



**UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE RIBEIRÃO PRETO**

ERICK RICARDO SILVA

**Estudo imunohistoquímico, histomorfométrico e microtomográfico
comparativo entre os ossos autógeno e xenógeno em cirurgias de enxerto
onlay em mandíbulas de coelhos.**

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Versão corrigida.

Tese apresentada ao Programa de Pós-Graduação em Odontologia (Periodontia), área de concentração Cirurgia Buco-Maxilo-Facial, da Faculdade de Odontologia de Ribeirão Preto da Universidade de São Paulo, para obtenção do título de Doutor em Ciências.

Orientador: Prof. Dr. Samuel Porfirio Xavier

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Dedico este trabalho a **Deus**, minha fonte de inspiração diária.

Aos meus pais, **João Batista da Silva** e **Silvana Sueli Silva**, meus amores para toda a vida.

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“Os dias prósperos não vêm por acaso; nascem de muita fadiga e persistência.”

Henry Ford

RESUMO

SILVA, E. R. **Estudo imunohistoquímico, histomorfométrico e microtomográfico comparativo entre os ossos autógeno e xenógeno em cirurgias de enxerto *onlay* em mandíbulas de coelhos.** [Tese]. Ribeirão Preto: Universidade de São Paulo, Faculdade de Odontologia de Ribeirão Preto, 2019. 118f.

A busca por substitutos ósseos como alternativa ao osso autógeno tem aumentado nos últimos anos. Os blocos de osso xenógeno são considerados uma opção para reconstrução horizontal de rebordos alveolares atróficos. O objetivo deste estudo foi comparar, por meio de histomorfometria, imunohistoquímica e microtomografia, os processos de incorporação e remodelação de enxertos de osso autógeno e xenógeno em blocos, utilizados para aumento lateral de mandíbula em coelhos. Dezoito coelhos machos da raça *New Zealand*, com 3,5 – 4,0 kg de peso e 4 – 5 meses de idade, foram incluídos neste estudo randomizado, prospectivo, do tipo boca dividida. A face vestibular do ângulo mandibular dos foi enxertado bilateralmente com blocos de osso autógeno ou xenógeno (Heket Biomaterials, Trento, Itália). Os animais foram eutanasiados após 7, 20 e 60 dias de reparo, respectivamente. Foram realizadas biópsias para cada período e as amostras foram submetidas a escaneamento microtomográfico. O volume total do enxerto (VT, mm³) e o volume ósseo (VO: enxerto residual + osso neoformado, mm³) foram aferidos para ambos os grupos utilizando o *software* CTAn (Bruker, Kontich, Bélgica). Cortes histológicos em parafina foram preparados e corados em hematoxilina e eosina e tricrômico de Masson. Para avaliação histomorfométrica, uma grade de 80 quadrados foi sobreposta à imagem das lâminas histológicas para contagem das estruturas, em aumento de 200x. As porcentagens de enxerto residual e osso foram avaliadas. Os eventos moleculares de incorporação e remodelação do enxerto foram analisados por meio da imunomarcagem das proteínas VEGF, Col I, OPN, ALP, OC e Casp 3. Os testes ANOVA e *t* de Student foram realizados para análise estatística. Os blocos de osso autógeno e xenógeno apresentaram porcentagens semelhantes de osso neoformado ao longo do tempo. No entanto, o volume e a qualidade do osso neoformado foram superiores no grupo autógeno quando comparado ao xenógeno.

Palavras-chave: imunohistoquímica, histomorfometria, microtomografia, osso autógeno, osso xenógeno, coelhos.

ABSTRACT

SILVA, E. R. **Immunohistochemical, histomorphometric and microtomographic comparative study between autogenous bone and xenogenic bone in onlay graft surgery in rabbit mandibles.** [PhD Thesis]. Ribeirão Preto: University of São Paulo, Faculty of Dentistry of Ribeirão Preto, 2019. 118p.

The search for bone substitutes as an alternative to autogenous bone has increased in recent years. Xenogenous bone blocks are considered an option for horizontal reconstruction of atrophic alveolar ridges. The aim of this study was to compare, by means of histomorphometry, immunohistochemistry and microtomography, graft incorporation and remodeling processes of autogenous and equine xenogenous (Heket Biomaterials, Trento, Italy) bone blocks used for mandibular lateral augmentation in rabbits. Eighteen New Zealand male rabbits, with 3.5 – 4.0 kg of weight and 4 – 5 months of age, were included in this split-mouth randomized study. The lateral side of the angle of the mandible was augmented bilaterally with either block autografts or xenografts. Animals were euthanized after 7, 20 and 60 days of healing, respectively. Biopsies were taken for each period and specimens underwent microtomographic scanning. Total graft volume (TV, mm³) and bone volume (BV: residual graft plus new bone, mm³) were measured for both autogenous and xenogenous groups using CTAn software (Bruker, Kontich, Belgium). Paraffin sections were prepared and stained in hematoxylin and eosin and Masson's trichrome. For histomorphometric evaluation, a grid consisting of 80 squares was superposed to the image of histological slides for counting the structures at a magnification of ×200. The percentages of residual graft and new bone were assessed. The graft incorporation and remodeling molecular events were evaluated by means of VEGF, Col I, OPN, ALP, OC and Casp 3 immunolabelling. ANOVA and the Student's *t* test were performed. Both autogenous and xenogenous bone blocks presented similar percentages of newly formed bone over time. However, the volume and the quality of the grafted area was superior at the autogenous compared to the equine xenogenous graft group.

Keywords: immunohistochemistry, histomorphometry, microtomography, autogenous bone, xenogenous bone, rabbits.

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LISTA DE ABREVIATURAS E SIGLAS

ALP	Fosfatase Alcalina
Casp3	Caspase 3
CEUA	Comissão de Ética no Uso de Animais
COL I	Colágeno Tipo I
FORP	Faculdade de Odontologia de Ribeirão Preto
i.m.	Intramuscular
OMBD	Osso Mineral Bovino Desproteinizado
PVPI	Polivinil Pirrolidona Iodo
OC	Osteocalcina
OPN	Osteopontina
ROI	Região de Interesse
s.c.	Subcutânea
TRAP	<i>Tartrate Resistant Acid Phosphatase</i>
USP	Universidade de São Paulo
VT	Volume Total do Enxerto
VEGF	<i>Vascular Endotelial Growth Factor</i>
VO	Volume Ósseo
VOI	Volume de Interesse

LISTA DE SÍMBOLOS

% Por cento

® Registrada

mm³ Milímetros cúbicos

Nº Número

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1 INTRODUÇÃO

1 INTRODUÇÃO

O prognóstico dos implantes osseointegráveis está diretamente relacionado à presença de um volume ósseo adequado para sua instalação.

Com a evolução da Implantodontia, diversas técnicas cirúrgicas têm sido propostas no intuito de restabelecer o volume ósseo alveolar perdido, tornando viáveis o planejamento e execução de reabilitações implantossuportadas adequadas (BUSER et al., 1993; 1996; ODA et al., 2000; CORDARO et al., 2002; DONOS et al., 2002; CHIAPASCO et al., 2006).

A reconstrução de rebordo alveolares atróficos continua sendo um desafio para cirurgiões e implantodontistas dos dias atuais (SCHWARZ et al., 2010). Embora esses procedimentos sejam previsíveis e bem-sucedidos (HÄRMMELE & KARRING, 1998), eles são considerados de difícil execução técnica, podendo estar relacionados à algumas complicações pós cirúrgicas (CHIAPASCO et al., 2006; DONOS et al., 2008). Apesar da dificuldade técnica, implantes instalados nos sítios regenerados apresentaram excelente sobrevida em longo prazo (BUSER et al., 1996; NEVINS et al., 1998).

O osso autógeno, coletado a partir de sítios doadores extra ou intrabucais, permanece como “padrão ouro” para as reconstruções ósseas alveolares devido às suas propriedades osteoindutoras, osteocondutoras e osteogênicas (DONOVAN et al., 1994; VON ARX et al., 2001a; 2001b; CHRIAC et al., 2005; NOWZARI & AALAM, 2007).

O reparo sequencial de blocos ósseos autógenos utilizados para aumento lateral de mandíbula foi descrito detalhadamente em estudos experimentais publicados anteriormente (FARIA et al., 2008; PEDROSA et al., 2009) e em estudos clínicos (ZERBO et al., 2003; ACOCELLA et al., 2010).

No entanto, a enxertia óssea autógena tem sido associada a diversas desvantagens, tais como: morbidade cirúrgica no sítio doador, infecção, parestesia, dor pós-operatória, perda de sangue exarcebada, disponibilidade óssea limitada, tempo cirúrgico aumentado e custo financeiro com hospitalização (CHIAPASCO et al., 2006; NKENKE et al., 2001; 2004; VON ARX et al., 2005; MCALLISTER & HAGHIGHAT, 2007). Embora tenha sido comprovada a eficácia dos blocos de osso autógeno em cirurgias para aumento ósseo horizontal (BUSER et al., 1996), estudos experimentais e clínicos mostraram redução volumétrica substancial ao longo do

tempo, especialmente quando foram considerados os primeiros cinco anos após a instalação do implante nas áreas enxertadas (CHIAPASCO et al., 2006). Por essas razões, as pesquisas têm concentrado o seu foco no desenvolvimento de substitutos ósseos que possam ser utilizados como alternativa ao osso autógeno nos procedimentos reconstrutivos.

Os blocos de osso xenógeno são uma opção no sentido de transpor as limitações apresentadas pelo osso autógeno. Esse biomaterial propicia a eliminação da morbidade cirúrgica decorrente de um sítido doador, funcionando como um excelente arcabouço osteocondutor (ARAÚJO et al., 2002; JENSEN et al., 2006; ROTHAMEL et al., 2009)

O osso mineral bovino desproteinizado (OMBD) é o substituto ósseo de natureza xenógena mais estudado e empregado nos procedimentos de reconstrução óssea contemporâneos (SCHWARZ et al., 2010; HÄMMERLE et al., 1998; ZITZMAN et al., 1997; 2001; HOCKERS et al., 1999).

Em estudo clínico (HÄMMERLE et al., 2008), foram relatados 12 casos em que os blocos ósseos de OMBD cobertos por uma membrana de colágeno foram utilizados para aumento lateral de rebordos alveolares. Os autores observaram que o procedimento constitui uma opção eficaz para o aumento ósseo horizontal, uma vez que possibilita a formação de osso novo em quantidade e qualidades adequadas para a instalação de implantes após um período de 9 a 10 meses de reparo.

DE SANTIS et al. (2012; 2015) investigaram histológica e histomorfometricamente o comportamento do OMBD em comparação com o osso autógeno de ramo mandibular em blocos, ambos cobertos por uma membrana de colágeno reabsorvível e enxertados para aumento lateral em mandíbulas de cães.

Em modelo experimental também envolvendo cães, SCHWARZ et al. (2010) avaliaram histologicamente o reparo de dois blocos de osso xenógeno (bovino vs eqüino) cobertos por uma membrana de colágeno reabsorvível. No entanto, nesse estudo, o comportamento volumétrico e imunohistoquímico dos enxertos, da sua instalação à remodelação, não foi investigado.

O presente estudo, portanto, foi o primeiro a comparar histomorfométrica, imunohistoquímica e microtomograficamente o comportamento biológico de enxertos de osso xenógeno equino com enxertos autógenos de osso ilíaco em blocos, em cirurgias para aumento ósseo lateral em mandíbulas de coelhos.

2 PROPOSIÇÃO

2 PROPOSIÇÃO

O objetivo deste estudo foi comparar histomorfométrica, imunohistoquímica e microtomograficamente os ossos autógeno e xenógeno equino quanto aos processos de incorporação, neoformação e remodelação ósseas em cirurgias de enxerto *onlay* em mandíbulas de coelhos.

3 MATERIAL E MÉTODOS

3 MATERIAL E MÉTODOS

O protocolo experimental foi aprovado pela Comissão de Ética no Uso de Animais da Faculdade de Odontologia de Ribeirão Preto da Universidade de São Paulo, Brasil (CEUA nº: 2016.1.666.58.8).

3.1 Seleção da amostra

Neste estudo, prospectivo, randomizado, do tipo boca dividida, foram incluídos dezoito coelhos adultos machos da raça *New Zealand*, com aproximadamente 3,5 – 4,0 kg de peso e 4 – 5 meses de idade. Os animais foram divididos em três grupos de seis indivíduos cada, de acordo com os períodos experimentais propostos, ou seja, 7, 20 e 60 dias.

A adoção de um modelo experimental do tipo boca dividida permitiu a realização do estudo com um número reduzido de animais. O tamanho da amostra ($n = 6$) foi considerado suficiente para revelar possíveis diferenças entre os grupos com base em dados apresentados em trabalhos anteriores (FARIA et al., 2008; PEDROSA et al., 2009; HAWTHORNE et al., 2013), nos quais amostras idênticas foram utilizadas.

3.2 Randomização

A face vestibular do ângulo mandibular do coelho foi enxertada bilateralmente com blocos de osso autógeno ou xenógeno, com os lados direito e esquerdo distribuídos aleatoriamente. A randomização foi realizada eletronicamente (<http://www.randomization.com>) por um colaborador que não participou dos procedimentos cirúrgicos, sendo revelada ao cirurgião somente após a coleta do bloco de osso autógeno proveniente da crista ilíaca.

3.3 Procedimento cirúrgico

Um cirurgião buco-maxilo-facial treinado realizou todas as cirurgias. A anestesia foi realizada com acepromazina (1,0 mg / kg, s.c., Acepran®, Vetnil, Louveira, São Paulo, Brasil) e com uma solução de xilazina (3,0 mg / Kg; i.m.; Anasedan®, Sespo Indústria e Comércio LTDA, Paulínia, São Paulo, Brasil) e

cetamina (50,0 mg / Kg, i.m., Dopaser®, Sespo Indústria e Comércio LTDA, Paulínia, São Paulo, Brasil). Após a anestesia, os animais receberam uma dose profilática de oxitetraciclina di-hidratada (40 mg / kg, i.m., Terramicina LA®, Zoetis Indústria e Produtos Veterinários, Campinas, São Paulo, Brasil).

Os blocos de osso autógeno foram coletados da crista ilíaca anterior. Após a tricotomia, foi realizada antissepsia do sítio cirúrgico com PVPI a 1% (Riodeíne® Tintura, Rioquímica, São José do Rio Preto, São Paulo, Brasil). O local da incisão foi infiltrado com ½ tubete de mepivacaína a 2% e epinefrina a 1:100.000 (Mepiadre®, Nova DFL, Rio de Janeiro, Rio de Janeiro, Brasil). Uma incisão de ~ 3,0 cm de comprimento foi realizada ao longo da protuberância da crista ilíaca anterior. Pele, músculos e periósteo foram rebatidos para exposição do osso ilíaco. Uma broca trefina de 10,0 mm (Neodent, Curitiba, Paraná, Brasil) foi utilizada para remoção de um bloco ósseo de espessura bicortical. O enxerto ósseo foi mantido em uma gaze embebida em solução salina estéril refrigerada. Ao final da coleta, no leito doador, foram utilizados fios reabsorvíveis (Poliglactina 910 5-0, Vicryl® 4-0, Ethicon, Johnson & Johnson, São José dos Campos, Brasil) para sutura dos músculos e periósteo, e fios de nylon para sutura da pele (Ethilon 4-0®, Ethicon, Johnson & Johnson, São José dos Campos, Brasil).

Previamente à instalação dos blocos de osso autógeno, uma das corticais foi removida com auxílio de uma ponta diamantada para peça reta e o enxerto foi modelado, de modo que pudesse ser adaptado passivamente ao leito receptor. Esses blocos possuíam aproximadamente 10 mm de diâmetro e 3 mm de espessura. Os blocos de osso xenógeno esponjoso (Heket Biomaterials, Trento, Itália), de origem equina, foram preparados com dimensões idênticas à dos blocos de osso autógeno.

Com o intuito de melhorar a perfusão dos enxertos, nove perfurações monocorticais equidistantes de 1,0 mm de diâmetro foram realizadas no leito receptor (Figura 1A), de acordo com um modelo pré-fabricado em aço inoxidável.

Os enxertos foram fixados com um único parafuso de titânio de 1,5 x 10,0 mm (Neodent, Curitiba, Paraná, Brasil, Figura 1B e C) e cobertos com membranas colagenosas de pericárdio bovino (Exaflex matrix®, Exaflex, Andazeno, Itália, Figura 1D). A sutura do leito receptor foi realizada com Vicryl® 4-0 (Ethicon, Johnson & Johnson, São José dos Campos, São Paulo, Brasil) nos planos periosteal e muscular, e com Nylon 4-0 (Ethicon, Johnson & Johnson, São José dos Campos,

São Paulo, Brasil) na pele. No pós-operatório, os animais receberam cetoprofeno (3,0 mg / kg, i.m., Ketofen® 1%, Merial, Monte-Mor, São Paulo, Brasil) e cloridrato de tramadol (Tramadol® 2%, s.c., 1,0 mg / kg, Cronidor, Agener União Saúde Animal, Apucarana, Paraná, Brasil) duas vezes ao dia por dois dias.

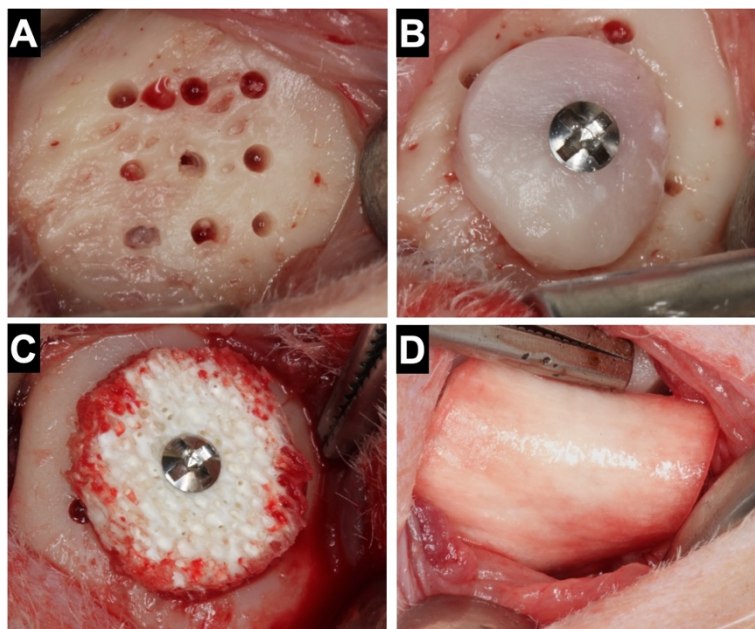


Figura 1. Fotografias transcirúrgicas do procedimento de enxerto *onlay*: A. Nove perfurações monocorticais equidistantes foram realizadas na cortical vestibular do leito receptor, com o intuito de possibilitar a perfusão sanguínea dos enxertos; B. Fixação do bloco de osso autógeno na face lateral do ângulo mandibular; C. Fixação do bloco de osso xenógeno equino (Heket Biomaterials, Trento, Itália) na face lateral do ângulo mandibular contralateral; D. Recobrimento do enxerto com membrana colagenosa de pericárdio bovino (Exaflex matrix®, Exaflex, Andazeno, Itália).

3.4 Manutenção dos animais

Os animais foram mantidos nas instalações do Biotério da Faculdade de Odontologia de Ribeirão Preto da Universidade de São Paulo (FORP – USP), sob apropriados cuidados veterinários.

As salas possuíam gaiolas metálicas individuais (1 animal / 4500 cm²), ar-condicionado *split*, exaustor (27 a 34 trocas de ar / hora) e controle de iluminação automático (ciclo claro-escuro de 12 horas). Todos os animais receberam ração e água filtrada *ad libitum*.

Um rigoroso protocolo de monitorização dos animais foi realizado durante todo o período experimental, por meio de atenção diária às funções biológicas

básicas de alimentação e excreção, aos sinais de comportamento em relação à dor pós-operatória, e observação das feridas cirúrgicas quanto à deiscência de sutura, sangramentos e / ou sinais de infecção.

3.5 Eutanásia

Cada grupo contendo seis coelhos foi eutanasiado aos 7, 20 e 60 dias de pós-operatório, respectivamente. Foi utilizada uma sobredose intravenosa de 2,0 mL de tiopental 1,0 g (Thiopentax®, Cristália, Itapira, São Paulo, Brasil). As áreas enxertadas da mandíbula foram reduzidas a espécimes de aproximadamente 12,0 mm x 12,0 mm. O parafuso de fixação foi utilizado como referência para osteotomias.

3.6 Processamento e análise microtomográfica

Os espécimes removidos foram fixados em uma solução tamponada de formaldeído a 10%. Após a fixação, as peças foram escaneadas em um microtomógrafo de alta resolução (SkyScan 1172®, Bruker, Kontich, Bélgica) (Figura 2).

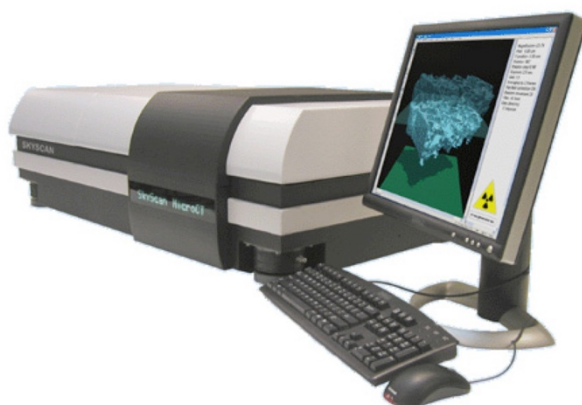


Figura 2. O microtomógrafo SkyScan® 1172 (Bruker, Kontich, Bélgica) do Laboratório Multiusuário para Análises de Imagens Tridimensionais de Tecidos Biológicos e Biomateriais (LAB 3D BIO) da FORP- USP foi utilizado para escaneamento das peças. Fonte: <https://www.bruker.com>

Os seguintes parâmetros de escaneamento foram utilizados: exposição de 1280 ms por movimento, voltagem de 100 kV, corrente de 100 μ A, resolução

isotrópica de 8,70 μm , 360° de rotação em torno do eixo vertical com passo de rotação 0,6°, média de 4 quadros, utilizando um filtro de Al + Cu.

A próxima etapa consistiu na reconstrução das imagens utilizando o *software* NRecon® 1.6.10 (Bruker, Kontich, Bélgica), aplicando-se os parâmetros a seguir: redução de artefatos em forma de anel (*Ring Artifact Correction*) no valor de 12, endurecimento de feixe (*Beam Hardening*) de 32% e suavização (*Smoothing*) no valor de 2.

As imagens reconstruídas foram reposicionadas tridimensionalmente por meio do *software* DataViewer® 1.5.4.6 (Bruker, Kontich, Bélgica), no qual foi determinado o volume de interesse (VOI) dos espécimes (Figura 3).

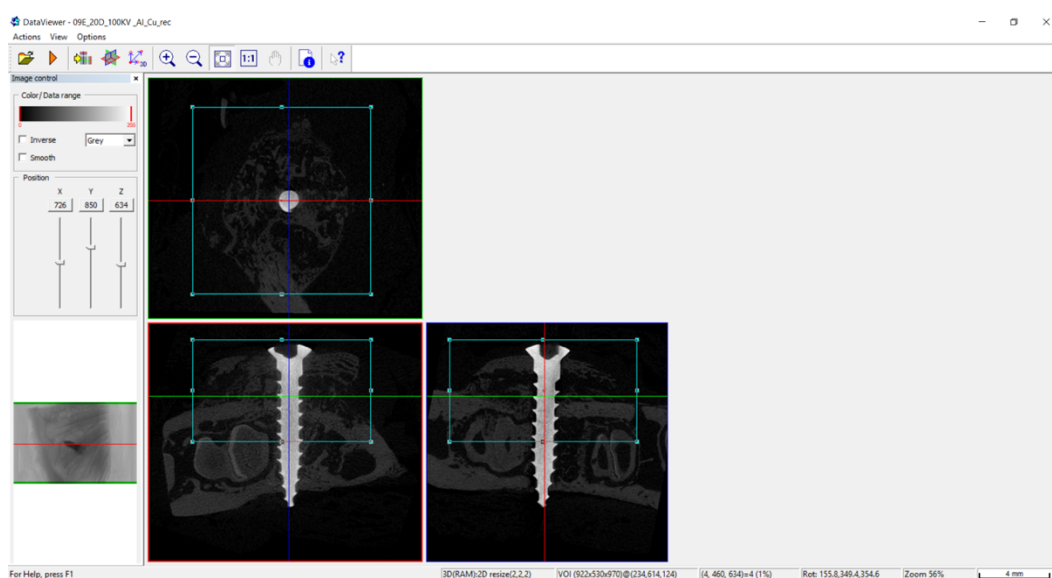


Figura 3. Determinação do volume de interesse (VOI) das amostras no *software* DataViewer® 1.5.4.6 (Bruker, Kontich, Bélgica).

Após a obtenção do VOI, o *software* CTAn® 1.17.7.2 + (Bruker, Kontich, Bélgica) foi utilizado para binarização das imagens e determinação da escala de cinza (*thresholding*). Para identificação de estruturas mineralizadas, os limites dessa escala foram fixados nos valores de 30 a 180 (Figura 4).

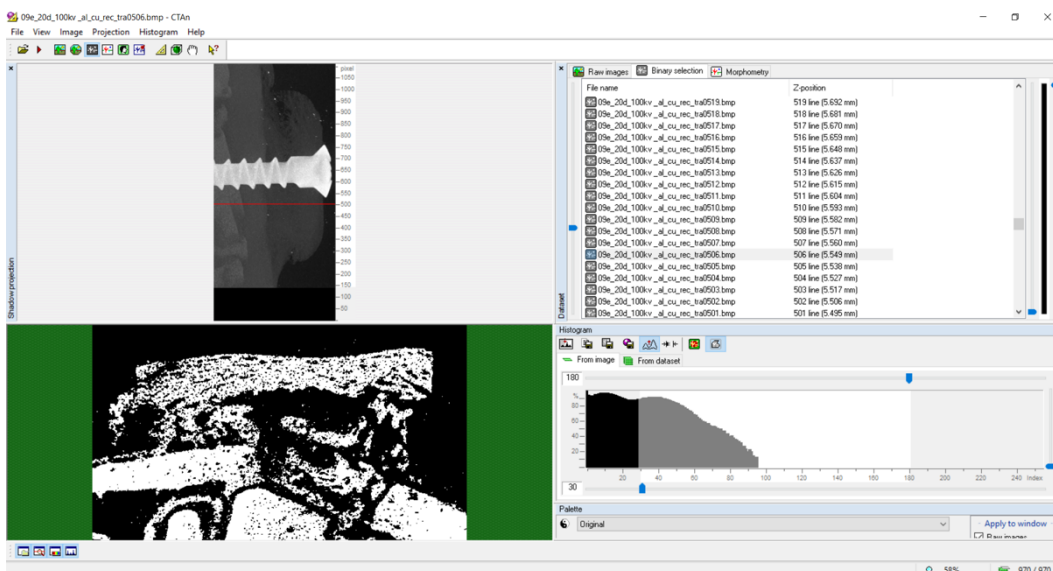


Figura 4. O software CTAn® 1.17.7.2 + (Bruker, Kontich, Bélgica) foi utilizado para binarização das imagens e determinação da escala de cinza (*thresholding*), para posterior quantificação do volume total do enxerto e volume ósseo.

O volume total do enxerto (VT) e volume ósseo (VO: novo osso mais enxerto residual) foram obtidos pela análise morfométrica tridimensional da interpolação total das regiões de interesse (ROI) de cada amostra (Figura 5). Um único examinador calibrado realizou todas as análises.

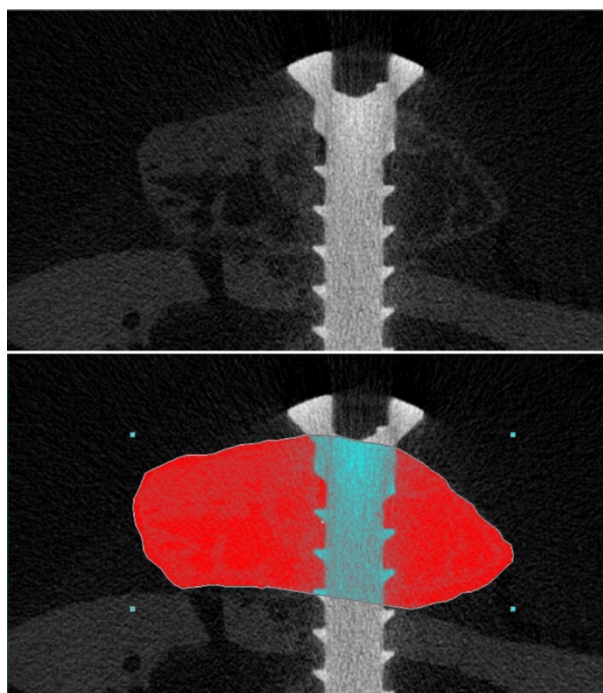


Figura 5. Determinação da região de interesse (ROI) (CTAn®, Bruker, Kontich, Bélgica). O ROI dos sítios enxertados está representado em vermelho. As imagens foram interpoladas para a obtenção do volume total do enxerto e do voume ósseo. O parafuso de fixação dos enxertos foi eliminado durante as mensurações.

3.7 Processamento histológico

Após o escaneamento microtomográfico, os espécimes foram lavados em água corrente para remoção do fixador. Em seguida, as peças foram descalcificadas em solução de EDTA a 7% (Merck, Darmstadt, Alemanha) até adquirirem consistência borrachóide, verificada por meio de um teste semanal realizado com o auxílio de um alfinete.

Na sequência, os espécimes foram desidratados por meio da imersão em uma série de álcoois com concentrações crescentes (30%, 50%, 70%, 80%, 90% e em álcool absoluto por duas vezes consecutivas). Cada imersão teve duração de uma hora. As peças foram, então, diafanizadas por meio de duas imersões sequenciais em xilol, de 40 minutos cada, para permitir melhor penetração da parafina.

Posteriormente, foi realizada a inclusão dos espécimes por meio de três banhos de uma hora de duração cada em parafina líquida a 60° C. As etapas compreendidas entre a desidratação e a inclusão final das peças foram realizadas no Processador de Amostras Leica TP1020® (Leica, Wetzlar, Alemanha) (Figura 6). O emblocamento das peças foi realizado no Emblocador de Amostras Leica EG1150H® (Leica, Wetzlar, Alemanha) (Figura 7A). As peças foram resfriadas no Resfriador Leica EG1150C® (Leica, Wetzlar, Alemanha) (Figura 7B).



Figura 6. O Processador de Amostras Leica TP1020® (Leica, Wetzlar, Alemanha) do Laboratório de Histotecnologia da FORP-USP foi utilizado para processamento histológico das peças. Fonte: <https://www.leicabiosystems.com>

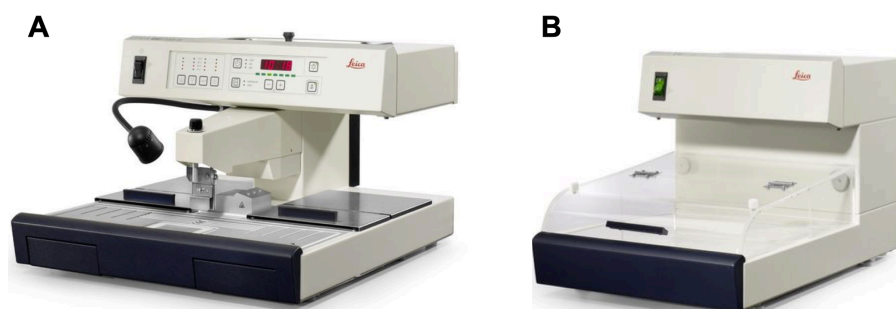


Figura 7. A: Emblocador de Amostras Leica EG1150H® (Leica, Wetzlar, Alemanha); B: Resfriador Leica EG1150C® (Leica, Wetzlar, Alemanha), ambos do Laboratório de Histotecnologia da FORP-USP. Fonte: <https://www.leicabiosystems.com>

Após 24 horas de resfriamento, os blocos foram cortados em micrótomo rotativo Leica RM2255® (Leica, Wetzlar, Alemanha) (Figura 8), de modo que fossem obtidas lâminas de 6 µm de espessura.



Figura 8. Micrótomo rotativo Leica RM2255® (Leica, Wetzlar, Alemanha) do Laboratório de Histotecnologia da FORP-USP utilizado para confecção dos cortes histológicos.

Em seguida, os cortes foram divididos em dois grupos e montados em lâminas convencionais (Knittel, Waldemar Knittel, Braunschweig, Alemanha) para histologia e em lâminas silanizadas (StarFrost®, Waldemar Knittel, Braunschweig, Alemanha) para imunohistoquímica. Na sequência, as lâminas foram colocadas em estufa Odontobrás® 1.1 (Biodont, Brodowski, Brasil) a 50° C, para remoção do excesso de parafina e auxílio da fixação do corte histológico à lâmina (Figura 9).



Figura 9. Após a montagem, as lâminas foram mantidas a 50°C em estufa (Biodont, Brodowski, Brasil), para fixação do corte histológico e remoção do excesso de parafina.

3.7.1 Coloração histológica

A próxima etapa consistiu na coloração das lâminas. O primeiro passo foi desparafinizá-las por meio de dois banhos de 10 minutos cada em xilol puro. Em seguida, as mesmas foram hidratadas em uma série de álcoois com concentrações decrescentes (álcool absoluto por duas vezes consecutivas, 90%, 80%, 70% e 50%). Cada imersão teve duração de 5 minutos. As lâminas foram, então, lavadas em água corrente. Neste momento, foram realizados banhos contendo os reagentes para cada uma das colorações (hematoxilina-eosina ou tricrômico de Masson). Realizou-se nova lavagem em água corrente, desidratação com álcool 70% e absoluto por 3 minutos cada e, por fim, a fixação do corante e conservação do material com xilol.

Ao final do processamento, foi realizada a montagem das lamínulas sobre as lâminas utilizando resina líquida (Entellan®, Merck, Darmstadt, Alemanha).

3.7.2 Marcação imunohistoquímica

A marcação imunohistoquímica foi realizada pelo método da imunoperoxidase. Os seguintes anticorpos primários foram empregados: anti-Col I (COL1), anti-OPN (AKM2A1), anti-VEGF (SC1881), anti-ALP (B-10), anti-OC, TRAP,

anti-Casp 3 (31A1067) produzidos em caprinos (Santa Cruz Biotechnology, Santa Cruz, EUA). Como anticorpo secundário, foi utilizado o anticorpo biotilado anti-IgG em uma concentração de 1:200 (Jackson ImmunoResearch Laboratories, West Grove, EUA). A reação foi revelada utilizando a diaminobenzidina (Dako Laboratories, Santa Clara, EUA). Ao final das reações, procedeu-se com a contra-coloração de Harris-Hematoxilina. A determinação dos níveis de marcação para cada anticorpo foi realizada de forma semi-quantitativa, utilizando escores de 0 a 3 (0 = ausência de marcação; 1 = marcação leve, em que até 25% da área analisada apresentou marcação positiva; 2 = marcação moderada, em que 50% da área analisada apresentou marcação positiva; 3 = marcação intensa, em que 75% da área analisada apresentou marcação positiva, modificado de PEDROSA et al., 2009).

As análises histológica e imunohistoquímica foram realizadas em um microscópio de luz Leica DMLB® (Leica, Wetzlar, Alemanha) conectado a um computador por meio de uma câmera de vídeo digital Leica DC300F®, (Leica, Wetzlar, Alemanha). As imagens analisadas foram obtidas em lente objetiva de 20x para histologia e em 10x para imunohistoquímica.

3.7.3 Calibração do examinador

As análises foram realizadas por um único examinador treinado, sem conhecimento prévio das amostras, após calibração com um profissional experiente. Um teste *Kappa* intra-examinador para reconhecimento de estruturas histológicas e das imunomarcações foi aplicado, tendo alcançado um índice superior a 0,90.

3.7.4 Análises histológica e histomorfométrica

O *software* ImageJ® 1.50i (National Institutes of Health, EUA) foi empregado para as mensurações. Uma grade de 80 quadrados foi sobreposta às imagens das lâminas histológicas (Figura 10). Os pontos de intersecção entre os quadrados foram utilizados como referência para contabilizar as estruturas encontradas.

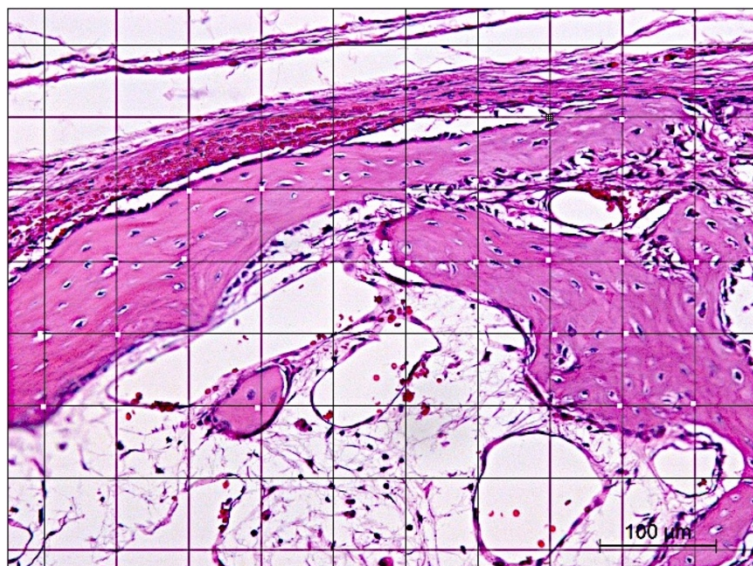


Figura 10. Uma grade contendo 80 quadrados foi sobreposta às fotomicrografias das lâminas histológicas com o auxílio do *software* ImageJ® 1.50i (National Institutes of Health, EUA), para determinar a composição tecidual de cada corte histológico. Os pontos de intersecção entre os quadrados foram utilizados para contabilizar as estruturas histológicas encontradas.

Duas áreas distintas foram determinadas e utilizadas para as análises. A área próxima à membrana de colágeno foi chamada de “região de membrana” e a área próxima ao leito receptor chamada de “região de base” (Figura 11).

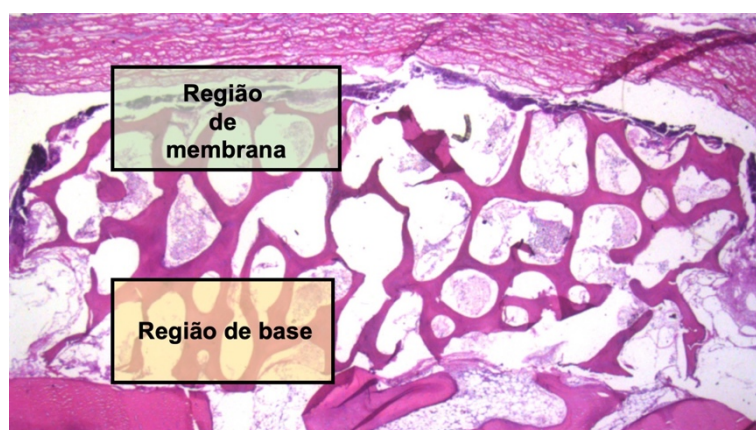


Figura 11. Duas áreas distintas do corte histológico foram utilizadas para análises histológica e histomorfométrica, como mostram os retângulos coloridos. Em amarelo: região de base (próxima ao leito receptor); em verde: região de membrana (próxima à membrana de colágeno).

As medidas histomorfométricas foram tomadas separadamente para cada área. Valores de média e desvio padrão foram calculados para avaliar a

neoformação óssea (variável primária) e a reabsorção do enxerto. As estruturas avaliadas incluíram porcentagens de osso novo mineralizado e de enxerto autógeno ou xenógeno remanescentes.

3.8 Análise estatística

Os dados coletados foram submetidos à análise estatística utilizando o *software* IBM SPSS Statistics (IBM Inc., Chicago, EUA). Os dados foram expressos como média \pm desvio padrão. Para verificar a distribuição normal, foi realizado o teste de Shapiro-Wilk. Para comparar a neoformação óssea entre os grupos de autógeno e xenógeno e entre os três períodos de avaliação, foi utilizado um teste ANOVA *two-way*. Para comparação entre grupos do mesmo período de avaliação, foi realizado o teste *t* de Student. O nível de significância foi de 5% ($p < 0,05$). Na ocorrência de diferença estatística, o pós-teste de Tukey foi aplicado.

4 RESULTADOS

4 RESULTADOS

Um animal do grupo de 20 dias veio a óbito durante a anestesia e necessitou ser substituído. Nenhum dos animais apresentou complicações pós-operatórias, como deiscência de sutura e / ou infecção.

4.1 Resultados microtomográficos

A avaliação tridimensional dos enxertos revelou uma contração volumétrica ao longo do tempo para ambos os grupos. O volume total (VT) do grupo autógeno diminuiu entre 7 e 60 dias, de $258,1 \pm 15,8 \text{ mm}^3$ para $107,2 \pm 17,5 \text{ mm}^3$ ($p < 0,05$), enquanto o volume do grupo xenógeno diminuiu de $166,9 \pm 26,0 \text{ mm}^3$ para $96,0 \pm 19,1 \text{ mm}^3$ ($p < 0,05$), como mostrado na Tabela 1 e na Figura 12. Taxas de contração de 58,5% e 42,5% foram observadas para os grupos autógeno e xenógeno, respectivamente.

O volume ósseo (VO: osso novo + enxerto residual) para o grupo autógeno foi de $52,8 \pm 7,7 \text{ mm}^3$, $27,2 \pm 11,1 \text{ mm}^3$ e $33,1 \pm 2,8 \text{ mm}^3$ após 7, 20 e 60 dias de reparo, respectivamente. No grupo xenógeno, os volumes ósseos foram de $4,2 \pm 0,4 \text{ mm}^3$, $18,8 \pm 9,5 \text{ mm}^3$ e $7,6 \pm 3,7 \text{ mm}^3$, respectivamente (Tabela 1). A análise entre grupos revelou maior volume ósseo para o osso autógeno após 60 dias de reparo ($p < 0,05$), o que também pode ser observado qualitativamente na Figura 13.

Tabela 1. Volume total do enxerto (VT, mm^3) e volume ósseo (VO: volume ósseo osso novo + enxerto residual, mm^3) após 7, 20 e 60 dias de reparo.

Tempo	Grupo	VT (mm^3)	VO (mm^3)
7 dias	Autógeno	$258,3 \pm 15,8^*$	$52,8 \pm 7,7^*$
	Xenógeno	$166,9 \pm 26,0^*$	$4,2 \pm 0,4$
20 dias	Autógeno	$159,5 \pm 77,1$	$27,2 \pm 11,1$
	Xenógeno	$166,4 \pm 50,3$	$18,8 \pm 9,5$
60 dias	Autógeno	$107,2 \pm 17,5^*$	$33,1 \pm 2,8^{***}$
	Xenógeno	$96,0 \pm 19,1^*$	$7,6 \pm 3,7^{***}$

Valores expressos como média \pm desvio padrão, $*p < 0,05$: análise intragrupo; $***p < 0,05$: análise entre grupos.

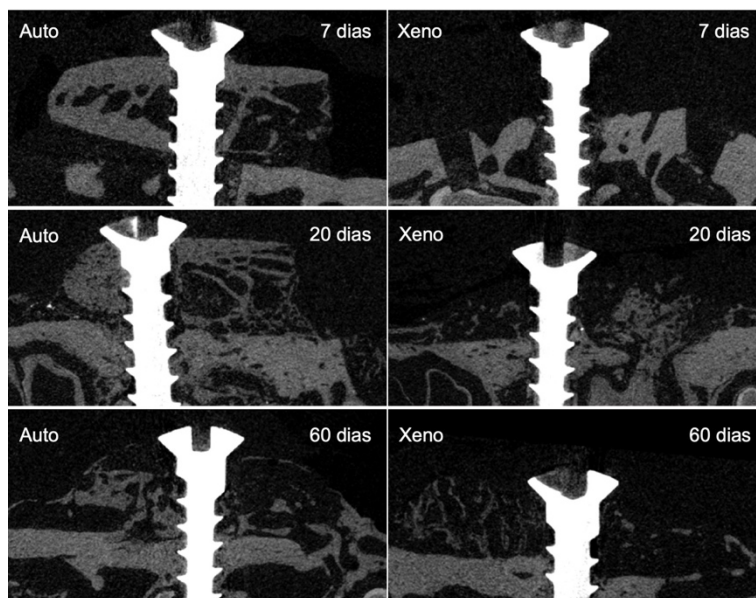


Figura 12. Cortes microtomográficos transversais dos grupos autógeno (controle) e xenógeno (teste) ilustrando as alterações volumétricas dos enxertos após 7, 20 e 60 dias de reparo.

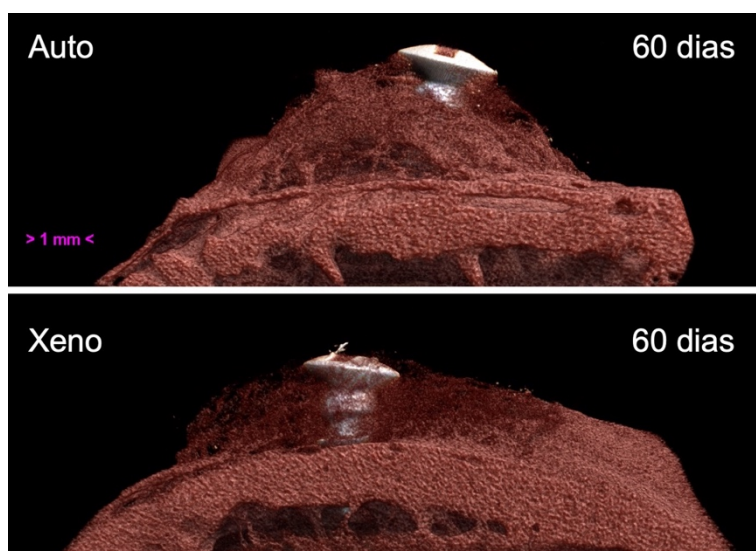


Figura 13. Reconstrução tridimensional de um bloco de enxerto autógeno e xenógeno após 60 dias de reparo, realizada por meio do software CTVox® (Bruker, Kontich, Bélgica).

4.2. Resultados histológicos e histomorfométricos

Após 7 dias de reparo, não foi possível observar osso novo em nenhum dos grupos avaliados (Gráfico 1, Tabela 2). Na “região de base”, em ambos os grupos, foi encontrada uma matriz imatura contendo vasos e células osteoprecursoras entre o enxerto e a cortical externa dos locais receptores (Figuras 14A e B).

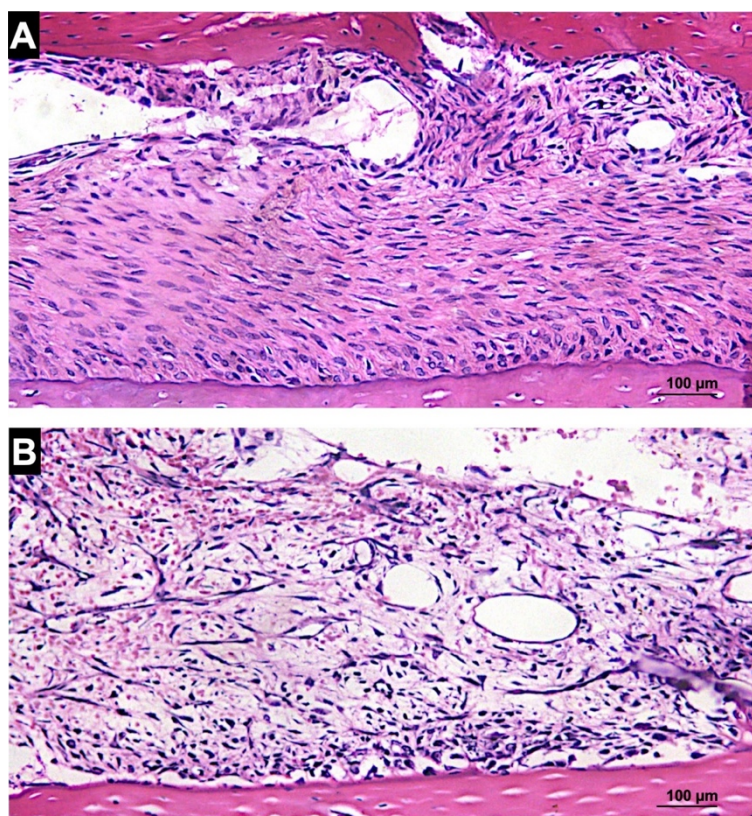


Figura 14. Fotomicrografia histológica da interface entre leito receptor e enxerto (“região de base”) dos grupos autógeno (A) e xenógeno (B) após 07 dias de reparo. Uma matriz rica em células semelhantes a fibroblastos foi observada em ambos os grupos. Hematoxilina e eosina, magnificação de x200.

Na “região de membrana”, a matriz de colágeno estava recobrendo os enxertos nos grupos autógeno e xenógeno, não sendo encontrado osso novo nessa região.

Após 20 dias de reparo, $20,3 \pm 13,8\%$ e $18,9 \pm 4,4\%$ de osso novo foram encontrados nos grupos autógeno e xenógeno (Gráfico 1, Tabela 2), respectivamente. Na “região de base” do grupo autógeno, o osso novo foi observado formando pontes que conectavam o enxerto à cortical externa do leito receptor (Figura 15A), bem como na “região de base” do osso xenógeno (Figura 15B).

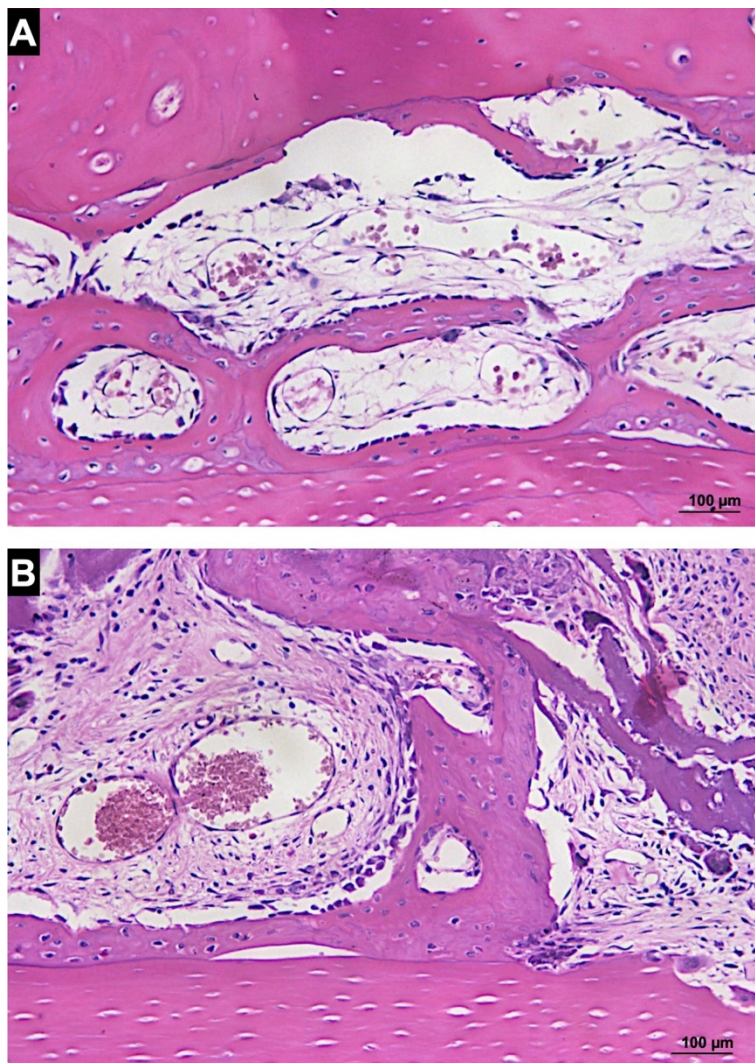


Figura 15. Fotomicrografia histológica da “região de base” dos grupos autógeno (A) e xenógeno (B) após 20 dias de reparo. O osso neoformado foi encontrado formando pontes entre os enxertos e a cortical externa do leito receptor em ambos os grupos. Hematoxilina e eosina, magnificação de x200.

Na “região de membrana”, verificou-se a presença de osso neoformado em ambos os grupos. No grupo autógeno, a formação óssea foi observada a partir da superfície do enxerto (Figura 16A), enquanto no grupo de xenógeno, o osso novo foi encontrado dentro das trabéculas do enxerto residual (Figura 16B).

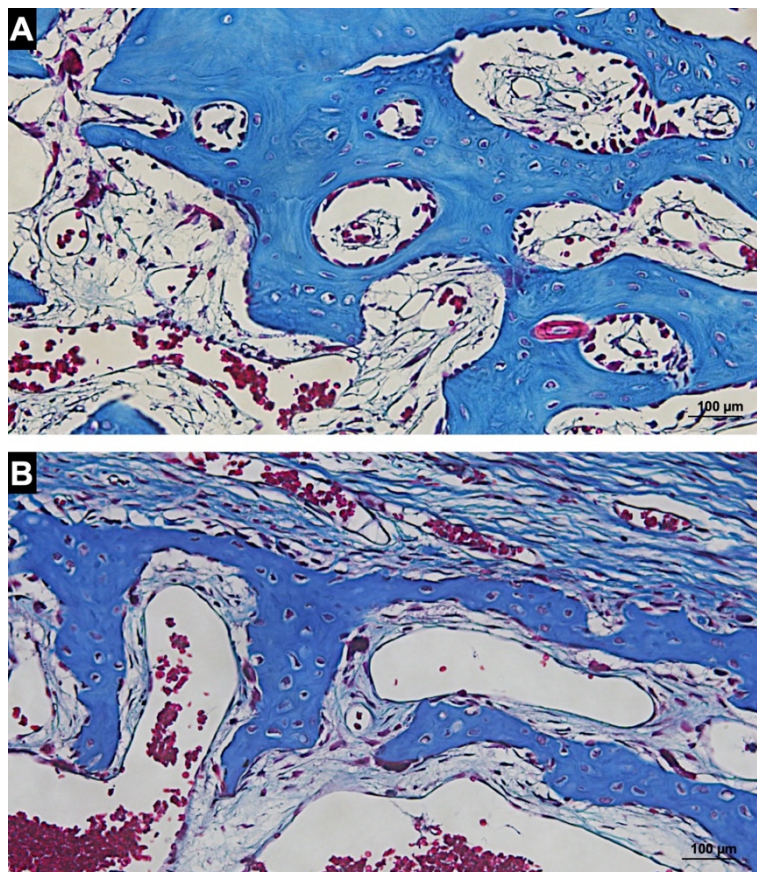


Figura 16. Fotomicrografia histológica da “região de membrana” dos grupos autógeno (A) e xenógeno (B) após 20 dias de reparo. O osso neoformado foi encontrado formando-se a partir da superfície do enxerto no grupo autógeno e em íntimo contato com as trabéculas do enxerto no grupo xenógeno. Tricrômico de Masson, magnificação de x200.

Após 60 dias de reparo, o osso novo aumentou em ambos os grupos, atingindo $24,2 \pm 11,2\%$ e $31,6 \pm 13,3\%$ nos grupos autógeno e xenógeno, respectivamente (Gráfico 1, Tabela 2). O osso autógeno apresentava um estágio avançado de remodelação na maioria dos locais, sendo possível observar lacunas osteocíticas em algumas regiões de enxerto remanescente. O osso xenógeno encontrava-se reabsorvido e substituído por osso neoformado e espaços medulares. No entanto, regiões contendo matriz óssea imatura ainda foram observadas.

Na “região de base” do grupo autógeno, os enxertos estavam bem integrados aos leitos receptores (Figura 17A). No grupo xenógeno, os enxertos estavam conectados aos leitos receptores por meio de pontes esparsas de osso neoformado (Figura 17B).

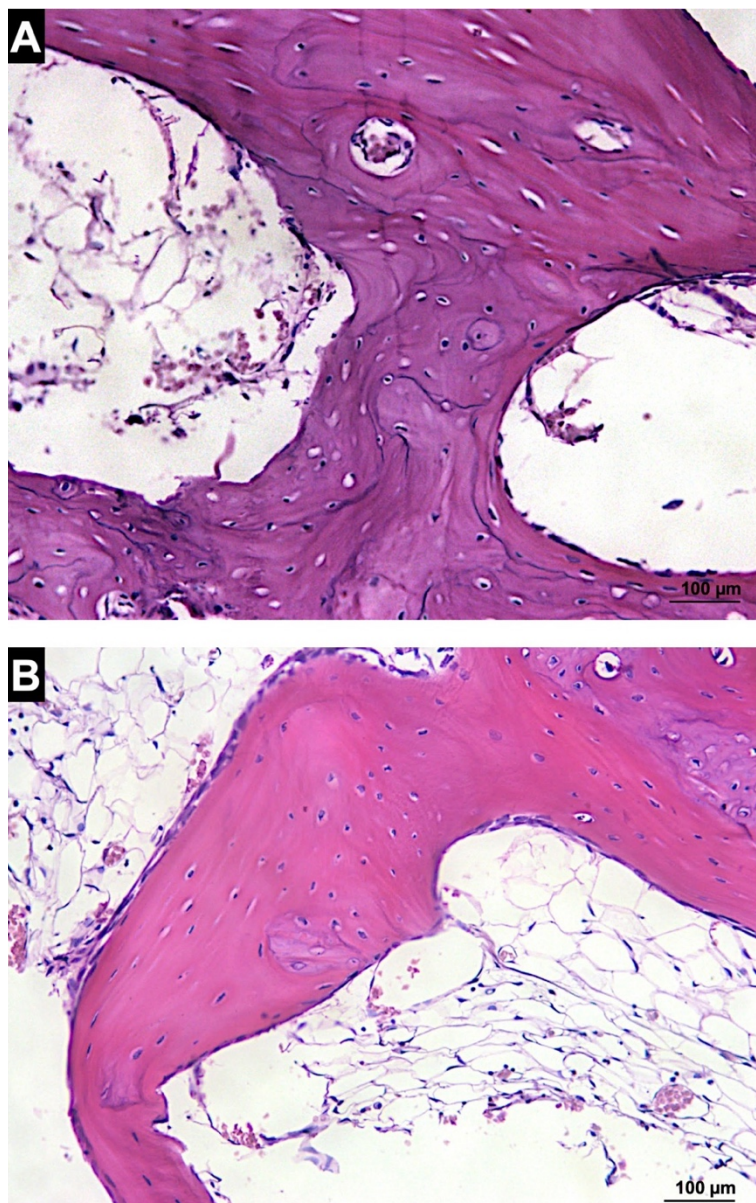


Figura 17. Fotomicrografia histológica da “região de base” dos grupos autógeno (A) e xenógeno (B) após 60 dias de reparo. Os enxertos autógenos encontravam-se bem incorporados ao leito receptor enquanto os enxertos xenógeno estavam conectados à cortical externa do leito receptor por meio de pontes ósseas esparsas. Hematoxilina e eosina, magnificação de x200.

Na “região de membrana”, o osso autógeno encontrava-se remodelado, apresentando ósteons secundários com lacunas osteocíticas ocasionais (Figura

18A). No grupo xenógeno, o osso neoformado constituía-se de ósteons primários, sendo possível observar pequenas quantidades de enxerto remanescente (Figura 18B).

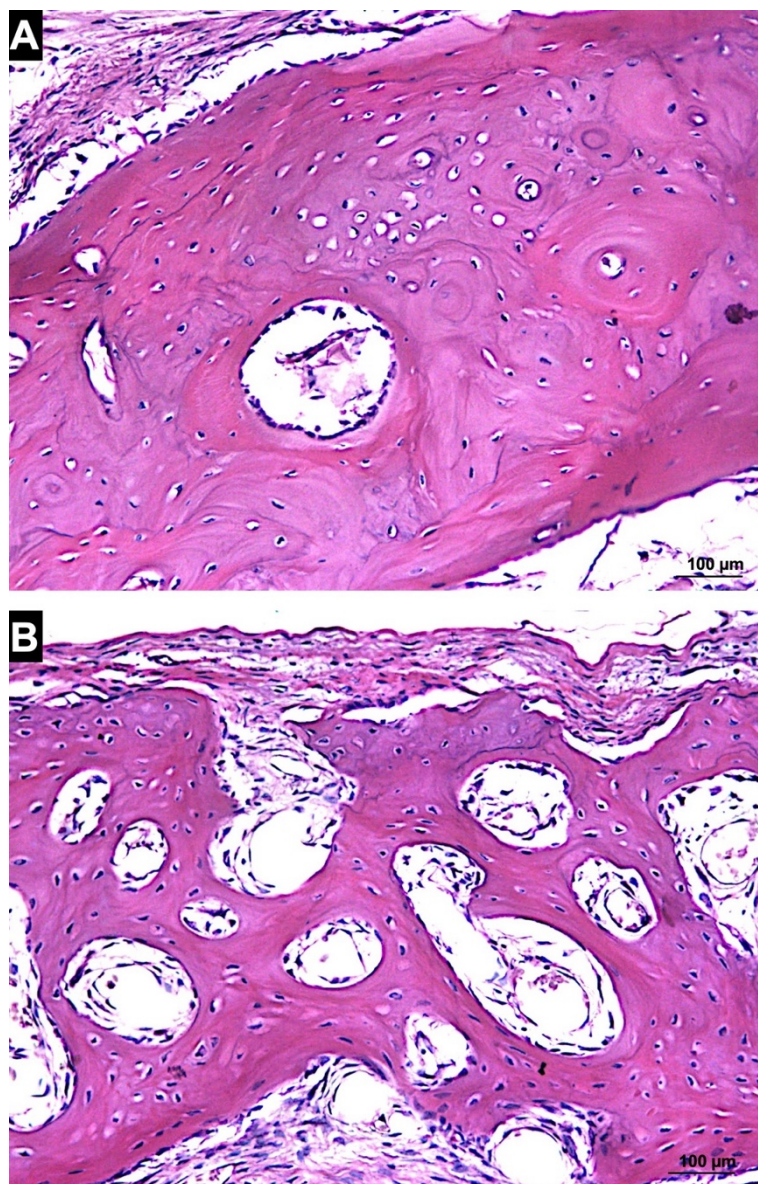


Figura 18. Fotomicrografia histológica da “região de membrana” dos grupos autógeno (A) e xenógeno (B) após 60 dias de reparo. O osso autógeno encontrava-se remodelado, apresentando ósteons secundários com raras lacunas osteocíticas. O osso neoformado nos grupo xenógeno era composto majoritariamente por ósteons primários. Pequenas quantidades de enxerto xenógeno residual foram detectadas. Hematoxilina e eosina, magnificação de x200.

As membranas de colágeno (pericárdio bovino) utilizadas para recobrir os enxertos foram gradualmente reabsorvidas. Aos 60 dias de reparo, remanescentes da membrana eram dificilmente identificados.

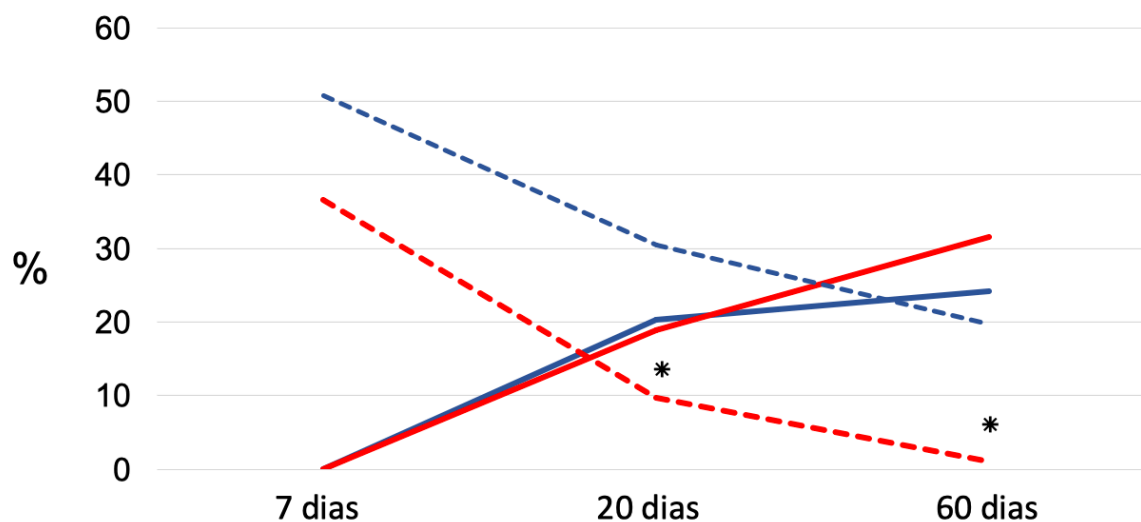


Gráfico 1. Porcentagens de osso novo e enxerto residual nos grupos xenógeno (teste) e autógeno (controle) após 7, 20 e 60 dias. Osso novo xenógeno: — ; osso novo autógeno: — ; enxerto residual xenógeno: - - - ; enxerto residual autógeno: - - -. Valores expressos como média ± desvio padrão são relatados, * $p < 0,05$.

Tabela 2. Percentuais de osso novo e enxerto residual obtidos a partir da análise histomorfométrica para os grupos autógeno e xenógeno após 7, 20 e 60 dias de reparo.

		Osso novo (%)	Enxerto residual (%)
7 dias	Autógeno	0	50,7 ± 24,5
	Xenógeno	0	36,7 ± 7,9
20 dias	Autógeno	20,3 ± 13,8	30,4 ± 15,4**
	Xenógeno	18,9 ± 4,4	9,7 ± 9,9**
60 dias	Autógeno	24,2 ± 11,2	19,7 ± 10,3**
	Xenógeno	31,6 ± 13,3	1,0 ± 1,7**

Valores expressos como média ± desvio padrão, * $p < 0,05$: análise intragrupo; ** $p < 0,05$: análise entre grupos.

4.3 Resultados imunohistoquímicos

Os resultados obtidos para as proteínas analisadas neste estudo encontram-se descritos a seguir.

A imunomarcção do anticorpo para o VEGF apresentou maiores escores para o grupo autógeno quando comparado xenógeno, principalmente nos períodos iniciais de incorporação dos enxertos, ou seja, 07 e 20 dias.

O COL I apresentou um padrão similar de imunomarcção entre os grupos autógeno e xenógeno, com um escore ligeiramente maior para o osso autógeno, principalmente aos 7 e 60 dias.

Para a OPN, não foi possível observar diferença entre os grupos em todos os períodos de avaliação.

A ALP apresentou pontuações ligeiramente maiores para o grupo xenógeno quando comparado ao autógeno, especialmente no último período de avaliação. O mesmo pôde ser observado para a Casp3, com o grupo xenógeno apresentando maiores escores para o período de avaliação inicial, ou seja, 07 dias.

A imunomarcção para TRAP, proteína estritamente relacionada à reabsorção óssea, evidenciou maiores escores para osso autógeno quando comparado ao xenógeno, principalmente após 60 dias de reparo. Os escores indicaram comportamento semelhante para OC.

Os resultados mencionados acima são mostrados na Tabela 3 e nas Figuras 19 e 20.

Tabela 3. Escores obtidos para as proteínas investigadas neste estudo.

		VEGF	COL I	OPN	ALP	Casp3	OC	TRAP
7 dias	Autógeno	2	2	1	2	1	1,5	1,5
	Xenógeno	1,5	1,5	2	1,5	2	2	1
20 dias	Autógeno	2	1,5	2,5	1,5	1,5	2	2
	Xenógeno	1	1,5	1,5	2	2	2	2
60 dias	Autógeno	1	2	2	1	1	2,5	2,5
	Xenógeno	1,5	1,5	1,5	1,5	2	2	1

0: ausência de marcação; 1: marcação leve; 1,5: marcação leve à moderada; 2: marcação moderada; 2,5: marcação moderada à intensa; 3: marcação intensa.

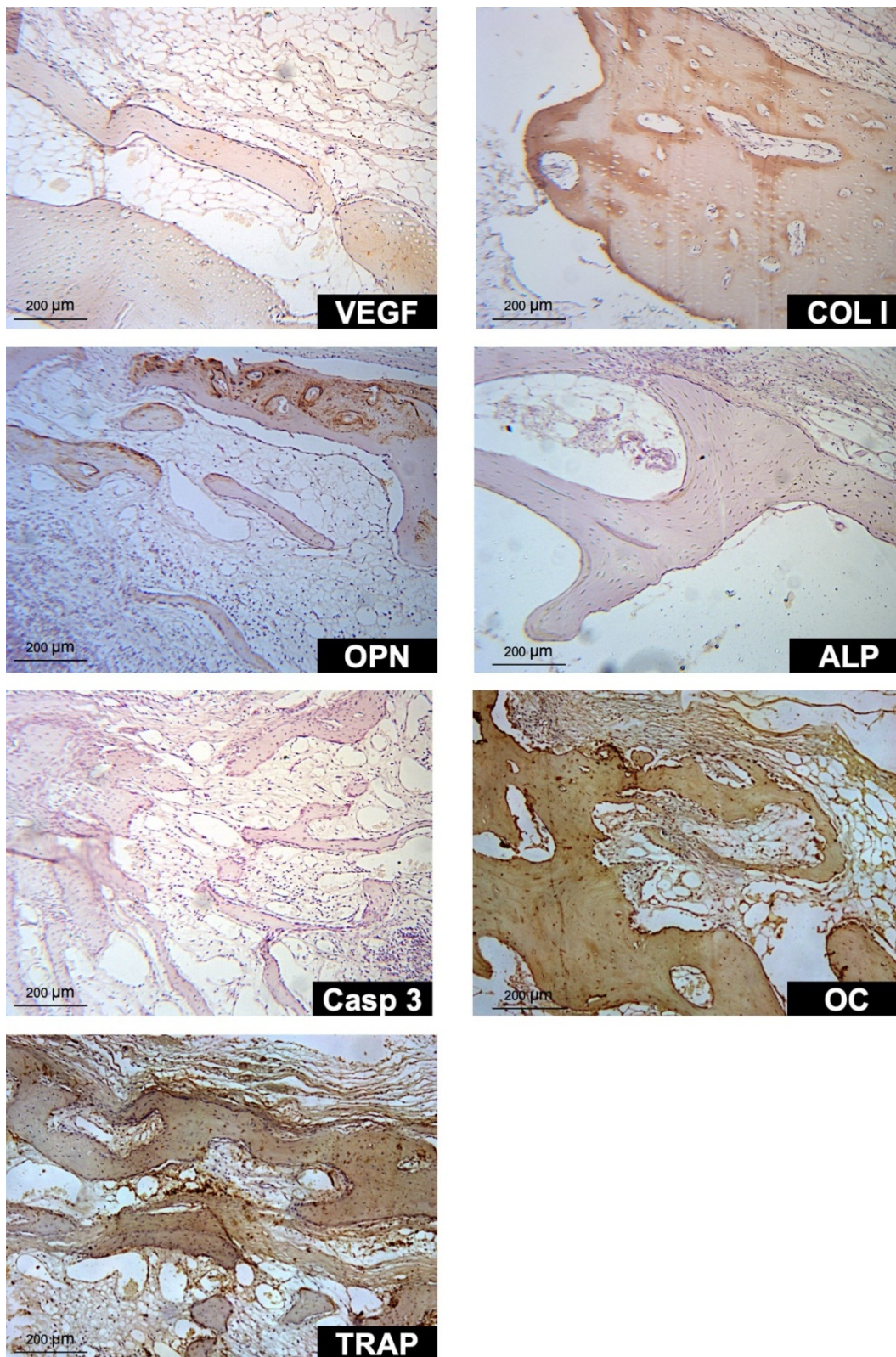


Figura 19. Fotomicrografia das imunomarcações do grupo autógeno após 60 dias de reparo. Harris-hematoxilina, magnificação de x100.

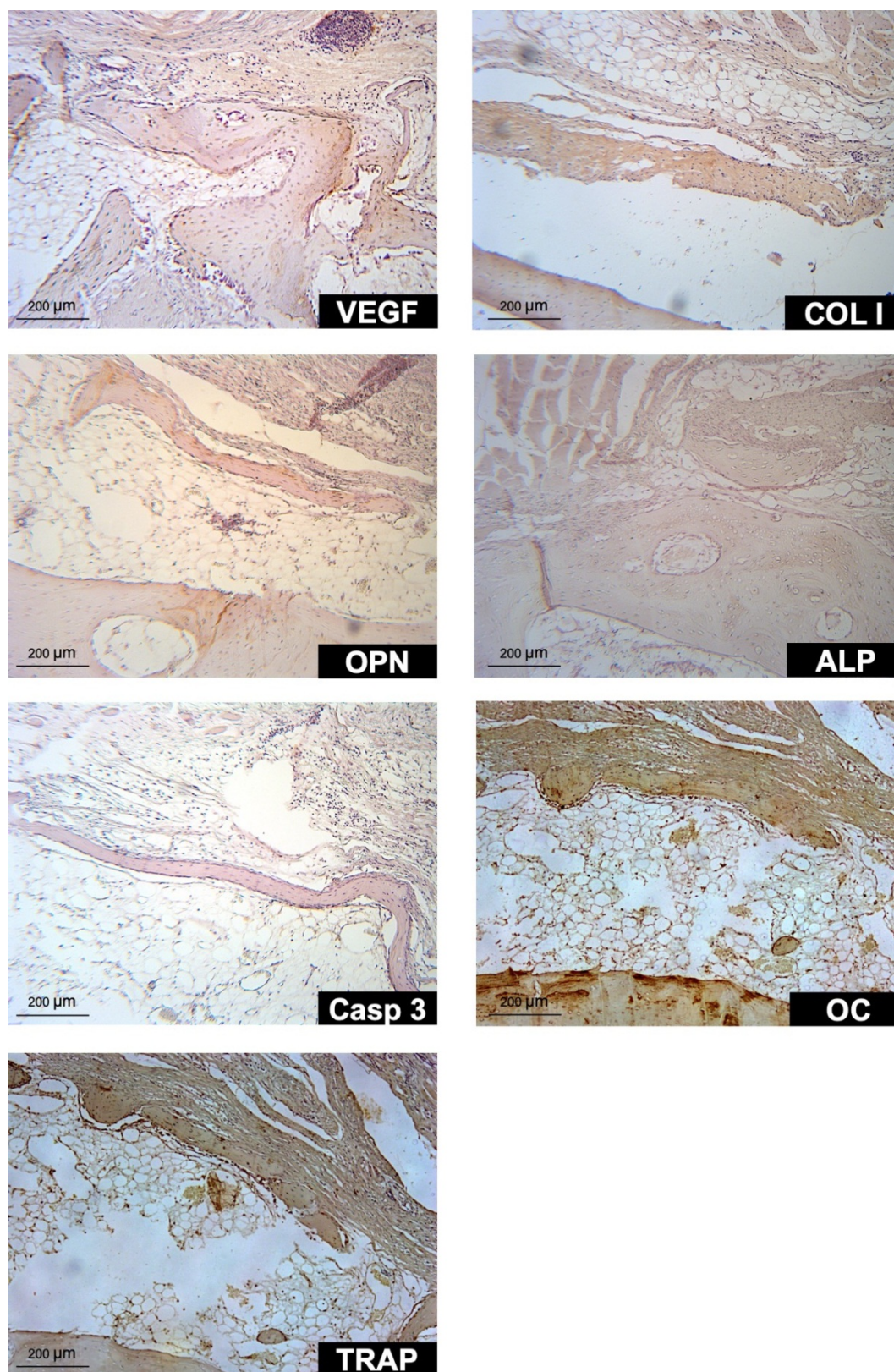


Figura 20. Fotomicrografia das imunomarcações do grupo xenógeno após 60 dias de reparo. Harris-hematoxilina, magnificação de x100.

5 DISCUSSÃO

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Após 60 dias de reparo, os enxertos de osso autógeno encontravam-se em estágio avançado de remodelação óssea, sendo que aproximadamente 60% do volume total dos enxertos haviam sido reabsorvidos. Essa reabsorção concentrou-se na região periférica dos blocos de osso ilíaco, próxima à membrana de colágeno utilizada para recobri-los. O processo de remodelação óssea, por sua vez, ocorreu predominantemente no centro dos blocos. Os enxertos xenógenos estavam praticamente reabsorvidos e parcialmente substituídos por novo osso mineralizado, especialmente na região da membrana e na interface enxerto / leito receptor. Ambos os enxertos estavam incorporados ao leito receptor ao final desse período.

As taxas de reabsorção dos enxertos autógenos obtidas para o grupo autógeno neste trabalho corroboram com as de outro estudo sobre aumento lateral mandibular com blocos de osso autógeno em coelhos. FARIA et al. (2008) avaliaram a influência das perfurações monocorticais realizadas nos leitos receptores previamente à instalação de enxertos autógenos de crista ilíaca na face vestibular do ângulo mandibular de coelhos. O lado teste foi perfurado de forma semelhante ao do presente estudo, enquanto no lado controle (contralateral) não foram realizadas perfurações. Os autores observaram que, após 60 dias de reparo, aproximadamente 50% do volume do total do enxerto haviam sido absorvidos no grupo teste, enquanto uma taxa de absorção de cerca de 70% pôde ser observada para o lado controle. Todavia, no estudo mencionado, o volume total dos enxertos foi avaliado por meio de tomografias computadorizadas e não com microtomografias.

Em estudo semelhante, PEDROSA et al. (2009) também investigaram a influência da perfuração do leito receptor sobre o comportamento de blocos de enxerto autógeno utilizados para aumento lateral de mandíbula em coelhos, porém tendo a calvária como sítio doador. Esses autores concluíram, à semelhança do ocorrido neste trabalho, que as perfurações do leito receptor contribuíram para que houvesse uma vascularização precoce dos enxertos, acelerando o processo de incorporação. Os autores também observaram melhor manutenção volumétrica para o grupo contendo as perfurações no leito receptor.

Diante disso, é possível afirmar que a preparação do leito receptor por meio de perfurações melhora o processo de incorporação e diminui as taxas de reabsorção dos enxertos.

No presente trabalho, após 7 dias de reparo, foi observada uma matriz óssea imatura contendo células semelhantes a fibroblastos, vasos e fibras nos locais das perfurações. Esses resultados estão em concordância com os obtidos pelo estudo de CANEVA et al. (2017), no qual enxertos autógenos coletados da calvária foram fixados face vestibular do ângulo mandibular de coelhos por meio de parafusos posicionais ou pela técnica de *lag screws*. A incorporação na interface enxerto / leito receptor foi avaliada após 3, 7, 20 e 40 dias. Similarmente a este trabalho, após 7 dias, um tecido ricamente vascularizado e com células semelhantes a fibroblastos foi observado no interior das perfurações.

Após 20 dias de reparo, em ambos os grupos deste estudo, observou-se o início da incorporação dos enxertos pela neoformação óssea ocorrida a partir das perfurações realizadas na cortical dos leitos receptores, evento este descrito pelos trabalhos de FARIA et al. (2008), PEDROSA et al. (2009) e CANEVA et al. (2017).

Após 60 dias de reparo, os enxertos autógenos encontravam-se intimamente conectados ao leito receptor; no grupo xenógeno, pontes ósseas esparsas conectavam o enxerto à cortical do leito receptor. A análise entre grupos revelou uma maior quantidade de enxerto remanescente para o autógeno, sendo que aproximadamente 20% do osso pré-existente ainda podia ser observado. Cerca de 24% de osso neoformado estavam ocupando a região enxertada. Esses dados indicam que um intervalo de tempo maior é necessário para que os enxertos estejam totalmente remodelados.

Os blocos de osso xenógeno equino deste trabalho foram amplamente reabsorvidos ao longo do tempo. SCHWARZ et al. (2010), em um estudo em cães, investigaram comparativamente a viabilidade de blocos xenógenos de origem bovina com outros de origem equina, ambos esponjosos, em cirurgias de aumento ósseo lateral. Os autores também relataram uma notável reabsorção dos blocos de osso equino, ocorrida pela degradação do material por osteoclastos e células gigantes multinucleadas.

Altas taxas de reabsorção não ocorrem somente com os enxertos de natureza xenógena. Em um estudo prospectivo em humanos, SILVA et al., (2017) utilizaram blocos de osso alógeno fresco congelado para aumento vértico-lateral na região posterior de mandíbula de vinte pacientes. Tomografias computadorizadas de feixe cônico foram realizadas antes da cirurgia, após 1 semana, 6 meses e 18 meses de pós-operatório. A perda volumétrica dos enxertos foi de 41%.

No presente estudo, não foi possível observar diferença estatística entre os grupos autógeno e xenógeno no que se refere à quantidade de osso neoformado ($24,2 \pm 11,2$ vs $31,6 \pm 13,3$, respectivamente). Todavia, esses achados contrariam os resultados obtidos por estudos sobre enxerto xenógeno para aumento ósseo lateral. DE SANTIS et al. (2015) investigaram o reparo de blocos de OMBD em comparação com blocos de osso autógeno coletados da região de ramo mandibular, em cirurgias de aumento ósseo lateral em mandíbula de cães. Os autores observaram uma taxa de osso novo mineralizado de $77 \pm 6,2\%$ para o grupo autógeno e de $5,9 \pm 7,5\%$ para o grupo xenógeno. Além da quantidade limitada de osso xenógeno observada, os enxertos estavam embebidos por tecido conjuntivo em sua maioria e pouco incorporados aos leitos receptores. Em outro trabalho, os autores mostraram também os blocos de OMBD não promoveram a osseointegração dos implantes instalados nos sítios enxertados com esse biomaterial e que os implantes se encontravam estáveis em decorrência de seu travamento em osso autógeno nativo (DE SANTIS et al., 2012).

Como discutido previamente, neste estudo, a incorporação dos enxertos xenógenos ocorreu de forma parcial. Esse padrão de remodelação apresentado pelo grupo xenógeno era completamente distinto daquele observado no grupo autógeno, em que uma conexão mais efetiva entre os enxertos e os leitos receptores pôde ser verificada. Nos estudos mencionados anteriormente (DE SANTIS et al., 2012; 2015) os enxertos de osso autógeno também se mostraram completamente incorporados ao leito receptor. No entanto, os enxertos xenógenos encontravam-se separados dos sítios receptores por tecido conjuntivo, não apresentando contato direto com a cortical óssea.

Os resultados microtomográficos mostraram melhor manutenção volumétrica ao longo do tempo para os blocos de osso autógeno quando comparados ao grupo xenógeno. Esses resultados podem estar relacionados à presença de uma quantidade maior de enxerto remanescente ao final de 60 dias no grupo autógeno, quando comparado ao grupo xenógeno. Deve-se considerar que os blocos de osso xenógeno utilizados neste estudo possuíam uma estrutura pouco mineralizada, o que pode ter contribuído para a reabsorção volumétrica apresentada por esses enxertos. Além disso, no grupo xenógeno, o osso neoformado foi encontrado principalmente na região da membrana e na interface enxerto / leito receptor,

enquanto a porção central dos enxertos estava ocupada por um tecido similar a espaços medulares.

Convém ressaltar que, durante as avaliações microtomográficas, foi difícil determinar uma escala de cinza (*thresholding*) que pudesse diferenciar com precisão osso neoformado de enxerto remanescente, razão pela qual os resultados microtomográficos foram expressos apenas como volume total do enxerto (VT: osso novo + enxerto remanescente) e volume ósseo (VO). As dificuldades encontradas aqui são compatíveis com as que foram discutidas em um estudo sobre avaliação microtomográfica de um enxerto xenógeno particulado no seio maxilar de coelhos (OMORI et al., 2018).

As marcações imunohistoquímicas confirmaram os resultados encontrados durante as análises histológica e histomorfométrica. Os escores obtidos para VEGF sugerem uma melhor vascularização para o grupo autógeno, o que pode ter contribuído para melhorar a incorporação dos enxertos e acelerar a remodelação óssea (OC, COL I e TRAP). Esses resultados são similares aos observados para o osso autógeno por FARIA et al. (2008) e PEDROSA et al. (2009).

6 CONCLUSÃO

6 CONCLUSÃO

Os resultados do presente estudo sugerem que tanto osso autógeno quanto xenógeno apresentam percentuais similares de osso neoformado ao longo do tempo. No entanto, volume e qualidade do osso neoformado, e grau de incorporação do enxerto ao leito receptor mostraram índices superiores para o grupo autógeno quando comparado ao xenógeno.

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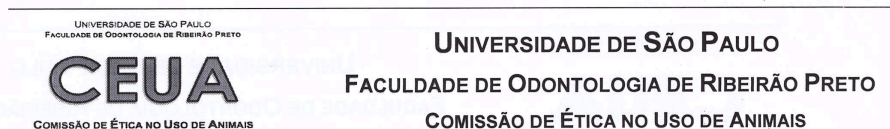
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ANEXOS

ANEXOS

ANEXO I


OFÍCIO DE APROVAÇÃO PELA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

**CERTIFICATE CEUA – FORP/USP**

We hereby certify that the project entitled “Immunohistochemical, histomorphometric and microtomographic comparative study between autogenous bone and xenogeneic bone in onlay graft surgery in rabbit mandibles” Protocol 2016.1.666.58.8, under the responsibility of Prof. Dr. Samuel Porfírio Xavier – involving the production, maintenance and/or use of animals from the phylum Chordata, subphylum Vertebrata (except human), for purposes of scientific research (or teaching) – is in accordance with the provisions of Law No. 11.794, of October 8th, 2008, Decree No. 6899 of July 15th, 2009, and the rules issued by the National Board of Animal Experimentation Control (CONCEA), with the Ethical principles in animal research adopted by the Animal Research Ethics Committee of the School of Dentistry of Ribeirão Preto, University of São Paulo, Brazil, and was approved in 16/11/2016.

Duration of the Project	16/11/2016-16/11/2017
Species/Lineage	Rabbits / New Zealand
Nº of animals	48
Weight / age	3,5 - 4,5 kg
Gender	Male
Origin	Central Animal Research Facilities of Campus Ribeirão Preto – University of São Paulo

Ribeirão Preto, November 16th, 2016.


Prof. Dr. Michel Reis Messoria
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CEUA - FORP/USP

ANEXO II

VERSÃO RESUMIDA EM INGLÊS DO ARTIGO SUBMETIDO I

Evaluation of bone graft incorporation and remodeling dynamics in an animal model: xenograft vs autograft.

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Keywords: histomorphometry; microtomography; immunohistochemistry; xenograft; rabbit.

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Abstract

The aim of this study was to compare, by means of histomorphometry, immunohistochemistry and microtomography, graft incorporation and remodeling processes of autogenous and equine xenogenous bone blocks used for mandibular lateral augmentation in rabbits. Eighteen rabbits, approximately 3.5-4.0 kg of weight and 4-5 months of age, were included in this split-mouth randomized study. The lateral side of the angle of the mandible was augmented bilaterally with either block autografts or xenografts. Animals were euthanized after 7, 20 and 60 days of healing, respectively. Biopsies were taken for each period and specimens underwent microtomographic scanning. The percentage of total volume (TV, mm³) and bone volume (BV: residual graft plus new bone, mm³) were measured for both autogenous and xenogenous groups using CTAn software. Paraffin sections were prepared and stained in hematoxylin and eosin and Masson's trichrome. For histomorphometric evaluation, a grid consisting of 80 squares was superposed to the image of histological slides for counting the structures at a magnification of 200x. The percentages of residual graft and new bone were assessed. The graft incorporation and remodeling molecular events were evaluated by means of VEGF, Col I, OPN, ALP, OC and Casp3 immunolabelling. ANOVA and the t test were performed. Both xenogenous and autogenous bone blocks presented similar percentages of newly formed bone over time. However, the volume and the quality of the grafted area was superior at the autogenous compared to the equine xenogenous graft groups. Before undergoing to clinical studies, more pre-clinical data are required.

Introduction

Predictable bone augmentation of severely atrophied alveolar ridges remains a challenge in regenerative efforts¹. The long-term prognosis of dental implants is directly related to an adequate bone volume at the implant placement site². As dental implants have evolved, several surgical techniques have been proposed to increase the alveolar bone volume before implant installation²⁻⁷. Although these techniques were reported to be associated with clinical success and implant survival rates⁷, they are surgically demanding and frequently followed by wound dehiscence^{7,8}.

Guided bone regeneration (GBR) has been proven to be highly successful for bone augmentation of localized defects⁹. Implants installed in regenerated sites present excellent long-term survival^{10,11}.

Autogenous bone harvested either from extra- or intra-oral donor sites is considered the gold standard for GBR due to its osteoconductive properties¹²⁻¹⁶. The sequential healing of autogenous bone blocks used for mandibular lateral augmentation has been previously described in experimental^{17,18} and in clinical studies^{19, 20}. Nevertheless, autografts have been associated with several disadvantages, such as surgical morbidity at the donor site, infection, paresthesia, postoperative pain, blood loss, limited bone availability, longer surgical time and increased financial cost^{7,21-24}. Although it has been demonstrated that autogenous onlay bone blocks promoted horizontal bone gain³, experimental and clinical studies have also shown substantial volumetric contraction over time, especially in the first five years after implant placement⁷. For these reasons, researches have focused on the development of bone substitutes.

Xenogenous bone blocks are considered to serve as an alternative to overcome the disadvantages mentioned above. They offer reduced morbidity and act as a scaffold for new bone formation²⁵⁻²⁹. The deproteinized bovine bone mineral (DBBM) is the most widely used xenogeneic graft material, which has also been investigated for bone augmentation in association with GBR procedures^{1,30-33}. In a clinical study³⁴, twelve cases were reported in which DBBM bone blocks in combination with collagen membrane were used for lateral ridge augmentation. The authors concluded that this combination is an effective treatment option for horizontal bone augmentation since it provides adequate newly formed bone to place implants after of 9 to 10 months of healing. More recently¹, in a dog model, the healing of two blocks of xenografts (bovine vs equine) covered with a resorbable membrane was evaluated. However, the volumetric and biological behavior of the grafts was not assessed.

Hence, the aim of the present study was to compare, by means of histomorphometry, immunohistochemistry and microtomography, graft incorporation and remodeling processes of autogenous and equine xenogenous bone blocks used for mandibular lateral augmentation in rabbits.

Material and methods

The experimental protocol was approved by the Ethical Committee of the Faculty of Dentistry of Ribeirão Preto of the University of São Paulo, Brazil (CEUA n°: 2016.1.666.58.8). The study was reported according to the ARRIVE guidelines.

Animal sample

Eighteen New Zealand male white rabbits, approximately 3.5-4.0 kg of weight and 4-5 months of age, were included in this study. Three groups of six animals each were selected, and each group was assigned to a different period of evaluation, *i.e.* 7, 20 and 60 days, respectively. The guidelines for animal care used in Brazil were followed.

The adoption of a split mouth experimental design allowed reducing the number of animals. The sample size $n = 6$ was considered sufficient to disclose possible differences between groups based on the data presented previous studies^{17,18,35} in which a statistically significant difference in bone formation was observed using the same number of animals.

Randomization and allocation concealment

The lateral side of the angle of the mandible was augmented bilaterally with either blocks of autograft or xenograft, randomly assigned. The randomization was performed electronically (randomization.com) by an author that did not participate in the surgical procedure (DB). The surgeon was informed about the allocation of the graft type after the iliac bone graft had been removed.

For the histological and microtomographic analyses, no information was provided to the assessors regarding the assignment of the block type or periods of evaluation.

Surgical procedure

An experienced specialist surgeon (ERS) performed all surgeries. The anesthesia was carried out using acepromazine (1.0 mg/kg; Acepran®, Vetril, Louveira, São Paulo, Brazil), administrated subcutaneously and a mix of xilazine (3.0 mg / Kg; Anasedan®, Sespo Indústria e Comércio LTDA, Paulínia, São Paulo, Brasil) and ketamine (50.0 mg / Kg; Dopaser®, Sespo Indústria e Comércio LTDA, Paulínia, São Paulo, Brazil) injected intramuscularly. After anesthesia, the animals received an intramuscular prophylactic antibiotic dose of oxytetracycline (40 mg/kg, Biovet, Vargem Grande Paulista, São Paulo, Brazil).

Autogenous bone blocks were harvested from the anterior iliac bony crest. After shaving, the experimental site was disinfected with PVPI 1% (Riodeíne® Tintura, Rioquímica, São José do Rio Preto, São Paulo, Brazil). The surgical area was infiltrated with mepivacaine 2% and epinefrine 1:100.000 (Mepiadre®, Nova DFL, Rio de Janeiro, Brazil). An incision of ~3.0 cm length was performed along the anterior

iliac crest protuberance. Skin, muscles and periosteum were elevated, and the iliac bone was exposed. A trephine bur of 10.0 mm (Neodent, Curitiba, Paraná, Brazil) was used to remove a bicortical bone block. After removal, the bone graft was kept in a gauze soaked in sterile saline. Resorbable sutures were used for the periosteum (Poliglactyn 910, Vicryl® 4-0, Ethicon, Johnson & Johnson, São José dos Campos, Brazil) while nylon sutures were used to close the skin flaps (Ethilon 4-0®, Ethicon, Johnson & Johnson, São José dos Campos, Brazil) at the donor sites. Before fixation, the inner cortical layer of the bone block was removed with a diamond bur, and the graft was shaped to adapt passively at the recipient site. Equine spongy bone blocks (10 x 10 x 20 mm, Heket Biomaterials, Trento, Italy) were prepared with identical dimensions of the autogenous bone blocks.

Before graft fixation, nine perforations of 1.0 mm in diameter were distributed equidistantly throughout the recipient bed (Figure 1A) according to a prefabricated template. These perforations aimed at providing blood and cellular perfusion from the marrow spaces. Grafts were fixed with a single 1.5 x 10.0 mm titanium screw (Neodent, Curitiba, Paraná, Brazil, Figure 1B and C) and covered with bovine pericardium membranes (Exaflex matrix®, Exaflex, Andazeno, Italy, Figure 1D). The wound closure was performed with Vicryl® 4-0 (Ethicon, Johnson & Johnson, São José dos Campos, São Paulo, Brazil) in the periosteum and muscular planes, and Nylon 4-0 (Ethicon, Johnson & Johnson, São José dos Campos, São Paulo, Brazil) on the skin, with simple sutures. In the post-operative period, ketoprofen twice a day for 2 days (3.0 mg/kg, Ketofen® 10%, Merial, Monte-Mor, São Paulo, Brazil) was injected *i.m.* tramadol hydrochloride 2% twice a day for 2 days (1.0 mg/kg, Cronidor®, Agener União Saúde Animal, Apucarana, Paraná, Brazil) was injected subcutaneously.

Maintenance care

The animals were kept in individual cages in acclimatized rooms at the animal facilities of Faculty of Dentistry of Ribeirão Preto – USP. A strict monitoring protocol of the wounds and of the biological functions was established for the whole period of experiment.

Euthanasia

Six rabbits from each group were euthanized at 7, 20 and 60 postoperative days by intravenous overdose (2.0 mL) of thiopental 1.0 g (Thiopentax®, Cristália, Itapira, São Paulo, Brazil). Each side of the mandible was reduced to a specimen of approximately 12.0 mm x 12.0 mm. The fixation screw was used as a reference for bone sections.

Micro-CT evaluations

After fixation in 10% buffered formalin, the specimens were scanned in a high-resolution micro-CT SkyScan 1172 (Bruker, Kontich, Belgium) using a resolution of 8.70 μm isotropic pixel at 100 kV/100 μA and a Al + Cu filter. The exposure was 1280 ms, the rotation step amounted to 0.6°, the frame averaged 4, and the random movement was 10.

The micro-CT cross-sectional images were repositioned using the software DataViewer (Bruker, Kontich, Belgium, Figure 2). The software CTAn (Bruker, Kontich, Belgium) was used to evaluate the volume of the grafted region at the various periods of healing. The fixation screw was eliminated from the measurements. The thresholds of gray levels to identify total graft volume (TV) and bone volume (BV: new bone plus residual graft) was set at 30-180 gray levels.

Histological preparation

Following the micro-CT analyses, specimens were decalcified with 7% EDTA (Merck, Darmstadt, Germany) and subsequently dehydrated through a series of alcohols with increasing concentrations. Following this, the specimens were diaphanized with xylol immersions and impregnated with paraffin (Leica TP1020, Wetzlar, Germany). Paraffin blocks containing the samples were cut in a microtome to obtain slides of 6 μm of thickness. The slides were divided in two groups for either histological or immunohistochemical analyses. The slides for histological analysis were stained with hematoxylin and eosin or Masson's Trichrome stain.

Calibration of the histomorphometric evaluation

The measurements were performed by a well-trained examiner (ERS) after a calibration with an experienced professional (DB) and the inter-examiner test for recognition of histological structures reached a $K > 0.90$.

Histological and histomorphometric analyses

All histological evaluations were performed using a light microscope (Leica, Wetzlar, Germany) connected to a computer through a digital video camera (Leica DC 300F, Leica, Wetzlar, Germany). The software ImageJ 1.50i (National Institutes of Health, USA) was used for measurements. A point-counting procedure was applied to determine the tissue composition (Schroeder and Münzel-Pedrazzoli, 1973). A grid consisting of 80 squares was superposed to the image of the histological slides (Figure 3).

The area delineated by the end of the graft in close contact to the collagen membrane was defined as *Membrane region* and the area close to the recipient bed was defined as *Base region* (Figure 4). The evaluations were performed using an objective x20.

The histomorphometric measurements were taken separately for each area and mean values were calculated to evaluate new bone formation and graft resorption. The structures assessed included proportions of new mineralized bone and remaining graft (composed of pre-existing mineralized bone and marrow spaces).

Immunohistochemical processing

The immunohistochemical labeling was carried out using the immunoperoxidase detection method with the following primary antibody: anti-Col I (COL1), anti-OPN (AKM2A1), anti-VEGF (SC1881), anti-ALP (B-10) and anti-Cas 3 (31A1067) polyclonal antibodies produced in goats (Santa Cruz Biotechnology, Santa Cruz, USA). As a secondary antibody, the anti-IgG biotinylated antibody at a 1:200 concentration (Jackson ImmunoResearch Laboratories, West Grove, USA) was used. The reaction was revealed using Diaminobenzidine (Dako Laboratories, Santa Clara, USA). At the end of the reactions, the Harris-Hematoxylin counter-staining was performed. The determination of labeling levels for each antibody was performed semi-quantitatively, using scores from 0 to 3 (0=absence of labeling; 1=light labeling, in which up to 25% of the analyzed area showed positive labeling for the protein investigated; 2=moderate labeling, in which 50% of the analyzed area presented positive labeling for protein; 3=intense labeling, in which up to 75% of the analyzed area showed positive labeling for the analyzed protein, modified from a previous study¹⁹.

The analysis was performed using a light microscope (Leica, Wetzlar, Germany) connected to a computer through a digital video camera (Leica DC300F, Leica Microsystems, Wetzlar, Germany), using objectives x10 and x25. The analyzer (RO) had no previous knowledge of the samples and was submitted to the Kappa intra-examiner test ($K > 0.90$).

Statistical analysis

The data collected were submitted to statistical analysis using the software IBM SPSS Statistics software (IBM Inc., Chicago, IL, USA). Data were expressed as mean \pm standard deviation. To check normal distribution the Shapiro-Wilk test was performed. To compare new bone formation between autogenous and xenogenous graft groups and among the three evaluation periods a two-way ANOVA test was used. For comparison between groups of the same evaluation period, the Student's t-test was performed. The level of significance was 5% ($p < 0.05$). In the occurrence of statistical difference, the Tukey post-test was applied.

Results

One animal of the 20-day group died during anesthesia and had to be replaced. None of the animals presented postoperative complications, such as suture dehiscences and / or infections.

MicroCT evaluation

Three-dimensional reconstructions of the bone blocks were performed for their characterization. The three-dimensional evaluation of grafts revealed a volumetric contraction over time for both groups. The total volume (TV) of autografts decreased between 7 and 60 days from $258.13 \pm 15.3 \text{ mm}^3$ to $107.2 \pm 17.5 \text{ mm}^3$ ($p < 0.05$) while the xenograft volume decreased from $166.9 \pm 26.0 \text{ mm}^3$ to $96.0 \pm 16.1 \text{ mm}^3$ ($p < 0.05$), as shown in Figure 5 and Table 1. Contraction rates of 58.5% and 42.5% were observed for autogenous and xenogenous bone respectively.

Bone volume (BV: new bone plus residual graft) for autograft was $52.8 \pm 7.7 \text{ mm}^3$, $27.2 \pm 11.1 \text{ mm}^3$, and $33.1 \pm 2.8 \text{ mm}^3$ after 7, 20, and 60 days of healing, respectively. In the xenograft group, the bone volumes were $4.2 \pm 0.4 \text{ mm}^3$, $18.8 \pm 9.5 \text{ mm}^3$, and $7.6 \pm 3.7 \text{ mm}^3$, respectively (Table 1). The intergroup analysis revealed higher bone volume for autografts after 60 days of healing ($p < 0.05$), which could also be qualitatively observed in Figure 6.

Histomorphometric evaluation

After 7 days of healing, new bone was not observed in either group (Table 2, Figure 7). In the *Base region*, in both groups, provisional matrix, rich in fibroblast-like cells, vessels and fibers was found interposed between graft and the cortical bone of the recipient sites (Figures 8A and B). In the *Membrane region*, the collagen membrane was lining the grafts in both autogenous and xenogenous grafts, and no new bone was found either.

After 20 days of healing, $20.3 \pm 13.8\%$ and $18.9 \pm 4.4\%$ of new bone was found at the autogenous and xenogenous graft groups (Table 2, Figure 7), respectively. In the *Base regions* of the autogenous bone, new bone formed bridges connecting the graft to the recipient cortical bone (Figure 9A) as well as in the *Base region* of xenogenous bone (Figure 9B). In the *Membrane regions*, new bone was found in both groups. In the autogenous group, bone formed from the graft surface (Figure 10A) while in the xenograft group, new bone was found within the trabeculae of the xenograft (Figure 10B).

After 60 days, new bone increased in both groups reaching fractions of $24.2 \pm 11.2\%$ and $31.6 \pm 13.3\%$ in the autograft and xenograft groups, respectively (Table 2, Figure 7). The bone graft was remodeled in most sites, while regions with empty lacunae were seen in the residual bone graft. The xenograft appeared to be mainly resorbed and substituted by new bone and marrow spaces. Nevertheless, regions presenting immature tissues (provisional matrix) were still observed. In the *Base region* of the

autogenous bone, the grafts were connected and well-integrated to the recipient sites (Figure 11A). The xenograft was connected by means of sparse bridges of newly formed bone to the recipient sites (Figure 11B). In the *Membrane region*, the autogenous bone was remodeled and presented secondary osteons with occasional empty lacunae (Figure 12A) while, in the xenograft group, new bone contained primary osteons and small amounts of remnants of xenograft could be detected (Figure 12B).

The collagen (bovine pericardium) membranes used to cover the grafts were gradually resorbed. At 60 days, remnants of the membrane were hardly visible.

Immunohistochemical evaluation

The results obtained for the immunohistochemical evaluation are described below. VEGF labeling showed higher scores for the control (autogenous) when compared to the test group, mainly in the initial periods of incorporation of the grafts, *i.e.*, 07 and 20 days. Collagen Type I presented a similar pattern of labeling between the test and control groups, with a slightly higher score for autogenous bone, especially at 7 and 60 days.

For osteopontin, it was not possible to observe differences between the test and control groups for all the evaluation periods.

ALP presented slightly higher scores for the test when compared to the control group, especially in the last evaluation period. The same was observed for Caspase 3, in which the test group also presented higher scores for the initial evaluation period, *i.e.*, 07 days.

TRAP, a protein strictly related to bone resorption, presented higher scores for autogenous bone when compared to the xenogenous bone, especially after 60 days of healing. A similar immunolabelling pattern was also observed for OC. The results mentioned above are shown in Figures 13 and 14 and Table 3.

Discussion

After 60 days of healing, the autografts were well integrated into the recipient sites and were remodeled with new bone, presenting about 53% loss of volume. The xenograft was almost completely resorbed and only partly substituted by new mineralized bone, mainly at the base and in the periphery.

The autogenous grafts appeared to be resorbed for the periphery while, in the body of the graft, remodeling processes were occurring. In the interface between the graft and the recipient sites, bone formation was incorporating the graft over time.

The results obtained at the autogenous bone sites are in agreement with other studies that used autogenous bone for onlay bone augmentation. In a similar experiment in rabbits¹⁷, autografts were collected from the iliac crest and fixed to the lateral aspect of the mandible. The recipient site at one side of the mandible was multiple perforated, similarly to the present study, while the contralateral side was left untreated. After 60 days, the grafts were well integrated onto the perforated recipient

sites and about 50% of the volume was lost. At the untreated sites, the graft presented less integration onto the recipient bed and about 70% of loss of volume. In another similar experiment in rabbits¹⁹, bicortical autogenous bone was harvested from the calvaria and applied to the lateral aspect of the mandible, either perforated or not. After 60 days, the resorption was higher at the not-perforated sites (~22%) compared to the perforated sites (~6%), even though both grafts were incorporated into the recipient bed.

The recipient bed preparation by means of perforations has been shown to accelerate and improve the incorporation process. In fact, in the studies previously mentioned^{17,18}, the 20 days interval was evaluated, and a faster bone formation and graft incorporation was observed at the perforated sites. Moreover, after 3 days of healing, VEGF was only present at the perforated sites. In the present study, after 7 days of healing, a tissue including fibroblast-like cells, vessels and fibers was observed within the perforations, and the first bone formation was seen after 20 days. These results agree with another similar study³⁶ in which autografts from the calvaria were fixed to the lateral aspect of the mandible. The healing at the interface region between the autograft and the recipient site was assessed after 3, 7, 20 and 40 days. Similarly to the present study, after 7 days of healing, a tissue rich in fibroblast-like cells, vessels and fibers was observed within the perforations at the recipient bed, and new bone was detected after 20 days.

In the present study, a high rate of remodeling of the autograft was observed. The remodeling process of the autogenous graft has been reported in previous studies^{17,18}. In an experiment in dogs²⁹, an autogenous bone graft was collected from the ascending ramus of the mandible and fixed within buccal defects of the mandible. After 6 months of healing, the grafts were analyzed histologically. Very little content of non-vital bone was found, meaning that a high rate of remodeling occurred during healing that substituted the pre-existing bone with newly formed bone. In the present study, after 60 days of healing, only ~20% of pre-existing bone was still present, while ~24% of new bone was occupying the augmented region. This, in turn, means that additional time was needed to complete the remodeling process.

Micro-CT results showed better volumetric maintenance over time for the autogenous blocks when compared to the xenogenous group. This result might be related to the higher content of residual graft at the autogenous compared to the xenogenous groups. It has to be considered that the xenograft used in the present study was composed of a poorly mineralized structure, which may have contributed to the contraction of the grafts. Moreover, in the xenograft group, new bone was mainly found at the *base region* and in the periphery of the membrane region, while the most central regions were occupied by soft tissue resembling marrow.

It should be noted that during micro-CT assessments, it was difficult to determine an appropriate threshold that could accurately differentiate new bone from remaining graft, which is why microtomographic results were expressed only as total bone volume (new bone + remaining graft). The difficulties found here are consistent with those discussed in another study on micro-CT evaluation of a xenogenous particulate graft³⁷.

The material used in the present study was extensively resorbed. Other biomaterials have shown a high resorption rate. In a previous prospective study³⁸, a fresh frozen allograft was used to augment the lateral aspect of the mandible in twenty patients. CBCTs were taken before surgery and after, 1 week, 6 months, and 18 months. The volumetric loss of volume was 41%. Nevertheless, another biomaterial presented much lesser rate of resorption. In an experiment in dogs already mentioned, at one side of the mandible a DBBM block was used to augment a buccal bone defect²⁹. After 3 months of healing, the volumetric measurements did not reveal changes compared to the initial surgical stage. In the present study, the incorporation of the xenograft was partial and was composed of few bridges of newly formed bone connecting the graft to the recipient sites. This feature of healing at the xenograft sites was completely different from that observed at the autogenous sites that presented a more compact connection between grafts and recipient beds. In the previously described studies^{28,29} at the autogenous sites, the graft was completely incorporated into the recipient site. However, at the DBBM sites, the blocks were separated from the recipient sites by connective tissue, presenting no direct contact to the recipient sites. Occasionally the grafts were incorporated into the recipient bed, and only in a limited region of the base of the grafts.

In conclusion, the findings from the present study suggest that both xenogenous and autogenous bone blocks present similar percentages of newly formed bone over time. However, volume, quality of the grafted area and graft incorporation to the recipient sites was superior at the autogenous compared to the equine xenogenous graft sites.

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Conflict of interests

All the other authors declare no conflict of interest regarding this study.

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ANEXO III

VERSÃO RESUMIDA EM INGLÊS DO ARTIGO SUBMETIDO II

Osteoconductivity of Bio-Oss granules of different dimensions used for maxillary sinus augmentation. A histo-morphometric study in rabbits.

Osteoconductivity of Bio-Oss granules of different dimensions

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Abstract

The aim of this study was to evaluate the osteoconductivity of deproteinized bovine bone mineral with granules of different size applied for maxillary sinus floor elevation. Maxillary sinus floor elevation was performed bilaterally in 18 rabbits. Deproteinized bovine bone mineral (DBBM) with particle dimensions of either 0.125 – 1.0 mm or 1 – 2 mm was used as a filler material. The antrostomy was covered using a collagen barrier. The animals were euthanized in groups of 6 after 2, 4, and 8 weeks of healing. MicroCT and histological analyses were performed. New bone developed between 2 and 8 weeks from 7.0% to 27.6% in the small granule sites, and from 6.1% to 27.6% in the large granule sites. After 2 weeks of healing, bone-to-graft contact was 10.9% and 11.9% for the small and large granule sites, respectively. The highest values were found close to the sinus bony walls. After 8 weeks of healing, the bone-to-graft contact increased to 65% and 62% in the small and large granule sites, respectively. The highest values were located close to the bony walls and to the bony window. Thus, similar outcomes in osteoconductivity and bone formation were found at both small and large DBBM granule sites.

Key words: animal study, sinus floor elevation, bone healing, osteoconductivity, histology, morphometry, collagen membrane, xenograft.

Introduction

When the bone volume in the posterior regions of the maxilla is not sufficient to allow the installation of implants, the vertical bone height beneath the maxillary sinus is often augmented. Due to the tendency of the sinuses to re-pneumatize over time after the elevation of the Schneiderian membrane,¹⁻⁵ various biomaterials have been applied.⁶

Xenografts in various particle sizes are widely used as filler materials.⁷⁻¹⁰ Depending of their structure, the particles of xenografts will be either resorbed over time at different rates or embedded into newly formed tissues.¹¹⁻¹⁴ A deproteinized bovine bone mineral (DBBM) has been applied in several clinical^{7,8,15,16} and experimental studies^{11,17,18} showing a high volumetric stability compared with other biomaterials.

The influence on the size of the particles on the clinical outcomes have also been evaluated.

In a randomized clinical study,¹⁹ a ridge preservation technique was applied after molar extraction. Human demineralized bone matrix putty was used as a filler. Either the putty contained particles of small (0.125 – 0.710 mm) or a mixture of larger dimension (2 – 4 mm) and small particles. No clinical and histological statistically significant differences were found between the two augmentation sites.

In a randomized controlled split-mouth clinical study,²⁰ 10 partially edentulous patients were recruited for a bilateral sinus floor augmentation. Granules of different sizes (0.125 – 1.0 mm or 1 – 2 mm) of DBBM were used to fill the elevated space in the sinus. After 8 months of healing, 25 implants were installed, and implant stability was measured applying resonance frequency analysis (RFA). After further 6 months of healing, RFA values were similar in both augmentation sites. The histological data on biopsies retrieved at the time of implant installation from the same patients were subsequently reported in another article.¹⁶ No statistically significant differences between the two augmentation sites were found regarding residual biomaterial and newly formed bone proportions.

These results are in agreement with the outcomes from other clinical²¹ and animal¹⁷ studies on sinus floor augmentation. However, a further clinical study⁸ reported larger amounts of new bone formation at large compared to small particle size sites.

The integration of DBBM particles (bone-to-graft contact) has been evaluated in augmented sinuses.^{11,17} In a study in minipigs,¹⁷ only the total bone-to-graft contact was evaluated, without providing data divided for each region. In a study in rabbits,¹¹ bone-to-graft contact in various regions of the augmented sites were evaluated after different periods of healing. This allowed the demonstration of bone formation proceeding from the sinus bone wall towards the most central regions of the elevated space. However, only sites with small granules of DBBM were evaluated.

Due to the uncertainty in the selection of the dimensions of the xenograft to be used and the lack of information about the osteoconductivity of large granules studied in different sites of the augmented sinus, the study of the influence of dimensions of xenograft particles on healing outcomes appears to be justified.

Hence, the aim of the present experimental study was to evaluate the osteoconductivity of deproteinized bovine bone mineral with granules of different sizes applied in maxillary sinus floor augmentation.

The null hypothesis was that of no difference in osteoconductivity and in bone formation in the sites augmented with either small or large granules of deproteinized bovine bone mineral (DBBM).

Material and methods

The experimental protocol was submitted and approved by the Ethical Committee of the Faculty of Dentistry in Ribeirão Preto of the University of São Paulo (USP, SP-Brazil; 2017.1.278.58.9). The study is reported according to the ARRIVE guidelines. The guidelines for animal care adopted in Brazil were strictly followed.

Animal sample

Eighteen New Zealand white rabbits, approximately 3.5 – 4.0 kg and 4 – 5 months of age, were used. Three groups of six animals each were randomly assigned to a different period of healing, *i.e.* 2, 4, and 8 weeks, respectively.

To adhere to the Three R requirements for animal research, a rabbit model was selected owing to the simplicity of the surgical treatment. Moreover, the use of a split mouth design reduced the variability among animals, a fact that decreased the number of animals needed. Nevertheless, for sample calculation, the data from an experiment in minipigs¹⁷ was used. In that experiment, a difference of 9.1% in bone-to-graft contact was found after 6 weeks of healing in favor of small compared to large granule sites. With a standard deviation of 6%, six rabbits were calculated to be sufficient to reject the null hypothesis with a power of 0.8 and an $\alpha = 0.05$.

Randomization and allocation concealment

The randomization for the placement of xenograft granules of different dimensions was performed digitally (www.randomization.com) by one author not involved in the surgeries (DB). The surgeon (ERS) was informed about the side (right or left) where to place the xenograft after the elevation of both sinuses.

No indications were reported on the histological slides regarding test and control sites. However, the different dimensions of the granules may have provided clues to differentiate the two sites, even though no indications about the aim of the study were given to the histological assessor (KAAA).

Surgical procedures

A maxillofacial surgeon specialist (ERS) performed all surgeries. The anesthesia was induced using acepromazine (1.0 mg/kg; Acepran®, Vetnil, Louveira, São Paulo, Brazil), administered subcutaneously and a mix of xylazine (3.0 mg/Kg; Dopaser®,

Hertape Calier, Juatuba, Minas Gerais, Brazil) and 50mg/kg ketamine hydrochloride (Ketamin Agener, União Química Farmacêutica Nacional S/A, Embu-Guaçú, São Paulo, Brazil) injected i.m. Local anesthesia was added in the experimental regions. After having shaved and disinfected the experimental area, an incision ~2.5 cm long was carried out along the midline of the nasal dorsum. Skin, muscles and periosteum were elevated and the nasal bone was exposed bilaterally at the nasal-incisal suture. A squared antrostomy, of about 4 mm in dimensions, was prepared with diamond drills in both sides, laterally to the nasal-incisal suture, and anteriorly to the nasal-frontal suture (Figure 1A). The sinus mucosa was detached from the bony walls and elevated at both sides. A small screw was placed in the nasal-incisal suture as a landmark for the histological processing to identify the central position of the antrostomies. Deproteinized bovine bone mineral (DBBM) granules (Bio-Oss®, Geistlich Biomaterials, Wolhusen, LU, Switzerland), either 0.250-1.0 mm or 1.0-2.0 mm, were randomly allocated and grafted within the elevated space in similar volumes (Figure 1B). The antrostomies were subsequently covered with collagen barriers (Bio-Gide® Geistlich Biomaterials, Wolhusen, LU, Switzerland) (Figure 1C). Resorbable sutures were used for the periosteum (Poliglactyn 910 5-0, Vicryl®, Ethicon, Johnson & Johnson, São José dos Campos, Brazil) while nylon sutures were used to close the skin flaps (Ethilon 4-0®, Ethicon, Johnson & Johnson, São José dos Campos, Brazil).

Maintenance care

Each animal was kept in an individual cage and within an acclimatized room. The wounds and the biological functions were carefully monitored by veterinarians during the full period of the experiment. The animals had access to food and water *ad libitum*.

Euthanasia

The same procedures to induce anesthesia during surgery were also applied for the euthanasia. An overdose of sodium thiopental (1.0 g, 2 mL, Thiopentax®, Cristália Produtos Químicos Farmacêuticos, Itapira, São Paulo, Brazil) was added to euthanize the animals. Biopsies were retrieved in blocks and were fixed in 10% buffered formalin.

MicroCT evaluations

A microCT analysis was performed using a microCT 1172 equipment (Bruker, Kontich, Belgium). The parameters were as follows: 9.92 µm isotropic pixel, 60KV/165 µA, filter Al 0.5 mm, exposure time 596 ms, rotation step 0.4 degrees, frame average 4, and random movement 10. The software DataViewer® (Bruker, Kontich,

Belgium) was used to reposition the cross-sectional images and measurements were performed with the software CTAn (Bruker, Kontich, Belgium).

Histological preparation

The experimental region was reduced and the biopsies were dehydrated in increasing concentrations of ethanol. Subsequently, the biopsies were infiltrated in resin (LR White™ hard grid, London Resin Co Ltd, Berkshire, UK). After polymerization, two ground sections were prepared using the small screw as reference, and following a transverse plane.

The ground sections were first prepared at a width of about 100-150 μm using a precision slicing equipment (Exakt®, Apparatebau, Norderstedt, Germany) and then, they were ground to about 50-60 μm using a cutting–grinding machine (Exakt®, Apparatebau, Norderstedt, Germany). The sections were stained with either toluidine blue or Stevenel's blue and alizarin red.

Calibration for morpho-histometric evaluations

All histological measurements were made by a trained assessor (KAAA) after a calibration with another professional (DB) performed until the inter-rater agreement in the recognition of the tissues reached a $K > 0.90$.

Histomorphometric evaluations

The histological measurements were carried out using the software NIS-Elements D (v 4.0, Laboratory Imaging, Nikon Corporation, Tokyo, Japan) on an Eclipse Ci microscope (Nikon Corporation, Tokyo, Japan) equipped with a video camera (Digital Sight DS-2Mv, Nikon Corporation, Tokyo, Japan).

The area of the augmented space was evaluated in all three periods of healing while the residual defects on the antrostomy were measured at the 8-week period of healing.

The following regions within the augmented space were analyzed (Figure 2): (i) close to the medial and lateral bony walls (Bone wall regions), (ii) in the center of the elevated space (Middle region), (iii) subjacent the sinus mucosa (Sub-mucosal region), (iv) and in close vicinity to the antrostomy, still within the sinus (Close-to-window region). The antrostomy (Antrostomy region) was evaluated in three different zones: close to the lateral and medial margins and in the center of the antrostomy.

To perform morphometric measurements, a point-counting procedure was used.²² Lattices with squares of 75 μm in dimensions were superposed onto the image of the histological slide at x100 magnification. The proportions of the following tissues were included in the analyses: mineralized bone, marrow spaces, dense and loose matrix tissues, connective tissue, xenograft, inflammatory cells, osteoclasts, vessels and membrane residues.

As linear measurements, the following tissues in contact with the xenograft surface were evaluated at a magnification of x100: mineralized bone, marrow spaces, dense and loose matrix tissues, osteoclasts, and vessels. Moreover, the area of the augmented sinus and of the residual defects in the outer part of the antrostomy were measured.

As an explorative aim, the intersection point²³ was evaluated between dense tissue and newly formed bone in contact with the graft surface (bone-to-graft contact).¹⁷

Data analysis

The primary outcome variable was the osteoconductivity as expressed by bone-to-graft contact. The total mineralized bone was used as secondary variable. Mean values and standard deviations were reported for each outcome. Mean values were obtained for the two histological slides. All calculations were carried out using the software Excel 2013 (Microsoft Corporation, Redmond, WA, USA). Statistical analyses were performed for both primary and secondary variables using the IBM SPSS Statistics software (IBM Inc., Chicago, IL, USA). The Wilcoxon test was used to evaluate differences between large and small particle sites. The level of significance was set at 5%.

Results

No perforations of the mucosa were noticed during the surgical procedures. All biopsies were collected and histologically processed, and an $n = 6$ was achieved for each period of healing.

The volumes evaluated at the microCT (Figure 3A – C) were about 132 mm³, 114 mm³, 115 mm³ at the sites with large granules, 123 mm³, 104 mm³, 118 mm³ at the sites with small granules after 2, 4 and 8 weeks, respectively. No statistically significant differences were found between large and small granule sites at any of the three healing periods analyzed.

The histological analyses (Figure 4A – C) showed that the augmented area slightly decreased over time in both augmentation sites, from 17.5 ± 3.8 mm², 16.5 ± 2.0 mm² and 14.6 ± 1.0 mm² at the small particles sites, and from 17.1 ± 2.3 mm², 16.6 ± 2.5 mm², and 15.3 ± 3.1 mm² at the large particles sites, after 2, 4, and 8 weeks, respectively. No statistically significant differences were found between sites and between healing periods. Small residual defects in the outer part of the antrostomy were present after 8 weeks of healing in both the small (0.3 ± 0.2 mm²) and the large (0.4 ± 0.3 mm²) particle sites. No statistically significant differences were found between sites either.

After 2 weeks of healing (Figure 5A,B), at the histological analyses of the content of the augmented sinuses (Table 1), bone was found at percentages of $7.0 \pm 4.5\%$ in the small granule sites and $6.3 \pm 3.4\%$ in the large granule sites ($p = 0.686$). Xenograft was occupying about 50% of the area in both sites ($p = 0.753$). Dense

matrix tissue was found at about 20% in both sites ($p = 0.917$) surrounding the xenograft granules, while loose matrix tissue was interposed among the granules. New bone was found growing within the granules and in close contact with the xenograft surfaces (Table 2). The percentages of tissues in contact with the xenograft were mainly represented by dense tissue (~70%), while newly formed bone was covering 11 – 12% ($p = 0.753$) of the surfaces. Bone was found at higher percentages in the bone wall regions (Table 3). Osteoclasts were found at percentages of ~5% ($p = 0.462$).

After 4 weeks of healing (Table 1; Figure 6A,B), newly formed bone was occupying 17-18% ($p = 0.173$) of the areas analyzed, and xenograft was still occupying ~44-49% of this area ($p = 0.116$). The dense tissue proportions decreased to ~13% ($p = 0.917$). The tissues in contact with the xenograft surfaces (Table 2) were now mainly represented by newly formed bone (~49%; $p = 0.753$) while the dense tissue proportions were reduced to ~35-36% ($p = 0.674$). Osteoclast proportions also decreased to ~2% ($p = 0.395$).

After 8 weeks of healing (Table 1; Figure 7A,B), the new bone within the augmented area further increased to ~28% ($p = 0.753$) in both the small and large granule sites, while the dense tissue was reduced to 4-6% ($p = 0.173$). The percentages of xenograft were similar to those of the previous periods of healing. New bone in contact with the xenograft (Table 2) was coating $65 \pm 7.3\%$ of the surface at the small granule sites and $62 \pm 8.7\%$ at the large granule sites ($p = 0.345$). The corresponding percentages of the dense tissue were $15.0 \pm 8.1\%$ and $21.0 \pm 10\%$ at the small and large granule sites, respectively. Osteoclasts were found at percentages < 1%.

In both granule augmentation sites, the intersection point between new bone and dense tissue (Table 4) occurred earlier at the Bony wall region and later at the Schneiderian membrane region (Figure 8).

Discussion

The aim of the present experiment was to evaluate the osteoconductivity of deproteinized bovine bone mineral (DBBM) with granules of different sizes used for maxillary sinus floor augmentation. As a secondary aim, bone formation within the augmented sites was to be evaluated. No differences were found in terms of new bone formation and bone-to-graft contact.

After 2 weeks of healing, the tissues in contact with the DBBM surfaces were represented mainly by dense tissue that was surrounding up to about 70% of the surfaces of both grafts, while new bone was present at a percentage of 11-12%. The highest amounts of bone-to-graft contact were seen close to the bony walls (20-24%) in both augmentation sites. The new bone in contact with the xenograft increased during the following periods of healing reaching fractions of 62-65% after 8 weeks, while the dense tissues were reduced correspondingly. These outcomes are in agreement with those of another study in rabbits in which small DBBM granules or a collagen sponge were used to augment the sinus floor in rabbits. In that study, healing was evaluated after 7, 14, 21, and 40 days.¹¹ After 40 days of healing, 68.1% of bone-to-graft contact was found. It was also shown that the highest bone contact to the DBBM granules in the early phases of healing was located close to the bony walls. Moreover, when the data representing the percentage of total bone (mineralized bone and marrow spaces) and that of the soft tissues (dense and loose tissues) were illustrated in a graph, the lines representing the percentages in the various periods analyzed were intercepting each other at different periods. The earliest intersection occurred at the regions close to the bony walls followed by the middle and the sub-mucosal regions. This may be interpreted as if bone formation on the DBBM surfaces started from the bony walls and then, proceeded towards the other regions owing to the osteoconductive properties of the biomaterial. Also in the present study, similar graphs were prepared, however, using only the data of new bone and dense tissues. At the small granule sites, analogous outcomes to the previous study¹¹ were observed. However, for the large granule sites, a delayed intersection point for the middle region was found compared to the small granule sites, so that the intersection point was similar to that observed in the Sub-mucosa region. This, in turn, may indicate a higher osteoconductivity of small *versus* large DBBM particles in the central portion of the grafted region. Nevertheless, the total amount of bone-to-graft contact in all regions was similar in both groups. These findings are not in agreement with those of a study in minipigs¹⁷ in which large or small DBBM particles, like those applied in the present experiment, were used for maxillary sinus floor augmentation. Implants were immediately installed and the animals were euthanized after 6 or 12 weeks. The full augmented area was analyzed, and a higher proportion of bone-to-graft contact was observed at the small granule compared to the large granule sites in the early phases of healing. This disagreement in the outcomes compared to the present study might be due to the different animal model used and to the different method applied for the microscopic

analysis. Moreover, no dental implants were used in the present study, fact that might have altered the results.

Osteoconductivity has been defined as the process by which bone grows on a surface.²⁴ This may depend on the material onto which bone will grow. In that respect, the surface of implants has of great influence on osteoconduction as well.^{23,25} In an analysis of various experimental and clinical studies,²³ new bone apposition on the implant surface in relation to the resorption of the pre-existing bone (old bone) in contact with the implant surface was evaluated. Graphs were prepared with lines representing new bone formation and old bone resorption. The point at which the lines were crossing was defined as the intersection (or interception) point of osseointegration, and it was expressed as time of occurrence and percentage (%) of osseointegration. The intersection point strongly depended on the model used, the bone density and the surface characteristics of the implant surface.

In the present study, within the augmented area, the pattern of healing of the various tissues examined at the various periods of healing was similar in both augmentation sites. New bone increased in both sites from 6-7% to about 28% between 2 and 8 weeks of healing. During the same period of observation, the graft was reduced in percentages from about 51-53% to 44-46%. These findings supported those reported by other experimental studies that showed an increased bone formation and a decreased percentage of deproteinized bovine bone mineral (DBBM) graft over time.^{11,17,18}

A histological evaluation of the healing at maxillary sinus augmented with either small or large granules of DBBM was performed both in experimental¹⁷ and clinical studies.^{8,16,21}

In a minipig experiment,¹⁷ the fraction of new bone found after 6 weeks was 39.0% for the small granule sites, and 40.0% for the large granule sites. These fractions increased to 44.3% and ~45.1% for the small and large granule sites after 12 weeks, respectively. In turn, the percentages of DBBM decreased from 25.1% to 21.3% for the small granule sites, and from 24.6% to 19.8% for the large granule sites. In agreement with that study the results of the present study allowed the conclusion that the size of the granules did not influence new bone formation or DBBM degradation in augmented sites.

In a randomized clinical study (RCT),²¹ after 6 to 9 months of healing, the fraction of bone and DBBM was 28.0% and 34.6% at the small granule and 27.1% and 33.7% at the large granule sites, respectively. No differences were found between sites.

In another RCT study,¹⁶ 10 patients were recruited for a bilateral sinus floor elevation. After 8 months of healing, the new bone fraction was 36.1% and 23.8% and the DBBM fraction was 32.3% and 38.6%, at the small and large granule sites, respectively. Again, the differences were not statistically significant.

However, in another multicenter RCT,⁸ different results were obtained for the fraction of new bone. After 6-8 months, biopsies were collected from 11 patients. The small granule sites presented with 18.8% of new bone and 21.7% of DBBM. The large granule sites displayed significantly higher proportions of new bone of 26.8% and 20.0% of DBBM. Obviously, the biopsies retrieved did not represent the entire

spectrum of the augmented area. Moreover, the results of the observation period of that study cannot really be compared to the results of the present study.

Conclusion

In conclusion, the results of the present study have demonstrated that similar osteoconductive performances and similar proportions of new bone were observed in both augmentation sites and hence, both small and large granules of DBBM xenografts may be recommended for sinus floor augmentation.

The main limitation of the present study is the use of phylogenetically lower animals than human.

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Conflicts of interests

The authors declare no conflict of interest.

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ANEXO IV

RESUMO DA PRODUÇÃO CIENTÍFICA DURANTE O DOUTORADO

1. Artigos completos em periódicos:

1. MASUDA, K.; **SILVA, E.R.**; BOTTICELLI, D., APAZA ALCCAYHUAMAN, K.A., XAVIER, S.P. Antrostomy preparation for maxillary sinus floor augmentation using drills or a sonic Instrument: A microcomputed tomography and histomorphometric study in rabbits. **Int J Oral Maxillofac Implants**, v. 34, n. 4, Jul/Ago, 2019.

2. IIDA, T., **SILVA, E.R.**, LANG, N.P., APAZA ALCCAYHUAMAN, K.A., BOTTICELLI, D., XAVIER, S.P. Histological and micro-computed tomography evaluations of newly formed bone after maxillary sinus augmentation using a xenograft with similar density and mineral content of bone: An experimental study in rabbits. **Clin Exp Dent Res**, v. 23, n. 6, Nov, 2018.

3. DE SANTIS, E., **SILVA, E.R.**, MARTINS, E.N.C., FAVERO, R., BOTTICELLI, D., XAVIER, S.P. Healing at the interface between autologous block bone grafts and recipient sites using n-butyl-2-cyanoacrylate adhesive as fixation: Histomorphometric study in rabbits. **J Oral Implantol**, v. 43, n. 6, Dez, 2017.

2. Resumos publicados em anais de congresso:

1. BALAN, V.F.; CHAVES, L.B.; **SILVA, E.R.**; XAVIER, S.P. Comparative analysis of autogenous and xenogenous onlay bone blocks for rabbit mandible augmentation. In: Anais do VII Congreso Región Latioamericana LAR-IADR, 2018.

2. CHAVES, L.B.; BALAN, V.F.; **SILVA, E.R.**; XAVIER, S.P. Histological and histomorphometrical evaluation of autogenous and xenogenous onlay bone blocks in rabbits. In: Anais do VII Congreso Región Latioamericana LAR-IADR, 2018.

3. BALAN, V.F.; CHAVES, L.B.; **SILVA, E.R.**; BOTTICELLI, D.; XAVIER, S.P. Comparação entre os enxertos autógeno e xenógeno do tipo *onlay*: estudo microtomográfica em mandíbulas de coelhos. In: Anais da 35ª Reunião Anual da Sociedade Brasileira de Pesquisa Odontológica, 2018.

4. CHAVES, L.B.; BALAN, V.F.; **SILVA, E.R.**; BOTTICELLI, D.; XAVIER, S.P. Avaliação histomorfométrica entre os enxertos autógeno e xenógeno em mandíbulas de coelho: estudo piloto. In: Anais da 35ª Reunião Anual da Sociedade Brasileira de Pesquisa Odontológica, 2018.

5. XAVIER, S.P.; **SILVA, E.R.**; PITOL, D.L.; CHAVES, L.B.; BALAN, V.F.; BOTTICELLI, D. Comparative analysis of autogenous vs xenogenous bone grafts in rabbit mandible: Preliminary histological results. In: Anais da 26th International Conference on Advanced Dental Care, 2018.

6. **SILVA, E.R.**; CHAVES, L.; BALAN, V.; PITOL, D.; BOTTICELLI, D.; XAVIER, S. Histomorphometric study on onlay xenograft remodeling: preliminary results in rabbits. In: Anais da 27th Annual Scientific Meeting of the European Association for Osseointegration, 2018.

7. **SILVA, E.**; BALAN, V.; CHAVES, L.; BOTTICELLI, D.; XAVIER, S. Microtomographic study on onlay grafts in rabbits – autograft vs xenograft: preliminary results. In: Anais da 27th Annual Scientific Meeting of the European Association for Osseointegration, 2018.

3. Apresentações de trabalho:

1. BALAN, V.F.; CHAVES, L.B.; **SILVA, E.R.**; XAVIER, S.P. Comparative analysis of autogenous and xenogenous onlay bone blocks for rabbit mandible augmentation. In: VII Congreso Región Latioamericana LAR-IADR, Montevidéo, 2018.

2. CHAVES, L.B.; BALAN, V.F.; **SILVA, E.R.**; XAVIER, S.P. Histological and histomorphometrical evaluation of autogenous and xenogenous onlay bone blocks in rabbits. In: Congreso Región Latioamericana LAR-IADR, Montevidéo, 2018.

3. BALAN, V.F.; CHAVES, L.B.; **SILVA, E.R.**; BOTTICELLI, D.; XAVIER, S.P. Comparação entre os enxertos autógeno e xenógeno do tipo *onlay*: estudo microtomográfica em mandíbulas de coelhos. In: 35^a Reunião Anual da Sociedade Brasileira de Pesquisa Odontológica, Campinas, 2018.

4. CHAVES, L.B.; BALAN, V.F.; **SILVA, E.R.**; BOTTICELLI, D.; XAVIER, S.P. Avaliação histomorfométrica entre os enxertos autógeno e xenógeno em mandíbulas de coelho: estudo piloto. In: 35^a Reunião Anual da Sociedade Brasileira de Pesquisa Odontológica, Campinas, 2018.

5. XAVIER, S.P.; **SILVA, E.R.**; PITOL, D.L.; CHAVES, L.B.; BALAN, V.F.; BOTTICELLI, D. Comparative analysis of autogenous vs xenogenous bone grafts in rabbit mandible: Preliminary histological results. In: 26th International Conference on Advanced Dental Care, Moscou, 2018.

6. **SILVA, E.R.**; CHAVES, L.; BALAN, V.; PITOL, D.; BOTTICELLI, D.; XAVIER, S. Histomorphometric study on onlay xenograft remodeling: preliminary results in

rabbits. In: 27th Annual Scientific Meeting of the European Association for Osseointegration, Viena, 2018.

7. **SILVA, E.**; BALAN, V.; CHAVES, L.; BOTTICELLI, D.; XAVIER, S. Microtomographic study on onlay grafts in rabbits – autograft vs xenograft: preliminary results. In: 27th Annual Scientific Meeting of the European Association for Osseointegration, Viena, 2018.