



UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”

FACULDADE DE ODONTOLOGIA DE ARARAQUARA



SUZANE CRISTINA PIGOSSI

**Avaliação de compósitos à base de celulose bacteriana-
hidroxiapatita com peptídeo osteogênico para reparação óssea**

ARARAQUARA

2014



UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”

FACULDADE DE ODONTOLOGIA DE ARARAQUARA



SUZANE CRISTINA PIGOSSI

**Avaliação de compósitos à base de celulose bacteriana-
hidroxiapatita com peptídeo osteogênico para reparação óssea**

Dissertação apresentada ao Programa de Pós-Graduação em Odontologia - Área de Periodontia da Faculdade de Odontologia de Araraquara da Universidade Estadual Paulista Júlio de Mesquita Filho, para obtenção do título de Mestre em Odontologia.

Orientadora: Prof^ª. Dr^ª. Raquel Mantuaneli Scarel-Caminaga

Co-orientadora: Dr^ª. Sybele Saska Specian

ARARAQUARA

2014

SUZANE CRISTINA PIGOSSI

**Avaliação de compósitos à base de celulose bacteriana-
hidroxiapatita com peptídeo osteogênico para reparação óssea**

COMISSÃO JULGADORA

DISSERTAÇÃO PARA OBTENÇÃO DO GRAU DE MESTRE

Presidente e Orientador: Prof^ª. Dr^ª. Raquel Mantuaneli Scarel Caminaga

2º Examinador: Prof^º. Dr^º. Sérgio Roberto Peres Line

3º Examinador: Prof^º. Dr^º. Carlos Rossa Júnior

Araraquara, 21 de Março de 2014

SUZANE CRISTINA PIGOSSI

Dados curriculares

Nascimento: 22 de Setembro de 1989

Naturalidade: Rio Claro - SP

Filiação: Antonio Pigossi
Sandra Aparecida Buccioli Pigossi

2007-2011 Curso de Graduação em Odontologia -
Faculdade de Odontologia de Araraquara - FOAr
Universidade Estadual Paulista - UNESP

2012-2014 Curso de Pós-Graduação em Odontologia,
Área de Concentração em Periodontia
Nível Mestrado
Faculdade de Odontologia de Araraquara - FOAr
Universidade Estadual Paulista – UNESP

Dedico esse trabalho..

Aos **meus pais**, Antonio Pigossi, Sandra Ap. B. Pigossi, pois sem eles, NADA seria possível. Agradeço por todo amor, carinho e dedicação pela nossa família desde SEMPRE.

Pela educação e ensinamentos que levarei para toda minha VIDA.

Ao **meu irmão**, Vitor Pigossi, um anjinho nas nossas vidas, por toda paciência e companheirismo, estaremos juntos para sempre.

Aos **meus avôs**, João Buccioli e Ilda de Souza Buccioli, pelo amor incondicional por nossa família e por estarem SEMPRE presentes.

Aos **meus tios**, Adilson Nodari e Solange Buccioli Nodari, por me amarem como filha, estando sempre presentes me apoiando em todas as escolhas.

A minha amiga, **Livia Sertori Finoti**, pelo seu apoio em TODOS os momentos. Minha irmã de coração e uma das pessoas mais maravilhosas e importantes da minha vida. Não importa a distância, as dificuldades e o tempo, sempre estaremos juntas!

Agradeço a **DEUS** por todas as coisas boas que tenho na minha vida. Por iluminar meus dias, minha família e meus amigos.

Agradeço a minha orientadora, Profa. Dra. **Raquel Mantuaneli Scarel Caminaga**, pela dedicação ao nosso grupo de pesquisa, por me apoiar nas minhas escolhas acadêmicas e principalmente por estar sempre disposta a fazer o melhor.

Agradeço a minha co-orientadora, Dra. **Sybele Saska Specian**, por nos apoiar desde o início nessa pesquisa compartilhando sua experiência e conhecimentos, pela paciência nos momentos difíceis e principalmente por ter se tornado uma amiga.

Agradeço a minha amiga-irmã, **Livia Amato**, simplesmente por TUDO! Por estar do meu lado desde o começo em todos os momentos, escutando e apoiando todas as minhas escolhas, pelo amor e carinho incondicional e por ser essa pessoa maravilhosa que fez e sempre fará parte da minha vida.

Agradeço ao meu amigo, **Guilherme Oliveira**, uma das pessoas mais dedicadas que eu conheço, por estar presente na reta final de execução desse trabalho compartilhando seus conhecimentos e me apoiando nos momentos difíceis.

Agradeço as minhas amigas, **Livia Bueno, Máira Cristo Daitx, Thamires Reis, Priscila Quintino e Mariana Reis**, companheiras de SEMPRE presentes mesmo quando a correria do dia-a-dia nos impede de estarmos juntas, por todas as risadas, histórias e momentos especiais que compartilhamos. Sem dúvidas são as MELHORES amigas que eu poderia ter.

Agradeço minha amiga, **Flavia Cera**, uma pessoa iluminada que mesmo longe, está sempre presente, que me escuta e apoia em todos os momentos e deixa meus dias muito mais felizes.

Agradeço meus amigos e parceiros de trabalho, **Rafael Nepomuceno Oliveira** e **Sâmia Corbi Tfaile**, não só pelo apoio essencial nas etapas desse trabalho, mas também pelo carinho, apoio e amizade.

Agradeço minha amiga e futura companheira de trabalho **Mayara Terenzi**, que em pouco tempo se tornou essencial, por estar cada vez mais presente me apoiando em todos os momentos. Sem dúvidas, um presente do ano de 2013.

Agradeço as minhas amigas e companheiras de trabalho, **Sâmara Tfaile Corbi** e **Ticiania Sidorenko de Oliveira Capote**, as melhores conselheiras, por me ouvir nos momentos difíceis e estarem sempre dispostas a ajudar.

Agradeço a **Milena Barreto Albuquerque**, minha eterna dupla da faculdade companheira desde o ano 2007 quando tudo começou, por toda paciência, ajuda e carinho em todos esses anos de amizade.

Agradeço as minhas amigas **Giovana Anovazzi** e **Luana Pires**, companheiras do ano de 2012, pela amizade, carinho e apoio de sempre. Nunca esquecerei os momentos que passamos juntas.

Agradeço minha amiga, **Natalia Moreira**, que mesmo à distância está sempre presente, me apoiando e aconselhando nos momentos mais importantes. Obrigada pelo carinho.

Agradeço minha amiga, **Margot Fabiana Pereira**, companheira de colégio presente até hoje na minha vida, por toda ajuda, apoio e carinho desde sempre.

Agradeço aos meus amigos mais que especiais do Mestrado, **Adinael Trindade Junior**, **Letícia Mestieri** e **Vinicius Paiva**, pelos momentos de descontração, alegria e apoio nesses dois anos de trabalho.

Agradeço a **Família Pigossi** e **Chagas** por estarem sempre presentes na nossa vida nos apoiando nas dificuldades e comemorando as conquistas.

Agradeço a **Família Rosa**, minha família de coração e companheiros desde sempre, pelo apoio nos momentos difíceis e por encherem nossas vidas de felicidade. A minha amiga, **Tais Fini Rosa**, que mesmo distante está sempre presente nos meus pensamentos, parceiras desde pequenas estaremos sempre juntas.

Agradeço minha tia de coração, **Vera Sertori Finoti**, a pessoa mais guerreira que conheci até hoje, pela preocupação, dedicação, carinho, amor e por ser essa pessoa única que alegra nossas vidas. Tia Vera você é um exemplo.

Agradeço aos meus tios Vladimir e Josilene Buccioli pelo apoio, carinho e amor que sempre tiveram por mim e por nossa família.

Agradeço a *Faculdade de Odontologia de Araraquara*, na pessoa de sua Diretora, Profa. Dra. **Andreia Affonso Barretto Montandon**, e da Vice-Diretora, Profa. Dra. **Elaine Maria Sgavioli Massucato**, responsável pela minha formação, pela estrutura oferecida para a realização dessa pesquisa.

Agradeço o Coordenador do Curso de Pós-Graduação em Odontologia, Área de Periodontia, Prof. Dr. **Carlos Rossa Júnior**, e a todos os docentes do Curso de Pós-Graduação do Programa de Periodontia, sempre dispostos a ensinar, pela disponibilidade e dedicação ao programa e aos seus alunos.

Agradeço aos docentes do curso de Especialização em Periodontia, Profa. Dra. **Rosemary Adriana Chiérici Marcantonio**, Prof. Dr. **Carlos Rossa Júnior**, Prof. Dr. **Joni Augusto Cirelli** e Prof. Dr. **José Eduardo César Sampaio** pela dedicação ao curso.

Agradeço ao Prof. Dr. **José Scarso Filho**, pela dedicação, apoio e ensinamentos clínicos nos anos de estágio na clínica de Cirurgia na Graduação. Um exemplo de Professor.

Agradeço ao Prof. Dr. **Luis Carlos Spolidorio**, pelo apoio e dedicação durante a elaboração e realização desse trabalho.

Agradeço a Fundação de Amparo à Pesquisa do Estado de São Paulo – **FAPESP**, pelo apoio financeiro.

Agradeço aos meus amigos do Mestrado e Doutorado, **Andressa, Adriana, Chaine, Cássio, Fabiana, Fausto, Lélis, Lígia, Marcel, Mario** e **Paula** pelo carinho, amizade, apoio e pelos momentos de descontração nesses dois anos.

Agradeço aos funcionários da Disciplina de Periodontia, **D. Maria do Rosário, Maria José, Isabela, Claudinha, Ester e Leandro**, sempre dispostos a ajudar, pelo carinho e apoio aos alunos.

Agradeço aos funcionários do Departamento de Morfologia, especialmente o **Luis Antonio Potenza**, por ensinar e ajudar na confecção dos cortes histológicos.

Agradeço aos funcionários da Seção de Pós-Graduação, principalmente a **Mara**, pela paciência e dedicação aos alunos.

Agradeço aos funcionários da **Biblioteca** pela disposição de sempre.

Agradeço a todos que, direta ou indiretamente, colaboraram e tornaram possível a realização deste trabalho.

Pigossi SC. Avaliação de compósitos à base de celulose bacteriana-hidroxiapatita com peptídeo osteogênico para reparação óssea [Dissertação de Mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2014

RESUMO

A celulose bacteriana (CB) é um biopolímero sob a forma de hidrogel nanoestruturado, o qual é uma excelente matriz para incorporação de outros compostos, e nos últimos anos a CB tem demonstrado resultados promissores para reparação tecidual. Além disso, compósitos baseados em CB e hidroxiapatita (CB-HA) são osteocondutores, e quando este compósito associado a peptídeos como OGP (osteogenic growth peptide) e a sua sequência C-terminal o pentapeptídeo OGP(10-14) poderão proporcionar-lhe propriedade osteoindutora. O presente estudo avaliou os compósitos CB-HA associados ou não a estes peptídeos para reparação óssea de defeitos críticos em calvária de camundongos. Os grupos experimentais avaliados foram CB-HA, CB-HA OGP, CB-HA OGP(10-14) e controle, nos respectivos períodos de análise: 3, 7, 15, 30, 60 e 90 dias. Após 3 dias de pós-operatório, as membranas CB-HA OGP e CB-HA OGP(10-14) promoveram maiores níveis de expressão dos principais genes marcadores de formação óssea. Além disso, após 7 dias foi observado na análise histomorfométrica que as membranas CB-HA e CB-HA OGP(10-14) promoveram maior neoformação óssea. Semelhantemente, essas membranas apresentaram maiores porcentagens de neoformação óssea como foi observado na microtomografia computadorizada nos períodos de 60 e 90 dias. Maiores níveis de expressão de alguns biomarcadores ósseos, como *Alpl*, *Spp1* e *Tnfrsf11b* também foram observados para as mesmas membranas em 60 e 90 dias de pós-operatório. Conclui-se que os materiais CB-HA e CB-HA OGP(10-14) apresentaram melhor potencial na reparação óssea em defeitos críticos em calvária de camundongos, destacando o papel osteoindutor nos períodos iniciais de formação óssea da membrana de CB-HA OGP (10-14).

Palavras-Chave: Materiais Biocompatíveis, Celulose, Osteogênese

Pigossi SC. Evaluation of bacterial cellulose/hydroxyapatite nanocomposite with osteogenic growth factor for bone repair [Dissertação de Mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2014

ABSTRACT

Bacterial cellulose (BC) is a biopolymer in the form nanostructured hydrogel, which is an excellent matrix for the incorporation of others compounds, and at last years, BC has demonstrated promising results for tissue repair. In addition, composites based on BC and hydroxyapatite (BC-HA) is osteoconductor; and when this BC-HA composite associated with the osteogenic growth peptide (OGP) or its C-terminal sequence the pentapeptide OGP (10-14) could be provide osteoinductive properties. This study aimed to evaluate the potential of BC-HA composites with or without OGP and OGP (10-14) in bone repair in critical-size calvarial defect in mice. The analyzed experimental groups were BC-HA, BC-HA-OGP, BC-HA-OGP (10-14) and control group in the respective periods: 3, 7, 15, 30, 60 and 90 days of postoperative. After 3 days of postoperative, bone biomarkers were upregulated mainly by BC-HA OGP and BC-HA OGP (10-14) membranes. In addition, a higher new bone formation was observed in the histomorphometric analysis for BC-HA and BC-HA OGP (10-14) membranes 7 days of postoperative. Similarly, in 60 and 90 days, high percentage of bone formation could be observed in micro-computed tomography analysis for BC-HA and BC-HA OGP (10-14) membranes. High expression of some bone biomarkers, such as *Alpl*, *Spp1* and *Tnfrsf11b* were also observed for the same membranes 60 and 90 days of postoperative in mice calvarial. In conclusion, the BC-HA and BC-HA OGP (10-14) membranes promoted a better bone formation in critical-size calvarial defects in mice, mainly the BC-HA OGP (10-14) nanocomposite in the early periods of bone repair.

Keywords: Biocompatible Materials, Cellulose, Osteogenesis.

SUMÁRIO

INTRODUÇÃO	14
PROPOSIÇÃO	21
CAPÍTULO 1	22
CONCLUSÃO	67
REFERÊNCIAS	69
APÊNDICE	75
ANEXO	87

INTRODUÇÃO

A perda óssea associada a traumas, neoplasias, cirurgias reconstrutivas, defeitos congênitos ou a doença periodontal pode levar a grandes déficits de tecido ósseo ⁴². Além disso, a perda óssea pode ser considerada um problema de saúde, uma vez que em casos de doença periodontal, quase metade dos adultos entre as idades de 45 e 65 anos tem periodontite moderada ou avançada com perda óssea associada, que se não tratada pode levar a perda 6.5 dentes/indivíduo ^{34, 42}. Sendo assim, nesses casos em que o processo de reparação óssea fisiológico é insuficiente, é necessário realizar procedimentos de regeneração óssea para reabilitar a função mastigatória e a estética bucal do paciente. Por conseguinte, na última década, inúmeros esforços foram direcionados para desenvolver biomateriais seguros e eficazes para substituir e promover a reparação óssea ^{42, 49}.

A reparação óssea é um processo fisiológico complexo envolvendo muitos fatores regulatórios locais e sistêmicos, como fatores de crescimento e diferenciação celular, hormônios, citocinas e proteínas da matriz extracelular ²⁷. Após a implantação, os biomateriais de forma imediata e espontaneamente adquirem uma camada de proteínas adsorvidas do hospedeiro tais como albumina, fibrinogênio complemento, fibronectina, globulina entre outros, que irão interagir com as células inflamatórias e de adesão ¹. Assim, macrófagos/fagócitos e células gigantes multinucleadas são atraídos para o sítio de implantação. Essas células podem controlar a dissolução dos biomateriais e estimular a formação de novos vasos sanguíneos por meio da produção e liberação de mediadores inflamatórios e fatores de crescimento ²². Rápida e extensa vascularização tem sido mostrada ser um elemento-chave para a formação de tecido ósseo ^{21, 22}. Portanto, o número de células gigantes multinucleadas e o grau de vascularização no sítio de implantação são responsáveis pela degradação e integração do biomaterial e sucesso na reparação do defeito ²².

Os osteoblastos são células presentes no tecido ósseo, capazes de sintetizar matriz mineralizada, colágeno tipo I (COL-1), osteopontina (OPN), fosfatase alcalina (ALP) e sialoproteína óssea (BSP) entre outras proteínas, contribuindo com um vasto potencial regenerativo do tecido ósseo. Mudanças nos níveis de expressão dessas moléculas são característicos de células em diferentes graus de desenvolvimento ósseo ou de maturação. A citodiferenciação em osteoblastos começa a partir de células-tronco mesenquimais multipotentes localizadas na superfície periosteal e na medula óssea ^{3,43}. Na fase intermediária de pré-osteoblastos, expressam-se várias proteínas como ALP, COL-1 e receptor do hormônio da paratireoide (PTHrP). ALP também é expressa pelo osteoblasto maduro, além de BSP e Osteocalcina (OCN) e encontra-se adjacente ao osteóide recém-sintetizado ⁴³.

Em células de calvária de fetos de ratos verificou-se que a expressão de COL-1 foi relativamente alta no início da formação do tecido ósseo, diminuindo em seguida; OPN foi expressa antes de outras proteínas da matriz óssea, incluindo a BSP, que foi detectada pela primeira vez em osteoblastos diferenciados, e a OCN expressou-se na fase de deposição mineral na matriz óssea, sendo que a ALP diminuiu quando a fase de mineralização estava bem avançada. Um pico de expressão de COL-1 foi observado no estágio de proliferação e um pico de expressão de OPN, no estágio de diferenciação do tecido ósseo ⁴.

Todavia, os estágios de migração, proliferação, quimiotaxia, diferenciação celular e síntese de proteínas extracelulares podem ser afetados no processo de reparação óssea, já que cada uma dessas etapas é dependente das condições no local da injúria, como os níveis de fatores de crescimento, hormônios, nutrientes e pH ⁴⁴. Os fatores de crescimento são polipeptídeos biologicamente ativos que afetam a proliferação, a quimiotaxia e diferenciação das células do epitélio, do tecido conjuntivo e ósseo ¹⁶. Dentre os mais importantes fatores de crescimento para o tecido ósseo estão proteínas da superfamília do fator de transformação do crescimento tipo beta (TGF- β) incluindo as proteínas morfogenéticas ósseas (BMPs), o fator

de crescimento similar a insulina (IGF-1 e 2), o fator de crescimento fibroblástico (FGF-2), o fator de crescimento derivado de plaquetas (PDGF) e o fator de crescimento endotelial vascular (VEGF) ¹⁷. O VEGF promove a angiogênese, um pré-requisito para o recrutamento local de células precursoras de osteoblastos durante a formação óssea ¹⁴. A angiogênese, é um processo crítico nas respostas fisiológicas e patológicas em uma variedade de condições, incluindo a regeneração periodontal ^{11, 20, 28}. A habilidade das plaquetas promoverem a cicatrização tem sido explorada recentemente na terapia periodontal regenerativa ¹¹, principalmente devido à ação de VEGF.

Com base nos conhecimentos sobre o processo de regeneração óssea, a engenharia tecidual tem buscado desenvolver um biomaterial ideal que se assemelhe ao osso natural, seja biocompatível e que promova adequadamente o processo de reparação óssea. Nesse contexto, vários materiais à base de polímeros naturais e sintéticos têm sido desenvolvidos para serem utilizados como biomateriais na reparação óssea. Destaque pode ser dado aos polímeros que estão presentes naturalmente em organismos vivos e trazem consigo certas propriedades nativas que são úteis no processo de regeneração tecidual ³⁵.

A celulose é o biopolímero mais abundante no mundo, com uma produção anual de mais de 50 bilhões de toneladas ⁴⁸, sendo que a maior parte da produção ocorre a partir das paredes celulares das plantas. Entretanto, a celulose também pode ser obtida química e enzimaticamente por ação bacteriana. A celulose é um homopolissacarídeo linear formado por unidades de β -D-glicopiranosose unidas em cadeias longas, não ramificadas, por ligações glicosídicas β (1 \rightarrow 4). Este polímero pode ser dividido em duas formas nativas: celulose pura ou celulose complexa. Exemplos de celulose pura são aquelas obtidas diretamente de seu estado natural, como do algodão, da celulose bacteriana e da celulose produzida por algumas algas. A celulose complexa inclui a maior parte da celulose presente na natureza, componente fundamental da parede celular de plantas superiores, mas contém impurezas como lignina, pectina e hemicelulose².

Atualmente acredita-se que a CB possa se tornar um dos produtos biotecnológicos com maior potencial tanto para área da saúde quanto para o setor industrial, pois pode ser obtida em larga escala com baixo custo de fabricação a partir de rotas biossintéticas de bactérias dos gêneros *Gluconacetobacter*, *Rhizobium*, *Sarcina*, *Agrobacterium*, *Alcaligenes*. Como mencionado, esta celulose é quimicamente pura, livre de componentes biogênicos como ligninas, hemiceluloses e outros polissacarídeos encontrados embebidos na celulose vegetal, fator que diminui o custo final da produção.

Gluconacetobacterxylinus (*Acetobacterxylinum*) é uma bactéria gram-negativa quimioheterotrófica, que secreta celulose como camada de exopolissacarídeos; que está disposta em forma de fitas compostas de microfibrilas flutuantes organizadas de forma linear ao longo do eixo axial da bactéria ¹⁰. Vigorosos tratamentos com bases fortes em altas temperaturas permitem a remoção das células bacterianas embebidas na trama permitindo obter um biomaterial não pirogênico, atóxico e biocompatível ¹⁵.

A membrana formada em condições de cultura estática resulta numa estrutura tridimensional constituindo em um sistema ultrafino de nanofibras (10-50 nm), cujas fibras são orientadas uniaxialmente, o que não ocorre em celulose vegetal, cuja dimensão das fibras é micrométrica. Esta estrutura 3-D da CB resulta numa celulose altamente cristalina (60-80%) quando comparado à celulose vegetal (~40%), similar à cristalinidade do algodão (~70%) ⁴⁸, e com maior resistência mecânica. As ligações de hidrogênio inter e intra-molecular mantêm as cadeias de celulose juntas conferindo essas propriedades, além da baixa solubilidade e alta retenção de água.

Com base no conhecimento científico disponível, sabe-se que a membrana de CB pode ser empregada na regeneração óssea guiada, em defeitos periodontais ou peri-implantares ^{18,31,33}, como substituto da dura-máter ³⁰, potencial substituição do menisco ⁹ e cartilagem articular ⁴¹. Nos casos dos defeitos periodontais e periimplantares a CB funciona como uma barreira

mecânica impedindo que células fibroblásticas e tecido conjuntivo fibroso invadam o local dos defeitos ósseos, inibindo a fibrose e promovendo neoformação óssea efetiva no local agredido¹⁸. Novaes et. al.³² (1990) avaliou o potencial de membranas de CB em lesões de furca Classe II e relatou histologicamente que a membrana de CB promoveu regeneração tecidual completa em animais. Semelhantemente, um estudo clínico prévio comparou o potencial das membranas de CB e politetrafluoretileno expandido na regeneração tecidual guiada e demonstrou que ambas as membranas foram efetivas no tratamento de lesões de furca Classe II em molares inferiores¹⁸. Além disso, um relato de caso demonstrou clínica e histologicamente que o uso da membrana de CB associada a hidroxiapatita porosa foi eficaz para o reparo completo do defeito ósseo associado a um implante TiAl6v4 (IMZ)³¹. Portanto, a CB, além de suas excelentes propriedades já descritas, tem sido útil como uma eficiente matriz para o desenvolvimento de novos compósitos para aplicação na regeneração e engenharia tecidual.

Além disso, os polímeros podem estar associados a outros biomateriais como o colágeno tipo I, hidroxiapatita e outros fosfatos de cálcio, que são comumente utilizados na engenharia tecidual óssea⁴⁶. Esses materiais promovem a adesão e diferenciação de células osteoprogenitoras aumentando a formação óssea^{46, 47}. No entanto, esses materiais têm limitações nas suas propriedades mecânicas e dificuldades de degradação, prejudicando sua utilização. Sendo assim, para superar essas limitações, esses têm sido combinados a outros materiais naturais/sintéticos como a CB, melhorando suas propriedades mecânicas⁴².

Desses materiais utilizados, destacam-se os fosfatos de cálcio, que são materiais cerâmicos com razões molares Ca/P variadas, sendo a forma mais conhecida a hidroxiapatita (HA), a qual está presente nos ossos e dentes. A química estrutural da hidroxiapatita biológica é muito complexa, devido a sua composição não ser totalmente pura (não estequiométrica). Frequentemente esta é uma hidroxiapatita deficiente em cálcio ou enriquecida com íons carbonatos, formando a carbonatoapatita²⁹. Como mencionado, a HA é um dos componentes

do tecido ósseo e dentes, e há muitos anos diferentes formas de sínteses e aplicação desses fosfatos de cálcio tem sido avaliada na reconstrução e regeneração de estruturas ósseas. As hidroxiapatitas sintéticas são muito utilizadas para esta finalidade por serem materiais bioativos e com razões molares Ca/P geralmente menor que 1,67. Desta forma, são mais eficazes clinicamente devido a suas similaridades com a composição do tecido ósseo e pela sua propriedade de osteocondução ³⁶.

Fang et al. ¹⁹ (2009) realizaram estudo in vitro com células mesenquimais derivadas da medula óssea de humanos (hBMSC) e verificaram que o compósito à base de CB e hidroxiapatita (HA) não foi citotóxico, promovendo proliferação e diferenciação celular. Estudos revisados por Grande et al (2009) revelaram por avaliações in vitro que compósitos CB-HA têm um ótimo potencial para aplicação em reparação óssea ²³, devido a HA conferir à celulose propriedades bioativas e osteocondutivas ³⁸.

As membranas de CB também podem ser impregnadas com moléculas como fatores de crescimento e proteínas da matriz extracelular que estimulam a proliferação das células ósseas³⁹. O peptídeo de crescimento osteogênico (OGP – *osteogenic growthpeptide*), descoberto no início dos anos 90, é um peptídeo de ocorrência natural cuja estrutura primária é idêntica à sequência C-terminal da histona H4. Está presente fisiologicamente no soro humano, de ratos, e aparentemente de outras espécies de mamíferos em concentração micromolar ⁶. Esta conservação evolucionária indica a importância biológica do OGP ²⁴. A maioria (cerca de 90%) do OGP sérico encontra-se especialmente na forma de um complexo de proteína de ligação OGPBP-OGP (OGPBP: OGP *bindingprotein*) ⁶.

A concentração do OGP sérico é aumentada transitoriamente durante uma injúria local ao tecido ósseo, medula óssea ou a reações osteogênicas sistêmicas, e também quando baixas doses de OGP exógeno são administradas, já que ele é requerido para a estimulação da formação óssea. Isso sugere uma função auto-regulativa para os complexos OGPBPs ^{6, 24, 25}. Bab et al.⁷

(1999) verificaram que a sequência PreOGP, é traduzida do RNA mensageiro da própria H4 [85-103], ou seja, configurando o PreOGP contendo os aminoácidos [85-103], o qual é convertido em OGP endógeno [90-103] após a clivagem proteolítica que resulta na remoção de cinco aminoácidos terminais (e.g. H4 [90-103]). Este mecanismo biossintético, aparentemente está presente em muitas, se não em todas, as células, podendo desempenhar uma função crucial no *feedback* positivo do OGP, controlando a proliferação e diferenciação de células ósseas e hematopoiéticas. Defeitos em ossos longos de coelhos tratados com *scaffolds* de PLGA (poly(lactic-co-glycolic acid) com OGP sintético adsorvido demonstraram, através da análise radiográfica e histológica, menor tempo de reparo ósseo em relação aos animais que receberam o OGP por via sistêmica, indicando um importante resultado na via de administração destes peptídeos, já que o efeito terapêutico foi mais eficaz quando aplicado localmente ⁴⁰.

A clivagem proteolítica do OGP pode gerar o pentapeptídeo OGP (10-14), que também tem como função estimular proliferação e diferenciação de células osteoprogenitoras e células progenitoras hematopoiética. A regeneração hematopoiética está relacionada ao efeito secundário do OGP ^{7, 12, 26, 39}. In vitro o peptídeo OGP (10-14) possui excelente potencial mitogênico para células de linhagem fibroblástica e osteoblástica ^{6, 8, 24}, favorecendo a neoformação óssea ^{13, 45}. Também favorece o aumento da atividade da fosfatase alcalina (ALP) e da mineralização da matriz óssea ^{5, 37}. Desta forma, este peptídeo apresenta um grande potencial para utilização na medicina regenerativa, empregando-o na funcionalização de biomateriais.

A metodologia detalhada desse trabalho científico está presente no Apêndice.

PROPOSIÇÃO

O objetivo do presente estudo foi avaliar o potencial do compósito à base de celulose bacteriana-hidroxiapatita associado ou não aos peptídeos OGP e OGP (10-14) para reparação óssea em defeitos críticos de calvária de camundongos por meio das análises de microtomografia computadorizada, histologia, histomorfometria e expressão gênica de biomarcadores ósseos.

Evaluation of bacterial cellulose/hydroxyapatite nanocomposite with osteogenic growth peptides for bone repair.

Suzane C. Pigossi^{*†}, Guilherme J. P. L. Oliveira^{*}, Livia S. Finoti^{*†}, Rafael N. Oliveira^{*†}, Luis Carlos Spolidorio[✧], Carlos Rossa Jr^{*}, Sidney J. L. Ribeiro[‡], Sybele Saska[‡], Raquel M. Scarel-Caminaga^{†**}.

* Department of Oral Diagnosis and Surgery, School of Dentistry, UNESP- Univ. Estadual Paulista, Humaita St, 1680, CEP 14801-903, Araraquara, SP, Brazil.

† Department of Morphology, School of Dentistry, UNESP- Univ. Estadual Paulista, Humaita St, 1680, CEP 14801-903, Araraquara, SP, Brazil.

✧ Department of Physiology and Pathology School of Dentistry, UNESP- Univ. Estadual Paulista, Humaita St, 1680, CEP 14801-903, Araraquara, SP, Brazil.

‡ Department of General and Inorganic Chemistry, Institute of Chemistry, UNESP- Univ. Estadual Paulista, Prof. Francisco Degni St, 55 CEP 14800-900, Araraquara, SP, Brazil.

O artigo será submetido para a revista Biomaterials

**Correspondence: Prof. Dr. Raquel M. Scarel-Caminaga. UNESP- Univ. Estadual Paulista, School of Dentistry, Department of Morphology, CP. 331, CEP 14801-903, Araraquara, SP, Brazil. E-mail: raquel@foar.unesp.br

ABSTRACT

This study aimed to evaluate the potential of bacterial cellulose/hydroxyapatite (BC-HA) composites associated with osteogenic growth peptide (OGP) or pentapeptide OGP (10-14) in bone repair in critical-size calvarial defects in mice. In this study were utilized 264 animals, randomly distributed in four groups (BC-HA; BC-HA-OGP; BC-HA-OGP (10-14); control group) which were analyzed in the periods: 3, 7, 15, 30, 60 and 90 days. In each analysis period, the specimens were evaluated by micro-computed tomography, histology, histomorphometric, gene expression of bone biomarkers and VEGFR-2 (vascular endothelial growth factor) quantification by ELISA immunoassay. After 3 days of postoperative, bone biomarkers were upregulated mainly by BC-HA OGP and BC-HA OGP (10-14) membranes. New bone formation could be observed in the histomorphometric analysis for BC-HA and BC-HA OGP (10-14) membranes 7 days of postoperative. Similarly, in 60 and 90 days, high percentage of bone formation could be observed in micro-computed tomography analysis for BC-HA and BC-HA OGP (10-14) membranes. High expression of some bone biomarkers, such as *Alpl*, *Spp1* and *Tnfrsf11b* were also observed for the same membranes 60 and 90 days of postoperative in mice calvarial. In conclusion, the BC-HA and BC-HA OGP (10-14) membranes promoted a better bone formation in critical-size mice calvarial defects, mainly the BC-HA OGP (10-14) nanocomposite in the early periods of bone repair.

Keywords: Biomaterial, Bacterial Cellulose, Bone repair, Osteogenic Peptide, Gene Expression

1. INTRODUCTION

The increase in lifespan and interest in quality of life have concurrently stimulated the development of new biomaterials for various clinical applications. Bone loss due to trauma, neoplasia, periodontal disease or to other damage, is an important human health problem. Based on this problem, the goal of tissue engineering is to offer effective materials to replace and promote bone repair [1]. In this context, natural polymers as collagen, elastin, alginate, chitosan, starch, and cellulose have all been investigated as biomaterials to promote bone healing [2].

Among these biopolymers, bacterial cellulose (BC) is an emerging biocompatible polymer with good physical and chemical properties characterized by high tensile strength, elastic modulus and hydrophilicity [3, 4]. Furthermore, BC has a unique nano fibril network morphology, similar to collagenous fibers, which mimics the properties of the extracellular matrix and exhibits tissue integration [2, 5]. This feature, makes this polymer even more promising for tissue engineering/regenerative medicine applications, such as in wound healing, artificial skin, coverings for nerve surgery, dura mater prostheses, and as an arterial stent coating [4]. In addition, BC has also been successful used as a physical barrier in the repair of periodontal tissue [6, 7].

Type I collagen, hydroxyapatite (HA), and other calcium phosphate biomaterials have been used to promote osteoblast and osteoprogenitor attachment and differentiation to enhance bone formation [8, 9]. However, these materials alone have mechanical limitations [5]. However, these materials can be made into composites with synthetic/natural polymers to improve their mechanical properties. The incorporation of HA into BC (BC-HA) as an *in vitro* substrate for osteoblasts allowed cells to adhere, proliferate and mineralize better as compared to the BC polymer alone [5, 10-12]. Furthermore, *in vivo* BC-HA nanocomposite membranes

have been shown to be effective in bone repair by accelerating new bone formation at defects on rat tibiae [13].

Osteoblasts are cells completely differentiated responsible by osteogenesis, whose function is to synthesize the organic compounds of organic matrix such as, collagenous proteins (type I and V collagen) and non-collagenous proteins (fibronectin, bone sialoprotein, osteocalcin, osteopontin, alkaline phosphatase, bone morphogenetic proteins, among other) [14]. Thus, osteoblasts regulate the gene expression of these proteins, promoting a dynamic equilibrium of ionic exchanges, mainly of Ca^{2+} and PO_4^{3-} ions and initiating apatite crystal nucleation into bone matrix.

BC membranes can be impregnated with molecules such growth factors and extracellular matrix proteins to encourage cell proliferation, cell attachment, spreading, proliferation and osteoinduction [15-17]. One interesting option is osteogenic growth peptide (OGP), an H4 histone-related peptide. A via alternative translational of H4 genes predicts the synthesis of a 19-amino acid peptide, H4-(85-103), hereby designated Pre OGP, which may be converted to OGP by removal of its five amino-terminal residues [18]. Its primary sequence contains a highly conserved 14-amino acid motif ($\text{NH}_2\text{-ALKRQGRTLYGFGG-OH}$). This peptide was isolated from blood during osteogenic remodeling of post ablation marrow regeneration. [19].OGP is proteolytically cleaved, thus generating the C-terminal pentapeptide ($\text{NH}_2\text{-YGFGG-OH}$, OGP (10-14)).OGP (10-14) may be the physiologically active form of OGP inasmuch as it is this C-terminal pentapeptide, not the full length OGP, that activates the cytoplasmic OGP signaling pathway [20] . Therefore, this suggests that OGP (10-14) is the bioactive form of OGP [21, 22].

Previous studies have established that OGP and OGP (10-14), as a soluble peptide, stimulate the proliferation, differentiation, alkaline phosphatase activity and matrix mineralization in osteoblastic lineage cells [19, 23]. Furthermore, OGP regulates the expression

of transforming growth factor, insulin-like growth factor and basic fibroblast growth factor [24], and also increasing bone formation and trabecular bone density *in vivo*. OGP peptide systemically administered increases the rate of ossification repair in distraction osteogenesis [17,[25]. Using the same approach, OGP and OGP (10-14) have stimulated fracture healing when administered in normal or osteoporotic experimental animals [26, 27]. In addition, OGP can stimulate hematopoiesis *in vivo* [28].

Therefore, based on these biological properties, OGP and OGP (10-14) are considered good candidates for tissue engineering applications. Previous studies have been carried out to evaluate the effectiveness of OGP and OGP (10-14) incorporated into tissue engineered scaffolds/membranes, in which the peptides demonstrated a positive effect on the osteogenic process [15, 29].

Based on the excellent physicochemical properties of BC and the important participation of HA, OGP and OGP (10-14) peptides in bone repair, the aim of this study was to evaluate the potential of a BC-HA nanocomposite associated with OGP and OGP (10-14) for bone repair at critical-size defects in the mice cranial bone by microcomputed tomography, histological and histomorphometric analysis, as well to investigate the gene expression of bone biomarkers in this process.

2. MATERIALS AND METHODS

2.1 In vivo experiments.

All experiments followed protocols approved by the Research Ethics Committee of the Araraquara Dental School, UNESP. Two hundred sixty-four male Balb/c mice (10-weeks-old) were used in this study. The mice were randomly allocated in four groups: a control and three experimental groups, i.e. BC-HA, BC-HA OGP and BC-HA OGP (10-14). General anesthesia was induced using intramuscular injections of ketamine hydrochloride (25 mg/kg; Agener

União, Brazil) and xylazine hydrochloride (5 mg/kg; Bayer, Brazil). Surgery was performed using standard aseptic techniques. After shaving and preparation of the fronto parietal region, an incision (approximately 20 mm long) was made over the interparietal suture. Bone defects not associated with the parietal suture were executed on the left parietal bone (lateral osteotomy) in each animal with a 4-mm-diameter trephine bur (Neodent®, Brazil), under copious saline irrigation. The resulting bone fragments were carefully elevated with a Freer elevator (Quinelato, Schobell Industrial Ltda), thereby maintaining the integrity of the dura mater and brain.

Bone defects in the control group were filled with a blood clot only. Bone defects of the other experimental groups (Group I: BC-HA; Group II: BC-HA OGP; Group III: BC-HA OGP (10-14)) received the respective membrane. Before implantation, membranes were immersed in saline solution and were positioned in order to cover the defect. The flaps were sutured with 4-0 vicryl® (polyglactin 910) and 4-0 mononylon (Ethicon, Johnson & Johnson, Brazil). In the immediate postoperative period, all animals received an intramuscular administration (single dose) of 0.1 ml/kg of sodium dipyrone (Ibasa, Porto Alegre, RS-Brazil).

The animals were evaluated in the 3, 7, 15, 30, 60, and 90 postoperative days. Specimens were harvested and reduced, preserving the periosteum, dura mater and part of the encephalus. One hundred and twenty animals (n=5 per group; n=20 per period) were utilized for the microcomputed tomography analysis (μ CT), histological and histomorphometric analysis; one hundred and twenty animals (n=5 per group; n=20 per period) were utilized to assess the gene expression of bone biomarkers. At least, 24 animals (n=3 per group; n=12 per period) were used to analyze the expression of vascular endothelial growth factor receptor 2 (VEGFR-2) by an enzyme-linked immunosorbent assay (ELISA) at days 3 and 7.

2.2 VEGFR-2 Expression Analysis

The ELISA for growth factor receptor detection was carried out to assess VEGFR-2 expression. Tissue samples were obtained from the calvarial bone defects and the harvested tissue samples (per group/ per period) were macerated and homogenized in a cocktail containing tissue protein extraction reagent buffer (T-PER, Pierce, Rockford, IL). The samples were centrifuged for 5 minutes at 13,000 rpm at 4 °C, and the supernatant was transferred to another tube and stored at -20 °C. The total amount of protein was quantified according to the Lowry method (DC Protein Assay, Bio-Rad). The concentration of the VEGFR-2 protein was measured using a commercially available ELISA kit following the manufacturer's instructions (PathScan® Total VEGFR-2 Sandwich ELISA kit, Cell Signaling Technology, MA, USA). In order to normalize the VEGFR-2 protein concentration, we utilized the values obtained by Lowry methods.

2.3 Gene expression analyses

Transcriptional changes in osteogenic factors were performed in mice calvarial bone defects in the control group and in the respective treatment groups were assessed on postoperative days 3, 7, 15, 30, 60 and 90. After the induction of anesthesia, an 8-mm-diameter trephine bur (Neodent®, Brazil) was centralized on the defect site and a bone sample was harvested from each mouse. Bone samples were stored in RNAlater (AMBION) to conserve RNA integrity. Samples were crushed in dry ice and mRNA was extracted from the bone with TRIzol® (Invitrogen, Carlsbad, CA) and the corresponding cDNA was synthesized using Capacity cDNA Reverse Transcription (Invitrogen™). Messenger RNA (mRNA) levels of osteogenic factors were determined in duplicate by quantitative PCR (qPCR) performed on a real-time PCR system. TaqMan Gene Expression Assay kits (Applied Biosystems, Foster City, CA, USA) were utilized, and the primers and probe sets employed were specific for *Spp1*

(osteopontin; Applied Biosystems catalog number Mm00436767_m1), *Alpl* (alkaline phosphatase; catalog number Mm00475834_m1), *Tnfrsf11b* (osteoprotegerin; catalog number Mm01205928_m), *Runx2* (Runx; catalog number Mm00501584_m1), *Bglap* (osteocalcin; catalog number Mm00649782_gH), *Vegf* (vascular endothelial growth factor; catalog number Mm01281449_m1), and *Gapdh* (catalog number Mm99999915_g1). The optimization of PCR conditions indicated an ideal volume of 0.625 µl for all primers and probes and 3ng of cDNA in each qPCR reaction. All qPCR reactions were performed in a final volume of 12.5 µl in 96-well optical plates using the following cycling parameters: 2 minutes at 50 °C, 10 minutes at 95 °C and 40 cycles of 15 seconds at 95 °C and 60 seconds at 60 °C. The negative control included nuclease-free water instead of cDNA. Calculations to determine the level of gene expression were performed in reference to the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase - *Gapdh*) in the sample using the cycle threshold (Ct) method. The mean Ct values from duplicate measurements were used to calculate expression of the target gene, with normalization to the *Gapdh* gene using Expression Suite Software (Applied Biosystems®).

2.4 Microcomputed tomography analysis (µCT)

At the designated time points, the animals were sacrificed by an overdose of ketamine and xylazine. The heads of five animals in each experimental group were removed and fixed in 10% buffered formalin for 24h; afterwards, the samples were transferred and maintained in a 70% ethanol solution. Subsequently, the samples were scanned and reconstructed with 17.48-µm isotropic voxels on a µCT analysis system (SkyScan 1174, SkyScan, Aartselaar, Belgium), coupled with a 1.0-mm Al filter in the beam. Reconstructions of the images were performed using specific software (NRecon 1.6.1.5 – SkyScan N. V. Belgium). After the reconstruction, the images were three-dimensionally repositioned using Dataviewer software (Skyscan 1174,

Aartselaar, Belgium) with the following parameters: spatial plan: anteroposterior; ROI: round with 120 x 120 pixels; threshold: 64–183.

2.5 Histological analysis

Subsequent to μ CT analysis, the specimens were reduced and decalcified with a solution containing equal parts 50% formic acid and 20% sodium citrate. Routine histological processing for light microscopy was carried out, and 6 μ m thick sections were stained with hematoxylin-eosin (H&E). Sections were analyzed under a DIASTAR optical microscope (Leica Reichert & Jung products, Germany) coupled to a DXC-1107A/107AP digital camera (Sony Electronics Inc, Japan). A researcher (S.C.P) blinded to the experimental groups analyzed the following parameters: mineralized bone quality, evidence of fibrotic tissue formation within the defect site, angiogenesis, inflammatory reactions, degradation, and encapsulation of the membranes. The inflammatory reaction observed in the specimens was measured using a score table according to the ASTM F981-04 standard [30].

2.6 Histomorphometric analysis

This analysis was performed in order to evaluate the percentage of critical- size bone defect closure and to assess the percentage of newly formed bone in the defect using the software Image J (U.S. National Institutes of Health, Bethesda, Maryland, USA). The percentage of bone defect closure was obtained by a linear measurement performed on the middle portion of the bone defect, the initially measured size (TO) and the remaining defect size (TR) were recorded. From these data, the percentage closure was obtained by the equation: $TR \times 100/TO$. Moreover, to evaluate the percentage of newly formed bone, a measurement of the total defect area (TA) and the area of bone formation (AO) was performed. Then, the percentage of newly formed bone was obtained by the equation: $AO \times 100/AT$. In both

analyses, three sections of each sample were randomly selected for analysis. All measurements were performed by a blinded, trained, and calibrated examiner for analysis.

2.7 Statistical Analyses

Quantitative data obtained for VEGFR-2 protein levels, gene expression, μ CT and histomorphometric analyses were tested for normality with the Kolmogorov-Smirnov test. Afterward, for VEGFR-2 protein levels, μ CT and histomorphometric analyses the t test was used, while for gene expression, the Mann Whitney test was used to evaluate differences between each experimental group and the control group. For analysis of the inflammatory reaction the data were tested for normality with the Lilliefors test. Subsequently, the Kruskal-Wallis test and Dunn post-hoc test were used to evaluate differences among groups and regarding the effect of the follow-up time in each group. Moreover, intra-examiner calibration (for histomorphometric analysis and microcomputed tomography analysis) was evaluated by repeating, with a one-week interval, the measurements of 10% of the previously measured samples. The Paired t-tests showed no statistically significant differences between the measurements. In addition, the Pearson correlation test provided a correlation coefficient of $R=0.90$ and $R=0.80$ for the histomorphometric and microcomputed tomography analysis, respectively. All statistical analyses were performed using GraphPad Prim 5 (San Diego, CA, USA) and the statistical differences were considered significant if their p values were less than 0.05 (* $p<0.05$).

3. RESULTS

Postoperative day 3

VEGFR-2 protein concentration associated with BC-HA OGP membrane was found statistically significant lower than the control (Figure 1). Regarding the gene expression analysis, the BC-HA OGP nanocomposite was the material that seemed to induce the greatest gene expression of bone biomarkers, since, excepting the *Spp1* gene, all genes were significantly upregulated in comparison with the control group ($p < 0.05$) (Figure 2A). Similarly, the BC-HA OGP (10-14) nanocomposite induced the expression of more genes involved in bone regulation than the other nanocomposites ($p \leq 0.05$). The investigated material which seemed to induce less expression of bone genes was BC-HA, since only the *Bglap*, gene was upregulated by treatment with this material.

Histological analysis of all experimental groups revealed incipient granulation tissue formation mainly composed of mononuclear leukocytes, supported by thin collagenous fibers, fibroblasts and blood vessels associated with viable osteocytes on the edges of the critical-size defect (Figure 8). Furthermore, in the central portion of the bone defect, no granulation tissue was observed, but many blood clots were observed. A moderate inflammatory reaction associated with the nanocomposites was also observed in relation to the control group ($p < 0.005$). In addition, early bone formation, i.e. an osteoid matrix, was observed in the BC-HA OGP and BC-HA OGP (10-14) groups adjacent to the edges of the bone defects (Figure 8).

Postoperative day 7

There were no significant differences in the mRNA levels of the investigated genes in any of the groups (Figure 2B). The μ CT analysis showed that the critical calvarial defects

(CCD) covered with the BC-HA OGP (10-14) membrane presented statistically significant highest percentage of new bone formation volume than the control group (Figure 5). The histomorphometric analysis revealed that the CCD covered with BC-HA and BC-HA OGP (10-14) membranes presented significantly greater new bone formation than the CCD covered with BC-HA OGP or filled with a blood clot ($p < 0.05$) (Figure 6). At this time point, the granulation tissue was composed of chronic inflammatory cells, newly formed blood vessels and fibroblasts filling the bone defect; moreover, a moderate inflammatory reaction was observed with the BC-HA and BC-HA OGP (10-14) nanocomposites, and a very slight reaction was seen with the BC-HA OGP membrane and in the control group. Although, the BC-HA OGP (10-14) membrane promoted a moderate inflammatory reaction, no statistically significant difference was observed in relation to the control group ($p = 0.0248$). At the edges of the bone defects, viable osteocytes, an osteoid matrix and newly formed immature bone were observed to a greater extent in the treatment groups, i.e. BC-HA, BC-HA OGP and BC-HA OGP (10-14) (Figure 8).

Postoperative day 15

Regarding the gene expression of bone biomarkers, in this period of analysis, the BC-HA OGP was the nanocomposite which promoted significantly higher expression of the majority of genes, such as *Runx 2*, *Alpl*, *Tnfrsf11b* and *Bglap* genes (Figure 3A). Following, the BC-HA OGP (10-14) nanocomposite induced significantly higher expression of the *Alpl*, and *Tnfrsf11b* genes ($p \leq 0.05$).

The μ CT analysis showed a higher percentage of new bone formation in the CCD treated with the BC-HA membrane when compared with control group. (Figure 5). Furthermore, no differences in bone formation were observed between the groups in the histomorphometric analysis (Figure 6).

In the histological analysis of critical-size bone defects, granulation tissue was found to fill the defect, along with the presence of connective tissue that was more fibrous than the previous periods. At the edges of the bone defect, regions with newly formed immature bone were observed, along with bone remodeling and osteoclasts, in all treatment groups. A decrease in the inflammatory response was observed in the treatment groups in comparison to the 7 day time point, except for the BC-HA OGP group, in a significant increase in the inflammatory reaction was observed (mild to moderate) ($p=0.0064$). However, this response was only significant in relation to the control group ($p<0.005$) (Figure 9).

Postoperative day 30

Interestingly, at this time point, BC-HA and BC-HA OGP (10-14) induced significantly higher expression of all genes in relation to the control group. (Figure 3B)..

The μ CT analysis showed that CCD covered with the BC-HA OGP (10-14) nanocomposite presented a higher percentage of new bone formation than all the other materials and the control group, although there were no statistically significant differences among groups (Figure 5). Moreover, no differences were observed between the groups in the histomorphometric analysis (Figure 6).

Moreover the histological analysis demonstrated, the presence of fibrous connective tissue on the edges of the defects with collagenous fibers in a regular pattern in all groups. A mild inflammatory reaction was observed in the treatment groups and the control group, but no statistically significant differences were measured among groups ($p=0.0739$). Newly formed bone was identified in all treatment groups, which was apparently more organized and mineralized in relation to the previous time point. In the defects treated with BC-HA and BC-HA OGP areas of mineralized bone could be identified beneath the membrane (Figure 9).

Postoperative day 60

In relation to the qPCR analysis, BC-HA at this time point continued to demonstrate good induction of gene expression of bone biomarkers, since five of the six investigated genes were upregulated in comparison with the control group (Figure 4A). BC-HA OGP also significantly induced higher expression of four of the six investigated genes: *Runx2*, *Vegf*, *Alpl* and *Tnfrsf11b*. All materials induced downregulation of the *Bglap* gene at this time point, in comparison with the control group.

The μ CT analysis showed that the CCD covered with the BC-HA, BC-HA OGP and BC-HA OGP (10-14) membranes presented a significantly higher percentage of bone formation than the control group ($p < 0.05$) (Figure 5). This result also confirmed by the histomorphometric analysis for BC-HA and BC-HA OGP (10-14) (Figure 6).

At this time point, the critical-size bone defects were not fully repaired yet and newly formed bone appeared in various stages of maturation in all analyzed specimens. However, in the treatment groups, the membranes promoted greater bone formation than in the control group, mainly for the BC-HA OGP (10-14) nanocomposite (Figure 10). The periosteum covering the defect was similar to that observed at the previous time point and the absence of an inflammatory reactions was seen in all groups.

Postoperative day 90

Genes associated with bone mineralization, such as *Alpl* and *Tnfrsf11b* were upregulated by the BC-HA OGP (10-14) membrane (Figure 4B). In addition, the BC-HA membrane triggered a significant increase in *Spp1* gene expression.

Similar to the 60 day follow-up period, μ CT analysis showed that the CCD treated with the BC-HA, BC-HA OGP and BC-HA OGP (10-14) membranes presented a significantly higher percentage of bone formation than the control group ($p < 0.05$) (Figure 5). The volumetric

analysis of newly formed bone also showed greater bone formation and better CCD closure when the BC-HA and BC-HA OGP (10-14) membranes were used, but no statistically significant differences were found between the groups. (Figure 6).

At the final time point, the histological analysis showed that the critical-size bone defects were not fully repaired. However, when compared with μ CT data, these results show that bone formation increased in relation to the previous time point period of 30 days in the specimens treated with BC-HA and BC-HA OGP [10-14] (Figure 5). Newly formed bone also appeared in various stages of maturation in all analyzed specimens at this time point; mainly mature bone was seen, with several osteocytes and blood vessels. No inflammatory response was observed in the treatment groups. Areas of mineralized bone tissue were observed adjacent to the membranes at this time point (Figure 10).

4. DISCUSSION

BC has been widely targeted in the development of new materials due to its excellent physiochemical and biological properties and its high potential for applications in tissue engineering/regenerative medicine [7, 31-38]. Indeed, several researchers have proposed BC for applications in tissue engineering as a scaffold for cartilage [39-41], bone repair [16, 17, 41-44], vascular grafts [45-49] and neural repair [50-52], or as a barrier membrane for guided bone regeneration [7, 13, 31, 32, 44, 53]. Composites or biomaterials based on BC have demonstrated that these materials are excellent at maintaining a cellular niche for stem cell-mediated tissue regeneration. Additionally, stem cells or primary lineage cells on BC alone or BC composites (HA, collagen, gelatin or chitosan) have also demonstrated excellent results regarding cell adhesion and proliferation [43, 54-58]. These materials can be used in various applications such as, bone, cartilage and neural repair, vascular grafts and tracheal grafts.

In this study, the potential of BC-HA nanocomposites associated with OGP and OGP (10-14) were evaluated for bone repair in critical-size defects in mice cranial bone. The results observed to different analyses, mainly bone formation from the histomorphometric analysis, revealed that both the BC-HA and BC-HA OGP (10-14) nanocomposites promoted better bone repair of critical-size defects in mice cranial bone than the BC-HA OGP nanocomposite and the control group.

On postoperative day 3, the histological results showed the presence of an osteoid matrix around the bone defects treated with the OGP and OGP (10-14) membranes. This result is in agreement with the gene expression analysis for bone biomarkers genes, since the BC-HA OGP and BC-HA OGP (10-14) membranes induced the expression of more genes (Figure 2). In this early period of bone repair, gene expression of *Runx2* and *Vegf* could be highlighted because they are related to osteoblast growth and differentiation [59-63]. Similarly, Matsumoto et. al., (2012) demonstrated that the use of a bioactive vitroc ceramic (biosilicate) in rabbit calvarial defects permitted new bone formation and showed intense Runx-2 and VEGF immunoexpression [64]. Runx-2 is an essential transcription factor for osteoblast differentiation and subsequent bone formation [59, 60]. Otto et. al., (1997) demonstrated an interruption of the osteoblasts development that impaired the skeletal formation in Runx2 *-/-* mice [65]. VEGF and their receptors play important roles in the regulation of bone remodeling by inducing neovascularization, attracting endothelial cells, and osteoclasts, and stimulating osteoblast differentiation [62, 66-68]. VEGFR-2 is an endothelial specific receptor tyrosine kinase and is considered as a major transducing receptor of the effects of VEGF [69]. Because of the indirect importance of VEGFR-2 in bone repair, VEGFR-2 protein production was investigated on postoperative day 3. Thus, this study evaluated whether the membranes could have different effects in terms of the VEGFR-2 production, since this protein plays an important role in angiogenesis and could favor bone tissue formation in this early period. Even though the results

were not statistically significant, treatment with the BC-HA OGP (10-14) membrane led to a higher level of VEGFR-2 protein induction on postoperative day 3. This may have occurred due to the potential of OGP (10-14) to stimulate hematopoiesis, thereby increasing blood and bone marrow cellularity *in vivo* and *in vitro* [28, 70, 71]. In addition, OGP (10-14) peptide acts on human hematopoietic stem cells by enhancing the activity of some growth factors *in vitro* [70].

On postoperative day 7, the histomorphometric analysis showed greater bone formation associated with the BC-HA and BC-HA OGP (10-14) membranes (Figure 6). In agreement with this, Saska et al. 2011 observed new bone formation associated a BC-HA membrane used to treat noncritical-size bone defects in the rat tibia after 7 days [13]. In relation to gene expression, no differences were found for any of the investigated materials in comparison to the control group.

On postoperative days 15 and 30, significant differences in the percentage of bone formation were found between BC-HA membrane and control group only for μ CT analyses at 15 days. With regard to the gene expression analysis, at 15 days, the *Bglap* gene mRNA level continued to be high, as well as in the previous time points at 3 and 7 days. The *Bglap* gene is translated into the osteocalcin protein which is the most abundant non-collagenous bone matrix protein. It is associated with the regulation of osteoblast/osteoclast activity during bone mineralization [72-74].

BC-HA and BC-HA OGP (10-14) membranes significantly induced higher *Tnfrsf11b* mRNA levels at the 30 day time point in comparison with control group. This finding confirms the occurrence of bone remodeling at this time point, since the *Tnfrsf11b* gene is translated into the osteoprotegerin protein, which is a key molecule in the interaction between osteoblasts and osteoclasts during bone remodeling [75-77]. The gene expression inductive behavior observed for the BC-HA membrane was interesting; at 15 days lower expression of the investigated genes

was found, but at 30 days, this nanocomposite induced high expression of all the investigated genes. He, (2012) tested an HA-coated hybrid silk scaffold and showed an increase in osteogenic differentiation by the upregulation of *Runx2* and *Spp1* gene after 3 weeks [78]. Moreover, these results show that HA acts as a nucleation site for mineralization and promotes further deposition of HA from seeded cells [78, 79].

The histomorphometric analysis showed a significantly higher percentage of bone formation for the BC-HA and BC-HA OGP (10-14) membrane on postoperative day 60 days (Figure 6), while the μ CT analysis showed that the BC-HA OGP membrane also induced a significantly higher percentage of bone formation in comparison with the control group. Comparative studies evaluating the accuracy of 3D μ CT morphological measurements with traditional 2D measurements have reported a highly significant correlation between the two methods. However, μ CT usually over estimates bone volume [80, 81]. In this study, the results obtained using both techniques were complementary, since differences between the two methods were observed in relation to the percentage of bone formation. Furthermore, the efficiency of the BC-HA and BC-HA OGP (10-14) membranes was confirmed by the histological analysis since bone formation was observed on the membranes surface, indicating the potential of these membranes for inducing bone formation. Regarding the gene expression analysis, at 60 days, the BC-HA membrane demonstrated good effectiveness for inducing significantly higher expression of five of the six investigated genes. It was noted, at 60 days, the mRNA levels of *Alpl* were higher in comparison with the control group for all the membranes tested, but most notably for the BC-HA membrane. This reflects the function of the *Alpl* gene in this time point of the bone repair process since the alkaline phosphatase (ALP) is a key enzyme in osteogenesis. ALP acts as a mineralization promoter by increasing the local concentration of inorganic phosphate, thereby promoting hydroxyapatite deposition [82]. Fang et. al., (2008) found a significant increase in ALP activity after treatment with BC-HA

nanocomposites [11]. On postoperative day 90, the BC-HA membrane induced a decrease in the mRNA levels of *Alpl*, since at this time point, the bone matrix usually is mineralized. On the other hand, this membrane induced significantly higher mRNA levels of *Spp1*, which is important at this time point in bone repair. *Spp1* is a marker of mature osteoblasts produced during by osteogenic differentiation, and is expressed at higher levels at later stages during mineralization when it helps in with bone remodeling [78]. This gene is translated into osteopontin (OPN), an extracellular matrix protein present in several tissues that is strikingly upregulate under conditions of inflammation and bone remodeling [83]. In mineralized tissues, OPN is associated with osteoclastic activity [84, 85], osteoblastic/osteoclastic attachment mediated by $\alpha v/\beta 3$ integrin [86, 87] and the inhibition of HA formation [88]. The BC-HA OGP (10-14) membrane also induced significantly higher expression of *Alpl*, *Runx2* and *Tnfrsf11b* at 90 days. Considering the significant results found for the BC-HA and BC-HA OGP (10-14) membranes regarding gene expression, these results are in agreement with greater percentage of bone formation shown in the μ CT analysis (Figure 5).

The effectiveness of BC-HA membranes in bone repair gradually increased during this study (Figure 7). *In vitro* studies have shown that nanostructured HA incorporated into biomaterials mimics the chemical and morphological nanostructure of natural bone, promoting osteoblast adhesion and proliferation and the deposition of calcium-containing minerals [89, 90]. Furthermore, HA, as a bioceramic, upon contact with tissues and fluid body, i.e. the material tissue interface, initiate reactions on the molecular scale such as the dissolution of Ca^{2+} and PO_4^{3-} ions. Subsequently, there is an increase of the local pH promoted by the liberation of Ca^{2+} ions. This increase in pH stimulates alkaline phosphatase (ALP) activity in pre-existing osteoblast. Simultaneously, newly differentiated active osteoblasts synthesize more ALP, type I collagen and other non-collagenous proteins. Therefore, the pH of the material-tissue interface is gradually reestablished by the action of biological buffers and by decreasing the local

chemical mediators produced by leukocytes, in addition to the liberation of PO_4^{3-} ions from molecules such as, adenosine-5'-triphosphate (ATP) and pyrophosphate, or from adjacent tissue by ALP activity thereby promoting apatite crystal nucleation on collagen fibers. [91]. According to the literature, the use of HA for bone repair have demonstrated efficient results *in vivo* [13, 92, 93]. Moreover, to improve the potential of the BC-HA nanocomposite, the OGP and OGP (10-14) peptides were incorporated into the nanocomposite based on previous reports showing that OGP is useful in bone repair. OGP stimulates cell attachment, proliferation and alkaline phosphatase activity in osteoblastic cells *in vitro* [15, 19, 94, 95] and increase bone mass in rats when injected *in vivo* [19, 25, 94]. In the present study, the BC-HA OGP membranes showed similar performance regarding bone formation (Figure 7), but only at earlier time points (7 and 15 days) compared with the BC-HA and control group. This was also observed for the expression of bone biomarkers at 3 days. This result corroborates a study that utilized OGP incorporated into poly(lactic-co-glycolic) scaffolds, in which more abundant new bone formation was found in non-critical defects in the rabbit radius, on postoperative day 7 [29]. Thus, it can be suggested that an increase in the efficiency of locally administered peptides in the early period can be attributed to the greater availability of peptides during the first events in tissue repair. Therefore, early local release and action of these peptides, i.e. OGP and OGP (10-14), associated with BC membranes or nanocomposites can promote a more rapid response regarding cell proliferation/differentiation. However, other methods of analysis need to be applied to confirm this hypothesis.

The BC-HA OGP (10-14) membranes induced greater new bone formation from the initial periods until day 60 of the analysis (Figure 7). *In vitro* studies have demonstrated that OGP (10-14) promotes the proliferation/differentiation of osteogenic cells, increases alkaline phosphatase activity and enhances mineralized nodule formation [15, 23]. Moreover, the BC-

OGP and BC-OGP (10-14) membranes demonstrated no *in vitro* cytotoxicity, genotoxicity or mutagenicity[15].

All membranes evaluated, in this study, were biocompatible and did not promoted an inflammatory reaction after 60 days. The BC-HA membranes promoted a moderate inflammatory reaction at the early time points, i.e. 3 and 7 days, but a significant decrease in the reaction was observed after 15 days ($p<0.05$). In the BC-HA OGP group, between the periods 3 and 7 days of postoperative occurred a significant decrease of inflammatory degree ($p=0.0064$); however, after 7 days this membrane promoted a significant increase in inflammation ($p=0.0064$), while at 15 days a slight inflammatory reaction was observed ($p=0.0284$). Finally, the BC-HA OGP (10-14) membrane promoted a moderate inflammatory reaction at 3 and 7 days, which was significantly different in relation to the 15 and 30 day time point (mild inflammatory reaction, $p<0.05$). According to the literature, studies using subcutaneous BC membrane application in rats [33] or vascular grafts in pigs [96] demonstrated that BC membranes do not promote foreign body or acute inflammatory reaction postoperative day 90. In this study, the foreign body reaction was not observed at any time point.

Some questions remain regarding the use of these membranes, since this study had some shortcomings. It is unknown how long the OGP peptide is released into the organism induce its effects on bone repair. Moreover, it is worth considering that this model of critical-size calvarial bone defects impair optimum bone healing with membranes without a filling biomaterial. There is a tendency of invasion of the connective tissue due to the membrane collapse or even the invasion of the brain tissue that was not isolated by the membranes into the defect.. In clinical situations, the treatment of a bone defect involves filling the defect with biomaterials, which provides better space maintenance, then covering the defect with a membrane. Therefore, the effects of the nanocomposite membranes tested here on bone repair in critical-size bone defects could be evaluated associated with different types of biomaterials for bone filling. We did not

utilize biomaterials to fill the defects in this study because we intended to challenge the efficacy of the tested membranes. In general, we were satisfied with the results, which demonstrated that even in adverse clinical situations (i.e. without using other biomaterials for bone filling), the nanocomposite membranes are materials with potential applications in bone repair.

The nanocomposite membranes investigated here were biocompatible and promoted new bone formation. In particular, mainly the BC-HA and BC-HA OGP (10-14) membranes were efficient for bone repair in critical-size bone defects, in particular the BC-HA OGP (10-14) nanocomposite in the early periods of bone repair.

REFERENCES

- [1] Zaborowska M, Bodin A, Backdahl H, Popp J, Goldstein A, Gatenholm P. Microporous bacterial cellulose as a potential scaffold for bone regeneration. *Acta Biomater.* 2010;6:2540-7.
- [2] Petersen N, Gatenholm P. Bacterial cellulose-based materials and medical devices: current state and perspectives. *Appl Microbiol Biotechnol.* 2011;91:1277-86.
- [3] Helenius G, Backdahl H, Bodin A, Nannmark U, Gatenholm P, Risberg B. In vivo biocompatibility of bacterial cellulose. *J Biomed Mater Res A.* 2006;76:431-8.
- [4] Czaja WK, Young DJ, Kawecki M, Brown RM, Jr. The future prospects of microbial cellulose in biomedical applications. *Biomacromolecules.* 2007;8:1-12.
- [5] Tazi N, Zhang Z, Messaddeq Y, Almeida-Lopes L, Zanardi LM, Levinson D, et al. Hydroxyapatite bioactivated bacterial cellulose promotes osteoblast growth and the formation of bone nodules. *AMB Express.* 2012;2:61.

- [6] Novaes AB, Jr., Novaes AB. Bone formation over a TiAl6V4 (IMZ) implant placed into an extraction socket in association with membrane therapy (Gengiflex). *Clin Oral Implants Res.* 1993;4:106-10.
- [7] dos Anjos B, Novaes AB, Jr., Meffert R, Barboza EP. Clinical comparison of cellulose and expanded polytetrafluoroethylene membranes in the treatment of class II furcations in mandibular molars with 6-month re-entry. *J Periodontol.* 1998;69:454-9.
- [8] Wahl DA, Sachlos E, Liu C, Czernuszka JT. Controlling the processing of collagen-hydroxyapatite scaffolds for bone tissue engineering. *J Mater Sci Mater Med.* 2007;18:201-9.
- [9] Wang H, Li Y, Zuo Y, Li J, Ma S, Cheng L. Biocompatibility and osteogenesis of biomimetic nano-hydroxyapatite/polyamide composite scaffolds for bone tissue engineering. *Biomaterials.* 2007;28:3338-48.
- [10] Zimmermann KA, LeBlanc JM, Sheets KT, Fox RW, Gatenholm P. Biomimetic design of a bacterial cellulose/hydroxyapatite nanocomposite for bone healing applications. *Materials Science and Engineering: C.* 2011;31:43-9.
- [11] Fang B, Wan YZ, Tang TT, Gao C, Dai KR. Proliferation and osteoblastic differentiation of human bone marrow stromal cells on hydroxyapatite/bacterial cellulose nanocomposite scaffolds. *Tissue Eng Part A.* 2009;15:1091-8.
- [12] Grande CJ, Torres FG, Gomez CM, Bano MC. Nanocomposites of bacterial cellulose/hydroxyapatite for biomedical applications. *Acta Biomater.* 2009;5:1605-15.
- [13] Saska S, Barud HS, Gaspar AM, Marchetto R, Ribeiro SJ, Messaddeq Y. Bacterial cellulose-hydroxyapatite nanocomposites for bone regeneration. *Int J Biomater.* 2011;2011:175362.
- [14] Anselme K. Osteoblast adhesion on biomaterials. *Biomaterials.* England2000. p. 667-81.

- [15] Saska S, Scarel-Caminaga RM, Teixeira LN, Franchi LP, Dos Santos RA, Gaspar AM, et al. Characterization and in vitro evaluation of bacterial cellulose membranes functionalized with osteogenic growth peptide for bone tissue engineering. *J Mater Sci Mater Med*. 2012;23:2253-66.
- [16] Shi Q, Li Y, Sun J, Zhang H, Chen L, Chen B, et al. The osteogenesis of bacterial cellulose scaffold loaded with bone morphogenetic protein-2. *Biomaterials*. 2012;33:6644-9.
- [17] Saska S, Teixeira LN, Tambasco de Oliveira P, Minarelli Gaspar AM, Lima Ribeiro SJ, Messaddeq Y, et al. Bacterial cellulose-collagen nanocomposite for bone tissue engineering. *Journal of Materials Chemistry*. 2012;22:22102-12.
- [18] Bab I, Smith E, Gavish H, Attar-Namdar M, Chorev M, Chen YC, et al. Biosynthesis of osteogenic growth peptide via alternative translational initiation at AUG85 of histone H4 mRNA. *J Biol Chem*. 1999;274:14474-81.
- [19] Bab I, Gazit D, Chorev M, Muhlrad A, Shteyer A, Greenberg Z, et al. Histone H4-related osteogenic growth peptide (OGP): a novel circulating stimulator of osteoblastic activity. *EMBO J*. 1992;11:1867-73.
- [20] Gabarin N, Gavish H, Muhlrad A, Chen YC, Namdar-Attar M, Nissenson RA, et al. Mitogenic G(i) protein-MAP kinase signaling cascade in MC3T3-E1 osteogenic cells: activation by C-terminal pentapeptide of osteogenic growth peptide [OGP(10-14)] and attenuation of activation by cAMP. *J Cell Biochem*. 2001;81:594-603.
- [21] Greenberg Z, Chorev M, Muhlrad A, Shteyer A, Namdar M, Mansur N, et al. Mitogenic action of osteogenic growth peptide (OGP): role of amino and carboxy-terminal regions and charge. *Biochim Biophys Acta*. 1993;1178:273-80.
- [22] Chen YC, Muhlrad A, Shteyer A, Vidson M, Bab I, Chorev M. Bioactive pseudopeptidic analogues and cyclostereoisomers of osteogenic growth peptide C-terminal pentapeptide, OGP(10-14). *J Med Chem*. 2002;45:1624-32.

- [23] Chen ZX, Chang M, Peng YL, Zhao L, Zhan YR, Wang LJ, et al. Osteogenic growth peptide C-terminal pentapeptide [OGP(10-14)] acts on rat bone marrow mesenchymal stem cells to promote differentiation to osteoblasts and to inhibit differentiation to adipocytes. *Regul Pept.* 2007;142:16-23.
- [24] Brager MA, Patterson MJ, Connolly JF, Nevo Z. Osteogenic growth peptide normally stimulated by blood loss and marrow ablation has local and systemic effects on fracture healing in rats. *J Orthop Res.* 2000;18:133-9.
- [25] Zhao ZY, Shao L, Zhao HM, Zhong ZH, Liu JY, Hao CG. Osteogenic growth peptide accelerates bone healing during distraction osteogenesis in rabbit tibia. *J Int Med Res.* 2011;39:456-63.
- [26] Sun YQ, Ashhurst DE. Osteogenic growth peptide enhances the rate of fracture healing in rabbits. *Cell Biol Int.* 1998;22:313-9.
- [27] Chen YC, Bab I, Mansur N, Muhlrud A, Shteyer A, Namdar-Attar M, et al. Structure-bioactivity of C-terminal pentapeptide of osteogenic growth peptide [OGP(10-14)]. *J Pept Res.* 2000;56:147-56.
- [28] Gurevitch O, Slavin S, Muhlrud A, Shteyer A, Gazit D, Chorev M, et al. Osteogenic growth peptide increases blood and bone marrow cellularity and enhances engraftment of bone marrow transplants in mice. *Blood.* 1996;88:4719-24.
- [29] Shuqiang M, Kunzheng W, Xiaoqiang D, Wei W, Mingyu Z, Daocheng W. Osteogenic growth peptide incorporated into PLGA scaffolds accelerates healing of segmental long bone defects in rabbits. *J Plast Reconstr Aesthet Surg.* 2008;61:1558-60.
- [30] ASTM F981-04. Standard practice for assessment of compatibility of biomaterials for surgical implants with respect to effect of materials on muscle and bone. 2010.

- [31] Novaes Jr AB, Moraes N, Novaes AB. Uso do BioFill como membrana biológica no tratamento de lesão de furca com e sem a utilização de hidroxiapatita porosa. *Revista Brasileira de Odontologia*. 1990;47:29-32.
- [32] Simonpietri CJ, Novaes AB, Jr., Batista EL, Jr., Filho EJ. Guided tissue regeneration associated with bovine-derived anorganic bone in mandibular class II furcation defects. 6-month results at re-entry. *J Periodontol*. 2000;71:904-11.
- [33] Helenius G, Backdahl H, Bodin A, Nannmark U, Gatenholm P, Risberg B. In vivo biocompatibility of bacterial cellulose. *Journal of Biomedical Materials Research Part A*. 2006;76A:431-8.
- [34] Mendes PN, Rahal SC, Pereira-Junior OCM, Fabris VE, Lenharo SLR, de Lima-Neto JF, et al. In vivo and in vitro evaluation of an *Acetobacter xylinum* synthesized microbial cellulose membrane intended for guided tissue repair. *Acta Veterinaria Scandinavica*. 2009;51.
- [35] Mello LR, Feltrin LT, Fontes Neto PT, Ferraz FA. Duraplasty with biosynthetic cellulose: an experimental study. *J Neurosurg*. 1997;86:143-50.
- [36] Klemm D, Heublein B, Fink HP, Bohn A. Cellulose: fascinating biopolymer and sustainable raw material. *Angew Chem Int Ed Engl*. 2005;44:3358-93.
- [37] Fontana JD, de Souza AM, Fontana CK, Torriani IL, Moreschi JC, Gallotti BJ, et al. *Acetobacter* cellulose pellicle as a temporary skin substitute. *Appl Biochem Biotechnol*. 1990;24-25:253-64.
- [38] Lin S-P, Calvar IL, Catchmark JM, Liu J-R, Demirci A, Cheng K-C. Biosynthesis, production and applications of bacterial cellulose. *Cellulose*. 2013;20:2191-219.
- [39] Gaspar AMM, Abdalla CDM, Ribeiro SJL, Saska S, Messaddeq Y. Applying bacterial cellulose membrane intra-articularly on damaged surface of patella, and for reconstructing

ligament, tendon and synovial capsule, involves collecting bone cartilage cells and totipotent cells in musculoskeletal tissue. Univ Estadual Paulista; Univ Sao Paulo.

[40] Bodin A, Concaro S, Brittberg M, Gatenholm P. Bacterial cellulose as a potential meniscus implant. *Journal of Tissue Engineering and Regenerative Medicine*. 2007;1:406-8.

[41] Favi PM, Benson RS, Neilsen NR, Hammonds RL, Bates CC, Stephens CP, et al. Cell proliferation, viability, and in vitro differentiation of equine mesenchymal stem cells seeded on bacterial cellulose hydrogel scaffolds. *Mater Sci Eng C Mater Biol Appl*. 2013;33:1935-44.

[42] Gaspar AMM, Marchetto R, Messaddeq Y, Ribeiro SJD, Saska S. Obtaining reabsorbable composites based upon bacterial cellulose and collagen by obtaining bacterial cellulose, esterification of an amino acid or a derivative to the membrane of bacterial cellulose, and incorporation of collagen. *Fapesp Fundacao Amparo a Pesquisa Estado; Univ Estadual Paulista*; 2011.

[43] Zaborowska M, Bodin A, Backdahl H, Popp J, Goldstein A, Gatenholm P. Microporous bacterial cellulose as a potential scaffold for bone regeneration. *Acta Biomater*. England: 2010 Acta Materialia Inc; 2010. p. 2540-7.

[44] Saska S, Scarel-Caminaga RM, Teixeira LN, Franchi LP, dos Santos RA, Minarelli Gaspar AM, et al. Characterization and in vitro evaluation of bacterial cellulose membranes functionalized with osteogenic growth peptide for bone tissue engineering. *Journal of Materials Science-Materials in Medicine*. 2012;23:2253-66.

[45] Andrade FK, Costa R, Domingues L, Soares R, Gama M. Improving bacterial cellulose for blood vessel replacement: Functionalization with a chimeric protein containing a cellulose-binding module and an adhesion peptide. *Acta Biomaterialia*. 2010;6:4034-41.

[46] Klemm D, Schumann D, Udhardt U, Marsch S. Bacterial synthesized cellulose - artificial blood vessels for microsurgery. *Progress in Polymer Science*. 2001;26:1561-603.

- [47] Putra A, Kakugo A, Furukawa H, Gong JP, Osada Y. Tubular bacterial cellulose gel with oriented fibrils on the curved surface. *Polymer*. 2008;49:1885-91.
- [48] Malm CJ, Risberg B, Bodin A, Backdahl H, Johansson BR, Gatenholm P, et al. Small calibre biosynthetic bacterial cellulose blood vessels: 13-months patency in a sheep model. *Scand Cardiovasc J*. 2012;46:57-62.
- [49] Fink H, Hong J, Drotz K, Risberg B, Sanchez J, Sellborn A. An in vitro study of blood compatibility of vascular grafts made of bacterial cellulose in comparison with conventionally-used graft materials. *J Biomed Mater Res A*. 2011.
- [50] Kowalska-Ludwicka K, Cala J, Grobelski B, Sygut D, Jesionek-Kupnicka D, Kolodziejczyk M, et al. Modified bacterial cellulose tubes for regeneration of damaged peripheral nerves. *Arch Med Sci*. 2013;9:527-34.
- [51] Pertile R, Moreira S, Andrade F, Domingues L, Gama M. Bacterial cellulose modified using recombinant proteins to improve neuronal and mesenchymal cell adhesion. *Biotechnol Prog*. 2012;28:526-32.
- [52] Zhu C, Li F, Zhou X, Lin L, Zhang T. Kombucha-synthesized bacterial cellulose: Preparation, characterization, and biocompatibility evaluation. *J Biomed Mater Res A*. 2013.
- [53] Saska S, Teixeira LN, de Oliveira PT, Minarelli Gaspar AM, Lima Ribeiro SJ, Messaddeq Y, et al. Bacterial cellulose-collagen nanocomposite for bone tissue engineering. *Journal of Materials Chemistry*. 2012;22.
- [54] Bodin A, Ahrenstedt L, Fink H, Brumer H, Risberg B, Gatenholm P. Modification of nanocellulose with a xyloglucan-RGD conjugate enhances adhesion and proliferation of endothelial cells: implications for tissue engineering. *Biomacromolecules*. 2007;8:3697-704.

- [55] Backdahl H, Esguerra M, Delbro D, Risberg B, Gatenholm P. Engineering microporosity in bacterial cellulose scaffolds. *Journal of Tissue Engineering and Regenerative Medicine*. 2008;2:320-30.
- [56] Andersson J, Stenhamre H, Bäckdahl H, Gatenholm P. Behavior of human chondrocytes in engineered porous bacterial cellulose scaffolds. *Journal of Biomedical Materials Research - Part A*. 2010;94:1124-32.
- [57] Yadav V, Sun L, Panilaitis B, Kaplan DL. In vitro chondrogenesis with lysozyme susceptible bacterial cellulose as a scaffold. *J Tissue Eng Regen Med*. 2013.
- [58] Berti FV, Rambo CR, Dias PF, Porto LM. Nanofiber density determines endothelial cell behavior on hydrogel matrix. *Mater Sci Eng C Mater Biol Appl*. 2013;33:4684-91.
- [59] Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. *Osf2/Cbfa1*: a transcriptional activator of osteoblast differentiation. *Cell*. 1997;89:747-54.
- [60] Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, Yamaguchi A, et al. *Cbfa1* isoforms exert functional differences in osteoblast differentiation. *J Biol Chem*. 1999;274:6972-8.
- [61] Fujita T, Azuma Y, Fukuyama R, Hattori Y, Yoshida C, Koida M, et al. *Runx2* induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. *J Cell Biol*. 2004;166:85-95.
- [62] Hah YS, Jun JS, Lee SG, Park BW, Kim DR, Kim UK, et al. Vascular endothelial growth factor stimulates osteoblastic differentiation of cultured human periosteal-derived cells expressing vascular endothelial growth factor receptors. *Mol Biol Rep*. 2011;38:1443-50.
- [63] Deckers MM, Karperien M, van der Bent C, Yamashita T, Papapoulos SE, Lowik CW. Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. *Endocrinology*. 2000;141:1667-74.

- [64] Matsumoto MA, Caviquioli G, Bigueti CC, Holgado Lde A, Saraiva PP, Renno AC, et al. A novel bioactive vitroc ceramic presents similar biological responses as autogenous bone grafts. *J Mater Sci Mater Med*. 2012;23:1447-56.
- [65] Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, et al. *Cbfa1*, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*. 1997;89:765-71.
- [66] Street J, Bao M, deGuzman L, Bunting S, Peale FV, Jr., Ferrara N, et al. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc Natl Acad Sci U S A*. 2002;99:9656-61.
- [67] Chim SM, Tickner J, Chow ST, Kuek V, Guo B, Zhang G, et al. Angiogenic factors in bone local environment. *Cytokine Growth Factor Rev*. 2013;24:297-310.
- [68] Zhang W, Zhu C, Wu Y, Ye D, Wang S, Zou D, et al. VEGF and BMP-2 promote bone regeneration by facilitating bone marrow stem cell homing and differentiation. *Eur Cell Mater*. 2014;27:1-12.
- [69] Karkkainen MJ, Petrova TV. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. *Oncogene*. 2000;19:5598-605.
- [70] Fazzi R, Galimberti S, Testi R, Pacini S, Trasciatti S, Rosini S, et al. Bone and bone marrow interactions: hematological activity of osteoblastic growth peptide (OGP)-derived carboxy-terminal pentapeptide. II. Action on human hematopoietic stem cells. *Leuk Res*. 2002;26:839-48.
- [71] Hui Z, Yu L, Xiaoli Y, Xiang H, Fan Z, Ningbo H, et al. C-terminal pentapeptide of osteogenic growth peptide regulates hematopoiesis in early stage. *J Cell Biochem*. 2007;101:1423-9.

- [72] Neve A, Corrado A, Cantatore FP. Osteocalcin: skeletal and extra-skeletal effects. *J Cell Physiol.* 2013;228:1149-53.
- [73] Lopes HB, Santos TD, de Oliveira FS, Freitas GP, de Almeida AL, Gimenes R, et al. Poly(vinylidene-trifluoroethylene)/ barium titanate composite for in vivo support of bone formation. *J Biomater Appl.* 2013.
- [74] Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, et al. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J Cell Physiol.* 1990;143:420-30.
- [75] Ribeiro V, Garcia M, Oliveira R, Gomes PS, Colaco B, Fernandes MH. Bisphosphonates induce the osteogenic gene expression in co-cultured human endothelial and mesenchymal stem cells. *J Cell Mol Med.* 2014;18:27-37.
- [76] Galli C, Macaluso GM, Guizzardi S, Vescovini R, Passeri M, Passeri G. Osteoprotegerin and receptor activator of nuclear factor-kappa B ligand modulation by enamel matrix derivative in human alveolar osteoblasts. *J Periodontol.* 2006;77:1223-8.
- [77] Zong ming W, Jian yu L, Rui xin L, Hao L, Yong G, Lu L, et al. Bone formation in rabbit cancellous bone explant culture model is enhanced by mechanical load. *Biomed Eng Online.* 2013;12:35.
- [78] He P, Sahoo S, Ng KS, Chen K, Toh SL, Goh JC. Enhanced osteoinductivity and osteoconductivity through hydroxyapatite coating of silk-based tissue-engineered ligament scaffold. *J Biomed Mater Res A.* 2013;101:555-66.
- [79] Bhumiratana S, Grayson WL, Castaneda A, Rockwood DN, Gil ES, Kaplan DL, et al. Nucleation and growth of mineralized bone matrix on silk-hydroxyapatite composite scaffolds. *Biomaterials.* 2011;32:2812-20.

- [80] Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Muller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res.* 2010;25:1468-86.
- [81] Chappard D, Retailleau-Gaborit N, Legrand E, Basle MF, Audran M. Comparison insight bone measurements by histomorphometry and microCT. *J Bone Miner Res.* 2005;20:1177-84.
- [82] Allori AC, Sailon AM, Warren SM. Biological basis of bone formation, remodeling, and repair-part II: extracellular matrix. *Tissue Eng Part B Rev.* 2008;14:275-83.
- [83] Giachelli CM, Steitz S. Osteopontin: a versatile regulator of inflammation and biomineralization. *Matrix Biol.* 2000;19:615-22.
- [84] Razzouk S, Brunn JC, Qin C, Tye CE, Goldberg HA, Butler WT. Osteopontin posttranslational modifications, possibly phosphorylation, are required for in vitro bone resorption but not osteoclast adhesion. *Bone.* 2002;30:40-7.
- [85] Suzuki K, Zhu B, Rittling SR, Denhardt DT, Goldberg HA, McCulloch CA, et al. Colocalization of intracellular osteopontin with CD44 is associated with migration, cell fusion, and resorption in osteoclasts. *J Bone Miner Res.* 2002;17:1486-97.
- [86] Denhardt DT, Giachelli CM, Rittling SR. Role of osteopontin in cellular signaling and toxicant injury. *Annu Rev Pharmacol Toxicol.* 2001;41:723-49.
- [87] Katayama Y, House CM, Udagawa N, Kazama JJ, McFarland RJ, Martin TJ, et al. Casein kinase 2 phosphorylation of recombinant rat osteopontin enhances adhesion of osteoclasts but not osteoblasts. *J Cell Physiol.* 1998;176:179-87.
- [88] Hunter GK, Kyle CL, Goldberg HA. Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. *Biochem J.* 1994;300 (Pt 3):723-8.

- [89] Webster TJ, Ergun C, Doremus RH, Siegel RW, Bizios R. Enhanced functions of osteoblasts on nanophase ceramics. *Biomaterials*. 2000;21:1803-10.
- [90] Rajzer I, Menaszek E, Kwiatkowski R, Chrzanowski W. Bioactive nanocomposite PLLDL/nano-hydroxyapatite electrospun membranes for bone tissue engineering. *J Mater Sci Mater Med*. 2014.
- [91] Mann S. *Biomineralization: Principles and concepts in biorganic materials chemistry*. United Kingdom: Oxford - University Press; 2005.
- [92] Fricain JC, Schlaubitz S, Le Visage C, Arnault I, Derkaoui SM, Siadous R, et al. A nano-hydroxyapatite--pullulan/dextran polysaccharide composite macroporous material for bone tissue engineering. *Biomaterials*. 2013;34:2947-59.
- [93] Kim SS, Kim BS. Comparison of osteogenic potential between apatite-coated poly(lactide-co-glycolide)/hydroxyapatite particulates and Bio-Oss. *Dent Mater J*. 2008;27:368-75.
- [94] Fei Q, Guo C, Xu X, Gao J, Zhang J, Chen T, et al. Osteogenic growth peptide enhances the proliferation of bone marrow mesenchymal stem cells from osteoprotegerin-deficient mice by CDK2/cyclin A. *Acta Biochim Biophys Sin (Shanghai)*. 2010;42:801-6.
- [95] Moore NM, Lin NJ, Gallant ND, Becker ML. The use of immobilized osteogenic growth peptide on gradient substrates synthesized via click chemistry to enhance MC3T3-E1 osteoblast proliferation. *Biomaterials*. 2010;31:1604-11.
- [96] Wippermann J, Schumann D, Klemm D, Kosmehl H, Salehi-Gelani S, Wahlers T. Preliminary results of small arterial substitute performed with a new cylindrical biomaterial composed of bacterial cellulose. *Eur J Vasc Endovasc Surg*. England 2009. p. 592-6.

LEGENDS

Figure 1: Enzyme-linked immunosorbent assay (ELISA) analysis of VEGFR-2 protein levels after 3 (A) and 7 (B) days of postoperative. * $p < 0.05$ when compared to control group; mean and standard deviation values

Figure 2: Quantitative real-time RT-PCR analysis on *Runx2*, *Vegf*, *Alpl*, *Spp1*, *Tnfrsf11b* and *Bglap* mRNA after 3 (A) and 7 (B) days of postoperative. * $p < 0.05$ when compared to control group; mean and standard deviation values.

Figure 3: Quantitative real-time RT-PCR analysis on *Runx2*, *Vegf*, *Alpl*, *Spp1*, *Tnfrsf11b* and *Bglap* mRNA after 15 (A) and 30 (B) days of postoperative. * $p < 0.05$ when compared to control group; mean and standard deviation values.

Figure 4: Quantitative real-time RT-PCR analysis on *Runx2*, *Vegf*, *Alpl*, *Spp1*, *Tnfrsf11b* and *Bglap* mRNA after 60 (A) and 90 (B) days of postoperative. * $p < 0.05$ when compared to control group; mean and standard deviation values.

Figure 5: Percentage of new bone volume / tissue volume (BV/TV %) by Microcomputed Tomographic Analysis (μ CT) induced by the use of BC membranes during 7, 15, 30, 60 and 90 days. * $p < 0.05$ when compared to control group; mean and standard deviation values.

Figure 6: Volumetric percentage of bone volume / tissue volume (BV/TV %) by the use of the BC membranes observed in histomorphometric analysis after 7, 15, 30, 60 and 90 days. * $p < 0.05$ when compared to control group; mean and standard deviation values.

Figure 7: Percentage of bone repair by the use of the BC membranes observed in histomorphometric analysis after 7, 15, 30, 60 and 90 days. * $p < 0.05$ when compared to control group; median values.

Figure 8: Histological images of membranes and control group after 3 and 7 days. Inflammatory infiltrate (In); membrane (m); mature bone (B); granulation tissue (I); bone matrix (mo); osteoclasts (osc); Hematoxylin-eosin staining (HE); scale bar (100 μ m).

Figure 9: Histological images of membranes and control group after 15 and 30 days. Inflammatory infiltrate (In); membrane (m); mature bone (B); granulation tissue (I); New bone formation (NB); Hematoxylin-eosin staining (HE); scale bar (100 μ m).

Figure 10: Histological images of membranes and control group after 60 and 90 days. Membrane (m); mature bone (B); granulation tissue (I); New bone formation (NB); Hematoxylin-eosin staining (HE); scale bar (100 μ m)

Figure 1

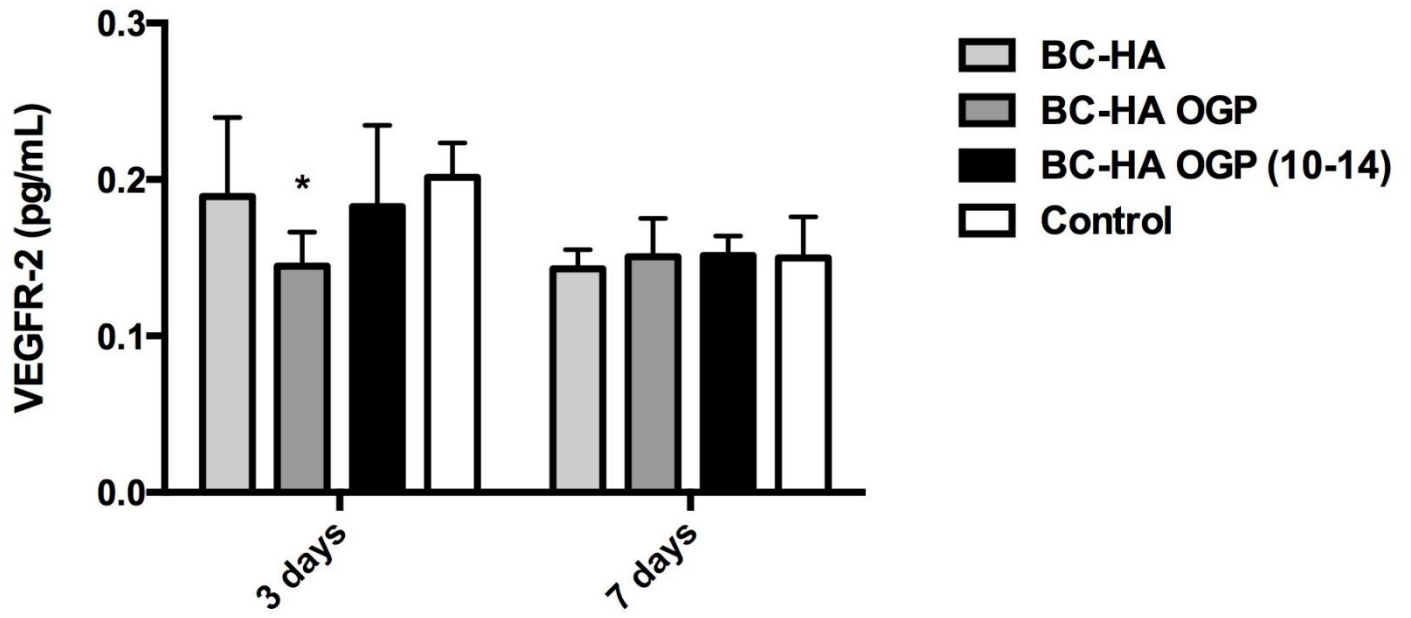


Figure 2

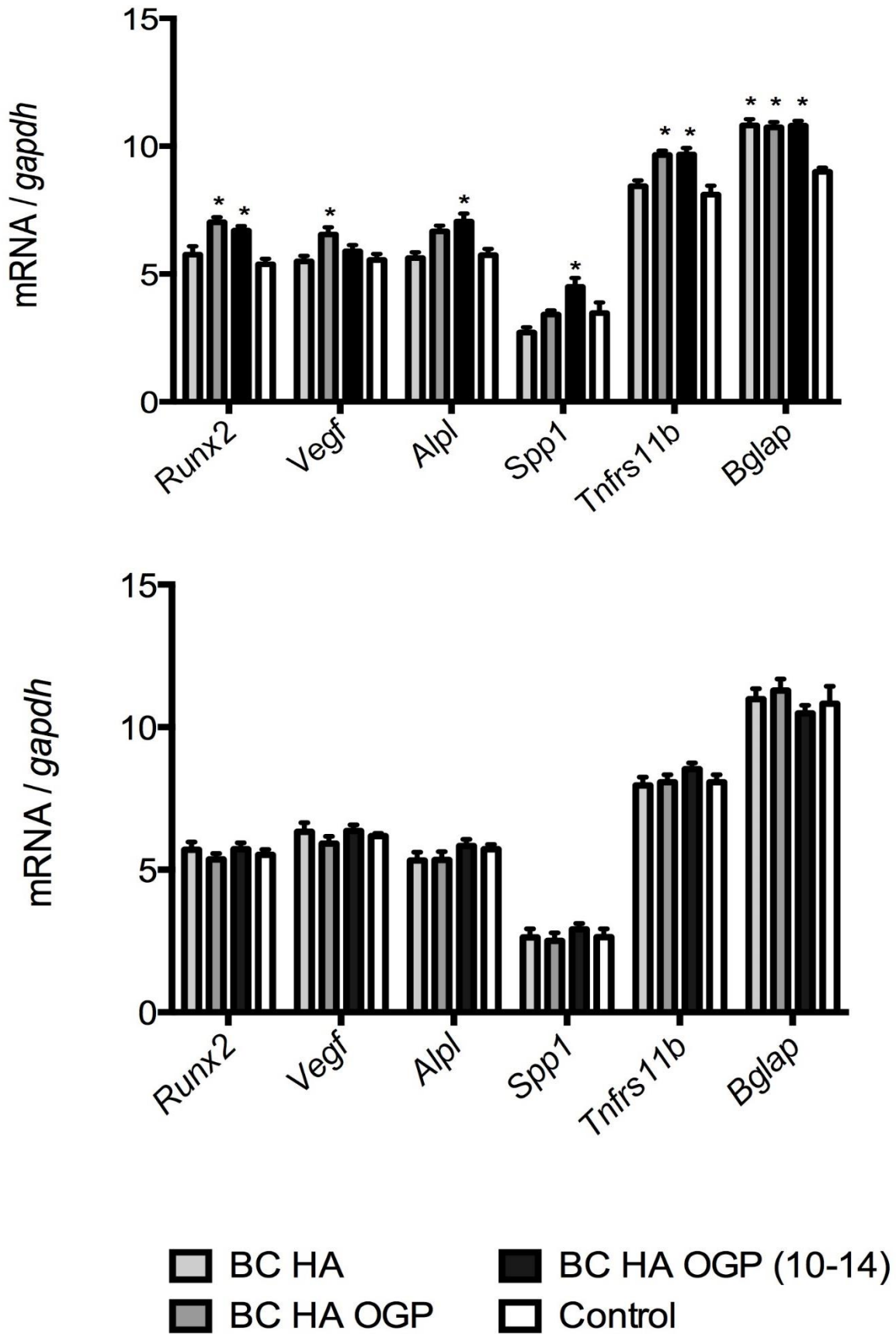


Figure 3

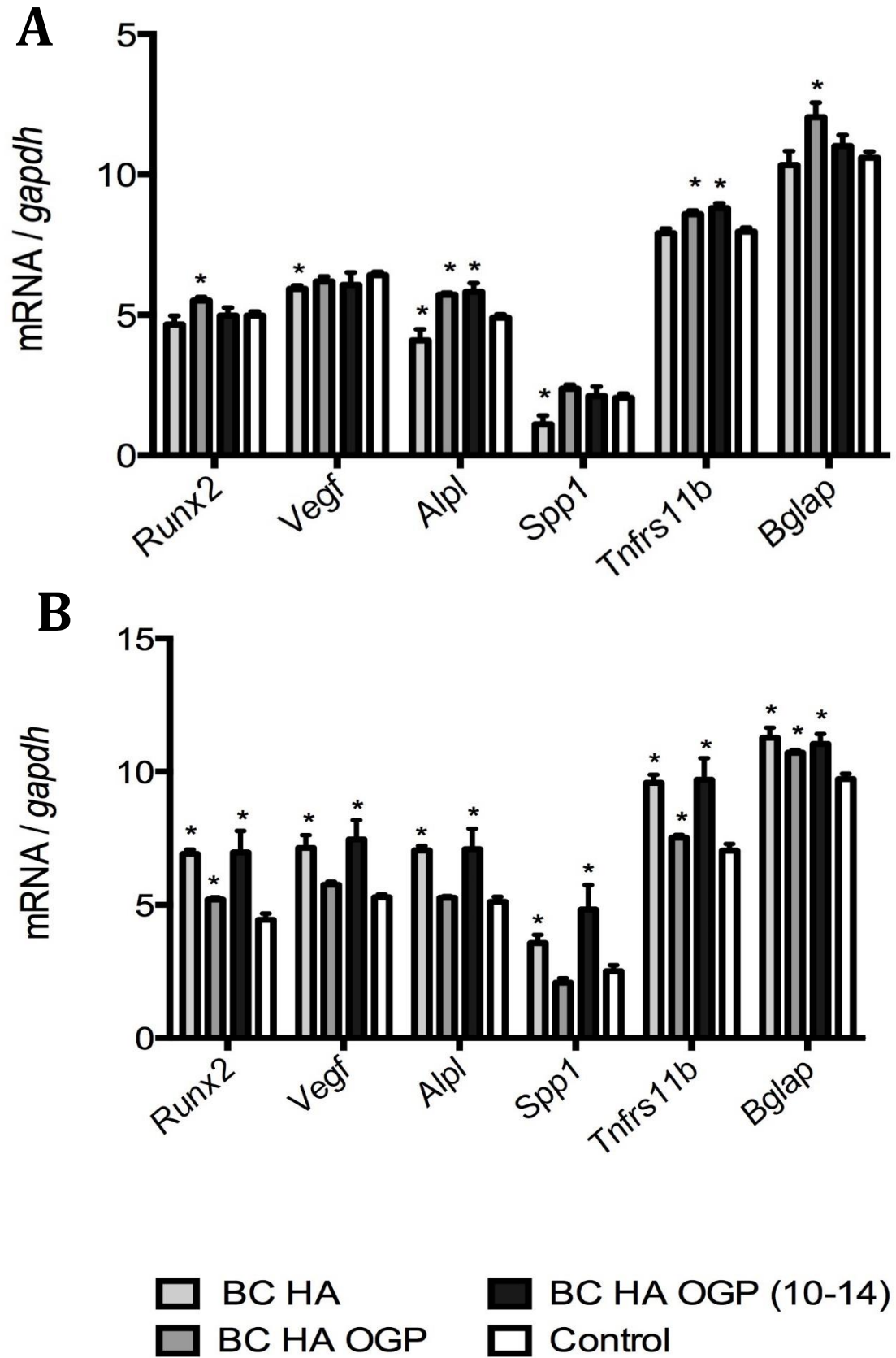


Figure 4

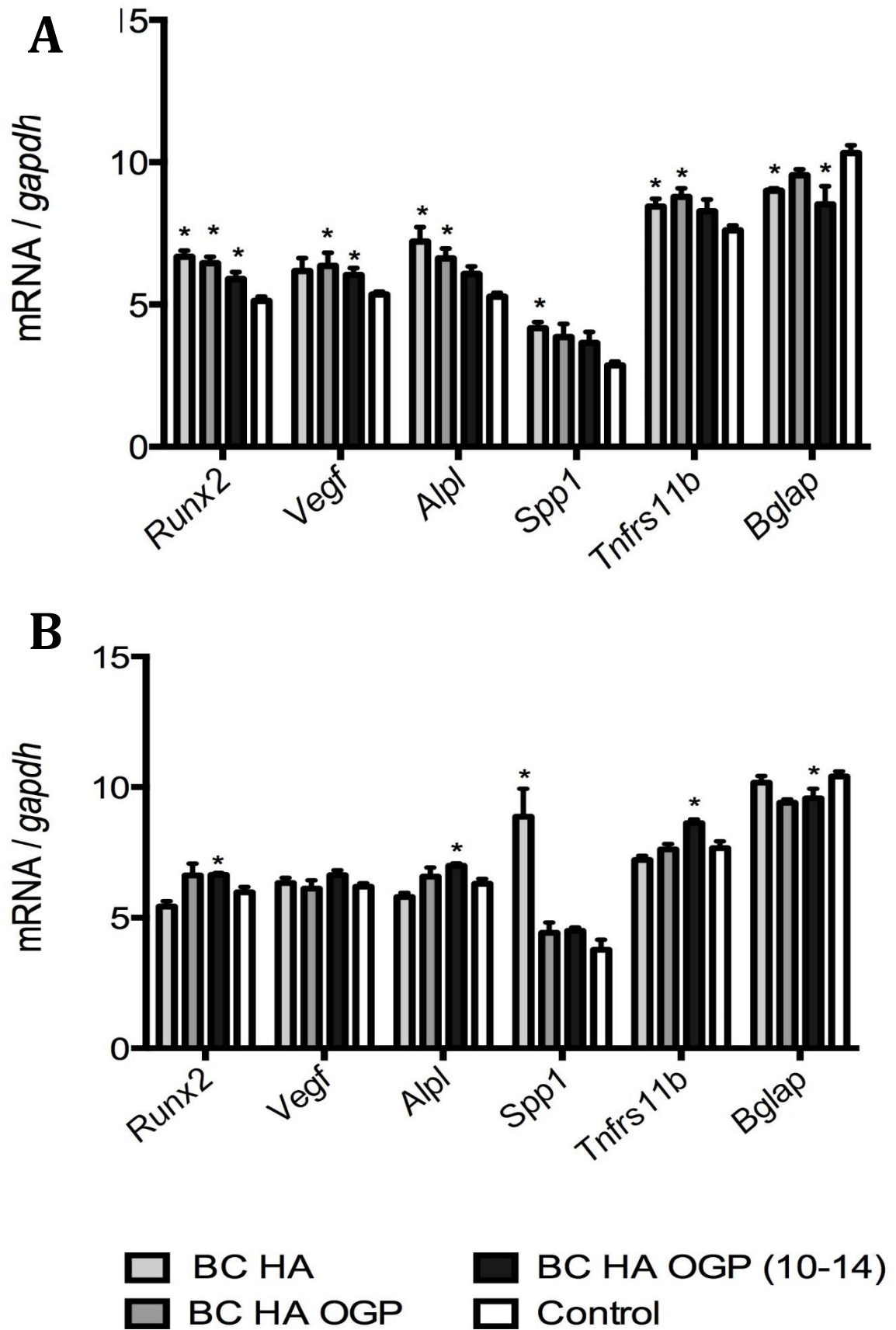


Figure 5

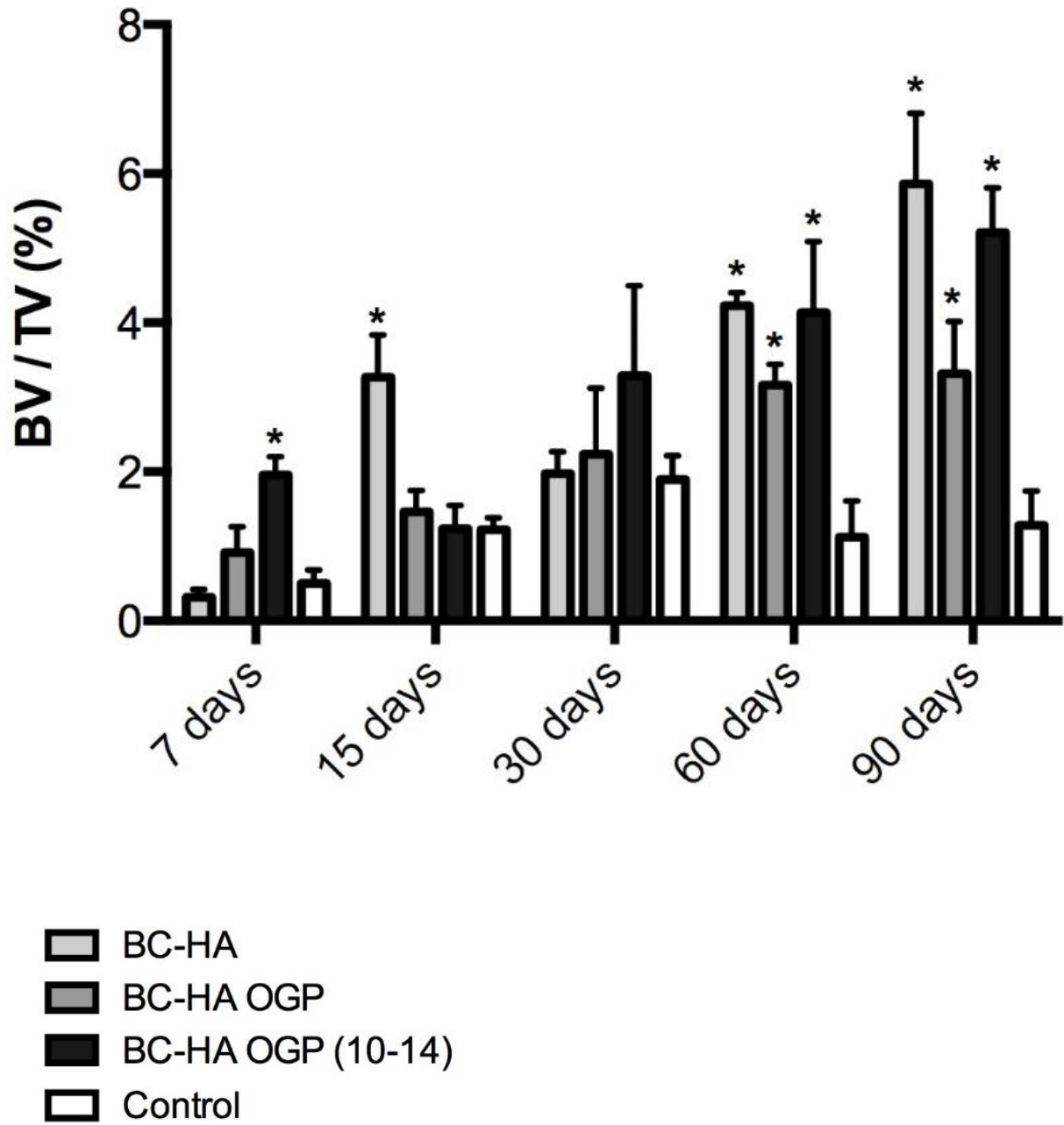
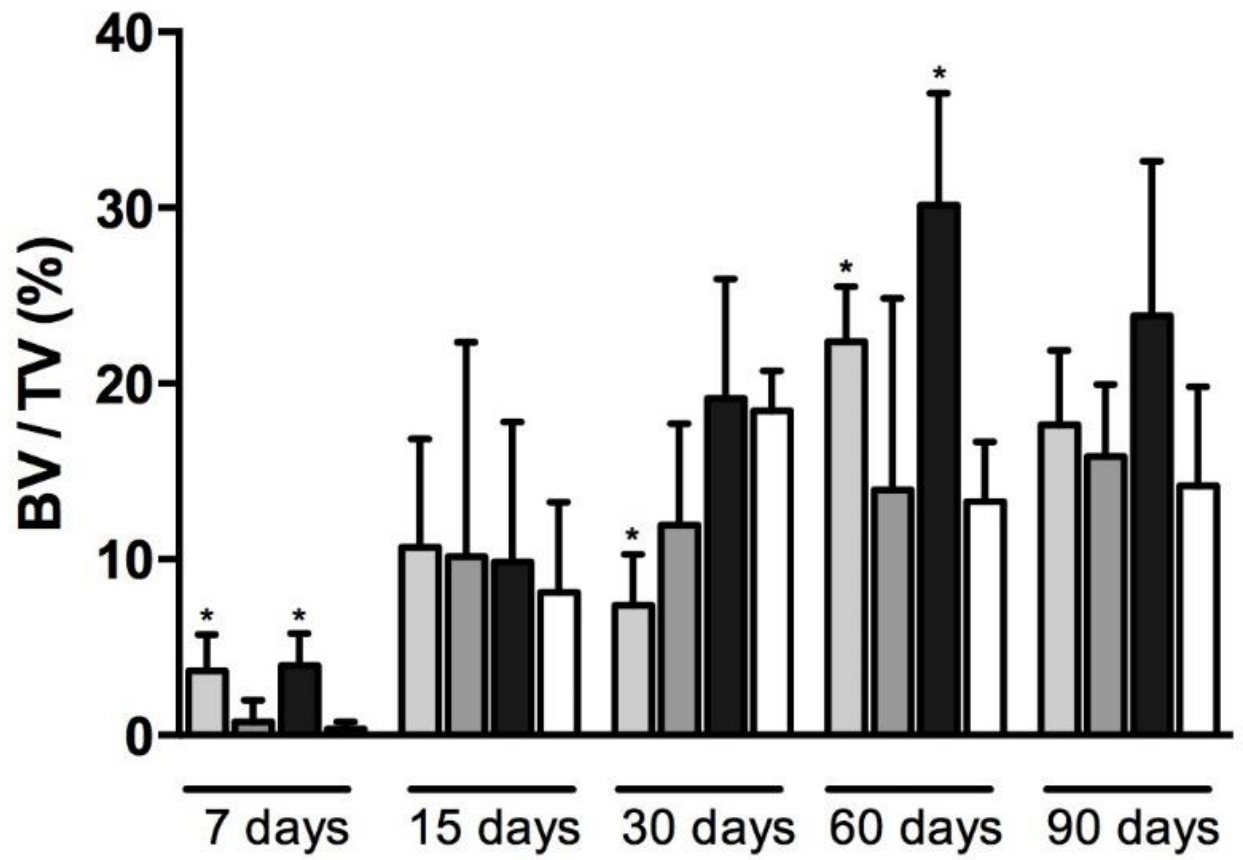
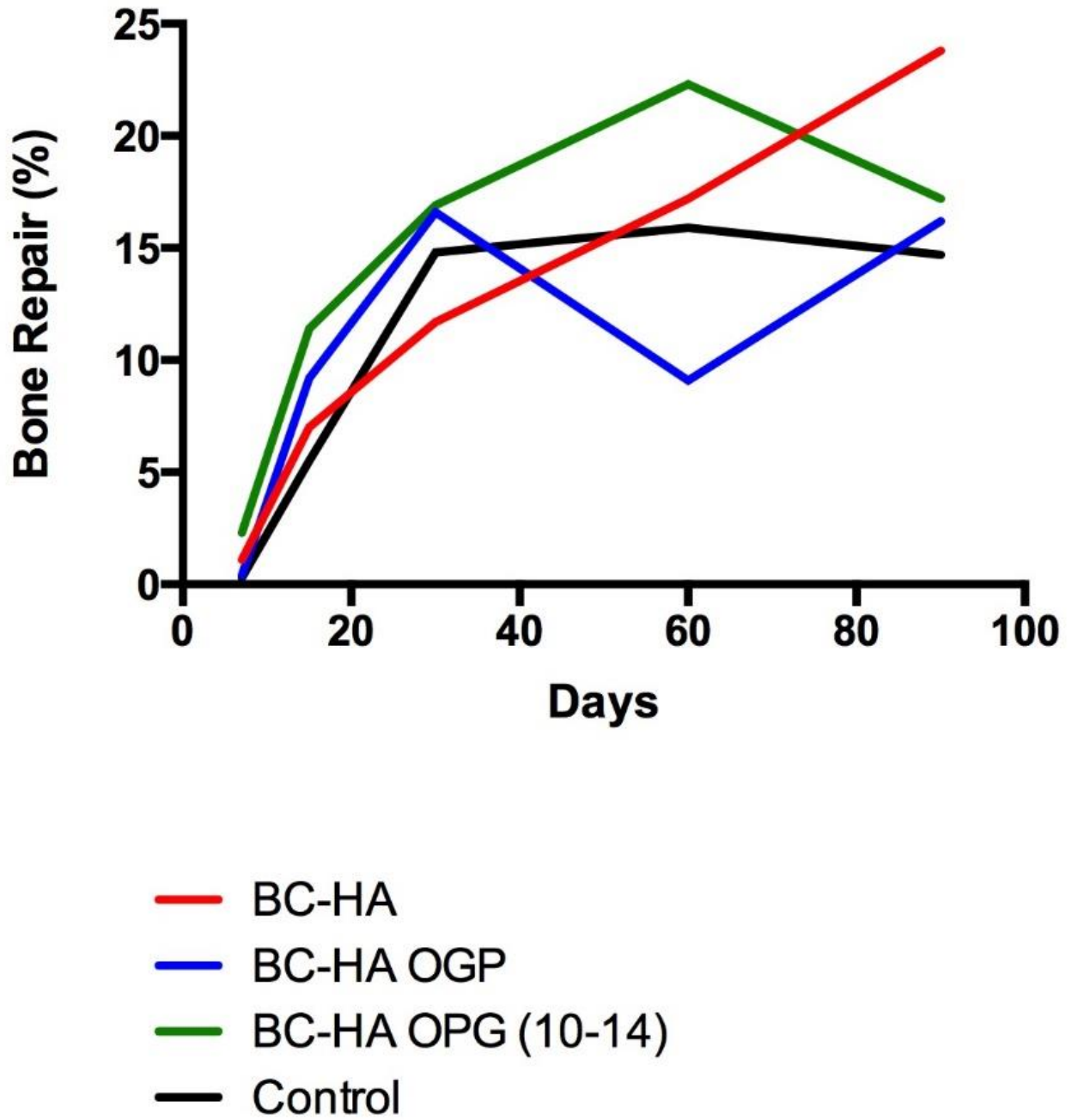


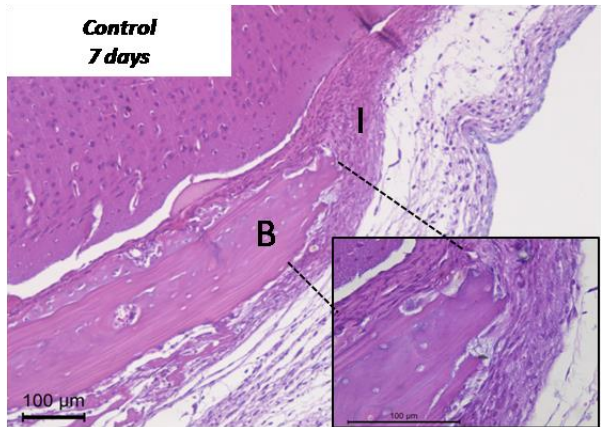
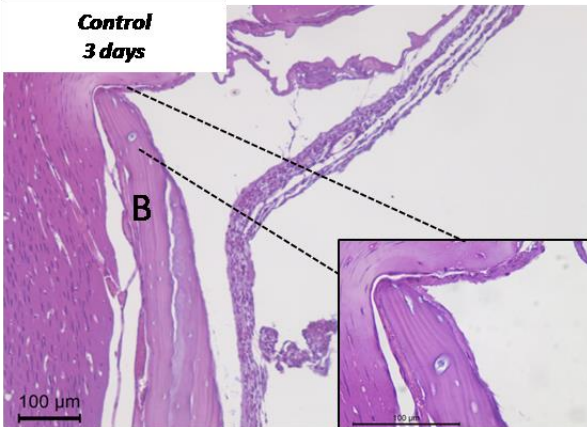
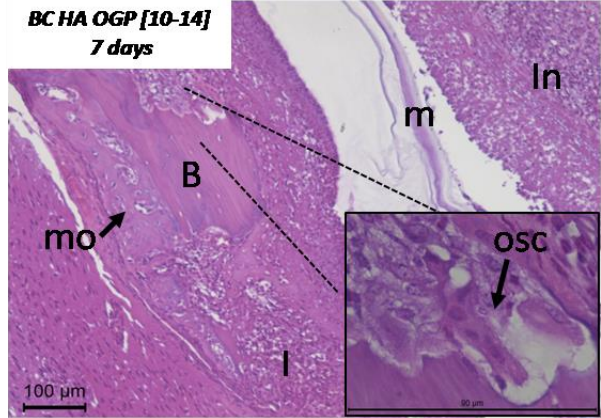
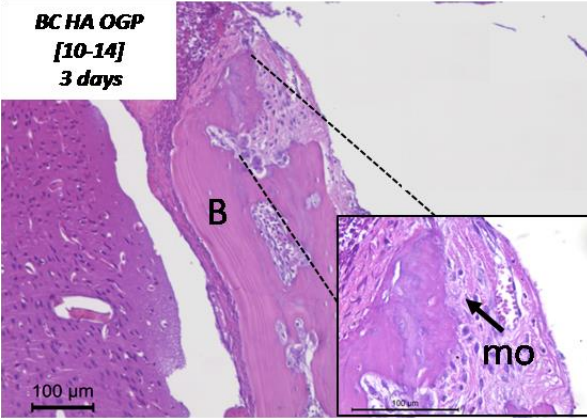
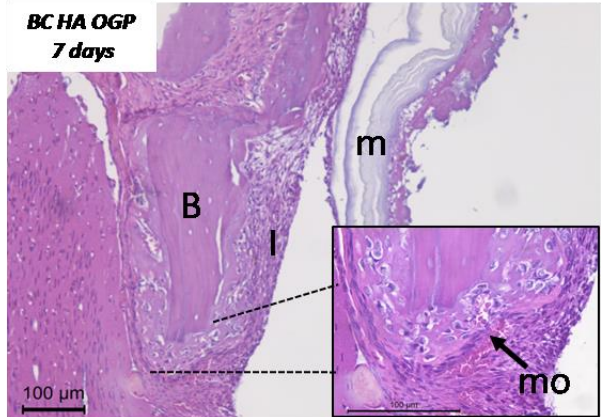
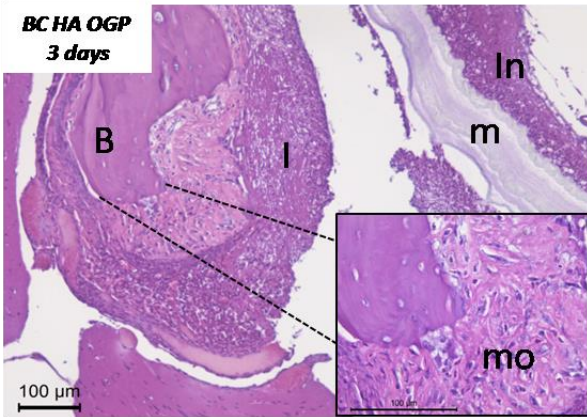
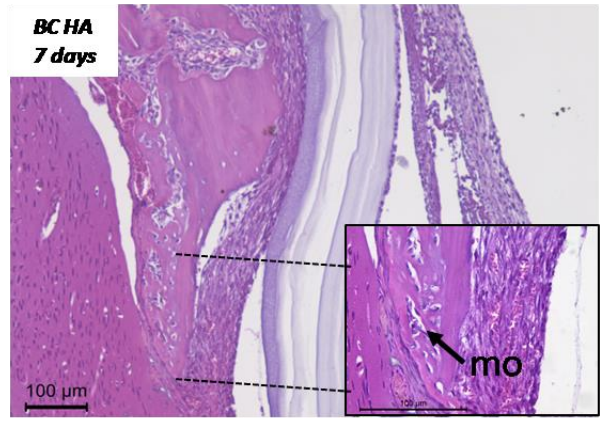
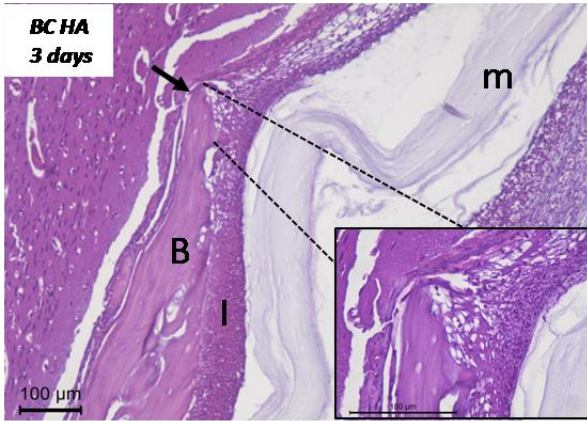
Figure 6

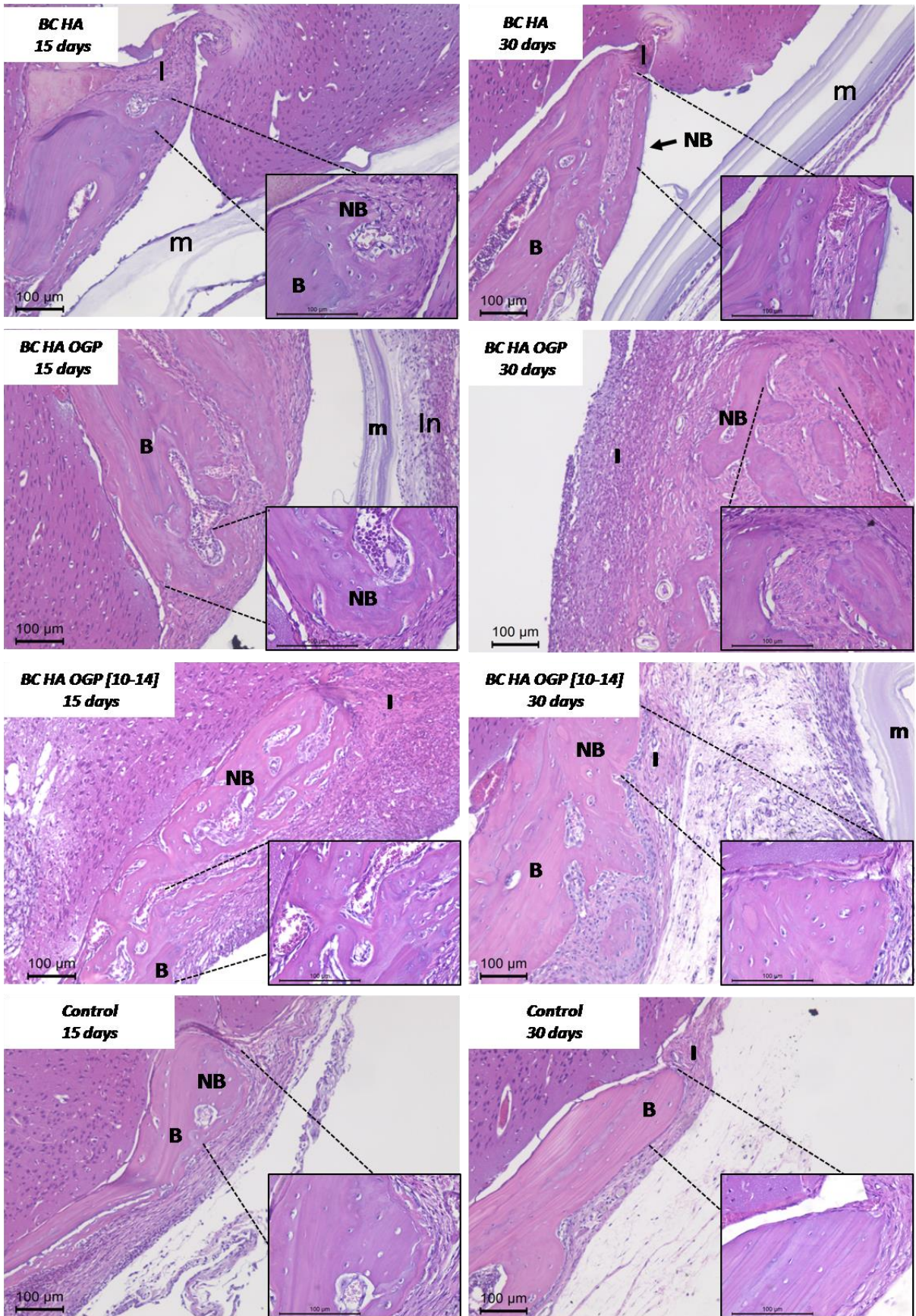


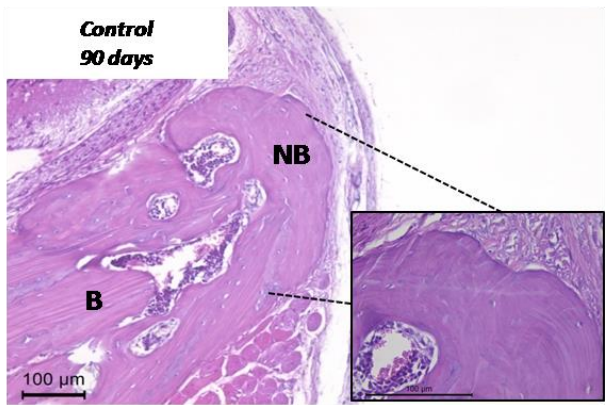
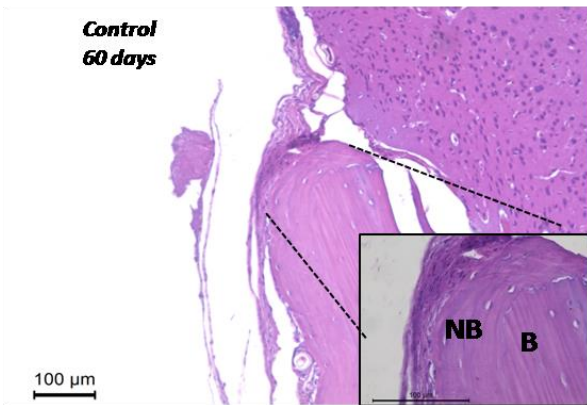
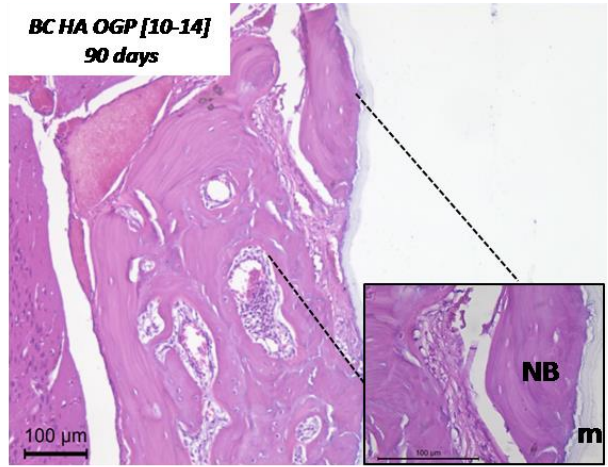
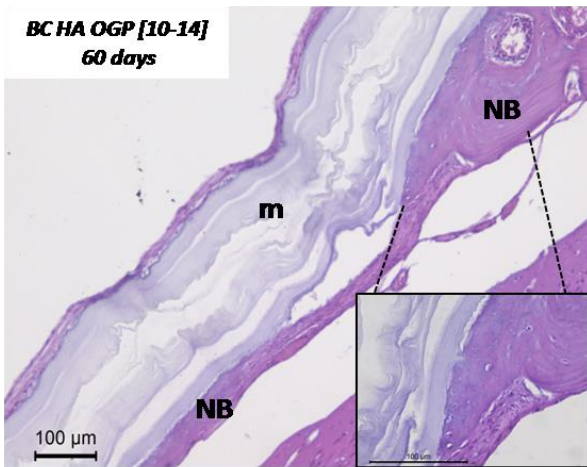
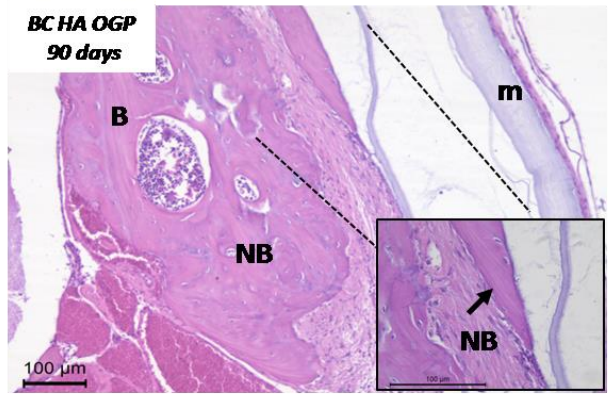
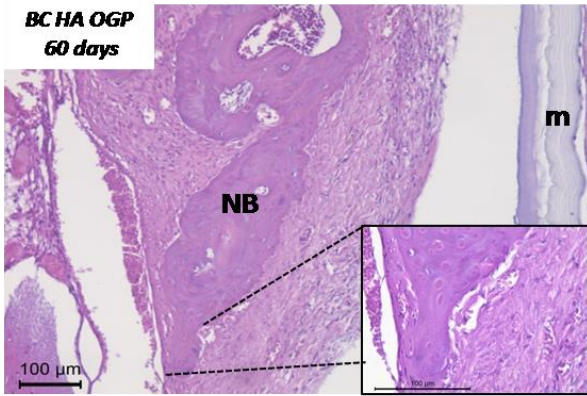
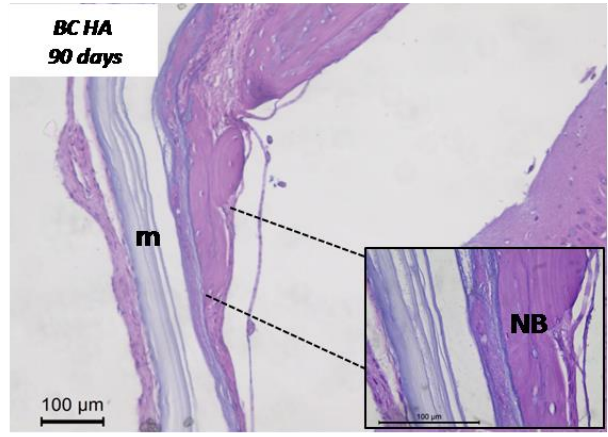
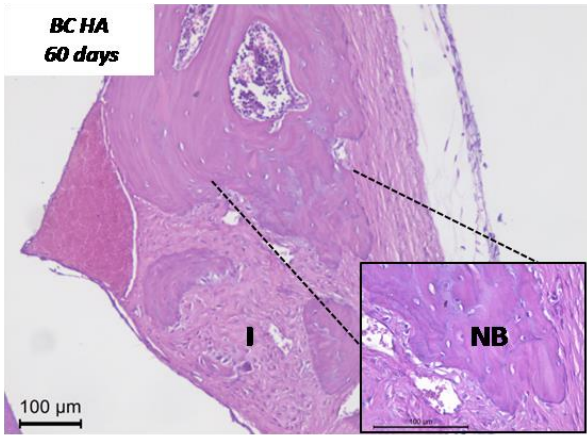
-  BC-HA
-  BC-HA OGP
-  BC-HA OGP (10-14)
-  Control

Figure 7









CONCLUSÃO

Conclui-se nesse estudo que:

- Os nanocompósitos CB-HA, CB-HA OGP e CB-HA OGP (10-14) investigados são biocompatíveis e promoveram maior formação óssea em relação ao grupo controle em defeito crítico em calvária de camundongo. Sendo que, o potencial indutor da membrana de CB-HA OGP foi inferior e limitou-se aos períodos iniciais do reparo ósseo.

- As membranas CB-HA e CB-HA OGP (10-14) parecem apresentar melhor potencial osteocondutor no reparo ósseo nesse estudo, destacando a eficácia indutora da membrana de CB-HA OGP (10-14) nos períodos iniciais da formação óssea.

- As membranas CB-HA, CB-HA OGP e CB-HA OGP (10-14) não foram reabsorvidas até 90 dias de pós-operatório.

- Os dados obtidos mostram que as membranas apresentam potencial para serem utilizadas na regeneração tecidual guiada uma vez que atuam como barreira mecânica e apresentam propriedades osteoindutoras na presença dos peptídeos, contudo estudos clínicos são necessários para avaliar o uso desses materiais no tratamento de defeitos ósseos.

PERSPECTIVAS FUTURAS

- Desenvolvimento de pesquisas científicas que possibilitem maior compreensão, a nível molecular, do mecanismos de ação e liberação dos peptídeos OGP e do OGP (10-14) in situ.
- Avaliar o desempenho das membranas de CB-HA e CB-HA OGP (10-14) associadas a biomateriais de preenchimento ósseo em defeito críticos.
- Estudos clínicos para avaliar o potencial osteoindutor dos nanocompósitos de CB associado

REFERÊNCIAS*

1. Anderson JM, McNally AK. Biocompatibility of implants: lymphocyte/macrophage interactions. *Semin Immunopathol.* 2011; 33(3): 221-33.
2. Atalla RH, Vanderhart DL. The role of solid state ¹³C NMR spectroscopy in studies of the nature of native celluloses. *Solid State Nucl Magn Reson.* 1999; 15(1): 1-19.
3. Aubin JE. Advances in the osteoblast lineage. *Biochem Cell Biol.* 1998; 76(6): 899-910.
4. Aubin JE, Liu F, Malaval L, Gupta AK. Osteoblast and chondroblast differentiation. *Bone.* 1995; 17(2 Suppl): 77S-83S.
5. Bab I, Chorev M. Osteogenic growth peptide: from concept to drug design. *Biopolymers.* 2002; 66(1): 33-48.
6. Bab I, Gazit D, Chorev M, Muhlrad A, Shteyer A, Greenberg Z, et al. Histone H4-related osteogenic growth peptide (OGP): a novel circulating stimulator of osteoblastic activity. *EMBO J.* 1992; 11(5): 1867-73.
7. Bab I, Smith E, Gavish H, Attar-Namdar M, Chorev M, Chen YC, et al. Biosynthesis of osteogenic growth peptide via alternative translational initiation at AUG85 of histone H4 mRNA. *J Biol Chem.* 1999; 274(20): 14474-81.
8. Bab IA, Einhorn TA. Polypeptide factors regulating osteogenesis and bone marrow repair. *J Cell Biochem.* 1994; 55(3): 358-65.
9. Bodin A, Concaro S, Brittberg M, Gatenholm P. Bacterial cellulose as a potential meniscus implant. *J Tissue Eng Regen Med.* 2007; 1(5): 406-8.

*De acordo com o manual da FOAr/UNESP, adaptadas das normas Vancouver. Disponível no site:

http://www.nlm.nih.gov/bsd/uniform_requirements.html

10. Brown RM, Jr., Willison JH, Richardson CL. Cellulose biosynthesis in *Acetobacter xylinum*: visualization of the site of synthesis and direct measurement of the in vivo process. *Proc Natl Acad Sci U S A*. 1976; 73(12): 4565-9.
11. Cetinkaya BO, Keles GC, Ayas B, Sakallioglu EE, Acikgoz G. The expression of vascular endothelial growth factor in a rat model at destruction and healing stages of periodontal disease. *J Periodontol*. 2007; 78(6): 1129-35.
12. Chen YC, Bab I, Mansur N, Muhrad A, Shteyer A, Namdar-Attar M, et al. Structure-bioactivity of C-terminal pentapeptide of osteogenic growth peptide [OGP(10-14)]. *J Pept Res*. 2000; 56(3): 147-56.
13. Chen ZX, Chang M, Peng YL, Zhao L, Zhan YR, Wang LJ, et al. Osteogenic growth peptide C-terminal pentapeptide [OGP(10-14)] acts on rat bone marrow mesenchymal stem cells to promote differentiation to osteoblasts and to inhibit differentiation to adipocytes. *Regul Pept*. 2007; 142(1-2): 16-23.
14. Chim SM, Tickner J, Chow ST, Kuek V, Guo B, Zhang G, et al. Angiogenic factors in bone local environment. *Cytokine Growth Factor Rev*. 2013; 24(3): 297-310.
15. Czaja W, Krystynowicz A, Bielecki S, Brown RM, Jr. Microbial cellulose--the natural power to heal wounds. *Biomaterials*. 2006; 27(2): 145-51.
16. Dereka XE, Markopoulou CE, Vrotsos IA. Role of growth factors on periodontal repair. *Growth Factors*. 2006; 24(4): 260-7.
17. Devescovi V, Leonardi E, Ciapetti G, Cenni E. Growth factors in bone repair. *Chir Organi Mov*. 2008; 92(3): 161-8.
18. dos Anjos B, Novaes AB, Jr., Meffert R, Barboza EP. Clinical comparison of cellulose and expanded polytetrafluoroethylene membranes in the treatment of class II furcations in mandibular molars with 6-month re-entry. *J Periodontol*. 1998; 69(4): 454-9.

19. Fang B, Wan YZ, Tang TT, Gao C, Dai KR. Proliferation and osteoblastic differentiation of human bone marrow stromal cells on hydroxyapatite/bacterial cellulose nanocomposite scaffolds. *Tissue Eng Part A*. 2009; 15(5): 1091-8.
20. Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev*. 1997; 18(1): 4-25.
21. Ghanaati H, Firouznia K, Vaziri Bozorg SM, Ghasemi Esfe AR, Motevallei M, Abedini MR, et al. Nintinol self-expandable metallic stenting in management of malignant obstructive jaundice: a case series. *Hepat Mon*. 2010; 10(1): 57-61.
22. Ghanaati S. Non-cross-linked porcine-based collagen I-III membranes do not require high vascularization rates for their integration within the implantation bed: a paradigm shift. *Acta Biomater*. 2012; 8(8): 3061-72.
23. Grande CJ, Torres FG, Gomez CM, Bano MC. Nanocomposites of bacterial cellulose/hydroxyapatite for biomedical applications. *Acta Biomater*. 2009; 5(5): 1605-15.
24. Greenberg Z, Chorev M, Muhlrad A, Shteyer A, Namdar-Attar M, Casap N, et al. Structural and functional characterization of osteogenic growth peptide from human serum: identity with rat and mouse homologs. *J Clin Endocrinol Metab*. 1995; 80(8): 2330-5.
25. Gurevitch O, Slavin S, Muhlrad A, Shteyer A, Gazit D, Chorev M, et al. Osteogenic growth peptide increases blood and bone marrow cellularity and enhances engraftment of bone marrow transplants in mice. *Blood*. 1996; 88(12): 4719-24.
26. Hui Z, Yu L, Xiaoli Y, Xiang H, Fan Z, Ningbo H, et al. C-terminal pentapeptide of osteogenic growth peptide regulates hematopoiesis in early stage. *J Cell Biochem*. 2007; 101(6): 1423-9.
27. Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. *Biomaterials*. 2000; 21(24): 2529-43.

28. Larrivee B, Karsan A. Signaling pathways induced by vascular endothelial growth factor (review). *Int J Mol Med*. 2000; 5(5): 447-56.
29. Mann S. *Biomaterialization: principles and concepts in bioinorganic materials chemistry*. New York: Oxford University Press; 2001.
30. Mello LR, Feltrin LT, Fontes Neto PT, Ferraz FA. Duraplasty with biosynthetic cellulose: an experimental study. *J Neurosurg*. 1997; 86(1): 143-50.
31. Novaes AB, Jr., Novaes AB. Bone formation over a TiAl6V4 (IMZ) implant placed into an extraction socket in association with membrane therapy (Gengiflex). *Clin Oral Implants Res*. 1993; 4(2): 106-10.
32. Novaes Jr AB, Moraes N, Novaes AB. Uso do BioFill como membrana biológica no tratamento de lesão de furca com e sem a utilização de hidroxiapatita porosa. *Rev Bras Odontol*. 1990; 47(5): 29-32.
33. Novaes Junior AB, Novaes AB. Immediate implants placed into infected sites: a clinical report. *Int J Oral Maxillofac Implants*. 1995; 10(5): 609-13.
34. Oliver RC, Brown LJ, Loe H. Periodontal diseases in the United States population. *J Periodontol*. 1998; 69(2): 269-78.
35. Petersen N, Gatenholm P. Bacterial cellulose-based materials and medical devices: current state and perspectives. *Appl Microbiol Biotechnol*. 2011; 91(5): 1277-86.
36. Reikeras O, Gunderson RB. Excellent results with femoral revision surgery using an extensively hydroxyapatite-coated stem: 59 patients followed for 10-16 years. *Acta Orthop*. 2006; 77(1): 98-103.

37. Robinson D, Bab I, Nevo Z. Osteogenic growth peptide regulates proliferation and osteogenic maturation of human and rabbit bone marrow stromal cells. *J Bone Miner Res.* 1995; 10(5): 690-6.
38. Saska S, Barud HS, Gaspar AM, Marchetto R, Ribeiro SJ, Messaddeq Y. Bacterial cellulose-hydroxyapatite nanocomposites for bone regeneration. *Int J Biomater.* 2011; 2011: 175362.
39. Saska S, Scarel-Caminaga RM, Teixeira LN, Franchi LP, Dos Santos RA, Gaspar AM, et al. Characterization and in vitro evaluation of bacterial cellulose membranes functionalized with osteogenic growth peptide for bone tissue engineering. *J Mater Sci Mater Med.* 2012; 23(9): 2253-66.
40. Shuqiang M, Kunzheng W, Xiaoqiang D, Wei W, Mingyu Z, Daocheng W. Osteogenic growth peptide incorporated into PLGA scaffolds accelerates healing of segmental long bone defects in rabbits. *J Plast Reconstr Aesthet Surg.* 2008; 61(12): 1558-60.
41. Svensson A, Nicklasson E, Harrah T, Panilaitis B, Kaplan DL, Brittberg M, et al. Bacterial cellulose as a potential scaffold for tissue engineering of cartilage. *Biomaterials.* 2005; 26(4): 419-31.
42. Tazi N, Zhang Z, Messaddeq Y, Almeida-Lopes L, Zanardi LM, Levinson D, et al. Hydroxyapatite bioactivated bacterial cellulose promotes osteoblast growth and the formation of bone nodules. *AMB Express.* 2012; 2(1): 61.
43. Thomas D, Kansara M. Epigenetic modifications in osteogenic differentiation and transformation. *J Cell Biochem.* 2006; 98(4): 757-69.
44. Tsiridis E, Upadhyay N, Giannoudis P. Molecular aspects of fracture healing: which are the important molecules? *Injury.* 2007; 38 Suppl 1: S11-25.

45. Vanella L, Kim DH, Asprinio D, Peterson SJ, Barbagallo I, Vanella A, et al. HO-1 expression increases mesenchymal stem cell-derived osteoblasts but decreases adipocyte lineage. *Bone*. 2010; 46(1): 236-43.
46. Wahl DA, Sachlos E, Liu C, Czernuszka JT. Controlling the processing of collagen-hydroxyapatite scaffolds for bone tissue engineering. *J Mater Sci Mater Med*. 2007; 18(2): 201-9.
47. Wang H, Li Y, Zuo Y, Li J, Ma S, Cheng L. Biocompatibility and osteogenesis of biomimetic nano-hydroxyapatite/polyamide composite scaffolds for bone tissue engineering. *Biomaterials*. 2007; 28(22): 3338-48.
48. Yu H, Matthew HW, Wooley PH, Yang SY. Effect of porosity and pore size on microstructures and mechanical properties of poly-epsilon-caprolactone- hydroxyapatite composites. *J Biomed Mater Res B Appl Biomater*. 2008; 86B(2): 541-7.
49. Zaborowska M, Bodin A, Backdahl H, Popp J, Goldstein A, Gatenholm P. Microporous bacterial cellulose as a potential scaffold for bone regeneration. *Acta Biomater*. 2010; 6(7): 2540-7.

APÊNDICE

METODOLOGIA DETALHADA

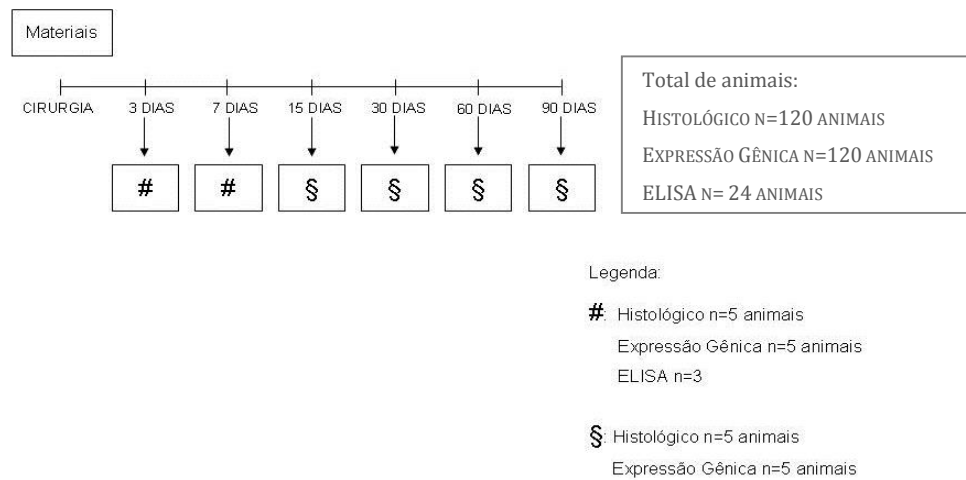
Os materiais foram confeccionados no Instituto de Química do Campus de Araraquara – UNESP, sob a responsabilidade do Prof. Dr. Sidney José Lima Ribeiro e da Pós-Doutoranda Dra. Sybele Saska Specian.

Foram investigados os seguintes materiais à base de celulose bacteriana sob a forma de membranas:

- Grupo I: CB-HA
- Grupo II: CB-HA OGP
- Grupo III: CB-HA OGP (10-14)
- Grupo IV: Coágulo

Foram utilizados 264 camundondos (balb/c), machos, 10 meses, com peso médio de 30g, provenientes do biotério da Faculdade de Ciências Farmacêuticas de Ribeirão Preto. Os animais foram aleatoriamente separados em grupos de igual número e mantidos em gaiolas plásticas em um ambiente com temperatura controlada ($21\pm 1^{\circ}\text{C}$), umidade (65-75%) e ciclos de luz (12h claro-12h escuro) e alimentados com água e ração *ad libitum*. Os grupos e condições experimentais estão dispostos na Figura A1. O protocolo experimental foi aprovado pelo comitê de ética de experimentação animal local (CEUA nº03/2012 – Anexo 1) e realizado de acordo com as normas do Colégio Brasileiro de Experimentação Animal (COBEA).

Figura A1 - Fluxograma dos grupos e condições experimentais.

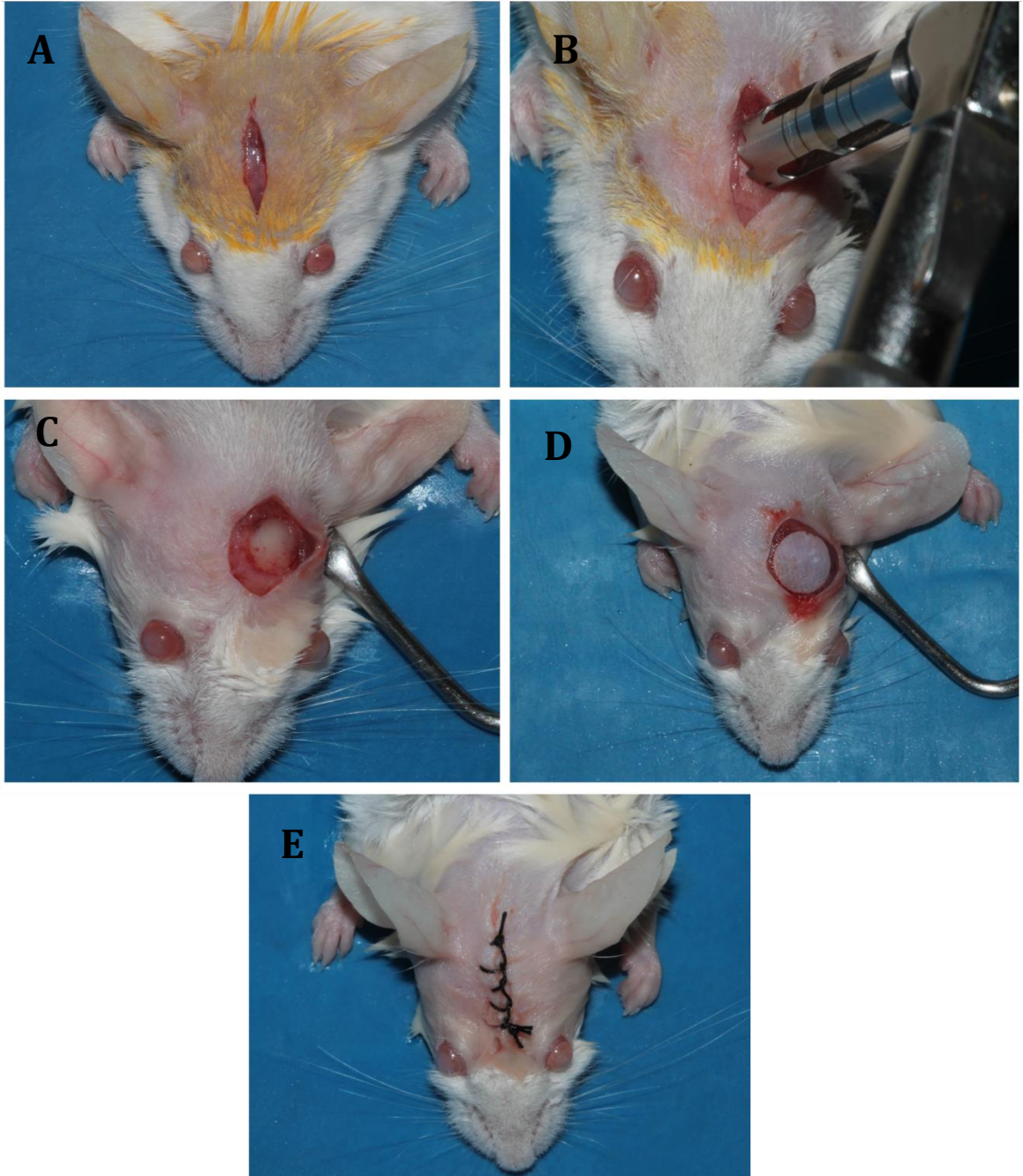


1 CONFECCÃO DO DEFEITO ÓSSEO

Em cada animal foi realizado um defeito ósseo no osso parietal. Cada defeito ósseo foi recoberto por um tipo de material (Grupo I ao Grupo III), exceto o defeito controle que foi preenchido somente com coágulo sanguíneo (Grupo IV). Os camundongos foram divididos em seis períodos de análise: 3, 7, 15, 30, 60 e 90 dias, sendo que os períodos de 3 e 7 dias foram utilizados treze (13) animais por grupo, e nos períodos restantes constaram de dez (10) animais por grupo dando um total de 264 camundongos (Figura A1). Os animais foram anestesiados por meio de administração intramuscular de cloridrato de ketamina (25 mg/kg; Agener União, Brasil) e de cloridrato de xilazina (5 mg/kg; Bayer, Brazil), dosagem esta suficiente para 30 minutos de anestesia. Todos os procedimentos cirúrgicos foram realizados sob rigoroso protocolo asséptico. Após a anestesia e tricotomia da região parietal, os animais foram acomodados em posição dorsal, onde receberam incisão sobre a sutura sagital (Figura A2A), seguida de descolamento e rebatimento dos tecidos contendo epiderme, tecido muscular e periósteo, até a exposição do osso parietal.

Os defeitos ósseos críticos foram realizadas com o auxílio de uma broca trefina de 4 mm de diâmetro (Neodent®, Brazil) ^{2, 3}, sob irrigação constante de soro fisiológico 0,9% (Figura A2B). A confecção dos defeitos constituiu na perfuração total do osso parietal mantendo-se a integridade da dura-máter (Figura A2C). As membranas a base de CB foram embebidas em soro fisiológico e posicionadas sob os respectivos defeitos (Figura A2D). A sutura foi realizada por meio de ponto contínuo com seda 4-0 (Ethicon, Johnson & Johnson, São José dos Campos, SP, Brasil) (Figura A2E).

Figura A2 - Obtenção das amostras biológicas; A: Incisão com lâmina de bisturi nº 15 sob o osso parietal; B: Confeção do defeito crítico no osso parietal com uma trefina de 4mm; C: Perfuração total do osso parietal mantendo-se a integridade da dura-máter; D: Posicionamento da membrana após ser hidratada em soro fisiológico; E: Sutura com fio de seda 4-0.



2 ANÁLISE DE MICROTOMOGRAFIA COMPUTADORIZADA (μ CT)

Após os períodos de acompanhamento de 7, 15, 30 e 60 dias, os animais foram sacrificados por sobredose anestésica. Após o sacrifício, os 5 animais de cada grupo dos respectivos períodos experimentais tiveram suas cabeças removidas e fixadas em formol a 10% durante 24 horas; posteriormente as cabeças foram transferidas para uma solução de álcool 70% sendo conservadas em temperatura ambiente. As cabeças foram escaneadas por meio de um sistema de microtomografia computadorizada (μ CT) (Skyscan 1174, Aartselaar, Belgium).

Os parâmetros utilizados para o escaneamento foram: filtro de Al 1.0 mm; tamanho do voxel: 17.48 μ m; voltagem: 65 kV; corrente elétrica: 385 μ A; espessura do corte: 18 μ m. As imagens foram reconstruídas, reorientadas e analisadas com auxílio de softwares específicos (NRecon 1.6.1.5, Data Viewer, CtanSkyscan 1174, Aartselaar, Belgium). As imagens foram analisadas tendo como referência a norma superior do crânio dos animais com região de interesse decircular com tamanho de 120 x 120 pixels com 20 secções de espessura *ethreshold* na faixa de 64–183 tons de cinza.

3 ANÁLISE HISTOLÓGICA

Após o escaneamento as peças foram desmineralizadas em solução de Morse (ácido fórmico a 50% e solução de citrato de sódio a 20% na proporção 1:1 trocada a cada 48 h durante 30 dias). Posteriormente os espécimes foram lavados em água corrente por 24 h e imersos em uma solução de sulfato de sódio a 5% por 48 h para neutralização do excesso de ácido. Em seguida, os espécimes foram novamente lavados em água corrente por mais 6 h e imersos nas seguintes soluções: álcool 70% por 12 h, álcool 90% por 1 h, álcool absoluto por 24 h (realizando trocas a cada 8 h), lavagem rápida com álcool-xilol (1:1), seguido de imersão em xilol por 3 h (realizando troca a cada 1h) e inclusão final em parafina.

Os espécimes foram preparados com cortes semi-seriados de $6\ \mu\text{m}$ para coloração em Hematoxilina & Eosina (HE) para análise descritiva. Utilizando-se um microscópio óptico DIASTAR (LeicaReichert& Jung products, Germany) com objetiva para aumento de 2,5/10 vezes e oculares com aumento de 10 vezes, as imagens foram captadas e enviadas para um microcomputador, com o auxílio de uma câmera de vídeo DXC-1107A/107AP (Sony EletronicsInc, Japão). Os parâmetros analisados foram: qualidade do osso mineralizado, viabilidade dos osteócitos, qualquer evidência de formação de tecido fibroso no interior do defeito, processo de angiogênese, reação inflamatória e degradação das membranas. A resposta inflamatória observada na análise dos cortes histológicos foi mensurada utilizando a tabela de escore (muito leve, leve, moderada e intensa) de acordo com a norma da ASTM F981-04¹.

4 ANÁLISE HISTOMORFOMÉTRICA

Essa análise foi executada com o intuito de avaliar a porcentagem de fechamento do defeito crítico, bem como avaliação da porcentagem de preenchimento ósseo do defeito crítico utilizando o Software Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA). Para avaliação da porcentagem de fechamento do defeito crítico, uma medida linear foi executada na porção mediana do defeito onde foi mensurado inicialmente o tamanho original (TO) e o tamanho remanescente do defeito (TR), sendo que a porcentagem de fechamento foi obtida por meio da fórmula: $\text{TR} \times 100/\text{TO}$ (Figura A3). Para avaliação da porcentagem de preenchimento ósseo, foi executada uma mensuração da área total do defeito (AT) e da área de formação óssea (AO), e a porcentagem de preenchimento ósseo foi obtida por meio da fórmula: $\text{AO} \times 100/\text{AT}$ (Figura A4). Em ambas as análises, três cortes de cada amostra foram randomicamente selecionados para serem avaliados. Todas as mensurações foram executadas por um examinador cego, treinado e calibrado para as análises.

Figura A3 - Porcentagem de Fechamento do defeito crítico; TO: Tamanho Original do defeito; TR: Tamanho Remanescente do defeito

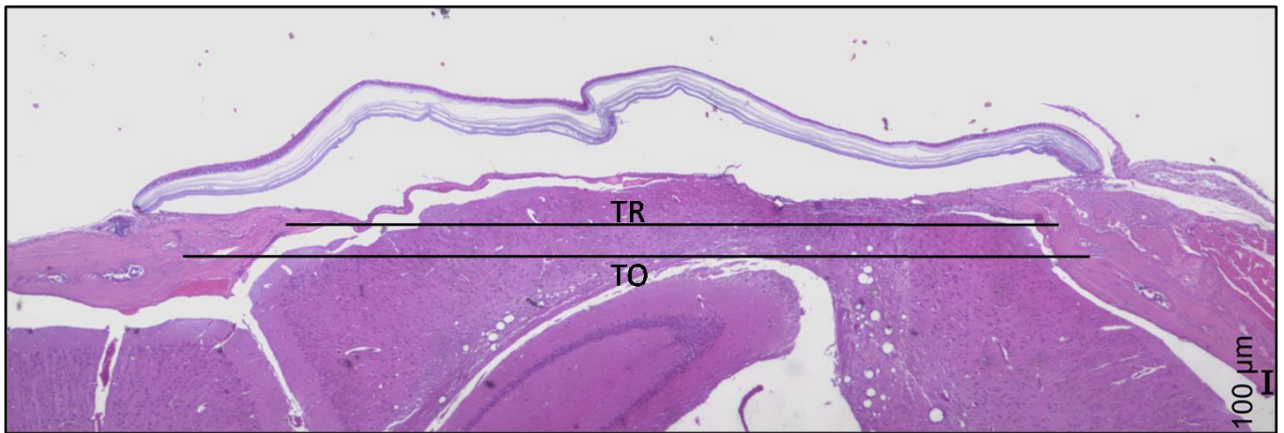
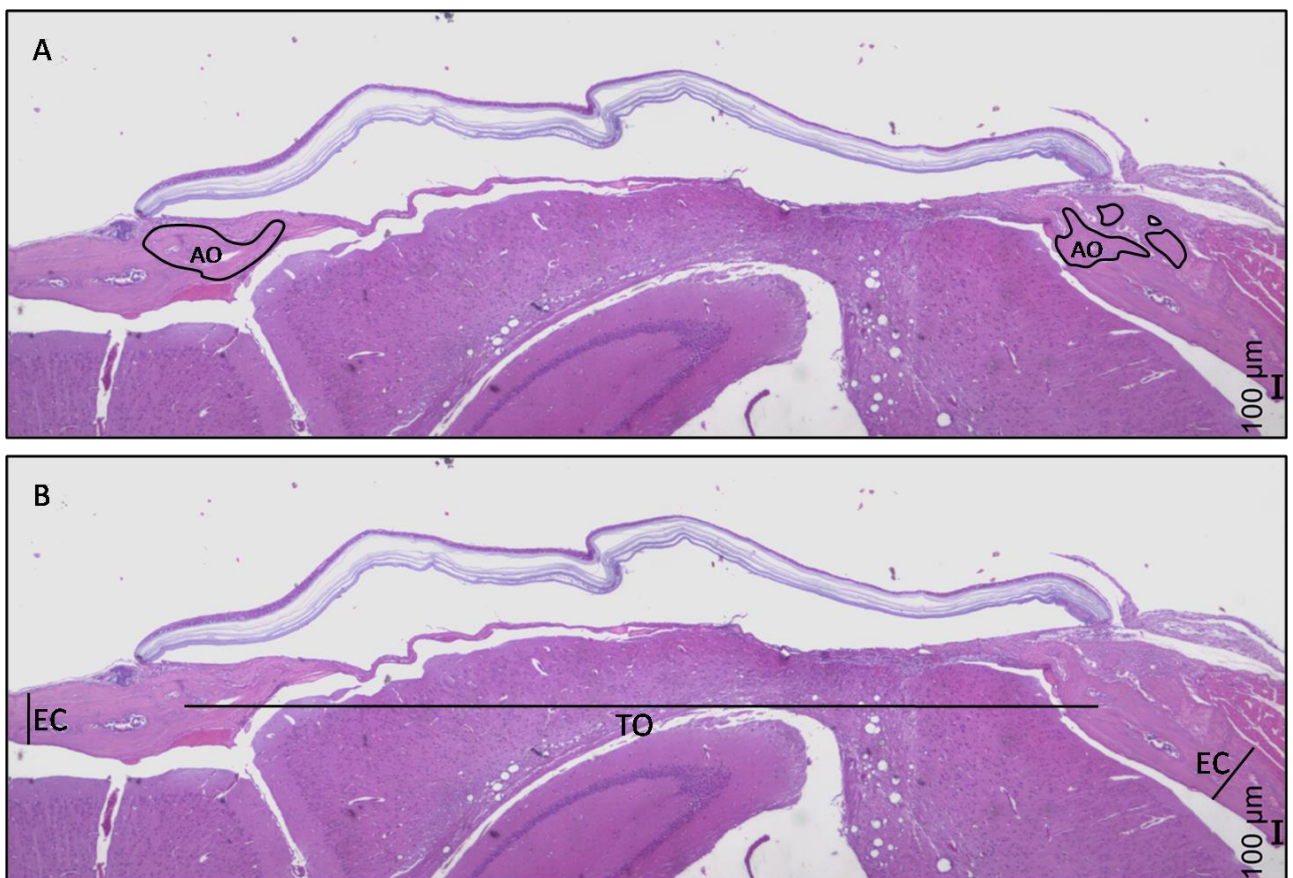


Figura A4 - Porcentagem de Preenchimento Ósseo. A) A área de formação óssea(AO) foi obtida através da soma das áreas onde observou-se formação óssea. B) A área total do defeito (AT) foi definida pela multiplicação da espessura do crânio do animal (EC) pelo tamanho original do defeito (TO).



5 ANÁLISE DA EXPRESSÃO DE VEGFR-2 POR ENSAIO IMUNOENZIMÁTICO (ELISA)

Foram utilizados 3 animais de cada grupo nos períodos de 3 e 7 dias para a análise da expressão do VEGFR-2. No momento do sacrifício dos animais, o tecido de granulação do interior da ferida cirúrgica foi removido com uma cureta para análise da concentração de VEGFR-2 por ensaio imunoenzimático ELISA. Amostras de proteína total do tecido coletado das feridas cirúrgicas foram extraídas com o reagente T-Per (Pierce, Thermo Fisher) e quantificadas segundo o método de Lowry (DC proteinassay, Bio-Rad).

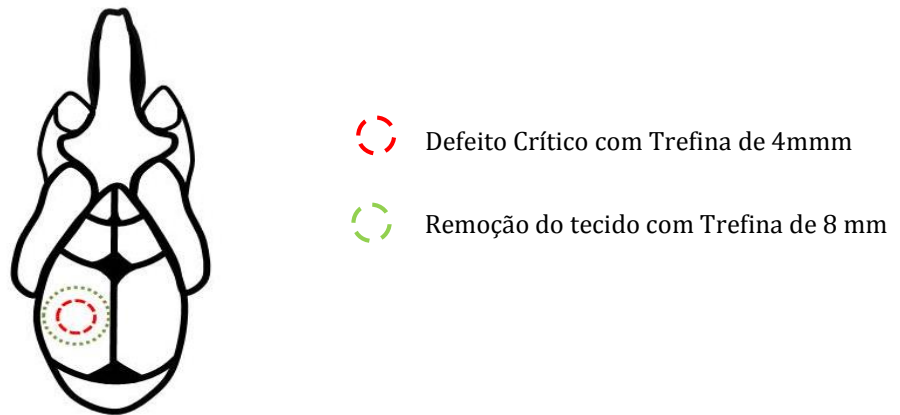
O procedimento foi realizado segundo instruções do fabricante, e as leituras foram realizadas em leitor de placas a 450 nm. A quantidade total de proteína presente em cada poço das placas de cultura de 96 poços foi determinada pelo método de Lowry (DC proteinassay, Bio-Rad) para normalização da concentração das proteínas-alvo.

A determinação da concentração do VEGFR-2 foi realizada seguindo rigorosamente as recomendações fornecidas pelo fabricante do Kit (PathScan® Total VEGFR-2 Sandwich ELISA kit, Cell Signaling Technology, Massachusetts, EUA). O curto período experimental proposto se justifica por haver maior concentração de fatores de crescimento no período inicial da reparação tecidual ⁴.

6 ANÁLISE DA EXPRESSÃO GÊNICA

Foram utilizados 5 animais por grupo para a análise da expressão gênica para cada grupo nos períodos de 3, 7, 15, 30, 60 e 90 dias. Imediatamente após os animais serem anestesiados como descrito acima, foi acessado o local onde foi realizado o defeito crítico. Uma trefina de 8mm de diâmetro foi centralizada na região de interesse de modo a obter o tecido para as análises de expressão gênica (Figura A5).

Figura A5 - Esquema ilustrativo do defeito crítico e da remoção do tecido de interesse



Esse tecido foi rapidamente transferido para o RNAlater (AMBION) para conservação da integridade do RNA. O tecido foi macerado em nitrogênio líquido, e em seguida a extração do RNA foi realizada utilizando *TRIzol* (Invitrogen, Carlsbad, CA) segundo o protocolo recomendado pelo fabricante. Em seguida, a concentração do RNA extraído de cada amostra foi medida por densidade óptica por meio do Nanodrop (*ThermoScientific*). As leituras no Nanodrop mostraram que as amostras extraídas de RNA apresentavam concentração suficiente e qualidade adequada para realização do qPCR (Média das Leituras: Concentração de RNA: 374.55 / Pureza: λ 260/280= 1.78; λ 260/230= 1.80).

Foi sintetizado o DNA complementar (cDNA) de todas as sequências gênicas expressas por meio do RT-PCR utilizando o OligodT₍₂₀₎ com o kit High CapacitycDNA Reverse Transcription (Invitrogen™), de acordo com as instruções do fabricante.

As reações de PCR em tempo real (RT-qPCR) foram realizadas pelo sistema *TaqMan*TM (Applied Biosystems, Foster City), que é constituído por um par de *primers* e uma sonda marcada com um fluoróforo. O gene para controle endógeno da reação foi gliceraldeído-3-fosfato desidrogenase (GAPDH ,cat. nº Mm99999915_g1), cujo nível de expressão foi usado para normalizar a expressão dos genes de interesse SPP1(*Osteopontin*; cat. nº

Mm00436767_m1), ALPL (*Alkaline Phosphatase*; cat. nº Mm00475834_m1), TNFRSF11B (*Osteoprotegerin*; cat. nº Mm01205928_m), RUNX2 (RUNX; cat. nº Mm00501584_m1), BGLAP (*Osteocalcin*; cat. nº Mm00649782_gH) e VEGF (*vascular endothelial growth factor*; cat. nº Mm01281449_m1).

Foi realizada a validação do sistema gene de interesse/controlado endógeno, a fim de verificar se as eficiências de amplificação de ambos os genes são semelhantes e próximas a 100%. Para a quantificação relativa do gene selecionado as reações de PCR em tempo real foram realizadas em duplicata a partir de: 6,25µL de *TaqMan Universal PCR Master Mix 2x*, 0,625µL da solução de primers e sonda, 1,625µL de água e 4,0µL de cDNA (50ng), sendo que no controle negativo, foi adicionado 4,0 µL de água ao invés do cDNA. As condições de ciclagem utilizadas foram: 50°C por 2 minutos, 95°C por 10 minutos e 40 ciclos de 95°C por 15 segundos e 60°C por 1 minuto.

Os valores da expressão gênica relativa dos genes de interesse foram analisados utilizando o software Expression Suite (Applied Biosystems®). O software utiliza o método comparativo C_t ($\Delta\Delta C_t$) e possibilitou quantificar com precisão a expressão gênica relativa dos genes analisados. Após normalização pela expressão do gene controle endógeno, foram obtidos os valores de expressão para cada gene alvo (ΔC_t). A média dos valores de ΔC_t de cada gene de interesse (alvo) referente à indução de cada material investigado foram comparados entre si para cada período analisado.

7 ANÁLISE DOS RESULTADOS

O software GraphPadPrism 6.0 (San Diego, CA, USA) foi utilizado para análise estatística desse estudo. Para avaliação da calibração intra-examinador com relação às análises histomorfométricas (porcentagem de formação óssea) e microtomográfica foram realizadas repetições das mensurações de 10% das amostras, com uma semana de intervalo entre as

mensurações. O teste t-pareado demonstrou que não houve diferenças estatisticamente significativas entre as mensurações. Adicionalmente, por meio do teste de correlação de Pearson, os obtidos índices de correlação $r=0.90$ na análise histomorfométrica e $r=0.80$ na análise microtomográfica, demonstrou que o examinador estava devidamente calibrado.

Os dados das análises de μ CT, histomorfométrica, expressão do VEGFR-2 e expressão gênica dos biomarcadores ósseo foram submetidos ao teste de normalidade de Kolmogorov-Smirnov test para avaliar se esses se distribuíram de acordo com o teorema da distribuição central. Para análise dos dados de μ CT, histomorfométrica, expressão do VEGFR-2 utilizado o teste t. Para análise dos resultados de expressão gênica foi utilizado o teste não paramétrico de Mann Whitney, comparando cada grupo experimental com o grupo controle. Na análise da reação inflamatória, os dados foram submetidos ao teste de normalidade Lilliefors. Em seguida, o teste de Kruskal-Wallis e o pós-teste de Dunn foram utilizados para avaliar a diferença entre os grupos e entre os períodos analisados. O nível de significância estatística estabelecido para todas as análises foi de 5% ($p<0.05$), sendo que foi utilizado o programa GraphPad Prim 5 (San Diego, CA, USA).

REFERÊNCIAS

1. ASTM F981-04. Standard practice for assessment of compatibility of biomaterials for surgical implants with respect to effect of materials on muscle and bone. 2010.
2. Cowan CM, Shi YY, Aalami OO, Chou YF, Mari C, Thomas R, et al. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. *Nature biotechnology*. 2004; 22(5): 560-7.
3. Kang YH, Jeon SH, Park JY, Chung JH, Choung YH, Choung HW, et al. Platelet-rich fibrin is a Bioscaffold and reservoir of growth factors for tissue regeneration. *Tissue engineering Part A*. 2011; 17(3-4): 349-59.

4. Ma L, Hollenberg MD, Wallace JL. Thrombin-induced platelet endostatin release is blocked by a proteinase activated receptor-4 (PAR4) antagonist. *Br J Pharmacol.* 2001; 134(4): 701-4.

ANEXO

Comitê de Ética em Experimentação Animal

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araraquara
FACULDADE DE ODONTOLOGIA

**DECLARAÇÃO**

DECLARO, para os devidos fins, que a CEUA - Comissão de Ética no Uso de Animais, desta Faculdade de Odontologia, recebeu o projeto "**AValiação HISTOMORFOMÉTRICA E DA EXPRESSÃO GÊNICA DE COMPÓSITOS À BASE DE CELULOSE BACTERIANA COM PEPTÍDEO REGULATÓRIO DE FATOR DE CRESCIMENTO NA REGENAERAÇÃO ÓSSEA**" (Processo CEUA nº 3/2012), apresentado pela Pesquisadora Profa. Dra. RAQUEL MANTUANELI SCAREL CAMINAGA. O referido projeto deu entrada nesta Comissão em 29/02/2012, não apresenta pendências até o momento e seu relatório final deverá ser entregue em ABRIL/2015.

Araraquara, 07 de fevereiro de 2014.

Prof. Dr. PAULO SÉRGIO CERRI
Coordenador da CEUA

Não autorizo a reprodução deste trabalho até 21/3/2017

(Direitos de publicação reservados ao autor)

Araraquara, 21 de Março de 2014

Suzane Cristina Pigossi