos também já tem sido sugerido como coadjuvante nos processos de enxertias e reparos de fraturas ósseas (Baheiraei *et al*., 2021). Alguns estudos têm investigado o uso tópico do Ranelato de Estrôncio (RE) na recuperação de perdas ósseas, incorporado ou não a biomateriais (Lourenço *et al*., 2017; Chiang *et al*., 2021).

O RE é uma droga antiosteoporótica que tem como ativo o íon estrôncio (Sr). Esse, por sua vez, é um metal divalente semelhante ao Ca. O Sr pode ser incorporado à matriz óssea por troca iônica com o elemento Ca nas superfícies dos cristais de hidroxiapatita ou por adsorção (Boivin *et al*., 2009).

No metabolismo ósseo, o Sr apresenta um efeito dual, com ação favorável na diferenciação de células mesenquimais em osteoblastos e inibição da atividade de osteoclastos (Kurssat *et al*., 2011). Isto ocorre por que o Sr estimula a expressão de diferentes fatores de diferenciação, sendo o principal o RUNX2 nas etapas de osteoblastogênese. Esse mineral também modula a transdução de sinal do Fator Nuclear Kappa- β Ligante (RANKL), produzido por osteoblastos, que age no receptor Fator Nuclear Kappa- β (RANK) de células pré-osteoclásticas para induzir a osteoclastogênese e reabsorção óssea (Wang e Yeung, 2017). Por apresentar interação com o Receptor Sensível a Ca (CaSR) em osteoblastos, o Sr aumenta a expressão de osteoprotegerina. Essa é uma glicoproteína solúvel que atua como sequestrante de RANKL, impedindo a sua ligação ao receptor RANK e inibindo a reabsorção do tecido ósseo, além de induzir a apoptose de osteoclastos (Lemaire 2004; Wang 2017).

Os efeitos do Sr no metabolismo ósseo chamaram a atenção para o desenvolvimento de pesquisas que evidenciam os benefícios de sua associação a biomateriais na enxertia óssea (Park *et al*., 2016). Zhao et al. (2013) adsorveu Sr a aloenxerto ósseo (enxerto entre indivíduos da mesma espécie) e seus resultados em cultura celular demonstraram efeitos não citotóxicos do Sr em células osteoblastos. O autor relata também maior taxa de deposição mineral comparada com grupos tratados apenas com a droga ou com o enxerto. Esse achado indica uma maior efetividade do substituto ósseo quando enriquecido com Sr. Todavia, não há dados na literatura em relação ao enriquecimento de enxertos bovinos com o RE.

Com base no contexto descrito nessa introdução, o presente trabalho, apresentado em capítulos no corpo da tese, propôs avaliar os efeitos de xenoenxertos de origem bovina em sua versão comercial (mineralizada) e em uma versão desmineralizada em ácido etilenodiaminotetracético (EDTA) - processada no laboratório, associados ou não ao Sr. Avaliou-se *in vitro* e *in vivo* um biomaterial de fonte nacional (LuminaBone), de custo mais acessível à população e cujos resultados da pesquisa anterior em anexo I foram potencialmente positivos.

Previamente aos ensaios *in vitro e in vivo*, o projeto validou o método de associação ou funcionalização dos biomateriais com Sr, proposto por Zhao *et al.* (2013). A adsorção do Sr aos biomateriais (ambas as versões mineralizadas e desmineralizadas) foi realizada por meio da incubação por 14 dias em solução saturada de RE. O nível de adsorção foi avaliado por meio da técnica de espectrometria de emissão atômica por plasma acoplado indutivamente (ICP-OES). A técnica de ICP-OES foi realizada no Centro de Desenvolvimento da Tecnologia Nuclear. Toda a metodologia e resultados estão descritos no Capítulo II. Em síntese, observou-se que os enxertos mineralizados retêm maior quantidade de Sr no processo de adsorção do que os enxertos desmineralizados. A técnica demonstrou também que o enxerto mineralizado liberou gradativamente o Sr adsorvido em meio aquoso ao longo de 14 dias.

Certificada a presença de Sr nos biomateriais, estes foram colocados como *scaffolds* para a cultura de células osteoblásticas imortalizadas da linhagem MC3T3-E1. Foram realizados testes de citotoxicidade e viabilidade celular por MTT (períodos de 3 e 7 dias de cultura celular) e Fosfatase Alcalina (FA - períodos de 7 e 10 dias de cultura celular, respectivamente). Os resultados mostraram maior viabilidade celular sobre os enxertos mineralizado e desmineralizado associados ao Sr nos períodos de 3 e 7 dias quando comparados aos seus controles. Quanto a atividade celular, os grupos controle (enxertos sem adsorção de Sr) demonstraram melhores resultados em todos os períodos. Nessa etapa *in vitro*, foi possível concluir que o Sr não compromete a proliferação celular. Passamos, então, para a fase dos experimentos *in vivo*. Todavia, antes de iniciarmos nossas avaliações, levamos em consideração uma das questões levantadas durante o mestrado (artigo anexo I) referente a confiabilidade dos dados obtidos pelas radiografias digitais. No mestrado utilizamos um modelo de análise morfométrica, com radiografias padronizadas, e avaliamos a radiopacidade pela medição de tons de cinza em 3 pontos dentro dos defeitos para cada período avaliado. O método de análise morfométrica das radiografias foi idealizado em parceria com professores do setor de radiologia da Faculdade de Odontologia da UFMG. Entretanto, há dados na literatura sobre uma outra opção para análises radiográficas, denominada “análise fractal” utilizada também para avaliação

de deposição óssea em radiografias digitais. A análise fractal tem como finalidade a reconstrução de imagem por meio da absorção de raios-x ao osso avaliado, segundo as diferenças de densidade. A avaliação utiliza de métodos matemáticos para análise estrutural do osso trabeculado, sendo comumente usada em estudos de osteoporose. Diante dessa possibilidade, a fim de seguir no doutorado com a análise radiográfica mais fidedigna e aplicável aos nossos defeitos, realizamos um estudo paralelo e preliminar, comparando as duas metodologias (tons de cinza e fractal) em nossas amostras de interesse, a saber, os defeitos ósseos submetidos aos xenoenxertos mineralizado e desmineralizado. Os resultados das análises radiográficas (radiopacidade por tons de cinza e análise fractal) foram também comparados aos dados histológicos dos mesmos animais, com o objetivo de validar nossas interpretações. A quantificação por escala de cinza (radiopacidade) pareceu ser mais efetiva na avaliação de grupos enxertados com biomaterial desmineralizado. Porém, ressalta-se que na avaliação de enxertias mineralizadas, os dois métodos de avaliação apresentaram eficácia. Toda a proposta, objetivos e resultados desse estudo foram recentemente publicados (doi: 10.1259/dmfr.20180466) e seguem também como parte do meu doutorado, aqui descritos no capítulo I.

Uma vez averiguado de que a análise radiográfica estava adequada, caminhamos para os ensaios *in vivo*, visando avaliar os efeitos dos biomateriais associados ao Sr (capítulo II). Para tais ensaios utilizamos o mesmo modelo de defeito ósseo apresentado no anexo I. Entretanto, o período final de avaliação pós cirúrgica foi estendido, passamos a coletar as amostras nos períodos de 1, 7, 14, 21 e 60 dias, uma vez que no estudo anterior, quando avaliamos os animais no período máximo de 49 dias, não observamos uma completa regeneração do defeito ósseo em nenhum dos grupos estudados.

As análises *in vivo* avaliaram os efeitos dos biomateriais no fechamento da mucosa sobre a área cirúrgica e a evolução do reparo ósseo avaliado nos períodos propostos após as enxertias. Os resultados revelaram uma possível interferência do Sr no processo de fechamento da mucosa sobre o sítio cirúrgico. Ao contrário das amostras de enxertos livres de Sr, a mucosa de animais tratados com Sr, aos 7 e 14 dias apresentaram-se mais abertas, com diferença estatística entre os grupos.

Na análise da deposição óssea nos sítios enxertados, as análises radiográficas revelaram que os enxertos mineralizado e desmineralizado associados ao Sr apresentaram elevada radiopacidade a partir dos 14 dias pós cirurgia. Corroborando com os achados radiográficos, os resultados histomorfométricos por tricrômico de masson mostraram melhores resultados na deposição

óssea para os grupos associados ao Sr, assim como o grupo desmineralizado sem Sr, quando comparados com o controle negativo (defeito preenchido com coágulo sanguíneo) e o biomaterial mineralizado sem Sr no período inicial de 14 dias e final de 60 dias.

Na avaliação de quantificação de osso maduro pela técnica de picrosirius red em relação a quantidade de osso neoformado apresentado na técnica de tricrômico de masson, observamos nos períodos de 14, 21 e 60 dias de avaliação pós cirurgia, que os grupos enxertados com biomateriais associados ao Sr apresentaram maiores valores de osso maduro quando comparados com os grupos CN e mineralizado.

O trabalho permitiu concluir que o enxerto mineralizado apresentou grande capacidade de adsorção de estrôncio e os xenoenxertos associados ao Sr comprometeram a cicatrização gengival. Entretando, para osteoblastos, os ensaios *in vitro* demonstraram ausência de citotoxicidade. Já os ensaios *in vivo* permitiram concluir que o uso do Sr adsorvido a xenoenxertos mineralizados pode ser uma alternativa na reconstrução óssea, com resultados significativos na deposição e maturação óssea avaliadas tanto por radiografia, quanto por histomorfometrias.

Por fim, a divulgação de informações fundamentadas em dados científicos referente aos xenoenxertos, nas versões mineralizada e desmineralizada, assim como a sua associação a elementos de impacto no metabolismo ósseo, como o Sr, podem cooperar para melhorias da qualidade dos biomateriais e uso de alternativas com melhor custo-benefício na reconstrução de defeitos ósseos nas clínicas odontológicas. Assim sendo, o presente estudo buscou reiterar a hipótese do potencial osteoindutor das matrizes ósseas orgânicas como adjuvantes no reparo ósseo e do uso tópico do Sr associado a biomateriais de diferentes versões (mineralizado e desmineralizado) em defeitos ósseos. Dessa forma, visou-se abrir perspectivas para subsidiar novas pesquisas com foco na melhoria das propriedades biológicas dos xenoenxertos, especialmente aqueles de origem nacional, que podem se tornar materiais de excelência como alternativas aos biomateriais importados, referenciados como melhores produtos do mercado, mas inacessíveis a maior parte da população.

PREFÁCIO DOS CAPÍTULOS

Os artigos aqui apresentados referem-se aos resultados obtidos dentro da tese e serão apresentados em 2 módulos o que denomino capítulos I e II:

Capítulo I - Artigo publicado

O artigo compara dois métodos morfométricos de avaliação de deposição óssea em radiografias digitais, obtidas do modelo de defeito ósseo (defeito intrabucal em ratos) enxertados com os biomateriais de interesse. Avaliou-se as mesmas radiografias pelo método de análise de radiopacidade por tons de cinza e pelo método de análise fractal. Os resultados foram recentemente publicados na forma de artigo original intitulado como *Comparison between fractal analysis and radiopacity evaluation as a tool for studying repair of an osseous defect in an animal model using biomaterials*.

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Capítulo II - Artigo submetido

O artigo aborda (i) o método de otimização dos enxertos ósseos com o Sr e os tipos de análises realizadas; (ii) os ensaios *in vitro*: análise da viabilidade das células pré-osteoblásticas imortalizadas (MC3T3-E1) cultivadas em *scaffolds* dos enxertos previamente tratados com Sr; (iii) as análises *in vivo*: Análise macroscópica do nível de epitelização gengival sobre a área enxertada com biomateriais associados ou não ao Sr, em comparação ao grupo controle (coágulo); análise radiográfica (por radiopacidade) e histomorfométrica (tricrômico de masson e picrosirius red) da neoformação óssea nos períodos de 1, 7, 14, 21 e 60 dias.

CAPÍTULO I – Artigo publicado Dentomaxillofacial Radiology (2019)

Comparison between fractal analysis and radiopacity evaluation as a tool for studying repair of an osseous defect in an animal model using biomaterials

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ABSTRACT

Objectives: To evaluate bone repair of an osseous defect in a rat animal model through fractal analysis and radiopacity analysis in radiographic images.

Methods: 120 rats were subjected to extraction of their first molar and divided into four groups (n = 6/group) according to the material used for bone grafting: mineralized bovine bone, demineralized bovine bone (DBB), blood clot (BC - control) or Bio-Oss® (BO). The animals were sacrificed after 1, 7, 14, 21 and 49 days and subjected to radiographic evaluation. For fractal analysis (FA), a square regionof interest of 30 × 30 pixels was used, and radiopacity was measured as the mean gray scale (MGS) value for three points of 5 × 5 pixels in the apical, medial and coronal regions of the defect. Histomorphometric evaluation was realized as the gold standard for bone neo-formation and maturation of the new osseous matrix.

Results: Histomorphometric evaluation suggested that DBB showed faster mineralized deposition and resulted in more mature bone at the final time point of evaluation. Mineralized bovine bone and Bio-Oss presented similar results. The mineralized groups did not show significant differences in bone maturation. The radiopacity analysis revealed a significant difference ($p < 0.05$) between the DBB and blood clot groups at the final time point. FA did not show any significant differences at the final time point.

conclusions: Mean gray scale seemed to be more effective for the quantification of bone repair than FA in the demineralized group in this animal model. Results for the mineralized groups did not reveal a significant difference, leading to the conclusion that both methods are effective.

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ReseaRch aRticle

comparisonbetweenfractalanalysisandradiopacityevaluation as a tool for studying repair of an osseous defect in an animal model using biomaterials

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Keywords: Fractals; Radiography; Biocompatible biomaterials

INTRODUCTION

The evaluation of jaw bone quality and quantitative characterization of structural changes in the jaw bone after tooth loss have been performed via various techniques to plan proper rehabilitation with prosthetics and implants.[1] The most accurate method, representing the gold-standard, is histological analysis; however, this method is not applicable in routine clinical practice.[2] The most common clinically used method is the analysis of radiographic images (periapical, panoramic and

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cone beam CT) that allow estimation of the integrity of mineralized structures and evaluation of bone regeneration.[3] However, periapical and panoramic radiography exhibit some limiting factors, such as different projections, distortion and superposition of images.[4]

Methods for determining the healing of the jaw bone based on the radiographic aspect of images have been studied. The traditionally most commonly used of these techniques is mean gray scale (MGS) analysis, while more recently, fractal analysis (FA) has played a role in these evaluations. MGS provides the mean gray level of each pixel of a plain image and has been applied to oral radiographs to assess bone quantity at implant sites.[5,6] FA is a method that mathematically describes the structural pattern of trabecular bone, since it exhibits self-symmetry and the image is the representation of X-ray absorption due to different structure densities.[7] This method has been widely used in the study of osteoporotic bone,[8–10] and there are some reports of its application in the evaluation of bone quantity and quality at implant sites as well as after bone grafts.[1,11–13]

FA has been reported to present some advantages, as it is independent of radiodensity, geometrical projection and alignment of bone trabeculae.[14] The method of box counting for bone analysis assesses the boundary of trabecular bone and marrow, meaning that a higher value indicates a more complex structure.[15]

Few studies have related FA and guided bone regeneration using autogenous or xenogenic bone grafts.[1,7,16] This type of analysis would be very beneficial for conducting this kind of treatment, as the visual assessment of bone roughness through radiographs alone is a subjective test. This visual interpretation cannot differentiate proper bone remodeling from a lack of material integration,[16] which is why the study of alternative methods for bone quantification in radiological images is necessary.

This study aimed to evaluate bone repair in osseous defects in animals after bone grafting using two variables of bovine bone (mineralized and demineralized versions), through two methods of radiographic analysis: fractal analysis and mean gray level analysis. The efficacy of both methods was evaluated using the morphometric results as a reference. Therefore, it was possible to assess and clarify some aspects of FA in relation to its use for qualitative bone analysis.

METHODS

This study was approved by the local Animal Ethics Committee (CEUA/UFMG number 07/2015).

Biomaterial for bone grafting

The materials used for bone grafting were two different brands of bovine bone: Lumina-Bone® (Criteria, São Carlos, Brazil) and Bio-Oss® (Geistlich, Switzerland). The mineralized commercialized versions of both types were employed. Bio-Oss® was used as a positive control because of its consistently good results both in research and clinical applications.[17,18] In addition to the mineralized versions, this study used a demineralized version to compare the influence of the organic matrix in bone grafts. The demineralized version was obtained by immersing a few blocks of Lumina-Bone® in 10% EDTA (ethylenediamine tetraacetic acid) for 72 h, which were then washed with water and kept in sterile PBS (phosphate buffered saline) solution. Prior to the surgical procedures, the blocks were portioned into 1–2 mmfragments.

Surgical procedures

The animals used in this research were 120 male, adult Wistar rats (Rattus norvegicus), with body weights between 280 and 350 g. The rats were evaluated in periods of 1, 7, 14, 21 and 49 days after graft surgery. Animals were anesthetized and positioned on a surgical table. The first left superior molar of the animals was extracted, and a defect was created with a cylindrical diamond drill, removing the remaining interradicular septum. The generated osseous defects were patterned to exhibit a diameter and profundity of 2.5 mm. The animals were divided into four groups (n = 6) according to the material used to fill the cavity: blood clot (control group—BC), Bio-Oss® (BO), mineralized Criteria's bovine bone (MBB) and EDTA-demineralized Criteria's bovine bone (DBB).

Radiographic evaluation (Digital X-ray)

The jaws of the rats were fixed in 10% neutral buffered formalin for 72 h. After this period, the maxillae were cut in half along the median line of the palate, between the central incisors, using a diamond disc. The pieces were washed and kept in alcohol 70% for radiographic procedures. Only the hemi-maxilla with the osseous defect (left side) was subjected to radiography. Images were captured using a 3 × 4 cm phosphor plate (Durr Dental, Bietigheim, Bissingen, Germany) and a Gendex 765DC® (Pennsylvania, USA) radiographic device. After a pilot study testing the exposure parameters, the standard was chosen as follows: 0,125 s exposure time, 65 kV, 7 mA and 10 cm distance from the end of collimator to the film. The plates were digitalized with a VistaScanPerio Plus® (Durr Dental, Bietigheim, Bissingen, Germany) scanner and processed using DBSWIN Imaging Software® (16 bits) (Durr Dental, Bietigheim, Bissingen, Germany). The images were converted to jpeg format with a 1080 dpi final resolution (Figure 1A).

Radiopacity evaluation (mean gray scale—MGS)

Using Adobe Photoshop CS5 software (16 bits), three regions of interest (ROIs) of 0.11 × 0.11 mm were determined in the apical, medial and coronal regions of the surgical site, 1 mm distant from the mesial root of the second molar (Figure 1B). For this purpose, a vertical line measuring 2.5 mm was positioned near the mesial

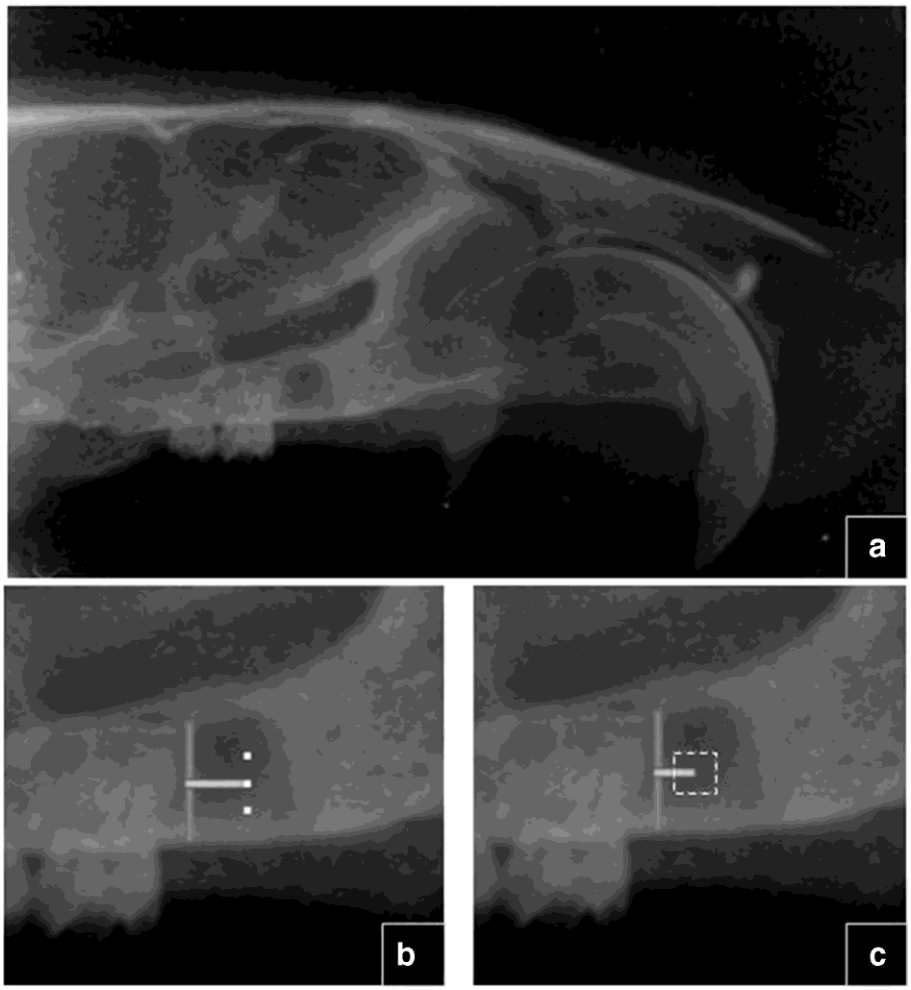

Figure 1 (a) Radiographic image of a rat hemi maxilla of the blood clot group at time one after graft surgery. (b) Vertical reference line and horizontal reference line. ROI used for measuring MGS, located in coronal, medial and apical regions of the osseous defect (white squares). (c) Square ROI with the center placed by the end of the horizontal line for FA. FA, fractal analysis; MGS, mean gray scale; ROI, region of interest.

root of the second molar, and a 1.0 mm horizontal line was then traced from the center of that first line. The end of that line defined the medial ROI and was used as a reference for determining the apical and coronal points, which were always at the limits of the bone defect. With the histogram tool, the gray scale was measured for each point, and the mean between the three points was calculated.

Fractal analysis

The fractal analysis, in which the results were expressed numerically as fractal dimension (FD), was realized based on the procedure described by White and Rudolph[10] using the box-counting method. The method for choosing the location of the ROI was as described for the MGS analysis, whereas for FA, the horizontal line measured 0.7 mm, and the end of the medial line defined the center of a square 0.71 × 0.71 mm ROI (Figure 1C).

Using ImageJ software, the ROI located in the center of the osseous defect was selected and blurred with a Gaussian filter (σ= 35). This stage was applied to remove brightness variations due to overlaying soft tissues and variation in bone thickness. The blurred image was subtracted from the original, and a 128 gray value was added to each pixel to discriminate bone marrow spaces and trabeculae. After binarization, the components were segmented in an image that visually outlined the trabeculae from the bone marrow. The next steps, erosion and dilatation, are performed with the aim of eliminating image noise and emphasizing structures, respectively. The last step, skeletonization, eroded the image until only the central line of pixels remained and prepared it for FA.[10,19] The box-counting method converts the image using a square grid of equally sized tiles and plots the number of counted tiles against the total number of tiles on a double logarithmic scale. Finally, the fractal dimensional values were calculated from the slope of the line.

Histological processing for histomorphometric analysis
Subsequent to radiography, the maxilla were demineralized in 10% EDTA, pH 7.2, then dehydrated with ethanol, diaphanized with xylol and embedded in paraffin. The blocks were sectioned at a 5 µm thickness along the frontal plane of the section and stained with hematoxylin and eosin, Masson's trichrome and Picro-Sirius Red.

Morphometric evaluation of osseous deposition was realized using ImageJ software. Three blinded and calibrated evaluators determined the percentage of the area occupied by newly formed bone, visualized as the trabeculae colored by Masson's trichrome. The histomorphometric measurements were realized in three antero posterior sections of the defect (one mesial, one central and one distally located). The mean of the obtained values was subjected to statistical analysis.

The morphometric analysis using PicroSirius Red wasconducted to investigatetheorganizationandmaturation of the new osseous matrix. Three sections of the paraffin block were again examined, and photographs were analyzed with polarized light, which enabled the study of collagen quality and organization. Collagen in the newly formed osseous matrix may form either finer fibers, exhibiting weaker green birefringence (Type III fibers), or moreorganized and thicker fibers, visualized as yellow and red fibers (Type I fibers). The red fibers showthemaximummatrixmaturation. ImageJsoftware was applied to determine the percentage of red fibers (more mature) using the canal colors tool in the images of the three slides, and the mean was used as the result of this analysis.

Statistical analysis
Statistics were plotted in Graph Pad Prism software using the *t* test and one-way ANOVA for parametric samples. Thegraphswereplottedusing MicrosoftExcel software.

RESULTS

Data obtained in the FA were compared with the results of the histomorphometric analysis of bonequantity and quality and with the radiopacity determined by MGS.

Comparisons were performed between the groups with mineralized bone grafts (BO and MBB) and between the demineralized bovine bone and blood clot groups (DBB and BC, respectively). Comparison between all groups is not possible, as the mineralized materials are slowly reabsorbed, leaving less space for bone repair.

Blood clot x demineralized bovine bone
The histomorphometry results revealed that DBB accelerated the healing process, showing statistical superiority compared to the control group in the periods of 14 and 21 days (Figure 2). Despite these results, final bone repair at 49 days was similar in the two groups, without a significant difference.

Regarding the maturation of collagen fibers in the new osseous matrix determined through PicroSirius Red analysis, the peak of maturation occurred from 21 to 49 days. Furthermore, at 49 days, the defects treated with DBB were considered to be significantly more mature than those of the BC group (Figure 3). When these results were compared with the histomorphometry results regarding osseous repair, 69.6% of the newly formed bone under DBB bone maturation consisted of mature collagen fibers, while this percentage was 52.2% in the BC group.

The mean FD and MGS values for all groups are shown in Table 1. Comparison of these histological evaluationswiththeradiographicmethodsrevealedthat radiopacity with MGS was the approach whose results were most closely related to the histomorphometry results. The MGS analysis of BC and DBB was consistent with bone repair at 14 and 49 days, and the DBB group even showed a significant difference compared with the BC group at the final time point of evaluation, as observed in theanalysisof thematuration of collagen fibers (Figure 3).

However, FA did not reveal this pattern. Instead, a gradual increase in values was seen with time in both groups, without a significant difference. In contrast, the FD values of the BC group were larger than those of DBB at 14 and 21 days, as compared with the histomorphometric results (Figure 2), which showed elevated bone deposition and more mature collagen fibers in the DBB samples (Figure 2). Thus, it was noted that the results of MGS analysis presented more similarity to those of the histological evaluation than to those of FA.

Bio-Oss® x mineralized bovine bone
Bone repair of defects with mineralized materials occurs along a different time curve in comparison with repair involving demineralized materials because the material has to be reabsorbed by the body to induce new bone formation. When bone repair was compared between the BO and MBB groups, it was observed that BO exhibited significant bone formation at day 14. However, by 49 days, there was no significant difference between the groups (Figure 4).

The PicroSirius Red analysis of BO and MBB did not shown any significant difference (Figure 5), although the percentage of red fibers was greater for the defects treated with MBB at 7, 21 and 49 days of evaluation. The final comparison of red fibers with newly formed bone showed that 28.4% of the bone in the MBB group and 23.8% in the BO group exhibited more mature fibers. Although MGS revealed inverted absolute values in the evaluations performed at 7 and 21 days, no significant difference was found. Thus, the results of both FA and MGS analysis were similar to those of the histomorphometric evaluations (Figure 4). One exception was observed for the period of 7 days, where BO

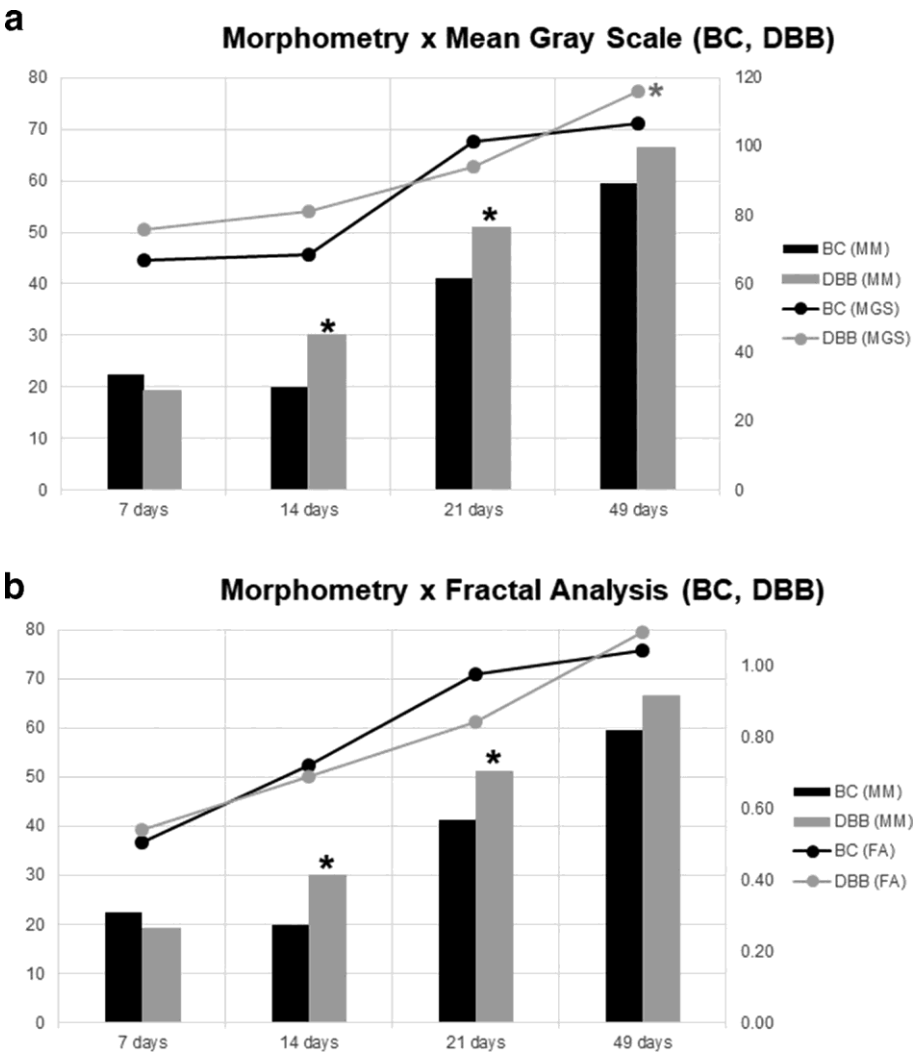

Figure 2 (a) The left axis represents the morphometric values (MM), and the right axis represents the MGS values. The columns show the results for bone formation determined through morphometric analysis, and the lines show the MGS results. Considering MGS, a significant difference was found between DBB and BC at the final time point ($p < 0.05$) (*t* test). (b) The left axis represents the morphometric values, and the right axis represents the FA values. The columns show the results of bone formation determined through morphometric analysis, and the lines show the results of FA. The comparison between the groups according to the morphometric analysis showed a significant difference at 14 and 21 ($p < 0.05$) days (*t* test). Under FA, no significant difference was found between the groups. BC, blood clot; DBB, demineralized bovine bone; FA, fractal analysis; MGS, meangray scale.

significantly outperformed MBB in the fractal analysis. This result can possibly be explained by the composition and complexity of the biomaterials, as BO appears to be more mineralized.

When analyzing the results of radiographic evaluations and bone maturation based on PicroSirius Red staining (Figure 5), FA was the method that best followed the pattern observed in the microscopic analysis, except for the period of 7 days. In contrast, MGS showed a continuous increase in values for both materials, with lower values obtained for MBB than BO, whereas the results for the two groups were similar at the final time point.

DISCUSSION

Since White and Rudolf[10] first described the use of the mathematical FA method for osteoporosis evaluation using radiographic images of the jaws, this method has been widely used in the detection of trabecular bone alterations in this region. Subsequently, studies using fractals for bone quantification and quality assessment started to draw attention toward this method.[11,14] As this approach is a recent type of evaluation, there are few studies[1] comparing the results of FA of images with those of histological analysis of the same site, as such comparisons are complicated *in vivo*.

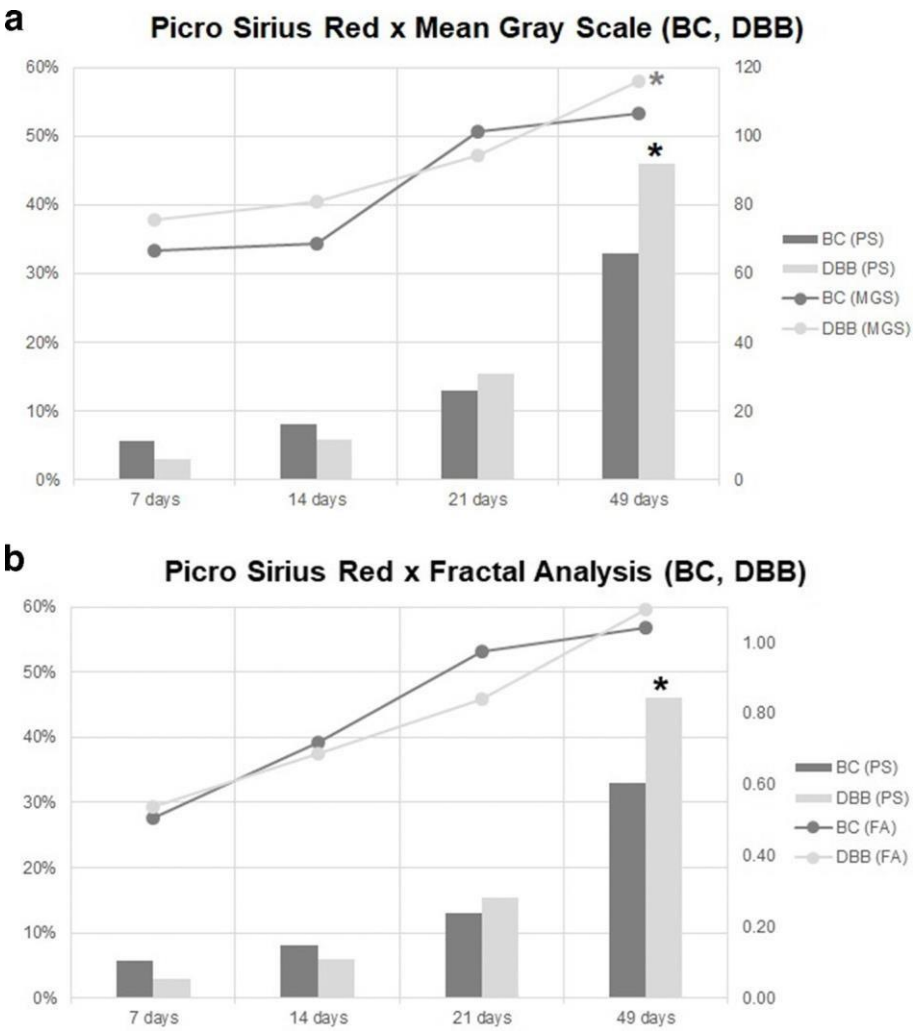

Figure 3 (a) The left axis represents the percentage of mature fibers acquired based on *PicroSirius Red* evaluation, and the right axis represents MGS values. The bone deposited in the DBB group was more mature by the final time point than that in the BC group, showing statistical significance ($p < 0.05$) (*t* test). MGS also revealed this result, with statistical significance. (b) The left axis represents the percentage of mature fibers acquired based on the *PicroSirius Red* evaluation, and the right axis represents the FA values. The columns show the quantitative analysis of collagen fibers base on *PicroSirius Red* staining. The bone deposited in the DBB group was more mature by the final time point than that in the BC group, showing statistical significance ($p < 0.05$) (*t* test). FA also revealed this result, although the results were not consistent at all other times of evaluation, and it was not statistically significant. BC, blood clot; DBB, demineralized bovine bone; FA, fractal analysis; MGS, meangray scale.

Table 1 Mean values and standard deviations of FD and MGS for all evaluation times (1, 7, 14, 21 and 49 days after graft surgery) and study groups

		1	*7*	*14*	*21*	*49*
FD	BC	0.7779 (±0.09)	0.5055 (±0.18)	0.7209 (±0.27)	0.9755 (±0.25)	1,0426 (±0.12)
	DBB	0.6578 (±0.22)	0.5394 (±0.34)	0.6893 (±0.20)	0.8420 (±0.26)	1.0921 (±0.13)
	BO	0.8674 (±0.16)	0.9693 (±0.12)	0.9186 (±0.09)	0.7923 (±0.22)	0.9042 (±0.24)
	MBB	1.0208 (±0.13)	0.6887 (±0.15)	0.8192 (±0.08)	0.8339 (±0.26)	0.9096 (±0.24)
MGS	BC	84.89 (±4.82)	66.79 (±3.75)	68.68 (±11.24)	101.34 (±6.05)	106.1 (±7.59)
	DBB	85.92 (±12.96)	75.62 (±5.05)	80.95 (±11.39)	94.22 (±4.64)	116.17 (±6.50)
	BO	97.67 (±9.12)	109.56 (±10.85)	96.77 (±5.39)	104.97 (±22.27)	106.05 (±5.16)
	MBB	99.30 (±9.91)	84.90 (±10.94)	76.29 (±9.00)	80.72 (±10.59)	105.71 (±4.10)

BC, blood clot; BO, Bio-Oss®; DBB, demineralized bovine bone; FD, fractal dimension; MBB, mineralized bovine bone; MGS, mean gray scale.

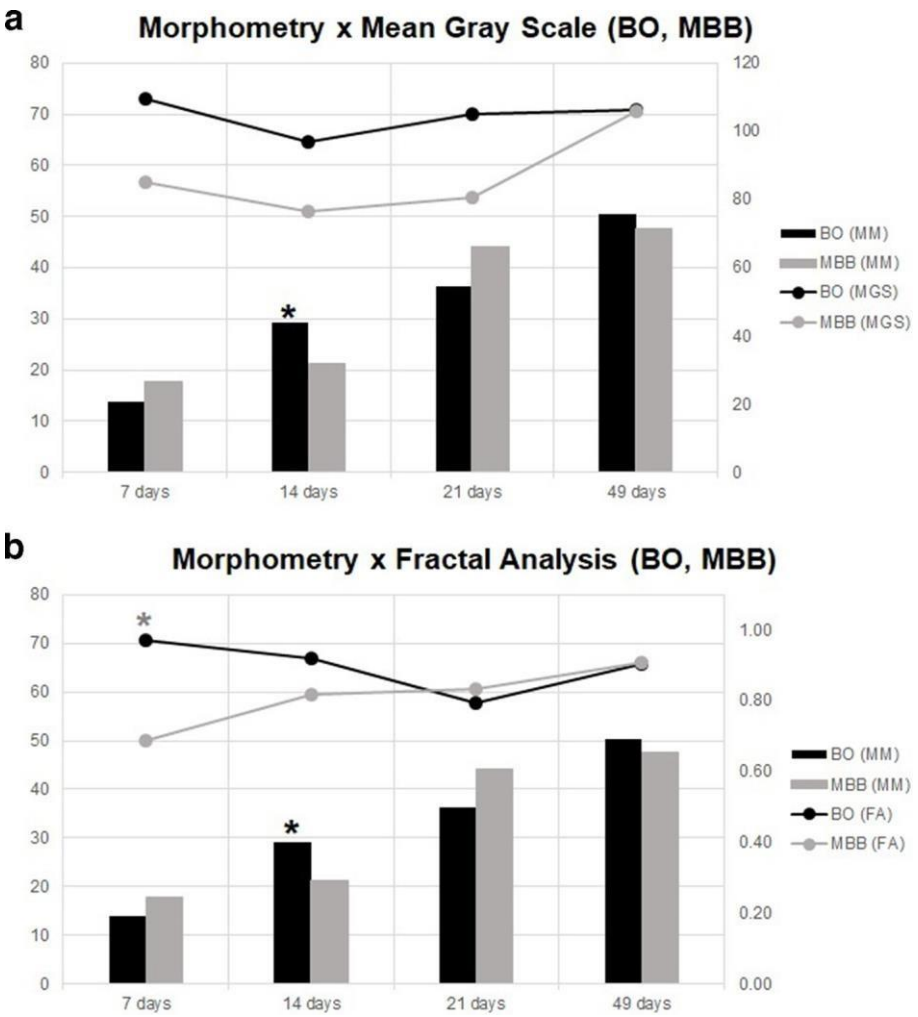

Figure 4 (a) The left axis represents the morphometric values, and the right axis represents the MGS values. No significant difference was found between the BO and MBB groups under MGS analysis; (b) the left axis represents the morphometric values, and the right axis represents the FA values. The columns show the results regarding bone formation determined through morphometric analysis, and the lines show the results of fractal analysis. The comparison between groups according to morphometric analysis showed a significant difference at 14 days (*t* test). A significant difference was found between the BO and MBB groups under FA at 7 days. BC, blood clot; BO, Bio-Oss; DBB, demineralized bovine bone; FA, fractal analysis; MGS, mean gray scale.

One of the challenges in dentistry is the acceleration of osseous repair and substitution by biomaterials. Therefore, it is necessary to develop clinical methods for assessing these therapies. In the present study, bone repair of an intrabuccal osseous defect in rats was evaluated after the introduction of organic (DBB) and inorganic (BO and MBB) bone grafts. The bone grafts used in clinical dentistry are usually of an inorganic nature. In the present study, BO was chosen for this purpose because it is considered a gold-standard material for bone-guided healing. Two versions of Lumina-Bone® were also employed: mineralized and demineralized. The use of a demineralized graft is based in the fact that inorganic components of the osseous matrix (such as BMPs, osteocalcin, osteopontin and collagen) retain osteogenic properties,[20] conferring osteoinduction and osteoconduction properties to the graft. The present study focuses on the analysis of radiographic methods forclinicallyfollowingbonerepairwithbonegrafts.The advantages of radiographic evaluation of bone grafts with organic material include the fact that every change in radiopacityobserved in imagesthroughoutheperiod of evaluationisconsidered torepresent bonedeposition and can be quantified. Radiographic evaluation of inorganic materials is more complex, as the radiopacity of thenewlyformedbonecanbemaskedbytheradiopacity of the biomaterials. Interestingly, the data presented in thisreportrevealed thattheresultsof radiographic evaluation by MGS were more closely related to the results of histomorphometry than those of FA.

Many reports indicate that FA is predictive of bone quality.[11,21,22] Primarily, it is necessary to clarify the definition of bone quality, which was proposed in 2000 by the National Institutes of Health[23] as “the sum of all

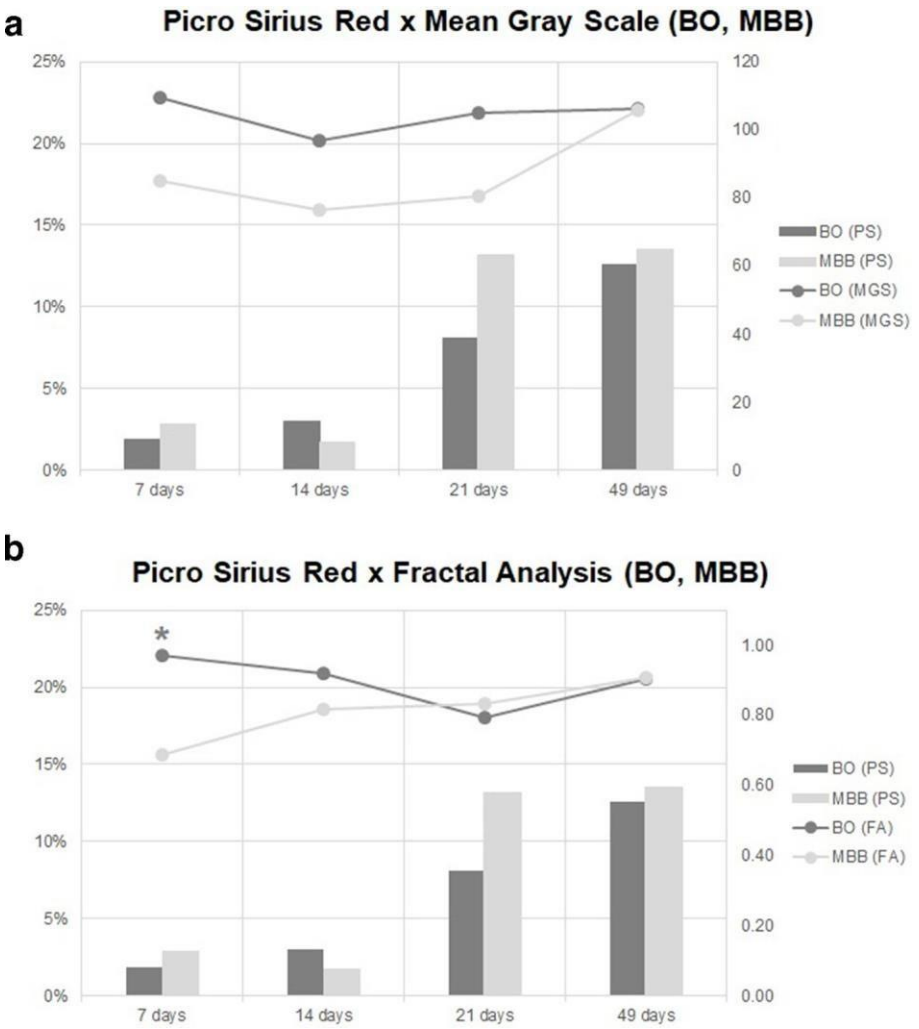

Figure 5 (a) The left axis represents the percentage of mature fibers acquired based on the *PicroSirius Red* evaluation, and the right axis represents the MGS values. The columns show the quantitative analysis of collagen fibers. MGS showed continuous growth of BO and MBB, with MBB presenting smaller values until the final time point, when the values were similar. This finding was only in accord with the maturation analysis at 14 and 49 days. (b) The left axis represents the percentage of mature fibers acquired based on *PicroSirius Red* evaluation, and the right axis represents the FA values. The bone deposited in the MBB group reached its peak of maturation before that in the BO group, at 21 days. However, there was no significant difference between the groups at the final time point. FA showed a significant difference between groups only after 7 days of evaluation ($p < 0.05$) (*t* test), and these results were in accord with the observed bone maturation in the other times. BC, blood clot; BO, Bio-Oss; DBB, demineralized bovine bone; FA, fractal analysis; MGS, mean gray scale.

characteristics of bone that influence the bone's resistance to fracture." Therefore, bone quality cannot be defined only by bone density and image radiopacity, as it comprises a more complex combination of bone turnover, bone mineralization, microdamage accumulation and bone architecture.[24] In this study, the superposition of morphometric analysis using PicroSirius Red with Masson's trichrome-stained samples produced a parameter indicative of the maturation of collagen fibers in the newly formed bone, which was considered a satisfactory qualitative evaluation approach for the bone for the purpose of the analysis. When those data were compared with the FA results for the radiographic images, no statistically significant correlation was found. Therefore, FA was not treated as being predictive of bone quality in the present study.

For the purpose of evaluation, radiopacity measured via MGS was more accurate than the FA for the prediction of bone repair in the demineralized and control groups. Many hypotheses can be formulated for this purpose. The first to be considered is the area of evaluation of each method. It was not possible to use the same acquisition method involving three points that was used in MGS analysis for FA, as no FA results was obtained with a ROI as small as five pixels. It has been reported that bone expresses self-similarity at sizes between 0.1 mm and 5 cm.[25] Five pixels represented 0.11 mm in our images, which is too close to the value determined

for the lack of these properties, which is probably why there were no results with a small ROI. The location of the three ROIs for MGS may also have played a role in the results, as alveolar repair occurs in an apical to coronal direction, and multiple points may provide a more detailed picture of the total repair than a single central ROI.

Another hypothesis is related to the use of blurs and filters in FA. In the description of this method from White and Rudolf,[10] it is emphasized that the program is designed to remove large-scale variations in brightness in the image to reflect particular types of images (trabeculae and marrow spaces) and so that brightness levels that may cause individual variations in the image can be eliminated. A limitation of this type of analysis for newly formed bone is that it can cause clearance of incompletely matured trabeculae, as the step involving the Gaussian filter removes structures that are considered to be fine-scale and medium-scale structures, retaining only large variations in density.

Although the results of FA were not in accord with the observations of bone repair in the demineralized and control groups, they were similar to the bone repair process observed during the assessed period and with the MGS results at the final time point when the mineralized materials were compared. It is suspected that defects treated with mineralized and demineralized grafts exhibit differences in the progression of healing. MBB and BO are initially reabsorbed by osteoclasts before the healing process of new bone formation begins,[26] which may explain the differences in the two groups in terms of bone repair. Additionally, the radiopacity and complexity of the materials may interfere in the evaluation of both methods, as these materials are not completely reabsorbed and cannot be differentiated from new bone in radiographic analysis.

Therefore,theuseof FAforbonerepairevaluationmust be performed with caution. Many mathematical formulas, such as those of the power spectral density, triangular prism surface area, blanket method, intensity difference scaling, variogram analysis and the box-counting method, for the evaluation of trabeculae in X rays have been described.[16] There is agreement in the literature that it is not possible to compare the results of different methods.[16,27] Molon et al[1] used the box-counting method and compared bone repair using autogenous bone grafts in sinus lifting with histomorphometric analysis. Although these authors did not observe significant differences between the fractal results for images and the results of microscopic methods, they found differences in fractals from the initial to final time points and this method to be reliable for the quantitative evaluation of bone.[1] Although Kozakiewicz et al[7] did not perform a histomorphometric evaluation, they used the Fourier power spectrum method, which operates in the frequency domain (while other methods operate in the spatial domain), and the considered this approach to be effective for describing the dynamics of bone remodeling and useful as a quantitative indicator.[7] In our study, the fractal dimension acquired via the box-counting method offeredan illustrationof bone repair,increasingwithtime, but the results were not equivalent to the histomorphometric results at all times of evaluation. This collection of literature shows that additional studies are necessary to compare different fractal methods and to determine whichmethod is idealforproperlyassessing bone repair.

Another concept that must be considered with caution regarding fractal dimensions is their capacity to measure bone quality. Many studies have associated FA with bone mineral density and considered it to be predictive of quality.[11,28,29] However, as discussed previously, this aspect should not be the only relevant factor in assessing bone quality. The present study used morphological methods to evaluate bone quality and did not find an association with FA.

While histomorphometric analysis considers the demineralized osseous matrix, radiographic analysis reads mineral deposition on that matrix. Therefore, histological analysis must be considered as a complementary evaluation that is still more reliable in terms of bone quantification, as it does not involve superposition of layers and is able to differentiate biomaterials from new trabeculae. Bone quality can be estimated based on the level of collagen fiber maturation, but it cannot be sufficiently determined based only on that criterion. In this context, radiography can be a useful tool. Radiographic evaluation presents limits as well, as it cannot differentiate biomaterials from osseous tissue during its formation, especially for mineralized forms, which present radiopacity similar to trabecular bone.

Although this study has some limitations, such as the use of an animal model, a small sample size, a brief period of evaluation and the small bone defect involved, it provides a better comparison of FA and MGS considering morphometric evaluations.

CONCLUSION

Although fractal analysis has been reported in the literature as a method for assessing bone repair, it did not exhibit significant differences compared to MGS, which is a simpler and more easily performed method for the same type of comparison. More studies are necessary to evaluate the use and benefits of FA in assessing bone repair, considering other experimental models and the different methods that can be used for this type of analysis.

acknowledgment

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CAPÍTULO II – Artigo submetido Journal of Periodontology (2021)

In vitro and *in vivo* evaluation of mineralized and demineralized bovine bone grafts incorporated with strontium ranelate

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ABSTRACT

Currently, mineralized bovine bone grafts are one of the most commonly used alternatives in dentistry for the replacement of bone loss. However, several studies have proposed the use of demineralized matrices, as well as the use of systemic drug therapies, such as strontium ranelate (SrRan), in the period of bone grafting for fracture treatment. This antiosteoporotic drug acts on bone remodeling, inhibiting osteoclastogenesis and stimulating osteogenesis. The present study evaluated the effects of the combination of SrRan on mineralized and demineralized bovine bone grafts in *in vitro* and *in vivo* experiments. The grafts were treated in saturated solution of SrRan for strontium (Sr) adsorption and evaluated by ICP-OES assay. *In vitro* assays were performed on culture of immortalized osteoblasts. The impact of this association on cell viability and activity was assessed by MTT and alkaline phosphatase (AP) assays. In *in vivo* assays, intrabuccal bone defects were constructed in rats and filled with blood clot; mineralized graft; demineralized graft; mineralized graft + Sr and demineralized graft + Sr. The healing of the surgical site was evaluated by means of macrophotographs and deposition of neoformed bone through radiographic and histomorphometric analysis at 1, 7, 14, 21 and 60 days after surgical procedures. The results evidenced a higher Sr adsorption capacity for the mineralized graft. In culture of immortalized osteoblasts, cell viability was greater in all evaluated periods in grafts treated with Sr, without cytotoxic effect. However, such grafts provided the lowest rates of cell differentiation. *In vivo* assays revealed Sr toxicity to the epithelial tissue, compromising gingival healing of the surgical site. However, animals grafted with Sr showed a higher rate of mineral deposition over time and in the final period of evaluation in the radiographic and histomorphometric analysis. In conclusion, the grafts associated to Sr promote bone deposition, without cytotoxicity to osteoblasts, but compromise gingival epithelization.

Key words: Xenograft, bioactive ion, osteoblast, bone reconstruction.

1. INTRODUCTION

Intrinsic or extrinsic factors, such as hormones and mechanical pressures, are responsible for promoting constant changes in bone remodeling. The capacity of tissue regeneration of the bone is provided by its metabolic characteristics. Osteoprogenitor cells similar to stem cells allow differentiation into osteoblastos which, in turn, initiate the process of bone matrix formation and play a role in regulating angiogenesis (Nahian and Davis, 2021). However, extensive bone defects promoted by different causes, as well as degenerative diseases, metabolic desorders and fractures, may present insufficient tissue regeneration and are a serious problem for the orthopedic and dental clinic (Walsh *et al*., 2017). For this reason, numerous researches have been developed involving grafting techniques, such as induction of tissue regeneration by osteoinductive and / or osteoconductive molecules and biomaterials (Buza and Einhom, 2019).

Biomaterials, such as bone grafts, used in the treatment of bone lesions have the function of attaching themselves to the patient's bone and allowing a physiological response adequate for the regeneration process (Lei *et al*., 2015). The combination of strategies such as the association of grafts with osteoinductive factors and organic bone matrix with bioactive molecules can improve graft properties and clinical outcomes (Watanabe et al., 2016).

Xenogenic grafting has been extensively used in dental clinics and is characterized as occurring among individuals of different species (Scarano *et al*., 2020). Mineralized bovine grafts, used *in vitro* and *in vivo* assays (Al-juboori *et al*., 2016; Godoi *et al*., 2021; Lee *et al*., 2021), have shown a good potential in bone lesions repair. In addition, demineralized xenografts rich in organic bone matrix constituents, such as Bone Morphogenetic Proteins (BMP), osteopontin and collagen, have also been investigated and suggested as potential accelerators of bone repair (Sierra-Garcia *et al*., 2016, Nam and Han, 2019). Some of these components have already been isolated from the bone matrix and are already available for clinical use, such as BMP-2, as osteogenesis enhancers in operated areas (Zhang *et al*., 2018).

The use of xenogenic origin demineralized scaffolds alone or in combination with other materials and drugs has shown beneficial results (Drosos *et al*., 2015). The osteogenic proteins exposed in a bone graft submitted to demineralization can represent a significant gain in the process of cell differentiation and proliferation. However, the clinical use of these organic matrices is still

restricted. It is suggested the need for higher quality clinical studies to favor or avoid the use of demineralized bone products in grafting procedures (Drosos *et al*., 2015; Russell *et al*., 2020).

Aiming at the acceleration of bone repair and the quality of the newly formed bone, researchers have invested in the use of anabolic drugs (Silva *et al*., 2018). Among them, strontium ranelate (SrRan) has been prominent, being a new generation drug among the anti-osteoporotic agents, which interferes with the bone remodeling process. The bone remodeling occurs through bone multicellular units, defined as aggregates of osteoblasts and osteoclasts that act in sequence to remodel bone (Henry and Bordone, 2021). SrRan has a dual effect, stimulating osteoblasts and inhibiting osteoclastogenesis (Park *et al*., 2013). Thus, SrRan decreases the resorption of bone tissue by inhibiting the differentiation of osteoclasts and promoting their apoptosis. This process occurs by stimulating the production of osteoprotegerin, a protein produced by osteoblasts that inhibits the osteoclastic differentiation activator, RANKL, produced by activated T lymphocytes and osteoblasts. As an anabolic effect, this drug induces Core-Binding Factor Alpha-1 (CBFA1) or Runt-Related Transcription Factor 2 (RUNX2) gene expression (Guo *et al*., 2016), favoring the activation and differentiation of preosteoblastic cells in osteoblastos (Henry and Bordone, 2021). The systemic use of SrRan as adjuvant therapy during the phases of bone repair and implant osseointegration has been evaluated (Scardueli *et al*., 2018). In 2017, Amaral *et al*. demonstrated the optimization of bone deposition in intraoral defects in rats treated systemically with SrRan. The animals treated daily with SrRan demonstrated acceleration of the bone neoformation process at the bone defect sites. The topical use of SrRan has also been suggested after the publication of data that ensure the viability and differentiation of osteoblasts, cultured on scaffolds of bone grafts enriched with Sr. In fact, Zhao *et al*. (2013) incorporated Sr into a bone allograft (graft between individuals of the same species) *in vitro* assay with osteoblastic cells. The results demonstrated non-cytotoxic effects in culture and higher rate of mineral deposition compared to groups treated with either the drug or the graft, indicating a greater effectiveness of the bone substitute when enriched with strontium ranelate. Similar results were obtained with the culture of osteoblasts on bovine bone scaffolds, maintained with culture medium added with SrRan (Silva *et al*., 2018). These data suggest the feasibility of topical use of SrRan directly in grafted areas under clinical conditions. Therefore, more *in vivo* studies must precede this indication.

In this line, the present study proposed to evaluate the effects of the association of SrRan to bovine bone in a mineralized commercial version and a demineralized version processed in the

laboratory, in culture of immortalized osteoblasts and in the repair of intrabuccal bone defect in rats.

2. MATERIALS AND METHODS

2.1. Biomaterials

Bovine bone blocks (LuminaBone - Critéria, Brazil) were used in two versions, mineralized (commercial) and demineralised in 10% EDTA (commercial modified in the laboratory). The grafts were employed in *in vitro* assays and *in vivo* experiments. Strontium ranelate PA (SrRan, BioX Corporation®, Ontario, Canada) used corresponds to the same as the production of osteoporosis drugs.

To obtain demineralized samples, gamma ray sterile bovine bone blocks (10x10x5mm) were immersed in 10% EDTA for 72 hours and washed in running water overnigth. The pieces were reduced to fragments of approximate dimensions of 2.5mm x 2.5mm x 2.5mm for both *in vitro* and *in vivo* experiments. All instruments and solutions used were sterile and the procedure performed in laminar flow.

For Sr adsorption to grafts, both mineralized and demineralized bone grafts, were treated in saturated solution of SrRan at 30 mM for 14 days according to Zhao et al (2013). Then, the fragments were processed to Inductively Coupled Plasma - Atomic Emission Spectrometry assays (ICP-OES) in order to quantify the adsorption of strontium in the samples. For that, the fragmets were subjected to acid digestion (hydrochloric acid + nitric acid 1: 3) for 24 hours to remove all the organic components and release the mineral contents. Then, the samples were read by a plasma atomic emission spectrometer and the quantitative data were statistically evaluated. Additionally, the dynamics of strontium release by the grafts when placed in a liquid medium was evaluated, simulating what should happen with the fragments in *in vitro* experiments (immersed in culture medium) or transplanted to sites of bone loss, *in vivo*. Então, The blocks were immersed in deionized water for 1, 7 and 14 days and a new analysis by ICP-OES was performed.

2.2. *In vitro* procedure – Sr effect on osteoblasts culture

Immortalized osteoblasts (MC3T3-E1 Subclone4 - American Type Culture Collection - ATCC® ™ CRL2593) were seeded onto the previously treated strontium ranelate grafts at a density of $5x10^4$ in αMEM medium (Life Technology ®) and 10% fetal bovine serum (Gibco ®). Cultures were maintained at 37 °C and 5% CO_2 for 3 and 7 days for cell viability assay (MTT); 7 and 10 days for

cell activity assay (alkaline phosphatase), on mineralized and demineralized grafts associated or not with Sr. The MTT assay was performed to determine whether strontium-adsorbed bone grafts compromised cell viability as a prior experiment to *in vivo* grafts. Cells cultured on the biomaterials for 3 and 7 days were incubated in MTT solution (3- (4,5-dimethylthiazol-2yl) -2,5-diphenyltetrazolium bromide - 0.05 mg / mL, Life Technologies®) for 2 hours at 37 °C and 5 % CO_2. After removal of the solution, the wells with the samples received 500µL of acid isopropanol (100ml of isopropanol: 134µL of hydrochloric acid). The reading was performed on a 96-well plate (100 µl) with absorbance of 595nm (ELX800, BioTek®). The alkaline phosphatase (AP) activity was detected using the NBT / BCIP kit (Thermo Fisher Scientific), according to the protocol established by the manufacturer. At 7 and 10 day, the grafts were rinsed with PBS and incubated with 200 µl / well of NBT / BCIP solution in the ratio 1: 1: 8 (NBT / BCIP / PBS) for 2 hr 37ºC and 5% CO_2. Then, 210 µl of 0.1 N SDS / 10% HCl was added per well, and the plate was incubated for 18 h. Subsequently, the solution was transferred to a 96-well plate (100 µl / well) and the absorbance was measured at 595nm.

2.3. *In vivo* Procedure:

A total of 150 male Wistar (Rattus novergicus) rats weighing 280-350g were used. The animals were divided into 5 groups (table 1), with n = 6 animals by period evaluated in each group, sacrificed after 1, 7, 14, 21 and 60 days of grafting procedures.

EXPERIMENTAL GROUPS

GROUPS	DESCRIPTION
BC	Bone defects filled with blood clot.
M	Bone defects filled with strontium-free mineralized bonive bone graft.
D	Bone defects filled with strontium-free demineralized bovine bone graft.
M + Sr	Bone defects filled with strontium-adsorbed mineralized bovine bone graft.
D + Sr	Bone defects filled with strontium-adsorbed demineralized bovine bone graft.

Table 1: Experimental groups.

2.4. Surgical procedure – grafting protocols

The animals were anesthetized with intramuscular injection of 2% Xylazine Hydrochloride associated with anesthetic base, 10% Ketamine Hydrochloride, both at 0.1ml / 100g. In order to create the bone defect, after the extraction of the first right maxillary molars, the remaining area of the alveoli of the four distal roots was drilled under irrigation using a cylindrical diamond tip KGS-2094, coupled to the dental micromotor (Driller). Thus, the osteotomy generated bone

defects of standard dimensions (2.5mm in diameter x 2.5mm in depth) in all animals (Figure 1), following the protocol of Filho *et al*. (2021).

The bone cavities were filled according to the 5 experimental groups proposed (Table 1). The mucosa on the defect was repositioned and sutured. The animals were kept under heating until the anesthesia returned and in the post-surgical phase they received three doses, one every 24 hours, of TRAMAL analgesic (4mg /kg) and antibiotic oxytetracycline (Terramycin Injection Solution) 10mg / kg. The animals were kept on a paste diet for 5 days. Surgical stitches were removed on the 7th postoperative day. Animals were euthanized at the end of each proposed period. The 1-day period was used for radiographic control of the grafted biomaterials. the other periods aimed for the morphometric analysis of bone deposition.

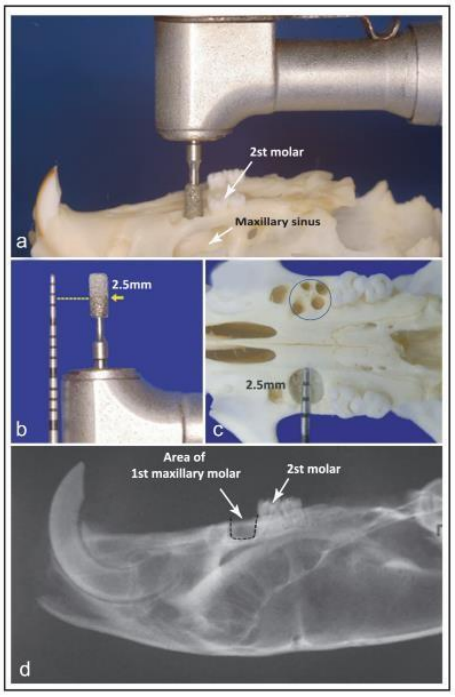

Figure 1. **Location and dimensions of the bone defect in a dry skull.** (a). Position of the bur perpendicular to the bone surface and parallel to the crown of the 2nd upper molar; (b). Arrow indicates the depth of the bur (2.5mm) used during the osteotomy; (c). The area circled in blue indicates the alveoli of the 4 distal roots of the upper 1st molar, which will be unified to create the defect. On the opposite side, the imagem illustrates the defect made with a diameter of 2.5mm; (d). Radiographic image of the defect. The radiograph was obtained with the animal in the surgical position. Dashed line indicates the limits of the bone cavity.

2.5. Macroscopic evaluation of the surgical site

The influence of biomaterials on wound closure (on gingival healing) was evaluated using macroscopic photographs obtained with macro lenses from the Panasonic DMC-TZ3, LUMIX photographic camera. The level of mucosal epithelization was measured by morphometric analysis of the areas of granulation tissue, using the Adobe Photoshop program. For this purpose, standardized macrophotographs were positioned under a grid with 117 points equally distributed in the area selected for evaluation (Albergaria *et al*. 2019). The points on the granulation tissue were counted and converted into percentages, in relation to the total number of points (total area selected). The greater number of spots on granulation tissue indicated a worse level of healing. In contrast, the lower the percentage of granulation tissue, the better the epithelization or closure of the mucosa on the surgical site.

2.6. Radiographic evaluation

O método de análise radiográfica foi previamente validado, segundo dados de Gomes *et al*. (2019). In summary, the right hemimaxils containing the bone defects were fixed in neutral buffered formalin and then transferred to 70% ethanol solution. The pieces were placed in a standardized way on the Durr Dental 3x4 match plates (Bietigheim, Bissingen - Germany). The continuous current Gendex 756DC (Pennsylvania, USA) radiographic device, with exposure time of 0.125 dm / s, 65 kV and 7 mA, and fixed focus / film distance of 10 cm was used. The plates were scanned by the DurS Dental VistaScanPerio Plus processor. With Adobe Photoshop CS5 software, three evaluation points were defined within the defect área (Figure 2). These points, called "ROIs - Regions of Interest", were standardized with size 5x5 pixels located in the apical, medial and cervical regions of the bone defect, positioned on the digital radiographs at 1mm distance from the mesial root of the upper second molar. The levels of gray tones (radiopacity) were recorded at the three points and then the mean values were calculated as a result of radiopacity for each animal. An initial radiograph, considered a "zero point" of the study, obtained 24 hours after surgical procedures, was taken as a reference of the natural radiopacity of the biomaterials within the grafted defects. This care eliminated interference from the natural radiopacity of biomaterials in calculations and interpretation of results. In the final analysis, the values of the radiopacity of biomaterials, recorded at zero point were discounted. The results obtained by the analysis of the digital radiographs were compared with the results of the histological analyzes. Thus, it was possible to evaluate if the radiopacity recorded in the radiographic images was due to the bone repair or only the radiopacity of the biomaterials.

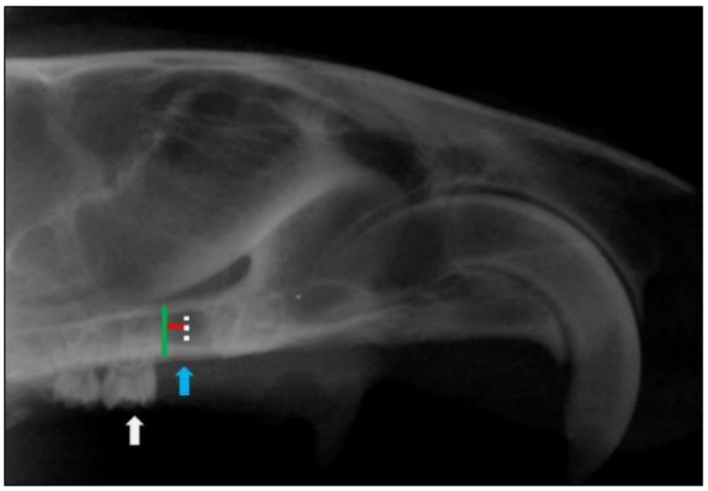

Figure 2: **Radiographic image of a rat hemi-jaw from a blood clot group.** The white arrow shows the second molar, while the blue arrow indicates the bone defect. The green line represents the reference point of the mesial root, the red line corresponds to the distance of 1 mm from the mesial root for definition of the ROI, represented by the white squares. The radiograph represents the animal in anatomical position.

2.7. Histomorphometry

Histological preparations were used to evaluate (i) the quantity and quality of the newly formed bone, (ii) the resorption and osseointegration pattern of grafts. For this, maxillae were dissected and fixed in neutral 10% buffered formalin for 72h, demineralized in EDTA 10% solution, dehydrated by means of graded ethanol solutions and embedded in paraffin. Three sections of 5µm obtained in the frontal plane, 120 µm equidistant from one another, representing the central area of bone defect, were selected from each of the 6 animals. Sections were stained with Masson's trichrome, Hematoxylin & Eosin (H&E) and picrosirius red. Images were captured with a 4x objective (Olympus BX-41, New York, USA) for morphometric analysis through the morphometric software Image J (open software, Laboratory for Optical and Computational Instrumentation, University of Wisconsin-Madison, Madison, WI, USA).

The percentage of the area occupied by bone neoformation, visualized by trabecular deposition, was defined by masson's trichrome staining in a blind analysis. The same principle was used for picrosirius red staining, evaluated in Bright-field and polarized light, allowing to quantify the mature bone matrix (collagen type I). H&E stained slides were used to evaluate biomaterials osseointegration and graft resorption.

2.8. Statistic

All results were analyzed using PrismStatiscal software (Graphpad, San Diego, CA) with one-way ANOVA and Tukey's test. Confidence levels <95% ($p < 0.05$).

3. RESULTS

3.1. Adsorption of Sr to biomaterials

The quantification of Sr adsorbed to biomaterials, after remaining immersed in a saturated solution of SrRan for 14 days, was evaluated by ICP-OES. This technique allows the quantification of chemical elements of metallic and semi-metallic characteristics in a liquid medium. The results showed that the mineralized graft presented higher Sr adsorption capacity than the demineralised biomaterial. The amount adsorbed on the mineralized grafts was 35mg/L, while demineralized grafts showed only 2,9mg/L of Sr (Figure 3A). Thus, demineralized grafts showed low affinity to Sr. This was also proven in the analysis of Sr release from biomaterials in aqueous media. The demineralized graft released 86.2% of all Sr adsorbed on the first day and 100% at 7 days (Figure 3B). Regarding the mineralized graft, 13.62%, 95.38% and 100% of the adsorbed Sr were released at 1, 7 and 14 days, respectively. The data suggest a more gradual release of Sr adsorbed to the mineralized graft.

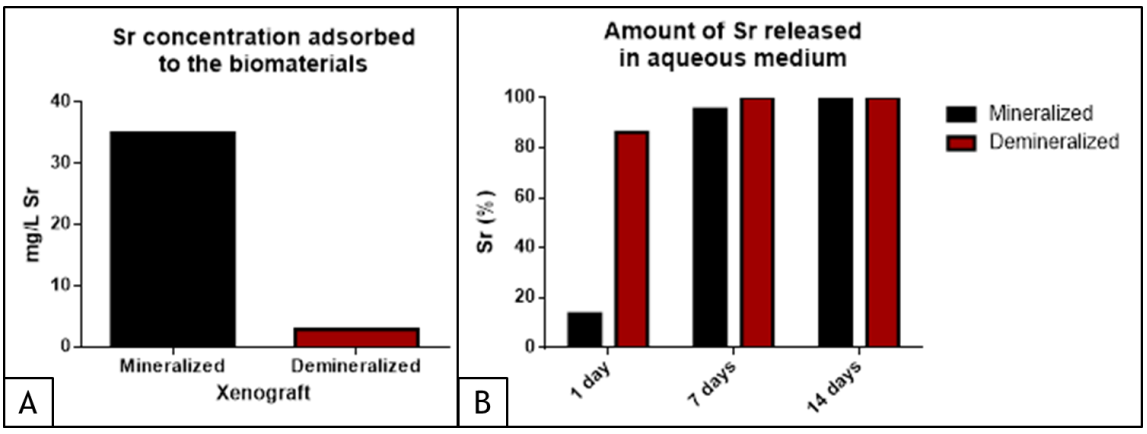

Figure 3: **Concentration of Sr adsorbed to the graft and amount released in aqueous medium**. (A) The mineralized graft presented higher amount of strontium adsorbed when compared to demineralized. (B) On the first day, the demineralized graft released more than 80% Sr. The rate of release of Sr by the mineralized graft in the same period was below 13%. At 7 days, 100% of all adsorbed Sr was released by the demineralized graft, and the same result was found for the mineralized graft only at 14 days.

3.2. *In vitro* Results - effects of Sr in osteoblast culture

In the periods of 3 and 7 days, the cells cultivated in grafts associated with Sr presented greater cell viability when compared with Sr-free grafts (Figure 4A). The comparison between M+Sr x M groups, revealed a greater viability of osteoblasts in the graft enriched with Sr at 3 and 7 days of culture. The same was observed in the comparison between groups D+Sr and D, where the D+Sr showed greater cell viability in all evaluation times. The evaluation of the cellular activity between the groups treated with Sr associated grafts and their controls was performed by the AP assay at the 7 and 10 days periods. The Sr-free mineralized groups, in both periods of evaluation, presented higher AP index. The same was observed for the demineralized groups Sr-free only in the 7 days period and no statistical difference was found at 10 days of cell culture (Figure 4B). In the comparisons between mineralized and demineralized groups (Figura 4C), the demineralized versions associated or not to Sr presented higher levels of AP at 7 days. In the 10 days there was no statistical difference between the comparisons.

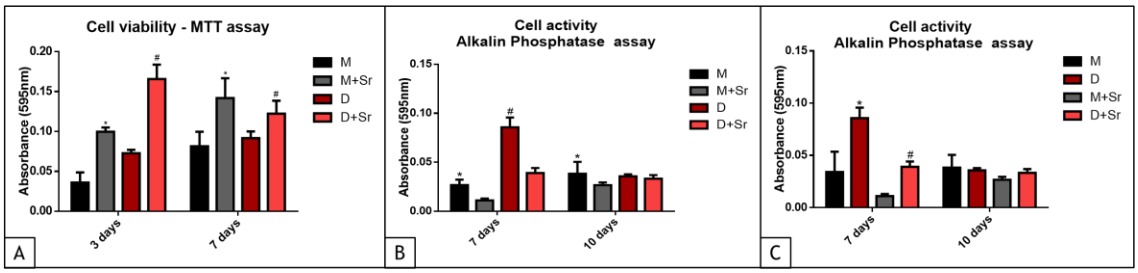

Figure 4: **Cell viability and AP activity in three-dimensional cultures of osteoblasts on grafts with and without Sr.** (A) Note greater cell viability of osteoblasts in the presence of Sr in both groups. (B) Note greater cellular activity of osteoblasts at 7 days in both Sr-free biomaterials. At 10 days only the M group showed greater activity. (C) Comparison between groups: M x D and M+Sr x D+Sr. Note that the demineralized group, regardless of Sr, had higher AP activity at 7 days. There was no difference between groups at 10 days.

3.3. *In vivo* Results

3.3.1. Mucosa epithelialization over the surgical site

In control animals (BC), within 7 days, the healing process of the mucosa over the surgical site is in an intermediate phase with about 60% of the wound closed. At 14 days, the mucosa is completely healed. Figure 5 shows that Sr-free biomaterials (M and D) did not interfere in this process, showing similar results to the BC group. However, it was evident that the presence of

Sr, regardless of the biomaterial, mineralized or demineralized, delayed the epithelialization process. At 7 days there was around 80% of granulation tissue present in both groups (M+Sr and D+Sr) and at 14 days, the surgical site remained in the healing process with around 50% of granulation tissue present. Figure 5 does not show data for subsequent periods because from 21 days onwards, all groups had the surgical site fully epithelialized.

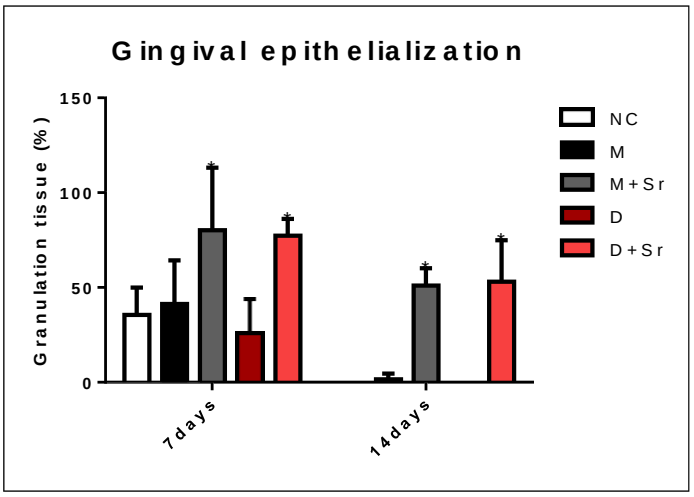

Figure 5: **Epithelialization of the surgical site**. At 7 and 14 days, grafts associated with Sr presented higher levels of granulation tissue in relation to the control group (*).

3.3.2. Radiographic evaluation

The radiographs allowed to estimate the amount of newly formed bone through bone density in the bone defects submitted to grafting with the proposed biomaterials. The results showed the radiopacity curve by measuring the gray tones in the periods of 1, 7, 14, 21 and 60 days. Figure 6 shows the results of the radiopacity gain of the 4 experimental groups and controls plotted within the grayscale range, with the value zero (0) being closer to black and the value of 150 closer to White. In the initial period the groups grafted with mineralized biomaterials presented larger radiopacity. However, the group grafted with mineralized biomaterial without Sr showed decreased radiopacity between the periods of 1, 7 and 14 days. From 14 days, the levels of radiopacity were ascending. Already the M + Sr group presented an upward curve from the 7 days of grafting.

The groups grafted with demineralized or non-grafted biomaterials (BC) presented similar levels of radiopacity in the initial period, being below the mineralized groups. The presence of Sr in biomaterials (D + Sr) generated low radiopacity, in addition to the control or Sr-free demineralized group (D). However, from the 7 days post grafting, the D + Sr group presented

higher levels of gray tones when compared to the D and BC groups. The groups associated to Sr, mineralized or demineralized, presented higher radiopacity in the final period when compared to all groups (Figure 6).

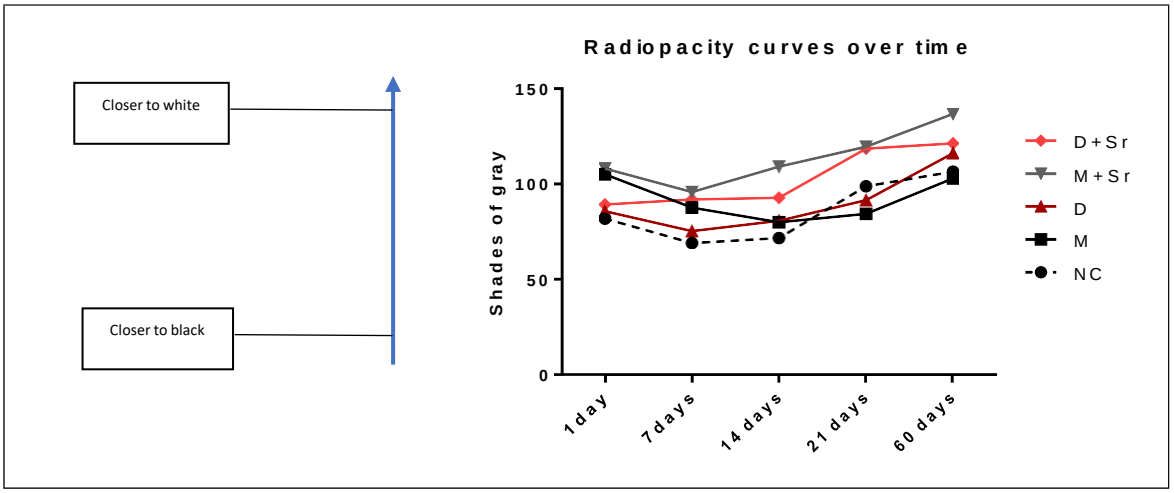

Figure 6: **Radiopacity in the defect area over time.** The mineralized groups presented higher radiopacity in the initial period of evaluation. At 60 days after grafting, the groups associated with Sr showed higher radiopacity, especially the M + Sr group. The lowest levels of radiopacity in the final evaluation period were found for the BC and M groups.

3.3.3. Histomorphometry results

3.3.3.1 Bone deposition level

Histomorphometry of bone deposition in the defect area was performed using Image Jey software. This software enabled an accurate reading of percentage of collagen on the osteid and new bone trabeculae, stained in blue by Masson's trichrome. Figure 7A summarizes the results. Within 7 days, the initial phase of bone deposition, there was no significant difference in bone deposition between groups. at 14 days, the groups associated with Sr (M + Sr and D + Sr), as well as the demineralized group only (D), showed better results when compared to the BC. At 21 days, although the numerical data show a greater trend of bone deposition in all grafted groups, this difference was not statistically supported. However, at 60 days (final evaluation period), the best results were revealed in the Sr enriched groups and in the demineralized matrix group (D). Among these three groups mentioned, there was no statistical difference, which showed similar performance among themselves and superior to the BC group. Only the group of Sr-free mineralized grafts (M), showed worse performance compared to the control group (BC) at 60 days (Figure 7A).

3.3.3.2 Bone maturation level of the newly formed bone

For the analysis of bone maturation, the percentage of mature bone in relation to the total bone formed in the defect area was considered (Figure 7B). That is, even if the defect was partially filled, these trabeculae could be 100% mature, visualized by the red color, generated by the birefringence of type I collagen under polarized light microscopy.

At 7 days the BC group showed bone maturation greater than the groups M, D and D + Sr and similar value to the group M + Sr. In the other periods, there was a gradual increase in the maturation of collagen fibers in all groups compared to the control (BC), except in the mineralized group (M), which showed lower levels of bone maturation up to 60 days. In the final period of repair, the gain in bone maturation was evident in both groups associated with Sr, D + Sr and M + Sr.

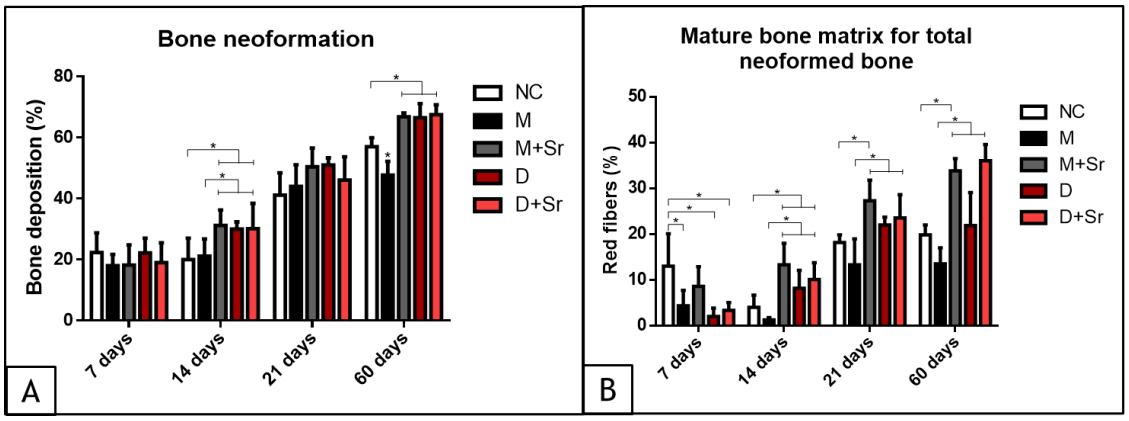

Figure 7: **Bone neoformation and amount of mature bone over total neoformed bone**. (A) The BC and M groups presented the lowest values of neoformed bone at 14 and 60 days when compared to the others. (B) BC group showed a higher rate of mature bone in the initial evaluation period, except when compared to M + Sr group. At 14, 21 and 60 days, the M + Sr, D + Sr and D showed high levels of mature bone compared to M group. In the same periods, the M + Sr group also presented higher results than the NC group.

4. DISCUSSION

The strontium ranelate (SrRan) is a salt composed of a carrier (ranelic acid) and an active formed by two stable Sr atoms (Sr^{2+}). This salt has been used systemically to increase bone volume and promote fracture regeneration in osteoporotic patients (Cianferotti *et al*., 2013; Marx *et al*., 2020). Sr ions are able to adsorb on the surface of hydroxyapatite crystals and replace Ca ions by heteroionic process due to similarity between minerals (Kourkoumelis, 2016). Newly formed

bone tissue tends to have a higher concentration of Sr incorporated into mineral granules when compared to mature bones, whose Sr present occurs by adsorption (Martin-del-Campo *et al.*, 2019). Although Sr is similar to Ca, its presence in hydroxyapatites may alter some structural properties of the crystal, such as crystal size, solubility and especially its density, since it has atomic weight approximately twice as high as Ca. These changes are discussed regarding their advantages and disadvantages. however, the increase in bone density makes it more resistant to fractures (Marx *et al*., 2020).

In therapeutic processes via systemic pathways, Sr has the ability to modify the physiology of bone tissue, favoring bone formation by stimulating osteogenesis and angiogenesis (Martin-del-Campo *et al.*, 2019). The continuous use of SrRan via systemic is also proposed to aid the body's responses in cases of bone grafting (Zhao *et al*., 2013). However, the form of administration is a relevant point to discuss when considering specific areas to be addressed. This is because SrRan can present cytotoxicity to the intestinal mucosa and systemic toxicity, such as increased risk of cardiovascular problems (Loca *et al.*, 2018). The topical use of Sr conveyed by SrRan or strontium chloride has been suggested in research (Chiang *et al*., 2021; Tohidnezhad *et al*., 2020). Preliminary studies have demonstrated the biocompatibility of Sr maintaining the viability and activity of osteoblasts *in vitro* (Romagnoli *et al*., 2017). However, the topical use of Sr is still poorly explored clinically. An alternative also discussed in the literature is the placement of the Sr using the biomaterials intended for bone grafting. Different studies have worked with the incorporation of Sr to biomaterials for bone defect grafts or *in vitro* tests (Raucci *et al.*, 2015; Silva *et al.*, 2018). The incorporation of SrRan into the grafts, in adequate concentration, may contribute to a more rapid rehabilitation of the patient without the need for daily systemic consumption of the drug (Neves *et al*., 2017). In the present work, we proposed the association of Sr in two types of biomaterials, mineralized bovine bone (M + Sr) and demineralized bovine bone (D + Sr). Through the ICP-OES technique it was possible to observe that mineralized grafts have higher Sr adsorption capacity when compared to demineralized grafts by immersion in saturated SrRan solution. These data show the importance of the presence of Ca for adsorption of the proposed drug through the methodology employed. Also reiterate the statements of Neves *et al.* (2017) that affirms Sr's affinity for hydroxyapatite crystals. Another finding from our work demonstrates the capacity of gradual release of Sr by the studied mineralized graft, being able to potentiate the biological properties of this biomaterial. The gradual delivery of the drug to the site of interest has the advantage of minimizing the risks of systemic effects (Jiménez-Holguín *et al*., 2020).

The contact of stem or osteoprogenitor cells with Sr-enriched biomaterials to the site of interest may favor their survival, proliferation and differentiation, stimulating osteogenesis (Li *et al.*, 2017). In our *in vitro* experiments we found a higher cell viability rate in MC3T3-E1 culture in Sr adsorbed mineralized grafts at 3 and 7 days of cell culture when compared to the control (Sr-free mineralized graft). The literature clarifies the form of action of Sr in cellular stimulation, which justifies our findings. According to Bakhit *et al*. (2018), Sr in osteoblast-like cells interacts with the calcium sensitive receptor (CaSR). *In vitro* experiments have shown that the Sr-CaSR interaction promotes mitogen-activated protein kinase activation and induction of cyclooxygenase-2 expression with consequent increase in prostaglandin-E2 production. These mechanisms are related to the regulation of mesenchymal and osteoblastic stem cell replication (Reitmaier *et al.*, 2017). Wnt and COX2 signaling pathways are also stimulated by the presence of Sr in inducing cell proliferation (Romagnoli *et al.,* 2017).

On the other hand, the culture of osteoblasts on demineralized grafts also revealed greater cell viability at 3 days, even with a low concentration of Sr adsorbed on these biomaterials shown by the ICP-OES (2.9 µg / ml). These results can be justified for two reasons: First, the release of almost all of the Sr on the 1st day, in aqueous release tests, allows us to suggest that the Sr released into the culture medium in the first 3 days may have played its role in inducing cell proliferation. It is known that Sr effects are triggered with small doses (Aimaiti *et al*., 2017), especially in culture conditions where there is no drug dissipation in tissues or competition by other cells in repair sites, as occurs *in vivo*. The second reason is the nature of the demineralized biomaterial. The demineralization process exposes collagenous and non-collagenous proteins from the organic bone matrix, such as collagen and BMPs, The demineralization process exposes collagenous and non-collagenous proteins from the organic bone matrix, which also induce cell proliferation. Our hypothesis can be confirmed by observing the cell viability pattern at 7 days. In this case, the D + Sr group still maintains a high viability, however, compared to the M + Sr group it presents a better performance in the cell viability assay. This is because this biomaterial still provides Sr, which acts as an inducer of osteoblastic proliferation.

Regarding cell activity, in both periods, at 7 and 10 days, we found lower rates of cell activity by quantifying alkaline phosphatase (AP) staining in Sr-adsorbed grafts, when compared to their respective controls (Sr-free grafts) even with better results in cell viability. It is understood that Sr can maintain Runx 2 activation in proliferating cells (Park *et al*., 2013), delaying the phase of differentiation and cell activity. Similar works have shown that activity of AP presents better

results for the control group in pre-osteoblast culture at low Sr concentrations (equal to or below 0.5mM) in culture periods of 7, 14. and 21 days. (Lourenço *et al.,* 2019). Regarding demineralized grafts, these have factors that can contribute to both phases of cell proliferation and differentiation. This fact justifies the superior cellular activity demonstrated by osteoblasts cultivated on demineralized biomaterials. These factors are type I collagen (aids in cell migration and adhesion), BMPs (favors cell differentiation) and osteopontin (favors mineral deposition by having calcium binding sites) (Martin-del-Campo *et al.,* 2019). This organic matrix proteins not only allows the migration of cells, but also increases the capacity for bone neoformation, favoring the expression of proteins involved in bone metabolism.

In vitro experiments are usually performed as preliminary tests for *in vivo* assays to ascertain cytotoxicity and other possible effects of drugs such as Sr. Our results showed that at the proposed concentration, Sr-enriched biomaterials could favor the performance of osteoblasts *in vivo*. Although the focus of this study was bone repair, we understand that the success of therapy also depends, in clinical conditions, on the closure of the mucosa in the operated area. Interestingly, we observed that animals grafted with materials associated with strontium had a delay in gingival epithelialization, which suggests a possible cytotoxicity of the drug towards fibroblasts. In fact, some studies using human periodontal ligament fibroblasts have shown that cytotoxicity is dose-dependent and inert at appropriate doses (Fiorilli *et al.*, 2018). Er *et al*. (2008) showed that Sr concentrations above 20mg / ml had extreme cytotoxic effects and doses of 2.5mg / ml did not compromise the viability of human fibroblastic cells of the periodontal ligament. However, in our studies, we used a lower concentration than that reported by Er *et al*. (2008) and observed a delay in initial healing, at 7 days. As for the level of bone neoformation in the defect area, this was not compromised. Therefore, our proposal is that resources be used for the isolation of the operated area submerged by Sr grafts. The use of collagen membranes in grafted bone defect sites may provide a support base for the epithelial tissue and avoid its contact with the biomaterial used (Al-Askar and Alsaffar, 2018).

Although the Sr has compromised gingival epithelialization, the results have already shown an acceleration in bone deposition for Sr adsorbed groups, evaluated by radiopacity on radiographic images, and histological assays. From 14 days on the M + Sr and D + Sr groups presented higher bone neoformation rates in histological and radiographic analysis, being even higher for the mineralized graft. Although Sr adsorbed demineralized graft also showed better results in our study, there was no statistical difference when compared to D group, but when compared to mineralized without Sr and BC.

In summary, the findings of this study reveal satisfactory results that can be compiled as follows: (i) the addition of strontium to mineralized bone grafts, due to its affinity to hydroxyapatite, is a strategy that can improve the performance of osteoblasts during bone repair; (ii) The use of demineralized grafts is a means of accelerating bone deposition, since exposure of organic matrix proteins from bovine grafts favors osteogenesis.

The indication of the best strategy for bone grafting will depend on the patient's clinical conditions. Demineralized grafts are quickly reabsorbed, they do not provide mechanical resistance in load-receiving areas. However, these biomaterials are excellent alternatives for filling alveoli for inducing bone neoformation, prior to the insertion of implants. In more extensive bone defects that can receive loads, the indication of mineralized xenografts associated with Sr seems the best alternative. Finally, this study opens perspectives for the use of Sr in partially demineralized grafts. The presence of residual Ca can favor Sr adsorption while organic components of the bone matrix remain available for osteoblastic activation. This is a hypothesis that deserves to be investigated.

Acknowledgements

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CONCLUSÕES

Síntese das conclusões na execução do projeto do doutorado:

1. O método de avaliação radiográfica da deposição óssea por meio de tons de cinza (radiopacidade avaliada em ROIs) é um método simples, confiável, especialmente fidedigno para análise de reparo ósseo em sítios enxertados com biomateriais não mineralizados.
2. O potencial de adsorção de estrôncio aos xenoenxertos mineralizados é maior que nos desmineralizados. Além disso, a liberação do estrôncio em meio aquoso é mais rápida nos enxertos desmineralizados comparativamente aos mineralizados. Estes dados sugerem que enxertos desmineralizados não são bons sistemas para a veiculação do estrôncio e liberação gradual de estrôncio nos sítios enxertados. Enxertos mineralizados cumprem melhor esse papel.
3. A presença de estrôncio associado aos xenoenxertos, mineralizados ou desmineralizados, não prejudica a viabilidade de osteoblastos em cultura.
4. A presença de estrôncio nos biomateriais parece induzir a proliferação celular, mas mantem as células em um estágio mais precoce, com menor atividade celular.
5. Enxertos desmineralizados favorecem a proliferação celular e também a diferenciação e atividade celular em cultura.
6. A presença de estrôncio nos sítios cirúrgicos *in vivo* atrasa a cicatrização, epitelização da mucosa que recobre o defeito ósseo, mas acelera a deposição óssea na área do defeito.
7. Biomateriais desmineralizados são mais rapidamente reabsorvidos não conferindo resistência mecânica nos sítios operados.
8. Dentre os biomateriais testados, os que demonstraram melhores resultados em relação a deposição e maturação óssea foram os enxertos desmineralizados livres de estrôncio (D) e os mineralizados associados ao estrôncio. Ambos possuem mecanismos distintos de atuação sobre osteoblastos, seja via ligação do estrôncio aos receptores de cálcio ou por indução de osteogênese por proteínas expostas (BMPs, Osteopontina, Colágeno tipo I) presentes na matriz orgânica dos enxertos desmineralizados.

Os resultados do presente estudo abrem perspectivas para a utilização clinica destes biomateriais, como aceleradores da deposição óssea.

PERSPECTIVAS

Este estudo, por meio dos resultados obtidos, tem como perspectiva (i) a realização de culturas de osteoblastos sobre enxertos associados ao Sr a fim de averiguar, por PCR quantitativa, o padrão de expressão de genes específicos ligados a diferenciação celular através da metodologia de tratamento proposto; (ii) a realização de cultura de fibroblastos sobre os enxertos desenvolvidos nessa tese a fim de averiguar a citotoxicidade do tratamento, uma vez que os experimentos *in vivo* demonstraram atraso na cicatrização do epitélio sobre o sítio cirúrgico da região de defeito ósseo enxertado; (iii) a valiação do padrão de expressão gênica por RNAseq da região de regeneração óssea após enxertias com biomateriais associados ou não ao Sr nos períodos iniciais de regeneração (7 e 14 dias). Dessa forma, espera-se contribuir para o entendimento e esclarecimentos sobre o uso de enxertos bovinos, mineralizado ou desmineralizado, adsorvidos com Sr como estratégia de otimização dos reparos ósseos em odontologia.

ANEXO I

Influence of mineralized and demineralized xenograft bone on the repair of intraoral bone defect in rats

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Abstract

Background and purpose

Mineralized Bovine bone grafts have been widely used as substitutes for bone losses in dental clinics. Studies have been showing that exposing the organic components of the bone matrix at surgical sites accelerates bone deposition. We evaluated the bone repair progress of an intrabuccal bone defect in rats after grafting with mineralized (MBB) and demineralized bovine bone (DBB).

Materials and methods

An intraoral bone defect was created after extraction of the right maxillary first molar, drilling the area of the alveoli of the four distal roots using a diamond tip. The defect was filled with the MBB or DBB graft. Grafting effects were evaluated after 1, 7, 14, 21 and 49 days, using radiography and histology data.

Results

After 14 days, all groups showed full mucosa epithelialization at the surgical site. Radiographic data showed an improvement in bone deposition in defects grafted with the organic matrix. This data was confirmed by the histological analysis. A higher level of bone maturation of the

neoformed trabeculae and a faster reabsorption rate were also observed to be a feature of the DBB graft.

Conclusion

Together, our in vivo data revealed that the DBB graft might represent an alternative to mineralized biomaterials.

Keywords: Xenograft; bone repair; demineralized bone matrix; intraoral bone defect.

1. Introduction

Extensive bone loss caused by fractures, tumor excision and metabolic or degenerative diseases usually require additional therapeutic resources for the restoration of the bone function (Watanabe *et al.*, 2016). In dentistry, the absence or deficiency of a support, bone grafts have been indicated as substitutes or as transient filling biomaterials (Shirmohammadi *et al.*, 2014). In this latter case, grafts tend to be partially or totally reabsorbed over time, while they are replaced by neoformed bone tissue (Oryan *et al.*, 2014).

Bone grafts have different origins and presentations. The xenografts, obtained from animal sources such as swine and bovine (Yaghobee *et al.*, 2018), are commercially available as fragmented (bone particulates) or as three-dimensional blocks, similar to the natural bone structure, with its porous and trabecular structure (Dasmah *et al.*, 2012).

Properties such as osteoconduction and osteoinduction should be considered as criteria for the selection of biomaterials used in bone grafts (Palachur *et al.*, 2014; Jo *et al.*, 2018). Furthermore, the osseointegration of biomaterials in the remaining bone walls can provide stability to dental implants, this stability being a basic condition to support all the functional demands of the tissue (Oryan *et al*., 2014; Lei *et al.*, 2015).

Biomaterials from bovine sources used in bone grafting are mostly osteoconductive, whereas the autogenous grafts are recognized as osteoinductive. However, autogenous fragments are obtained from surgical procedures and the amount of tissue that can be harvested from the donor area can result in increased morbidity and does not always meet the demand for bone loss. These limitations have encouraged the development of research in the search for alternative materials or improvement of grafts already available in a clinical setting in bone replacement therapies and are available as mineralized blocks or granulates (Khoshzaban *et al.*, 2011; Bahammam, 2016; de Assis Gonzaga *et al.*, 2017; Thorwarth *et al.*, 2006). They have been

shown to have suitable biocompatibility, osseointegration, mechanical resistance and present a good bone deposition inductor potential (Palachur *et al*., 2014; Berglundh *et al.*, 1997; Carmagnola *et al.*, 2003; Fickl *et al.*, 2008; Pichotano *et al.*, 2019; Naros *et al.*, 2019).

Although most commercial grafts are available in mineralized fragments, new research has now revealed the positive influences of the organic components of the bone matrix in the process of osteogenesis during bone repair. Different growth and differentiation factors are among these organic components, such as transforming growth factor – beta (TGF-β), insulin-like growth factors I and II, platelet derived growth factor (PDGF), fibroblast growth factor and bone morphogenetic proteins – BMPs; (Palachur *et al*., 2014; Drosos *et al.*, 2015; Fernandez de Grado *et al.*, 2018). In this way, the exposure of the organic matrix present in bone grafts may represent a strategy for optimizing bone repair at recipient sites. Therefore, the chemical treatment used to expose the organic phase of the biomaterials should maintain the integrity of the components associated with the osteogenic and osteoinductive properties. EDTA (ethylenediamine tetraacetic acid) with neutral pH can be an alternative to minimize damage to the bone structure during the demineralization process. According to Hülsmann *et al.* (2003), EDTA is an organic compound that acts as a chelating agent, forming very stable complexes with several metallic ions (such as calcium), removing them from the tissue with minimal histological changes.

In the present work, we have evaluated the in vivo influence of mineralized and demineralized xenografts during the bone repair of a jaw defect in a rat. The morphometric results (macroscopic, radiographic and histological analysis) reiterated the main hypothesis that the organic bone matrices exert an osteoinductive influence in the grafted sites.

2. Methods

2.1 Biomaterials

Bovine bone (LuminaBone® - Critéria, Brazil) was used as biomaterial in this work in both mineralized and demineralized forms. Mineralized bovine bone (MBB) was used in granules of approximately 3 mm. Demineralized bovine bone (DBB) was obtained by immersion of the mineralized blocks in EDTA 10% for 72 h.

2.2 Validation of the demineralization protocol – organic matrix preservation analysis after EDTA treatment

A stereomicroscope (EZ4D – Leica; and LAS EZ 2.1.0 software) with 35x magnification was used to assess the maintenance of the three-dimensional structure of the fragments selected for grafting. This analysis considered the trabeculae and pores preservation of biomaterials after the EDTA demineralization protocol. The preservation of bone matrix content was assessed by histology, immunohistochemistry and immunofluorescence analysis.

DBB was fixed in neutral buffered formalin and embedded in paraffin. Sections of 5µm were stained with Hematoxylin and Eosin (H&E) according to routine histology. The presence of BMP4 was confirmed by immunohistochemistry and osteopontin and collagen type I by immunofluorescence. For this, 5µm sections were deparaffinized in xylol, rehydrated in a gradual series of ethanol and washed in PBS. For immunohistochemistry the endogenous peroxidase was blocked by hydrogen peroxide. Non-specific binding sites were blocked with 2% BSA in Tris-HCl (pH 7.4) for 1 h for both techniques. Sections were incubated overnight at 4°C with anti-BMP4 (Santa Cruz Biotechnology anti-mouse), anti-osteopontin (Abcam anti-mouse) and anti-collagen type I (Abcam anti-rabbit), all polyclonal primary antibody and diluted 1:200 in PBS. After washing with PBS, sections were incubated in room temperature for 1 h with the specific secondary antibody for immunofluorescence (488 goat anti-rabbit and 488 goat anti-mouse, Alexa Fluor-Molecular Probes), diluted 1:700 in 2% BSA in PBS. For immunohistochemistry, the secondary antibody used was EnVision Dual Link System-HRP (DAKO). The DAB precipitation showed the presence of BMP4 protein. Hematoxylin was used to counterstain. In both techniques, the sections were washed with PBS and mounted with 80% glycerol. Images were captured using the Olympus BX-50 microscope (Olympus, Hamburg, Germany). Negative controls were performed by the omission of the primary antibodies.

2.3 Animals, surgical procedure and experimental groups

120 adult male Wistar rats (Rattus norvegicus; 3-4 month old) with body weight < 280 g were used in this work. The management of the animals and surgery protocols were previously approved by the Commission of Ethics in the Use of Animals of the Universidade Federal de Minas Gerais (Protocol 7/2015).

For the surgical procedures, the animals were anesthetized with intramuscular injection of 2% Xylazine Hydrochloride associated with 10% Ketamine Hydrochloride, both at 0.1ml/100g. The

bone defect was created by the extraction of the right maxillary first molar. The remaining area of the alveoli of the four distal roots was drilled under irrigation using a cylindrical diamond tip KGS-2094, coupled to the dental micromotor (Driller). The osteotomy generated a bone defect with approximate dimensions of 2.5 mm in diameter x 2.5 mm in depth in all animals.

The bone defects were filled as follows: group (i) – MBB, filled with mineralized bovine bone in granules; group (ii) – DBB, filled with demineralized bovine bone fragments; group (iii) – BO, filled with Bio-Oss® granulated bone (Geistlich, Switzerland); and group (iv) – NC, filled with blood clot. Bio-Oss® particulated bone (Geistlich, Switzerland) was used in its commercial version (mineralized granulate). As this is widely studied in the literature, Bio-Oss® was used in this work as a positive control. Blood clot was used as a negative control. All biomaterials were transplanted until the volume of the defect was completely filled. After filling, the mucosa on each defect was repositioned and sutured. Animals were kept under heating until recovery from anesthesia and TRAMAL (4mg/kg) and antibiotic oxytetracycline (Terramycin Injection Solution, 10mg/kg) was administered during the post-surgical stage every 24 h. Animals were kept on a paste diet for 5 days. Surgical stitches were removed on the 7th postoperative day. The animals of the 4 groups were euthanized at 1, 7, 14, 21 and 49 days after the surgical procedure, N= 6 animals per period, 30 animals in total for each group.

2.4 Macroscopic analysis

The effects of each biomaterial on wound closure (on gingival healing) were evaluated using macroscopic photographs obtained with macro lenses from the Panasonic DMC- TZ3, LUMIX photographic camera. Mucosal epithelization level was measured by morphometric analysis of the granulation tissue area, using Adobe Photoshop. For this, the selected area was obtained in standardized macrophotographs and positioned under a grid with 117 equally distributed points (Figure 1). The points on tissue granulation were counted and converted into percentages, related to the total number of points (total area selected). High numbers of spots on tissue granulation indicated a low level of gingival healing and vice-versa.

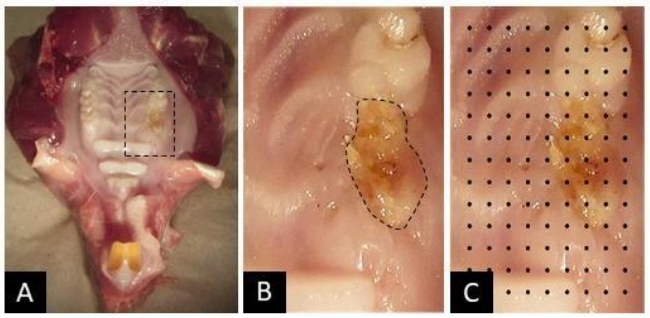

Figure 1. Gingival healing granulation analysis. (A) Standardized macrophotography. The black grid region represents the evaluation area for gingival healing, referenced in B. The black dotted line in B delimits the tissue granulation. (C) Equally distributed points over the evaluated area. The points on the tissue granulation were converted into percentages in relation to the total number of points in the area under consideration.

2.5 Radiopacity index evaluation

For radiographic evaluation, the right hemi-jaws containing the defects were fixed in neutral buffered formalin for 72 h and then transferred to 70% ethanol. Pieces were placed on the Durr Dental 3x4 match plates (Bietigheim, Bissingen - Germany), always in the same position. The Gendex 756DC (Pennsylvania, USA) radiographic device was used with exposure time of 0.125 dm/s, 65 kV and 7 mA, and fixed focus/film distance of 10cm. Plates were then scanned by the Durr Dental VistaScanPerio Plus processor. Using the Adobe Photoshop CS5 software, three points with 5x5 pixels each were defined within the defect area. These points, called "ROI - Regions of Interest" were positioned on the digital radiographs: (i) at the apical, (ii) medial and (iii) cervical regions of the bone defect, at 1 mm distance from the mesial root of the upper second molar. The radiopacity index was calculated as the mean of the gray tones recorded in each of the three points for each animal. Radiopacity index obtained 24 h after the surgical procedure and graft filling was used as reference of the natural radiopacity of the biomaterials and discounted as background.

2.6 Histomorphometric analysis

Bone repair evolution was evaluated in histological sections of the hemi-jaws 7, 14, 21 and 49 days after the surgical procedure and graft filling. Histological sections allowed evaluating aspects of bone maturation and graft osseointegration and reabsorption over time. For this,

jaws were fixed in neutral buffered formalin for 72 h and demineralized in 10% EDTA, pH 7.2. After washing in water, samples were dehydrated in rising baths of ethyl alcohol, diaphanized in three xylol baths and embedded in paraffin. Sections of 5µm obtained in the frontal plane were stained with Masson's Trichrome and H&E, for morphometric analysis; and with picrosirius red, for analysis of collagen fiber maturation by polarized microscopy. In order to facilitate the description and interpretation of the results, the bone defect area, which is delineated by the lateral walls, vestibular and palatine, and the bottom wall, was virtually divided into three equal parts: the apical third, middle third and cervical third (Figure 2A-B).

Bone deposition was determined by histomorphometric analysis performed in Masson's Trichrome stained sections. Images were captured under Olympus BX-50 light microscope, coupled to Q-color 3 camera and evaluated using the ImageJ software. Morphometry data was expressed as the mean of three sections, taken at different points of the bone defect. The percentage of the area occupied by bone neoformation, identified by the deposition of trabeculae stained by Masson's Trichrome, was determined using the mean of three measurements obtained in the three different depth, trained in a blind analysis.

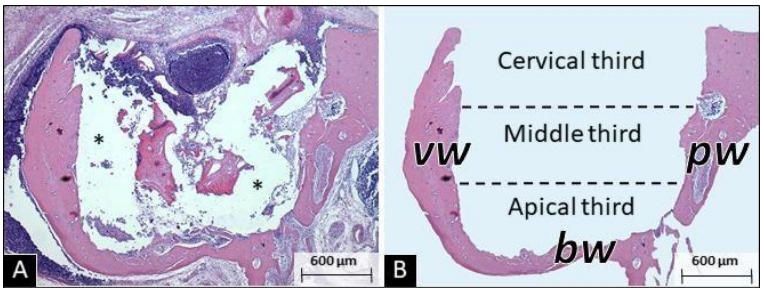

Figure 2. Schematic representation of the bone defect and the method used to the histomorphometric analysis. In (A), an original histological image of a section in the area of the bone defect. Blank areas (*) represent the area filled with the biomaterial. In (B), a representative image of the bone defect, showing the presence of the remaining bone walls (vw - vestibular wall and pw - palatal wall; bw = bottom wall/floor of the bone cavity).

2.7 Bone maturation analysis

The maturation of the neoformed bone trabeculae in the defect was determined in sections stained with picrosirius red. We used 5µm histological sections selected at 3 different depths of serial cuts of the bone defect. Briefly, sections were dewaxed in two xylol baths (20 min each),

hydrated with 3 baths of decreasing series of alcohols (100%, 90% and 70% for 5 min each) and immersed in water for another 5 min. Sections were stained with picrosirius red for 45 min in an oven at 60°C and submerged in acidic solution (hydrochloric acid 0,01N) for 5 min. Sections were subsequently stained with Harris Hematoxylin for 5 min, washed for an additional 5 min in running water and dehydrated in an increasing sequence of alcohol for one minute in each solution (90%, 100% and 100%). Finally, the slides were mounted using Entellan (Merck - Germany). In the microscopic evaluation, polarized light emission on the sirius red dye allows distinguishing the collagen quality by the birefringence and organization of its bundles. Collagen fibers from newly formed bone matrix (immature collagen) are thin, with poor green birefringence; while organized fibers appear in yellow and red (type I fibers). Red fibers represent the maximum maturation of the matrix. Images were obtained using the Olympus BX-50 polarized microscope. Data were collected only on neoformed bone trabeculae and quantified using ImageJ software.

2.8 Biomaterials resorption evaluation

Biomaterial resorption rate was estimated calculating the area occupied by the biomaterial on the 49th day, related to the area observed after 24 h of the grafting procedure, by histomorphometry of H&E stained sections.

2.9 Statistical Analysis

All results were analyzed using PrismStatiscal software (Graphpad, San Diego, CA). Data were represented as mean standard deviation and statistically compared with confidence levels > 95% ($p<0.05$), using ANOVA One-way ANOVA and Tukey's test. The direct comparison between two groups was performed by Student t-test.

3. Results

3.1 Effects of the demineralization protocol on the bone graft

The EDTA demineralization process shows the potential to preserve the three-dimensional bone structure of xenografts. Stereomicroscope image revealed trabeculae and interconnected pores

similar to mineralized graft, demonstrating the preservation of bone structures (Figure 3A and 3B).

Three Non-collagenous bone proteins (BMP4, Collagen type I and osteopontin) were evaluated for Immunohistochemistry and Immunofluorescence analysis as references for monitoring the preservation of bone structures and organic matrix. BMP4 and collagen typo I showed a more diffuse expression pattern (Figure 4A and D, respectively); Osteopontin revealed a more localized expression at the edges of the trabeculae (Figure 4C).

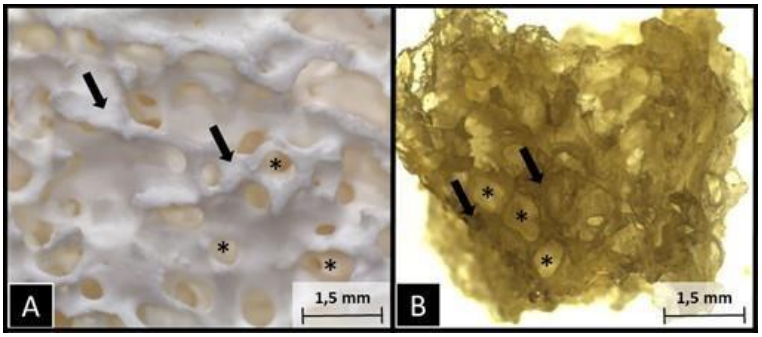

Figure 3. Stereomicroscope image. In A and B, the asterisks show the porosity of the mineralized and demineralized biomaterials. Black arrows show bony trabeculae.

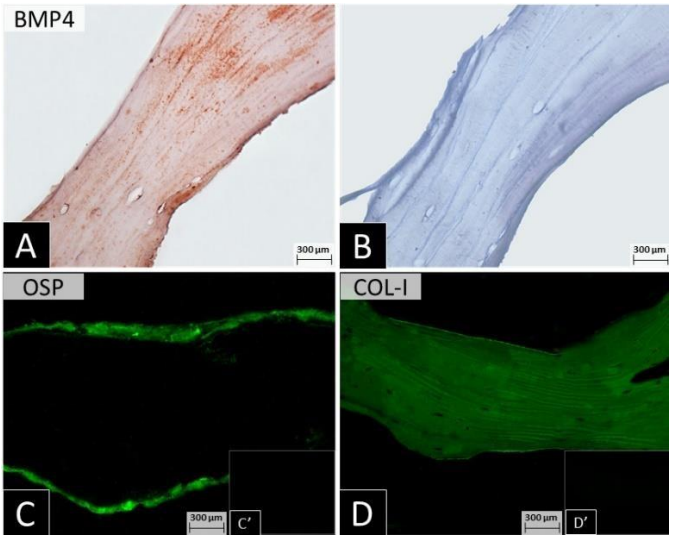

Figure 4. Bone proteins staining in the DBB organic matrix. In (A), BMP4 staining; in (C), osteopontin; and in (D), collagen type I. (B), (C') and (D') are the negative controls.

3.2 Effects of biomaterials on mucosal healing

Gingival epithelialization at the surgical site was measured by the amount of granulation tissue visible over the operated area, after 7 and 14 days of grafting. We could not observe any interference of the MBB, DBB and the positive control at the wound closure; the evolution of the process was similar to the observed in control group. After 7 days, the mucosa was under repair, with granulation tissue present in all groups. The DBB group showed a lower percentage of granulation tissue - 31.2%, followed by MBB - 34%; NC control - 35.6% and BO - 41.2% (Figure 5A-D, I). Although the DBB group presented the highest epithelial area and the BO group the smallest one, we could not observe any significant difference between groups. All groups showed full mucosa epithelialization after 14 days of grafting (Figure 5E-H).

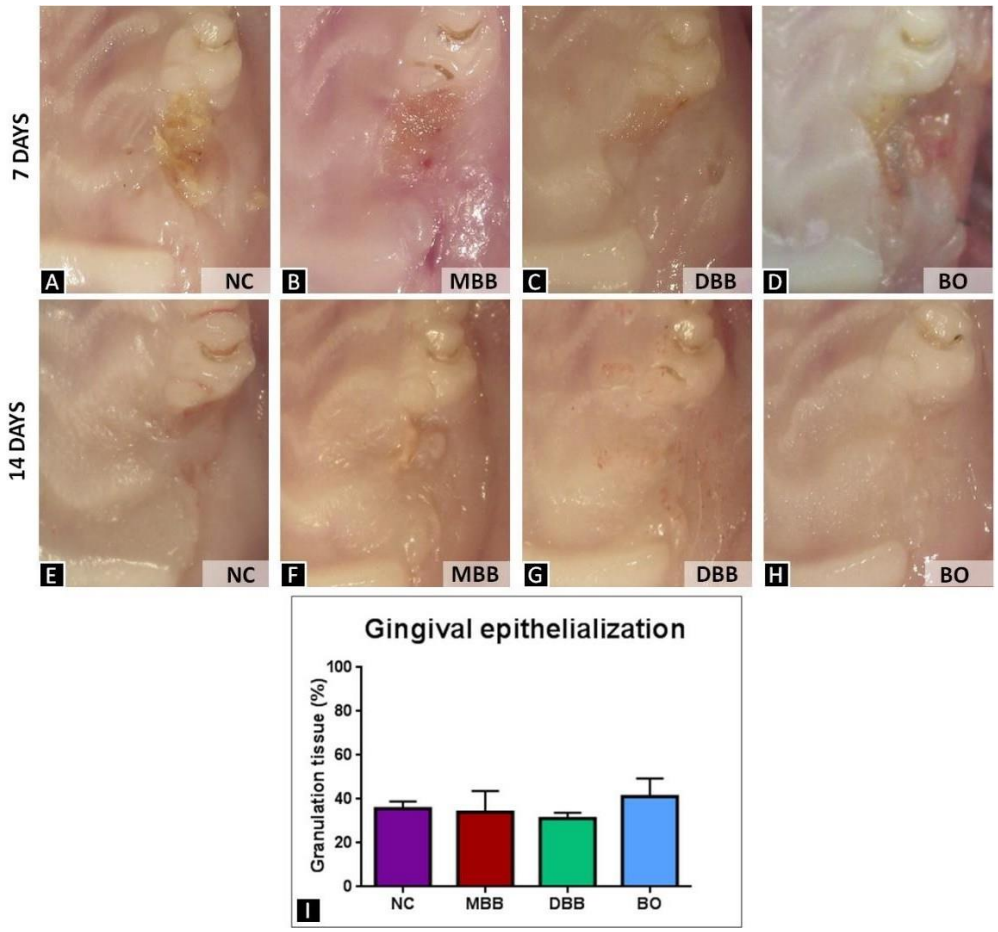

Figure 5. Macroscopic aspects and quantitative granulation tissue data. From A to D illustrates the presence of granulation tissue at the surgical site at 7 days. From E to H at 14 days, all groups show an absence of granulation tissue. In (I), the graph shows similarity in gingival epithelization among all groups, with no statistical difference.

3.3 Radiographic analysis of bone deposition

BO group showed the highest natural radiopacity after 1 day of grafting, followed by MBB, DBB and NC (Figure 6A). A decrease in the radiopacity index was observed after 7 and 14 days of MBB and DBB grafting and also in the NC group (Figure 6A); while no significant decrease was observed when the BO was used. After 49 days, the DBB graft showed the highest radiopacity index, compared to the others and to the background (obtained after 1 day; Figure 6A).

Histological analysis showed the presence of biomaterial granules after 49 days in both MBB and BO grafting (Figure 6B and C), implicating in a smaller area destined for new bone formation. On the 49th day, the bone defects filled with DBB showed 100% of biomaterial reabsorption (Figure 6D).

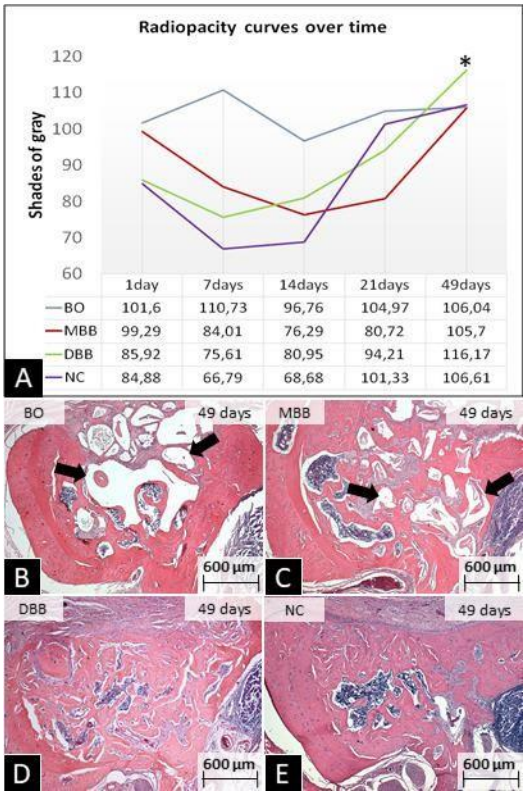

Figure 6. Radiopacity index for each biomaterial over time. In (A), radiopacity index for each biomaterial over time. The BO biomaterial showed the highest radiopacity index after 1 day of grafting, while the DBB showed the highest index at 49 days. In (B) and (C) a representative image of the histological analysis performed in defects filled with BO and MBB grafts, respectively. Black arrows indicate the presence of remaining biomaterial granules even after 49 days of grafting. In (D) showed the absence of demineralized graft (totally reabsorbed). (E) Negative control. *$p<0.05$, ANOVA with correction factor in Tukey's Test.

3.4 Histomorphometric analysis of bone deposition

Compared to the NC group (control), the DBB graft revealed a higher percentage of neoformed bone trabeculae, after 14 and 21 days of grafting (Figure 7A). Comparisons between DBB and MBB groups showed higher bone deposition for the group filling with demineralized biomaterial after 14 and 49 days of grafting (Figure 7A). Referring to BO group, the DBB group revealed a higher bone formation in the period of 49 days. In the 14 days after grafting, the BO group presented better results when compared to negative control (Figure 7A).

The DBB group showed an ascending line in the bone deposition curve from 7 days after grafting (Figure 7B), suggesting an acceleration of the repair process. Although the quantitative analysis has revealed no statistical difference in the final period (49 days), it is worth mentioning that it was possible to observe the concave bone surface only in the NC group, suggesting a small loss of bone volume in the cervical third of the ungrafted defects (Figure 8A). A more regular bone deposition in the DBB group could be observed in the final stage, with a flat aspect, recomposing the entire upper limit of the bone cavity (Figure 8B).

In the comparison between MBB and BO, a higher bone deposition was observed in the BO group after 14 days of grafting, compared to the MBB one (Figure 7A). Both grafts showed an ascending line of bone deposition over time, but no significant difference was observed between MBB and BO (Figure 7B).

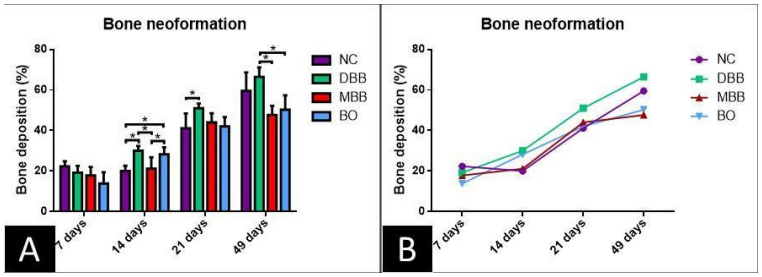

Figure 7. Bone deposition curve throughout time in all groups. In (A), the percentage of bone deposition was higher when DBB graft was used, compared to the other groups, after 14 days of grafting. BO group showed a higher percentage of bone deposition at 14 and 49 days of grafting, compared to the MBB group. In (B), the bone deposition curve established over time showed an acceleration of the bone deposition process to the DBB group when compared to the other groups. * $p<0.05$ t-test.

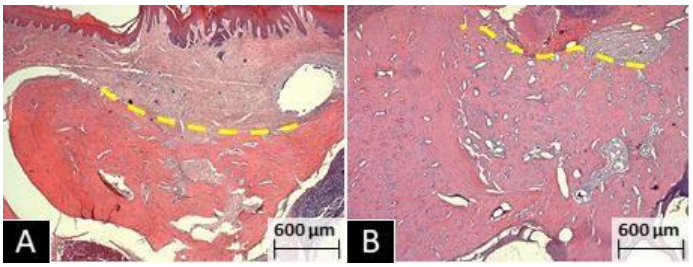

Figure 8. Histological images of the final repair stage. Limit of bone deposition in the cervical 1/3. In (A), the control group (NC). Note the concave surface, dashed line in yellow. In (B), data obtained using the DBB group.

3.5 Maturation level of neoformed trabeculae

The collagen fiber maturation was one of the criteria used as an indicative of bone maturation. After 49 days, the percentage of mature collagen in the neoformed trabeculae was higher in the DBB group, followed by the control, MBB and BO groups (Figure 9). The mineralized groups (BO and MBB) showed less than 30% of bone maturation in the newly formed bone, with no statistical difference between them.

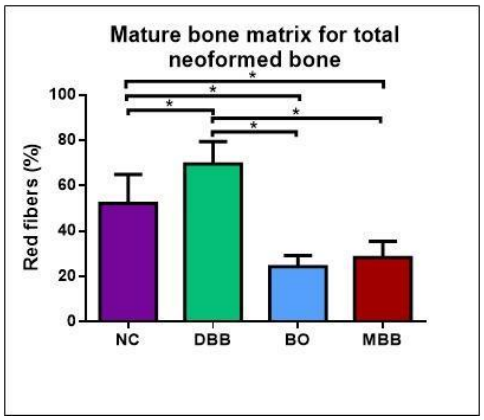

Figure 9. Quantification of the red collagen fibers. Higher bone maturation was observed in the neoformed trabeculae of the DBB group, followed by the NC group. The bone trabeculae deposited in the mineralized groups presented lower bone maturation index without statistical differences between them. * $p<0.05$, Anova with correction factor in Tukey Test.

3.6 Biomaterial reabsorption

The DBB graft showed a rapid rate of reabsorption, compared to the mineralized biomaterials (Figure 10A). After 14 days, few residues of the DBB organic matrix could be histologically detected (Figures 10B and C); and after 21 days, the material had already been completely resorbed (Figure 10D). The mineralized biomaterials, on the other hand, could still be observed in the defect site after 49 days, integrated to the neoformed bone matrix (Figures 6B and C). The BO group showed a greater resistance to resorption than MBB (Figure 10A).

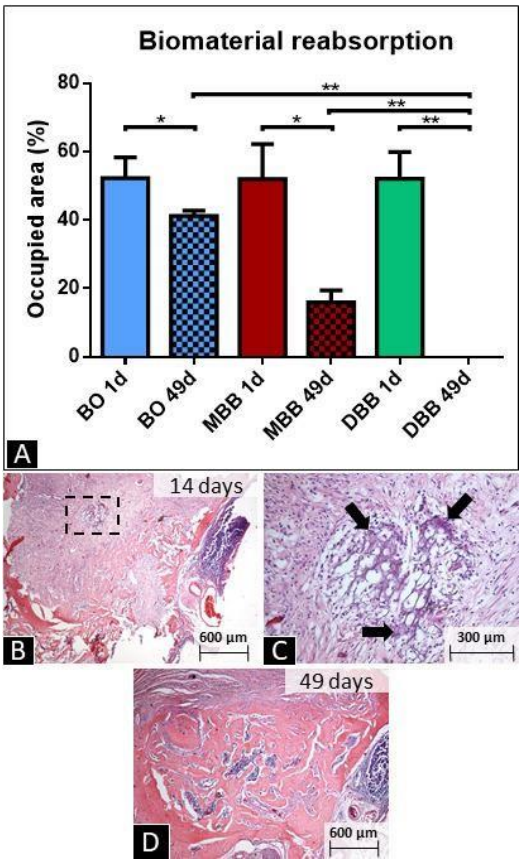

Figure 10. Analysis of the biomaterial reabsorption. The DBB was almost completely reabsorbed at 49 days of grafting. In mineralized group (BO and MBB) the area occupied by the granules was significantly reduced. However, the area occupied by BO at 49 days was statistically larger than the area occupied by MBB, which indicates its relative resistance to resorption. In (B), few biomaterial residues could be observed in the defect area at 14 days. In (C), the magnified image of the black grid region observed in (B), with the evidence of organic matrix xenograft residues (black arrows). In (D), the area of the defect completely free of DBB

biomaterial, filled with connective tissue and trabeculae in formation. *P<0.05 and ** P<0.01 t-test.

4. Discussion

Bovine bone matrices available in different forms (mineralized, deproteinated, partially demineralized or completely demineralized) have been widely evaluated as biomaterials for bone grafts (Watanabe *et al*., 2016; Saima *et al*., 2016; Shirmohammadi *et al*., 2014; Lei *et al*., 2015; Carmagnola *et al*., 2003; Hulsmann *et al*., 2003; Tomasi *et al.*, 2018). Bone grafts should enable the proliferation of mesenchymal and endothelial cells, as well as induce the differentiation of osteoprogenitor stem cells.

Studies show that the efficiency of xenografts in bone repair can be improved by exposing organic components of their bone matrix (Drosos *et al*., 2015; Hinsenkamp *et al.*, 2015; Katz *et al.*, 2009; Labutin *et al.*, 2018). According to Huber *et al.* (2017), the demineralization of bone grafts allows the collagen matrix and other proteins to be exposed, such as BMP and growth factors, favoring the osteoinductive potential.

The osteoinductive capacity of demineralized bone matrix depends on the preparation technique. The demineralizing active used must maintain the osteoinductive agents (Roberts *et al.*, 2012; Kumar *et al.*, 2013; Zhao *et al.*, 2021). Hydrochloric acid has been used in some works in graft demineralization (Hammoudeh *et al.*, 2017; Huber *et al.*, 2017). However, a chelating solution (10% EDTA), in place of acidic pH solutions, facilitates the application of the technique with better time control and material preservation (Gomes *et al.*, 2019; Bertassoli *et al.*, 2020). Here, demineralized xenografts obtained after treatment by 10% EDTA solution, demonstrated the preservation of the collagenous trabeculae bypassing regular medullary spaces, as well as the presence of BMP4, osteopontin and collagen type I.

The hypothesis that these organic components exposed by the demineralization process can accelerate bone repair was confirmed by both the qualitative and quantitative results obtained in this work. Bone defects filled with the DBB revealed a higher percentage of bone deposition and neoformed trabeculae, with a higher level of bone maturation. Similar results had been observed in Mahyudin *et al*. (2017) work. In this work, the authors compared the effectiveness of freeze-dried xenograft, freeze-dried allograft, hydroxyapatite xenograft, and demineralized bone matrix xenograft as bone graft to fill bone defects in femoral diaphysis of white rabbits. The results found showed a high VEGF formation, inducing acceleration of angiogenesis, in the

first week of grafting with allograft freeze-dried cortical New Zealand white rabbits and xenograft demineralized bone matrix bovine. Regarding the bone tissue formation, all groups showed an increase from the second week onwards, except in demineralized bone matrix bovine. In this group the highest woven bone was found in week 1. In conclusion, the bone healing process in demineralized bone matrix bovine group occurred faster when compared to other groups, with greater osteoinduction

When considering the performance of bone grafts their influence on wound closure/soft tissue healing should also be taken into consideration. Open wounds for a prolonged period of time allow the access of physical or biological agents and may compromise the restoration of specialized epithelial and connective tissue function (Mittal *et al.*, 2016; Al-Fotawei *et al.*, 2014). Here, we demonstrated the complete epithelialization of the mucosa after 14 days of grafting, regardless of the type of grafted biomaterial, revealing that bovine xenografts do not compromise gingival healing. Additionally, the underlying epithelial and connective tissue can invade the defect area, leading to a loss of space for bone replacement. In fact, histological sections of ungrafted animals revealed a superficial concavity at the end of the bone repair process, suggesting a small loss of bone volume in the cervical region of the defect. Pippi (2017) claims that in spontaneous healing after tooth extraction flapless, the socket is filled by a blood clot. The next step, the clot is replaced by an increasing granulation tissue density in the first week. The top surface of the post-extraction socket remains concave due to soft tissue invasion, with general reduction in bone volume. After 1 year, the residual alveolar bone is triangular-shaped due to higher bone resorption. Socket grafting procedures with xenograft and allograft seem to reduce alveolar bone loss after tooth extraction (Pippi, 2017). In line with Pippi's (2017) data, our results reiterate the importance of filling the cavities with biomaterials that can guide the regeneration while inhibiting early epithelial invasion.

Digital radiographs are commonly used to assist the definition of the best strategy for the restoration of the bone functions. The level of bone neoformation can be obtained from Digital radiograph images, as an estimative of the radiopacity gain observed in the end of the treatment, related to the starting day (Yaghobee *et al*., 2018; Pichotano *et al*., 2019; Al-Fotawei *et al*., 2014; Dimitriou *et al.*, 2012; Wang *et al.*, 2017). The radiographic evaluation technique used by us was validated in a previous study where Gomes *et al.* (2019) used radiographic images to assess bone repair, of intraoral bone defect in rats, by both fractal analysis (FA) and radiopacity analysis. The authors used the same type of bone defect presented in our work, grafted with mineralized bovine bone and demineralize bovine bone. The aim of this study was

to comparatively evaluate the methods of radiographic analysis in order to validate the best way to estimate bone deposition through digital radiographs. For fractal analysis (FA), a square region of interest of 30 × 30 pixels was used. This analysis mathematically describes the structural pattern of the trabecular bone based on radiographic images and has the advantage of not interfering with radiodensity and geometric projection factors in the evaluation results. For radiopacity analysis, was measured as the mean gray scale (MGS) value for three points of 5 × 5 pixels in the apical, medial and cervical regions of the defect. The radiopacity analysis method was considered adequate since the MSG measurements corresponded to the data from the histomorphometric analyses. Such results add credibility to the MGS radiographic analysis method, which was used in the present study.

Our work revealed that the DBB graft showed the best result in terms of radiopacity. Radiopacity only reflected bone deposition in DBB group, since the biomaterial has an organic nature, with no significant impact on the radiopacity curve, unlike mineralized grafts. Additionally, histological analysis demonstrated that the organic graft was quickly reabsorbed, eliminating the possibility of its interference in the records of shades of gray during the analysis performed after 49 days. Both histological and radiographic analyzes revealed a progressive gain of neoformed bone in the DBB groups from 7 days, with bone deposition peaks superior to the control group (NC). After 21 days, the trabeculae were thicker, present in all thirds of the defect, suggesting a more advanced bone repair. After 49 days, however, the induction of bone deposition of the DBB was similar to the control in histological analyzes and superior in radiographic evaluation. For this reason, the quantitative histological data might be underestimated, as the “area of interest” used in this work was previously defined as below this boundary area with the oral mucosa, being restricted to the center of the bone cavity.

Concerning mineralized grafts (BO and MBB), a lower radiopacity gain after 49 days of grafting, compared to the control, was observed. The histological analysis performed in parallel revealed, however, that this result reflects a smaller free area for bone neoformation, compared to the control. BO was found to be resistant to reabsorption, meaning that the area of the defect remains largely occupied by the biomaterial granules. Similarly, MBB granules were also seen after 49 days of grafting. The literature is consistent with these results by stating that Bio-Oss® becomes integral to the structure of the newly formed bone (Galindo-Moreno *et al.*, 2010; Haas Junior *et al.*, 2016).

The osseointegration of inorganic biomaterials, which means the structural connection between the remaining bone, the newly formed bone and the grafts, is of great relevance for the therapy

success (Watanabe *et al*., 2016). Here, both MBB and BO showed osseointegrated granules in the apical and middle thirds, after 49 days of grafting. No osseointegrated granules were observed in the cervical third. Defects filled with BO or MBB have also revealed the absence of the bony coating on the remaining granules in the superficial third of the defect. A study claims that Bio-Oss® always requires a longer regeneration time before successful osseointegration (Liu *et al.*, 2016), suggesting that 49 days were insufficient for the complete repair of the defect when filled with BO or MBB in our work.

In a rehabilitated patient, not only the amount of bone formed is relevant, but the quality of the regenerated bone is a importante factor for bone-implant integration (Bracey *et al*., 2018). Our work revealed that DBB group presented bone trabeculae with a higher level of bone maturation after 49 days of grafting, compared to the other groups. The presence of proteins such as BMP4 and osteopontin, available at the surgical site grafted with demineralized matrix (DBB), might have contributed, respectively, to the results of higher levels of bone deposition and greater bone maturation recorded with the histomorphometric analysis. BMP4 exposure favors cell differentiation and osteogenesis (Sawkins *et al.*, 2013; Glowacki, 2015; Sierra-Garcia *et al.*, 2016; Park *et al.*, 2016; Durham *et al.*, 2018), which might explain the accelerated bone deposition process observed after 21 days in the DBB groups. The presence of osteopontin assists in mineral deposition during bone repair and might influence the maturation stage of the tissue (Nam *et al.*, 2016; Lund *et al.*, 2009), which could respond for the better performance of the DBB group in terms of bone maturation. Molecules isolated from organic matrices, such as BMPs, have already been used in the clinics (Zhang *et al.*, 2018; Liu *et al.*, 2013); but costs are still a limiting factor for their wide application. The natural bone matrix is a site of growth factors and osteogenic signaling molecules, such as transforming growth factor beta (TGF-beta), platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and BMPs, which act together to improve cellular performance in the bone repair phases (Kumar et al., 2013).

In conclusion, in this experimental model in rats, the demineralized version of the graft, obtained by a simple EDTA demineralization protocol, induced faster bone deposition that culminated in a more mature bone tissue. MBB and BO biomaterials in their commercial mineralized versions presented similar performances regarding their influence on soft tissues, bone deposition level, osseointegration and quality of the neoformed bone matrix. These results open new perspectives for the development of alternative (demineralized) bovine grafts, indicated for the acceleration of the osteogenesis process in clinical conditions that do not demand the immediate mechanical resistance provided by the traditional mineralized grafts.

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