UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

Guilherme dos Santos Trento

Avaliação histomorfométrica, imuno-histoquímica e microtomográfica da neoformação óssea ao redor de implantes de titânio com diferentes superfícies instalados em áreas de defeitos ósseos preenchidos ou não com biomaterial

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Orientador: Prof. Dr. Valfrido Antonio Pereira Filho

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DADOS CURRICULARES

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Trento GS. Avaliação histomorfométrica, imuno-histoquímica e microtomográfica da neoformação óssea ao redor de implantes de titânio com diferentes superfícies instalados em áreas de defeitos ósseos preenchidos ou não com biomaterial [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2019.

RESUMO

O objetivo desse estudo foi avaliar a resposta óssea peri-implantar de implantes osseointegrados de titânio com dois tipos de tratamento de superfície, na presença ou não de substituto ósseo. Para tanto, foram utilizados 92 implantes dentários que foram divididos em quatro grupos de acordo com a superfície do implante e a presença de hidroxiapatita/beta-tricálcio fostato (HA/TCP) ou coágulo sanguíneo (BC) no defeito peri-implantar: (BC-A = implantes de superfície porosa e hidrofílica sem enxerto de substituto ósseo; HA/TCP-A = implantes de superfície porosa e hidrofílica com enxerto de substituto ósseo; BC-N = implantes de superfície porosa sem enxerto de substituto ósseo; HA/TCP-N = implantes de superfície porosa com enxerto de substituto ósseo). Os implantes foram instalados nas tíbias de 46 coelhos, sendo que cada animal recebeu dois implantes. Os animais foram submetidos a eutanásia em três diferentes períodos: 15 (n=10), 30 (n=10), e 60 (n=26). Após, as amostras foram submetidas a avaliação da qualidade óssea por meio das análises histológica por coloração Hematoxilina-Eosina, imuno-histoquímica para detecção dos anticorpos RUNX2, OPN, OCN, OPG, RANK-L e proteínas presentes na matriz óssea, utilizando imunoperoxidase para detecção e 3,3-diaminobenzidiana e de micro-tomografia computadorizada (micro-CT). Ainda, foi também realizada análise e avaliação histomorfométrica para quantificação do contato osso-implante (BIC) e a expressão gênica foi verificada por meio dos marcadores Runx2 e BSP, por meio da reação da transcriptase reversa, seguida de reação em cadeia da polimerase (RT-PCR). Os dados foram tabulados e submetidos a análise estatística por meio dos testes de Shapiro-Wilk, Kolgomorov-Smirnov e ANOVA. Em todos os períodos, o grupo HA/TCP-A apresentou uma porcentagem maior de neoformação óssea. No entanto, não houve diferenças estatisticamente significante para a neoformação óssea entre os grupos ($p < 0,05$). Para os dois tipos de superfície, a presença do substituto ósseo apresentou valores mais altos em todos os parâmetros avaliados da micro-CT, exceto no parâmetro superfície/volume ósseo. A análise descritiva da imuno-histoquímica apresentou resultados semelhantes entre os grupos. Além disso, as superfícies porosas e hidrofílicas apresentaram uma expressão mais elevada de RUNX2. O grupo BC-A apresentou o maior valor médio do BIC (46,43%). As superfícies porosas e hidrofílicas também apresentaram melhores resultados de BIC quando comparadas à superfície porosa nas duas condições experimentais. Não foram encontradas diferenças estatisticamente significantes entre todos os grupos ($p < 0,05$). Esses resultados sugerem que a superfície porosa e hidrofílica testada pode melhorar e acelerar a formação óssea, bem como a expressão de proteínas que desempenham papel importante na atividade osteogênica. E na presença de substituto ósseo, esta pode acelerar a formação de tecido ósseo peri-implantar. No entanto, após a osseointegração, ambas superfícies testadas tem comportamento similar tanto na presença quanto na ausência dos substitutos ósseos.

Palavras chave: Implantação Dentária Endo-Óssea. Xenoenxertos. Osteogênese. Interface Osso-Implante

Trento GS. Histomorphometric, immunohistochemical, and microtomographic evaluation of bone neoformation around two different surfaces of titanium implants placed in areas of bone defects filled or not with biomaterial [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2019.

ABSTRACT

The aim of this study was to evaluate the peri-implant bone response of osseointegrated titanium implants with two types of surface treatment, with or without bone substitute. Ninety-two titanium implants were divided into four groups according to implant surface and presence of hydroxyapatite/β-tricalcium phosphate (HA/TCP) or blood clot (BC) in the peri-implant defect: (BC-A = porous-hydrophilic surface implants without bone substitute material; HA/TCP-A = porous-hydrophilic surface implants with bone substitute material; BC-N = porous surface implants without bone substitute material; HA/TCP-N = porous surface implants with bone substitute material). The implants were installed in the tibias of 46 rabbits, and each animal received two implants. The animals were euthanized in three different periods: 15 (n=10), 30 (n=10), and 60 (n=26). After, the samples were submitted to bone quality evaluation by means of histological analysis by Hematoxylin-Eosin staining, immunohistochemical analysis for detection of antibodies: RUNX2, OPN, OCN, OPG, RANK-L and bone matrix proteins using immunoperoxidase for detection and 3,3-diaminobenzidian and computerized tomography (micro-CT). In addition, histomorphometric analysis was also performed to quantify bone-implant contact (BIC), and gene expression was verified by RUNX2 and BSP markers by means of reverse transcriptase – polymerase chain reaction (RT-PCR). The data were tabulated and submitted to statistical analysis using Shapiro-Wilk, Kolgomorov-Smirnov and ANOVA tests. In all periods, the HA/TCP-A group presented higher percentage of bone formation. However, there were no statistically significant differences for bone formation among all groups ($p < 0.05$). For both types of surface, the presence of bone substitute material presented higher values in all evaluated micro-CT parameters, except in the bone surface/volume parameter. Immunohistochemistry descriptive analysis showed similar results between groups. In addition, porous and hydrophilic surfaces showed a higher expression of Runx2. The BC-A group presented the highest mean value of BIC (46.43%). The porous-hydrophilic surfaces also showed better BIC results when compared to the porous surface in both experimental conditions. No statistically significant differences were found among all groups ($p < 0.05$). These results suggest that porous-hydrophilic surface may improve and accelerate bone formation, as well as the expression of proteins that play an important role in osteogenic activity. And in the presence of bone substitute, it can accelerate the formation of periimplant bone tissue. However, after osseointegration, both surfaces have similar behavior both in the presence and absence of bone substitute materials.

Keywords: Dental Implantantion, Endosseous. Heterografts. Osteogenesis. Bone-Implant Interface

SUMÁRIO

1 INTRODUÇÃO

Desde a descoberta do processo de osseointegração por Brånemark et al.[1] (1969), diversos estudos envolvendo diferentes ferramentas metodológicas têm sido realizados para o melhor entendimento deste processo[2,3], visto que, nos implantes osseointegrados, as cargas oclusais são transmitidas diretamente ao osso circunjacente[4]. Neste processo, a formação óssea pode ocorrer de duas formas, a saber: osteogênese à distância e por contato. Na primeira, a formação óssea ocorre em direção ao implante a partir do osso já existente. Na segunda forma, o crescimento ósseo se inicia na superfície do implante[5-7]. Entretanto, é sabido que o principal fator responsável pela osseointegração ocorre pelo contato do osso neoformado junto a superfície do implante[8].

A integração entre superfície do implante dentário e tecido ósseo adjacente desempenha um papel fundamental na longevidade e função da reabilitação oral[9]. Um importante pré-requisito para a osseointegração e sucesso dos implantes é estabilidade primária[10] descrita como a ausência de movimentação do implante logo após sua inserção, sendo determinada principalmente pelas condições mecânicas, tais como quantidade e qualidade óssea, geometria do implante e técnica cirúrgica[10,11]. Segundo Grassi et al.[9] (2006), após a instalação de implantes, o evento biológico mais importante na fase de reparo é a adesão celular junto à interface do implante entre o tecido do hospedeiro e o próprio implante. A união estrutural e funcional decorrente da adesão celular é influenciada pelas propriedades da superfície dos implantes[9].

Metodologias variadas têm sido utilizadas para verificar a ossointegração, e demonstrando sua alta previsibilidade[12]. De fato, o sucesso dos implantes dentários é altamente dependente das características químicas, físicas, mecânicas e topográficas da sua própria superfície. Essas diferentes propriedades interagem e determinam a atividade celular próxima a superfície do implante[9]. A topografia de superfície dos implantes tem demonstrado influência na diferenciação e proliferação de osteoblastos, além de aumentarem os fatores de transcrição responsáveis pela expressão de genes formadores de matriz óssea[13].

Uma modificação significante que afeta positivamente a estabilidade mecânica é a criação de irregularidades na superfície do implante as quais retém o

coágulo sanguíneo por completo e estimula o reparo ósseo[14]. Segundo alguns autores, favorece esse processo a combinação de jateamento abrasivo e ataque ácido, em que o primeiro é responsável pela criação de macro-rugosidade e o segundo é responsável pela formação de micro e/ou nanorugosidade[15,16]. Estudo comparativo, em norma histológica e histomorfométrica, entre implantes que apresentavam diferentes texturas nanométricas de rugosidade à textura convencional de rugosidade instalados em coelhos, concluiu que as amostras apresentaram diferenças estatisticamente significativas nas taxas de contato osso-implante quatro semanas após a instalação dos mesmos, porém, oito semanas após não houve diferença estatisticamente significante entre as amostras[17].

O contato osso-implante, definido pela porcentagem da superfície do implante coberta por osso neoformado, é uma medida crítica usada para quantificar o grau de osseointegração[18]. O valor do percentual é obtido pela avaliação histomorfométrica por meio de coloração convencional de áreas teciduais descalcificadas e não-descalcificadas[19], e está relacionado a inúmeros fatores, tais como superfície do implante, sítio anatômico, tempo de reparo, entre outros[20]. Esses fatores influenciam na adesão de células osteoblásticas, na adsorção de proteínas e na neoformação óssea na superfície do implante[19]. Entretanto, na prática há uma certa dificuldade de determinar com precisão esse contato osso-implante devido a limitação técnica na preparação das amostras para expor a interface entre osso e implante. Além disso, não há informação suficiente para afirmar o tipo de osso formado ao redor do implante. Uma melhor avaliação e entendimento da natureza da interface entre o tecido ósseo e o implante são necessários para guiar o desenho/geometria dos implantes, melhorando a estabilidade inicial e acelerando o processo de reparo[18].

Botzenhart et al.[21] (2015), compararam duas superfícies de implantes, avaliando a osseointegração em implantes de titânio de 9mm de comprimento. O grupo controle apresentava superfície com jateamento cerâmico, enquanto no grupo experimental a superfície associava ataque ácido ao jateamento cerâmico. O grupo controle obteve aproximadamente 49% de contato osso-implante após oito semanas de reparo sem carga. Esse valor de contato é o mesmo encontrado em um estudo realizado por Bressan et al.[22] (2012) em implantes de titânio com superfície moderadamente rugosa, no entanto, após quatro meses de reparo sem carga. Em relação ao grupo experimental, a média obtida do valor de contato osso implante foi de 77,2% após oito semanas[21]. Stadlinger et al.[23] (2009) e Stadlinger et al.[24] (2009),

encontraram valores de 62% e 64%, e Buser et al.[25] (2004), o valor de 74.5% no contato entre osso e implante no mesmo período e em implantes de titânio com superfície tratada por jateamento e ataque ácido.

Outra propriedade importante que influencia na interação do material com o meio fisiológico que o circunda é a molhabilidade do substituto ósseo e da superfície do implante[26-27]. Materiais hidrofílicos podem aumentar a absorção da fibronectina, uma glicoproteína extracelular que auxilia na organização da matriz proteica e promove a atividade de ligação dos osteoblastos e a osteogênese[28]. Ainda, quanto mais hidrofílica for a superfície, maior será a quantidade de osteoblastos aderidos durante a fase inicial da osseointegração[29] e pode resultar em um contato maior das proteínas sanguíneas com o implante[30-31].

As superfícies porosas e hidrofílicas são mais desejáveis que superfícies apenas porosas, por apresentarem melhores resultados devido a melhor integração dos tecidos duros e moles[25,32]. Quando comparadas, em tíbias de coelhos, foi observado um valor no contato entre osso e implante 53% maior, após quatro semanas[33].

Buser et al.[34] (1991) compararam implantes instalados em porcos, com topografia semelhante e com diferentes índices de hidrofilia. Foi observado um aumento do contato osso implante de 20% nas superfícies hidrofílicas nas primeiras duas semanas após a instalação dos implantes, 15% em quatro semanas e nenhuma diferença foi encontrada entre as superfícies após oito semanas. Em outro estudo semelhante, Bornstein et al.[35] (2008) utilizaram cães e verificaram um aumento de 6% no contato osso implante nas superfícies hidrofílicas após duas semanas da instalação, porém não foi observada nenhuma diferença após quatro semanas. Ainda, Lang et al.[36] (2011) relataram diferença de 16% em quatro semanas pós-operatórias, sem encontrar diferenças nos períodos de duas e oito semanas.

Apesar de toda tecnologia para desenvolvimento de novas superfícies de implantes osseointegráveis, a quantidade e qualidade óssea presentes para a instalação de implantes devem ser levadas em consideração para a obtenção de melhores resultados funcionais e estéticos. Defeitos ósseos alveolares podem apresentar alterações morfológicas significativas, e necessidade de enxertos ósseos e/ou utilização de substitutos ósseos para a instalação de implantes[37-38]. A perda de

um elemento dentário é um dos fatores que levam ao desenvolvimento de defeitos ósseos a partir de diferentes mecanismos[39].

A instalação imediata de implantes, ou seja, logo após a extração do elemento dentário, é amplamente discutida[40-41]. Botticelli et al.[42] (2003) consideraram essa técnica um desafio, entretanto são observadas vantagens significativas como a melhora da percepção do posicionamento do implante, a diminuição do tempo total do tratamento e, principalmente, uma menor perda óssea periimplantar. No entanto, na instalação de um implante no alvéolo fresco, fica claro que não há o preenchimento total do alvéolo pelo implante, dessa maneira, haverá um defeito ósseo, acarretando uma osteogênese de contato e à distância[6,42-43]. É observado que implantes instalados imediatamente após a extração dentária apresentam taxas de sucesso similares quando comparados a implantes instalados em alvéolos raparados[44-47].

Segundo de Rouck et al.[48] (2008), o procedimento de instalação imediata do implante deve ser limitado a quantidade de osso suficiente para que haja a estabilidade primária, pela inserção de 3 a 5 mm na região apical do implante ou por uma largura do implante maior que o alvéolo receptor. Ainda, em alguns casos, não existe disponibilidade óssea necessária para a instalação de implantes, sendo requeridos procedimentos de regeneração óssea guiada. Alternativamente, para a instalação imediata de implantes, podem ser realizados enxertos ósseos concomitantes para preenchimento[49-50]. Porém, a distância entre a parede alveolar e a superfície do implante, também chamada de "*gap"*, é um fator importante a ser considerado. Estudos mostraram que, após seis meses, "*gaps"* com 2mm ou menos apresentaram reparo ósseo adequado sem a utilização de enxerto[51].

Dois estudos histológicos[52,53] apresentaram resultados positivos em relação ao reparo de alvéolos preenchidos com enxerto heterógeno e substitutos ósseos, enquanto outros estudos reportaram resultados pobres com enxerto heterógeno de origem bovina e autógeno[54,55]. Um desses estudos concluiu que os enxertos heterógenos bovino e autógeno interferem no reparo normal do alvéolo e, portanto, não deveriam ser utilizados[54]. Por outro lado, outros resultados demonstram que a presença de substitutos ósseos osteocondutores em contato com superfície dos implantes aumenta a osseointegração[56-58]. Segundo alguns autores, após oito semanas, ainda há processos de neoformação óssea ativa na interface osso-

implante[59]. Ainda, outro estudo demonstra que, após o mesmo período, ainda não há completa substituição de substituto ósseo por neoformação óssea[60].

Nevins et al.[61] (2006) verificaram que a reabsorção da crista alveolar pós-extração diminui consideravelmente quando utilizado osso bovino para preenchimento do "*gap"* quando comparado ao reparo normal apenas com o coágulo. No entanto, em outros dois estudos que compararam alvéolos preenchidos com osso bovino e coágulo, observaram taxas significativamente altas de neoformação óssea nos alvéolos preenchidos apenas com coágulo sanguíneo, após doze semanas, sugerindo retardo na reoformação óssea dos alvéolos preenchidos com este tipo de enxerto[62-63]. Entrentato, Artzi et al.[53] (2000) observaram altas taxas de neoformação óssea em alvéolos humanos preenchidos com osso bovino quando comparado ao período de seis semanas e três meses de reparo.

Em um estudo clínico, foi divido a forma de tratamento do alvéolo imediatamente após a extração em três grupos, no grupo 1 preencheram os alvéolos com osso bovino e após 10 meses instalaram implantes, no grupo 2 preencheram os avéolos com osso bovino mas sem instalação futura de implantes, e, finalmente, no grupo 3 mantiveram os alvéolos apenas com o coágulo sanguíneo. No primeiro ano, notaram leve reabsorção óssea apenas nos grupos 2 e 3. No terceiro ano, essa mesma reabsorção continuou maior no grupo 2 e menor no grupo 3. Enquanto que no grupo 1, ao longo dos três anos, houve manutenção do tecido ósseo[64].

O processo do reparo ósseo depende da condição vascular e celular tecido do ósseo[65]. Portanto, a presença de proteínas da matriz extracelular se torna um ponto importante, pois elas desempenham uma função no processo de ossificação contribuindo para o aumento da atividade celular ao redor dos implantes e osseointegração[66-67].Dessas proteínas, a osteoprotegerina que é secretada por osteoblastos, células do estroma, fibroblastos e linfócitos T, merece uma atenção especial ao passo que ela é considerada um regulador fisiológico da reabsorção óssea mediada pelos osteoblastos. Sua presença está envolvida na dinâmica da remodelação do tecido ósseo do processo de formação óssea[68-70]. Outra proteína que é produzida pelos osteoblastos e é importante no processo de ossificação é a osteocalcina. Além de ser a proteína mais abundante presente no osso, essa proteínanão-colagenosa desempenha funções nos estágios precoces do reparo ósseo e é essencial na regulação das atividades dos osteoblastos[71-73]. Em um estudo recente, foi concluído que o osso bovino permite a expressão dessas

proteínas da matriz óssea em um padrão semelhante quando comparado ao preenchimento de defeitos ósseos com enxerto ósseo autógeno, promovendo a mobilização de células formadoras de osso no defeito existente[74].

A osteocondução pode ser gerada a partir de reações em cadeia pelo recrutamento e migração de células osteogênicas pela iniciação da ativação plaquetária pela própria superfície do implante, enquanto que a neoformação óssea resulta em uma matriz mineralizada nessa superfície. Além disso, alguns estudos relatam que a osteogênese de contato depende da rigidez da superfície do implante[5-6,75]. Isto se deve ao fato de que a rigidez pode aumentar as possibilidades de adesão das fibrinas a superfície do implante e auxiliar a migração de células ósseas. Em estágios tardios da osseointegração, o novo osso formado pela osteogênese distante do implante e pela osteogênese de contato se fusionam estabelecendo uma interface justa entre o osso e o implante. A observação sobre esses dois tipos de formação óssea indica que ambas se iniciam precocemente e rapidamente, sendo que nas primeiras duas semanas as duas diferentes estruturas ósseas podem ser identificadas e a osseointegração pode ser estabelecida dentro de quatro semanas[18].

Desta forma é observada uma diversidade de estudos avaliando superfícies de implantes, substitutos ósseos e seu uso em defeitos ósseos. Contudo, não há estudo avaliando a viabilidade celular imediata e tardia com superfícies hidrofílicas em situação clínica simulando o alvéolo de extração com e sem substituto ósseo associado. Sendo assim, a proposta desse projeto é verificar a presença e viabilidade celular associada a superfícies hidrofílicas ao redor de implantes instalados em tíbias de coelho, com a criação de defeitos ósseos e a colocação ou não de substitutos ósseos.

2 PROPOSIÇÕES

O presente estudo teve por objetivo:

Artigo 1 – Avaliar por meio de análise microtomográfica e histológica o reparo ósseo peri-implantar, em duas superfícies de implantes de titânio, instalados em defeitos ósseos, preenchido ou não com substituto ósseo.

Artigo 2 – Avaliar por meio de análise molecular, imuno-histoquímica e microtomográfica, o reparo ósseo peri-implantar, em duas superfícies de implantes de titânio, instalados em defeitos ósseos, preenchido ou não com substituto ósseo.

Artigo 3 – Avaliar por meio de análise histomorfométrica, o reparo ósseo peri-implantar, em duas superfícies de implantes de titânio, instalados em defeitos ósseos, preenchido ou não com substituto ósseo

3 PUBLICAÇÕES

O desenvolvimento desse projeto resultou na produção de três artigos com objetivos específicos distintos.

1) “Bone formation around two titanium implant surfaces placed in bone defects with and without a bone substitute material: a histological and radiographic evaluation” encaminhado e aceito no periódico Clinical Implant Dentistry and Related Research;
2) “Gene expression, immunohistochemical and microarchitectural evaluation of bone formation around two implant surfaces placed in bone defects filled or not with bone substitute material” encaminhado para o periódico Clinical Oral Implants Research;
3) “Histomorphometric analysis of bone tissue formation around two titanium implants surfaces placed in bone defects filled or not with bone material substitute“ encaminhado e aceito no periódico Clinical Implant Dentistry and Related Research.

3.1 Artigo 1* – Bone formation around two titanium implant surfaces placed in bone defects with and without a bone substitute material: a histological and radiographic evaluation

Bone formation around two titanium implant surfaces placed in bone defects with and without a bone substitute material: a histological and radiographic evaluation

Short title: Histological and microtomographic evaluation of bone formation around two Ti implant surfaces

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Key-words: Osseointegrated dental implantation, bone grafting, cell viability

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* *Artigo submetido e aprovado para publicação no periódico Clinical Implant Dentistry and Related Research (ANEXO B – Versão pré-revisão por pares)*

Abstract

Objective: to evaluate the histological and microtomographic response of peri-implant bone tissue around titanium implants with different surface treatments, placed in bone defects filled or not filled with bone substitute materials. Materials and Methods: thirty rabbits were divided into two groups according to the implant surface treatment. A bone defect was created in both tibias of all the rabbits, followed by the placement of one implant in each of these defects. On the left tibia, the defect was filled with a blood clot (BC), and on the right tibia, the defect was filled with biphasic hydroxyapatite/β-tricalcium-phosphate (HA/TCP); thus, there were four groups in total: BC-N: bone defect filled with a blood clot and porous surface titanium implant (control group); BC-A: bone defect filled with a blood clot and porous-hydrophilic surface titanium implant; HA/TCP-N: bone defect filled with a bone substitute material and porous surface titanium implant; HA/TCP-A: bone defect filled with a bone substitute material and porous-hydrophilic surface titanium implant. The animals were submitted for euthanasia at three distinct periods: 15, 30, and 60 days after implant installation. The samples were evaluated histologically and histometrically, to assess the quantity and quality of cells and the remaining bone substitute material in the grafted areas. The bone quality was assessed by micro-computed tomography. Results: at all periods, the HA/TCP-A group had a higher percentage of new bone formation. No significant statistical difference was found for new bone formation between the four groups. For both surface types, the presence of a bone substitute material led to higher values in all evaluated micro-CT parameters, except in the bone surface/volume ratio parameter. Conclusion: these results suggest that a porous hydrophilic surface in the presence of bone substitute material can accelerate peri-implant bone tissue formation.

Keywords: Osseointegrated dental implantation, bone grafting, cell viability

Introduction

Dental implant installation immediately after a tooth extraction has become a very common surgical technique in clinical practice, with advantages such as a shorter rehabilitation treatment time and less surgical procedures. Moreover, this technique has shown successful osseointegration at a similar rate, when compared to implants placed in healed sites that are independent of the presence of bone substitutes.[1,2]

Although a blood clot by itself can lead to socket repair after tooth extraction, there is normal diminishing of the alveolar crest due to physiological resorption.[3] It is suggested that dental implants positioned immediately after the tooth extraction in a fresh socket, filled with bone substitute material can prevent this physiological resorption.[4,5] However, immediate placement of an implant may lead to a space between the implant broders and the marginal borders of the fresh socket. This space is called a gap or jumping distance, and can occur on a buccal, lingual or proximal aspect.[6] However, bone substitute materials have been used in the gaps associated with dental implants to help prevent bone defects and aid implant stability.[7]

Osborn & Newesely[8] described two distinct mechanisms leading to bone formation: distance and contact osteogenesis. However, it has not been elucidated whether both mechanisms occur independently or if there is an interaction between them, leading to peri-implant bone healing.[9] The integrity of the bone and implant interface precept the oral rehabilitation success. A chain of biological reactions associated with numerous factors and surgical techniques, are extremely important for wound healing after implant placement.[10]

Currently, there are a vast number of dental implant systems with different characteristics, such as dimension, shape, thread design, and surface modifications.[11] Surface features such as topography, coatings, and wettability can mediate the direct adhesion of host osteoblasts in bone formation, that affect biological processes during osseointegration.[12] Furthermore, studies have shown that microscopic modifications to titanium implant surfaces can stimulate blood proteins, that direct bone formation from the implant surface toward the host bone.[13,14]

The development and improvement of bone substitute material that can accelerate a quality bone formation process, is essential to optimize patient oral rehabilitation. Therefore, the aim of this *in vivo* study was to evaluate, through histologic, histometric, and radiographic (micro-CT) assessment, the bone formation at different time periods, of two different titanium implant surfaces placed in bone defects, simulating alveolar sockets, either filled with biphasic ceramics of hydroxyapatite/β-tricalcium phosphate (HA/TCP), or not.

Materials and Methods

This study was submitted to and approved by the Ethics Committee in the Use of Animals of the São Paulo State University (Unesp), School of Dentistry, Araçatuba, Brazil (Protocol number FOA: 465-2016). Thirty *Oryctolagus cuniculus* male rabbits, weighting between 300 to 400g, and approximately 5 months in age, provided by Central Vivarium of the São Paulo State University (Unesp), Botucatu Campus, were used. The animals were kept in separate cages with rabbit chow and water *ad libitum*.

The animals were randomly divided into two groups according to the type of implant surface. Before implant placement, a defect was created on both tibias of all the rabbits. The groups were divided according to how the created defect was to be filled: BC-N: the bone defect was filled with a blood clot, and implants with a porous surface (Neoporos surface, Neodent®, Curitiba, Brazil) (control group), BC-A: the bone defect was filled with a blood clot, and implants with a porous-hydrophilic surface (Acqua surface, Neodent®, Curitiba, Brazil), HA/TCP-N: the bone defect was filled with biphasic ceramics of hydroxyapatite/β-tricalcium-phosphate (Straumann® Bone Ceramic, Straumann AG, Basel, Switzerland), and implants with a porous surface (Neoporos surface, Neodent®, Curitiba, Brazil), HA/TCP-A: the bone defect was filled with biphasic ceramics of hydroxyapatite/β-tricalcium-phosphate (Straumann® Bone Ceramic, Straumann AG, Basel, Switzerland), and implants with a porous-hydrophilic surface (Acqua surface, Neodent®, Curitiba, Brazil).

Euthanasia of the rabbits occurred at three distinct experimental periods: 15, 30, and 60 postoperative days, generating 12 groups. Each group was composed of 5 tibias (Figure 1). The selection of the tibias, the type of implant, and how the defect was filled were determined by simple randomization.

Surgical procedure

After 8 hours of fasting, the animals were sedated and anesthetized via an intramuscular injection, using the combination of ketamine 50mg/ml/kg (Fracontar, Vibrac do Brasil Ltda., São Paulo, Brazil), and xylazine 5mg/ml/kg (Rompum, Bayer AS, Saúde Animal, São Paulo, Brazil). Subsequently, the rabbits underwent trichotomy of the internal region of the left and right hind paws, antisepsis was also performed. Local anesthesia was administered using 0.3ml/kg of mepivacaine with 2% epinephrine 1:100.000 (Mepiadre – Nova DFL, Rio de Janeiro, Brazil), for analgesia and hemostasis.

A 30-mm incision was created in the plane of the tuberosity of both tibias. After delicate dissection of the soft tissues, and a complete detachment and exposition of the bone tissue in the area of interest, an osteotomy was performed with a 6.1-mm trephine drill (Neodent®, Curitiba, Brazil), creating a defect in the tibias. The internal cortical was removed, but the external cortical of each tibia was preserved. Subsequently, the drilling for implant installation was performed in the defect center, initially with a pilot drill, followed by helicoidal drills with a thickness of 2.0mm, 2.8mm, and 3.0mm (Neodent®, Curitiba, Brazil), to accommodate a 8-mm-high titanium implant, with a 3.5-mm-diameter (Drive CM Neoporos and Acqua surfaces, Neodent®, Curitiba, Brazil). Both osteotomies were made under abundant irrigation with sterile saline. According to each group, the implants were installed achieving primary stabity in the external cortical of the tibia, and the bone defect was then filled with either a bone substitute or a blood clot. The tissue was repositioned and sutured by planes.

Fifteen, 30, and 60 days post-surgery, the animals were submitted to euthanasia by an overdose of anesthetic. The tibias were removed, maintained, and fixed in 10% formalin solution for 48 hours, washed in running tap water for 24 hours, and later stored in 70º ethanol.

Micro-CT

The samples were scanned with a micro-CT (μCT) scanner (SkyScan 1176 Bruker MicroCT, Aatselaar, Belgium), with the following parameters: camera pixel, 12.45; X-ray tube potency, 50 kilovoltage peak (kVp); X-ray intensity, 500μA; integration time, 300ms; filter, 1mm aluminum (Al); voxel size, 9μm^3. The images obtained from the X-ray projections were filed, reconstructed, spatially repositioned, and analyzed using specific software (NRecon, Data Viewer, CTAnalyser, Aatselaar, Belgium). Three possible planes were obtained and evaluated: coronal, transaxial, and sagittal. Each plane had a defined region of interest (ROI): coronal: 0.5mm circular region around the entire diameter of the implant, transaxial and sagittal: a rectangular shape vertically from the top of the implant to the end of the second big thread, and 0.5mm horizontally from the border of the implant. This ROI was defined as the total volume (0.5 mm margin around the implants, with a height of two entire big threads) (Figure 2). The threshold used in the analysis was 25-90 in grayscale, and the values of the mineralized tissue around the implants were assessed using five parameters: bone volume (BV), percent bone volume (BV/TV), bone surface

(BS), bone surface/volume ratio (BS/BV), and bone surface density (BS/TV). A trained and blinded examiner performed this analysis.

Histological analysis

After µCT analysis, the samples were submitted for decalcification processing. As soon as the decalcification was verified as completed, the implants were removed from the specimens. The samples were embedded in paraffin, sliced to a thickness of 6µm, placed on slides, and stained with hematoxylin and eosin.

Next, samples were evaluated by light microscopy (Diastar Leica Reichert & Jung Products, Germany), and images were captured using the attached digital camera (DFC-300-FX, Leica Microsystems, Germany), at 2.5x and 20x magnification. Images were analyzed at their original size, using ImageJ 1.45s (National Institutes of Health, Bethesda, MD, USA). Areas of new bone formation, connective tissue, and remaining bone substitutes around the implant threads were captured and calculated. A single researcher trained in advance for this work performed all analyses and data collection.

Histometric analysis:

The same images acquired for the histological analysis, were used for the histometric analysis. The images were stored as TIFF files and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The area of bone tissue present around the first three threads of the implant was assessed. The data obtained from the analyses were transformed into absolute values of pixels, and then to percentages for statistical tests, to minimize any interference by the negative size difference. To reduce bias a single researcher blinded to the study performed all the analyses and collected the necessary data.

Statistical analysis

The data generated by the histological and microtomographic analyses was examined for normality using the Shapiro-Wilk test. The data was normally distributed, and the group differences were examined using a parametric test either, one-way analysis of variance (ANOVA) (microtomographic), or two-way ANOVA (histologic and histometric), complemented by the Tukey's test. The bone matrix and adipocytes data presented a non-normal distribution and were examined with the Kruskal-Wallis test. Moreover, histological data was submitted to descriptive analysis.

Results

Histological and histometric analysis

Since the implants were removed from the tibia samples before paraffin embedding, the samples suffered some damage to their quality, however, descriptive and statistical analyses continued to be performed. During histological analysis several cell types were observed and recognized, such as adipocytes, fibroblasts, bone matrix, endothelial and inflammatory cells. Also, particles of bone substitute material were identified. All results are in Tables 1 and 2.

Descriptive qualitative histological analyses showed that the presence of bone matrix cells were time-dependent from the initial to the last period in three groups (HA/TCP-A, HA/TCP-N, and BC-A). Curiously, the same three groups had a similar cell response, with the number of cells decreasing from day 15 to 30, and then increasing at day 60. The control group (BC-N) presented a cell response peculiar, but bad with regard to qualitative analysis.

The total number of cells in each group (adipocytes, fibroblasts, bone matrix, endothelial, and inflammatory cells) was statistically significant between the different implant surfaces. These differences could be seen at all time periods. The HA/TCP-A group presented a statistically higher quantity of cells when compared to the other groups. The HA/TCP-N group presented a statistically higher quantity of cells when compared to both porous surface groups. No significant statistical differences were found between the porous surface groups. The presence of fibroblasts was statistically higher in the HA/TCP-A group when compared to the other groups all over the periods. No significant statistical differences were found regarding the other cell types (Figures 3 and 4).

Table 3 shows the mean percentages and standard deviation, of the new bone formation values for each group at all time periods. At all time periods, the HA/TCP-A group had higher values. Similar to the qualitative histological analysis, the HA/TCP-A, HA/TCP-N, and BC-A groups had a similar cell response to new bone formation, the percentage decreased from day 15 to 30, and then increased at day 60. At first the control group (BC-N) presented an increase in percentages, which then decreased to below the initial period. There were no statistically significant differences between the groups.

Micro-CT

Five different parameters were used to assess the mineralized bone of the samples. All results are in Table 4. Figure 5 shows the graphics of all parameters.

Although, no significant statistical differences were found between the time periods for all groups, the quantitative differences are evident.

Bone volume (BV): The BV values related to the porous-hydrophilic surface decreased over all the time periods, while the BV values related to the porous surface increased from 15 to 30-days, and subsequently decreased to the same value observed on day 15. The porous-hydrophilic surface associated with bone substitute material, had higher values of BV at the earliest and latest periods. At 15-days, there was a significant statistical difference in the BV, between the surfaces in the presence of bone substitute material ($p \leq 0{,}001$).

Percent bone volume (BV/TV): Over the time periods, the porous-hydrophilic surface presented a decrease in BV/TV values, whereas the values related to the porous surface increased from day 15 to 30, and then decreased to almost the same BV/TV value recorded on day 15. The porous surface associated with a bone substitute material, had a higher BV/TV at 30 and 60 days. Again, at the later period of 60 days, a significant statistical difference in BV/TV between the surfaces in the presence of bone substitute material was observed ($p \leq 0{,}05$).

Bone surface (BS): Although porous-hydrophilic surfaces presented an overall progressive decrease in BS over the time periods, when this surface was associated with a bone substitute material, higher BS values were observed at day 15 compared to all other groups. Similar to other parameters, the porous surface had an increase in BS from 15 to 30 days, followed by a subsequent decrease. At all time periods, the porous hydrophilic surface groups presented a significant difference in BS ($p \leq 0.05$ for 15-days, $p \leq 0.01$ for 30-days, and $p \leq 0.001$ for 60-days).

Bone surface/volume ratio (BS/BV): Higher BS/BV values were detected in the blood clot groups. The BS/BV for the porous-hydrophilic surface was maintained over all the time periods, while the porous surface BS/BV oscillated, with lower values being observed at day 60. At all time periods a significant statistical difference was observed in BS/BV between the same surface groups, regarding the presence of bone substitute material ($p \leq 0.001$).

Bone surface density (BS/TV): All but one group presented a progressive decrease in BS/TV values over all time periods. The porous surface associated with a blood clot initially had a decrease in BS/TV, followed by an increase. A significant difference was only observed on day 15 ($p \leq 0.05$; $p \leq 0.001$).

Discussion

Tooth extraction is an undesirable experience that can affect oral function and esthetics of patients. The absence of the extracted tooth, becomes a complaint that needs to be treated as soon as possible.[15] However, surgical procedures, such as socket graft filling, bone augmentation, or the complete healing of the alveolar bone, delays patient rehabilitation.[4] Therefore, protocols were proposed that took into account the timing of implant placement.[16,17] Furthermore, different studies were performed to assess the size of defects, implant designs, and the use of bone substitutes to accelerate the bone formation process, with the aim of shortening, and optimizing the treatment time.[1,6,18] This study also researched how bone formation at tooth extraction sites could not only be improved, but occur faster by different titanium implant surfaces, whether associated with bone substitute material or not.

In vitro and animal studies have already demonstrated that alterations to the biomaterial surface, improves the interaction between the titanium implant and the human body.[19,20] Implants with a high-energy surface present a better wettability (or hydrophilicity) and higher affinity for the adsorption of blood proteins, resulting in a faster bone formation process. Therefore, wettability results in positive features during the early stages of wound healing.[21] In this study, a porous-hydrophilic surface resulted in higher values of bone volume, with significant statistical differences among all the groups during the initial stages of bone formation (15-day period). Histologically, a higher number of cells were observed on the porous-hydrophilic surface after a 15-day period. This difference was statistically significant when compared to the porous surface. However, it was maintained throughout all of the time periods.

Schwarz et al. (2007)[22] compared two different nanostructured dental implant surfaces. The authors showed that hydrophilic surfaces have a higher affinity to form an initial blood clot, with improved angiogenesis, and a greater bone density after a 14-day period of bone healing. In this study, histological and histometric results showed that porous-hydrophilic surfaces had a higher number of endothelial cells on day 15 when compared to days 30 and 60. Also, the results of this study were similar to Schwarz et al. (2007)[22] with the group HA/TCP-A presenting with a greater bone density, with a significant statistical difference, than the groups without bone substitute material. However, no significant statistical differences were found between the surfaces that were associated with bone substitute material on day 15.

In this study some differences among the groups were found in the descriptive qualitative histological analysis, however the HA/TCP-A, HA/TCP-N, and BC-A groups followed a similar cell presence response. The only obvious difference was the presence of bone substitute material. According to Lang et al. (2010)[23], the bone healing process of a hydrophobic or modified hydrophilic surface, has a similar histological pattern with many common features. The use of bone substitutes is another critical point in dental implant osseointegration. Santos et al. (2013)[7] performed a literature review on bone substitute materials, that were applied to the surrounding area of dental implant, that had been immediately placed following tooth extraction, and concluded that this is a reliable treatment, that reduces the length of the patient's rehabilitation time. Therefore, the use of bone substitute materials, for improving osseointegration and increasing bone implant contact is required. However, in the present study there were no significant statistical differences between the implant surfaces in the presence of bone substitute materials, and there were no significant statistical differences between the surfaces when they were compared without bone substitute material in the early time period. Furthermore, there are no statistical significant differences regarding the presence of bone matrix cells.

El Chaar et al. (2019)[24] performed a similar study to the present one but filled the defect with two different bone substitute materials and did not have a blood clot group. The authors suggest that the presence of bone substitute material may facilitate blood clot stabilization and support the osteoprogenitor cells invasion and access to the implant surface. Schwarz et al. (2007)[22] described these processes as crucial events for faster ossointegration of hydrophilic surfaces. The results from the present study support these conclusions, because faster bone formation was observed in the porous-hydrophilic surface and bone substitute material group (HA/TCP-A) at the earliest period (15-days). Moreover, Chen & Buser[16] recommended the filling of an implant periphery gap with a bone replacement graft in order to avoid alterations to the dimensional crestal bone, leading to a negative aesthetics outcome.

In contrast, Stavropoulos et al. (2004)[25] investigated newly formed bone in association with two different bone substitute materials placed inside a capsule, which was in contact with the mandibular bone in rats and compared the bone growth to an empty capsule. The results were opposite to those found in this study. The

authors concluded that the use of bone substitute material is stable on a long-term basis, but obstructed bone formation.

Philipp et al. (2014)[26] performed a preclinical study in sheep, comparing hydrophilic and hydrophobic surface implants installed in the maxillary sinuses grafted with HA/TCP. The results did not show any differences between the surfaces. However, these results can be attributed to the experimental time period of 3 to 6 months once bone formation tends to be observed within a shorter timeframe than the cited experimental period. Pinotti et al. (2018)[27] evaluated the effect of a hydrophilic surface on the osseointegration in the grafted areas, with two different bone substitute materials, including HA/TCP. The results showed an increasing volume of mineralized tissues (BV/TV) around the implants in the grafted areas from day 15 to day 45. The present study showed contrasting results, presenting with a decrease in the volume of mineralized tissue from day 15 to day 60. Furthermore, the authors presented statistically higher results from a hydrophilic surface, when compared to a hydrophobic surface in the presence of HA/TCP at day 45, while in the present study this difference was only observed at day 15. One interesting conclusion by the authors is that, the behavior of hydrophilic surfaces is more positively noticeable in areas grafted with bone substitute materials with a lower osteoconductive potential.

Although the effects of hydrophilic surfaces of dental implants in grafted defects with bone substitutes on bone integration remain unclear[27], the results suggest that the association between porous-hydrophilic surfaces and HA/TCP has an optimizing effect on the osseointegration process. However, further analysis is required to fully understand this process, and to reach concrete conclusions.

Conclusion

In a rabbit tibia model, implants with porous-hydrophilic surfaces accelerated bone tissue formation in association with bone substitute material in created defects compared to porous surface implants.

Acknowledegments

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TABLES

Table 1. Mean and standard deviation of each cell/0.1mm^2 according to all groups and time periods

		CELLS					
	GROUP	**Bone matrix**	**Fibroblasts**	**Adipocytes**	**Endothelial**	**Inflammatory**	**Total**
15 days	**HA/TCP-A**	4.2 (9.39)	17.6 (8.53)^B	-	2.4 (1.51)	32.6 (11.92)	**57 (12.62)**
	HA/TCP-N	0.6 (1.34)	9.4 (5.5)^A	2.4 (1.51)	1.6 (1.34)	57.2 (29.6)	**71.2 (26.78)**
	BC-A	5 (11.18)	8.8 (3.42)^A	2 (1)	1.4 (0.89)	37.8 (10.84)	**50 (10.12)**
	BC-N	1 (1.73)	7.2 (3.27)^A	0.6 (1.34)	1.6 (1.14)	23.4 (11.67)	**33.8 (12.23)**
30 days	**HA/TCP-A**	1.4 (3.13)	9.4 (3.78)^B	2.8 (2.58)	1.6 (1.14)	49 (41.8)	**64.2 (44.75)**
	HA/TCP-N	-	5.2 (1.78)^A	2.8 (0.83)	1 (0.7)	23 (15.89)	**32 (13.83)**
	BC-A	0.6 (1.34)	5 (1)^A	1.4 (0.54)	1.4 (1.14)	52.8 (39.06)	**61.2 (40.65)**
	BC-N	1.4 (3.13)	7.4 (1.95)^A	2.2 (1.64)	1.6 (1.51)	24 (15.16)	**36.6 (17.17)**
60 days	**HA/TCP-A**	5 (7.54)	12.2 (5.8)^B	5 (4)	0.6 (0.54)	42.8 (48.39)	**65.6 (48.43)**
	HA/TCP-N	3.8 (7.42)	7.4 (3.43)^A	3 (2.54)	1.4 (0.54)	42.4 (20.48)	**58 (18.43)**
	BC-A	8.4 (11.05)	6.2 (1.64)^A	1,6 (0,54)	1,4 (1,67)	48,2 (20,74)	**60,4 (25,22)**
	BC-N	-	6.2 (4.09)^A	3,8 (1,64)	1,2 (0,83)	25,4 (20,99)	**36,6 (23,43)**

Uppercase letter means statistically significant difference.

Table 2. Mean and standard deviation of the total amount of cells according to all groups and time periods.

	PERIODS			
GROUPS	**15 days**	**30 days**	**60 days**	**Total**
HA/TCP-A	57 (12.63)^{Aa}	64.2 (44.75)^{Aa}	65.6 (48.44)^{Aa}	**62.27 (36.1)^{A}**
HA/TCP-N	71.2 (26.79)^{Ba}	32 (13.84)^{Ba}	58 (18.44)^{aa}	**53.73 (25.32)^{B}**
BC-A	50 (10.12) ^{Aa}	61.2 (40.66)^{Aa}	60.4 (25.22)^{Ab}	**57.2 (26.67)^{C}**
BC-N	33.8 (12.24)^{Ba}	36.6 (15.18)^{Ba}	36.6 (23.44)^{Bb}	**35.67 (16.35)^{B}**
Total	**53 (20.7)^{a}**	**48.5 (32.78)^{a}**	**55.15 (30.72)^{a}**	

Uppercase letter means statistically significant difference among columns; lowercase letter means statistically significant difference among rows.

Table 3. Mean percentages and standard deviation of new bone formation values in each group at all time periods.

	GROUPS			
PERIODS (days)	**HA/TCP-A**	**HA/TCP-N**	**BC-A**	**BC-N**
15	31.73 (7.9)	27.22 (6.1)	25.08 (3.9)	24.1 (2.6)
30	30.32 (2.7)	26.32 (2.1)	24.67 (2.7)	26.2 (2.1)
50	33.54 (6.2)	30.76 (5.2)	27.68 (3.7)	22.42 (2.35)

Table 4. Mean and standard deviation of all parameters in all groups and time periods.

µCT	Period (days)	Groups			
		HA/TCP-A	HA/TCP-N	BC-A	BC-N
BV (mm^3)	15	49.00 (9.85)	29.18 (5.92)	16.59 (4.13)	14.14 (2.57)
	30	38.17 (3.47)	39.76 (8.92)	12.66 (2.94)	15.53 (4.79)
	60	29.98 (10.88)	24.16 (9.36)	9.7 (2.36)	12.64 (2.60)
BS (mm^2)	15	811.6 (79.71)	657.3 (134.7)	474.8 (64.13)	387.9 (34.18)
	30	634.2 (77.9)	681.7 (95.22)	374.2 (69.74)	453.9 (115.1)
	60	493.4 (125.8)	366.8 (82.24)	296.1 (62.21)	331.6 (63.35)
BS-BV (mm^{-1})	15	20.99 (1.65)	23.05 (1.18)	30.59 (2.07)	29.02 (1.06)
	30	17.14 (3.2)	17.87 (1.41)	30.1 (1.94)	30.18 (3.1)
	60	17.7 (3.52)	16.52 (1.95)	30.8 (2.44)	27.04 (1.79)
BV-TV (%)	15	37.33 (1.97)	30.78 (3.66)	19.93 (2.4)	20.98 (4.57)
	30	35.57 (6.17)	36.94 (2.70	16.79 (3.68)	17.21 (4.39)
	60	32.55 (7.56)	35.84 (6.24)	15.82 (3.81)	19.28 (3.51)
BS-TV (mm^{-1})	15	7.69 (0.27)	6.93 (0.84)	5.86 (0.28)	5.77 (0.59)
	30	6.02 (1.43)	6.52 (0.42)	5.01 (0.85)	5.03 (0.82)
	60	5.43 (0.55)	5.6 (0.38)	4.82 (0.98)	5.12 (0.89)

FIGURES LEGENDS

Figure 1. Groups flowchart.

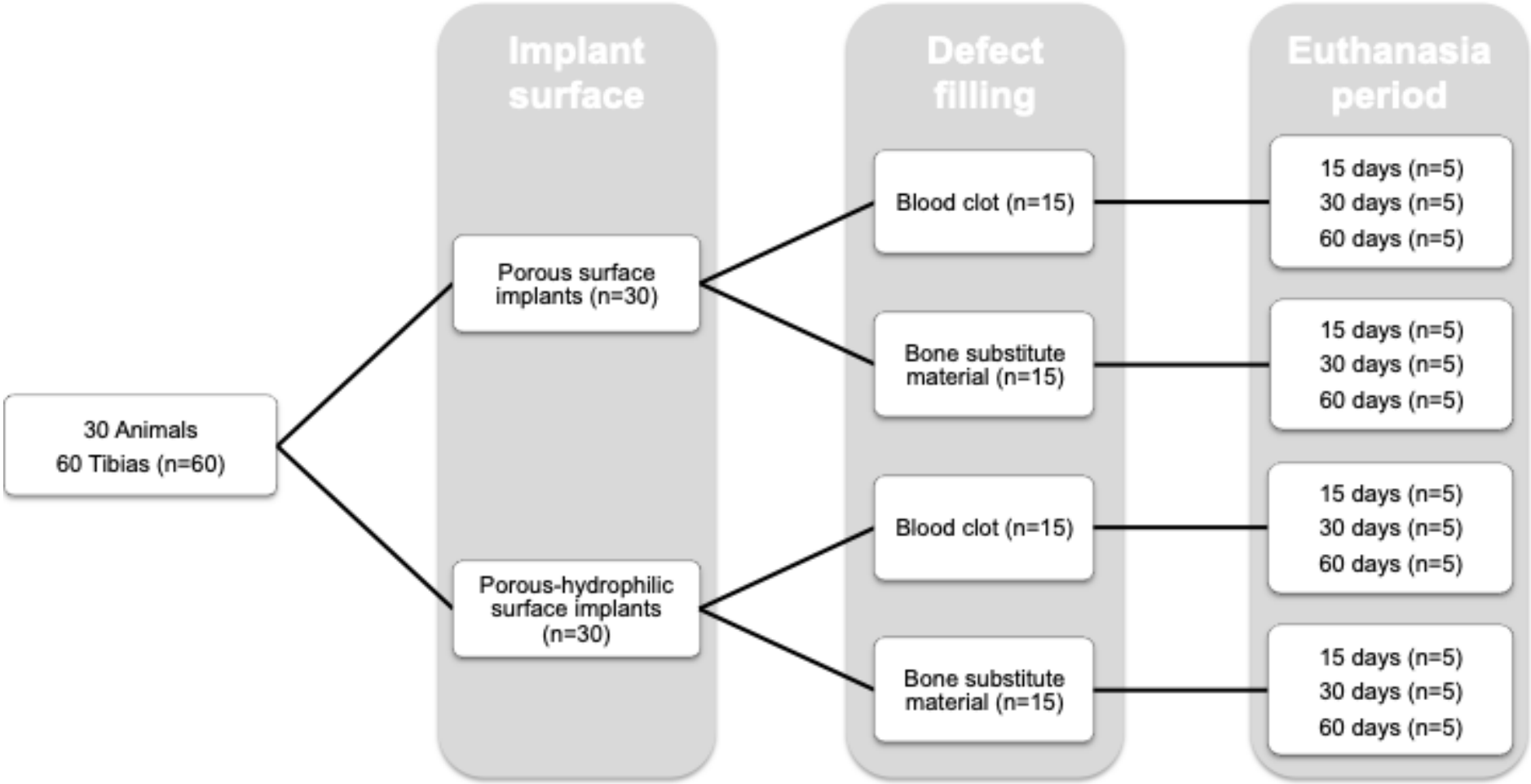


Figure 2. Model of the micro-computed tomography (CT) analysis. The region of interest (ROI) was defined as the total volume (white circle and rectangular form).

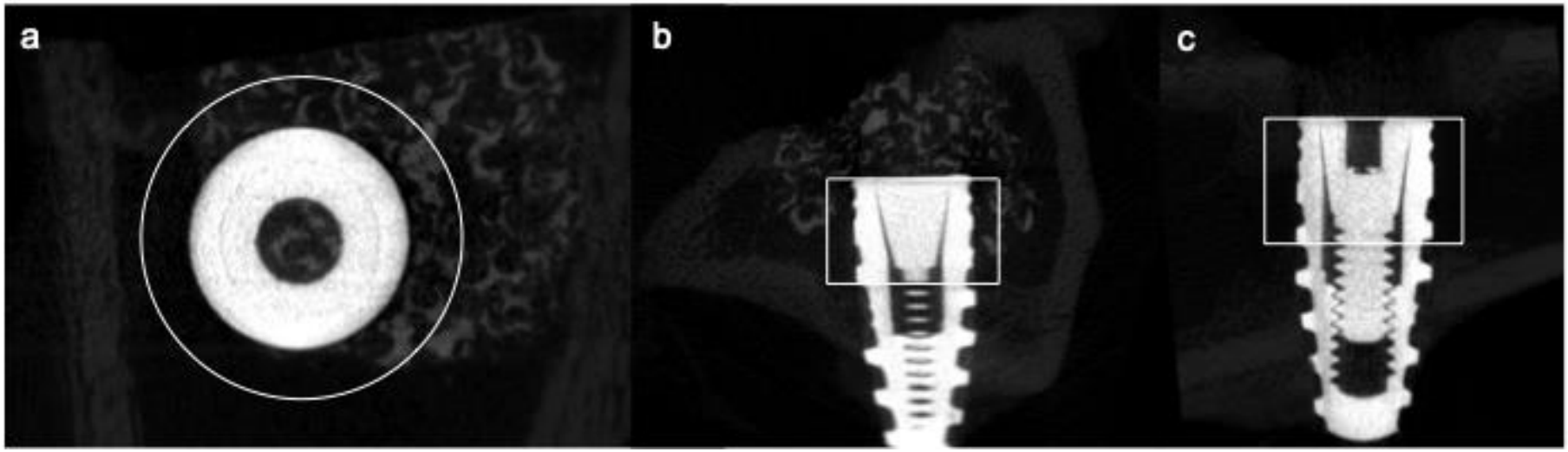


Figure 3. Histological slices stained with hematoxylin and eosin (20x) of areas around the implant threads.

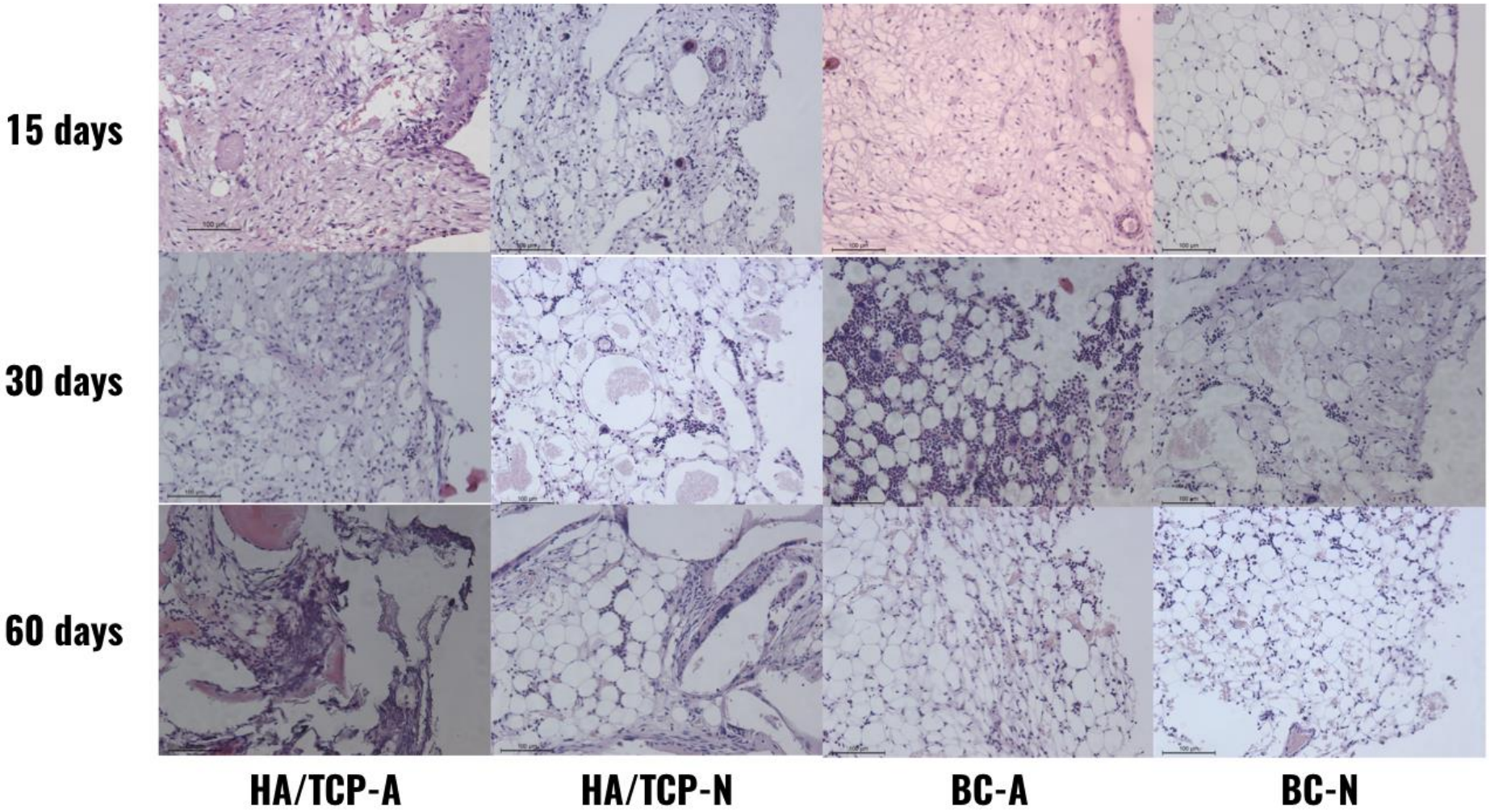
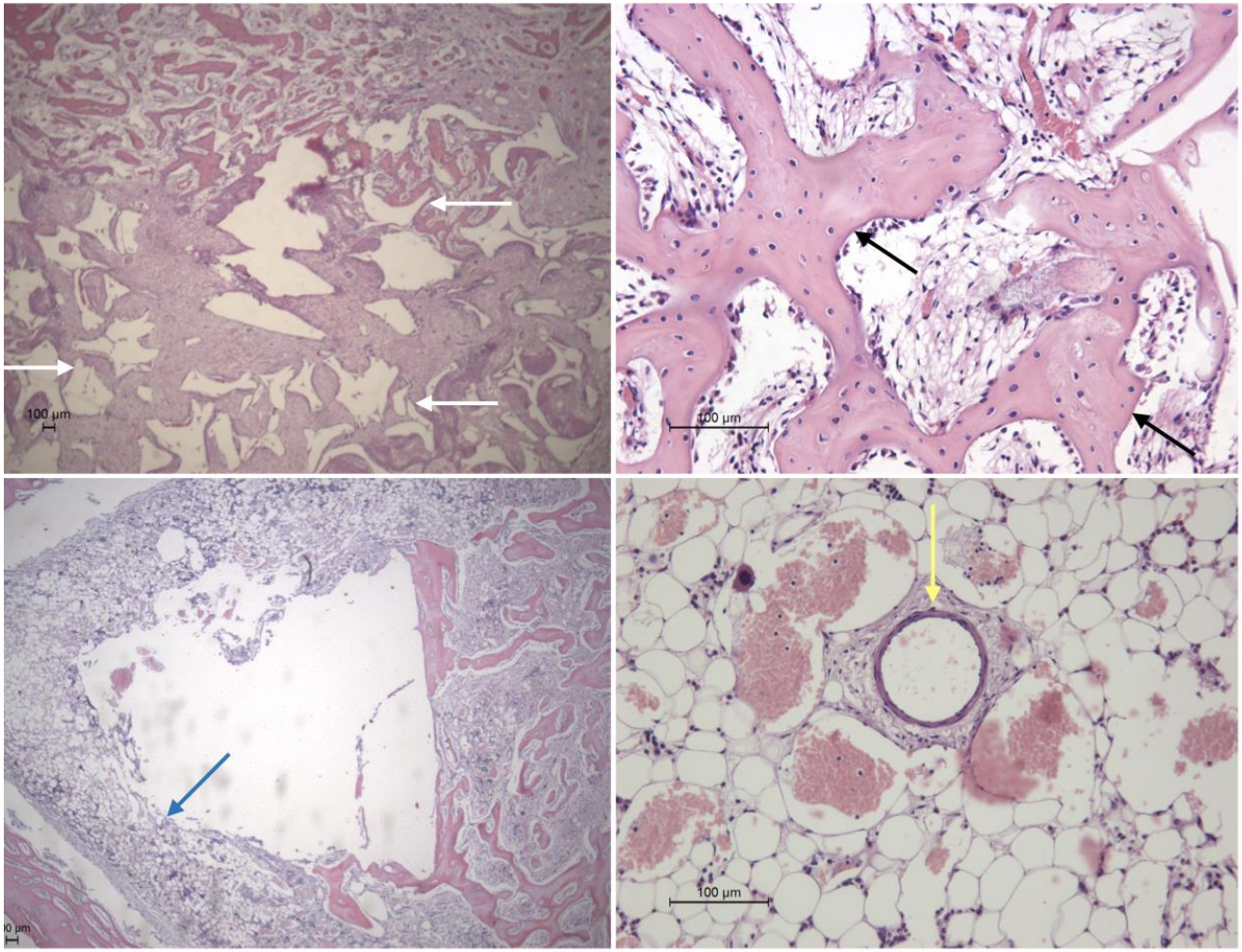


Figure 4. Recognized cells in samples (hematoxylin and eosin, 5x and 20x) at 15-day period.



Bone substitute material (white arrow); Bone formation (black arrow); Adipocytes (blue arrow); Endothelial cells (yellow arrow). Fibroblasts and inflammatory cells can be also observed.

Figure 5. Graphics of all micro-computed tomography (CT) parameters (* p≤0.05; ** p≤0.01; *** p≤0.001).

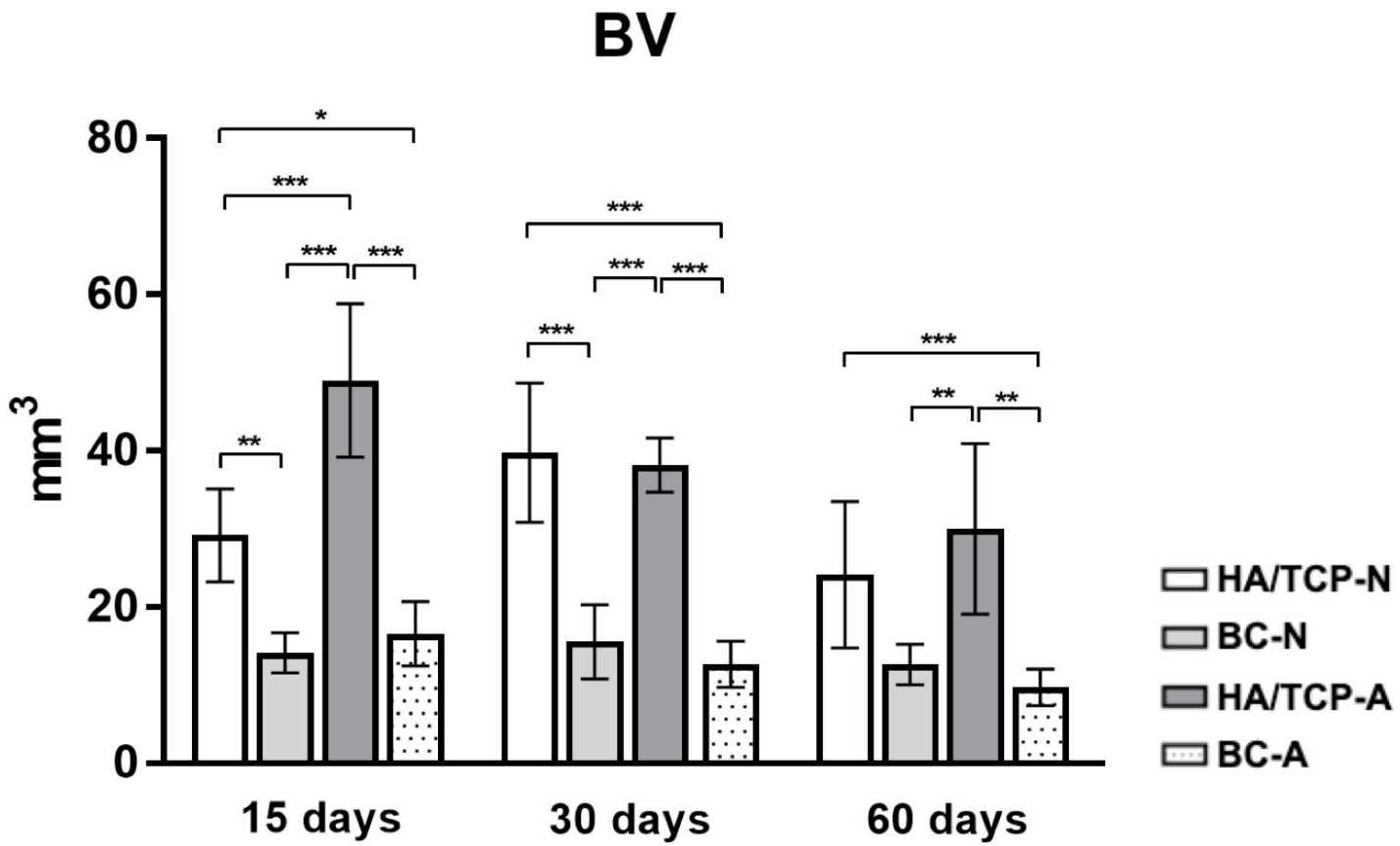


Figure 5. Graphics of all micro-computed tomography (CT) parameters (* p≤0.05; ** p≤0.01; *** p≤0.001) (*continuation*).

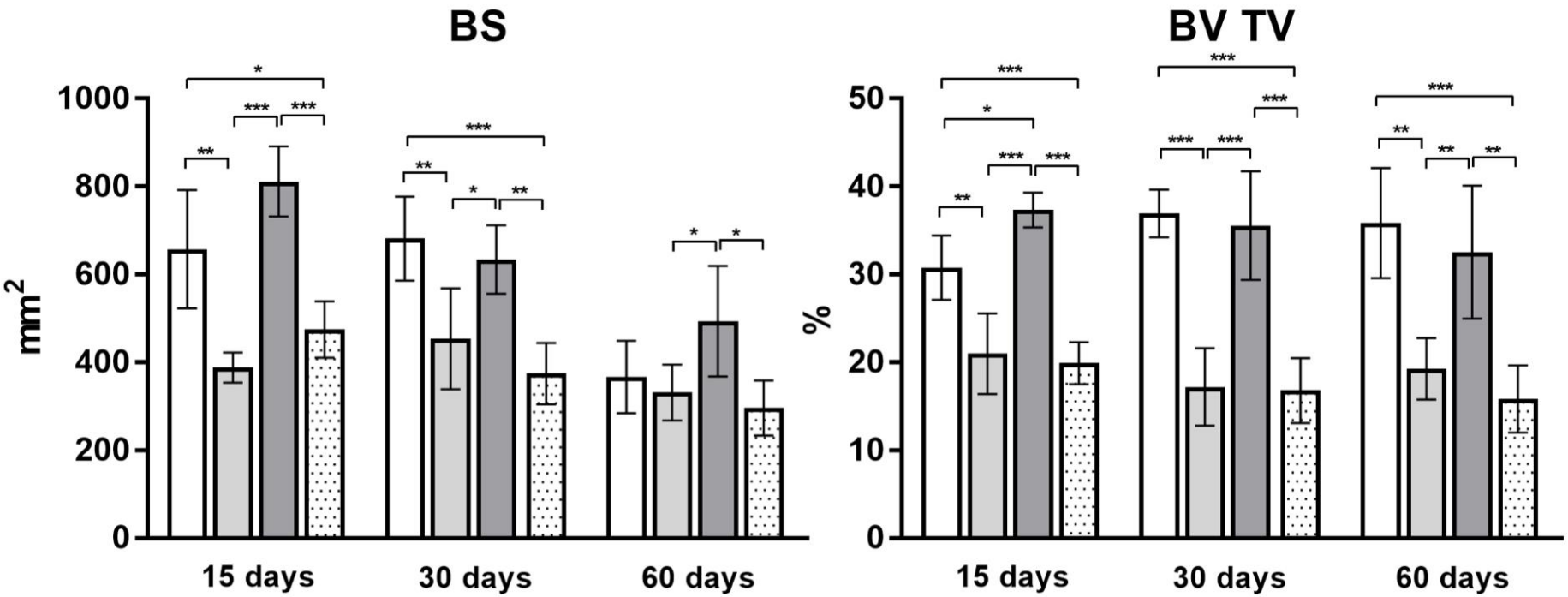
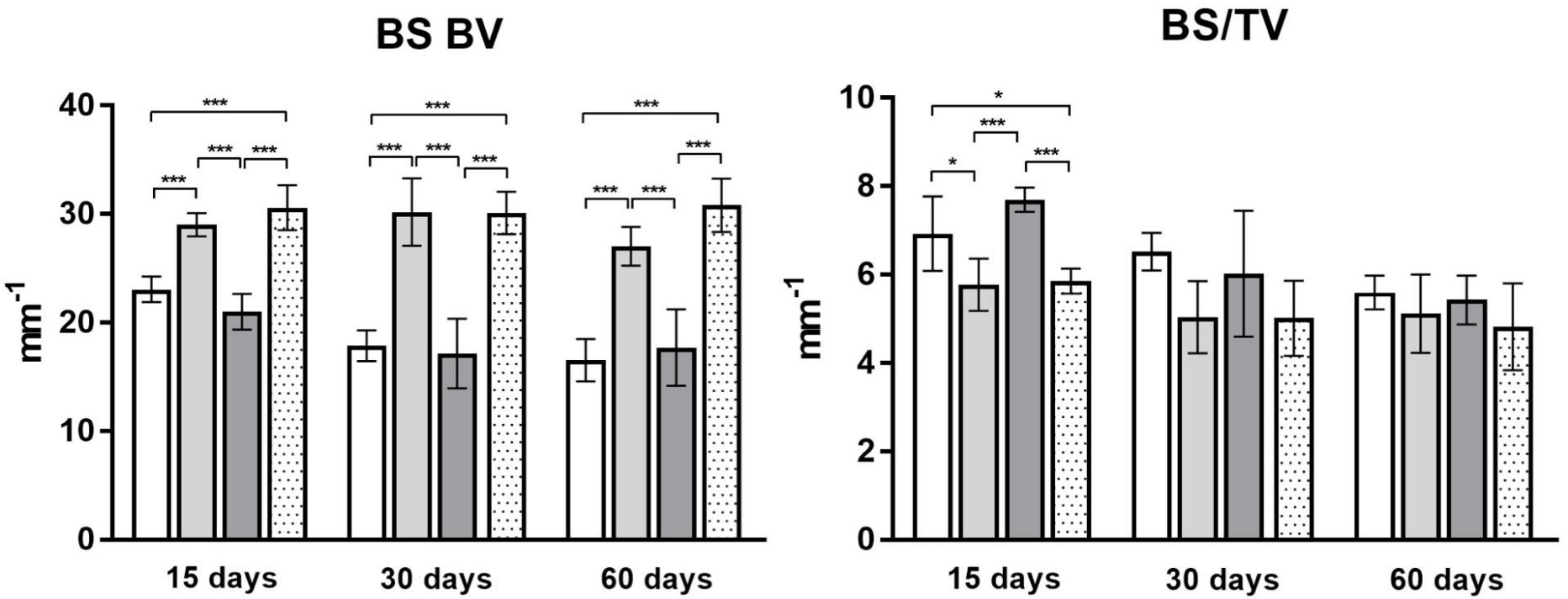


Figure 5. Graphics of all micro-computed tomography (CT) parameters (* p≤0.05; ** p≤0.01; *** p≤0.001) (*continuation*).



3.2 Artigo 2* – Gene expression, immunohistochemical and microarchitectural evaluation of bone formation around two implant surfaces placed in bone defects filled or not with bone substitute material

Gene expression and immunohistochemical evaluation of bone formation around two implant surfaces placed in bone defects filled or not with bone substitute material

Short title: Gene expression and immunohistochemical analysis of bone formation around two different implant surfaces

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Key-words: osseointegrated dental implantation; bone grafting; cell viability

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1. ABSTRACT

The objective of this study was to evaluate through gene expression, immunohistochemical and microtomographic (micro-CT) analysis the response of peri-implant bone tissue around titanium implants with different surface treatments, placed in bone defects filled or not with bone substitute materials. Twenty-six animals were divided into two groups according to implant surface treatment. In each tibia, a bone defect was created followed by the placement of one implant. On the left tibia, the defect was filled with blood clot (BC), and on the right tibia, the defect was filled with biphasic hydroxyapatite/β-tricalcium-phosphate (HA/TCP) generating four subgroups: BC-N: bone defect filled with blood clot and porous surface titanium implant installed; BC-A: bone defect filled with blood clot and porous-hydrophilic surface titanium implant installed; HA/TCP-N: bone defect filled with bone substitute material and porous surface titanium implant installed; HA/TCP-A: bone defect filled with bone substitute material and porous-hydrophilic surface titanium implant installed. The animals were submitted to euthanasia 15, 30, and 60 days after implant installation. The expression of two genes was evaluated: runt-related transcription factor 2 (RUNX2) and bone sialoprotein (BSP). Immunohistochemical analyses were performed to detection of run-related transcription factor 2 (RUNX2), osteopontin (OPN), osteocalcin (OCN), osteoprogesterin (OPG), and receptor activator of nuclear factor kappa B ligand (RANKL) antibodies and bone matrix proteins by immunoperoxidase detection and 3,3'diaminobenzidine. Finally, four parameters were chosen for micro-CT analysis: trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and connectivity density (Conn.Dn). The descriptive analysis showed similar findings among the experimental groups. Moreover, porous-hydrophilic surfaces present a higher expression of RUNX2, what it seems to be a better indicative of osteogenesis. In conclusion, porous hydrophilic surface can improve and accelerate the bone formation such as protein expression.

Key-words: osseointegrated dental implantation; bone grafting; cell viability

2. INTRODUCTION

Currently, the use of osseointegrated titanium implants for oral rehabilitation of partially or fully edentulous patients is a reliable treatment therapy with high success rates.[1,2] The integrity of bone-implant contact is an important factor that dictate the success of dental implant rehabilitation. After implant installation, several biological reactions dependent by numerous factors occur for bone repair.[3] Implant design, physico-chemical implant surface modifications and the presence of bone substitute materials may influence these events.[4,5]

In order to reduce the time of oral rehabilitation, some techniques have been reported such as immediately placement of dental implant in fresh extracted socket. However, this socket can be considered a bone defect and may exist a gap between the implant and the remaining alveolar crest after its placement.[6,7] According to Quirynen et al.[8], the immediate dental implant placement generates some conditions that may or may not require guided bone regeneration (GBR).

Due to its biological properties of bone formation such as osteogenesis, osteoinduction, and osteoconduction, autogenous bone graft is still considered as gold standard for bone grafting procedures. However, the harvesting of this graft is not always possible and bone substitute material may be necessary for adequate GBR.[9,10] By this reason, some bone substitute materials with strictly osteoconductive properties have been used. Biphasic ceramics of hydroxyapatite/β-tricalcium phosphate is one of them and its importance has been described for its high predictability in bone gain and longevity in oral rehabilitation.[9,11,12]

Also as a contribution for a faster osseointegration process, modifications on the implant surface have been described and developed. In vitro studies already have presented positive properties o hydrophilic surfaces on the mesenchymal stromal cells and osteoblast differentiation and on the earl matrix mineralization induction.[13,14] According to Hamlet et al.[15], hydrophilic surface of dental implants modulates the inflammatory response by inducing down-regulation of inflammatory cytokines gene expression in monocytes. Besides, Hong et al.[16] affirmed that hydrophilic surfaces carries thrombogenic features and improves bone healing. Moreover, it was presented in a clinical study that this surface increase osteogenesis by either osteogenic and angiogenic effects on gene expression.[17]

In the process of osteoblast differentiation, several proteins of bone matrix are produced. Notably, bone sialoprotein (BSP) is a bone matrix protein besides being a

marker of osteogenesis.[18] Runt-related transcription factor 2 (RUNX2) upregulates the expression of bone matrix genes, including BSP. Furthermore, RUNX2 is a critical gene either for osteoblast differentiation and function.[19]

Although several studies[3-5,7,11-13] have compared different implant surfaces and bone substitute materials, there is a lack of information regarding the behavior of these surfaces in the presence of bone substitute materials in different periods. Therefore, the purpose of this preclinical study was to evaluate in a rabbit model through immunohistochemical and molecular analysis the cellular responses around titanium implants with different surface treatment installed in previously created defects filled or not with a bone substitute material.

3. MATERIALS AND METHODS

This study was submitted and approved by Ethics Committee in the Use of Animals of the São Paulo State University (Unesp), School of Dentistry, Araçatuba, Brazil (Protocol number FOA: 465-2016). Twenty-six *Oryctolagus cuniculus* male rabbits, weighting between 300 to 400 mg and aging approximately 5 months, were selected. The animals were kept in separate cages with rabbit chow and water *ad libitum*.

The animals were randomly divided into two groups according to the type of implant surface. Before the implant placement, a defect was created on both tibias of the animals. The groups were divided according to how the previous created defect was filled: BC-N: the bone defect was filled with blood clot, and implants with a porous surface (Neoporos surface, Neodent®, Curitiba, Brazil) were installed; BC-A: the bone defect was filled with blood clot, and implants with a porous-hydrophilic surface (Acqua surface, Neodent®, Curitiba, Brazil) were installed; HA/TCP-N: the bone defect was filled with biphasic ceramics of hydroxyapatite/β-tricalcium-phosphate (Straumann® Bone Ceramic, Straumann AG, Basel, Switzerland), and implants with a porous surface (Neoporos surface, Neodent®, Curitiba, Brazil) were installed; HA/TCP-A: the bone defect was filled with biphasic ceramics of hydroxyapatite/β-tricalcium-phosphate (Straumann® Bone Ceramic, Straumann AG, Basel, Switzerland), and implants with a porous-hydrophilic surface (Acqua surface, Neodent®, Curitiba, Brazil) were installed.

Finally, the euthanasia of the animals occurred in three distinct experimental periods: 15, 30, and 60 postoperative days. The 15 and 30-days groups were composed by 5 tibias while the 60-day group was composed by 3 tibias (Figure 1).

The selection of the tibiae, the type of implant, and how the defect was filled was determined at random.

3.1. *Surgical procedure*

After 8 hours fasting, the animals were sedated and anesthetized via intramuscular by the combination of 50mg/ml of ketamine (Fracontar, Vibrac do Brasil Ltda., São Paulo, Brazil) and 5mg/ml of xylazine (Rompum, Bayer AS, Saúde Animal, São Paulo, Brazil). Subsequently, the animals were submitted to a trichotomy of the internal region of the left and right hid paws, and antisepsis was performed. Local anesthesia was made using 0,3ml/kg of mepivacaine 2% with epinephrine 1:100.000 (Mepiadre – Nova DFL, Rio de Janeiro, Brazil) obtaining analgesia and hemostasis.

A 30-mm incision was performed in planes on the tuberosity of both tibias. After delicate dissection of soft tissues and complete detachment and exposition of interest area of bone tissue, an osteotomy with an 6.1-mm trephine drill (Neodent®, Curitiba, Brazil) mounted in a 20:1 low-speed driller (SG20, Neodent®, Curitiba, Brazil) with the aid of an electric motor adjusted (NeoSurg XT Plus, Neodent®, Curitiba, Brazil) in 800 rpm creating a defect of 8-mm length in the tibiae. The internal cortical was removed preserving the external cortical of each tibiae. After that, the drilling for implant installation was performed in the defect center initiating by the pilot drill followed by helicoidal drills with a thickness of 2.0mm, 2.8 mm and 3.0mm (Neodent®, Curitiba, Brazil) to accommodate a 8-mm-high titanium implant with a 3.5-mm-diameter (Drive CM Neoporos and Acqua surfaces, Neodent®, Curitiba, Brazil). Both osteotomies were made under abundant irrigation with sterile saline. According to each group, the implants were installed achieving primary stability in the external cortical of tibiae and the bone defect was then filled with the bone substitute or with blood clot.

The tissue was repositioned and sutured by planes. The muscular portion was closed with resorbable suture (5-0 Vicryl Ethicon, Johnson & Johnson, São José dos Campos, Brazil) and the skin portion was closed with silk thread (4-0 Ethicon, Johnson & Johnson, São José dos Campos, Brazil) obtaining primarily close of the wound. The animals received a single dose of pentabiotic (0,1ml/kg, Fort Dodge Saúde Animal Ltda., Campinas, Brazil) and dipyrone (1mg/kg, Ariston Indústria Químicas e Farmacêuticas Ltda., São Paulo, Brazil).

After 15 and 30 days post surgery, twenty animals were submitted to euthanasia by an overdose of anesthetic. Then, the tibias were removed and

maintained and fixed in 10% formalin solution for 48 hours, washed in running tap water for 24 hours, and later stored in 70º ethanol. On the sixtieth day, the last six animals, were sedated and anesthetized to the removal of entire tibia still alive following the surgical procedure previously described. These samples were maintained in liquid nitrogen and designated to molecular biology analysis. After that, the animals were submitted to euthanasia by an overdose of anesthetic.

3.2. *Micro-CT*

A micro-CT (μCT) scanner (SkyScan 1176 Bruker MicroCT, Aatselaar, Belgium) was used to scanned the samples, with particular specifications: camera pixel, 12.45; X-ray tube potency, 50 kilovoltage peak (kVp); X-ray intensity, 500μA; integration time, 300ms; filter, 1mm aluminum (Al); voxel size, 9μm^3. All the images obtained were filed and prepared for analyses using specific software (NRecon, Data Viewer, CTAnalyser, Aatselaar, Belgium). Three plans were obtained and evaluated: coronal, transaxial, and sagittal. A region of interest (ROI) was determined for each plan. For the coronal plan: 0.5mm circular region around the entire diameter of the implant; for the transaxial and sagittal plans: a rectangular shape vertically from the top of the implant to the end of the second big thread, and 0.5mm horizontally from the border of the implant. The threshold used in the analysis was 25-90 in grayscale, and the values of the mineralized tissue around the implants were assessed using four parameters: trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and connectivity density (Conn.Dn).

3.3. *Immunohistochemical analysis*

Immunohistochemical analyses were performed to identify and localize the expression of proteins related to bone remodeling: run-related transcription factor 2 (RUNX2), osteopontin (OPN), osteocalcin (OCN), osteoprotegerin (OPG), receptor activator of nuclear factor kappa B ligand (RANKL). After the micro-CT scan, the samples were removed from 70º ethanol and washed in running tap water and decalcified in 10% ethylenediaminetetra-acetic acid (EDTA) solution for 90 days, changing the solution every 72 hours. As soon as the decalcification was verified as completed, the implants were removed from the specimens. After that, samples were embedded in paraffin, sliced to a thickness of 6μm, and placed on silanized slides. The immunolabeling protocols starts with procedures for deparaffinization and rehydration of the slides. Following, the slides were subjected for to blockade of endogenous peroxidase by the application of 3% hydrogen peroxide for 30 minutes.

The antigen retrieval was achieved by citrate phosphate buffer in humid heat. Unspecific link blockade was performed by 1% albumin bovine serum and skim milk. Subsequently, the primary antibody incubation was performed against the following proteins: RUNX2, OPN, OCN, OPG, and RANKL (Santa Cruz Biotechnology, Dallas, Texas, USA). Negative control was performed through the serum of primary antibody host. Secondary antibody used was the biotinilated donkey anti mouse (Jackson Immunoresearch Laboratories). Avidin Biotin was used in order to amplify the immunolabeling signal and diaminobenzidine (Dako, Glostrup, Denmark) was the cromogen. The samples were counterstained with Mayer Hematoxylin solution for visualization of the cell nuclei. The images were obtained using a camera coupled to a light microscope (Leica Reichert Diastar Prodcuts & Jung, Wetzlar, Germany). The expression levels of the proteins were assessed through a protein labeling extension index: (-) without labeling (0% of cells/matrix); (+) mild/discrete labeling (<25% of cells/matrix); (++) moderate labeling (25%-50% of cells/matrix); (+++) strong labeling (<50 of cells/matrix).[30] A experienced examiner performed the descriptive analyses.

3.4. *Molecular analysis*

In the 60-day period of implant installation, three samples from each group were submitted to explantation of the implants by means of a digital torque wrench, the torque values (N) were colected and then the bone tissue adhered to the implants was collected for molecular biology experiments. The reverse transcription polymerase chain reaction (RT-PCR) was performed with the objective of evaluating the gene expression of different markers in a certain stage of osteoblastic cells: Runx2 and BSP. For this purpose, primers designed by Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA) were used.

Each bone fragment was carefully washed in PBS solution and subsequently frozen in liquid nitrogen so that the total RNA was isolated with the aid of the Trizol reagent (Life Technologies: Invitrogen, Carlsbad, CA, USA). RNA was quantified on a NanoDrop® 2000 spectrophotometer (Thermo Scientific NanoDrop Technologies, Wilmington, DE) for analysis of RNA integrity, purity and concentration. Afterwards, the cDNA was made using 1µg of RNA by the reverse transcriptase reaction (M-MLV: reverse transcriptase: Promega Corporation, Madison, Wisconsin, USA). The sequence of the primers was amplified and their products were electrophoresed with 1.5% agarose gel stained with ethidium bromide and visualized using Quantity One software (Bio-Rad Laboratories, Philadelphia, PA, USA).

Finally, RT-PCR was conducted using the StepOne system (Applied Biosystems, Foster City, CA, USA) using the SybrGreen (Primer Design Ltd, Southampton, UK) fluorescent dye system under the following cyclic conditions: 95° for 10 minutes for primary denaturation, and 35 cycles of 95° for 15 seconds for tape opening, and finally 60° for 60 seconds for annealing of the primers and extension of the fragments. The relative gene expression was calculated by reference to the expression of the mitochondrial ribosomal proteins and normalized by the gene expression of the bone fragments removed from the implants in the given period of 60 days.[20,21]

3.5. *Statistical analysis*

Collected data were tabulated using Microsoft Excel and submitted to statistical analysis. Data were submitted to Shapiro-Wilk test for and ANOVA two-way followed by Kurskal-Wallis test. The confidence interval was 95% and $p<0.05$. The softwares used were R 3.6.0 version and RStudio 1.2.1144 version.

4. RESULTS

4.1. *Micro-CT*

Trabecular number (Tb.N): the results suggest that there is a significant effect of the interaction between the presence bone substitute material or blood clot and the experimental period for the trabecular number ($p=0.001559$). This means that the presence of the bone substitute material and the period of bone formation have their influence over the quantity of trabeculas, independently the implant surface. Table 1 shows the effect of the interaction between bone substitute material presence and period. Figure 1 complements the information about the interaction of Table 1.

Trabecular separation (Tb.Sp): the results of this parameter analysis also suggest the existence of a significant effect of the interaction between the presence of bone substitute material or blood clot and the experimental period ($p = 0.00731$). Table 2 associated to Figure 4 show that the trabeculas are “more separated” (higher separation means) mainly due to the presence of blood clot after 15 and 30 days when compared to the presence of bone substitute material. “Closer” trabeculas are achieved with bone substitute material after 15 days.

Trabecular thickness (Tb.Th): The results of this parameter analysis also suggest the existence of a significant effect of the interaction between the presence of bone substitute material or blood clot and the experimental period ($p = 0.010$). Table 3

associated to Figure 3 show that the use of bone substitute material increases the trabecula thickness values compared to situation with blood clot in the same period and that the longer the experimental period associated with bone substitute material, the “thicker” the trabeculae are. However, there are no significant differences between the use of bone substitute material within 15 days and with blood clot in 30 days. Therefore, it may be more advantageous with bone substitute material in 30 days or more if the objective is thicker trabeculas.

In this analysis, one of the groups did not present normal distribution (porous surface implant, with bone substitute material and period of 15 days) whose p=0.04419 (Shapiro-Wilk test). The homoscedasticity assumption was also violated (p = 0.02777, Brown-Forsythe test). Then, was opted for the transformation of trabeculae thickness data into logarithm. It was observed that the groups showed homoscedasticity after the transformation (p = 0.05172, Brown-Forsythe test).

Connectivity density (Conn.Dn): The results suggest that there is no significant effect of the interaction between implant surface, presence of bone substitute material or blood clot and experimental period and neither effect of each of the variables in isolation in this parameter, even if the graphic (Figure 4) apparently demonstrated that implants in the presence of blood clot generally have a greater connectivity density.

4.2. *Immunohistochemical analysis*

The indirect immunoperoxidase technique was used with the diaminobenzidine as chromogen, which brownish staining labeled the cells and extracellular matrix positive for the antigens evaluated. During the description of the results, the labeling was categorized as mild/discrete (+), moderate (++) or intense (+++) according to the area/quantity of positive cells for each antigen (Table 4). The Figures 5 and 6 show the labeled samples for all antibodies according to all groups at both periods.

Porous surface groups

RUNX2: at 15 days, it was observed in both groups the presence of immature osteoblasts in intense proliferation and differentiation process. It was also observed the presence of extracellular matrix around cells intensely labeled throughout the region of interest. At 30 days, a reduction of the presence of RUNX-2-positively labeled cells is observed in the BC-N group, as expected at this stage. Thus, the labeling for RUNX-2 is considered mild. In the HA/TCP-N group, more RUNX-2-

labeled cells were observed and there was a delay in the development of repair bone tissue. Thus, immunolabeling for RUNX-2 is categorized as moderate.

OPN: in the BC-N groups, labeling is moderate for the presence of osteoblasts, but the repair process is well developed in both periods. In HA/TCP-N groups, at 15 days, an intense labeling for this protein is observed on osteoblasts around the new bone tissue formation, and positive labeling on cells located around the non-mineralized extracellular matrix. However, despite the intense immunostaining for this protein, the repair stage is delayed compared to the BC-N group at 15 days. At 30 days, it is observed that the bone repair process has evolved and a slight labeling for osteoblasts around the newly formed bone tissue is observed.

OCN: in the BC-N groups, it was observed intensely labeled in osteoblasts located around the newly formed bone tissue as well as in cells located in the non-mineralized extracellular matrix at 15 days. At 30 days, there is an important labeling, considered moderate, for osteocytes trapped within the newly formed bone tissue, characterizing its maturity. In the HA/TCP-N groups, at 15 days, the immunostaining showed similar results to that observed in the BC-N group in the same period. At 30 days, there is a delay in the repair stage, mainly evidenced by the moderate osteocalcin labeling in cells located in the non-mineralized extracellular matrix. There is a slight labeling for osteocytes trapped in the formed bone tissue, therefore, in smaller quantity when compared to the BC-N group at the same period.

OPG: In the BC-N groups, the labeling was observed moderately at both periods, being positively labeled in osteoblasts present around the formed bone tissue. In the HA/TCP-N groups, at 15 days, there was an intense positive labeling for this protein; however, most of the labeled cells are located in the non-mineralized extracellular matrix, which characterizes the delay of the repair stage when compared to the BC-N group at 15 days. At 30 days, a moderate labeling for osteoprotegerin was observed, with remnant of non-mineralized extracellular matrix, once again showing a delay in the repair stage when compared to the BC-N group in the same period.

RANKL: IN BC-N groups, RANKL labeling was intense at 15 days, where labeled cells were observed next to the non-mineralized extracellular matrix. At 30 days, a moderate labeling was observed for the cells located near the formed bone tissue. In the HA/TCP-N groups, intense RANKL labeling was observed at 15 days in cells located in the non-mineralized extracellular matrix. At 30 days, moderate labeling was observed for cells located near the non-mineralized extracellular matrix. It is

noteworthy that the repair stage is delayed at both 15 and 30 days of the HA/TCP-N groups compared to the BC-N groups.

The associated evaluation of OPG and RANKL shows a balance between the bone formation and resorption stages, represented by OPG and RANKL respectively, in both BC-N and HA/TCP-N groups.

Porous-hydrophilic surface groups

RUNX2: at 15 days, the BC-A group showed an intense positive labeling for RUNX2, observed in cells surrounded by the non mineralized extracellular matrix. The organization of bone tissue was noted, still incipient, near the negative area of the implant thread. At 30 days, in this same group, it was still possible to observe an intense positive labeling for this transcription factor in many regions of the evaluated area of interest and also areas of bone formation and immature osteoblasts were observed near the newly mineralized bone matrix. In the HA/TCP-A groups, at 15 days, it was possible to observe moderate positive labeling for pre-osteoblasts and immature osteoblasts. The repair stage is advanced when compared to the BC-A group at 15 days. At 30 days, moderate positive RUNX-2 labeling was observed for pre-osteoblasts and immature osteoblasts in a formed and organized bone tissue.

OPN: in the BC-A groups, at 15 days, the OPN labeling was moderate for osteoblasts. However, the presence of intense labeling for this protein in cementing lines in the bone tissue formed near the implant thread is highlighted. Moreover, positive labeling was also observed for osteoblasts located near the newly formed bone tissue. At 30 days, was moderately labeled for osteoblasts located near the newly formed bone tissue. In the HA/TCP-A groups, moderate osteopontin labeling was observed in cells located near the non-mineralized extracellular matrix at both periods.

OCN: in BC-A groups, at 15 days, the OCN labeling was moderate. Its presence was evidenced in cells located in the non-mineralized extracellular matrix and also in osteocytes trapped in newly formed bone tissue. At 30 days, moderate osteocalcin labeling was also observed in osteocytes trapped in the formed bone tissue, showing a higher degree of maturity in the repair stage when compared to the previous period. In the HA/TCP-A groups, moderate osteocalcin labeling was observed at 15 days. At 30 days, moderate immunostaining for osteocalcin was evidenced, highlighting the presence of many cells positive for this protein located in the non-mineralized

extracellular matrix, characterizing a delay in the repair process when compared to group BC-A at 30 days.

OPG: in BC-A groups, at 15 days, the labeling was moderate, especially in few osteoblasts and in cells located in the non-mineralized extracellular matrix. At 30 days, it was possible to observe moderate osteocyte labeling for this protein, characterizing the degree of maturity of the formed bone tissue. In the HA/TCP-A groups, at 15 days, moderate labeling was observed for cells located in the non-mineralized extracellular matrix, especially around fragments of the bone substitute material. At 30 days, moderate labeling was observed for cells located in the non-mineralized extracellular matrix, highlighting the presence of large amount of blood vessels.

RANKL: in the BC-A groups, at 15 days, the labeling was moderate in cells located in the non-mineralized extracellular matrix. It remained moderate in osteoblasts near the newly formed bone tissue, at 30 days. For the HA/TCP-A groups, moderate RANKL labeling was observed in osteocytes surrounded by newly formed bone tissue, at 15 days. At 30 days, moderate labeling in osteoblasts and osteocytes close to bone tissue was observed.

Again, the associated evaluation of OPG and RANKL shows a balance between bone formation and resorption stages, in both BC-A and HA/TCP-A groups.

4.3. *Molecular analysis*

Twelve samples were submitted to RT-PCR to verify the expression of two different proteins: Runx2 and BSP. Figure 7 is the graphic that represents the relative expression of the Runx2 protein. Figure 8 is the graphic that represents the relative expression of the BSP protein.

The expressions of the other groups were evaluated in relation to the BC-N group (control). For this reason, the control group always presents a value (average) of the expression close to 1. The value (average) presented by the other groups represents how many times the expression of such evaluated protein is greater in relation to the control group.

Analyzing the graphics, it is possible to observe that Runx2 protein expression was better in the groups of porous-hydrophilic surface implants. The comparison between the groups HA/TCP-N and BC-A resulted in $p < 0.0001$, that is, a very significant difference between them. Furthermore, the comparison between HA/TCP-N and HA/TCP-A resulted in $p = 0.0361$.

In relation to BSP protein, when we analyzed the graph, its expression, as well as that of the Runx2 protein, was better in the groups of porous and hydrophilic surface implants. When compared to the control group, the group without bone substitute material resulted in a p=0.0025, that is, a very significant difference, and the bone substitute material group resulted in a p=0.0289. However, the HA/TCP-N group had a better expression of the BSP protein when compared to all the other groups, resulting in very significant differences related to the BC-A group and very significant related to the BC-N and HA/TCP-A groups: p<0.0001 (BC-N), p=0.0018 (BC-A), p<0.0001 (HA/TCP-A).

In porous and hydrophilic surface implants there is a better expression of the Runx2 protein. In addition, the bone substitute material does not appear to increase the expression of this protein when compared to blood clot groups. Still, within the bone material substitute groups, we observed a better expression of the protein in the group of porous-hydrophilic surface implants. Regarding the expression of the BSP protein, we can observe a better expression in the groups of porous-hydrophilic surface implants when compared to the control group (BC-N).

5. DISCUSSION

The better understanding of the behavior of biological cells and biomaterials such as titanium implant surfaces can provide important information useful for the development of new clinical techniques for patient rehabilitation. The objective of this study was to evaluate through an animal model through the cellular responses around titanium implants with different surface treatment installed in previously created defects filled or not with a bone substitute material.

Immunostaining was performed to evaluate the activity of proteins that play an important role in osteogenic activity, from the osteoblast differentiation stage (RUNX2), to the synthesis of the organic matrix (OPN), mineralization (OCN) and finally the bone remodeling (New Tumor Necrosis Factor Members: OPG / RANKL). Besides this is a ordinary qualitative analysis, through scores attribution, the information about protein expression in the area close to the implant surface is very important, specially, when close to that, there is a biomaterial filling the perimplantar defect. The descriptive analysis showed similar findings among the experimental groups. Moreover, it was observed a protein activity more intense during the first period (15 days) with a diminishing of their activity to the final period (30 days). These results suggest that porous-hydrophilic surfaces can accelerate the bone formation in

defects filled with bone substitute material or blood clot with the activating of extracellular matrix proteins that have an important role in osteogenic activity. These findings are supported by several studies.[14,26,27] In contrast, the immunohistochemical results also suggest that porous surface can provide similar results in similar conditions.

Bone defects healing starts from cell proliferation allowed by a stable fibrin clot promoting the granulation tissue. This tissue forms new connective tissue with mature collagen that will promote the intramembranous ossification, leading to the bone defect to be filled by the newly formed bone. In this condition, the basic multicellular unit (BMU) is accurate. The BMU is a balance of bone formation and resorption, and osteoblasts and osteoclasts.[28,29] Following such concepts, during the defect healing, the newly formed bone must develop mature bone aspects, increasing trabecular thickness and decreasing trabecular space. Microscopically, the trabecular thickness (Tb.Th) increased in this study corroborating to the information above. Regarding to the trabecular separation (Tb.Sp), the results did not in accordance to that information; however, the porous-hydrophilic surface in the presence of bone substitute material strictly followed the concept. Moreover, by the immunohistochemical analysis, it is possible to affirm that this study rigidly follows the BMU concepts.

Studies already presented by means of in vitro data the positive influences of hydrophilic surfaces over the differentiation of osteoblasts.[13,14] Besides, Hong et al.[16] stated that hydrophilic surfaces can improve bone healing. The wettability of hydrophilic surfaces favors initial phases of wound healing leading to a faster osteogenesis.[22] In this study, according to the molecular analysis performed at 60 days post operative, when remodeling step of repair process is in evidence, porous-hydrophilic surfaces present a higher expression of RUNX2, what it seems to be a better indicative of osteogenesis as well as an important role on pre-osteoblastic differentiation process. This expression is not influenced by the presence of bone substitute material. Once the Runx2 induces the differentiation of osteoblasts, these results suggest that hydrophilic surfaces have an individual influence over the osteogenesis independently the presence of bone substitute material.

In contrast, the BSP expression is higher when the association between porous surface and bone substitute material. Although BSP function remains unclear, it is a initial marker of osteoblasts and its ability of bind in hydroxyapatite crystals

plays a important role in early stages of mineralization.[23] The results of this study suggest that the presence of bone substitute material can increase the BSP expression but the porous-hydrophilic surface decrease its expression by presenting a faster bone formation. On the other hand, the expression in high levels of BSP on the extracellular matrix indicates mature or in formation bone suggesting that, after 60 days, HA/TCP-N presents more mature bone or more bone formation activity.[24,25]

Taken all the information together, it is possible to state that porous-hydrophilic implant surface has similar behavior of porous implant surfaces in defects filled with blood clot or bone substitute material. However, some features demonstrate that porous hydrophilic surface can improve and accelerate the bone formation such as protein expression. By the fact of this methodology be innovative with different experimental variables, the comparison with others studies is challenging. The conclusions must be supported by future studies.

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TABLES

Table 1 – Multiple comparisons of trabecular number parameter.

Defect filling x Experimental period	Mean differences	p value
HA/TCP:15-BC:15	0.48610	***< 0.001***
BC:30-BC:15	-0.22915	0.14657
HA/TCP:30-BC:15	0.27665	**0.04507**
BC:30- HA/TCP:15	-0.71524	***< 0.001***
HA/TCP:30- HA/TCP:15	-0.20944	0.22277
HA/TCP:30-BC:30	0.50580	***< 0.001***

Table 2. Multiple comparison of trabecular separation parameter.

Defect filling x Experimental period	Mean differences	p value
HA/TCP:15-BC:15	-0.08421	0.14311
BC:30-BC:15	0.07431	0.25210
HA/TCP:30-BC:15	-0.05552	0.56954
BC:30- HA/TCP:15	0.15852	***< 0.001***
HA/TCP:30- HA/TCP:15	0.02868	0.95560
HA/TCP:30-BC:30	-0.12984	**0.00430**

Table 3. Multiple comparisons of trabecular thickness (log) parameter.

Defect filling x Experimental period	Mean differences (log)	p value
HA/TCP:15-BC:15	0.197130390	***0.02356***
BC:30-BC:15	0.034846390	0.99212
HA/TCP:30-BC:15	0.407547270	***< 0.001***
BC:30- HA/TCP:15	-0.162284000	0.09709
HA/TCP:30- HA/TCP:15	0.210416880	**0.01293**
HA/TCP:30-BC:30	0.372700880	***< 0.001***

Table 4. Representative scores (most frequently observed) of the immunolabeling, categorized as mild/discrete (+), moderate (++) or intense (+++), according to the area/quantity of positive cells for each antigen in all groups in both periods.[30]

	RUNX2	OPN	OCN	OPG	RANKL
15 days					
BC-N	+++	++	+++	++	+++
HA/TCP-N	+++	+++	+++	+++	+++
BC-A	+++	++	++	++	++
HA/TCP-A	++	++	++	++	++
30 days					
BC-N	+	++	++	++	++
HA/TCP-N	++	+	++	++	++
BC-A	+++	++	++	++	++
HA/TCP-A	++	++	++	++	++

FIGURE LEGENDS

Figure 1. Interactions among implant surfaces, bone substitute material or blood clot and experimental period on trabecular number parameter. Error bars represent the standard deviation around the means.

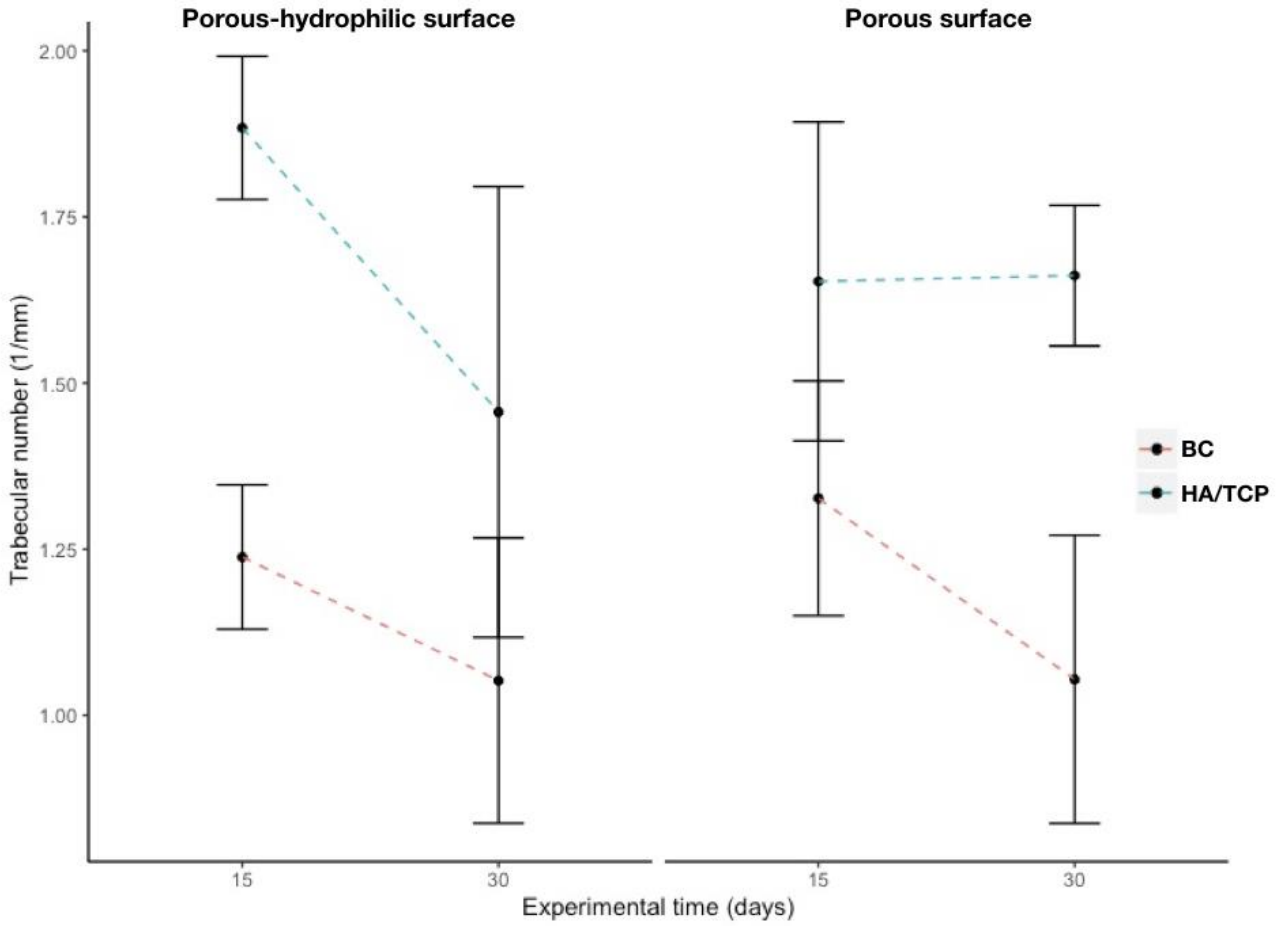


Figure 2. Interactions among implant surfaces, bone substitute material or blood clot and experimental period on trabecular separation parameter. Error bars represent the standard deviation around the means.

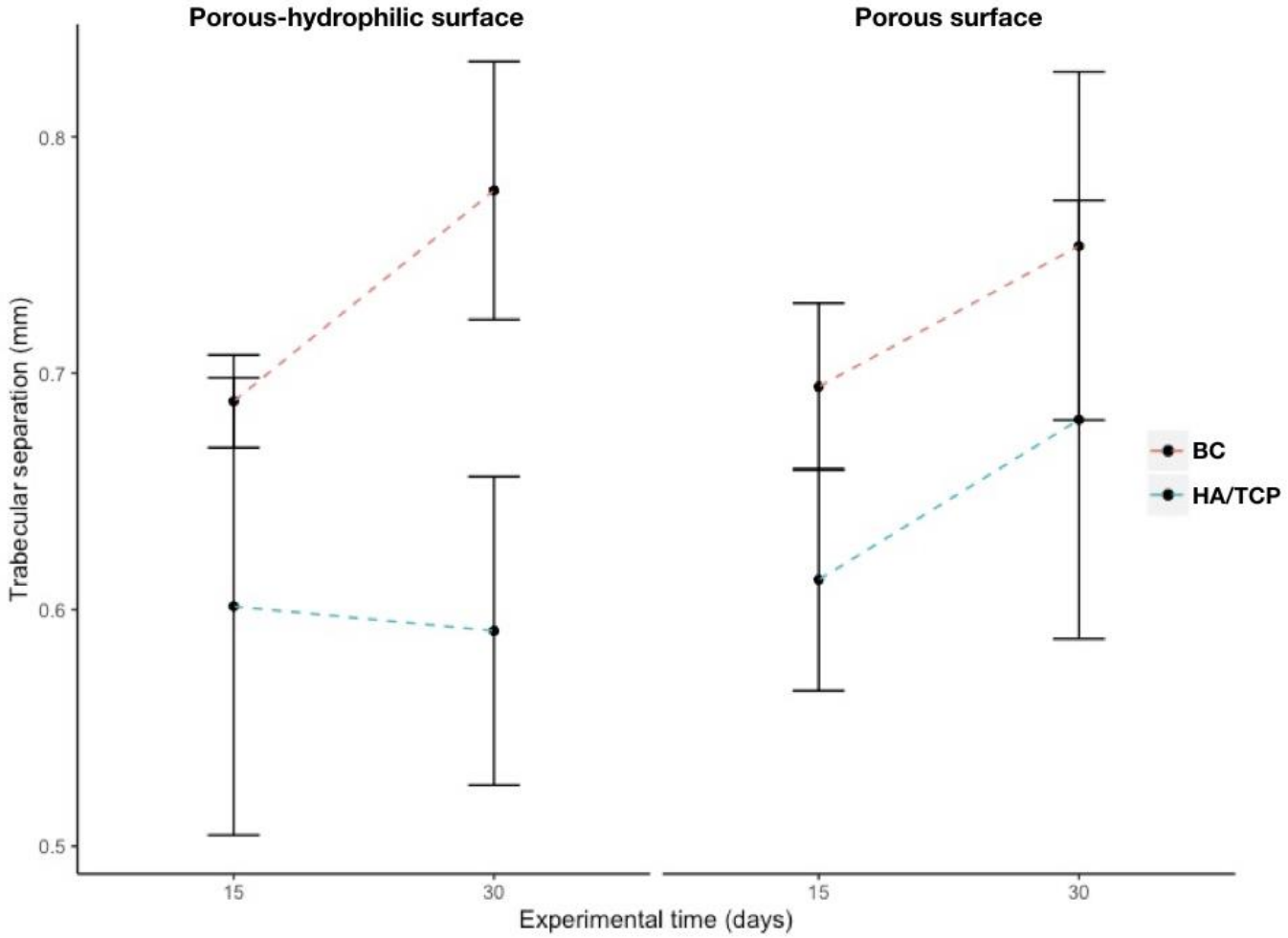


Figure 3. Interactions among implant surfaces, bone substitute material or blood clot and experimental period on trabecular thickness parameter. Error bars represent the standard deviation around the means.

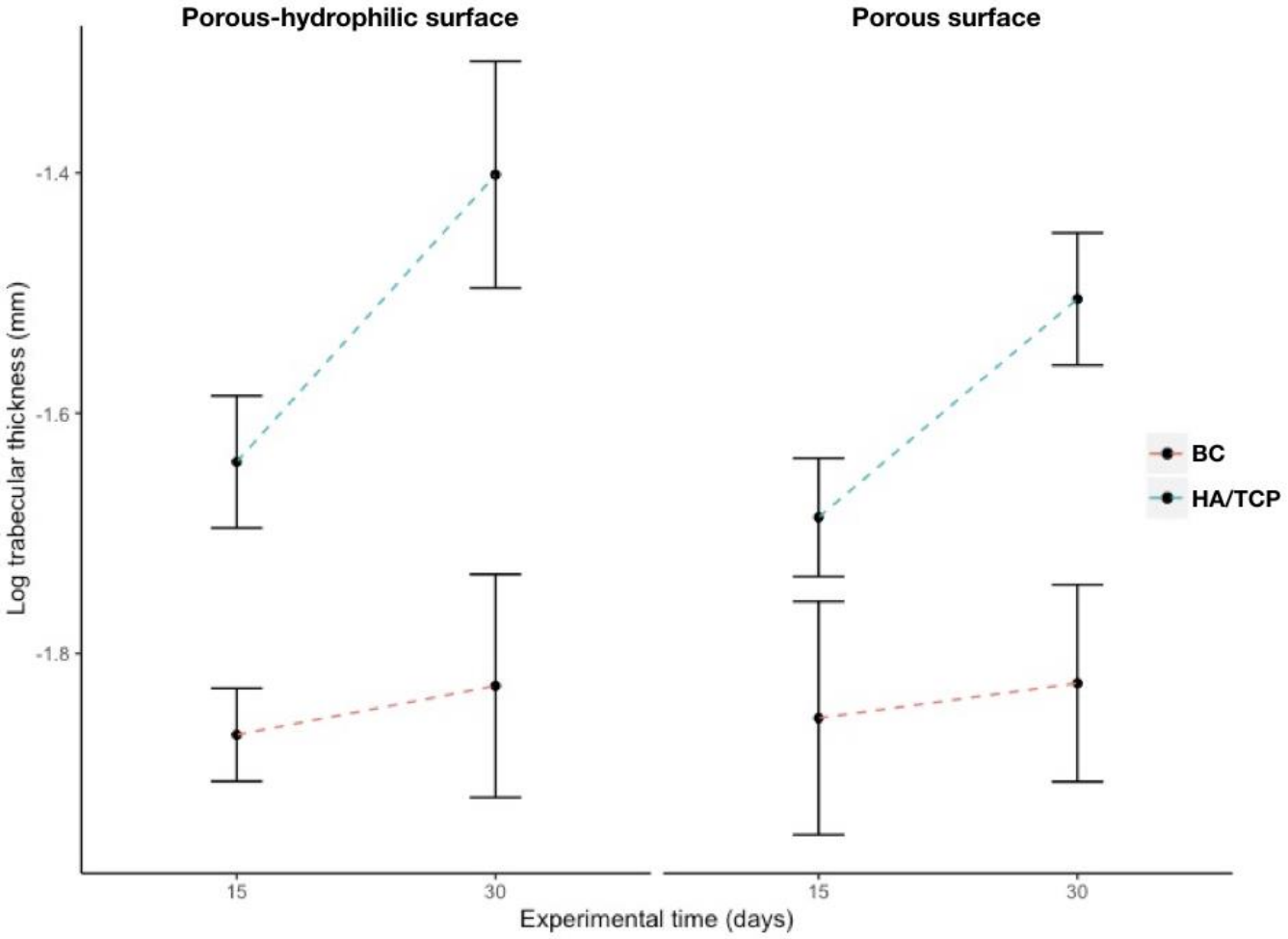


Figure 4. Interactions among implant surfaces, bone substitute material or blood clot and experimental period on connectivity density parameter. Error bars represent the standard deviation around the means.

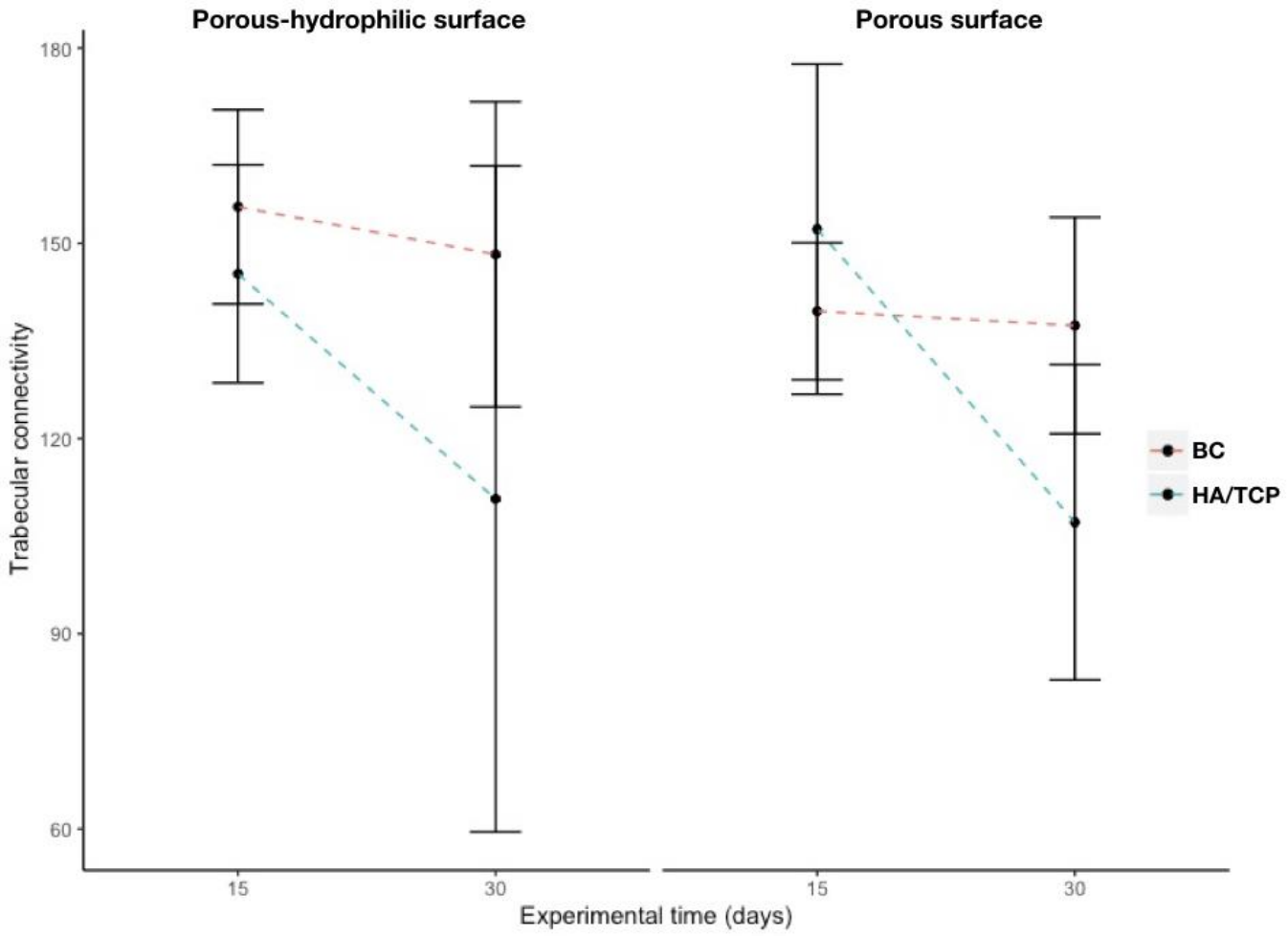
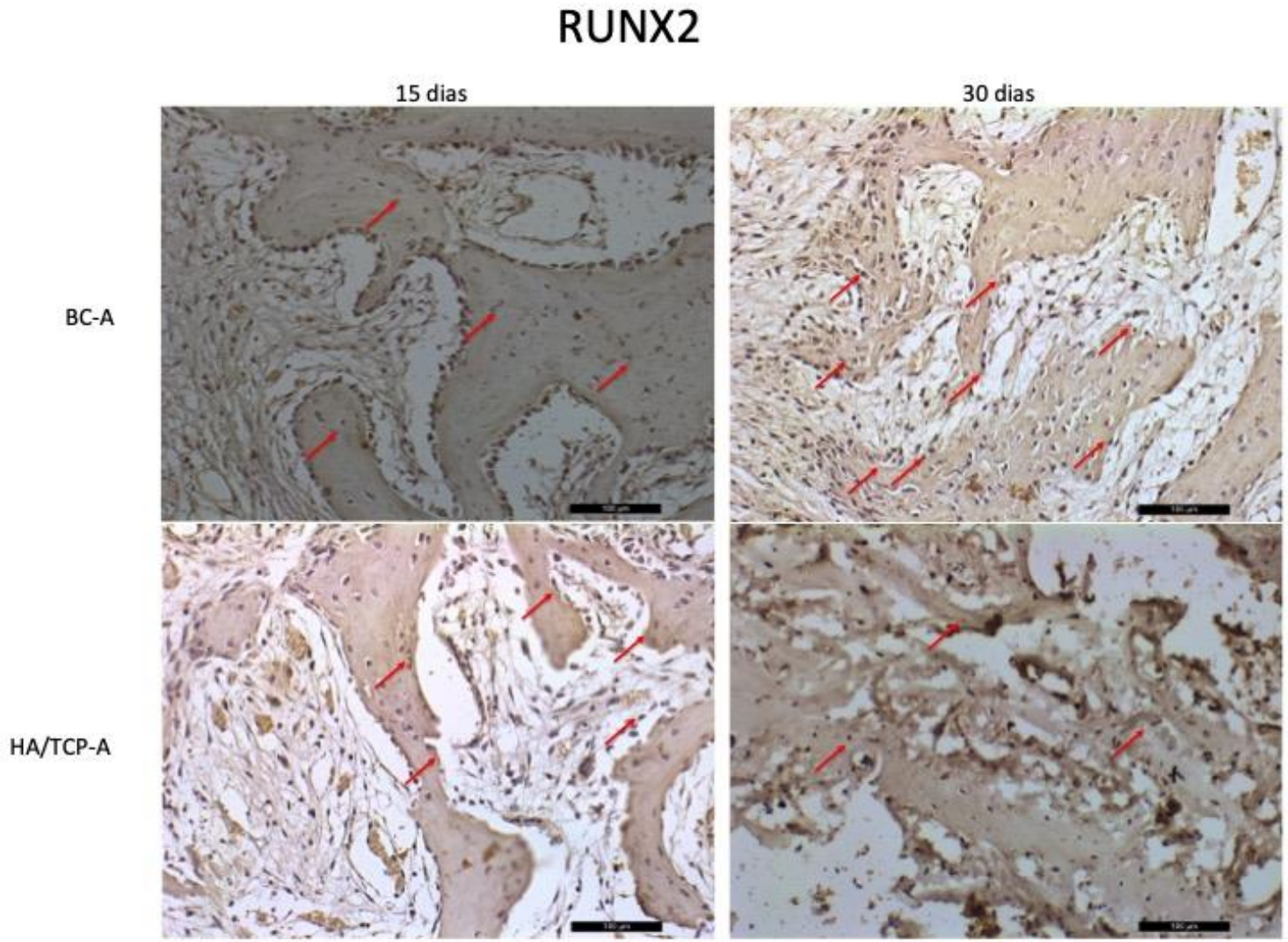
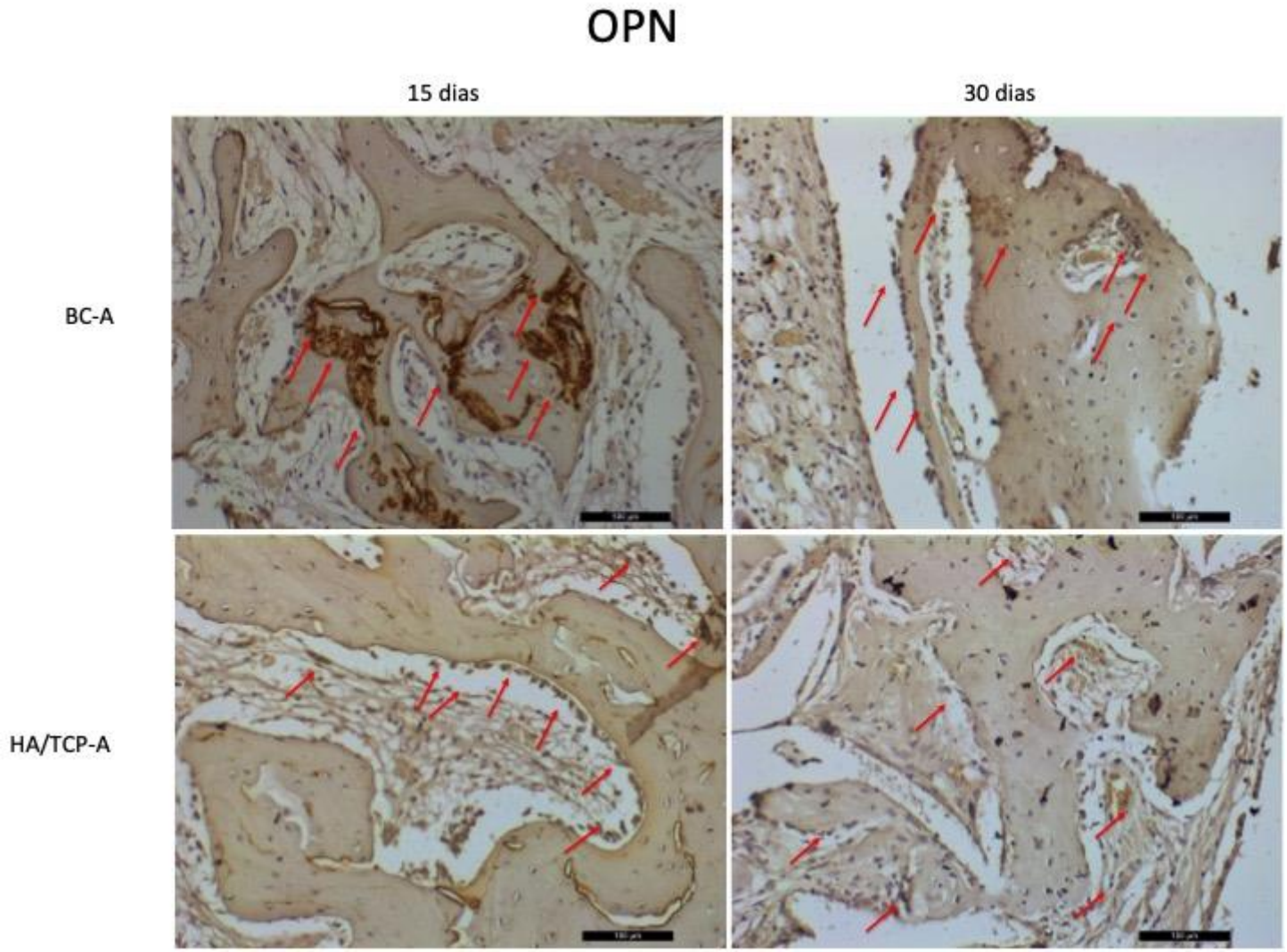


Figure 5. Labeled samples for all antibodies according to porous-hydrophilic surfaces groups at both periods.



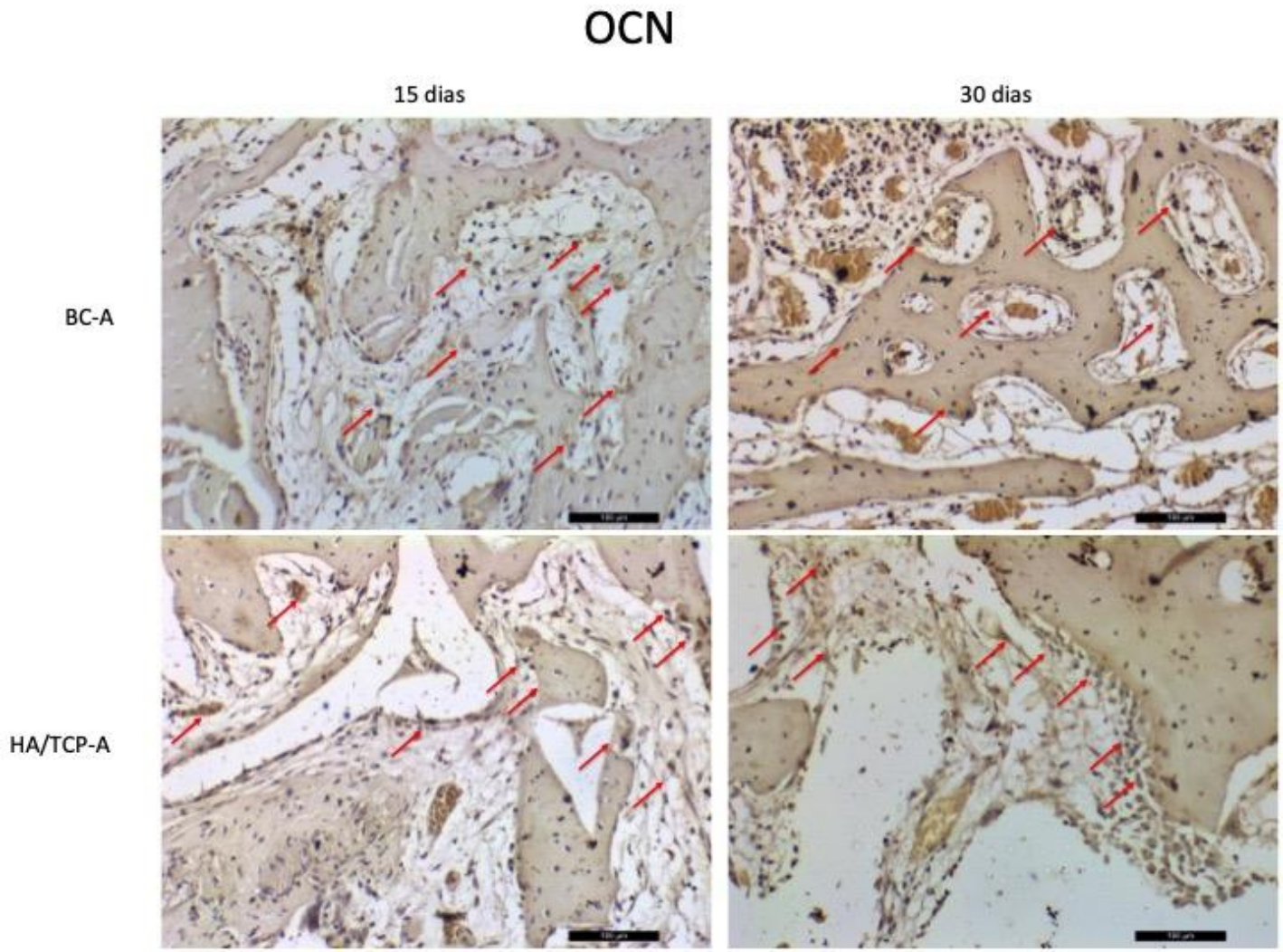
Immunolabeling of proteins (red arrow).

Figure 5. Labeled samples for all antibodies according to porous-hydrophilic surfaces groups at both periods (*continuation*).



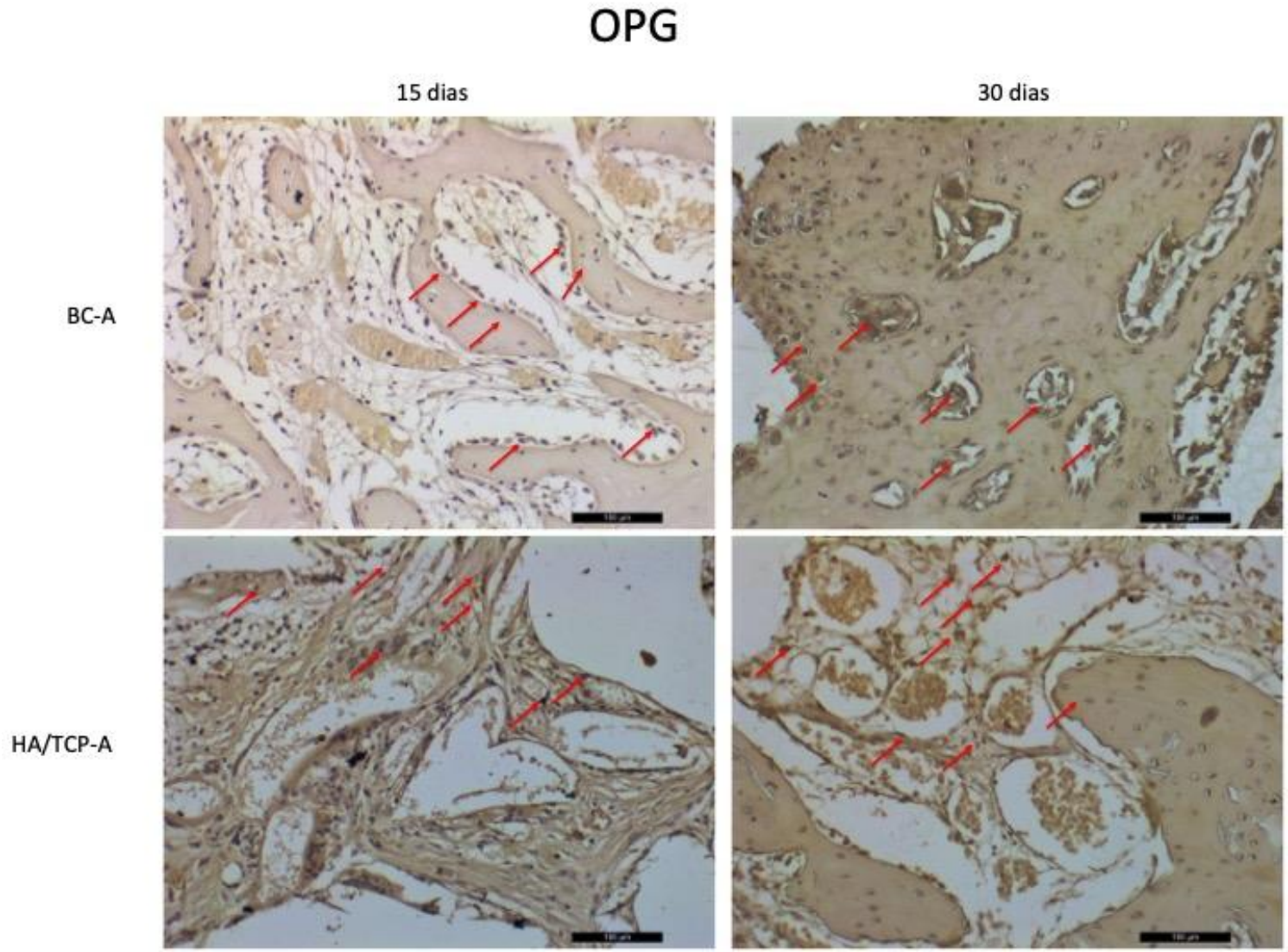
Immunolabeling of proteins (red arrow).

Figure 5. Labeled samples for all antibodies according to porous-hydrophilic surfaces groups at both periods (*continuation*).



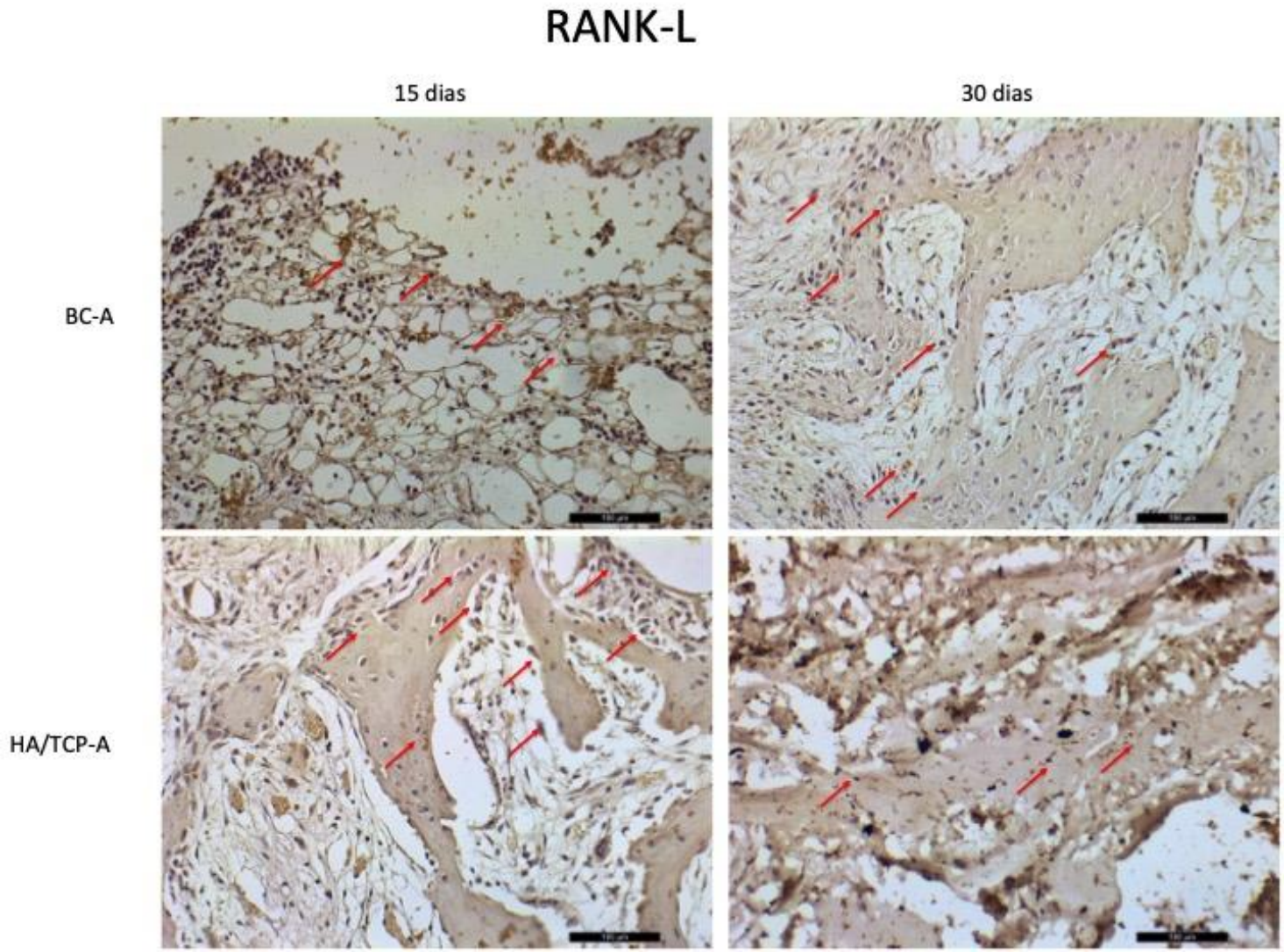
Immunolabeling of proteins (red arrow).

Figure 5. Labeled samples for all antibodies according to porous-hydrophilic surfaces groups at both periods (*continuation*).



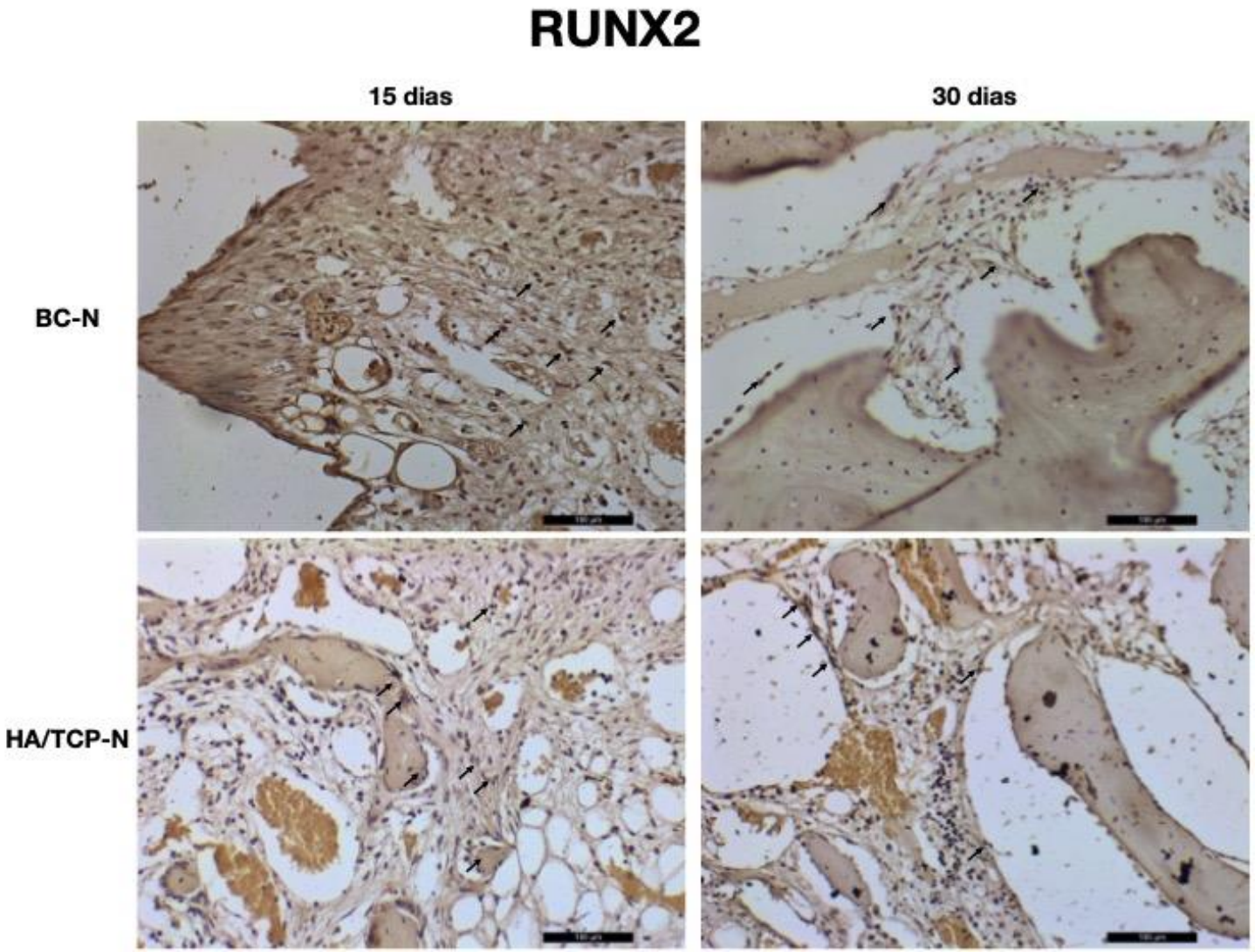
Immunolabeling of proteins (red arrow).

Figure 5. Labeled samples for all antibodies according to porous-hydrophilic surfaces groups at both periods (*continuation*).



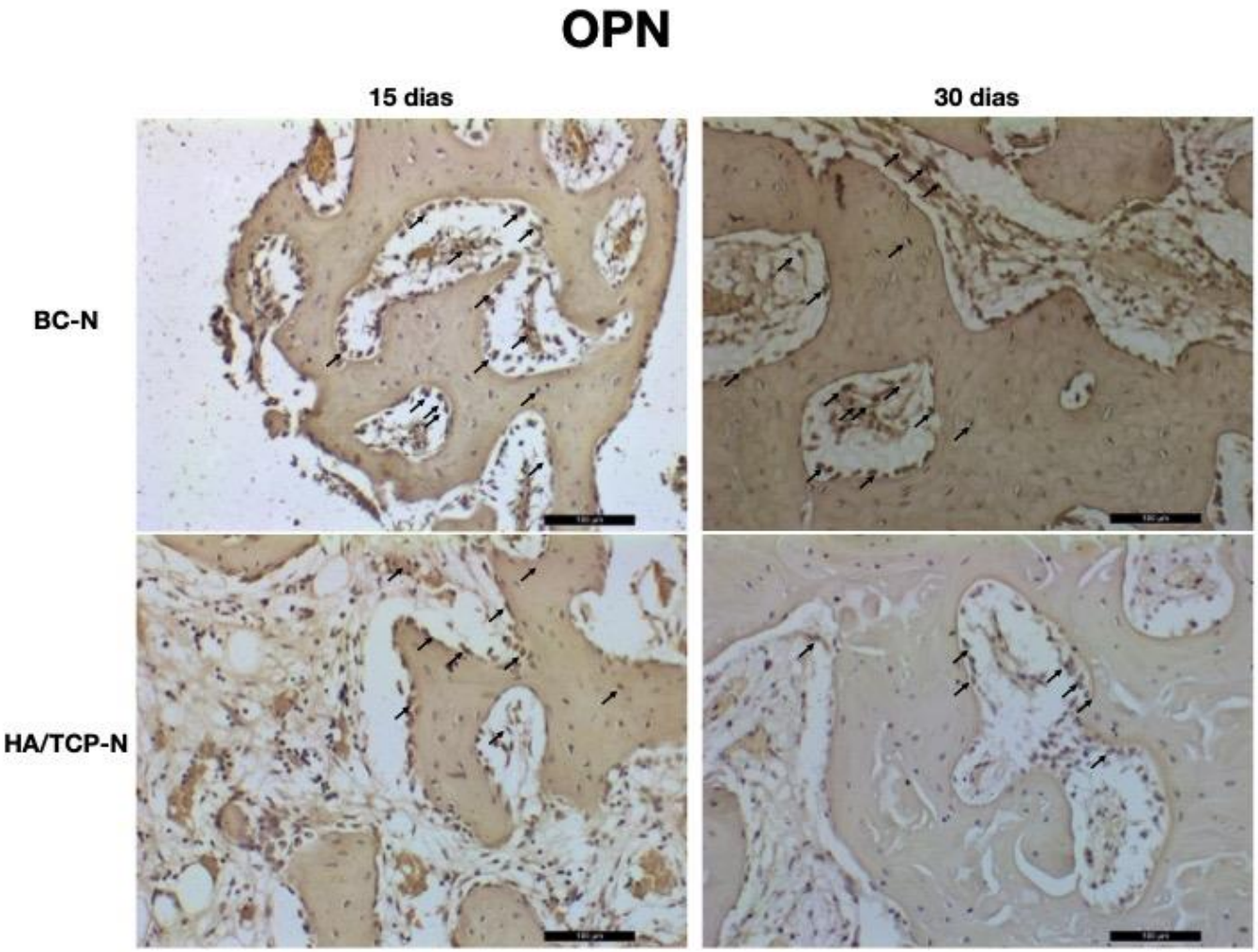
Immunolabeling of proteins (red arrow).

Figure 6. Labeled samples for all antibodies according to porous surfaces groups at both periods.



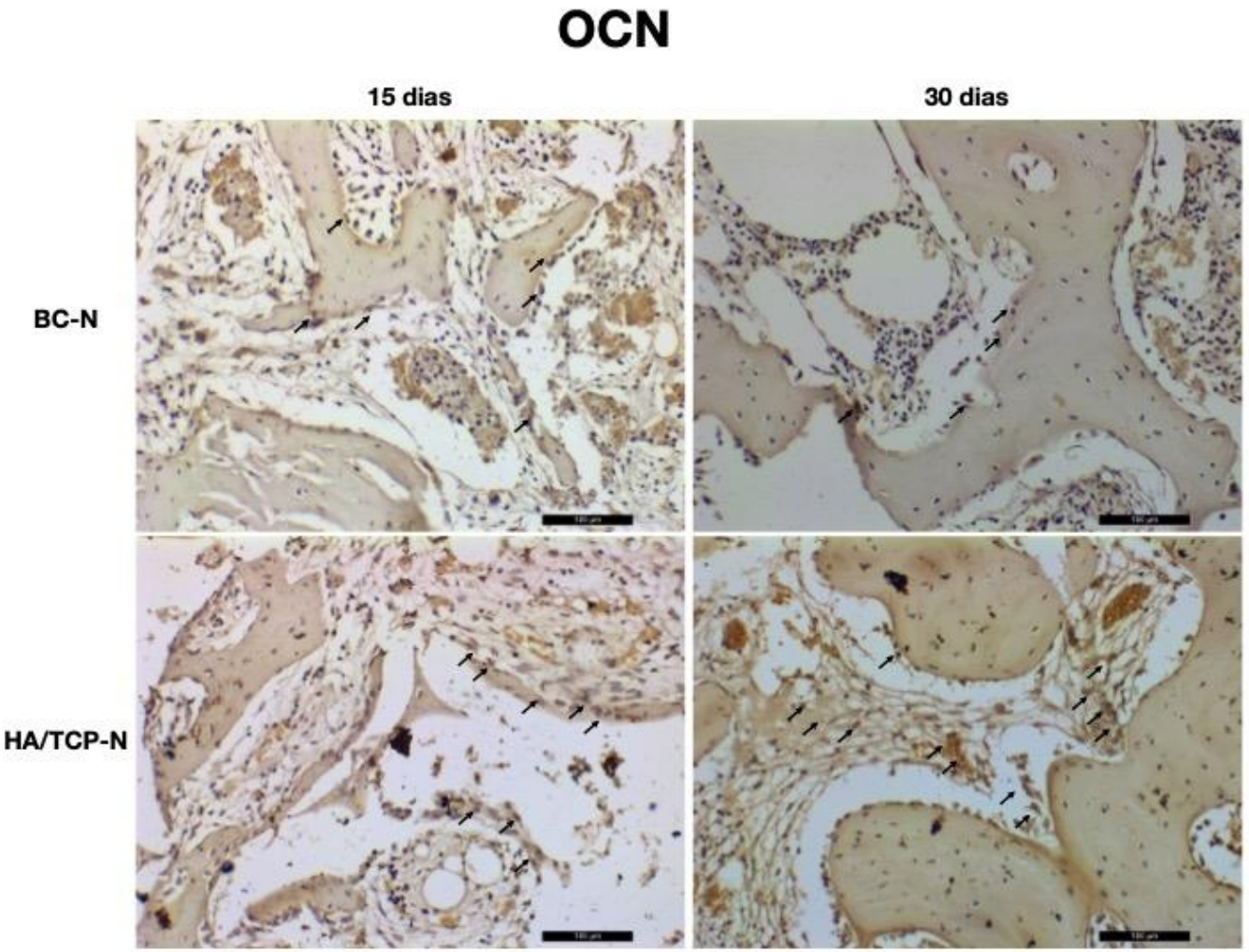
Immunolabeling of proteins (black arrow).

Figure 6. Labeled samples for all antibodies according to porous surfaces groups at both periods (*continuation*).



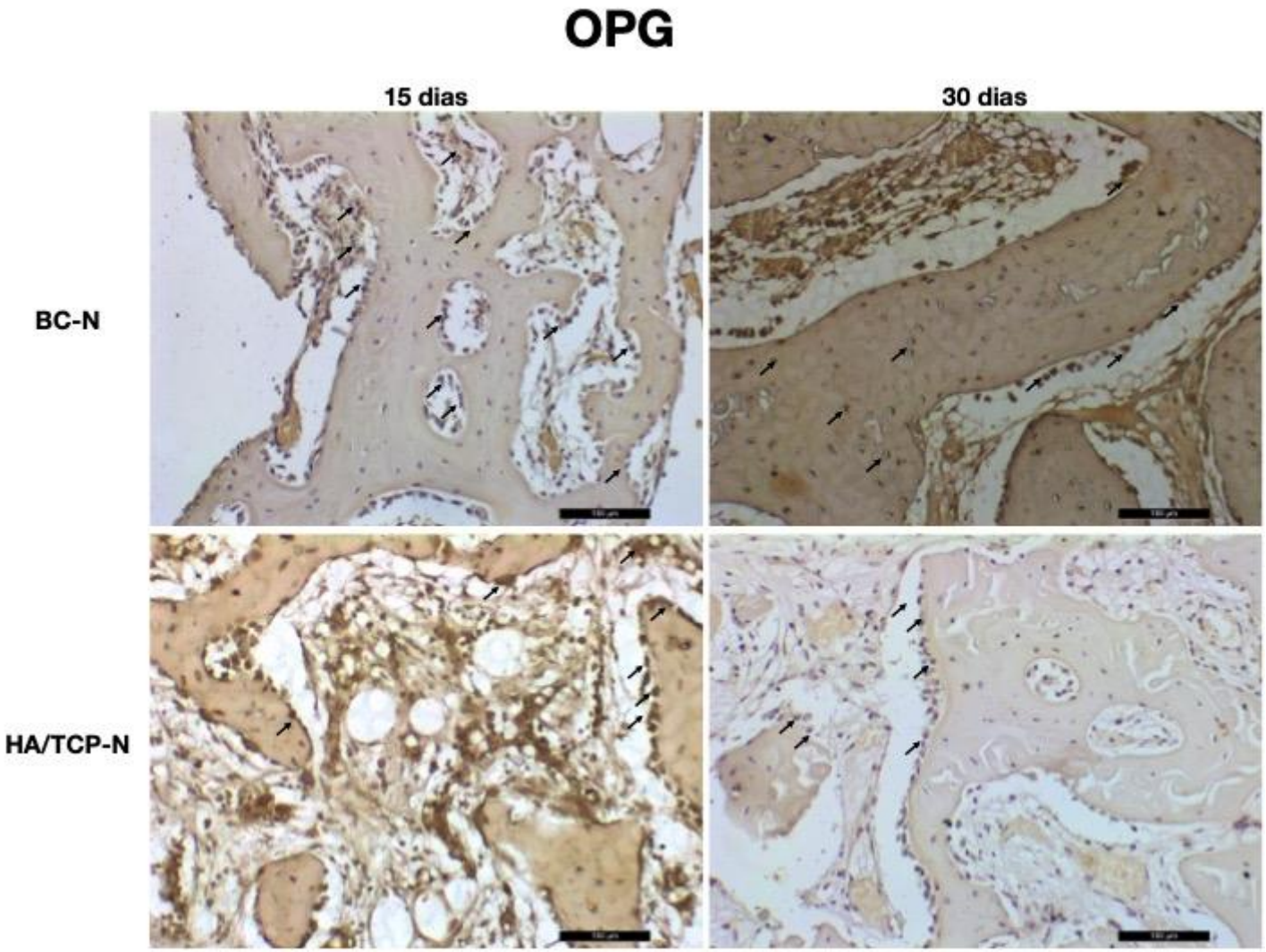
Immunolabeling of proteins (black arrow).

Figure 6. Labeled samples for all antibodies according to porous surfaces groups at both periods (*continuation*).



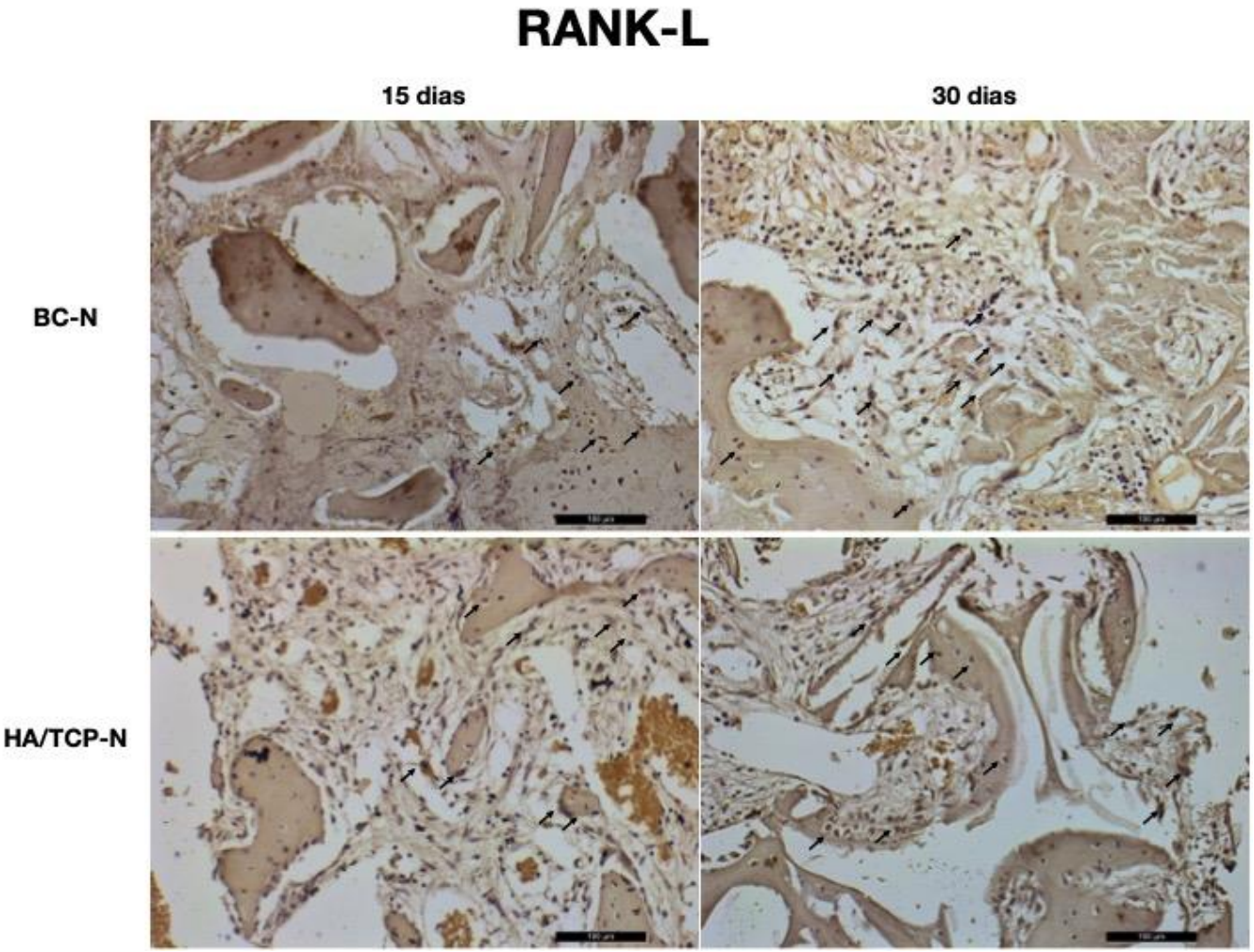
Immunolabeling of proteins (black arrow).

Figure 6. Labeled samples for all antibodies according to porous surfaces groups at both periods (*continuation*).



Immunolabeling of proteins (black arrow).

Figure 6. Labeled samples for all antibodies according to porous surfaces groups at both periods (*continuation*).



Immunolabeling of proteins (black arrow).

Figure 7. RUNX2 relative expression.

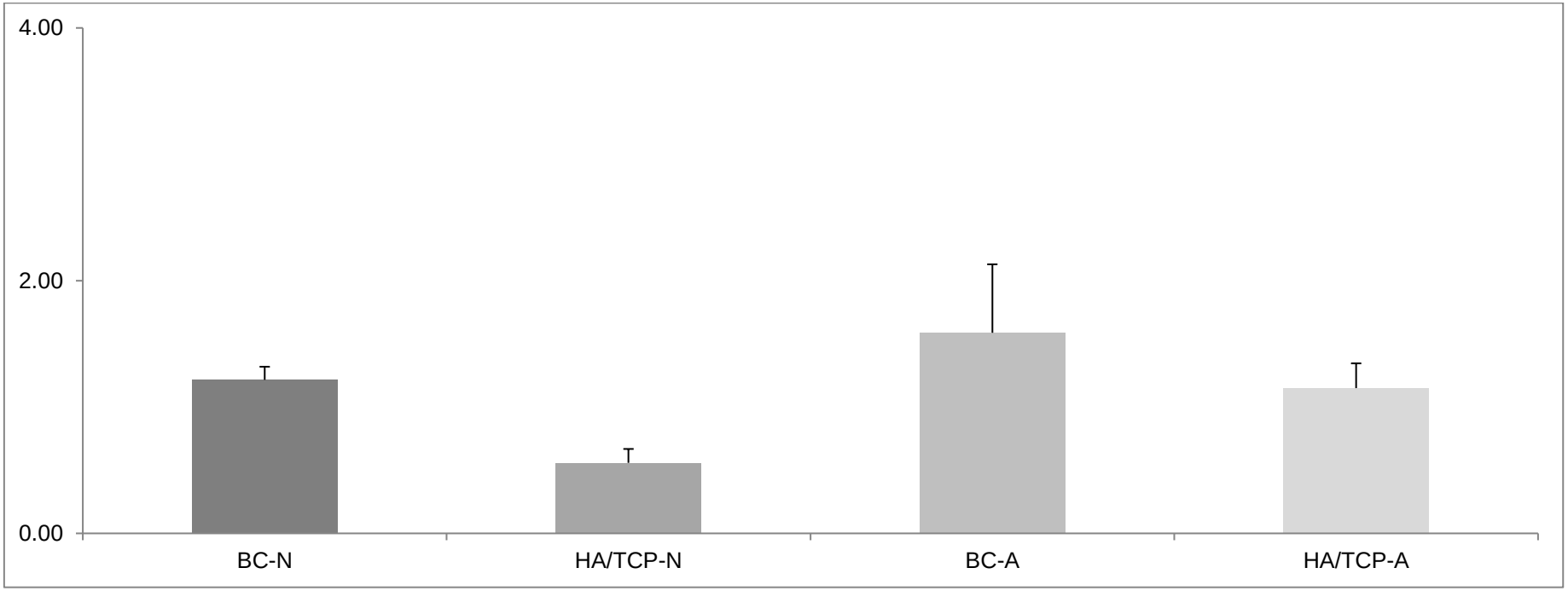
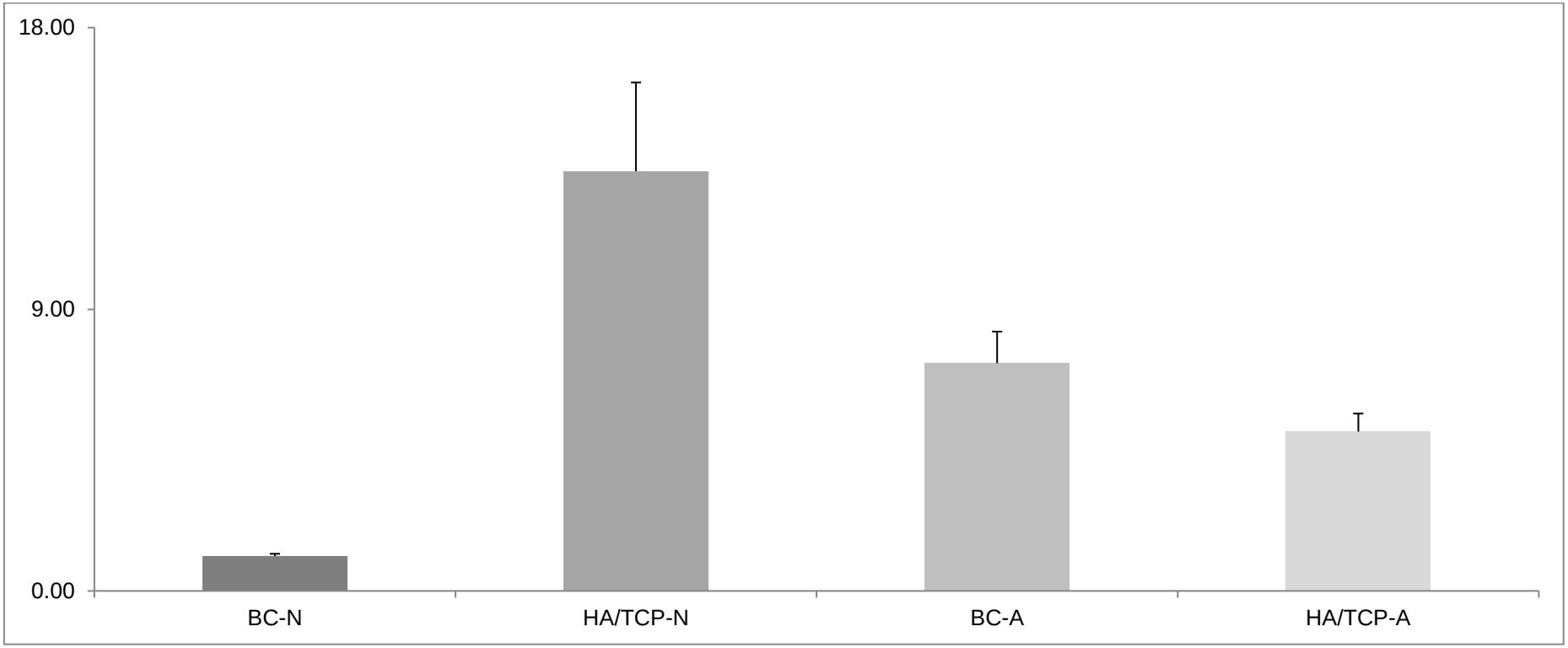


Figure 8. BSP relative expression.



3.3 Artigo 3* – Histomorphometric analysis of bone tissue formation around two titanium implants surfaces placed in bone defects filled or not with bone material substitute*

Histomorphometric analysis of bone tissue formation around two titanium implant surfaces placed in bone defects filled or not with bone substitute material

Short title: Histomorphometric analysis of bone formation around two Ti implant surfaces

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Key-words: osseointegrated dental implantation; bone grafting; cell viability

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ABSTRACT

Objectives: The objective of this study was to evaluate the peri-implant bone tissue formation around titanium implants with different surface treatments, placed in bone defects filled or not with bone substitute material (BSM).

Material and methods: Ten animals were divided into two groups according to implant surface treatment. In each tibia, a bone defect was created followed by the placement of one implant. On the left tibia, the defect was filled with blood clot (BC), and on the right tibia, the defect was filled with biphasic hydroxyapatite/β-tricalcium-phosphate (HA/TCP) generating four subgroups: BC-N: blood clot and porous surface; BC-A: blood clot and porous-hydrophilic surface; HA/TCP-N: BSM and porous surface; HA/TCP-A: BSM and porous-hydrophilic surface. The animals were submitted to euthanasia 60 days after implant installation. After light-curing resin inclusion, the blocks containing the implant and the bone tissue were stained and evaluated by means of histomorphometry to assess the percentages of bone implant contact (% BIC). Data was normally distributed and the group differences were examined using the parametric tests of Two-Way ANOVA.

Results: The BC-A group presented the higher mean value of BIC (46.43%). The HA/TCP-A group presented the higher mean value of BIC. The porous-hydrophilic surfaces presented better results of BIC when compared to the porous surface in both conditions of defect filling. No statistically significant differences were found among all groups (95% confidence interval and $p<0.05$).

Conclusion: According to histomorphometric analysis, after 60-days in a rabbit model, hydrophilic and hydrophobic surfaces have the same behavior in the presence or absence of HA/TCP.

Key-words: dental implant; bone substitute material; implant surfaces; bone formation

Word count: 249

INTRODUCTION

Over the past years, the replacing of missing teeth by dental implants has increased vigorously leading to development of different treatment planning options.[1] Naturally, it is very well established by different authors that the use of dental implants to restore oral function and aesthetics of partially or edentulous patients is a reliable method with predictable results.[2,3]

The osseointegration process, firstly described by Branemärk, involves a complex array of events and several experimental studies with animals had been performed in recent years, and a lot of them in order to investigate the stages of osseointegration regarding type of titanium implant designs and surface modifications.[4-7]

Albrektsson et al. (2008)[8] and Wennerbeg & Albrektsson (2009)[9] stated that one of the most critical factor for osseointegration is the configuration of implant as its topography, chemical, surface charge, and wettability. While macrostructure modifications are related to primary stability, microstructures modifications are involved to the implant secondary stability. These modifications contribute to better quality osseointegration.[10,11] In contrast, adequate bone quality and quantity still are pivotal for success and long-term stability for dental implants.[12]

In fresh extraction sockets, there is a lack in bone quantity and the implant diameter frequently is smaller than the alveolar ridge. Thus, immediate implant placement results in a gap between the implant surface and alveolar walls. About this gap, also called jumping distance, relay a controversial discussion regarding its filling. Potential benefits from bone substitute material remain unclear and its indication should to improve primary stability and stimulate bone support for dental implants.[13-16]

Verket et al. (2018)[17] compared two different bone substitute materials used to filling narrow marginal circumferential bone defects followed by implant placement in minipigs. Philipp et al. (2014)[18] compared two implant surfaces placed in sheep maxillary sinus with and without synthetic bone graft materials. Lee et al. (2016)[19] performed a study to determine healing patterns in peri-implant gap defect grafted with two different bone substitute materials. However, there is no evidence about the use of bone substitute material associated to different implant surface in created defects simulating fresh extraction sockets.

The objective of this study was to elucidate whether the bone formation around titanium implants, in created defects simulating a fresh socket, is influenced by the implant surface and the presence or not of bone substitute material.

MATERIALS AND METHODS

This study was submitted and approved by Ethics Committee in the Use of Animals of the São Paulo State University (Unesp), School of Dentistry, Araçatuba, Brazil (Protocol number FOA: 465-2016). Moreover, this study was conducted in strict accordance the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. Ten *Oryctolagus cuniculus* male rabbits, weighting between 300 to 400 mg and aging approximately 5 months, were selected. The animals were kept in separate cages with rabbit chow and water *ad libitum*.

The animals were randomly divided by manual draw into two groups according to the type of implant, each group was subdivided in two subgroups according to the way the previous created defect was filled: BC-N: the bone defect was filled with blood clot, and implants with a machined surface were installed (Neoporos surface, Neodent®, Curitiba, Brazil); BC-A: the bone defect was filled with blood clot, and implants with a hydrophilic surface were installed (Neoporos surface, Neodent®, Curitiba, Brazil); HA/TCP-N: the bone defect was filled with biphasic ceramics of hydroxyapatite/β-tricalcium-phosphate (Straumann® Bone Ceramic, Straumann AG, Basel, Switzerland), and implants with a machined surface were installed (Neoporos surface, Neodent®, Curitiba, Brazil); HA/TCP-A: the bone defect was filled with biphasic ceramics of hydroxyapatite/β-tricalcium-phosphate (Straumann® Bone Ceramic, Straumann AG, Basel, Switzerland), and implants with a hydrophilic surface were installed (Neoporos surface, Neodent®, Curitiba, Brazil). Finally, the euthanasia of the animals occurred at 60 postoperative days. Each group was composed by five tibias (Flow chart 1). The selection of the tibiae, the type of implant, and the way of the defect was filled was determined at random.

Surgical procedure

After 8 hours fasting, the animals were sedated and anesthetized via intramuscular by the combination of 50mg/ml of ketamine (Fracontar, Vibrac do Brasil Ltda., São Paulo, Brazil) and 5mg/ml of xylazine (Rompum, Bayer AS, Saúde Animal, São Paulo, Brazil). Subsequently, the animals were submitted to a trichotomy of the internal region of the left and right hid paws, and antisepsis was performed. Local

anesthesia was made using 0,3ml/kg of mepivacaine 2% with epinephrine 1:100.000 (Mepiadre – Nova DFL, Rio de Janeiro, Brazil) obtaining analgesia and hemostasis.

A 30-mm incision was performed in planes on the tuberosity of both tibiae. After delicate dissection of soft tissues and complete detachment and exposition of interest area of bone tissue, an osteotomy with an 6.1-mm trephine drill (Neodent®, Curitiba, Brazil) mounted in a 20:1 low-speed driller (SG20, Neodent®, Curitiba, Brazil) with the aid of an electric motor adjusted (NeoSurg XT Plus, Neodent®, Curitiba, Brazil) in 800 rpm creating a defect of 8-mm length in the tibiae. The internal cortical was removed preserving the external cortical of each tibiae. After that, the drilling for implant installation was performed in the defect center initiating by the spear drill followed by helicoidal drills with a thickness of 2.0mm, 2.8 mm and 3.0mm (Neodent®, Curitiba, Brazil) to accommodate a 8-mm-high titanium implant with a 3.5-mm-diameter (Drive CM Machined and Acqua surfaces, Neodent®, Curitiba, Brazil). Both osteotomies were made under abundant irrigation with sterile saline. According to each group, the implants were installed and stabled in the external cortical of tibiae and the bone defect was then filled with the bone substitute or with blood clot.

The tissue was repositioned and sutured by planes. The muscular portion was closed with resorbable suture (5-0 Vicryl Ethicon, Johnson & Johnson, São José dos Campos, Brazil) and the skin portion was closed with silk thread (4-0 Ethicon, Johnson & Johnson, São José dos Campos, Brazil) obtaining primarily close of the wound. The animals received a single dose of pentabiotic (0,1ml/kg, Fort Dodge Saúde Animal Ltda., Campinas, Brazil) and dipyrone (1mg/kg, Ariston Indústria Químicas e Farmacêuticas Ltda., São Paulo, Brazil).

After 60 days post surgery, the animals were submitted to euthanasia by an overdose of anesthetic using 105mg/kg of ketamine and 15mg/kg of xylazine as recommended by the ”American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals”. The tibias were removed and maintained and fixed in 10% formalin solution for 48 hours, washed in running tap water for 24 hours, and later stored in 70º ethanol.

Histometric analysis

The samples were dehydrated in increasing series of ethanols (70-100º) and infiltrated and polymerized using light-curing resin (Technovit 7,200 VLC, Kulzer Heraus GmbH & CO, Wehrheim, Germany). The blocks containing the implant and

the bone tissue were cut at a central point using a cut and wear system for nondecalcified samples (Exakt Apparatebeau, Hamburg, Germany). The final sections were approximately 45-µm thick and were stained with Stevenel's blue associated with acid fuchsin. Then, sections were analyzed using an optical microscope (DIASTAR – Leica Reichert & Jung products, Wetzlar, Germany) at a magnification of 2.5x. The histomorphometric evaluation was performed using software for image analysis (ImageJ, San Rafael, CA, USA). The percentages of bone implant contact (% BIC) were evaluated in the first three implant threads. A blinded and trained examiner performed these analyses (Figure 1).

Statistical analysis

The SPSS Statistics v23 software (IBM, New York, USA) was used considering a 95% confidence interval. The data generated by the histomorphometric analysis were examined for normality using Shapiro-Wilk test. Data was normally distributed and the group differences were examined using the parametric tests of Two-Way ANOVA considering the type of surface treatment and the presence of bone substitute material.

RESULTS

The porous-hydrophilic surface in the absence of bone substitute material (BC-A group) presented the higher mean value (46.43%±9.52) of BIC. Meanwhile, the porous surface in the presence of bone substitute material (HA/TCP-N) presented the lower mean value (37.82%±9.66) of BIC. The higher value (65.47%) of BIC was presented by porous-hydrophilic surface in the presence of bone substitute material (HA/TCP-A). Curiously, the lower value (18,81%) was also presented by the HA/TCP-A group.

Porous-hydrophilic surface without bone substitute material (BC-A) presented higher mean value of BIC when compared to the same surface with bone substitute material (HA/TCP-A). Likewise, porous surface presented higher mean value without bone substitute material (BC-N) when compared to the same surface with bone substitute material (HA/TCP-N).

When comparing the surfaces, the presence of bone substitute material associated to a porous-hydrophilic surface generates the higher value of BIC (65.47% for HA/TCP-A and 52.71% for HA/TCP-N). In contrast, the absence of bone substitute material associated to a porous surface generates the higher value of BIC (63.18% for BC-N and 60.49% for BC-A).

There were no statistically significant differences among all groups ($p<0.05$). All results are shown in Table 1. Figure 1 show, microscopically, the bone-implant contact. Figure 3 brings the representative graphic mean and standard deviation of all groups.

DISCUSSION

Several studies addressed the performance of implant surfaces and what it may improve in bone formation.[9] An in vitro data evidenced that the contact of a hydrophilic surface with the human blood might enhance the differentiation of mesenchymal stromal cells and osteoblasts and favors cell-implant contact and a cascade of events leading to the osteogenesis. These features are capable to facilitate the induction of early matrix mineralization and bone healing.[20,21] Bone cell-implant contact was evaluated by this study comparing two different surfaces in two different conditions.

Sand-blasting and acid-etching processes are subtraction methods of surfaces treatment that create microroughening on dental implant surfaces. These methods have been used productively to increase osteoblastic lineage cell differentiation *in vitro*, and bone formation *in vivo*.[5,20,22] The conditioning of these previously modified surface with an isotonic solution of 0.9% sodium chloride will chemically generate a property called wettability or hydrophilicity. As demonstrated by enhanced osteoblast maturation *in vitro*, and improved clinical success rates, hydrophilic surface of dental implants promote an environment conducive to bone formation.[21,23,24] The quantitative results of this study suggest that hydrophilic surfaces, independently the presence or not of bone substitute material, have a better bone formation around titanium implants in a long-term. However, all groups presented similar results statistically.

Sartoretto et al. (2017)[25] conducted a histomorphometric analysis comparing the same implant surfaces as did in this study but installed in sheep tibia. The experimental periods were four: 7, 14, 21, and 28 days. One important result from this study was that BIC is time-dependent for both surfaces. This evaluation could not be found in this study; however, the values found for hydrophilic surfaces are higher for both studies. Moreover, no bone defect was created and the implants were installed in direct contact to the bone different from this study. Nevertheless, this study presented BIC values, after 60 days installation, similar to those presented by the previously cited authors after 14, 21, and 28 days.[25] And those findings are

similar to those found in a clinical trial.[11] Lang et al. (2011)[11] found a superior degree of bone healing around hydrophilic surface implant after 14 days of implantation suggesting osseointegration. The findings in this study suggest that, after 60 days, there is a great possibility of osseointegration of these implants, even with the created defects.

In contrast, a pre-clinical study performed by Buser et al. (2004)[5] analyzed bone apposition on hydrophilic and hydrophobic surfaces and observed differences after 14 and 28 days, but similar results between the two surfaces after 42 days. Lang et al. (2011)[11] also found similar results presenting differences between surfaces at 28 days, but not after 42 days. In this study, no differences are noted between surfaces or situations regarding the presence or absence of bone substitute material corroborating to the studies previously cited.

Some authors[26-28] explained that the faster stimulation of the osseointegration promoted by hydrophilic surfaces occurs by the fact of a saline substance that the implant was previously immersed protects it from the air, preventing contamination by carbon molecules and thus avoiding the loss of surface energy and improving its wettability. Philipp et al. (2014)[18] performed a preclinical study and evaluated the osseointegration of hydrophilic surface implants placed in sheep maxillary sinuses grafted with osteoconductive bone substitute material (HA/TCP) and showed no superiority of hydrophilic surfaces compared to hydrophobic surfaces. The authors evaluated after 12 and 26 weeks. For this reason, the absence of differences between the experimental surfaces may be due the long evaluation periods. These findings are in agreement with the findings in this study when comparing to the literature. The bone healing period of 60 days may be too extend leading to results without any differences between the experimental groups.

Furthermore, Pinotti et al. (2018)[29] performed a study in a rat tibia model. The authors evaluated the effect of a hydrophilic surface on the osseointegration in grafted areas with HA/TCP and concluded that the hydrophilic surface improves the osseointegration. The study had two experimental periods (15 and 45 days). After 45 days, the author found statistically significant differences between hydrophilic and hydrophobic surfaces with HA/TCP (BIC values of 44,33% and 11.21%, respectively). After 60 days, the findings in this study are numerically similar to hydrophilic surface but not to hydrophobic surfaces in the same situation (BIC values of 42.75%±20 and

37.82%±9.66, respectively). Again, these differences may be explained by the long evaluation periods.

Smeets et al. (2016)[30] discussed on a recent review article about the impact of dental implant surface modifications on osseointegration. The authors agree that there are limited controlled, clinical trials on hydrophilic implants but enough to show better outcomes when compare this surface to hydrophobic. The results presented in this study show differences but not statistically suggesting that, after 60 days, both surfaces have the same behavior. However, there is a lack of information about the behavior of these surfaces on bone defects filled or not with bone substitute material. Other studies with this analysis must be conducted with lower healing period to clarify the differences between both surfaces in the conditions proposed in this study.

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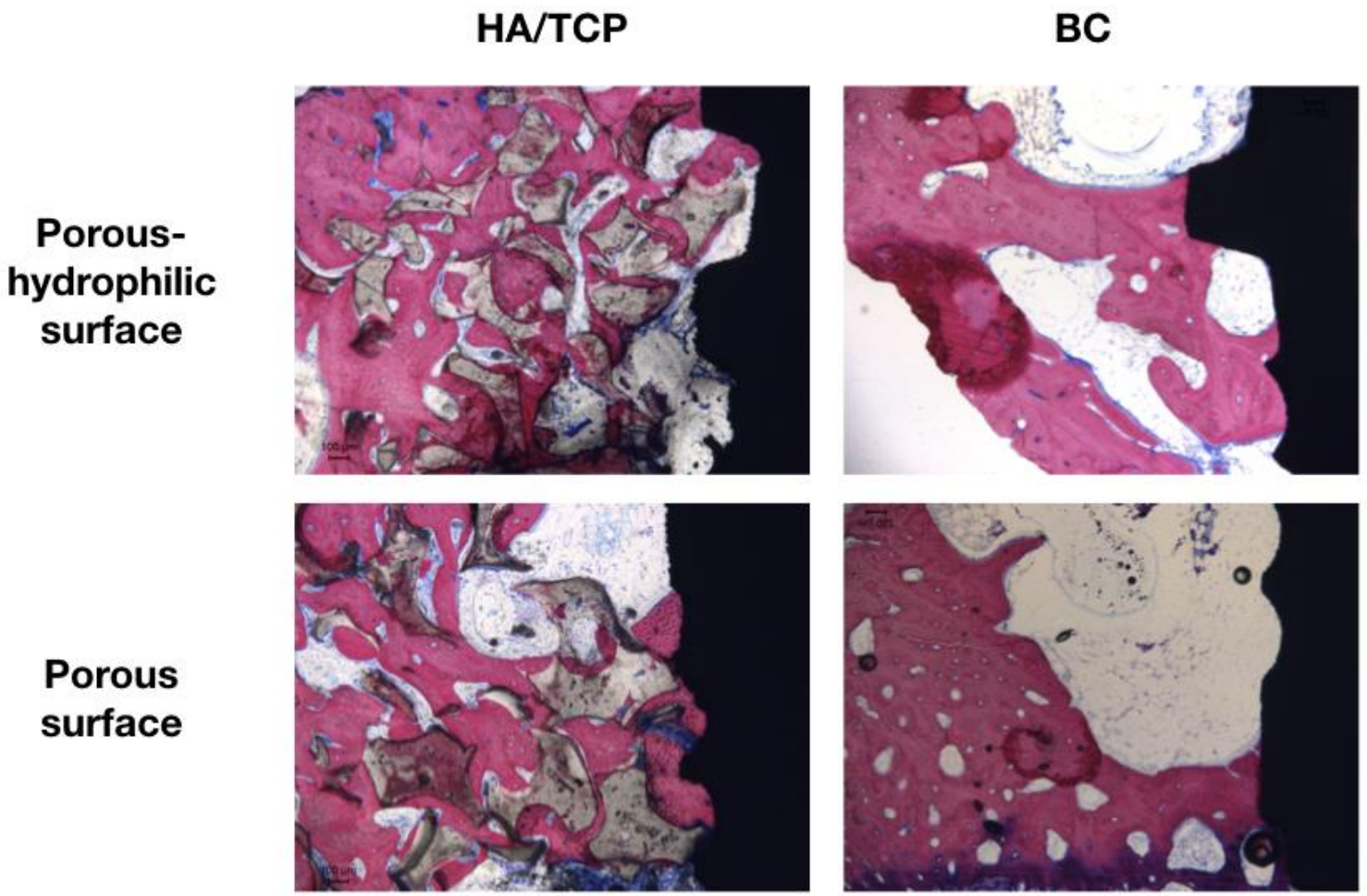
TABLES

Table 1. Bone-implant contact percentages of all samples according to groups. Also, mean (standard deviation) of BIC (%) of all groups (95% CI and p<0.05).

	BIC (%)			
Groups	**HA/TC-A**	**HA/TCP-N**	**BC-A**	**BC-N**
Samples	18,81	29,46	33,91	21,03
	26,42	30,05	43,84	37,36
	44,85	35,23	46,32	48,71
	58,19	41,67	47,57	50,42
	65,47	52,71	60,49	63,18
Mean (SD)	42,75 (19,99)	37,82 (9,66)	46,43 (9,51)	44,14 (15,83)

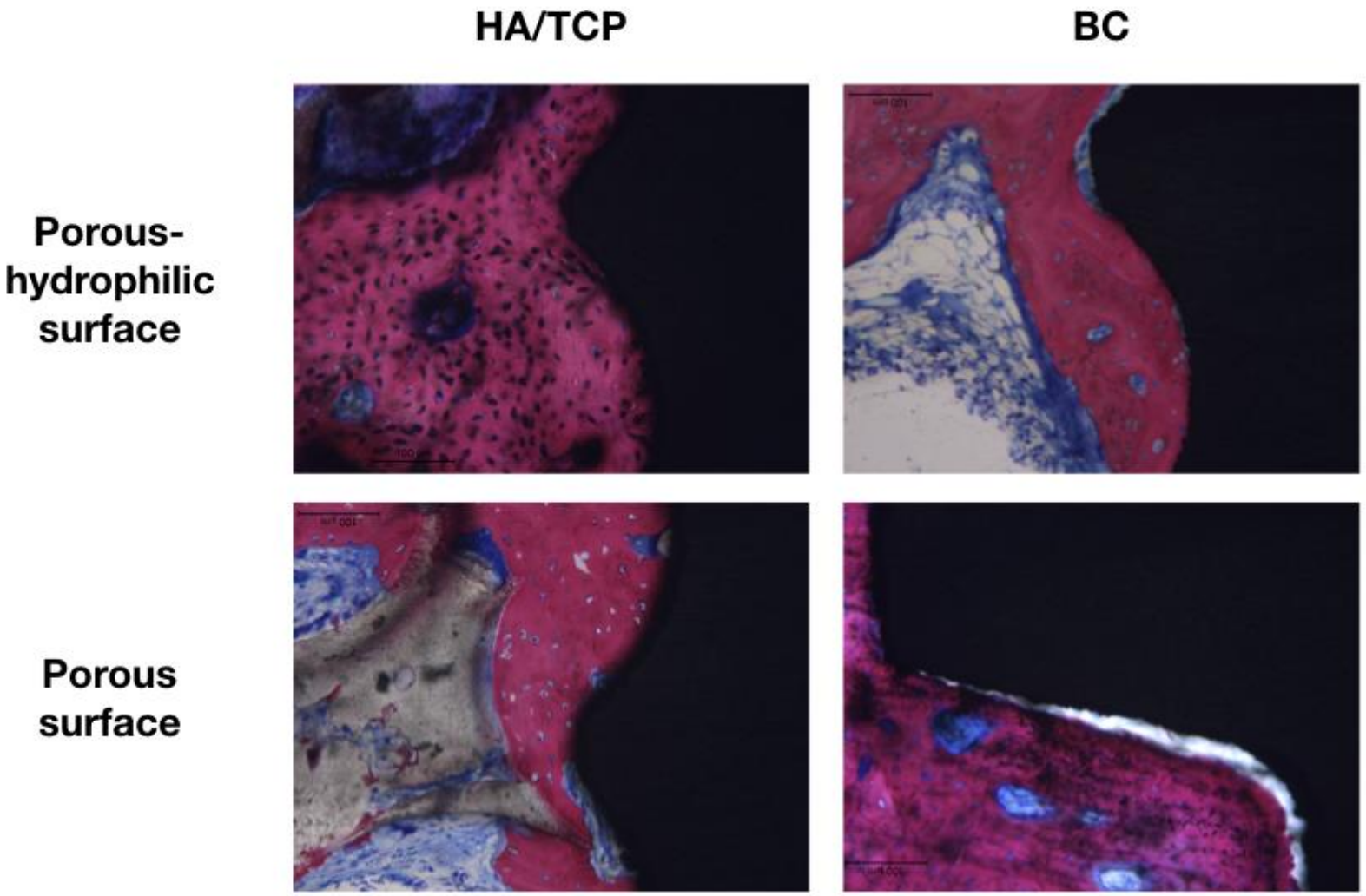
FIGURES LEGNDS

Figure 1. Representative figures of the nondecalcified sections. (Stevenel's blue associated to acid fuchsin – original augmentation 2.5x and 20x).



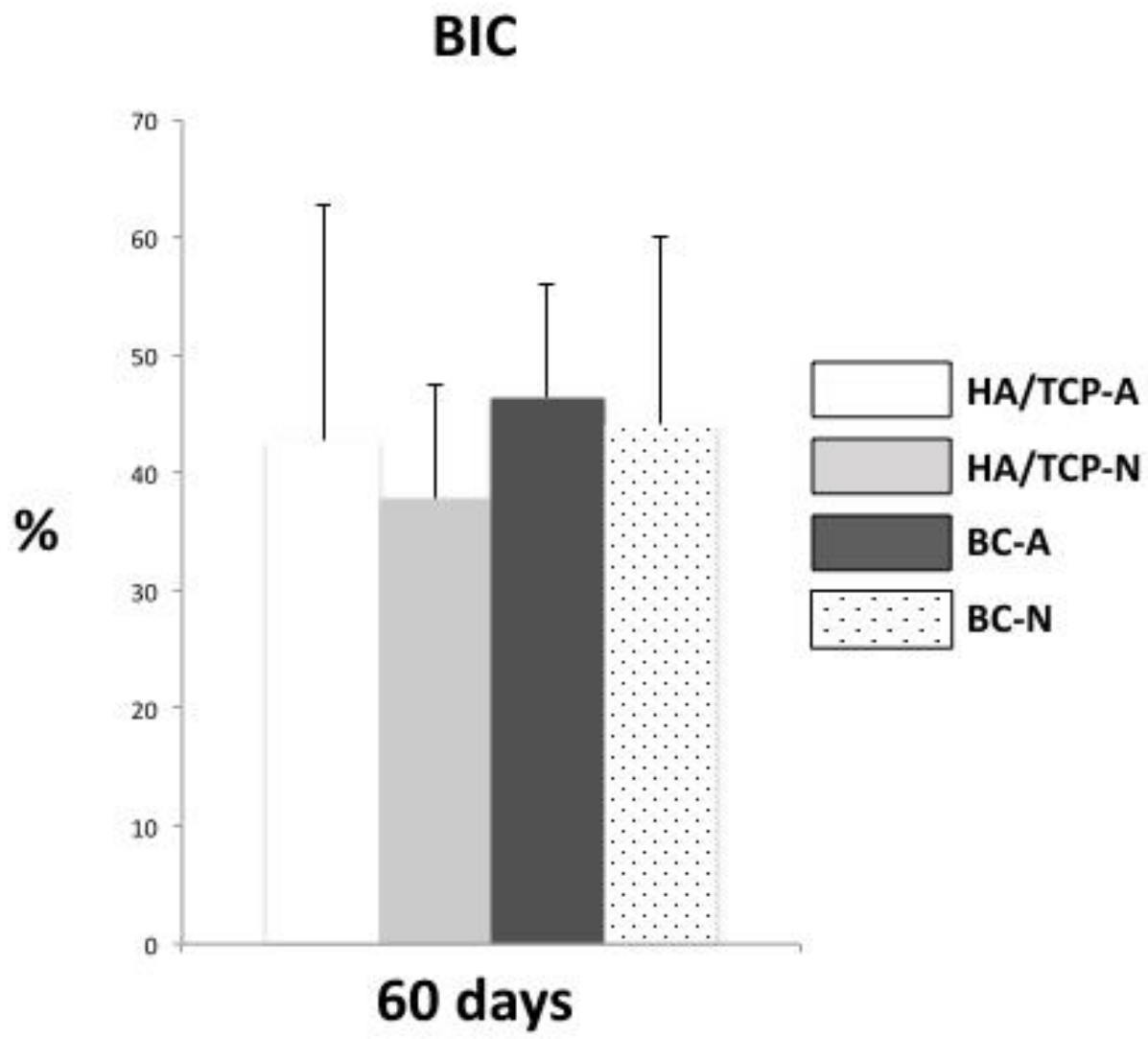
HA/TCP: biphasic hydroxyapatite/β-tricalcium-phosphate; BC: blood clot

Figure 1. Representative figures of the nondecalcified sections. (Stevenel's blue associated to acid fuchsin – original augmentation 2.5x and 20x) (*continuation*).



HA/TCP: biphasic hydroxyapatite/β-tricalcium-phosphate; BC: blood clot

Figure 2. Graphic of mean and standard deviation of all groups. There was no statistically difference between the groups ($p<0.05$).



HA/TCP: biphasic hydroxyapatite/β-tricalcium-phosphate; BC: blood clot; A: Acqua (porous-hydrophilic surface); N: Neoporos (porous surface)

4 CONCLUSÃO

Dentro das limitações da metodologia empregada neste estudo, foi possível concluir que:

1) Superfícies porosas e hidrofílicas em associação com substitutos ósseos podem acelerar a neoformação de tecido ósseo, somente nos períodos iniciais.
2) Quando da não há a necessidade de preenchimento do “gap” com substituto ósseo, as superfícies porosas e não hidrofílicas devem ser a primeira escolha.
3) Após a osseointegração, em períodos tardios, ambas superfícies testadas tem comportamento similar tanto na presença quanto na ausência de substitutos ósseos.

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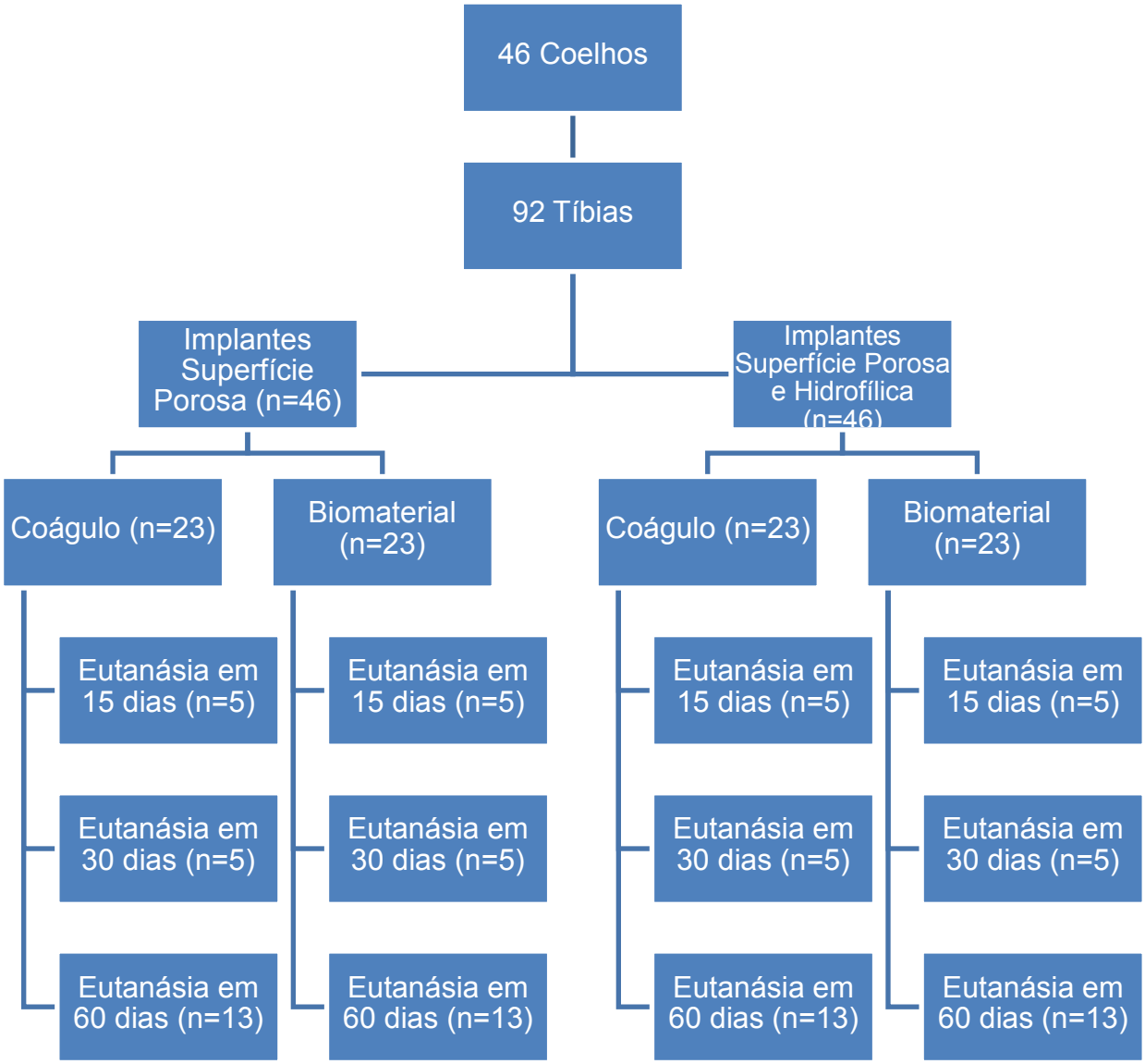
APÊNDICE A – METODOLOGIA DA PESQUISA

Foram selecionados 46 coelhos da espécie *Oryctolagus cunilicus* (New Zealand), com peso corporal médio entre 3 a 4kg, e aproximadamente 5 meses de idade. Os animais foram mantidos e assistidos no biotério do Campus da Faculdade de Odontologia de Araçatuba – UNESP. A pesquisa foi submetida ao Comitê de Ética no Uso de Animais para apreciação e aprovada (Processo FOA nº 00465-2016 – ANEXO A). Os animais foram mantidos em gaiolas individuais com dieta sólida padrão (Procoelho, Primor) e água “ad libitum”.

Os animais foram divididos em dois grupos de acordo com o tipo de implante utilizado. Cada grupo foi subdividido em dois grupos de acordo com o preenchimento do defeito criado. Por fim, a eutanásia dos animais ocorreu em três períodos experimentais distintos, a saber: 15, 30 e 60 dias pós-operatórios. Cada grupo foi composto por 23 tíbias, sendo que no último período de cada grupo foi composto por 13 tíbias dos animais, pois uma parte (n=5) serviu para a análise do BIC (corte do implante com Exakt, sem a descalcificação das peças e análise da micro CT), outra parte (n=5) foi realizado o processamento laboratorial com descalcificação das peças para análise histomorfométrica e imuno-histoquímica e a outra parte (n=3) foi submetida a RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) para avaliação da expressão gênica dos marcadores Runx2 e Sioloproteína Óssea. A determinação da tíbia que foi utilizada, tipo de implante e de defeitos, foi realizada por meio de sorteio aleatório (Fluxograma 1).



Fluxograma 1. Distribuição da amostra por grupos.

1. Procedimentos cirúrgicos (Figuras 1 a 4)

Após jejum de oito horas, os animais foram sedados por via intramuscular pela combinação de 50mg/kg de Ketamina (Fracontar – Vibrac do Brasil Ltda., São Paulo, São Paulo) e 5 mg/ml de cloridrato de Xilazina (Rompum - Bayer AS – Saúde Animal, São Paulo, São Paulo). Em seguida, foi realizada tricotomia e assepsia do sítio cirúrgico com Polivinil Pirrolidona Iodo Degermante (PVPI 10%, Riodeine Degermante, Rioquímica, São José do Rio Preto, São Paulo) associado ao Polivinil Pirrolidona Iodo Tópico (PVPI 10%, Riodeine Tópico, Rioquímica, São José do Rio Preto, São Paulo). Foi realizada infiltração anestésica local utilizando-se 0,3ml/kg de cloridrato de mepivacaína 2% com epinefrina 1:100.000 (Mepiadre – Nova DFL, Rio de Janeiro, Rio de Janeiro), obtendo-se analgesia e hemostasia do campo operatório.

O acesso cirúrgico foi realizado por meio de uma incisão em pele, fáscia e periósteo, utilizando-se de lâmina 15 (Lamedid – Osasco, São Paulo), e medindo aproximadamente 3 cm de comprimento. Os tecidos foram divulsionados para exposição total do tecido ósseo na área de interesse. Com uma broca trefina de 6.1mm (Neodent – Curitiba, Paraná), sob irrigação constante de cloreto de sódio a 0,9% (IsoFarma – Eusébio, Ceará), foi realizado o defeito ósseo na tíbia de cada coelho removendo-se a cortical superior e delimitando a localização dos implantes. A cortical inferior foi preservada. Após preparação, no centro do defeito foi iniciada a perfuração referente a instalação dos implantes. Sob irrigação constante, foram utilizadas, sequencialmente e na velocidade máxima de perfuração de 800RPM, as fresas lança, de 2.0mm e de 3.5mm (Neodent – Curitiba, Paraná) instaladas em contra-ângulo 20:1 (SG20 - Neodent – Curitiba, Paraná) montado em motor elétrico (Neorsurg XT Plus – Neodent – Curitiba, Paraná)

Em seguida, foi instalado um implante cônico de 3.5mm de diâmetro por 8mm de comprimento (Drive CM – Drive CM Acqua – Neodent, Curitiba, Paraná), por meio do motor e contra-ângulo a 30RPM e torque de 40N, em cada alvéolo cirúrgico. No total, foram utilizados 92 implantes do tipo Drive, sendo 46 com a superfície porosa e hidrofílica (Drive CM Acqua – Neodent, Curitiba, Paraná) e 46 com a superfície porosa (Drive CM – Neodent, Curitiba, Paraná). De acordo com sorteio prévio, os defeitos ósseos criados anteriormente e que permaneceram após a instalação dos implantes foram preenchidos por coágulo sanguíneo ou por substituto ósseo – 60% de hidroxiapatita e 40 % de beta tricálcio fosfato (BoneCeramic® – Straumann, Suiça). Após instalação, foi posicionado o parafuso de cobertura.

Os tecidos foram reposicionados e suturados em planos. No plano muscular foi realizada sutura contínua com fio reabsorvível (Poligalactina 910 – Vycril 4.0, Ethicon, Johnson Prod., São José dos Campos, São Paulo) e na derme foi realizada sutura interrompida simples com fio monofilamentar não reabsorvível (Nylon 4.0, Ethicon, Johnson Prod., São José dos Campos, São Paulo), obtendo-se fechamento primário da ferida. No período pós-operatório, os animais receberam medicação antibiótica e analgésica, conforme o segundo protocolo: Pentabiótico (0,1ml/kg, Fort Dodge Saúde Animal Ltda., Campinas, São Paulo) e Dipirona Sódica (1mg/kg/dia, Ariston Indústrias Químicas e

Farmacêuticas Ltda., São Paulo, São Paulo) uma dose pós-operatória imediata. Nenhuma restrição alimentar ou de mobilidade será imposta.

A eutanásia foi realizada nos períodos experimentais de 15, 30 e 60 dias. Foi realizada após sedação via intramuscular pela combinação de 50mg/kg de Ketamina (Fracontar – Vibrac do Brasil Ltda., São Paulo, São Paulo) e 5mg/kg de cloridrato de Xilazina (Rompum – Bayer AS – Saúde Animal, São Paulo, São Paulo) e perfusão de 1,8ml de formaldeído 4% no ventrículo esquerdo durante 40 minutos, usando a bomba de perfusão.

2. Obtenção das peças cirúrgicas

Após os procedimentos experimentais, as amostras foram removidas das tíbias dos coelhos para análise. As amostras foram removidas com o auxílio de material rotatório cortante, fixadas em paraformaldeído por 48 horas, lavadas em água corrente por 24 horas e armazenadas em álcool 70%. Então, as amostras foram avaliadas por análise radiográfica tridimensional (Micro-CT). Após a análise por micro CT as peças foram desidratadas em álcool etílico e preservadas em resina pura (LR White Hard Grade, London, UK) com o objetivo de inclusão com posterior polimerização das amostras. Por meio do sistema Exakt (Exakt Cuttingand Grinding system, Apparatebau Gmbh, Hamburgo, Alemanha) os blocos foram seccionados ao meio, fixados e polidos por lixas específicas (Hermes Schleifmittel Gmbh & Co., Hamburgo, Alemanha) de microdesgaste, o que resultou em lâminas de 40 a 60µm. Ao final, as peças foram submetidas a coloração com azul de Stevenel e vermelho de Alizarina. Para os experimentos de RT-PCR, três amostras de cada grupo, no período final de 60 dias, foram separadas e o tecido ósseo em contato com o implante foi submetido a análise molecular por meio da reação em cadeia da polimerase em tempo real utilizando o sistema StepOne (Applied Biosystems, Warrington, UK) com o objetivo de avaliar a expressão gênica de marcadores presentes na formação óssea: Runx2, Sialoproteína Óssea.

3. Escaneamento Micro-CT

As amostras ósseas armazenadas em álcool 70%, foram primeiramente submetidas à análise por varredura de feixe de raios-X em um sistema de microtomografia digital computadorizada. As peças foram escaneadas pelo microtomógrafo SkyScan (SkyScan 1176 Bruker MicroCT, Aatselaar, Bélgica, 2003) utilizando cortes de 9µm de espessura (50Kv e 500µ), com filtro de cobre

e alumínio e passo de rotação de 0,3mm. As imagens obtidas pela projeção dos raios-x nas amostras foram armazenadas e reconstituídas determinando a área de interesse pelo software NRecon (SkyScan, 2011; Versão 1.6.6.0). No software Data Viewer (SkyScan, Versão 1.4.4 64-bit) as imagens foram reconstruídas para adequação do posicionamento padrão para todas as amostras, podendo ser observada em três planos (transversal, longitudinal e sagital). Após, foi utilizado o software CTAnalyser – CTAn (2003-11SkyScan, 2012 Bruker MicroCT Versão 1.12.4.0) para determinação da área de interesse (ROI). No software CTAn foi realizada a análise e mensurações da imagem de acordo com a escalas de cinza (*threshold*). O *threshold* utilizado na análise será de 25-90 tons de cinza, possibilitando a obtenção do volume de osso formado no defeito e sua caracterização.

4. Análise histomorfométrica

Parte das peças que serviram para a análise histomorfométrica foram armazenadas em álcool 70%, lavadas por 2 horas em água corrente e descalcificadas em solução de EDTA a 20%, trocada a cada 48 horas, durante 60 dias. Verificada a correta descalcificação das peças, as mesmas foram utilizadas para avaliação histológica e histométrica. Foram obtidos cortes de 6µm de espessura de cada peça, divididos em lâminas com 5 cortes cada, corados por hematoxilina-eosina, e permitindo a correta avaliação histológica das estruturas teciduais.

A avaliação histológica foi realizada utilizando-se um microscópio de luz DIASTAR (Leica Reichert & Jung Products, Germany), com objetivas para aumento de 5 até 100 vezes, e oculares com aumento de 10 vezes, onde as imagens foram observadas e avaliadas. As imagens representativas à avaliação feita foram enviadas para um microcomputador, utilizando-se de uma câmera fotográfica digital Leica Microsystems DFC-300-FX (Leica Microsystems – Alemanha), com resolução de 1.3 megapixels, acoplada ao microscópio de luz comum, para sua posterior utilização na descrição dos resultados obtidos. Na análise histológica descritiva foram avaliados o tipo e a qualidade dos tecidos neoformados, além da reação tecidual e presença de reação inflamatória.

Na sequência, foi feita a avaliação histométrica das peças, com base nas mesmas lâminas. As mensurações foram realizadas utilizando-se imagens

obtidas com aumento final de 20x, unidas de forma a evidenciar a região como um todo. As imagens foram enviadas para um microcomputador, através de uma câmera fotográfica digital Leica Microsystems DFC-300-FX (Leica Microsystems, Alemanha), com resolução de 1.3 megapixels, acoplada ao microscópio de luz comum, e salvas sob a extensão TIF, permitindo que fossem salvas e unidas. A determinação dos valores foi feita empregando-se um software analisador de imagens (ImageJ 1.45s, NIH, USA). Os valores para a área do defeito ocupado por tecido ósseo foram obtidos em mm e mm^2. Adicionalmente à mensuração das áreas ocupadas por cada tecido, também foi empregado o método de contagem diferencial de pontos, utilizando-se o mesmo software analisador de imagens. Nesse momento, o software foi utilizado para delinear um "grid" de pontos com distância conhecida, sobre o qual a ferramenta de contagem de pontos por multi-canais foi empregada, quantificando: tecido ósseo, tecido conjuntivo, vasos sanguíneos e remanescente de substituto ósseo (quatro canais de contagem). O tamanho adequado do "grid" foi definido por meio de um piloto com algumas lâminas do próprio estudo, definindo o menor número de pontos possível para gerar dados representativos da amostra.[76,77]

5. Análise imuno-histoquímica

A análise imuno-histoquímica foi realizada por meio da técnica de detecção pela imunoperoxidase. Após a desparafinização dos cortes histológicos, a atividade da peroxidase endógena foi inibida com peróxido de hidrogênio. A seguir, as lâminas passaram pela etapa de recuperação antigênica com tampão fosfato citrato (pH 6.0) e o bloqueio da biotina endógena através de leite desnatado. Os anticorpos primários foram utilizados contra fator de transcrição relacionado a Runt 2 (Runx2, Goat anti-runx2 – Santa Cruz Biotechnology), Osteopontina (OPN), Osteocalcina (OCN), Osteoprotegerina (OPG, Goat anti-OPG, Santa Cruz Biotechnology, SC 21038) e Ligante do receptor ativador do fator nuclear kappa-B (RANK-L, Goat anti-RANK-L – Santa Cruz Biotechnology, SC18319).

Foram realizados procedimentos de controle negativo por meio da omissão de anticorpos primários. Ainda, como controle positivo, foi utilizada a cortical inferior da tíbia dos animais, para verificação da efetividade dos anticorpos utilizados.

Foram atribuídos escores para verificar a expressão das proteínas na região das espiras dos implantes, a saber: hiperpositivo, superpositivo, positivo e negativo. Ainda, foi obtida a razão entre RANK-L e OPG para avaliar a tendência à inibição ou estímulo para a formação dos osteoblastos.

6. Análise Molecular

No período de 60 dias da instalação dos implantes, três amostras de cada grupo foram submetidas a remoção dos implantes das tíbias por meio do torquímetro e, então, foi coletado o tecido ósseo aderido aos implantes para realização dos experimentos de biologia molecular. A reação da transcriptase reversa, seguida de reação em cadeia da polimerase (RT-PCR) foi realizada com o objetivo de avaliar a expressão gênica de diferentes marcadores em um determinado estágio de células da linhagem osteoblástica: Runx2, Sioaloproteína Óssea. Para tanto, foram utilizados primers desenhados pela Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA).

Cada fragmento ósseo foi cuidadosamente lavado em uma solução tampão fosfato-salino (PBS) e posteriormente congelado em nitrogênio líquido para que o RNA total fosse isolado com o auxílio do reagente Trizol (Life Technologies: Invitrogen, Carlsbad, CA, USA). O RNA foi quantificado em um espectrofotômetro NanoDrop®2000 (Thermo Scientific NanoDrop Technologies, Wilmington, DE) para análise da integridade, pureza e concentração do RNA. Após, foi confeccionado o cDNA utilizando 1µg de RNA por meio da reação da transcriptase reversa (M-MLV: transcriptase reversa: Promega Corporation, Madison, Wisconsin, USA). A sequência dos primers foi amplificada e seus produtos foram submetidos a eletroforese com gel de agarose a 1.5% corados com brometo de etídeo e visualizados utilizando o software Quantity One (Bio-Rad Laboratories, Philadelphia, PA, USA).

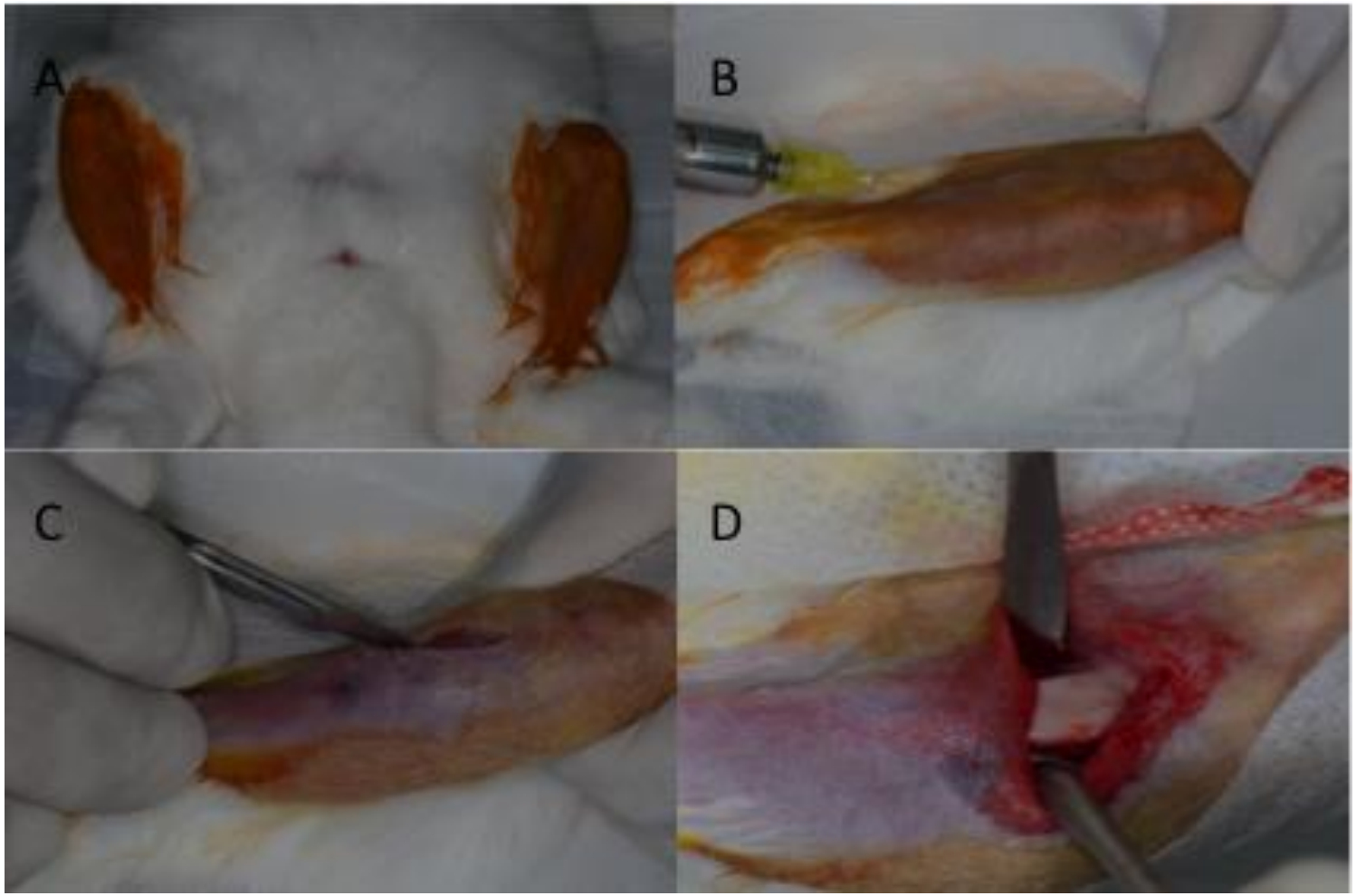
Por fim, a RT-PCR foi conduzida com base no sistema StepOne (Applied Biosystems, Foster City, CA, USA) utilizando o sistema de corantes fluorescentes para detecção SybrGreen (Primer Design Ltd, Southampton, UK) sob as seguintes condições cíclicas: 95º durante 10 minutos para a desnaturação primária, e 35 ciclos de 95° por 15 segundos para abertura das fitas e, finalmente, 60° por 60 segundos para anelamento dos primers e extensão dos fragmentos. A expressão gênica relativa foi calculada em referência a expressão das proteínas ribossômicas mitocondriais e normalizada

pela expressão gênica dos fragmentos ósseos removidos dos implantes no determinado período de 60 dias.[2,3]

7. Análise dos resultados

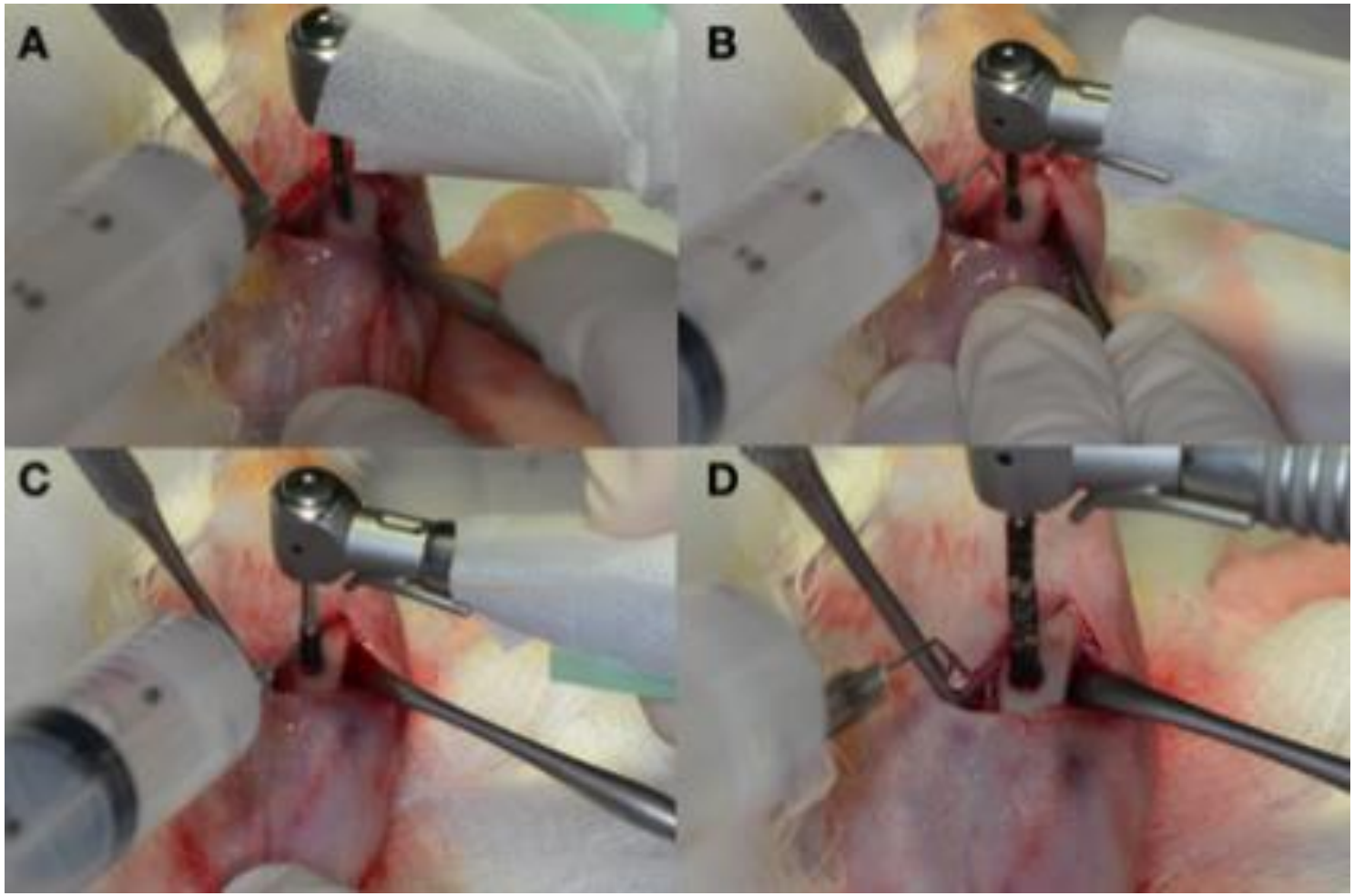
As mensurações foram tabuladas para avaliação estatística. Foram utilizados testes de comparação múltipla, com repetição. Após a confirmação do modelo de distribuição dos dados, por meio dos testes de normalidade Shapiro-Wilk e Kolgomorov-Smirnov, foi selecionado o teste estatístico adequado.

Figura 1. A) Assepsia do campo operatório. B) Anestesia local após sedação do animal. C) Incisão. D) Descolamento e exposição do local de interesse.



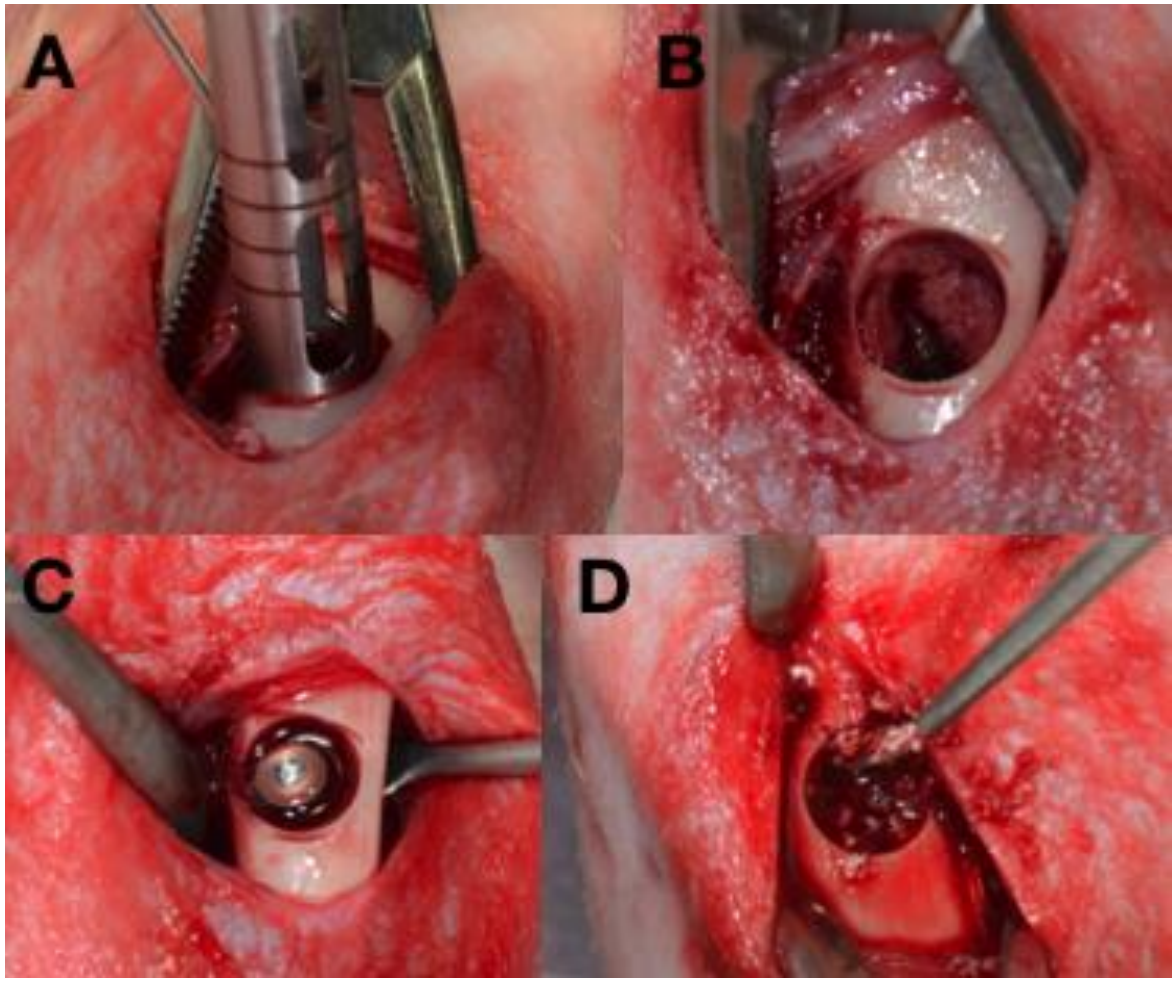
Fonte: Arquivo pessoal do autor.

Figura 2. Uso das brocas do sistema Neodent para a fresagem e posterior instalação dos implantes. A) Broca lança. B) Broca 2.0. C) Broca 2/3. D) Broca 2.8.



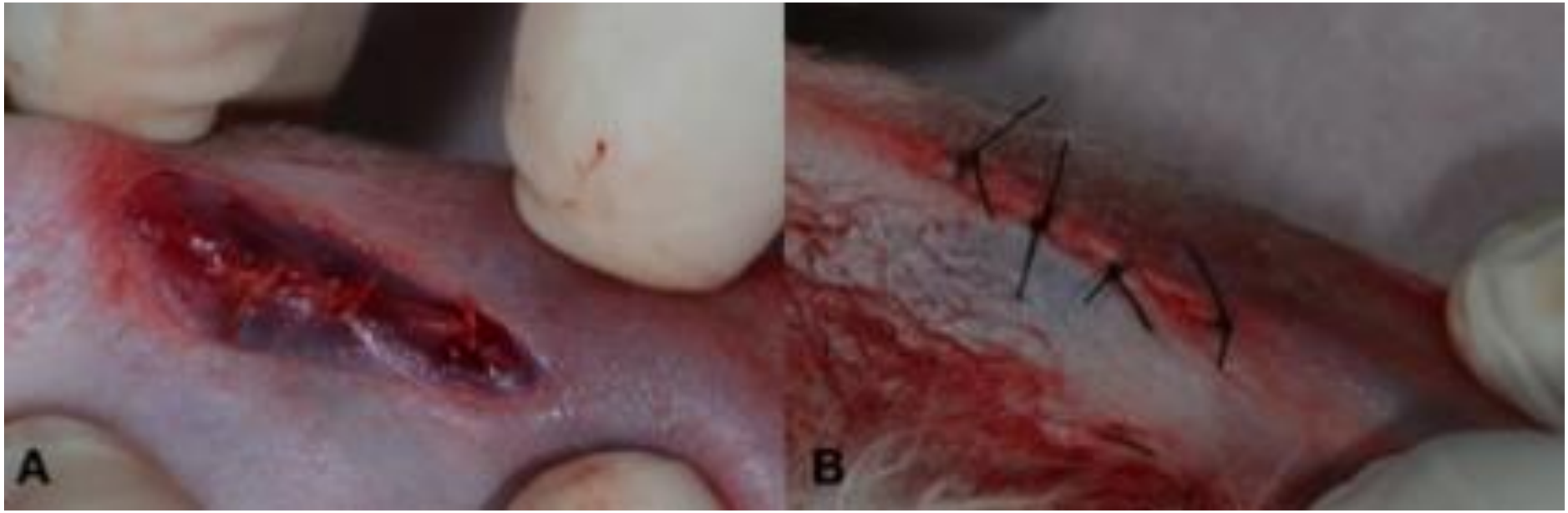
Fonte: Arquivo pessoal do autor.

Figura 3. A) Broca trefina de 6.1mm para realização do defeito. B) Defeito criado. C) Implante instalado com parafuso de cobertura. D) Biomaterial aposicionado nos grupos onde se fez necessário.



Fonte: Arquivo pessoal do autor.

Figura 4. Sutura por planos. A) Vycril 4-0. B) Nylon 4-0.



Fonte: Arquivo pessoal do autor.

ANEXO A – APROVAÇÃO DA COMISSÃO DE ÉTICA PARA USO DE ANIMAIS (CEUA)

unesp **UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"**

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Avaliação da osseointegração e viabilidade celular ao redor de implantes de titânio de diferentes superfícies instalados em áreas de defeito ósseo preenchidos com biomaterial"**, Processo FOA nº 00465-2016, sob responsabilidade de Ana Paula Farnezi Bassi apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 02 de Setembro de 2016.

VALIDADE DESTE CERTIFICADO: 28 de Outubro de 2019.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 28 de Novembro de 2019.

CERTIFICATE

We certify that the study entitled **"Evaluation of osseointegration and cell viability around two different surfaces of titanium implants placed in bone defects filled with biomaterial"**, Protocol FOA nº 00465-2016, under the supervision of Ana Paula Farnezi Bassi presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on September 02, 2016.

VALIDITY OF THIS CERTIFICATE: October 28, 2019.
DATE OF SUBMISSION OF THE FINAL REPORT: November 28, 2019.

Profa. Ass. Dra. Maria Gisela Laranjeira
Coordenadora da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
Rua José Bonifácio, 1193 – Vila Mendonça - CEP: 16015-050 – ARAÇATUBA – SP
Fone (18) 3636-3234 Email CEUA: ceua@foa.unesp.br

ANEXO B – SHERPA ROMEO: Políticas de copyright e de auto-arquivo de editores

SHERPA/RoMEO . . . opening access to research

Pesquisa - Políticas de copyright e de auto-arquivo de editores

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Araraquara, 14 de Novembro de 2019

Guilherme dos Santos Trento