UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CÂMPUS DE JABOTICABAL

COMPÓSITO PROTOTIPADO 3D NA RECONSTRUÇÃO DE FALHA CRÍTICA EM RÁDIO DE COELHOS (*Oryctolagus cuniculus*)

Arícia Gomes Sprada

Médica Veterinária, Mestre

2018

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Orientador: Prof. Ass. Bruno Watanabe Minto

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus Jaboticabal, como parte das exigências para a obtenção do título de Doutora em Cirurgia Veterinária.

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TÍTULO DA TESE: COMPÓSITO PROTOTIPADO 3D NA RECONSTRUÇÃO DE FALHA CRÍTICA EM RÁDIO DE COELHOS (*Oryctolagus cuniculus*)

AUTORA: ARÍCIA GOMES SPRADA
ORIENTADOR: BRUNO WATANABE MINTO

Aprovada como parte das exigências para obtenção do Título de Doutora em CIRURGIA VETERINÁRIA, pela Comissão Examinadora:

Prof. Dr. BRUNO WATANABE MINTO
Departamento de Clínica e Cirurgia Veterinária / FCAV / UNESP - Jaboticabal

Prof. Dr. SILVIO HENRIQUE DE FREITAS
Departamento de Clínica e Cirurgia de Pequenos Animais-FZEA/USP / Pirassununga/SP

Profa. Dra. LIZANDRA AMOROSO
Departamento de Morfologia e Fisiologia Animal / FCAV / UNESP - Jaboticabal

P/ Pesquisador Dr. ALEXANDRE SCHMAEDECKE (Videoconferência)
Medico Veterinário / Centro Integrado de Especialidades Veterinárias / Curitiba/PR

Pós-doutorando LUIS GUILHERME DE FARIA
Departamento de Clínica e Cirurgia Veterinária / FCAV / Unesp - Jaboticabal

Jaboticabal, 14 de janeiro de 2019

DADOS CURRICULARES DO AUTOR

Arícia Gomes Sprada – Nascida em 08 de abril de 1988, no município de João Pessoa, estado da Paraíba. Ingressou no curso de Medicina Veterinária na Universidade Federal de Santa Maria (UFSM), Rio Grande do Sul em agosto de 2005. Durante o curso de Medicina Veterinária, atuou como estagiária no laboratório de cirurgia experimental do hospital veterinário da instituição de 2006 a 2009. Em setembro de 2010 obteve o título de Médica Veterinária. De fevereiro de 2011 a fevereiro de 2012 fez aprimoramento profissional em Clínica Cirúrgica de Pequenos Animais pela Universidade Federal do Paraná, Câmpus Curitiba. Em março de 2012 ingressou no Programa de Pós-graduação em Medicina Veterinária pela Universidade Federal de Santa Maria, recebendo o título de mestra em outubro de 2014 com a dissertação intitulada “Toxicidade e estresse oxidativo das células mesenquimais estromais de tecido adiposo de cão em diferentes passagens de cultura”. Após obter o título de mestre, em 2015, iniciou as atividades no curso de Doutorado em Cirurgia Veterinária na Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista “Júlio de Mesquita Filho” (UNESP), Câmpus de Jaboticabal, sob orientação do Professor Dr. Bruno Watanabe Minto. Concomitantemente, finalizou curso de pós-graduação em Neurologia Veterinária pelo instituto Bioéticus, Botucatu, São Paulo. Ao total, publicou 20 artigos em diversas revistas, foi autora de dois capítulos de livros e escreveu 30 resumos em anais de congressos.

Na vida, nada é em vão: ou é benção, ou é lição!

(Autor desconhecido)

Dedico este trabalho aos meus pais Eliane Maria Gomes Sprada e Jackson Roberto Pereira Sprada, que não mediram esforços para que eu chegasse até aqui.

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E, finalmente, gostaria de agradecer ao meu orientador, Bruno Watanabe Minto, pela oportunidade e confiança. Obrigada pelos conselhos, pelas palavras de tranquilidade e pelas cobranças. Obrigada pelo incentivo e pela compreensão ao longo desses 4 anos. Tu és um modelo a ser seguido. O presente trabalho foi realizado com apoio do Cnpq e Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001 e Fapesp (Número do processo: 2015/10139-1).

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CERTIFICADO

Certificamos que o Protocolo nº 9417/15 do trabalho de pesquisa intitulado **"Impressão tridimensional de substituto ósseo na reconstrução na falha segmental em rádio de coelhos"**, sob a responsabilidade do Prof. Dr. Bruno Watanabe Minto está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 07 de agosto de 2015.

Jaboticabal, 07 de agosto de 2015.

Prof.ª Dr.ª Paola Castro Moraes
Coordenadora – CEUA

COMPÓSITO PROTOTIPADO 3D NA RECONSTRUÇÃO DE FALHA CRÍTICA EM RÁDIO DE COELHOS (*Oryctolagus cuniculus*)

RESUMO - A proposta deste estudo foi utilizar a tecnologia de impressoras 3D para obtenção de substitutos ósseos para reconstrução de falhas segmentares críticas em rádio de coelhos. Foram utilizados 60 coelhos da raça Nova Zelândia nos quais realizou-se ostectomia segmental de 1,5 cm no rádio direito. Os animais foram divididos em três grupos, onde o grupo controle (GI n = 20) foi submetido à ostectomia e não recebeu nenhum tratamento. O grupo enxerto (GII n = 20) foi submetido à ostectomia e reconstrução da falha óssea por meio de enxerto autólogo proveniente da asa do ílio. O grupo implante (GIII n = 20) foi submetido à reconstrução óssea pela implantação de próteses impressas por impressora 3D constituídas de ácido polilático (PLLA) e hidroxiapatita (HA). Os implantes foram preparados com base na imagem de tomografia computadorizada obtidas do membro do próprio animal. Os animais foram avaliados clinicamente nos momentos pós-operatório 7, 15, 30, 60 e 90 dias. Radiografias em duas projeções foram efetuadas nos dias 15, 30, 60 e 90 após a cirurgia, de acordo com os subgrupos T1, T2, T3 e T4, respectivamente. Ao final das avaliações radiográficas, os animais foram submetidos à eutanásia e o segmento ósseo de interesse encaminhado para análise histopatológica. Clinicamente, o grupo enxerto (grupo II) apresentou menor claudicação, edema, dor e complicações nas feridas cirúrgicas quando comparado aos grupos controle e grupo implante. Nas avaliações radiográficas a reação periosteal, formação de calo ósseo e ponte óssea também foram superiores no grupo II quando comparado aos outros grupos. No estudo histopatológico fibrose, osteogênese e condrogênese foi similar em todos os grupos. No entanto, a presença de congestão, hemorragia e inflamação foram maiores no grupo implante. Esses resultados sugerem que os implantes impressos apresentam boas propriedades biomecânicas que em estudos futuros podem ser associados às propriedades biológicas do enxerto ósseo criando-se um novo implante que apresente características osteocondutivas e osteogênicas, como por exemplo, a associação de PLLA, HA e células vivas.

Palavras-chave: Ácido Polilático, Defeito ósseo segmental, Enxerto ósseo, Hidroxiapatita, Impressão 3D

3D PROTOTYPED COMPOSITE IN THE RECONSTRUCTION OF CRITICAL DEFECTS IN RADIUS OF RABBITS (*Oryctolagus cuniculus*)

ABSTRACT – The purpose of this study was to use the 3D printing technology to obtain a bone substitute for reconstruction of critical segmental bone failures of 1.5cm in rabbits' radio. The animals were divided into 3 groups: Group I (n=20) or control group, Group II (n=20) or graft group and Group III (n=20) or implant group. On group I, the rabbits were submitted to ostectomy receiving no treatment. On group II, the bone defect was treated with autologous graft from the ilium crest. On group III, reconstruction occurred using a three-dimensional printed hydroxyapatite and Poly-L-lactic acid (PLLA) implant. The implants were prepared using 3D imaging based on the computed tomography obtained from each animal. Clinical evaluation were conducted postoperatively on day 7, 15, 30, 60 e 90. Radiographs in two projections were taken on days 15, 30, 60 and 90 postoperative, according to the subgroups T1, T2, T3 and T4, respectively. After radiographic evaluation, the animals were submitted to euthanasia and the bone segment was referred for histopathological analysis. Clinically, group II presented less lameness, edema, pain and complications on the surgical wound when compared to the control and implant groups. On radiographic evaluation, periosteal reaction, bone callus formation and bone bridge were also superior in group II when compared to the others groups. Histopathological study showed that fibrosis, osteogenesis and chondrogenesis were similar in all groups. However, the presence of congestion, hemorrhage and inflammation were higher in the implant group. These results indicate that biomechanical capacity of the implant and biological properties of the graft bone should be associated by creating a new implant that presents osteoconductive and osteogenic characteristics, such as a bone substitute printed using PLLA and hydroxyapatite enriched with living cells.

Keywords: Polylactic acid, Segmental bone defect, Bone graft, Hydroxyapatite, 3D printing.

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CAPÍTULO 1 – Considerações Gerais

1. INTRODUÇÃO

As afecções ortopédicas representam grande parcela da rotina hospitalar na medicina veterinária. Cirurgiões ortopedistas são frequentemente desafiados com fraturas cominutivas de ossos longos, neoplasias ósseas, não uniões, união retardada ou ainda má uniões. Umas das principais opções para o tratamento dessas afecções é a substituição de um segmento ou o preenchimento da falha óssea utilizando-se enxerto ou implante (Morello et al., 2001). Os enxertos autógenos têm sido descritos como a melhor opção de material biológico, pois excluem os riscos de rejeição imunológica e dispensam a necessidade de um banco de ossos. Porém, seu uso está associado ao aumento da morbidade, dor, tempos cirúrgico e anestésico e, principalmente, volume insuficiente para reconstrução de grande falha óssea (Millis e Martinez, 2003).

Neste contexto, outras fontes de material ósseo têm sido estudadas. A engenharia de tecidos, ramo das ciências biomédicas que mais tem recebido atenção na atualidade, apresenta, entre outros objetivos, a possibilidade de restaurar a função de um órgão ou reparar tecidos danificados por meio do sinergismo de biomateriais e células (Amini, et al., 2012). Desse modo, diversos métodos têm sido propostos com este objetivo, dentre eles, a utilização de impressoras tridimensionais.

A impressão em três dimensões (3D), também chamada de produção aditiva, está disponível desde 1980, porém apenas recentemente tem seu uso mais amplificado devido à diminuição de seu custo e ao crescimento da ciência de engenharia de computação (Klein et al., 2013). Uma ampla possibilidade de materiais pode ser utilizada nessas impressões como o alumínio, superligas de cromo à base de níquel e cobalto, aço inoxidável, titânio, polímeros e cerâmica (Berman, 2012). A facilidade de criar peças simples ou complexas em uma variedade de materiais permitiu a utilização dessas impressoras no âmbito da medicina. As aplicações vão desde a fabricação de modelos para planejamento cirúrgico, produção de próteses e implantes até a engenharia de tecidos biológicos (Klein et al., 2013).

As impressoras 3D têm sido empregadas em diversos campos da medicina como planejamento de excisão tumoral, transplante de órgãos e cirurgias reconstrutivas. Porém, estudos adicionais devem ser conduzidos a fim de assegurar a eficácia e segurança desta tecnologia visando sua aplicabilidade clínica. Tendo este cenário em vista, o objetivo deste trabalho foi desenvolver um substituto ósseo utilizando impressora 3D e implantar em falhas segmentares críticas em coelhos avaliando clínica, radiográfica e histologicamente em um estudo comparativo com o autoenxerto utilizando a asa do ílio.

Na busca por métodos modernos para solucionar afecções ortopédicas complexas, esta pesquisa desenvolveu um compósito impresso com auxílio de uma impressora tridimensional para aplicação em lesões ósseas críticas. Esta nova alternativa de substituição óssea poderá futuramente ser aplicada no tratamento de perdas ósseas, seja ela por trauma ou por remoção cirúrgica decorrentes de neoplasias ósseas.

Os objetivos gerais deste projeto foram desenvolver e avaliar um substituto ósseo obtido por meio de impressão tridimensional constituído por ácido polilático e hidroxiapatita e aplicado em falhas ósseas segmentares de 1,5 cm no rádio de coelhos.

2. REVISÃO BIBLIOGRÁFICA

2.1 Perdas ósseas

Perdas ósseas graves representam um desafio para cirurgiões ortopedistas. Traumas, infecções, neoplasias e deformidades congênitas são as maiores causas de defeitos ósseos críticos na rotina. O tratamento dessas afecções geralmente envolve múltiplos procedimentos de alta complexidade, estendendo-se por meses, resultando em oneração financeira, longo tempo de recuperação, dor e impacto psicológico aos pacientes (Lasanianos et al., 2009). Além disso, a reconstrução óssea requer processos como resposta celular e humoral do paciente bem como a estruturação mecânica. Logo, fatores como idade, estado imunológico, doenças concomitantes, estado nutricional e uso apropriado de técnicas cirúrgicas influenciam no resultado do

tratamento. Complicações como não união, união retardada e má união são recorrentes na correção de defeitos críticos, e por esse motivo, tem sido o foco de pesquisas há, pelo menos, 45 anos (Jackson et al., 2004; Lasanianos et al., 2009).

Com o objetivo de observar o comportamento de restauração óssea e avaliar técnicas, implantes e biomateriais, vários modelos experimentais de falhas ósseas foram descritos. Inicialmente, o termo “falha óssea crítica” foi descrito como o menor defeito ósseo possíveis seja incapaz de consolidar espontaneamente durante sua vida (Rimondini et al., 2005). No entanto, a definição de menor defeito ósseo não é bem entendida dentro do contexto de falha crítica e alternativamente, define-se o termo como deficiência óssea segmental de comprimento de 2 a 2,5 vezes o diâmetro do osso afetado (Gugala et al., 2007; Reichert et al., 2009).

Contudo, o defeito ósseo crítico não deve ser baseado apenas no comprimento da lesão, mas também deve considerar a espécie, localização anatômica, grau de acometimento de tecidos moles, fatores mecânicos e condições físicas e metabólicas (Lindsey et al., 2006). Devido a essas variantes, a padronização de uma classificação abrangente torna-se inadequada e o estabelecimento de uma técnica cirúrgica como tratamento de escolha não é possível. Por esse motivo, nas últimas décadas novas perspectivas para o manejo de grandes defeitos ósseos tem sido levantada, tais como uso de implantes, biomateriais e o emprego de engenharia de tecidos (Lasanianos et al., 2009).

2.2 Tratamento de perdas ósseas

O objetivo do tratamento de falhas ósseas segmentares críticas consiste em obter integração do implante ou enxerto com o osso remanescente com resistência similar ao osso normal e capacidade de suporte do peso durante a locomoção (Perry, 1999). Nesses casos, as abordagens terapêuticas baseiam-se em transplante de enxertos ósseos, implantes de biomateriais e transporte ósseo por meio do método Ilizarov (Cancedda et al., 2007). Os enxertos ósseos são os mais utilizados na medicina veterinária e representam, até o momento, uma das melhores alternativas, pois apresentam características de osteocondutividade, osteoindução e osteogênese. No entanto, seu uso não está livre de desvantagens como não uniões, uniões

retardadas e infecções (Muramatsu et al., 2003; Soucacos et al., 2006). Assim, os biomateriais surgiram como opção aos enxertos sendo considerados substitutos ósseos mais acessíveis (Marcacci, 1999).

Na técnica de transporte ósseo é realizada uma corticotomia proximal ou distal de um fragmento ósseo viável que será conduzido lentamente por meio de um ou mais fios transversos ao membro e unidos a um anel de transporte móvel que funcionam como cabos de tração (Green et al., 1992). Apesar da técnica ser considerada segura, o transporte ósseo tem limitações como tempo prolongado de tratamento, complexidade do procedimento, o qual exige profissional e equipe altamente especializada, e complicações como contratura articular, luxações e subluxações e deformidades (Green et al., 1994).

2.2.1 Enxertos

Enxertos ósseos podem ser definidos como qualquer material contendo células viáveis que sozinho ou em combinação com outros materiais promovem a cicatrização óssea por intermédio de osteoindução, osteocondução e/ou osteogênese (Muscher et al., 1992). Materiais osteoindutivos são capazes de recrutar células mesenquimais dos tecidos adjacentes e induzir a diferenciação em cartilagem ou tecido ósseo. Materiais osteocondutores servem de *scaffold*, ou seja, oferecem suporte para deposição de tecido ósseo orientando a sua conformação. A osteogênese ocorre quando o material é fonte de células progenitoras ósseas (Bauer e Muschler, 2000).

Os enxertos são classificados de acordo com a sua morfologia em osso esponjoso, cortical ou corticoesponjoso. O osso esponjoso, ou trabecular, é composto por trabéculas finas com amplos espaços preenchidos por células mesenquimais e hematopoiéticas (Weigel, 1993). O osso cortical é obtido de córtex de ossos longos, são bem compactos e mineralizados (Goldberg e Stevenson, 1987).

Os enxertos também podem ser classificados quanto a sua imunogenicidade em autógenos, homógenos e xenógenos. Os enxertos autógenos, ou autoenxertos, são obtidos do próprio indivíduo e não apresentam incompatibilidade (Bloomberg et al., 1984). Existem também os enxertos autógenos vascularizados que são segmentos ósseos transplantados juntamente com seu pedículo vascular, que pela técnica de

anastomose microvascular são implantados no defeito (Perry, 1999). Os enxertos homógenos, aloenxertos ou aloimplantes, advêm de outro indivíduo da mesma espécie. Por possuírem genes diferentes do receptor os aloenxertos estão sujeitos à rejeição imunológica (Weigel, 1993). Os enxertos xenógenos, ou xenoenxertos, consistem em enxertos de doador de espécie diferente do receptor. Nesses casos, reações imunológicas importantes são esperadas tendo em vista que não existe compatibilidade genética (Alexandre, 1983).

A incorporação do enxerto, isto é, a interação entre o enxerto e o receptor que resulta na formação óssea, depende de uma série de fatores como o processo inflamatório e reação imune do paciente em relação ao enxerto, proliferação, migração e diferenciação celular, revascularização, tipo de enxerto, estado do tecido adjacente e condição geral do paciente (Bauer e Muschler, 2000). Durante o processo de incorporação há a formação de hematomas com liberação de citocinas e fatores de crescimento. Também há inflamação dos tecidos que enviam sinais para migração e proliferação de células tronco com subsequente desenvolvimento de tecido fibrovascular ao redor do enxerto, neovascularização e, por fim, formação óssea na superfície do enxerto (Bauer e Muschler, 2000). Para o sucesso da enxertia deve haver abundância de células endoteliais progenitoras e progenitores de tecido conjuntivo no leito do enxerto, pois na ausência destes, o organismo não é capaz de responder apropriadamente aos estímulos osteoindutivos, osteocondutivos ou osteogênicos. Fatores como pouca vascularização, longos defeitos ósseos, infecção e imunossupressão podem prejudicar a incorporação do enxerto (Bauer e Muschler, 2000).

- Autoenxertos de osso esponjoso

Os enxertos autógenos de osso esponjoso incorporam-se rapidamente ao leito, pois possuem alta capacidade osteogênica e osteoindutiva. Isto porque o osso esponjoso é rico em células mesenquimais e proteína de matriz extracelular que respondem rapidamente aos estímulos locais acelerando a angiogênese e formação óssea (Khan et al., 2005). Além disso, não apresenta incompatibilidade gênica, pois o osso é coletado do próprio paciente. No entanto, sua capacidade de osteocondução

é nula tendo em vista que não possui estrutura mecânica para sustentação óssea. Também, o volume de enxerto obtido do local doador é escasso, o que limita a utilização destes enxertos em falhas ósseas longas (Fleming et al., 2000). Os enxertos de osso esponjoso podem ser obtidos da metáfise de todos os ossos longos, porém os locais mais utilizados para coleta são a crista ilíaca, úmero proximal, tíbia proximal, fêmur distal e esterno (Slocum e Slocum, 1998).

2.2.2 Biomateriais

Biomateriais podem ser definidos como materiais capazes de interagir com os sistemas biológicos com o propósito de tratar, aumentar ou substituir tecidos, órgãos ou funções (lakshmi, 2007). Mais especificamente, os biomateriais são associados a dispositivos médicos temporários ou permanentes. Esses materiais, com o auxílio da engenharia de tecidos, combinam propriedades mecânicas, químicas, físicas e biológicas tornando viável sua implantação em organismos vivos (Oréfice, 2005).

-Polímeros sintéticos

Os polímeros sintéticos são representados pelos polietilenos, poliuretanos, politetrafluoretileno, poliacetato, polimetilmetacrilato, polietilenotereftalato, borracha de silicone, polisulfona e ácido polilático (Stevens, 2008), entre outros. Os polímeros são leves, isolantes elétricos e térmicos, flexíveis e resistentes à corrosão. Como são facilmente fabricados e estão disponíveis em diversas formas são muito usados como biomateriais. Os polímeros estão sujeitos a reações físicas e químicas no ambiente biológico e, portanto, estão propensos a sofrer degradações. Essas degradações, geralmente, são controladas e são capazes de serem substituídas por tecido novo (Rodrigues, 2013).

O ácido polilático (PLLA) é um polímero sintético biodegradável utilizado na medicina há mais de quatro décadas. Possui boa propriedade física e, além da sua dureza, alguns estudos sugerem que podem auxiliar na proliferação e diferenciação de osteoblastos (Yang et al., 2009). Ademais, quando comparado a outros polímeros,

apresenta menor resposta imunogênica, sendo considerado boa alternativa de biomaterial (Zhou et al., 2017).

-Biocerâmica

Biocerâmicos são sintetizados para fins médicos, como implantes cirúrgicos, próteses e órgãos artificiais, como válvulas cardíacas. Por possuírem constituintes mais semelhantes à matriz mineral do osso, é o material com melhor osteointegração, isto é, com melhor interação de interface tecido ósseo-implante (Rodrigues, 2013). Além disso, os materiais cerâmicos são porosos, o que permite a adesão, proliferação e diferenciação de células (Oréfice, 2005). Os materiais mais comuns são as cerâmicas apatitas como a Hidroxiapatita (HA), biovidros, vidro-cerâmicas. Apesar da sua alta bioatividade, as cerâmicas apresentam desvantagens no que se refere às suas características mecânicas. Sua estrutura possui baixa tenacidade e alta elasticidade quando comparadas ao osso cortical. Isso implica em restrição do uso desses materiais em ortopedia, principalmente pelo risco de fraturas e falhas do implante (Rodrigues, 2013).

Contudo, tecnologias se propõem a adicionar o fator mecânico às características bioativas das biocerâmicas, como a produção de compósitos que aliam componentes cerâmicos a outros componentes (Silva e Oréfice, 2001).

-Compósito

Os compósitos, como o próprio nome sugere, são a combinação de um ou mais materiais. Ou seja, em nível atômico, os compósitos são constituídos por diferentes grupos. Cada grupo permanece com sua característica, não se dissolve e não se descaracteriza, atuando em sinergismo. Como resultado, os compósitos apresentam superioridade quando comparados aos seus constituintes separados (Silvestre Filho, 2001). A aplicação de compósitos tem sido bem sucedida em dispositivos biodegradáveis de fixação de fraturas, cimentos ósseos mais resistentes e componentes femorais de baixa dureza para artroplastia de quadril. O compósito polímero-fibra é o que mais tem recebido atenção da comunidade científica para fins

biomédicos (Mudali et al., 2003). As biocerâmicas combinadas com polímeros têm sido investigadas pelo seu potencial promissor como biomaterial (Niemelä et al., 2005).

Quando associada ao PLLA, a hidroxiapatita neutraliza os produtos ácidos liberados pelo polímero, criando um ambiente mais favorável para as regeneraçãp celular (Agrawal e Athanasiou, 1997). Além disso, a hidroxiapatita auxilia na cinética da degradação e absorção do polímero e, consequentemente, sua integração tecidual (Hutmacher et al., 1998).

2.2.3 Prototipagem

Na impressão 3D os produtos são construídos camada a camada por meio de uma série de cortes transversais. Todas as impressoras 3D utilizam sistema computacional chamado “Desenho Assistido por Computador” (do inglês CAD, *computer aided design*) que mede diversos cortes transversais de cada produto para a determinação exata das camadas a serem construídas. A máquina 3D distribui uma fina camada de resina líquida e utiliza luz ultravioleta controlada por computador para endurecer cada camada do padrão previamente especificado pelo software CAD (Berman, 2012). Ao final do processo, o excesso de resina é removido com banho químico. Além do software CAD outros arquivos digitais, como a ressonância magnética, também podem ser integrados a essas impressoras. Ampla possibilidade de materiais pode ser utilizada nas impressões como o alumínio, superligas de cromo à base de níquel e cobalto, aço inoxidável, titânio, polímeros e cerâmica (Berman, 2012).

A facilidade de criar peças simples ou complexas em uma variedade de materiais permitiu a utilização dessas impressoras no âmbito da medicina. As aplicações vão desde a fabricação de modelos para planejamento cirúrgico e produção de próteses e implantes até a engenharia de tecidos biológicos (Klein, 2013).

Frequentemente cirurgiões se deparam com grandes desafios no planejamento e execução de cirurgias, principalmente quando estruturas anatômicas complexas estão envolvidas ou quando a relação anatômica entre musculaturas, vasos, nervos e

ossos está alterada. As imagens bidimensionais como radiografias, podem ser úteis em muitos casos, porém dificilmente a arquitetura anatômica de lesões complicadas será totalmente compreendida quando baseada apenas nesse tipo de recurso (Klein, 2013).

Por esse motivo o advento das impressoras 3D surge como alternativa acessível no desenvolvimento de modelos adequados para treinamento pré-cirúrgico ou ensino, diminuindo os riscos e melhorando a didática em centros de ensino (Waran et al., 2014). Esta tecnologia já está sendo empregada em diversos campos da medicina como no planejamento de excisão de tumorais, transplante de órgãos e cirurgias reconstrutivas (Klein, 2013; Waran et al., 2014). Mais recentemente, pesquisas em biotecnologia exploram o potencial das prototipagens na engenharia de tecidos com o objetivo de criar tecidos e órgãos funcionais (Marro et al., 2016). No âmbito da cirurgia ortopédica, a impressão de *scaffolds* é bastante promissora, pois proporciona suporte para proliferação celular sem a necessidade de coleta de enxertos sejam autólogos, homógenos ou xenógenos (Hutmacher, 2000).

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CAPÍTULO 2 - Three-dimensional printing in veterinary medicine: a reality in our routine?

Artigo a ser enviado para revista Investigação

Abstract

Three-dimensional (3D) printing, rapid prototyping or additive manufacturing uses a precise computer process to create virtual models into 3D objects. The aim of this article is to approach the use of 3D printers in the biomedicine field, presenting techniques and application of this technology, especially, in veterinary medicine. Rapid prototyping is gaining popularity in recent years, mainly due to the decrease in its cost and greater availability. But how and where are the 3D printers being used in veterinary medicine and what can we expect from the future? This article explores the possibilities of 3D technology in clinical, experimental and teaching purposes.

Key-words

3D-printer, Rapid prototyping, Additive manufactoring

Introduction

The 3D printing methodology consists on the construction of a 3D physical model from a virtual model. This printers use a computer system called Computer Aided Design (CAD) that measures several cross sections of the product and from there, builds the object layer by layer (PELTOLA et al. 2008; RENGIER et al. 2010). Despite becoming popular just recently, this technology has been available since 1986 when it was first described by Charles Hull. His patent, named "stereolithography" (SLA), uses photopolymers that can be cured by ultraviolet ray radiation (UV). The UV radiation is projected successively on the resin layers that solidify, creating the object in an additive system from the base to the apex (HESPEL et al. 2014).

Other additive manufacturing technologies were created in the 1980s, such as the fused deposition modeling (FDM) which extrudes small beads of fused thermoplastic materials or metals that bond to the layer below. The selective laser sintering (SLS), based on the fusion of small particles by a high power laser. The laminated object manufacturing (LOM), that uses plastic films in sheets glued together and shaped by laser cutter, and the Inkjet printing, that through a piston distributes a thin layer of powder which are bonded by a liquid deposited by another piston (HESPEL et al. 2014). With the development of the technology and costs reduction, the three-dimensional printers have gained ground in a wide variety of areas, including the biomedical field (RENGIER et al. 2010; HESPEL et al. 2014).

The possibility of using data from medical imaging in DICOM format to process 3D objects in several types of materials including polymers, metals and living cells, allowed its application in surgical planning, medical education and implant or tissue designing (RENGIER et al. 2010; MARRO et al. 2016). The use of this technology in veterinary medicine will be approached in this article.

Discussion

Printing 3D objects

Image acquisition is the first of four steps to create 3D printed models. The images may be obtained from computed tomography (CT), cone beam computed tomography (CBCT), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT) and ultrasonography (US) (RENGIER et al. 2010). The resolution of these datas affects directly the quality of the printed object. Therefore, slice thickness less than 1mm is recommended to obtain better accuracy and minimizing artifacts (MAHESH 2002; WHITE et al. 2008). In the author's opinion, the choice of the scanning method depends on the purpose of the printing and the material to be used. In general, computed tomography presents lower cost and are easier available than MRI. If there is no need for high resolution, as for example, in the printing of models for orthopaedics surgical planning, optimal results may be obtained using a helical acquisition and 2mm slices thickness. Some studies corroborate with the author's experience (LILL et al. 1992; BAKER et al. 1994; HOPPER et al. 1996, BIBB and WINDER, 2010).

After obtaining the image, the data is exported in DICOM format to softwares that allow manipulation and isolation of the segment of interest. For example, from the CT of the thoracic limb, it is possible to extract only a segment of the radius. There are many programs availabe on the market, including some softwares with open source. After manipulating the images, the final data is converted from DICOM into STL format (Surface Tessellation Language). If further handling is required, a third step may be added to the printing process. Once the image is in STL file, it is possible to modify its shape and form with the aid of modeling programs. The final step consists in transfer the STL images to the printer (HESPEL et al. 2014).

The choice of the softwares and the printer depends on the purpose of the prototyping, availability and compatibility of the equipament and level of expertise of the user. The authors of this paper have been using successfully the Invesalius program for converting slt format (Ministry of Science and Technology, Campinas, Brazil). The image manipulation through the softwares Blender and Autodesk Meshmixer. And, finally, the 3D printing is obtained using a FDM printer (Prusa i3) with direct drive extrusion.

Applications

The three main pillars of prototyping application in medicine are individual patient care, research and educational or training (RENGIER et al. 2010). In some hospitals the 3D printer has been used as pre-surgical planning tool (WARAN et al. 2014). Creating a replica of the anatomy allows the rehearsing of complex procedures and antecipation of some steps, such as contouring plates before the orthopaedic surgical procedure (OLSZEWSKI, 2013; LIU et al. 2014). Therefore, the surgeon becomes familiar with the approach, the operation time and anesthetic exposure reduce as well as blood loss and tissue trauma, decreasing costs and postoperative risks (COHEN et al. 2009).

In veterinary medicine, 3D printers have also been used for this purpose. Since 2013, the veterinary medicine school at the university of California has been printing 3D models from dogs and cats' skulls to plane mandibular reconstructions. According to the researchers, the models improve preoperative planning, intraoperative guidance, help veterinary students and residents training and facilitate communication with pet owners. In some cases, the surgery was not recommended based on the printed skull, where it was possible to observe the complexity of the defect and the low chance of success (WINER et al. 2017). In addition to assisting in the planning of maxillofacial surgery, 3D printing have also been employed in orthopaedic, ophthalmic, oncologic and neurosurgeries (CROSSE and WORTH, 2010; HESPEL et al. 2010; DORBANDT et al. 2016).

Besides pre-surgical planning and training, 3D printed models can also be employed in medicine and veterinary schools in association with traditional teaching method in cadavers, since it can provide detailed anatomical understanding of any organ or tissue (SUZUKI et al. 2004). In fact, some studies suggest that undergraduate students with access to three-dimensional anatomical models present better learning experience when compared to those using only digital models or textbooks (PREECE et al. 2013; O'REILLY et al. 2016). Furthermore, these accurate prototyping models reduce costs, the necessity of anatomic specimens and inconvenient conservation methods, such as the formaldehyde solution. (LI et al. 2017). At São Paulo University, printed skeletal models of dogs and horses were introduced in veterinary anatomy classes, contributing to the teaching process in addition to providing a greater number of anatomical bones without the need of cadavers (DOS REIS, 2017).

More recently, 3D printers have increased their popularity in the scientific community, especially, in the tissue engineering field. The same principles used in printing anatomial models are used for printing biocompatible and biological materials with the goal of creating tissues and organs (MURPHY and ATALA, 2014). In this context, emerges the 3D bioprinting that converges cells, biological factors and scaffods (MURPHY and ATALA, 2014). A variety of biomaterials have already been described as scaffolds, including natural or synthetic polymers and bioceramics. Its role in the regeneration is to provide a physical structure to cell infiltration, adhesion and proliferation (SPILLER et al. 2011; LI and KAWASHITA, 2011).

Orthopaedics is one of the first medical fields to employ 3D printed scaffolds to achieve bone regeneration (AURICCHIO and MARCONI, 2016). For bone printing, ceramic scaffolds, such as hydroxyapatite (HA) and tri-calcium phophate (TCP) are typically used due to their mechanical stiffness, low elasticity and hard britte surface. Moreover, the ceramics have similar constituents to the mineral matrix of the bone being highly biocompatible (O'BRIEN, 2011). Some studies also describe the role of HA and TCP in osteoblast diferentiation and proliferation (AMBROSIO et al. 2001; HENCH, 1998).

Synthetic polymers including polystyrene, poly-l-latic acid (PLLA), polyglycolic acid (PGA) and poly-dl-lactic-co-glycolic acid (PLGA) among others, also have been described as scaffolds. They present good physical property and a controlled degradation, which means that they can be replaced by a new tissue during their absorption in a controlled environment. In addition, they are unexpensive and easily fabricated in various forms, becoming popular as biomaterials (O'BRIEN, 2011). Polymers scaffolds have been applied, especially, in cartilaginous tissue repair both in articular cartilage and cartilaginous organs, such as the trachea (OLUBAMIJI et al. 2016). Zopf and colleagues (2013) used successfuly a printed bioresorbable polycaprolactone trachea for transplantation in a 2-month-old child with tracheobrochomalacia. Other studies assessed the application of synthetic polymers in segmental tracheal defects in rabbits, some of them with promising results, demonstrating that the artificial organs printing is a close reality (CHANG et al. 2014; LEE et al. 2015).

Apart from synthetic polymers, natural polymers are also used in the prodution of scaffolds. These biopolymers are derived from human, animals, plants and bacteria and, therefore, have great biological compatibility. Some examples of hydrogels used in tissue engineering are collagen, fibrin, proteoglicans, alginate and chitosan (BALDWIN and KIICK, 2010) applied in bone, cartilage, smooth muscles as space filling scaffolds and as cells delivery material (DRURY and MOONEY, 2003).

The challenge of the 3D bioprinting is to construct a complex micro-architecture similar to the extracellular matrix of the receptor tissue with biological and mechanical properties suitable to organ restauration (MURPHY and ATALA, 2014). As we can see, there is a wide variety of available biomaterials and many studies with this goal. However, each of these materials has advantages and disavantages. Ceramic presents good mechanical properties and excellent biocompatibility, however, its brittleness difficults shaping for implantation and control during degradation. The synthetic polymers, during their degradation, produce carbon dioxide lowering the local pH leading to cell and tissue necrosis. Natural polymers are biologically active, but generally have poor mechanical properties (O'BRIEN, 2011).

In view of this, the 3D composite scaffolds were developed. Composite scaffolds are the combination of two or more materials. Each group remains with its original characteristic acting in synergism. As result, the composite present superiority when compared to their separate constituent (O'BRIEN, 2011). Some studies, for example, combined synthetic polymer with natural polymers. The synthetic polymer enhances the 3D structure of the scaffold, maintaining the mechanical properties over several weeks while the natural polymer provides excellent biocompatibility (LIZARRIBAR et al. 2018). The association of ceramics and polymers has also been investigated, mainly in bone regeneration. In this type of hybrid scaffold, the polymer guarantees the physical support to cell adhesion and the ceramics, besides aiding in tissue integration, neutralizes the acid products released by the polymer during its degradation (AGRAWAL and ATHANASIOU, 1997; YUNOS et al. 2008).

The authors of this paper are involved in the development of a composite scaffold for bone replacement in rabbits. The research combines Poly-L-lactic acid (PLLA) and hydroxyapatite printed with Prusa i3 from tomographic images of the radius of the rabbits. The aim of this study is to create a bone substitute for the treatment of critical bone defects, either by complications of fractures or by bone neoplasm removal. The 3D scaffold creating process was simple and inexpensive, no great resources were needed during the printing. For this reason, we believe that the 3D printing technology will be accessible, not only in educational centers, but also in veterinary hospitals and clinics in a near future. In fact, 3D models for pre-surgical planning is already available in Brazil through some companies that perform the printing from CT images. Regarding the use of this technology for bioprinting, it is important that more studies are conducted in order to guarantee safety to our patients prior to routine use.

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CAPÍTULO 3 - Critical bone defect model in rabbits (*Oryctolagus cuniculus)*

Artigo a ser enviado a revista Arquivo Brasileiro de Medicina Veterinára e Zootecnia

Resumo

Engenharia de tecidos é um campo da medicina regenerativa de grande interesse da comunidade científica nas últimas décadas. Esse novo conceito da ciência alia estrategicamente materiais e biologia celular na substituição e reparação de tecidos. Na ortopedia, o objetivo da engenharia de tecidos é a regeneração óssea, especialmente em longos defeitos. No entanto, o grande desafio da pesquisa deste campo é a aplicação dos achados experimentais na rotina clínica. Assim, a definição de um modelo experimental de falha óssea crítica faz-se necessária. O objetivo deste estudo foi propor um modelo de defeito ósseo crítico em coelhos visando pesquisas com interesse em regeneração óssea. Ao total, 40 animais foram utilizados e divididos em dois grupos, contendo 20 coelhos cada. No grupo controle foi realizado ostectomia de 1,5cm na diáfise do rádio direito. No grupo enxerto, o mesmo defeito foi feito, seguido da implantação de autoenxerto da asa do ílio. Os animais foram avaliados radiograficamente aos 15, 30, 60 e 90 dias pós-operatório. No grupo enxerto foi observado consolidação aos 60 dias de pós-operatório, enquanto que no grupo controle apenas um animal apresentou resolução espontânea aos 90 dias. O defeito crítico proposto neste estudo foi considerado satisfatório. A técnica cirúrgica é de fácil execução e apresentando poucas complicações. O exame radiográfico do 15º dia pós-operatório não apresentou mudanças ósseas significativas. Assim, sugere-se que a primeira avaliação radiográfica deve ocorrer no 30º dia pós-operatório. Um período de avaliação mais longo é sugerido. The critical bone defect proposed in this study was satisfactory. The surgical technique is easy to perform, presenting few complications. Radiographic examinations demonstrated no significant bone changes at 15 days postoperative. Therefore, the first evaluation may be delayed to the 30th day. Longer evaluation time also may be proposed.

Palavras-chave: falha óssea longa, falha segmental, consolidação óssea, regeneração óssea.

Summary

Tissue engineering is a field of regenerative medicine that has aroused great interest from the scientific community in recent decades. This new science concept allies materials and cell biology strategically to replace and repair tissues. In orthopaedics, tissue engineering aims for bone regeneration, especially for long bone defects. However, the tranlation of research findings into clinical application remains a challenge. Thus, the definition of an experimental model of critical bone defect is necessary. The aim of this study was to propose a model of critical bone failure in rabbits for researchs on bone regeneration. In total, 40 animals were divided into two groups containing 20 rabbits each. In the control group, a 1.5cm ostectomy was performed on the diaphysis of the right radius. In the graft group, the same bone defect was created, followed by the implantation of iliac crest autograft. After surgical procedure, radiographic follow-up was performed at 15, 30, 60 and 90 days postoperative. In the graft group, bone consolidation was observed at the 60th postoperative day, whereas in the control group, only one animal has spontaneous resolution at the 90th day. The critical bone defect proposed in this study was satisfactory. The surgical technique is easy to perform, presenting few complications. Radiographic examinations demonstrated no significant bone changes at 15 days postoperative. Therefore, the first evaluation may be delayed to the 30th day. Longer evaluation time also may be proposed.

Keywords: long bone defect, segmental defect, bone consolidation, bone regeneration.

Introduction

In the last 60 years, the biomaterial field and tissue engineering have been made great advances. During the twentieth century, the materials used as implants were derived from industrial use such as chemistry, mechanical and aerospace (Navarro, et al., 2008). Since then, a variety of materials, called bioactive and biodegradable, have been developed for the purpose of aiding or even replacing organic tissues. Due to the great demand for these biomaterials and their application in the biomedical field, the need for regularization and characterization of the material and material/host tissue interactions arises (Chandramohan and Marimuthu, 2010). In this context, in order to investigate implants and biomaterials, especially in the orthopaedics area, an adequate animal model and experimental bone defect is required (Boer et al, 1999).

When the purpose of the study is to evaluate bone regeneration, critical bone defect models are preferable. Long bone segmental defects were first established in 1984 (Bonnarens and Einhorn, 1984) and are used in tissue engineering for its inability to heal spontaneously and

to lead to nonunion due to the size or to the unstable biomechanical properties (Clements Jr et al., 2003; Horner et al., 2010). In addition, other characteristics should be taken into account when choosing the experimental model, such as the location, size of the defect, fixing method, type of implant and animal species (Horner et al., 2010).

Animals used in orthopaedics researchs include rats and mouses, rabbits, dogs, sheep, goats, pigs and primates (Li et al., 2015). Among them, rabbits are the most commonly used due its similarities in bone mineral density and toughness of the middiaphyseal bone to humans (Neyt et al., 1998; Wang et al., 1998; Li et al., 2015). Furthermore, rabbits are easy to handle, present low maintenance cost and has fast skeletal change and bone turnover (Wang et al., 1998; Li et al., 2015), allowing to observe a multiplicity of study objects over a relatively short period of time (Egermann et al., 2005). The most common implantation site in rabbits are tibia and femur, followed by mandible, calvaria and ulna (Li et al., 2015). The purpose of this study is to describe a critical segmental bone defect in rabbits' radius for evaluation of biomaterials destinated to bone regeneration.

Material and Methods

This study was approved by the institution's ethics committee under the protocol number 9417/2015. To elect the location and size of the defect, an anatomical study of the forearm of five adult female New Zealand rabbits was carried out. From these anatomical pieces, it was determined the length of the defect based on the radius diameter in the region to be removed. The diameter was measured in three points: at the site of the proximal cut, the distal cut and between the two cuts (Fig.1). The mean value of the largest measure between the bones was obtained (0.56cm) and multiplied by 2.5 resulting in 1.5 cm (Lindsey et al., 2006; Gugala et al., 2007). The most distal incision of the ostectomy was estipulated at 2 cm from the radiocarpal joint. The radius was chosen in view of that rabbits present a broader ulna than radius. The ostectomy location was based on the anatomical conformation of the radius. In this area, the bone is thinner and less curved, facilitating the cut.

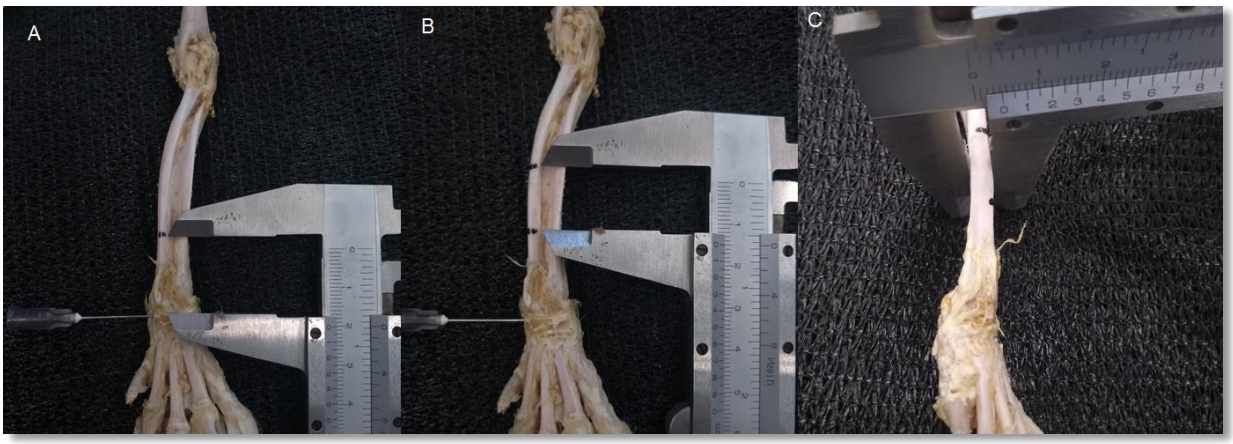

Fig 1. Forearm of a 7 months old, female, New Zealand Rabbit used for radius diameter measuring. A) Distal ostectomy cut determined at 2.0cm from the radiocarpal joint (Needle). B) Proximal ostectomy cut 1.5cm from the first cut. C) Measurment of the radius diameter between the proximal and distal ostectomy cuts.

Forty adults (> 7 months) female New Zealand Rabbits, weighing between 4 to 5.5Kg were divided randomly into two groups of twenty animals each, according to the treatment: Group 1 or control group and Group 2 or graft group. Preanesthetic medication consisted of ketamine hydrochloride (20mg/kg), midazolam maleate (2mg/kg) and morphine sulfate (2mg/kg) intramuscular. Induction and anesthetic maintenance were achieved with isofluorane using mask in open inhalation system vaporized in oxygen 100%. Afterwards, brachial plexus block was perfomed in all animals and sacroiliac regional anethesia in group 2 using lidocaine (6mg/kg) 2% without vasoconstrictor. With the animal in right lateral recumbency, a longitudinal skin incision of approximately 3.0 cm on the dorsomedial face of the limb was performed. Subcutaneous tissue and musculature were retracted to exposure the diaphysis of the radius and the periosteum was removed with blunt dissection. Then, the ostectomy was performed 2.0 cm above the carpus joint, removing a 1.5 cm segmental defect with the aid of an oscillating saw. The cut was carefully performed due to the proximity of the radius to the ulna. For bone fragment removal, the interosseous ligament was incised. In group 1, bone defect was left empty and subcutaneous tissue and skin were sutured in routinely fashion. In graft group, after bone defect creation, a segment of autologous corticocancellous bone was harvested from the left iliac crest. The bone graft was measured and, if necessary, cut with scissors to fit the defect. Postoperative medication consisted of Dipirona 25mg/kg Subcutaneously (SC) twice a day (BID), Tramadol hydrochloride 4mg/kg BID SC, Meloxican 0.1mg/kg once per day (SID) SC, all for three days and Enrofloxacin 5mg/kg BID, SC for five days.

For radiographic evaluation, both groups were subdivided into four subgroups containing 5 animals each. The subgroups were determined according to the time in T1 (15 days), T2 (30 days), T3 (60 days) and T4 (90 days). Radiographs were taken in cranio-caudal and mid-lateral incidence using mA 100 and Kv 70 for all animals.

Results

The technique of partial ostectomy of the radius' diaphysis in rabbits to create a critical bone defect model was easy to perform. However, the proximity of the radio to the ulna requires caution during the ostectomy. In one animal of the control group, during the defect creation, there was an injury in the distal sis-cortex of the ulna. On the 7th postoperative day, the animal presented complete transverse ulna fracture. With exception of this case, no other complication was observed during the trans-operative or postoperative period. Shortly after anesthetic recovery, the animals were already able to support the operated limb, although a mild degree of claudication was observed in some animals.

On the radiographic evaluation, all animals of the graft group presented bone callus formation from the 30 days of evaluation. Consolidation was observed at 60 days of evaluation in all animals of subgroups T3 and T4. In the control group, it was possible to visualize mild bone callus formation in three animals, two belonging to T4 group and one to T3 group. One animal from T4 presented bone consolidation. The other animals of control group presented no bone union or little or no biological activiy at the radiographic examination (Fig. 2).

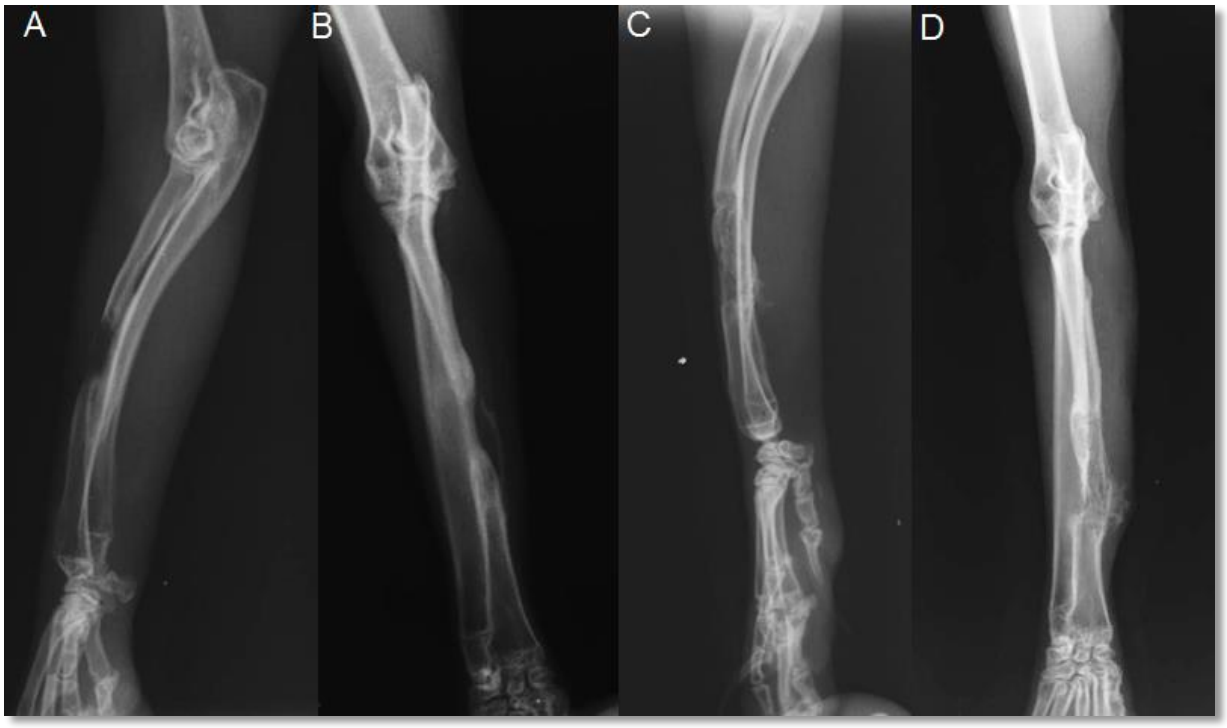

Fig 2. Mediolateral and craniocaudal radiographs of the right thoracic limb of two New Zealand rabbits. A and B) 90-day postoperative radiographic examination of a rabbit submitted to a 1.5cm ostectomy in the radius diaphysis resulting in a non-union. C and D) 90-day postoperative radiographic examination of a rabbit submitted to a 1.5cm bone defect treated with iliac crest autologous bone graft. Note the exuberante bone callus formation resulting in consolidation.

Discussion

To select a specific animal as model for orthopaedics research, a number of factors need to be considered, especially when the study aims translation to medicine (Reichert et al., 2009). Physiological and pathophysiological features should be similar between the studied species and humans (Liebschner, 2004). Bone macro and microstructure properties, for example, may be taken into account. In addition, the capacity of multiplicity of the study in a short period of time, acquisition and maintenance costs and ethical issues are important for defining the most appropriate model (Liebschner, 2004; Egermann et al., 2005). Bone microstructure of the rabbits are similar to human bones and its fast skeletal change and bone remodeling capacity encoraged us to propose this species as model for studies in bone regeneration (Wang et al., 1998; Castaneda et al., 2006). Furthermore, rabbits are docile animals, easy to house and handle and its acquisition and care costs are low. Usually, rabbits are easily available. However, animals from comercial vendors or research institutions normally are less than 3 months old.

Skeletal maturity, and therefore the ideal age of use in research, is achieved after 6 to 7 months which may be an disadvantage (Wang et al., 2013).

Due to these features, rabbits are widely used in research as model for bone defect. The sites of the failure, however, vary according to the purpose of the study and among research groups (Li, et al., 2015). We chose the radius based on the anatomy of the forearm of the rabbit and due the distribution of loads after the defect, which does not require stabilization (Nielsen et al., 1992). The defect size was also anatomically based according to that proposed in the literature. Critical bone failure consists on a large defect incapable of spontaneous healing. To achieve this goal, the length of the failure should exceed 2 to 2.5 times the diameter of the bone (Clements Jr et al., 2003; Lindsey et al., 2006; Gugala et al., 2007). In this study, 1.5cm segmental defect proved to be effective considering that 94.7% of the animals of control group presented non-union.

As previously mentioned, the fast bone turnover capacity of rabbits, allows studies to be performed in a shorter period when compared to other species (Wang et al., 1998). The time of evaluation of 3 months was based on previous studies (Boer et al, 1999; Azi et al., 2012). As a longer evaluation time was not established in our study, it is not possible to affirm that the non-union observed in control group would not progress to a delayed union. On the other hand, based on the results of the graft group, in which it was observed bone consolidation in 100% of the animals in the T3 and T4 subgroups at 60-day of evaluation, it may be suggested that the period of assessment was adequate. Also, considering the results of the subgroups T1 (15-day), in which there was no difference between the groups, it is suggested that radiographic exams of less than 30 days may not be necessary when the objective of the study is the visualization of bone activity.

Conclusion

Based on the results, the critical bone defect proposed in this study was satisfactory. The surgical technique is easy to perform and presents few complications. Radiographic examinations demonstrated no significant bone changes at 15 days postoperative. Therefore, the first evaluation may be delayed to the 30th day. Longer evaluation time may be proposed, however between 60 and 90 days postoperative bone consolidation was achieved in graft group, thus the authors suggest that this period is satisfactory, reducing the time of experimentation.

Acknowledgment

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CAPÍTULO 4 - Use of a three-dimensional printer to create a bone substitute scaffold for fracture repair: pilot study in a rabbit

Artigo a ser enviado para revista Veterinary and Comparative Orthopaedics and Traumatology

Summary

Objetive: The aim of this study was to evaluate the clinical and radiographic properties of a three-dimensional (3D) printed bone substitute made from Poly-L-lactic acid (PLLA) and hydroxyapatite in an experimental model of segmental bone defect in the radius of a rabbit. **Study design:** one adult, female, New Zealand rabbit was used in this pilot study. A 1.5cm ostectomy was made in the diaphysis of the right radius and the defect was repaired in the same surgical procedure with a 3D printed bone substitute. The implant was designed specifically for the animal based on the tomographic image of its own right limb. After implantation, the animal was evaluated both clinically and radiographically at: 15, 30, 60 and 90 days after surgery. **Results:** The bone substitute was successfully created using the 3D-printer. The implant placement was simple to perform and no complications occurred during the follow up period. **Conclusion:** a 3D bone substitute implant made from PLLA and hydroxyapatite may be an alternative treatment for the management of critical-size bone defects. **Clinical significance:** Scaffold creation using a 3D printer is a promising tool in orthopedic surgery since it provides support for cell proliferation without the need for grafts or complex surgical procedures, such as bone transport techniques.

Keywords: 3D printing; Polylactic acid; Hydroxyapatite; Bone graft, Segmental bone defect.

Introduction

Large bone defects are generally associated with high-energy trauma or bone neoplasia and represent a challenge, both in human and in veterinary, orthopedic surgery (1). Typical therapeutic approaches in these cases include bone graft, osteogenesis by distraction and biomaterial implants (2). Bone grafts and osteogenesis typically require multiple surgeries and a long recovery time and these procedures also have high complications rates due to their complexity (2).

Several biocompatible implants have been proposed as an alternative treatment, such as hydroxyapatite and synthetic and natural polymers (3). Hydroxyapatite (HA) is crystalline calcium phosphate, the same mineral found in the inorganic part of natural bone (4). HA has good osteoconductive properties since it provides mechanical rigidity allowing bony ingrowth within the scaffold. In addition, its porosity stimulates cell infiltration, migration, proliferation and differentiation (5).

Poly-L-lactic acid (PLLA) is a biocompatible synthetic polymer that has been widely investigated in recent years due its toughness and low immunogenicity in comparison with other polymers. When combined with HA, PLLA-based scaffolds act not only as osteoconductors during bone regeneration, but also promote osteogenic differentiation (6).

Three-dimensional (3D) printers are increasingly used in biotechnology research to create scaffolds in different materials (7). The ability to design 3D models from medical imaging data, such as computed tomography and magnetic resonance, introduced a new concept in the advancement of medicine (8). In the present study, we developed a 3D printed bone substitute using PLLA and HA to serve as a graft in an experimentally induced segmental bone defect in the radius of a rabbit. The objective was to use a bone substitute as an alternative to bone grafting and to evaluate clinical and radiographic response over 90 days.

Materials and methods

The study was approved by the institution's ethics committee under protocol number 9417/2015. The implant was created using data from computed tomograph (CT) images of the right thoracic limb of a female, adult, New Zealand rabbit. Sequential CT images were acquired (Speed GE helicoidal) at 120 kV and 130 mA. The 3D images were reconstructed in slice thicknesses of 1mm and saved in DICOM format. Using InVesalius 3.0 software, the images were manipulated, delimiting the area of interest for printing. A 1.5cm segment of the radius 2 cm above radiocarpal joint was defined for segmentation. Later, the data was converted into STL (sterolithografy) format creating a virtual replica of the bone segment which was printed using a Prusa I3 printer with direct drive extrusion modified for medical use only (Fig. 1).

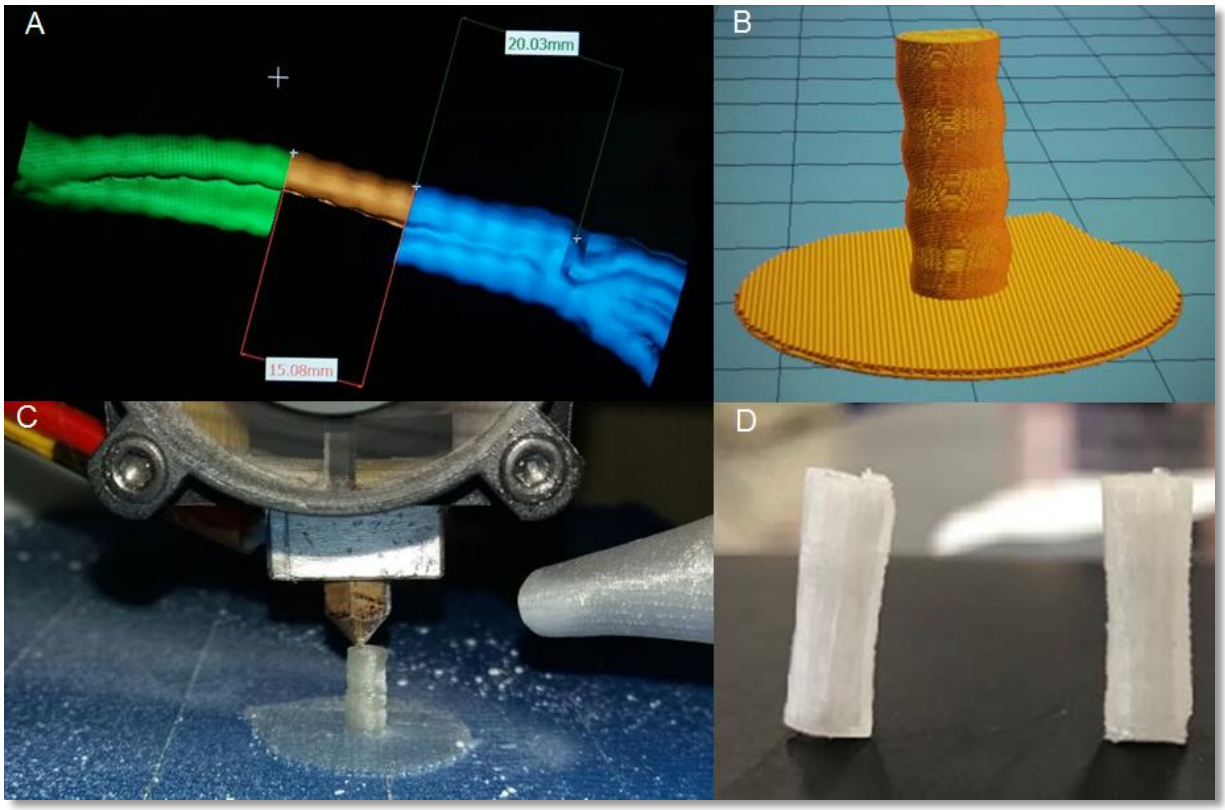

Fig. 1 Three-dimensional implant creation process. A) Three-dimensional modeling of the right radius from computed tomography. B) Virtual model of the segment of interest to be printed. C) Image from the bone substitute printing. D) Final printed model.

The 3D printer itself was printed on PETG, a material resistant to solvents so that it could be sterilised. Additionally, the printer was placed in a closed acrylic chamber with a bactericidal UV lamp. The extruder was adapted to allow injection of hydroxyapatite powder during the impression layer by layer (0.1mm) using PLLA (Fig.2). The extrusion temperature was 210º C and the printing table was heated to 50ºC. After printing, the implant was sterilized in ethylene oxide.

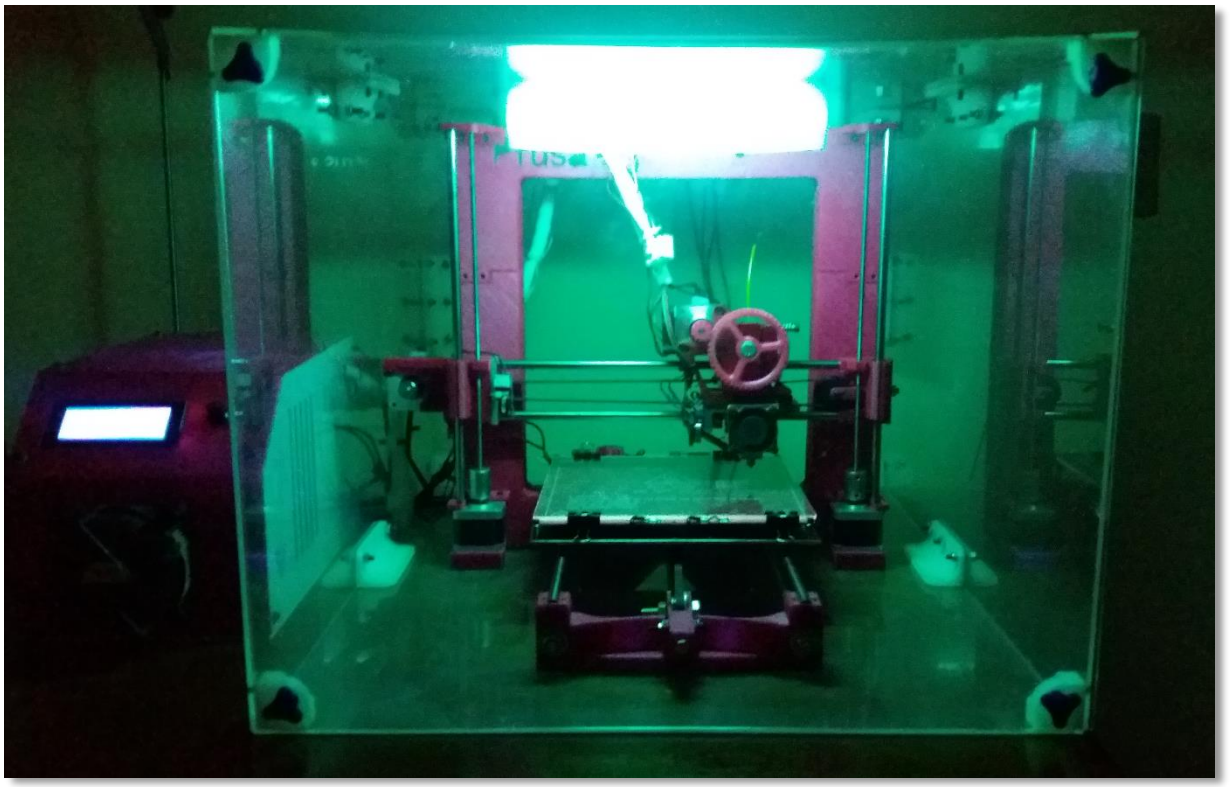

Fig. 2 Photographic image of Pruse I3 printer specially modified for medical implant printing. The machine was placed in a closed acrylic chamber. Note the ultraviolet lamp used to reduce contamination during the process.

The rabbit was prepared for surgery and a dorsomedial skin incision was made over the radius. After bone exposure, a radial ostectomy was performed 2.0 cm above carpus. A 1.5 cm bone segment was removed using an oscillating saw. The 3D printed bone substitute was placed into the defect so that its extremities remained in close contact with the bone edges, exerting

some compression on the surface between bone and scaffold, thus no implant fixation was required (Fig. 3). Subcutaneous tissue and skin were sutured routinely. Postoperative medication consisted of Dipirona 25mg/kg subcutaneously (SC) twice a day (BID), Tramadol hydrochloride 4mg/kg BID SC, Meloxican 0.1mg/kg once per day (SID) SC, all for three days and Enrofloxacin 5mg/kg BID, SC for five days. Radiographs were taken immediately postoperatively and at 15, 30, 60 and 90 days after surgery. The animals were examined on the 7th, 15th, 30th, 60th and 90th postoperative day to assess limb support, gait, presence of swelling and pain during palpation (Table 1). Natural behavior, activity inside and outside the cage, water and food intake were observed daily.

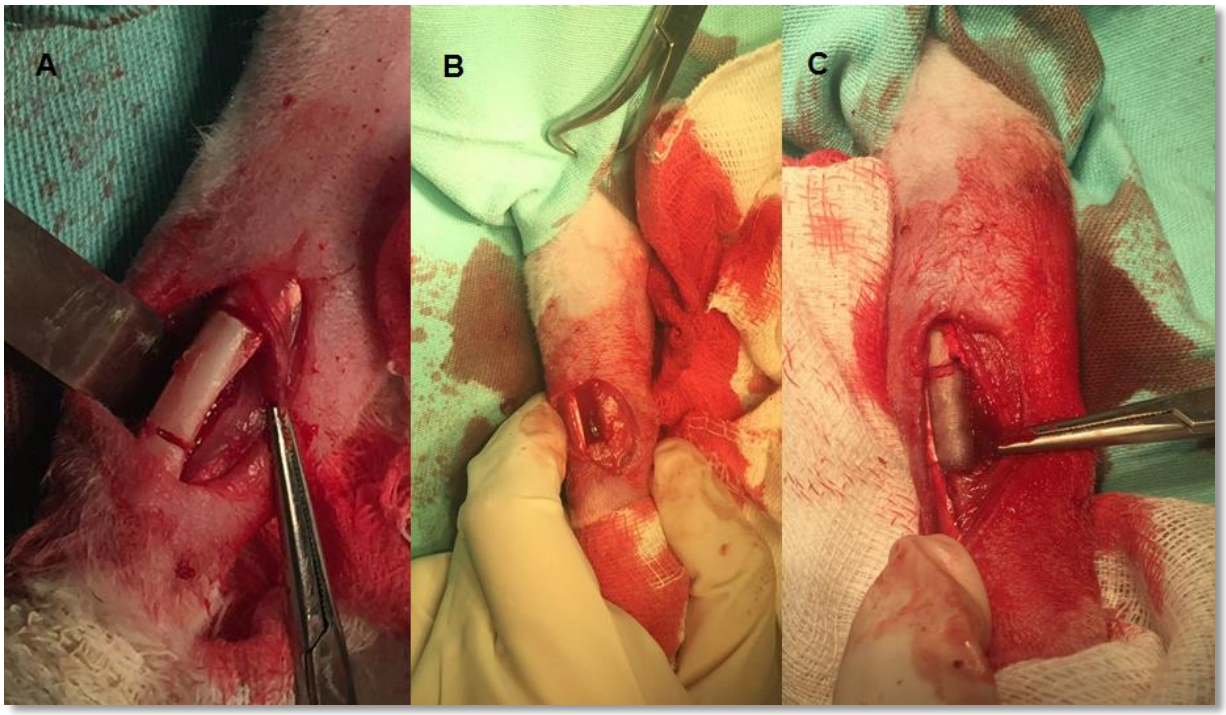

Fig. 3 Surgical procedure for 3d printed bone substitute implantation. A) Exposure of the right radius showing the proximal and distal ostectomy cuts measuring 1.5cm. B) Right radius after complete ostectomy. C) 3D bone scaffold implanted for defect repair.

Criteria		scores
Standing	Continuous weight-bearing	**0**
	Intermittent weight-bearing	**1**
	Completely non-weight-bearing	**2**
	N/A	**3**
Gait with movement	Continuous weight-bearing	**0**
	Intermittent weight-bearing	**1**
	Toe-touches non-weight-bearing	**2**
	Non-weight-bearing	**3**
Swelling	None	**0**
	Mild	**1**
	Obvious	**2**
	N/A	**3**
Pain during palpation	None	**0**
	Mild (ocasional vocalization)	**1**
	Moderate (frequent vocalization)	**2**
	Severe (vociferous vocalization, withdraws limb, bites, struggles)	**3**

Table 1. Pain scale used in the physical evaluation of the rabbit during the postoperative period. N/A Not applicable. Adapted from STASIAK, K.L. MAUL, D.; FRENCH, E. Species-specific assessment of pain in laboratory animals. *Contemporary topics*, v.42, p. 13-20, 2003.

Results

The bone substitute was successfully created from CT images. The implant was produced simply and cheaply.

The experimental surgery model was satisfactory and the bone defect was created without complications. However, the proximity of the ulna to the radius necessitated caution during the ostectomy. Implantation of the printed bone substitute was uneventfully. Shortly after anesthetic recovery, the animal was able to support weight on the right thoracic limb, although, a mild lameness was present. Analgesic drugs were administered for three days. The results of the physical examination are shown in Table 2. Food and water intake and behavior were considered normal during this period. Follow up radiographs were taken at 15, 30, 60 and 90 days postoperative (Fig.4).

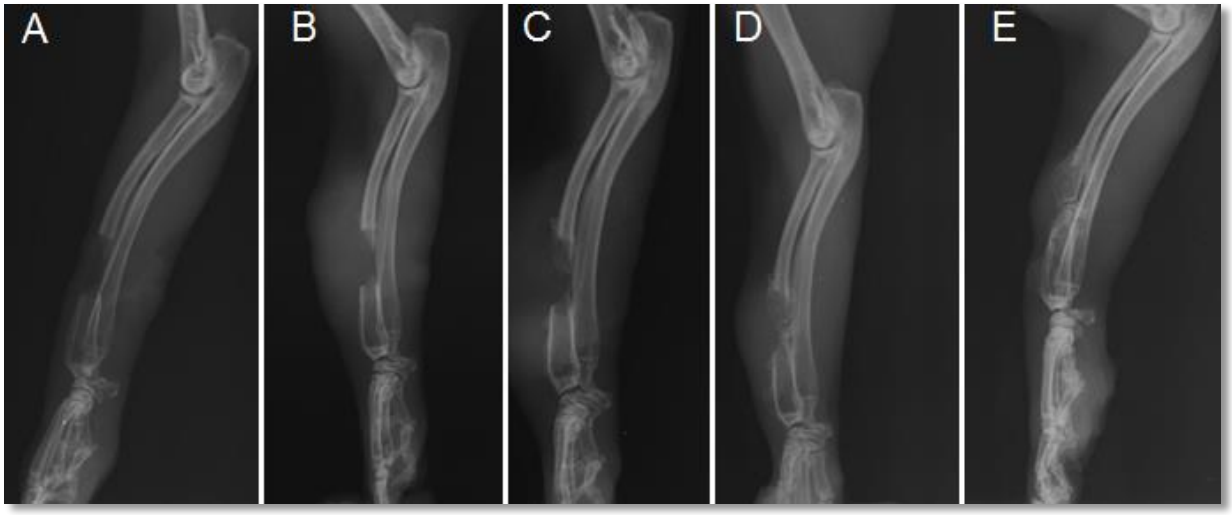

Fig. 4 Follow-up radiographs in lateral views of the right thoracic limb of a rabbit following ostectomy and implatation of scaffold printed on 3D printer. A) Immediate postoperative radiograph. B) 15 days after surgery. C) 30 days after surgery. D) 60 days after surgery. E) 90 days after surgery.

Radiographic images on day 15 showed a mild periosteal reaction and moderate soft tissue enlargement. At 30 days postoperative, soft tissue enlargement remained, swelling was still visible and periosteal reaction and bone formation extending from the osteotomy cuts towards the implant were visible. On the day 60 a large periosteal reaction was present and increased soft tissue was seen in the region of the defect. with bone bridge formation. Three months after surgery, bone consolidation was complete and there was a decrease in the adjacent

soft tissue volume. It was not possible to visualize the bone scaffold in the radiographs due to the different radiopacity of rabbit bone and the implant.

Postoperative day	Standing	Gait	Swelling	Pain
7	1	1	1	1
15	1	1	1	1
30	1	1	1	1
60	1	1	0	0
90	0	0	0	0

Table 2. Data from the physical evaluation of the rabbit following partial ostectomy of the radius and implantation of bone substitute on days 7, 15, 30, 60 and 90 postoperatively.

Discussion

Bone loss may result from trauma, infection or tumor excision and represents a challenge for orthopedic surgeons since treatment often extends for months and multiple surgical procedures may be needed. Following such prolonged treatment results may still be considered unsatisfactory (9). Due to the complexity of cases with critical size bone defects, several experimental models have been developed for assessment of treatments. In addition, many species including: rats, rabbits, dogs and monkeys have been used as models for research on bone regeneration, (10). We chose to use the rabbit in our pilot study due to its docile temperament and ease of handling. However, the rabbit is also larger than most rodents, making it easier to create a critical size defect, a requirement fundamental to this study. Furthermore, rabbits have a complete system of Harvers channels, similar to human bone, such that results of our research may be more confidently extrapolated (11).

Since the scaffold model completely filled the bone gap no orthopedic implant was required to stabilize the fracture. Other studies using a similar bone defect model in rabbits have been described without postoperative complications (12). During the surgery and the postoperative period, no instability of the radius in relation to the ulna was observed. This is probably due to the anatomy of the forearm of the rabbit, where there is a strong interosseous membrane that connects the radius to the ulna and the ulna has a large diameter when compared with the radius. Thus, the radius remains stabilized by the ligament, while the ulna supports most of the body weight (13).

The biomaterials were selected on 2 grounds: the need for a filament-laid biomaterial that could be printed using the Prusa i3 printer and the need for this material to be biocompatible. PLLA is a biocompatible synthetic polymer used in bone regeneration for its osteoconductive and osteogenic characteristics (6). However, it is described that during its degradation, acid formation changes the pH of the microenvironment which may hinder the regenerative process (14). However, hydroxyapatite, also widely used in orthopedic surgeries due its bioactivity, when associated with PLLA, neutralizes the acidic products released by the polymer creating a favorable cellular environment (15). In addition, hydroxyapatite also assists in the kinetics of PLLA degradation and absorption and tissue integration (16).

The follow-up period in this study was sufficient for radiographic evaluation of bone bridging. However, the biological behavior of the scaffold has not been assessed due to the experimental nature of the pilot study. The authors suggest that, in addition to the radiographic images, more complete analysis, including histopathological examination, should be conducted to confirm the use of this implant as an alternative to more conventional treatment of critical-size segmental bone defects.

Conclusion

Three-dimensional printed bone substitutes using PLLA and hydroxyapatite may be a feasible alternative tool in the treatment of large bone defects. However, in view of the pilot nature of this study, further investigations are needed to corroborate this finding. Histopatological examination, a larger number of cases and a longer evaluation period should be considered in planning future studies.

Acknowledgment

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CAPÍTULO 5 – 3D prototyped composite in the reconstruction of critical bone defects in rabbits' radius (*Oryctolagus cuniculus*)

Artigo a ser enviado para revista Veterinary and Comparative Orthopaedics and Traumatology

Summary

Objetive: The purpose of this study was to use the 3D printer technology to obtain a bone substitute for reconstruction of critical segmental bone failures in rabbits radio. **Study design:** The animals were divided into 3 groups: Group I (n=20) or control group, Group II (n=20) or graft group and Group III (n=20) or implant group. On group I, the rabbits were submitted to na 1,5cm ostectomy receiving no treatment. On group II, the bone defect was treated with autologous graft from the ilium wing. On group III, reconstruction occurred through three-dimensional printed hydroxyapatite and PLLA implant. The implants were prepared using 3D imaging based on the computed tomography obtained from each animal. Clinical evaluation were conducted postoperatively on day 7, 15, 30, 60 e 90. Radiographs in two projections were taken on days 15, 30, 60 and 90 postoperative, according to the subgroups T1, T2, T3 and T4, respectively. After radiographic evaluation, the animals were submitted to euthanasia and the bone segment was referred for histopathological analysis. **Results:** Clinically, group II presented less lameness, edema, pain and complications on the surgical wound when compared to the control and implant groups. On radiographic evaluation, periosteal reaction, bone callus formation and bone bridge were also superior in group II when compared to the others groups. Histopathological study showed that fibrosis, osteogenesis and chondrogenesis were similar in all groups. However, the presence of congestion, hemorrhage and inflammation were higher in the implant group.

Keywords: 3D printing; Polylactic acid; Hydroxyapatite; Bone graft, Segmental bone defect.

Introduction

Orthopedic conditions represent a large part of the routine in veterinary medicine. Orthopedics surgeons are often challenged with complex fractures, large bone defects caused by trauma or bone tumors resection, congenital deformities or complications such as delayed union or non-unions. The treatment of these conditions often includes bone grafting and distraction osteogenesis with ilizarov technique (1, 2). Autogenous grafts have been described as the best choice of biological material due the low risk of immunological rejection. However, their use is associated with increased morbidity and pain, longer surgical and anesthetic times, and in some cases, insufficient volume of bone. (3).

In this context, other sources of bone material have been studied. Tissue engineering, the field of biomedical sciences that has received the most attention at the present time, has, among other objectives, the aim of restoring the function of an organ or repairing damaged tissue through the synergism of biomaterials and cells. One of the tools of this new science is the technology of the three-dimensional printers (4).

The three-dimensional (3D) printing, also known as additive production, has been available since 1980, but only recently has its use amplified due to the cost decrease and growth of computer science engineering (5). A wide range of materials can be used in 3D printing, such as aluminium, stainless steel, titanium, polymers and ceramics (6). The possibility of creating simple or complex objects in a variety of materials enabled the use of 3D printers in medicine field. Applications include manufacturing of models for surgical planning, prostheses production and biological tissues implants (5).

The 3D printers have already been employed in medicine and veterinary medicine for planning tumor excision, organ transplantation and reconstructive surgeries. However, additional studies should be conducted to ensure the efficacy and safety of this technology for

clinical applicability. The objective of this study was to develop a bone substitute using a 3D printer and to implant in a critical bone defect in rabbits radius. Clinical, radiographic and histological evaluations were performed in a comparative study with ilium wing autograft.

Materials and methods

For this study, 60 female rabbits (O*ryctolagus cuniculus*), skeletically mature (>7 months) weighing between 4 to 5.5Kg were used. The animals were submitted to clinical and radiographic examination to evaluate general health conditions and the musculoskeletal system prior the study. Throughout the experiment, the rabbits remained in individual cages where they received food, water and the treatment necessary to their well-being.

The animals were divived into three groups according to the surgical treatment of the bone defect. Group I (control group) submitted to a 1.5 cm ostectomy in the right radius diaphysis, without treatment. Group II (graft group) submitted to the ostectomy and bone defect reconstruction with iliac crest autologous graft. Group III (implant group) received a 3D printed bone implant to reconstruction of the ostectomy. All groups were divided into four subgroups according to the radiographic and histopathological evaluation times. T1 – 15 days, T2 – 30 days, T3 – 60 days and T4 - 90 days postoperative.

In the period prior to the surgical procedure, all animals were weighed, remained in food and water fasting for 2 hours, and had ears, right front limb and left sacroiliac region (group II) hair clipped. The preanesthetic medication consisted of association of ketamine hydrochloride (20mg/kg), midazolam maleate (2mg/kg) and morphine sulfate (2mg/kg) intramuscularly (IM). The animals were, then, referred to the surgical block for induction and anesthetic maintenance with isofluorane with the use of an inhalation mask, vaporized in 100% oxygen with spontaenous respiration. After anesthetic plan was achieved, the right brachial plexus block was perfomed in all animals and sacroiliac regional anethesia in group II using lidocaine (6mg/kg)

2% without vasoconstrictor. Heart rate, oxygen saturation and oscillometric blood pressure were monitored throughout the procedure. Also, the animals remained in fluid therapy with lactated ringer's solution by cannulation of the caudal auricular vein with 24G cateter in a rate of 7ml/kg/hr. With the animal in right lateral recumbency, a longitudinal skin incision of approximately 3.0 cm on the dorsomedial face of the right limb was performed. Subcutaneous tissue and musculature were retracted to exposure the diaphysis of the radius and the periosteum was removed with blunt dissection. Then, the ostectomy was performed 2.0 cm above the carpus joint, removing a 1.5 cm segmental defect with the aid of an oscillating saw. The cut was carefully performed due to the proximity of the radius to the ulna. For bone fragment removal, the interosseous ligament was incised. After the bone defect was made, the treatment was conducted according to the animal's group.

In group I, bone defect was left empty and subcutaneous tissue and skin were sutured in routinely fashion (Fig. 1). In group II, after the radius defect was performed, a skin incision was made on the craniodorsal aspect of the ilium crest. Lateral and medial musculature were removed exposing the ilium bone. Using an oscillatory saw, a segment of corticalcancellous graft was harvested and soon after, implanted in the radius defect. The bone graft was measured and, if necessary, cut with scissors to fit the defect (Fig.2).

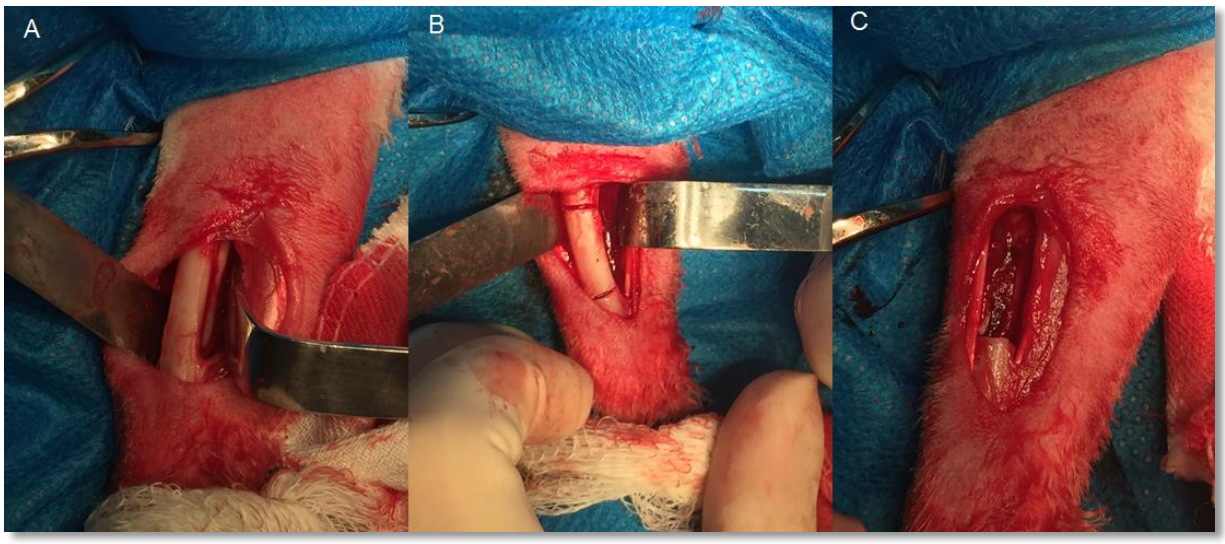

Fig 1. Critical segmental bone defect surgical procedure in the right radius of a New Zealand rabbit from the control group study. A) Radius exposure after skin incision and muscle reflecting. B) Osteotomies performed with oscillatory saw. The distal cut was made 2 cm above the radiocarpal joint. C) Removal of the radius segment of 1.5cm, creating a critical bone defect.

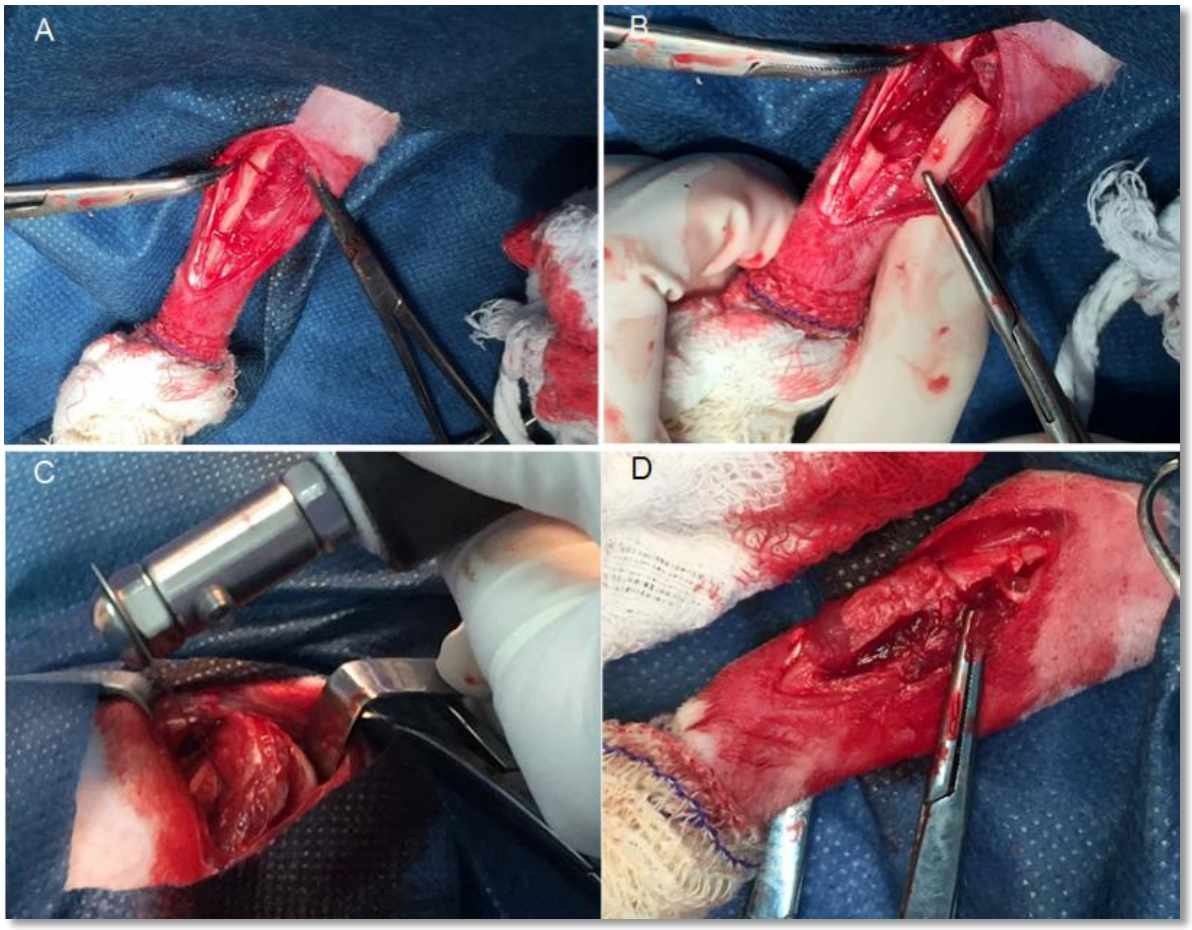

Fig 2. Surgical procedure of the graft group using autologous bone graft for treatment of a critical defect in the radius diaphysis of a New Zealand rabbit. A) After radius exposure, two osteotomies were performed with oscillatory saw. The distal cut was made 2 cm above the radiocarpal joint. B) Removal of the radius segment of 1.5cm, creating the critical bone defect. C) After the bone defect creation, a segment of the iliac crest from the same animal were harvest using an oscillatory saw. D) Implantation of the graft into the bone defect in the radius.

Through sequential computed tomography (GE Speed Helical) with 120kV, 130 mA and 1mm cut thickness, images of the right limb of all animals belonging to group III were obtained. These images were reconstructed in three-dimension images in DICOM format files

Sequentially, these files were converted into stl format using InVesalius software, allowing the manipulation of the images. Segment cuts and separation of the radius from the ulna were made with Blender and Autodesk Meshmixer modeling software, delimiting the area of interest for printing, a 1.5cm segment of the radius 2 cm above radiocarpal joint (Fig.3). With the virtual replica finished, the implant was printed with Prusa i3 printer with direct drive extrusion. The material used was the association of Poly-L-lactic acid (PLLA), an absorbable filament, and hydroxyapatite powder. The implants were sterilized with ethylene oxide at the end of the process. In the animals of group III, after the creation of the bone failure, the 3D printed bone substitute was placed into the defect so that its extremities remained in close contact with the bone edges, exerting some compression on the surface between bone and scaffold, thus no implant fixation was required (Fig. 4).

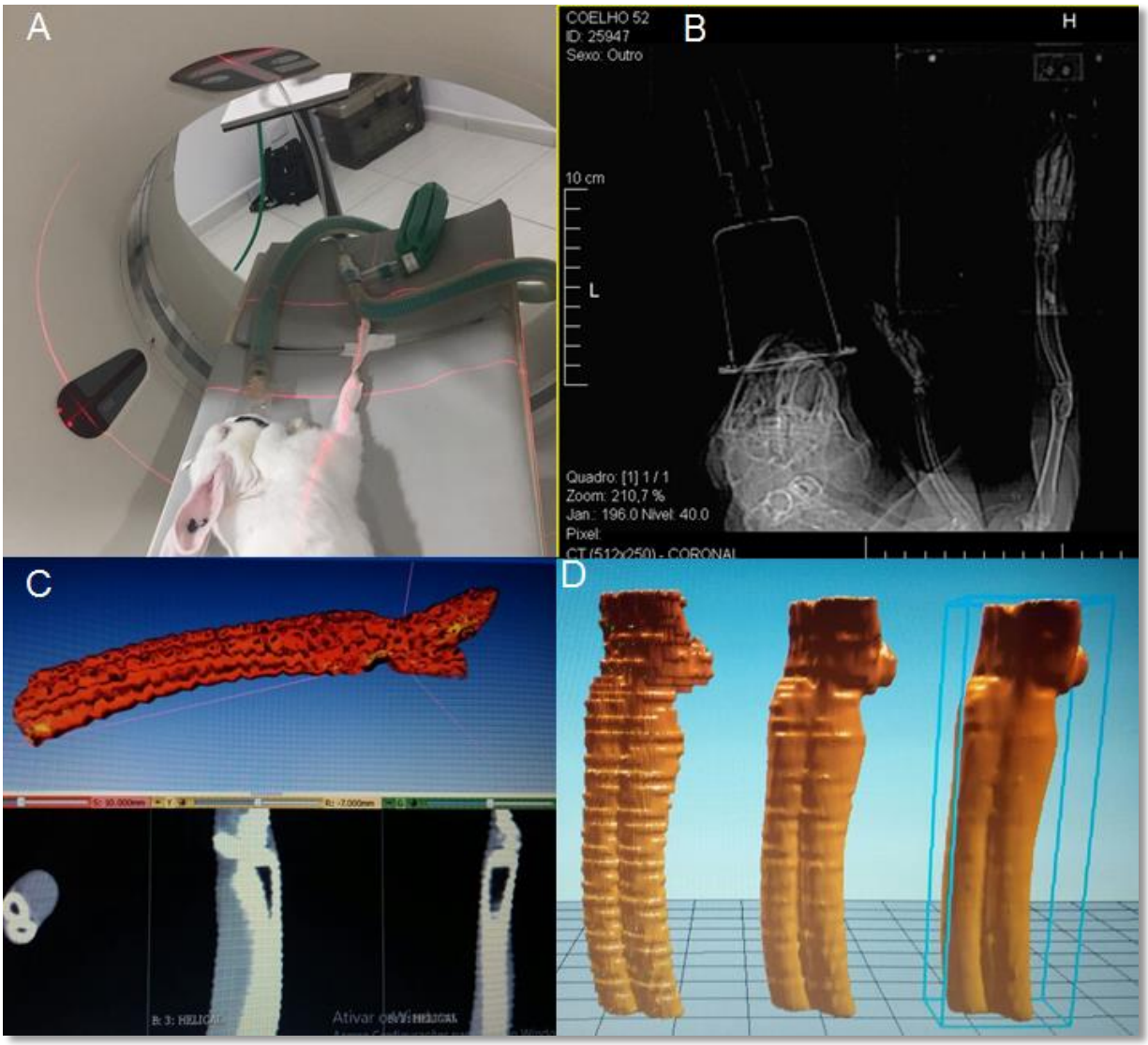

Fig 3. A) Female, New Zeland rabbit under sedation, submitted to computed tomography to obtain a three-dimensional image of the right thoracic limb. B) Scout image of the rabbit obtained by the computed tomography. C) Three-dimensional images of the forearm in the Invesalius software for image manipulation and file conversion from DICOM format to stl. D) 3D image of the forearm in the modeling software for further manipulation.

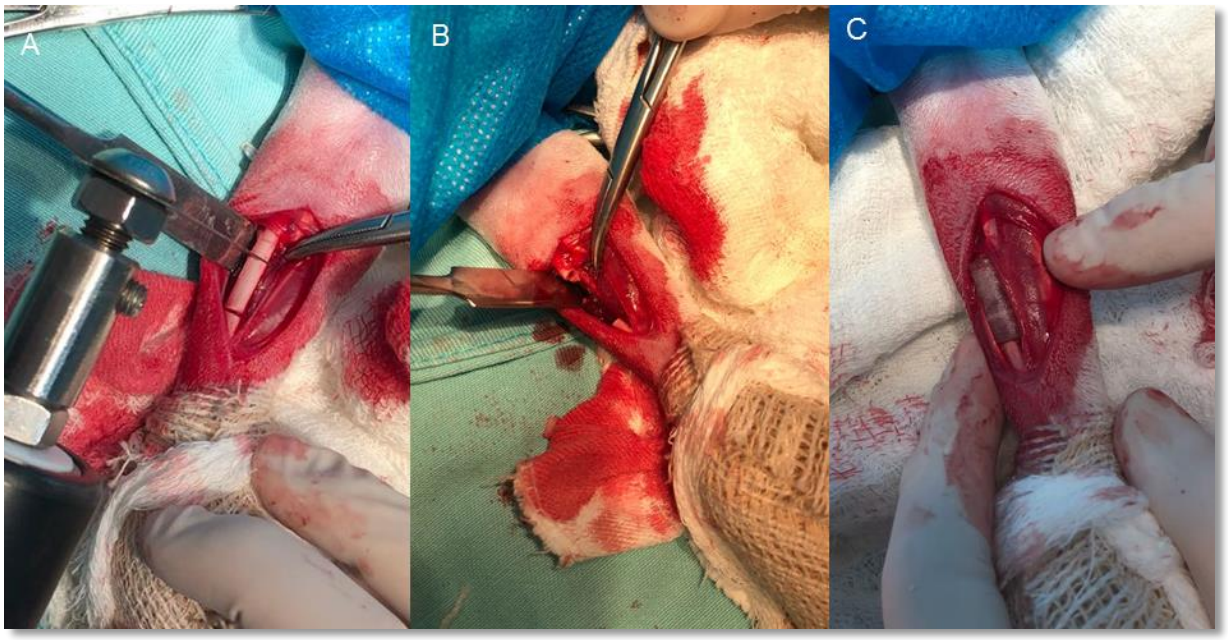

Fig 4. Surgical procedure of the implant group using a 3D printed bone substitute for treatment of a critical defect in the radius diaphysis of a New Zealand rabbit. A) After radius exposure, two osteotomies were performed with oscillatory saw. The distal cut was made 2 cm above the radiocarpal joint. B) Removal of the radius segment of 1.5cm, creating the critical bone defect. C) 3D bone substitute was implanted into the critical bone defect.

In the immediate postoperative period, the animals were monitored until complete restoration of consciousness, when they were sent to their individual cages. Postoperative medication consisted of Dipirona 25mg/kg subcutaneously (SC) twice a day (BID), Tramadol hydrochloride 4mg/kg BID SC, Meloxican 0.1mg/kg once per day (SID) SC, all for three days and Enrofloxacin 5mg/kg BID, SC for five days. The animals were clinically assessed for ambulation, limb support, presence of pain and inflammation in the affected limb. The observation was always perfomed by the same evaluator on the 7th, 15th, 30th, 60th and 90th day after surgery, according to each group and subgroup, following the classification listed in table 1.

Criteria		scores
Standing	Continuous weight-bearing	**0**
	Intermittent weight-bearing	**1**
	Completely non-weight-bearing	**2**
	N/A	**3**
Gait with movement	Continuous weight-bearing	**0**
	Intermittent weight-bearing	**1**
	Toe-touches non-weight-bearing	**2**
	Non-weight-bearing	**3**
Swelling	None	**0**
	Mild	**1**
	Obvious	**2**
	N/A	**3**
Pain during palpation	None	**0**
	Mild (ocasional vocalization)	**1**
	Moderate (frequent vocalization)	**2**
	Severe (vociferous vocalization, withdraws limb, bites, struggles)	**3**

Table 1. Pain scale used in the physical evaluation of the rabbit during the postoperative period. N/A Not applicable. Adapted from STASIAK, K.L. MAUL, D.; FRENCH, E. Species-specific assessment of pain in laboratory animals. *Contemporary topics*, v.42, p. 13-20, 2003.

Cranial-caudal and midlateral radiographs (mA 100, Kv 70) were taken in the immediate postoperative period, after 15 days, 30 days, 60 days and 90 days after surgery, according to the subgroups (T1, T2, T3 and T4, respectively) (Fig. 5). Radiographs were analyzed by three evaluators blinded in relation to the groups. The images were assessed for periosteal reaction, bone callus volume and bone bridge quality, receiving scores from 1 to 4, as described in tables 2, 3 and 4.

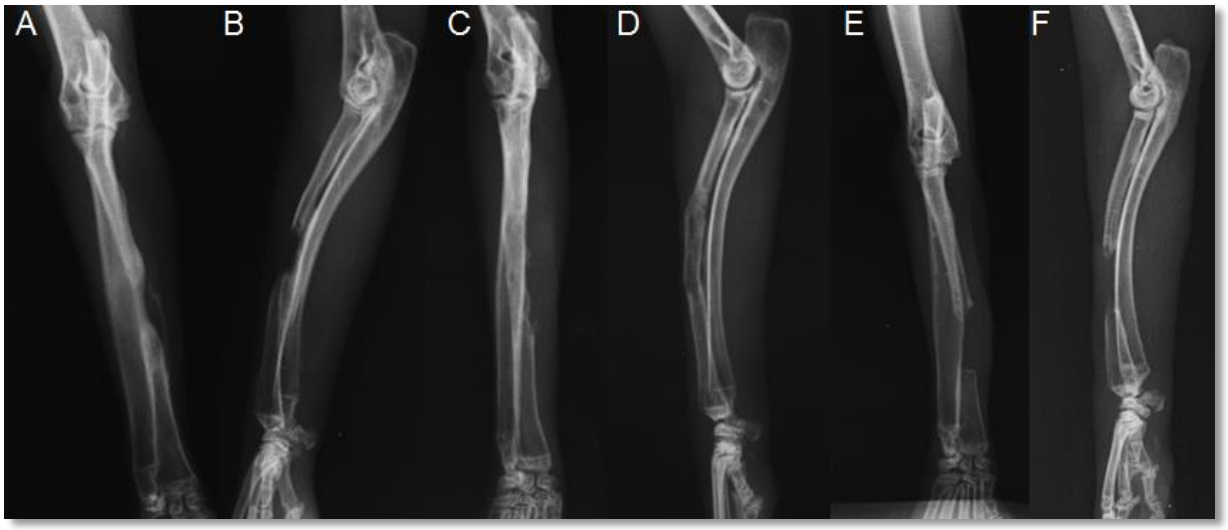

Fig 5. Cranio-caudal and midlateral postoperative radiographs of the right thoracic limb of three New Zealand rabbits from the study. A and B) Rabbit number 27 belonging to the control group at 90 days postoperative. C and D) Rabbit number 9 belonging to the graft group at 90 days postoperative. E and F) Rabbit number 59 belonging to the implant group at 90 days postoperative.

Score	Bone callus volume
1	0 a 25% of bone failure is filled by bone callus
2	25% a 50% of bone failure is filled by bone callus
3	50 a 75% of bone failure is filled by bone callus
4	75 a 100% of bone failure is filled by bone callus

Tabela 2. Table of scores classifying the volume of bone callus used in the postoperative radiographic evaluation of rabbits submitted to ostectomy of the right radius.

Score	Periosteal reaction
1	Abscence periosteal reaction
2	Discrete periosteal reaction
3	Moderate periosteal reaction
4	Intense periosteal reaction

Tabela 3. Table os scores classifying the periosteal reaction used in the postoperative radiographic evaluation of rabbits submitted to ostectomy of the right radius.

Scores	Bone bridge formation
1	Abscence of bone bridge between the fragments of radius
2	Discrete bone bridge formation in the ventrolateral region of the radius
3	Formation of a bone bridge of a thickness less than the diameter of the radius
4	Formation of bone brigde of thickness equal or greater than the diameter of the radius restoring the bone column

Tabela 4. Table os scores classifying the bone bridge formation used in the postoperative radiographic evaluation of rabbits submitted to ostectomy of the right radius.

For histopathological analysis, the radius and ulna bones of each experimental group were collected and dissected after eutanásia. Fixation was done in 10% buffered formaldehyde for four days. The samples were, then, washed overnight in running water to remove formaldehyde excess. After, the material was maintained in 10% nitric acid solution for three to five days for decalcification. At the end of this process, the fragments were treated with 5% sodium sulfate solution for 24 hours. Subsequently, the samples were dehydrated in alcohol 70%, 80% and absolute P.A for 60 minutes each one. Soon after, they were diaphanized in absolute xylene P.A I, II and III for 50 minutes each one. Finally, the samples were included in

histological paraffin I and II for 60 minutes each one. Afterwards, they were emblocated and histological sections were made in a semi-automatic microtome (LEICA RM 2155) with four micrometers thickness. The histological slides were stained by Masson's hematoxylin and eosin and trichrome techniques. The analyzes were performed using a light microscope to compare fibrous tissue, cartilaginous and osteoid tissue neoformation during the bone regeneration process. The evaluations were classified in scores (1 to 4) based on the presence of alterations, where one is the absence, two discrete, three moderate and four marked alterarions. The characteristics analysed were presence of congestion, hemorrhage, inflammatory infiltrade and collagen characterization. The evaluation was performed by a single experienced evaluator.

Statistical analysis was performed with software R® (R Foundation for Statistical computing, Vienna, Austria). Radiographic evaluations were compared among the observers by the Bland-Altman concordance test. Clinical, radiographic and histopathological parameters were subsequently compared between the treatment groups, the days of evaluation and the interaction of these factors by the Friedman test and Dunns post-test, presenting their results as mean ± IQR (interquartile range). Significance was set for all tests at 5%($p<0.05$).

Results

The critical segmental bone defect model used in this study was easy to perform, presenting only one case of complication in the control group, where one of the animals presented ulnar fracture 7 days after the procedure. Due to the proximity of the forearm bones, during the osteotomy of the radius, there was an injury to the sis-cortex of the ulna, causing the fracture in the postoperative period. However, no other complications were observed relating to the defect model. Support of the operated limb was observed in all animals soon after anesthetic recovery and, during the entire experimental period, no animal presented severe claudication, as discussed below.

The ilium crest autograft technique was also considered appropriate. The collection site was easily accessible with good availability of bone tissue for graft harvesting. Besides that, because it is a corticalcancellous bone, it was possible to adjust the size of the graft with scissors when needed, facilitating its implantation in the defect. Thus, there was no need for orthopaedic implant.

The manufactoring of the bone substitute using 3D printer is feasible. The obtaining process of these implants was fast and inexpensive, although some particularities should be improved. For this study, tomographic images of 1mm thickness were achieved from a 2-channel tomograph, therefore, the images resolution, and consequentely, the implants accuracy were affected. The printed implant had a robust architecture making it difficult to implant in the delicate bone structure of the rabbits. In three animals there was displacement of the material in the postoperative period observed in the radiographic examination.

Limb support was similar among groups (P=0,1954), increasing significantly after the 15th day of evaluation in all groups (p=0,0443). Lameness was greater (P=0,0243) in the implant group when compared with others groups on day 7 and 30 and decreased gradually (0,0225) with the advancement of the days (Fig. 6).

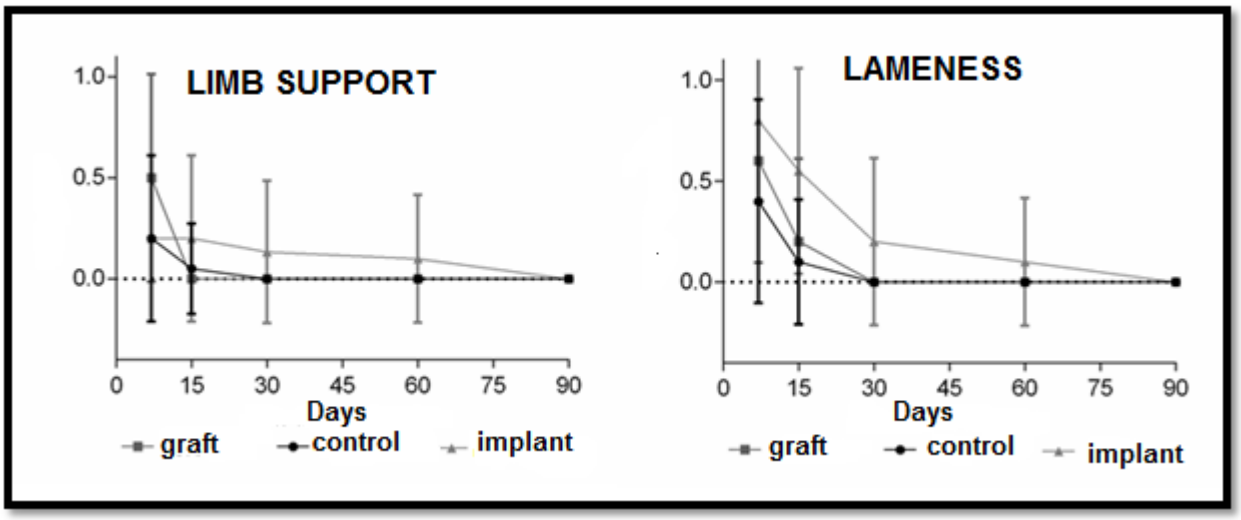

Fig. 6 Graphic showing limb support and lameness after segmental ostectomy of the radio in rabbits according to the treatment during the postoperative evaluation periods of 7 days, 15 days, 30 days, 60 days, and 90 days.

Edema was greater (P=0,0074) in the implant group than in others at 7 to 30 days of evaluation, and there was no influence of time (P=0,1496). Pain was greater (P=0, 0497) in the implant group at the 7th and 15th day and there was no evidence of time (P=0,4060). The presence of complications in the surgical wound was greater (P=0, 0308) in the implant group at the 7th and 15th day and there was no influence of time (P=0,4060) (Fig. 7).

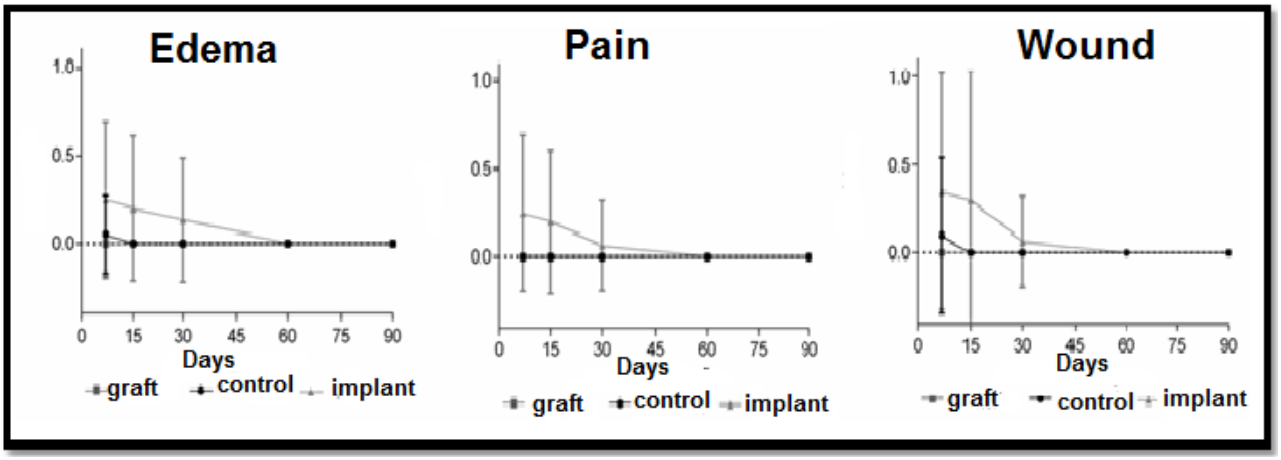

Fig 7. Graphic showing presence of edema, pain and wound complication after segmental ostectomy of the radio in rabbits according to the treatment during the postoperative evaluation periods of 7 days, 15 days, 30 days, 60 days, and 90 days.

In the radiographic evaluation, it was evidenced that the evaluator 3 underestimates (P=0.0001) the periosteal reaction with a bias of 26%. The evaluator 2 underestimates bone brigde (P=0.0001) with a bias of 22%, whereas bone callus evaluation was similar among evaluators (P=0.5161). The periosteal reaction was lower (P=0.0048) in the control group at day 90. Bone callus formation was smaller (P=0.0183) in the implant group during day 30, 60 and 90, and greater in the graft group at day 60 and 90. Bone brigde was smaller (P=0, 0421) in the implant group at 30, 60 and 90 days and greater in the graft group at 60 and 90 days.

Histopathological study showed bone consolidation in three animals of graft group T4, five animals of T3 and 1 animal of T2. Those animals that did not present consolidation had exuberante bone callus formation. In the implant group, it was possible to observe the presence of foreign body giant cells at the interface between the bone and the implant, mainly in the subgroups T4, T3 and T2. Also in these subgroups there was pseudocapsule formation involving the implant and in one animal belonging to T4, an abscess was present.

Statistical analyses of the microscopic features demonstrated that fibrosis was similar between days (P = 0.4835618) and treatments (P = 0.1353353), as well as chondrogenesis (days P = 0.7185168, treatments P = 0.1737739) and osteogenesis (days P= 0.5432912, treatments P=0.1737739). Congestion was similar between days (P= 0.1313505) and greater in the implant group when compared to control (P = 0.04688824). Hemorrhage was similar between days (P = 0.3916252) and greater in the implant group than in the other groups (P= 0.04978707). Collagen was similar between days (P = 0.40300738) and lower in the implant group than in the other groups (P=0.01831564). Inflammation was similar between days (P=0.4792326) and greater in the implant group than in the other groups and in the graft group than in the control group.

Discussion

The critical segmental defect in the radius of rabbits was chosen as the experimental model in this research based on previous studies of bone regeneration. Rabbits are widely used in researchs, especially in orthopaedics due their bone-like morphology when compared to humans, presenting a cortical bone with complete system of harvers channels, allowing the extrapolation of results for medicine (7). In addition, their docile temperament and low maintenance cost contribute to the management of a large number of animals during the study (8), as in our research in which 60 animals were used. Also based on previous studies, the

location of the bone defect in the radius was chosen due to the anatomy of the radius and ulna in this species. As seen in dogs and cats, the radius diaphysis has little muscle coverage, allowing surgical access with low morbidity of muscles, vessels and nerves. Another important factor considering the biomechanics of the rabbits forearm is the presence of the interosseous ligament between the radius and the ulna, which acts as a stabilizer (9). The choice of the radius instead of the ulna occurred because in rabbits the ulna is the main support of loads and presents a broader diameter than the radius (10). In view of these characteristics, some authors suggest that stabilization of the radius with orthopaedics implants is not necessary (9, 11, 12). In our study, the bone defect model presented few complications. The most important drawback was an ulna fracture in one animal of the control group due to an error in the surgical technique. Regarding the use of orthopaedic implants, both control group and graft group, there was no need for fixation. Three animals of the implant group showed displacement of the bone substitute, which may be avoided with the use of plates.

The implant failure in these animals may be due to the 3D printing process. Although the implants were made based on the tomographic examination of the animal's own limb, the quality of the images obtained did not allow an accurate printing of the implants, impairing their placement in the bone defect. To print small and detailed structures, it is recommended to obtain slice thickness smaller than 1mm using high definition images such as computed tomography with high channels or magnetic resonance (13).

In addition to these difficulties found during the implantation of the bone substitutes, the implant group presented greater claudication, edema and pain in the clinical evaluation. These clinical signs are in agreement with histopathological findings in which it was possible to observe presence of hemorrhage, congestion and inflammation more marked in this group when compared with control and graft groups. These results were expected in view of the

synthetic nature of the implants. Biomaterials implantation raise a foreign body reaction by the combination of two events: the binding of plasma proteins onto the biomaterial's surface and the histamine release by local mast cells associated to the tissue injury during the implantation (14, 15).

As the plasma proteins bind to the material, leukocytes recruitment occurs leading to differentiation into foreign body giant cells. The activated macrophages release cytokine and chemokines including interleucine 1, interleucine 8, tumor necrosis factor and metalloproteinases. The presence of these factors at the implant site increases neovascularization and inflammatory aggregates, leading to congestion and hemorrhage (16). The angiogenesis induced by the inflammatory response is critical to the granulation tissue development and, consequently, to the success of engraftment of the implant into the host (17). Thus, it may be consider that the presence of foreign body giant cells in the samples of the implant group are essencial to the implant degradation process. The accumulation of these macrophages is commonly observed during the first few weeks of implantation and at the end of the resorption process, depending on degradation time of the biomaterial (17). In our study a dense layer of giant cells were present in the interface of the implant with the bone, mainly in animals of subgroups T2, T3 and T4 (30 days, 60 days and 90 days after surgery, respectively). Some authors suggest that the rate of implant degradation is related to the chemistry of the biomaterial surface, its crystallinity, implant geometry and site of implantantion (18). Also the molecular weights of the polymers has been show to influence strongly in its half-life. Lower molecular polymers present faster resorption (19, 20). Unfortunately, in our study, the time of evaluation did not allow the observation of complete degradation of the implant. The authors suggest that due to the dense architecture of the bone implant obtained through the layer-by-

layer printing of the PLLA fillament, associated to the hydroxyapatite, has provided a high rigidity to the material increasing its half-life.

The challenge of tissue engineering, especially in orthopaedics, is to balance the mechanical properties of the implant with its biodegradability (21). Therefore, additional studies with longer evaluation times are necessary in order to fully understand the behavior of the implant and its degradation. The results obtained here, however, suggest that the bone substitute presents good mechanical properties and may be indicated in lesions where a strong structure is needed.

On the other hand, the graft group was considered radiographically and histologically superior to the implant and control group, showing faster bone callus and bridge formation, achieving bone consolidation in most animals of T3 and T4 subgroups. Autologous bone graft are considered the gold standard treatment for fusions, fracture repair and reconstruction of skeletal defects. Their osteogenic ability provides precursors cell such as osteoblasts and osteocytes important in the bone regeneration. In addition to the osteogenic properties, the porosity of trabecular bone found in corticocancellous graft is also highly angiogenic accelerating the revascularization into the host bone and its incorporation (22, 23). However, corticocancellous bone graft, such as the iliac crest, is structurally week with poor mechanical properties, limiting its use to small bone defects. Besides that, the bone harvest is restricted to the capacity of the donor site (23). In this study, a critical bone failure was performed in order to reflect the condition of a long bone injury or an invasive surgery for tumor removal. Based on the literature, the size of the segmental defect in the radius of rabbits should exceed 1cm to achieve a critical pattern (24, 25, 26). Even with the a 1.5cm defect, the bone graft group presented bone consolidation in the subgroups T3 and T4 or bridge formation in subgroups T1 and T2, where consolidation was not expected in view of the short time. This may indicates that

the osteogenic capacity excelled the biomechanical necessity in this experimental model. The authors suggest that the above mentioned radio stabilizing structures aided in these results.

Based on these data, it can be argued that the biomechanical capacity of the implant and the biological properties of the graft bone should be associated by creating a new implant that presents osteoconductive and osteogenic characteristics, such as a bone substitute printed using PLLA and hydroxyapatite enriched with bone marrow.

Conclusion

The experimental model adopted in this study allowed clinical, radiographic and histological evaluation comparing the three treatment groups. However, the evaluation time should be extended in view of the low biodegradability behavior of the implant. Also, it may be suggest a refinement of the 3D printing by using a more accurate imaging method, thus reducing the structure of the implant and consequently increasing its degradation rate. Furthermore, it may be indicated for future studies the association of biological materials such as bone marrow, platelet-rich plasma or stem cells in order to provide osteoinductive and osteogenic properties for the bone substitute.

Acknowledgment

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CAPÍTULO 6 – Considerações Finais

1. ASPECTOS RELEVANTES

Este estudo foi realizado no período de agosto de 2016 a maio de 2018. Os animais foram adquiridos através de um cunicultor da região de Londrina – PR com a proximadamente 3 meses de idade. Os animais eram, então, alojados no biotério da Universidade Unicesumar em Maringá- PR, onde permaneciam em gaiolas individuais até completarem 6 a 7 meses de idade. Neste período os coelhos eram submetidos à radiografia do membro torácico direito para visualização do fechamento da placa fisária.

Tendo em vista o caráter do estudo, apenas animais com maturidade esquelética foram submetidos à realização do defeito ósseo a fim de não haver influência na regeneração óssea e diminuir os riscos de fratura fisária ulnar.

Inicialmente foi realizado treinamento em cadáver de coelhos com o objetivo de determinar acesso cirúrgico, local do defeito e tamanho do defeito. Uma vez que o modelo de falha foi definido, foi realizado o projeto piloto em 3 coelhos.

A aquisição das imagens para confecção das próteses dos animais do grupo implante foi feita em um centro diagnóstico particular localizado na cidade de Maringá – PR. As imagens tomográficas do membro torácico direito eram enviadas à VetCraft 3D em Jaboticabal – SP onde todo o processo de impressão era realizado pelo pós-graduando Thiago de Sá Rocha. O filamento de PLLA foi importado da Holanda e a hidroxiapatita em pó obtida através do Laboratório de Biomateriais da UFMS.

As cirurgias foram realizadas no centro cirúrgico do Hospital Veterinário da Universidade Unicesumar, assim como as imagens radiográficas. As amostras histopatológicas foram processadas e avaliadas no Laboratório de Pesquisa do Serviço de Patologia Veterinária da FMZV – UNESP, Botucatu – SP pelo Prof. Ass. Dr. Alessandre Hataka.

2. DIFICULDADES ENCONTRADAS

A principal dificuldade encontrada foi a obtenção e manutenção dos animais do projeto. O biotério apresentava capacidade máxima de locação de 30 coelhos. Devido ao grande número de animais utilizado neste estudo e limitação no alojamento, os animais foram adquiridos em etapas. Aliado a isso, cada lote de animais recém chegados apresentavam idade máxima de 3 meses, resultando emu ma espera de, no mínimo 3 meses para início do experimento. Além disso, alguns animais eram avaliados até os 90 dias pós-operatório, totalizando uma estadia de 9 meses ao total.

Houve atraso no início dos procedimentos cirúrgicos do grupo III devido à quebra de equipamentos. Também, durante o decorrer do experimento, 16 animais operados morreram devido a um surto infeccioso de origem desconhecida. Os coelhos apresentavam apatia, hiporexia, diarreia e morte entre 24 a 48 horas após o início dos sinais clínicos. Todos animais foram substituídos, no entanto, contribuiu para o atraso na finalização do grupo enxerto.

Durante a avaliação histopatológica houve dificuldade na preparação das lâminas do grupo implante. Devido alta dureza do material não foi possível realizar o corte do implante com o micrótomo, tendo que ser removido da peça anatômica para posterior avaliação. Por não se tratar de tecido ósseo, o implante não sofreu ação do descalcificante, sendo impossível seu corte. Outros métodos foram pesquisados, porém sem sucesso.

3. LIMITAÇÕES DO ESTUDO

A maior limitação do estudo foi a indisponibilidade de método de imagem de melhor qualidade para obtenção e processamento do implante impresso. Como discutido neste trabalho, a qualidade da impressão é proporcional à definição da imagem. Além disso, outros métodos de avaliação como densitometria óssea, microscopia eletrônica e análise molecular das próteses poderiam ser associadas ao estudo, enriquecendo os dados obtidos.

4. PERSPECTIVAS

Como discutido ao longo desta tese, o objetivo deste trabalho foi o de produzir um substituto ósseo impresso em impressora 3D para o tratamento de falhas ósseas, diminuindo a necessidade de enxertos ósseos, procedimentos cirúrgicos longos e complexos ou o uso de biomateriais caros. A ideia é aproveitar as vantagens dessa tecnologia que, a cada dia, torna-se mais popular no meio biomédico, na resolução de problemas da rotina clínica-cirúrgica.

Tendo em vista a variedade de materiais disponíveis no mercado e a possibilidade de impressão de qualquer objeto, as impressoras 3D apresentam potencial para a substituição de tecidos e órgãos. Assim, pesquisas aplicando biomateriais e analisando seu comportamento biológico e biomecânico são necessárias.

Assim, a perspectiva é que novos estudos surjam deste protótipo inicial, com outros biomateriais, tempos de análises mais prorrogados e outros métodos de avaliação para melhor entendimento do processo de incorporação. Espera-se que esse projeto sirva como base de modelo experimental para pesquisas futuras em regeneração óssea.

ANEXO 1 – Normas do periódico Investigação Medicina Veterinária

PREPARATION OF MANUSCRIPT

The document must be presented in *Word* in "*docx*" format. The font Times New Roman 12 should be used (including tables and figure legend). Use single spacing (1.0) throughout the text. The pages must be numbered continuously centered on the bottom edge of the page and lines numbered continuously. Only English or Portuguese language will be accepted.

Use of animals - Manuscripts submitted for publications must contain a statement that the study protocol was approved by a local Ethics Commission.

FIGURES AND TABLES

They should be inserted throughout the text (eg Figure 1, Figure 2 ... / Table 1, Table 2, etc.).

Images from articles, books, etc. Must be sent separately with a letter of permission for the use of image / copyright for use signed by the author and / or publisher. If this is not possible, *only personal images may be used*.

STYLE AND FORMAT

Special Session

Invited Reviews will be sought by the Editorial Board for comprehensive review of a specific subject. This section is only for invited authors.

Original articles (full papers)

Consists on the presentation of unpublished data with a maximum of 15 pages (including the subtitles, tables and/or figures and references).

At the beginning of the text it may contain the word "ORIGINAL ARTICLE", just below it's necessary classify the area (eg. SMALL ANIMAL PRACTICE, LARGE ANIMALS). Both must be written in Caps Lock, bold and centered.

TITLE Caps Lock, bold and centered

AUTHOR(S): name and surname (middle name abbreviated) (eg. Geórgia M. Magalhães), and in case of more than one author, separate using commas. At the end of each name, superscript numbers corresponding to institution data, department, state and country must be used. Identified with "*" the corresponding author.

ABSTRACT (preferably starting with the objectives, with a maximum of 250 words),

KEY-WORDS (minimum of 3 and maximum of 6 words),

INTRODUCTION (900 to 3000 characters without spaces)

MATERIAL AND METHODS (Starting with local Ethics Commission protocol number. Should containing 1000-4000 characters without spaces).

RESULTS (presented clearly and concisely with 1000-4000 characters without spaces).

DISCUSSION (focused only on search results avoiding repetition of the results or revisions without character limit).

CONCLUSIONS (this element must be presented along with the discussion in the last paragraph)

ACKNOWLEDGEMENTS (when relevant)

REFERENCES (maximum of 35 references; IMPORTANT: in all forms of presentation, this topic should be avoided quotes from books, dissertations, theses, etc.; it is preferable to use scientific articles).

Paper review

Must be written in the first person and pertinently discussed, with the subject strictly related to the author's area of expertise and the journal's scope, limited to 15 pages (regardless of tables and/or figures).

At the beginning of the text it may contain the word "PAPER REVIEW", just below it's necessary classify the area (eg. SMALL ANIMAL SURGERY, LARGE ANIMALS). Both must be written in Caps Lock, bold and centered.

TITLE Caps Lock, bold and centered

AUTHOR(S): name and surname (middle name abbreviated) (eg. Geórgia M. Magalhães), and in case of more than one author, separate using commas. At the end of each name, superscript numbers corresponding to institution data, department, state and country must be used. Identified with "*" the corresponding author.

ABSTRACT (preferably starting with the objectives, with a maximum of 250 words),

KEY-WORDS (minimum of 3 and maximum of 6 words),

INTRODUCTION (900 -1,500 characters without spaces)

DISCUSSION is not necessary to follow the word "discussion". It may present topics or not. Must contain containing 3,000-15,000 characters without spaces).

FINAL CONSIDERATIONS or CONCLUSIONS (displayed along the "discussion" in the last paragraph),

ACKNOWLEDGEMENTS (when relevant)

REFERENCES must contain current and relevant articles of the topic in which it is inserted.

REFERENCES

Referring to quotations throughout the text. They should be presented in chronological order and they should follow the example:

1 author:

- At the beginning of the sentence:

"According to Freire (2009) variations on intra-abdominal pressure does not promote ..." or "Variations on intra-abdominal pressure does not promote hemodynamic changes, as described by Freire (2009) ..."

- At the end of the sentence:

"Variations on the intra-abdominal pressure does not promote hemodynamic changes (FREIRE, 2012)."

2 authors:

- At the beginning of the sentence:

"Based on the observations of Mangusto and Silva (1999), there is no glomerular alterations after the application ..." or "glomerular changes are observed after application of enrofloxacin, a fact verified by Mangusto and Silva (1999)."

At the end of the sentence:

"After the application of enrofloxacin glomerular changes are not observed (MANGUSTO and SILVA, 1999). "

More than 2 authors:

- At the beginning or middle of the sentence:

"Almeida et al. (2012) reported that the effects on ventilatory parameters ... "or" The effects on the ventilatory parameters have been described by Almeida et al. (2012) checking ... "

-At the end of the sentence:

"... Changes were not observed on the ventilatory parameters (SANTOS et al. 2011; ALMEIDA et al. 2012)

REFERENCES

Must be submitted at the end of the work (except in the form "What is your diagnosis?") In alphabetical order following the format below.

Complete scientific journal articles:

Examples

- 1 author:

Kästner SB. 2006. A2-agonists in sheep. *Veterinary Anaesthesia and Analgesia*. 32(2):79-96.

- 2 authors:

Bouchenafa O, Livingston A. 1987. Autoradiographic localisation of alpha 2 adrenoceptor binding sites in the spinal cord of the sheep. *Research Veterinary Science*. 42(3):382-386.

- More than 3 authors:

Crivellenti LZ, Silveira MP, Silva AN. et al. 2014. Transrectal bladder prolapse secondary to pelvic fracture in two dogs. *Journal of Small Animal Practice*. 55(8):424-426.

Abstracts published in Annals

The criteria for the inclusion of the authors follow the same pattern for scientific papers, first being the name (s) of author (s), year of publication, title of the work, event name, volume (when there is more than one), city, country and page.

Examples:

Crivellenti LZ, Silva GEB, Borin-Crivellenti S. et al. 2015. Glomerulopathies in dogs with erlichiosis - preliminary results. In. *40th World Small Animal Veterinary Association* – WSAVA. Bangkok, Tailândia, p. 76.

Honsho CS. 2013. Ocular effects of the retrobulbar block with different local anesthetics in healthy dogs. In 4*4th Annual Meeting of the American College of Veterinary Ophthaomologists*. Rio Grande, Puerto Rico. 16(6), p. 83.

Books:

The criteria for the inclusion of references to books, follow the same pattern for scientific papers, first being the name (s) of author (s) of the chapter, year of publication, chapter title, author (s) of the book , name of the book, edition, city editor, publisher and pages.

Examples:

Crivellenti LZ, Borin-Crivellenti S. 2015. *Casos de Rotina em Medicina Veterinária de Pequenos Animais*. MedVet Ltda, São Paulo, p. 840.

Book Chapter:

Example:

Cortopassi SRG, Mattos Junior E. 2014. Técnicas anestésicas utilizadas no exame ultrassonográgico. In: Carvalho C.F. *Ultrassonografia em pequenos animais*. 2. ed. São Paulo: Roca. pp. 41-60.

Electronic Documents:

Must inform the origin of the document (Internet or CD), with authors' names (if any, following the same criteria for scientific papers) or the name of the responsible institution, year, title of publication, website (internet) or bonds annals with event name, city and country of realiação (CD) and data access (internet).

Examples:

Doc. eletrônico (internet): *Veterinary Anesthesia & Analgesia Support Group*. 2003. Newer options for chronic pain management. 1 p. Disponível em: http://www.vasg.org [Acessado em 03/2015].

Doc. eletrônico (CD): Borges L.P. et al. 2014. Analgésicos opioides não potencializam os efeitos sedativos da dexmedetomidina em ovinos. In: *Anais do XI Congresso Brasileiro de Anestesiologia Veterinária*. (Águas de Lindóia, Brasil). 1 CD-ROM.

Theses / dissertations

Mattos Junior E. 2008. A*valiação biespectral, cardiorrespiratória e hemogasométrica em cadelas submetidas a ovariosalpingohisterectomia, tratadas com acepromazina associada ou não a meperidina e anestesiadas com halotano, isofluorano ou sevofluorano*. 175f. São Paulo, SP. Dissertação (Mestrado em Clínica Cirúrgica Veterinária) - Programa de Pós-graduação em Clínica Cirúrgica Veterinária, Universidade de São Paulo.

ANEXO 2 – Normas do periódico Arquivo Brasileiro de Medicina Veterinária e Zootecnia

Types of articles accepted for publication

Scientific article

This is a complete report of an experimental work. It is based on the premise that the results are posterior to the planning of the research.

Text sections: Title (Portuguese and English), Authors and Affiliation (only on the "Title Page – step 6), Resumo, Abstract, Introduction, Material and Methods, Results, Discussion (or Results and Discussion), Conclusions, Acknowledgements (when applicable), and References.

The number of pages must not exceed 15, including tables, figures **and References.**

The number of References must not exceed 30.

Sections of an article

Title. In Portuguese and in English. Must contemplate the essence of the article and not go beyond 50 words.

Authors and Affiliation. **Authors and Affiliation**. The names of the authors are placed below the title, with identification of the institution to which they belong. The corresponding author and their email must be indicated with and asterisk, only in the "Title Page" (step 6) in Word.

Resumo and Abstract. Must be the same presented in the registration, with up to 200 words and one paragraph. Do not repeat the text and do not add literature revision. Include the main numerical results, mentioning them without explanation, when applicable. Each sentence must contain a complete information.

Palavras-chave and Keywords. Up to five and at least two*.
***** in the submission use only the Keyword (step 2) and in the body of the article mention the keyword (English) and palavra-chave (Portuguese), regardless of the language the article is submitted in.

Introduction. Brief explanation in which the problem, its pertinence and relevance, and the aims of the work are established. It must contain few references, sufficient to define it.

Material and Methods. Mention the experimental design, the material involved, the description of the methods used or correctly reference the methods already published. In the work that involves animals and/or genetically modified organisms **there must be the number of the CEUA approval Certificate**. (verify the Ethics Committee Item).

Results. Present the results found in a clear and objective manner.

Table. Group of alphanumerical data ordered in lines and columns. Use horizontal lines in separating headers and at the end of the table. The title of the table receives the word Table, followed by an Arabic numeral and period (ex.: Table 1.). In the text the table must be referred to as Tab, followed by a period and order number (ex.: Tab. 1), even when referring to several tables (ex.: Tab. 1, 2, and 3). It may be presented with simple spacing and a font below size 12 (the smallest accepted size is 8). The Table legend must contain only that which is indispensable for its understanding. The tables must be inserted in the body of the text, preferably after the first citation.

Figure. Any illustration that presents lines and dots: drawing, picture, graphic, flow chart, scheme, etc. The legend initially receives the word Figure, followed by the Arabic numeral and period (ex.: Figure 1.) and is referred to in the text as Fig followed by a period and the order number (ex.: Fig.1), even when referring to more than one figure (ex.: Fig. 1, 2, and 3). Besides being inserted in the text, photos and images must be sent in high resolution jpg, in a zipped file, attached in the correct field in the submission screen for article registration. The figures must be inserted in the body of the text, preferably after the first citation.
***Note**: Every table and/or figure that has already been published must contain, below the legend, information regarding the source (author, authorization for use, date) and the corresponding reference must be in the References.*

Discussion. Discuss only the results obtained in the work. (Obs.: The sections Results and Discussion may be presented as one according to the author´s preference, without prejudice to the parts).

Conclusions. The conclusions must be supported by the results of the executed research and be presented in an objective manner, **WITHOUT** literature review, discussion, repetition of results or speculation.

Acknowledgements. Optional. Must be expressed briefly.

References. The references must be related in alphabetical order, preferring articles published in national and international magazines, and indexed. Books and thesis must be referenced as little as possible, and only when indispensable. The general ABNT norms are adopted, **adapted** for ABMVZ as the examples below:

How to reference:

1. Citations in the text

The indication of the source in parenthesis comes before the citation to avoid interruptions in the sequence of the text, as the examples:

- Single author: (Silva, 1971) or Silva (1971); (Anuário..., 1987/88) or Anuário... (1987/88)
- Two authors: (Lopes and Moreno, 1974) or Lopes and Moreno (1974)
- More than two authors: (Ferguson *et al*., 1979) or Ferguson *et al*. (1979)
- More than one article cited: Dunne (1967); Silva (1971); Ferguson *et al*. (1979) or (Dunne, 1967; Silva, 1971; Ferguson *et al*., 1979), always in ascending chronological order, and alphabetical order of the articles for articles of the same year.

Citation of a citation. Every effort must be made to consult the original document. In exceptional situations the reproduction of information already cited by other authors may be reproduced. In the text, cite the last name of the author of the non consulted document with the year of publication, followed by the expression **cited by** and the last name of the author and year of the consulted document. In the References only the consulted source should be mentioned.

Personal communication. These are not part of the References. The citation should include the last name of the author, the date of the communication and name of the Institution to which the author is linked.

2. Periodicals (up to 4 authors, cite all of them. More then 4 authors, cite 3 authors and *et al.*):

ANUÁRIO ESTATÍSTICO DO BRASIL. v.48, p.351, 1987-88.

FERGUSON, J.A.; REEVES, W.C.; HARDY, J.L. Studies on immunity to alphaviruses in foals. *Am. J. Vet. Res*., v.40, p.5-10, 1979.

HOLENWEGER, J.A.; TAGLE, R.; WASERMAN, A. et al. Anestesia general del canino. *Not. Med. Vet.*, n.1, p.13-20, 1984.

3. Single publication (up to 4 authors, cite all of them. More then 4 authors, cite 3 authors and *et al.*):

DUNNE, H.W. (Ed). Enfermedades del cerdo. México: UTEHA, 1967. 981p.

LOPES, C.A.M.; MORENO, G. Aspectos bacteriológicos de ostras, mariscos e mexilhões. In: CONGRESSO BRASILEIRO DE MEDICINA VETERINÁRIA, 14., 1974, São Paulo. *Anais*... São Paulo: [s.n.] 1974. p.97. (Resumo).

MORRIL, C.C. Infecciones por clostridios. In: DUNNE, H.W. (Ed). Enfermedades del cerdo. México: UTEHA, 1967. p.400-415.

NUTRIENT requirements of swine. 6.ed. Washington: National Academy of Sciences, 1968. 69p.

SOUZA, C.F.A. *Produtividade, qualidade e rendimentos de carcaça e de carne em bovinos de corte*. 1999. 44f. Dissertação (Mestrado em Medicina Veterinária) – Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte.

4. Electronic documents (up to 4 authors, cite all of them. More then 4 authors, cite 3 authors and *et al.*):

QUALITY food from animals for a global market. Washington: Association of American Veterinary Medical College, 1995. Disponível em: <http://www. org/critca16.htm>. Acessado em: 27 abr. 2000.

JONHNSON, T. Indigenous people are now more cambative, organized. Miami Herald, 1994. Disponível em: <http://www.summit.fiu.edu/ MiamiHerld-Summit-RelatedArticles/>. Acessado em: 5 dez. 1994.

ANEXO 3 – Normas do periódico Veterinary and Comparative Orthopaedics and Traumatology

General Guidelines

 You must submit a digital copy of your manuscript. Hard copy submissions are not accepted.

 Keep the format of your manuscript simple and clear. We will set your manuscript according to our style—do not try to “design” the document.

 The manuscript, including the title page, abstract and keywords, text, references, figure captions, and tables should be typewritten, double-spaced in 12-point font with 1-inch margins all around and saved as one file.

 Each figure should be saved as its own separate file. Do not embed figures within the manuscript file. This requires special handling by Schattauer’s Production Department.

 Keep abbreviations to a minimum and be sure to explain all of them the first time they are used in the text.

 The manuscripts should be written in UK English.

 The authors should use Système International (SI) measurements. For clarity, nonmetric equivalents may be included in parentheses following the SI measurements.

 Use generic names for drugs. You may cite proprietary names in parentheses along with the name and location of the manufacturer.

 Credit suppliers and manufacturers of equipment, drugs, and other brand-name material mentioned in the manuscript within parentheses, giving the company name and primary location.

MANUSCRIPT FORMAT

The following is a list of formatting requirements for submitted manuscripts. Papers that deviate from this will be returned with a request for changes, and will not undergo review until these changes have been made. Each of the following sections should be submitted as a separate document: a) Title Page b) Main document containing – Summary + Main Text + References, Legends, Tables, Figures. Word or Rich Text Format files should be used for the manuscript files; gif, jpeg, tif, or eps should be used

for all image files; and word or excel for all Tables. Do not embed Figures or Tables in the text of the manuscript.

Title Page

Should include all author names and affiliations, correspondence author and contact information, Acknowledgments, Funding, Author Contributions and Conflict of interest statements.

Formatting

Continuous line numbering should be used throughout the text along with doubleline spacing.

Blinding

All identification information should be removed from the paper for double blind peer review. This includes author names, initials, institutions countries and cities, as well as information which may appear in radiographs or other images. Either “Blinded” or “XX” can be used in the text for any places where this information was.

Animal Care

A section detailing the perioperative care which was given should be included, if relevant, as well as whether institutional approval was gained and what guidelines were followed. Please see the “Animal Care Guidelines”.

Character Count

Total character count for your main text should not exceed the allowed limits. Please see the “Article Types” section for this.

Author Contributions Form

Following the first online submission of your manuscript, the Corresponding Author should fill out the Author Contributions form on behalf of all

Abstract and Keywords

See the section Article Types for word limits.

The abstract should briefly outline the content of the article and any conclusions it may reach. It should be structured as follows: Objective, Study Design, Results, Conclusion.

The keywords should be words a reader would be likely to use in searching for the content of the article.

Main Document

 Please clearly distinguish the hierarchy of headings within the manuscript by using capital letters, underline, italic, and bold styles as necessary.

 As needed, use italic, superscripts, subscripts, and boldface, but otherwise do not use multiple fonts and font sizes.

 Do not insert page or section breaks except where noted in the Author Instructions.

 Use hard returns (the Enter key) only at the end of a paragraph, not at the end of a line. Allow lines of text to break automatically in your word-processing software. Do not justify your text.

 Use only one space, not two, after periods.

 Create tables using the Table function in Microsoft Word.

 For Original Research, Clinical Communications, & Brief Communications, the manuscript should divided into sections, including an Introduction, Materials and Methods, Results, and Discussion. The most important sections within each main section should be stressed by subheadings.

 Review Articles should have an Introduction, and then the appropriate section headings in bold.

 For Case Reports, please include an Introduction followed by a Discussion. Additional section headings can be included.

Formulas

Special care should be taken with the presentation of formulas, especially complex ones. In order to save formulas into your document in a manner that will ensure their accurate appearance in the proof generated by the system, create the formulas as text or use the “Formula” toolbar. Alternatively, upload as a separate document and refer to the formula as you would a Figure or Table.

Acknowledgments

Scientific advice, technical assistance, and credit for financial support and materials may be grouped in a section headed ’Acknowledgements’. Those who do not qualify for authorship should also be included here. This section will be placed at the very end of the text. For submission however, please place this information with the Title page.

Funding

Authors should provide all relevant information regarding the funding which was received, including any provision of experimental materials, equipment, writing

assistance, or related. It should also be stated what role the research funder had, for instance, whether they were also involved in other aspects of the study such as the design. This information will be published with the paper, should it be accepted. If no funding was received, please state this.

Conflict of Interest

Upon Submission, it is required to indicate on the Title page for each author if there is, or has been a situation where a conflict of interest could be construed. This includes both financial and personal relationships that might bias or be seen to bias their work. Each author should also acknowledge the source of any extra-institutional funds or support. Any financial interests in companies that market material that are, or have been, the subject of research reported in the manuscript should be acknowledged. Such information may or may not be held in confidence while the paper is under review, and should the article be accepted for publication, this information will be published with the paper.

Style Specifics

 Contributions should be submitted in UK English; this however is not a requirement, and if the paper is accepted, the Editorial Office will make all necessary changes. For non-English speaking authors, it may be of benefit to use a English editing firm to help in improving the English usage.

 Abbreviations should be spelled out for the first use followed by the abbreviation in parentheses; thereafter the abbreviation can be used. The use of abbreviations however should be kept to a minimum.

 Nomenclature should be done according to internationally approved rules. All anatomical nomenclature should be written in full and Anglicized.

 Units of measurement should be given in the metric system or in SI units and temperatures should be in °C

 For instruments, specific equipment, or drugs which are referred to in your paper, please cite the specific information (model number if relevant, generic and trade name, manufacturer and their location) as a footnote using roman letters at the end of the paper or as footnotes in the text.

 Figures and Tables should be cited in sequential order, in parentheses, in the text. The actual file for each figure and table should be named according to its number in the text (i.e. Figure 1, Table 2).

 Greek letters, special characters, & mathematical symbols should be insert using the “Symbol” or “Formula” toolbar menu in your word processing program. Before submitting your manuscript, please verify in the system-created pdf that all have converted correctly.