EMILY CORRENA CARLO REIS

DESENVOLVIMENTO DE MEMBRANAS RÍGIDAS E REABSORVÍVEIS E SUA APLICAÇÃO NA REGENERAÇÃO PERIODONTAL

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae*.

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RESUMO

REIS, Emily Correna Carlo, D. Sc., Universidade Federal de Viçosa, fevereiro de 2011. **Desenvolvimento de membranas rígidas e reabsorvíveis e sua aplicação na regeneração periodontal**. Orientadora: Andréa Pacheco Batista Borges. Co-orientadores: Ricardo Junqueira Del Carlo, Maria Verônica de Souza e Lissandro Gonçalves Conceição.

A doença periodontal apresenta alta prevalência em cães apresentando progressiva destruição dos tecidos periodontais de suporte, ou seja, osso alveolar, ligamento periodontal e cemento. Assim, diversos materiais objetivando a regeneração periodontal foram desenvolvidos e estão em uso, incluindo as membranas para regeneração tecidual guiada (RTG) e a aplicação de enxertos e biomateriais. Recentemente, novas possibilidades surgiram com o desenvolvimento das membranas multifuncionais para RTG. Neste trabalho, dois tipos diferentes de membranas multifuncionais foram desenvolvidos e avaliados *in vivo*. O primeiro tipo de membrana reabsorvível e rígida desenvolvida utilizou a associação da hidroxiapatita ao polihidroxibutirato. Neste, três membranas foram fabricadas pelo método de moldagem por injeção com diferentes proporções de hidroxiapatita, 25, 35 ou 50%. Após a conformação com broca odontológica, elas apresentaram uma superfície de complexidade microtopográfica com poros, estruturas lineares e grânulos de hidroxiapatita expostos. Em seguida, as membranas contendo 25 e 35% de hidroxiapatita foram testadas na regeneração de defeitos periodontais de furca classe II induzidos em cães. Os defeitos tratados com as membranas foram comparados a defeitos que não as receberam, coletando-se o dente com os tecidos periodontais nos dias 60 e 120 após a cirurgia para análise por tomografia micro-computadorizada e histologia. Observou-se, clinicamente, a exposição das membranas à cavidade oral em associação à recessão gengival em torno do oitavo dia após a cirurgia. Apesar de regeneração parcial ter sido observada no grupo tratado, não houve diferença significativa entre os grupos, provavelmente devido à contaminação dos defeitos tratados. O segundo tipo de membrana multifuncional desenvolvido foi denominado biomaterial em bicamada por seu design com uma camada lisa e outra de grande complexidade topográfica, apresentando macro, micro a nanoporos. Ela foi fabricada em constituição trifásica com a associação do ácido poli(láctico-co-glicólico) ao fosfato de cálcio e também foi avaliada em defeitos

periodontais de furca classe II induzidos em cães. A regeneração foi analisada clinicamente, seguida da coleta de material compreendendo o dente e tecidos de suporte aos 60 e 120 dias após a cirurgia para análise por tomografia micro-computadorizada e histologia, comparando-se defeitos tratados com o biomaterial em bicamada com defeitos sem a aplicação do biomaterial. Foi observada diferença significativa no volume de tecido ósseo regenerado e número, espessura e distância entre as trabéculas ósseas em ambas as datas de avaliação. A presença de ligamento periodontal e cemento foram observados juntamente com o tecido ósseo. Portanto, o biomaterial em bicamada favoreceu a regeneração periodontal, enfatizando-se neste trabalho a formação de osso alveolar na região vestibular do defeito.

ABSTRACT

REIS, Emily Correna Carlo, D. Sc., Universidade Federal de Viçosa, February of 2011. **Development of rigid resorbable membranes and their application on periodontal regeneration**. Adviser: Andréa Pacheco Batista Borges. Co-advisers: Ricardo Junqueira Del Carlo, Maria Verônica de Souza and Lissandro Gonçalves Conceição.

Periodontal disease is highly prevalent in dogs, which presents progressive destruction of the periodontal supportive tissues, i.e., alveolar bone, periodontal ligament and cementum. Thus, many materials aiming at regenerating the periodontium were developed and are now in clinical use, including membranes for guided tissue regeneration (GTR) and the application of grafts and biomaterials. Recently, new possibilities arose with the development of multifunctional membranes for GTR. In the present work, two different multifunctional membranes were developed and evaluated *in vivo*. The first type of resorbable and rigid membrane developed used the association of hydroxyapatite and polyhydroxybutyrate. With these materials, three membranes were manufactured by injection molding with different proportions of hydroxyapatite, 25, 35 or 50%. After grinding them with a dental bur, their surface was characterized as a complex microtopography by having pores, linear structures and exposed hydroxyapatite granules. Next, the membranes containing 25 and 35% hydroxyapatite were tested on the regeneration of periodontal class II furcation defects induced in dogs. Defects treated with the membranes were compared to the ones that did not receive them, collecting the teeth and periodontal tissues at 60 and 120 days after surgery for micro-computed tomography and histologic analysis. Clinically, membranes were exposed to the oral cavity in association to gingival recession around the 8th day after surgery. Although partial regeneration was observed in treated group, no significant difference between groups was found, probably due to the contamination of treated defects. The second type of multifunctional membrane developed was called bilayered biomaterial for its design: a flat layer coupled to another layer presenting a surface with a high topographical complexity with macro, micro and nanopores. It was manufactured in a three phase constitution with the association of polylactide-*co*-glycolide acid to calcium phosphate and was also evaluated on the regeneration of class II furcation defects induced in dogs. The regeneration was clinically evaluated and the teeth with the surrounding periodontium were collected at 60 and 120 days after surgery for micro-

computed and histologic analysis. Defects treated with the bilayered biomaterial were compared to defects that did not receive the biomaterial. Significant difference was observed for the volume of the regenerated bone and the number, thickness and distance of the trabeculae. Periodontal ligament and cementum were observed along with bone. Therefore, the bilayered biomaterial favored periodontal regeneration, here emphasizing the formation of alveolar buccal bone.

Capítulo I – Introdução geral

Regeneração periodontal em cães

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Regeneração de defeitos periodontais em cães

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Treatment of periodontal defects has gained importance in small animals veterinary medicine, mainly in dogs, as periodontal disease is highly prevalent among these animals which suffer its severe consequences. Periodontal regeneration is a complex process involving the formation of three tissues highly connected: alveolar bone, periodontal ligament and cementum. Therefore, several materials and techniques were and are constantly developed for regeneration of periodontal defects. These include membranes for guided tissue regeneration and the application of bone grafts and biomaterials, widely studied in human dentistry and already available for veterinary practice. Additionaly, new possibilities raise with the association of these techniques to growth factors and stem cells and the development of multifuncional membranes.

INDEX TERMS: veterinary dentistry, periodontal disease, guided tissue regeneration, membranes

RESUMO.- O tratamento de defeitos periodontais tem adquirido maior importância na medicina veterinária de pequenos animais, principalmente nos cães, visto que a doença periodontal apresenta alta prevalência nesta espécie com suas graves conseqüências. A

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regeneração periodontal é um processo complexo que envolve a formação de três tecidos intimamente ligados: osso alveolar, ligamento periodontal e cemento. Assim, diversos materiais e técnicas foram e são constantemente desenvolvidos para utilização em defeitos periodontais objetivando sua regeneração. Estes incluem as membranas na regeneração tecidual guiada e a aplicação de enxertos ou biomateriais, amplamente estudados na odontologia humana e já disponíveis para aplicação na rotina clínica veterinária. Adicionalmente, novas possibilidades surgem com a associação dessas técnicas a fatores de crescimento e células-tronco e o desenvolvimento das membranas multifuncionais.

TERMOS DE INDEXAÇÃO: odontologia veterinária, doença periodontal, regeneração tecidual guiada, membranas

INTRODUÇÃO

A doença periodontal tem ganhado importância na medicina veterinária, sendo amplamente discutida em encontros e congressos nacionais e internacionais (Marreta 2001). Conseqüentemente, sua forma de tratamento tem sido estudada e as técnicas disponíveis ampliadas, passando da única opção, a profilaxia periodontal com extrações dentárias, a cirurgias gengivais para impedir a progressão da doença até as mais recentes técnicas para regeneração periodontal. Cada vez mais, o médico veterinário deve estar ciente das novas opções de tratamento, que estão em constante evolução.

Assim, o objetivo desta revisão foi contextualizar o problema, ou seja, a doença periodontal, e abordar seu tratamento com ênfase na mais recente opção, a regeneração periodontal, abordando seus princípios e apresentando uma importante parcela do que já foi realizado, o que está hoje disponível e quais são as tecnologias em desenvolvimento.

A DOENÇA PERIODONTAL

A palavra “periodontal” se refere aos tecidos que dão suporte ao dente e o mantém na mandíbula ou maxila, incluindo o cemento, ligamento periodontal, osso alveolar e gengiva. A doença periodontal se inicia pelo acúmulo de bactérias imóveis, gram positivas e aeróbias na região cervical do dente, ou seja, a região da coroa dentária que se localiza mais próxima ao sulco gengival, formando a placa bacteriana (Harvey 1998, Marreta 2001). Em resposta a esse acúmulo, inicia-se um processo inflamatório no sulco gengival, com aumento da permeabilidade vascular (Dennison & Van Dyke 1997) e infiltração de neutrófilos, seguidos de macrófagos e linfócitos (Ohlrich et al. 2009), levando à gengivite que já é clinicamente detectável. Se o acúmulo da placa bacteriana persiste, bactérias predominantemente móveis, gram negativas e anaeróbias proliferam, persistindo a inflamação e liberação de citocinas e enzimas que levam à destruição dos tecidos periodontais, momento em que este processo é chamado doença periodontal (Harvey 1998, Wiggs et al., 1998). Alguns dos mediadores do processo inflamatório responsável pela destruição destes tecidos são interlecina 1, o fator de necrose tumoral e a prostaglandina E, que, por exemplo, estimulam osteoclastos e diminuem a proliferação de progenitores de osteoblastos levando à reabsorção óssea (Ohlrich et al. 2009), e estimulam a síntese de metaloproteinases que agem na degradação do ligamento periodontal (Reynolds & Meikle 1997).

A doença periodontal tem ganhado importância por sua alta prevalência, com um dado consagrado entre os profissionais da área: 85% dos cães acima de três anos de idade apresentam doença periodontal, sendo esta a doença mais comum na espécie (Harvey 1998, Marreta 2001). Estudos no Brasil demonstraram prevalências que corroboram esta afirmativa, 71,4% (Milken et al. 2003) e 92,5% (Venturini et al. 2007)

nas regiões da grande São Paulo e Uberlândia-MG, respectivamente, e 88,7% em análise na população do Hospital Veterinário da Universidade Federal de Viçosa (dado ainda não publicado). É importante notar sua alta prevalência em animais idosos, uma vez que a doença pressupõe o acúmulo de placa bacteriana, e por conseqüência, um período de tempo para tal, além de ser também mais prevalente em cães de raças de pequeno do que de grande porte (Harvey et al. 1994).

As conseqüências locais e sistêmicas da doença periodontal também contribuem para sua importância. Localmente, devido à destruição progressiva do periodonto são formados sulcos gengivais mais profundos, chamados bolsas periodontais, permitindo o maior acúmulo da placa bacteriana. Com a progressão da doença, a perda dentária é a conseqüência local mais comum, com prejuízo nas funções de preensão e mastigação de alimentos. Nos casos mais avançados da doença, onde existe grande reabsorção óssea, são comuns a formação de comunicações oronasais e a ocorrência de fraturas patológicas de mandíbula, principalmente, nos animais de raças pequenas (Harvey 1998, Wiggs et al. 1998). As fraturas patológicas de mandíbula são de especial importância por serem de difícil manejo clínico-cirúrgico (Legendre 2003) e apresentarem prevalência considerável, correspondendo a 13% das etiologias de fraturas de mandíbula em estudo realizado no Brasil (Lopes et al. 2005).

A doença periodontal também apresenta efeitos sistêmicos, uma vez que a placa bacteriana acumulada é uma constante fonte de bactérias que, penetrando na circulação sanguínea, pode afetar órgãos internos (Harvey 1998). A doença está associada a afecções cardíacas, pulmonares, articulares, renais e hepáticas (DeBowe et al. 1996). Estudo realizado por Pavlica e colaboradores (2008) analisou alterações anatomopatológicas em cães e estimou que, para cada centímetro quadrado de tecido

afetado pela doença periodontal, existe 40% mais chance da presença de alterações cardíacas e renais e 20% vezes mais chance de serem observadas alterações hepáticas.

TRATAMENTO

O tratamento da doença periodontal requer, inicialmente, a remoção da placa bacteriana causadora do problema e envolve uma série de manobras agrupadas na profilaxia periodontal. Esta compreende a curetagem supra- e sub-gengival (sulco gengival e bolsa periodontal), aplainamento radicular, polimento das superfícies dentárias e lavagem do sulco gengival ou bolsa periodontal para remoção de debris (Marreta 2001, Niemic 2008). A profilaxia periodontal pode ser complementada por diferentes técnicas como o acesso cirúrgico às raízes de dentes com bolsas periodontais maiores do que 5mm (Grove 1998) e a gengivectomia e gengivoplastia objetivando a redução da profundidade de bolsas periodontais (Marreta 2001). Apesar de efetivas na descontaminação local e na eliminação da inflamação, estas técnicas resultam na formação de um longo epitélio juncional recobrindo araiz dentária e na ligação da gengiva com o periodonto remanescente por tecido fibroso. Estes tecidos não são capazes de exercer as funções sensorial, nutricional e de suporte mecânico necessárias a um periodonto saudável. Portanto, ocorre reparação periodontal e não a regeneração, que deve compreender por definição, a formação de novos cemento, ligamento periodontal e osso alveolar (Christgau et al. 2007).

A principal técnica desenvolvida para promover a regeneração periodontal é a regeneração tecidual guiada (RTG), na qual uma membrana de origem natural ou sintética é implantada entre a gengiva e a raiz dentária e osso alveolar expostos,

cobrindo estes dois últimos (Wikesjö et al. 1995, Araújo et al. 1997, Araújo et al. 1999, Christgau et al. 2007). Inicialmente, acreditava-se que células provenientes dos tecidos epitelial e conjuntivo da gengiva ocupavam o defeito periodontal rapidamente, formando um longo epitélio juncional e tecido fibroso, o que impediria a formação de novo osso, ligamento periodontal e cemento. Assim, as membranas agiriam bloqueando os tecidos da gengiva e permitindo a regeneração (Robert & Frank 1994). Atualmente, acredita-se que as membranas atuam estabilizando o coágulo sanguíneo na região do defeito e mantendo este espaço até a migração das células progenitoras adequadas (Wikesjö et al. 2003; Christgau et al. 2007). Essas células progenitoras são as células-tronco mesenquimais localizadas nas proximidades e ao redor dos vasos sangüíneos em todo o organismo, neste caso, do ligamento periodontal e osso alveolar remanescentes próximos ao defeito. Assim, essa células podem migrar e se depositar tanto ao longo da raiz dentária para a formação de novos cemento e ligamento periodontal quanto em todo o volume da matriz provisória para a formação de novo osso (McCulloch et al. 1987, Araújo et al. 1999; Ivanovski 2009). Este conceito de “manutenção do espaço de um defeito” não é novo, ele já é amplamente conhecido quando se fala de regeneração óssea e está mais recentemente sendo abordado na regeneração periodontal (Wikesjö et al. 2003). Assim deve-se utilizar este conceito para a regeneração do osso alveolar pois este tecido necessita de um arcabouço na forma e volume adequados para sua regeneração, formado pela matriz provisória estável. Neste contexto, alguns autores testaram o preenchimento de defeitos periodontais com diferentes materiais osteocondutores. Contudo, acredita-se que no periodonto a estabilização do defeito é importante também para que a matriz provisória se mantenha aderida à superfície da raiz dentária, influenciando grandemente a formação do cemento e ligamento periodontal (Wikesjö et al. 1995, Christgau et al. 2007).

REGENERAÇÃO PERIODONTAL NA ODONTOLOGIA VETERINÁRIA

Não são muitos os trabalhos objetivando a regeneração periodontal na medicina veterinária e menor ainda é o número de trabalhos que realizaram análises histológicas, a única forma de comprovar a regeneração dos três tecidos periodontais de suporte (Araújo et al. 1999, Christgau et al. 2007).

Relatos de casos e estudos clínicos demonstraram bons resultados clínico-radiográficos, com a diminuição da bolsa periodontal pela diminuição do nível clínico de inserção (medida do fundo da bolsa periodontal até a junção cemento-esmalte) e aumento na radiopacidade e altura ósseas de defeitos periodontais. Foram utilizados nestes estudos o enxerto autógeno (Smith 1995), o biovidro particulado (DeForge 1997), a associação da matriz orgânica de osso associada ao peptídeo sintético de adesão celular P-15 (Ferro & Gioso 2009) e proteínas obtidas da matriz do esmalte (Watanabe et al. 2003). Os resultados permitem afirmar que houve melhora clínica resultante da formação de novo aparato de adesão ao dente, mas não se pode afirmar que houve regeneração, visto que a adesão pode ser proporcionada pelo longo epitélio juncional e tecido fibroso e não pelos tecidos periodontais (Araújo et al. 1999, Christgau et al. 2007).

Os resultados de trabalhos que analisaram histologicamente a regeneração periodontal são conflitantes, provavelmente, devido tanto à diferença dos materiais utilizados quanto aos tipos de defeitos periodontais analisados. Fófano et al. (2005) e Carlo et al. (2006) analisaram membrana de colágeno em defeitos periodontais de tamanho padronizado no osso alveolar, sendo que os primeiros associaram-na à matriz óssea bovina mineralizada. Ambos observaram que os tratamentos propostos auxiliaram a regeneração alveolar no início do processo. Já Shoukry et al. (2007) não observaram

benefício na utilização de enxerto ósseo autógeno associado a membrana amniótica canina em comparação com apenas a profilaxia periodontal de rotina. Ainda, Watanabe et al. (2001) analisaram a formação de cemento, osso alveolar e ligamento periodontal utilizando proteínas da matriz do esmalte em um defeito apical após a apicectomia experimental, observando o favorecimento da regeneração.

No cão e no homem a doença periodontal apresenta aspectos semelhantes, desde sua etiologia e fisiopatologia (Giannobile et al. 1994), como nos tecidos envolvidos, características clínicas como a formação de bolsas periodontais (Giannobile et al. 1994, Wikesjö et al. 2003), a morfologia dos defeitos periodontais ocorridos naturalmente e características de reparação e regeneração (Araújo et al. 1999, Christgau et al. 2007). Poucas características diferem, como por exemplo, a ocorrência mais freqüente de defeitos de furca classe III e o fato da remodelação óssea ocorrer em velocidade aproximadamente um terço (1/3) mais rápida no cão, mas, em geral, sem grandes influências na comparação entre as espécies (Giannobile et al. 1994). Portanto, numerosos estudos em cães têm sido realizados utilizando-os como modelos experimentais e ambasando a aplicação clínica de técnicas de regeneração periodontal.

RTG NO MODELO EXPERIMENTAL CÃO

A principal técnica estudada e utilizada para promover a regeneração periodontal é a RTG, algumas vezes associada a outros materiais, como o preenchimento do defeito com enxertos (Mardas et al. 2003) ou materiais sintéticos (Ivanovski 2009) e a aplicação de derivados da matriz do esmalte (Watanabe et al. 2003) ou fatores de crescimento (Wikesjö et al. 2003, Ivanovski 2009).

Para a RTG, as membranas mais estudadas até o momento são as de politetrafluoretileno expandido (ePTFE), utilizadas com sucesso na regeneração periodontal e de uso predominante na odontologia humana. Trata-se de um material com propriedades mecânicas adequadas, pois é facilmente posicionado sobre o defeito periodontal contornando suas bordas e ocluindo-as (Macedo et al. 2006, Roriz et al. 2006, Christgau et al. 2007). Em geral, são capazes de manter o espaço do defeito dependendo da sua morfologia, como defeitos intra-ósseos com três paredes ósseas (Sirgudsson et al. 1994, Kim et al. 2004). Para utilização naqueles que apresentam uma ou duas paredes ósseas e nos defeitos supra-alveolares, essas membranas estão também disponíveis reforçados por uma estrutura ou tiras de titânio mantêm a forma desejada, obedecendo ao princípio de manutenção do espaço do defeito (Sirgudsson et al. 1994, Peled et al. 2002, Kim et al. 2004, Sculean et al. 2008). Sua principal desvantagem é não ser reabsorvível, requerendo um segundo procedimento cirúrgico para sua remoção, tendo ainda o potencial de afetar os tecidos em regeneração (Roriz et al. 2006).

Para superar este problema, muitas membranas foram fabricadas com materiais reabsorvíveis como colágeno (Cirelli et al. 1997, Christgau et al. 2007), polidioxanona (Christgau et al. 2007), ácido polilático (Robert & Frank 1994, Amano et al. 2004, Christgau et al. 2007) e o ácido poli(láctico-co-glicólico) (PGLA) (Araújo et al. 1997, Araújo et al. 1999). Um fator de essencial importância para o desenvolvimento de membranas reabsorvíveis é seu tempo de reabsorção *in vivo,* que não deve ocorrer antes de oito a 12 semanas (Cirelli et al. 1997). Estudos demonstraram que o tempo de reabsorção de membranas de colágeno variou de acordo com o processo de fabricação, estando em torno de duas a 10 semanas (Minabe 1992) e quatro a oito semanas (Cirelli et al. 1997), sendo mais longo para membranas de ácido polilático, de três a quatro meses (Robert & Frank 1994).

Em geral, membranas reabsorvíveis apresentaram bons resultados depois que sua taxa de degradação foi estabelecida, resultados superiores aos obtidos apenas com a profilaxia periodontal (Bosshardt & Sculean 2009). Christgau et al. (2007) estudaram em cães a expressão de componentes da matriz extracelular na regeneração de defeitos de furca classe II (profundos mas mantêm a parede lingual) utilizando uma membrana não-reabsorvível e três reabsorvíveis. A regeneração periodontal foi observada, sendo que o tipo de membrana não influenciou o padrão de expressão dos componentes e a regeneração dos tecidos periodontais. Contudo, a quantidade de regeneração foi negativamente influenciada pela contaminação destas e pela sua falta de estabilidade. De fato, a capacidade de manutenção do espaço do defeito destas membranas é inadequada, com inúmeros relatos de colapso. Em geral as membranas reabsorvíveis apresentam suas propriedades mecânicas desfavoráveis, de modo que a membrana aplicada sobre o defeito colapsa e ocupa o espaço que deveria estar mantendo, o que prejudica a regeneração, sendo este um fator limitante para sua utilização (Cirelli et al. 1997, Chang & Yamada 2000, Wikesjö et al. 2003, Christgau et al. 2007). Com o objetivo de impedir este colapso, alguns autores utilizaram-nas com fixações ao osso alveolar por pinos e parafusos, com bons resultados (Imbronito et al., 2002, Amano et al. 2004).

Na tentativa de melhorar os resultados, procurou-se incorporar antibacterianos às membranas ou associá-las a os fatores de crescimento e enxertos e biomateriais utilizados para o preenchimento de defeitos, com resultados variados. Uma vez demonstrado que a contaminação bacteriana é um fator limitante para a regeneração periodontal, o metronidazol (Kurtis et al. 2002) e a doxiciclina (Chang & Yamada 2000, Lyons et al. 2008) foram incorporados a membranas reabsorvíveis de PGLA ou ácido poliláctico, objetivando a liberação lenta e constante destes antibacterianos. Contudo, o

benefício deste tipo de associação foi demonstrado apenas no estudo que utilizou a doxiciclina em membrana de PGLA (Chang & Yamada 2000).

O preenchimento do defeito periodontal com enxertos, autógeno ou alógeno, e biomateriais foi proposto com base nos princípios da regeneração óssea, com o objetivo principal de recuperar a altura óssea (Mardas et al. 2003, Taheri et al. 2009). Os resultados são variáveis dependendo muito da morfologia do defeito periodontal (Mardas et al. 2003, Duarte et al., 2006, Taheri et al. 2009, Tsiomis et al., 2010). O preenchimento de defeitos de furca com enxertos ou biomateriais não contribuiu para uma maior quantidade de tecidos regenerados, sendo freqüente a ocorrência de anquilose e reabsorção da raiz dentária. Contudo, defeitos com morfologia menos favorável à manutenção do espaço, como os intra-ósseos com uma ou duas paredes ósseas, podem se beneficiar com maior formação óssea (McClain & Schallhorn 2000, Mardas et al. 2003, Kim et al. 2004, Roriz et al. 2006, Taheri et al. 2009).

Diferentes substâncias foram utilizadas para favorecer a regeneração periodontal, também com resultados variáveis. As proteínas da matriz do esmalte em associação com membrana reabsorvível não proporcionou um aumento na quantidade de tecidos regenerados em relação ao uso apenas da membrana (Araújo & Lindhe 1998, Fernandes et al. 2005). Contudo, as características morfológicas do cemento na região apical do defeito se mostraram mais semelhantes às do cemento não afetado pela doença periodontal, incluindo a formação de ambas as camadas, celular e acelular do cemento (Araújo & Lindhe 1998). Já as associações de membranas com a proteína morfogenética óssea recombinante (rBMP-2) e com o fator de crescimento derivado de plaquetas demonstraram ser superiores à utilização apenas das membranas em defeitos de furca e supra-alveolares (Park et al. 1995, Wikesjö et al. 2003). Estes defeitos são considerados de difícil regeneração, pois tem uma morfologia desfavorável à estabilidade da

membrana além de apresentarem pouco tecido ósseo remanescente próximo a eles, uma das fontes de células progenitoras para este processo.

Apesar de todo o avanço em tais terapias com consequente obtenção de maiores quantidades de tecidos regenerados, ainda não se conseguiu a regeneração completa de defeitos periodontais, o que significaria todos os tecidos periodontais formados nas regiões vestibular, lingual, mesial, distal e furca em extensão igual ao que existia antes da doença (Araújo & Lindhe 1998, Wikesjö et al. 2003, Christgau et al. 2007, Bosshardt & Sculean, 2009).

PERSPECTIVAS

Técnica já estabelecida e amplamente utilizada na rotina, a RTG caminha agora para outro estágio, onde as membranas serão multifuncionais e não apenas com a função de estabilizar o defeito (Inanç et al. 2009, Park et al. 2009).

Diversos estudos realizados com biomateriais demonstraram a influência da topografia de superfície na regeneração óssea. Esta influência vai dos primeiros estágios, onde superfícies mais complexas aumentam a adesão de plaquetas e fibrina (Park & Davies 2000, Mendes et al. 2009), até os estágios finais de deposição de matriz óssea, onde estas superfícies também favorecem a migração de osteoblastos, por meio da osteocondução (Cho et al. 2009). Sendo assim, pesquisadores têm desenvolvido membranas com superfícies interna apresentando topografia complexa, onde a que estará em contato com o defeito deverá favorecer a osteocondução e a que estará em contato com a gengiva deverá favorecer sua adesão (Cho et al. 2009, Santana et al. 2010).

Estas novas membranas estão sendo chamadas multifuncionais porque sua estrutura física e constituição química são desenvolvidas para apresentar algumas, mas nem sempre todas, as seguintes funções: oclusão da borda superior do defeito para impedir a migração do epitélio juncional na direção apical; maior adesão da gengiva à sua superfície externa, diminuindo assim a possibilidade de ocorrer recessão gengival; propriedades mecânicas adequadas à manutenção do espaço e estabilização da matriz provisória; favorecimento da osteocondução; além de serem reabsorvíveis (Cho et al. 2009, Inanç et al. 2009, Park et al. 2009, Santana et al. 2010).

A estrutura e constituição dessas novas membranas diferem bastante. Algumas membranas foram desenvolvidas com materiais poliméricos como o PGLA, em um molde contendo ranhuras e sulcos de magnitude microscópica impressos em sua superfície (Owen et al. 2005). Outras membranas foram fabricadas com nanofibras com a combinação de diferentes biomateriais como o PGLA, a policaprolactona, nanotubos de carbono e hidroxiapatita (Mei et al. 2007, Cho et al. 2009). Outros pesquisadores optaram pela fabricação de membranas em duas ou três camadas com diferentes biomateriais, porosidades e espessuras, como as associações do PGLA com o ácido hialurônico (Park et al. 2009) e hidroxiapatita, colágeno e PGLA (Liao et al. 2005).

Mais recentemente, a utilização de terapias celulares tem ganhado atenção, com diferentes abordagens. Uma opção é a engenharia de tecidos com o cultivo de células diretamente sobre a membrana a ser aplicada sobre o defeito periodontal, como em estudo que analisou a obtenção de células do ligamento periodontal e sua cultura em membranas de nanofibras de PGLA (Inanç et al. 2009). Outra abordagem é o preenchimento do defeito periodontal pela aplicação direta de células-tronco mesenquimais, como nos recentes estudos de Wei et al. (2010) e Liu et al. (2010).

Células-tronco autógenas provenientes da medula óssea de cães foram cultivadas e aplicadas em defeitos periodontais agudos. Analisadas após seis semanas de tratamento, estas células foram observadas no osso alveolar, ligamento periodontal, cemento e vasos sanguíneos, exibindo marcadores de superfície típicos de osteoblastos e fibroblastos, mas a análise da quantidade de tecidos regenerados não foi realizada (Wei et al. 2010). De forma semelhante, Liu et al. (2010) utilizaram células-tronco do ligamento periodontal, também com resultados promissores.

Devido a gama de materiais utilizados e às diferentes morfologias de defeitos, a regeneração tecidual guiada apresenta resultados muito variáveis, mesmo assim, é a técnica que mostrou os melhores resultados até o momento. Atualmente, as membranas mais prontamente disponíveis são as de politetrafluoretileno expandido, seguidas de algumas membranas reabsorvíveis e biomateriais para o preenchimento do defeito. Apesar de ainda não ser possível a completa regeneração do periodonto, importante melhora clínica pode ser conseguida com tais técnicas. Sendo assim, há muito a ser realizado quando se fala de regeneração periodontal, observando-se boas perspectivas na utilização das membranas multifuncionais e na terapia com células-tronco.

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Capítulo II

Desenvolvimento e caracterização de membranas rígidas, osteocondutoras e reabosrvíveis de polihidroxibutirato e hidroxiapatita para regeneração periodontal

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Resumo

A regeneração tecidual guiada (RTG) é uma técnica que utiliza membranas para favorecer a regeneração tecidual, inculindo os tecidos periodontais perdidos devido à doença periodontal. As membranas hoje utilizadas ainda apresentam limitações, principalmente quanto à capacidade de manter o espaço do defeito. Assim, dois biomateriais de origem brasileira, a hidroxiapatita (HAP) e o polihidroxibutirato (PHB) foram utilizados para fabricar membranas rígidas para RTG, contendo 25, 35 ou 50% de HAP em matriz de PHB pelo método de moldagem por injeção. As membranas apresentaram alta cristalinidade, sendo que, inicialmente, a topografia de superfície era pouco complexa, sem exposição de grânulos de HAP. As membranas foram desgastadas com broca odontológica, como realizado nas cirurgias para implantação *in vivo.* Este procedimento resultou na exposição dos grânulos de HAP em uma superfície com poros e estruturas lineares de dimensões que variaram de 3,55µm ±1,14 a 75,98µm ±30,76, portanto. Observou-se, ainda, que a HAP é responsável por conferir uma topografia de superfície mais complexa às membranas. Portanto, espera-se que membranas com as características de rigidez e superfície microtopográfica possam proporcionar estabilidade ao defeito periodontal e permitir uma maior migração celular, assim, favorecendo a regeneração periodontal.

Palavras-chave: *regeneração tecidual guiada, hidroxiapatita, polihidroxibutirato, compósito, topografia de superfície.*

Development and characterization of rigid, resorbable and osteoconductive membranes made of polyhydroxybutyrate and hydroxyapatite for periodontal regeneration.

Abstract

Guided tissue regeneration (GTR) is a technique that applies membranes to favor the regeneration of periodontal tissues lost due to periodontal disease. However, they have some limitations, mainly related to the ability to stabilize the defect. Thus, two biomaterials of Brazilian origin, hydroxyapatite (HAP) and polyhydroxybutyrate (PHB) were used to make rigid membranes for GTR. Membranes were made of 25, 35 or 50% HAP in PHB matrix by injection molding. The membranes were highly crystalline, with an initial smooth surface topography where HAP particles were not exposed. Membranes were grinded with a dental bur, like it will be done during surgery for their implantation in vivo. This procedure resulted on the HAP granules being exposed on a surface with pores and linear features of dimensions varying from 3.55µm ±1.14 to 75.98µm ±30.76, thus, characterized as microtopography. Additionally, HAP was responsible for a greater surface complexity. Therefore, these characteristics of rigidity and surface microtopography may be able to provide stability to a periodontal defect and allow a greater cell migration, both favoring periodontal regeneration.

Keywords: *guided tissue regeneration, hydroxyapatite, polyhydroxybutyrate, composite, surface topography.*

Introdução

A regeneração tecidual guiada (RTG) é uma técnica consagrada no tratamento da doença periodontal visando a regeneração dos tecidos de suporte do dente perdidos no processo da doença, ou seja, osso alveolar, ligamento periodontal e cemento[1-3]. Ela pressupõe a utilização de membranas entre o retalho mucogengival e o osso alveolar e/ou raiz dentária expostos, cobrindo estes dois últimos e assim, protegendo o coágulo sanguíneo formado na região do defeito. Desta forma, com a organização do coágulo em matriz provisória, células progenitoras provenientes do ligamento periodontal e osso alveolar adjacentes ao defeito podem migrar. Estudos demonstram que essa origem das células é decisiva para a formação de novos tecidos periodontais e não de um tecido conjuntivo de reparação. Sendo assim, estas células são capazes de se depositar e se diferenciar em osteoblastos, fibroblastos e cementoblastos para o processo de regeneração periodontal[1-3].

Membranas de politetrafluoretileno são utilizadas na RTG com bons resultados, mas possuem como desvantagem a necessidade de uma segunda intervenção cirúrgica para sua remoção, pois não são reabsorvíveis[4]. Assim, membranas reabsorvíveis de diversos materiais, como colágeno e ácido polilático, têm sido desenvolvidas e utilizadas. Contudo, possuem como limitação a pequena capacidade de manter o espaço do defeito, ou seja, elas são excessivamente flexíveis e colapsam para dentro do defeito, ocupando-o e interferindo na formação do coágulo sanguíneo. Assim, a regeneração periodontal, principalmente do osso alveolar fica limitada, uma vez que as células não têm volume de matriz provisória necessário para migrarem, se depositarem e formarem novos tecidos. A regeneração dos tecidos periodontais é dependente da capacidade da membrana manter o espaço do defeito, sendo um fator decisivo para a regeneração periodontal[1-3].

Mais recentemente, membranas foram desenvolvidas com superfícies modificadas para favorecer a regeneração dos tecidos periodontais[5,6]. Em geral, a superfície interna da membrana, que estará em contato com o coágulo sanguíneo, é trabalhada química ou fisicamente para favorecer a adesão de fibrina da matriz provisória, plaquetas e osteoblastos[5-9]. Um exemplo é a membrana de ácido poli(láctico-co-glicólico) (PGLA) desenvolvida por Owen et al. [5] que utilizaram um molde contendo ranhuras e sulcos de magnitude microscópica para a dispersão do polímero líquido que, após a secagem e retirada do molde, dá origem a uma membrana com tais estruturas impressas em sua superfície. Ainda, pode-se citar a utilização do ataque ácido para aumentar a complexidade topográfica de uma superfície de titânio[7,8] e a adsorção de fibronectina para favorecer a adesão de osteoblastos[9].

Assim, diversos biomateriais têm sido utilizados no desenvolvimento de membranas, principalmente os polímeros. Ainda não utilizado para essa finalidade, o PHB é um polímero natural biodegradável e biocompatível, pertencente ao grupo dos polihidroxialcalonatos [9-12]. Suas características mais importantes para utilização como biomaterial são sua lenta reabsorção, compatível com a regeneração tecidual e ótimas propriedades mecânicas em relação ao tecido ósseo [12-14]. Tem sido testado principalmente na fabricação de arcabouços / suportes para a regeneração óssea e fabricação de parafusos e placas para osteossíntese, com bons resultados tanto no que diz respeito às propriedades mecânicas quanto às características de superfície e porosidade para a migração celular que são fatores depentes da forma de fabricação[9,11-13].

As associações de outros biomateriais com os polímeros têm sido cada vez mais utilizadas para agregar novas propriedades a eles, os polímeros, principalmente as cerâmicas. A hidroxiapatita (HAP) é o principal constituinte mineral do tecido ósseo,

podendo ser extraída desta fonte ou obtida sinteticamente. Desta forma, a HAP sintética, uma cerâmica biocompatível e osteocondutora, é amplamente utilizada para o preenchimento de defeitos ósseos, servindo como arcabouço para a migração de células osteoprogenitoras. Ela é considerada bioativa, formando uma ligação direta com o tecido ósseo, sem interposição de tecido fibroso[11,13,15,16]. A HAP é um material frágil devido à sua alta cristalinidade, o que também limita a fabricação de implantes constituídos somente dela. Assim, compósitos a partir da associação da HAP com polímeros, como PHB, têm sido desenvolvidos e analisados principalmente para a regeneração óssea servir como substituto temporário deste tecido, mostrando resultados promissores[11,12,17].

Desta forma, o presente trabalho relata o desenvolvimento e caracterização de membranas reabsorvíveis com dois biomateriais desenvolvidos com matéria-prima e tecnologia nacionais, a hidroxiapatita sintética e o polihidroxibutirato com as seguintes características: (i) uma membrana rígida capaz de manter sua forma, prevenindo seu colapso para dentro do defeito e mantendo o espaço para o desenvolvimento do coágulo; (ii) uma superfície interna de topografia complexa para permitir maior adesão de fibrina, plaquetas e células progenitoras, favorecendo assim, respectivamente, a manutenção do volume do coágulo sanguíneo, a sinalização que direciona a migração celular e a formação de novos tecidos. Assim, este trabalho objetivou desenvolver um novo biomaterial a partir da associação da HAP com o PHB para futuramente ser utilizado na regeneração de defeitos periodontais.

Material e métodos

Um protótipo foi desenvolvido com base as características anatômicas da face vestibular da mandíbula de cães, os futuros modelos experimentais *in vivo*, nas

seguintes dimensões: 10mm de largura x 10mm de comprimento x 1,5mm de espessura na extremidade inferior x 2,5mm de espessura na extremidade superior. Assim, postiços foram fabricados em aço ferramenta em cromo-níquel de alta resistência com cavidades usinadas por eletroerosão de acordo com estas dimensões. A hidroxiapatita sintética (HAP-91®, JHS Laboratório Químico, Belo Horizonte) foi previamente misturada ao PHB nas proporções em peso: 25% de HAP em 75% PHB (membrana 1), 35% de HAP em 65% de PHB (membrana 2) e 50% de cada biomaterial (membrana 3). Cada mistura foi separadamente inserida na máquina injetora para a fabricação das membranas pelo processo de moldagem por injeção, a pressão de 40 libras e temperatura entre 120 e 130°C.

A cristalinidade das membranas foi analisada por difração de raios X. As análises foram realizadas em três amostras de cada membrana em difratômetro da marca Rigaku D-Max modelo Geiger Flex equipado com tubo de cobalto (radiação Co-Kα, λ = 1,79026 Å), com um monocromador de cristal curvo de grafite no feixe difratado, operado com diferença de potencial de 40 kV e corrente elétrica de 30 mA. As varreduras foram realizadas no modo passo a passo em intervalo de 15 a 50º 2θ com 0.05º de incremento e 2 segundos de contagem de tempo em cada passo.

Para análise morfológica, foi utilizado inicialmente um microscópio eletrônico de varredura (MEV) da marca LEO 1430VP, a voltagens de 10 ou 15 kV. Para tal, as superfícies foram cobertas por uma camada de 20 nm de ouro, utilizando um sistema de deposição “sputtering balzers” (Electron Microscopy Sciences, modelo550x). Foi também utilizado MEV ambiental (Hitachi SU6600) para análise da topografia de superfície e micro-análise elementar por espectroscopia por energia dispersiva de raios-X (EDS). As análises morfológicas foram realizadas em cada uma das membranas 1, 2 e 3 nas superfícies (a) da forma como foi fabricada, (b) da superfície de fratura e (c) da

superfície após desgaste com broca odontológica. Esta última superfície foi incluída no estudo porque, no momento de implantação da membrana *in vivo*, o contorno interno das membranas será desgastado desta forma para obter-se a justaposição adequada entre a membrana e a superfície óssea da mandíbula.

Para estimar o diâmetro dos poros, comprimento e espessura de estruturas nas superfícies das membranas, foram obtidas 15 imagens em MEV por elétrons secundários de três amostras selecionadas de cada membrana 1, 2 e 3. Em imagens de 500x de magnificação, todas as estruturas existentes foram medidas no software Analisys 5 (Olympus BX51M). Com o mesmo software, mas em imagens obtidas à MEV por elétrons retroespalhados, as proporções de cristais de HAP e matriz de PHB presentes nas mesmas membranas foram estimadas utilizando-se a ferramenta de diferença de fases. Todos estes dados foram trabalhados na forma de média e desvio-padrão, comparando-se as membranas 1, 2 e 3 por ANOVA um critério complementado por Tukey quando necessário, com nível de significância a 5%.

Resultados e discussão

Os difratogramas das membranas 1, 2 e 3 são mostrados na figura 1. Notam-se os picos altos e agudos, todos de acordo com a literatura para a HAP e o PHB e consistentes com o CPDS Card para a HAP[17-19], demonstrando a alta cristalinidade dos materiais. Sabe-se que o PHB é um polímero semicristalino e que, portanto, dá origem a cristais e fases amorfas[19,20], ambas identificadas nos difratogramas e que a HAP, de estrutura cristalina hexagonal, apresenta normalmente alta cristalinidade[17-19]. A cristalinidade de um biomaterial é de grande importância uma vez que influencia seu desempenho *in vivo* por afetar a reabsorção e suas propriedades mecânicas. Sabe-se que suas regiões amorfas são reabsorvidas mais rapidamente do que as regiões cristalinas e

que, portanto, materiais mais cristalinos têm geralmente uma taxa de reabsorção mais lenta. Ainda, materiais altamente cristalinos, como a HAP, são geralmente mais frágeis por apresentarem menor módulo de elasticidade e assim, deformarem menos antes da fratura[11,14,16,20,21].

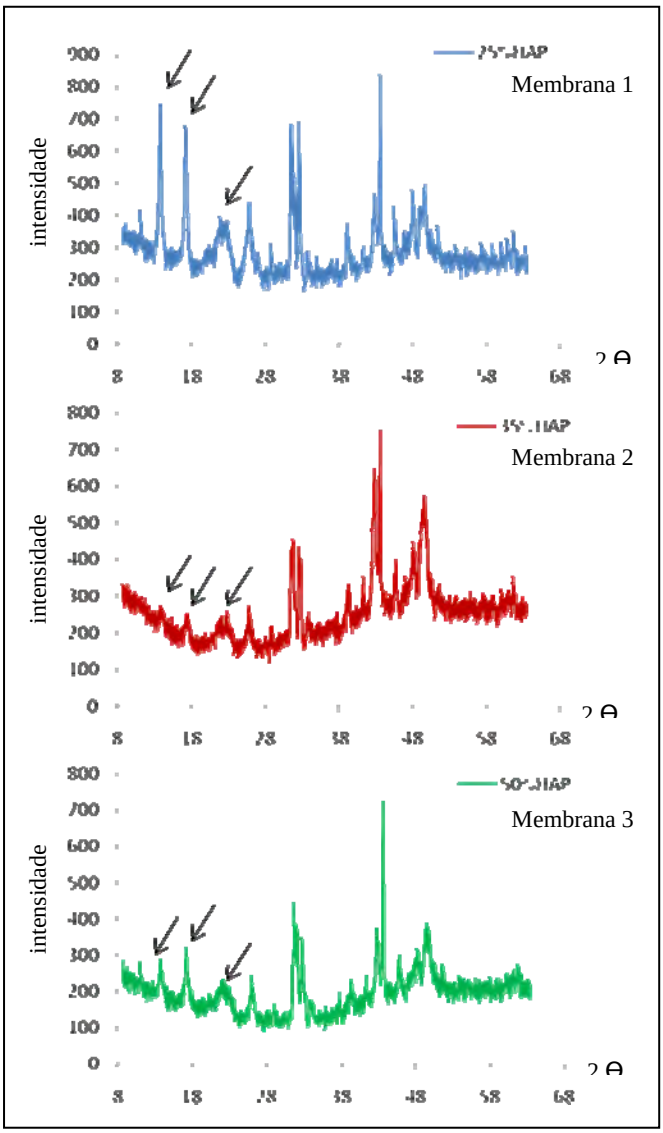

Figura 1. Difração de raios-X dos compósitos de HAP+PHB. As setas indicam os picos do PHB, os demais picos são da HAP.

A morfologia macroscópica da membrana pode ser vista na figura 2A. Para facilitar as manobras de desgaste com broca odontológica e a justaposição em uma região de anatomia muito variável (mandíbula, próxima ao dente em, um defeito periodontal), a superfície interna das membranas foi desenvolvida na forma côncava, com a extremidade superior mais espessa do que a inferior.

Na análise microscópcia da superfície (a), observou-se pequeno número de poros não interconectados (Figura 2B). Pode-se notar ainda, que os grânulos de HAP apesar de visíveis, não estavam expostos na superfície, pois estes estavam cobertos por uma fina camada do polímero (Figura 2C), assim como o observado por Mendonça et al.[9]. Esta superfície pode ser caracterizada como pouco complexa e, como tal, provavelmente não é a superfície ideal para a regeneração de tecidos por ser menos favorável à adesão do coágulo sanguíneo e adesão e atividades celulares[7,22].

As análises das superfícies de fratura das membranas (b) permitiram observar os grânulos de HAP em meio à matriz homogênea de PHB, sem a presença de poros internos em sua estrutura, ou seja, todas apresentavam uma estrutura compacta (Figura 2D). É importante notar que a estrutura interna do PHB pode apresentar poros com tamanhos que favorecem a osteocondução, definida como capacidade de um biomaterial de permitir ou favorecer a migração de células osteoprogenitoras[9,11,12,23] ou poros muito pequenos, que não permitem a osteocondução[13] e ainda pode não ser poroso[14] como aqui observado, características altamente influenciadas pelo método de fabricação[9,12,13].

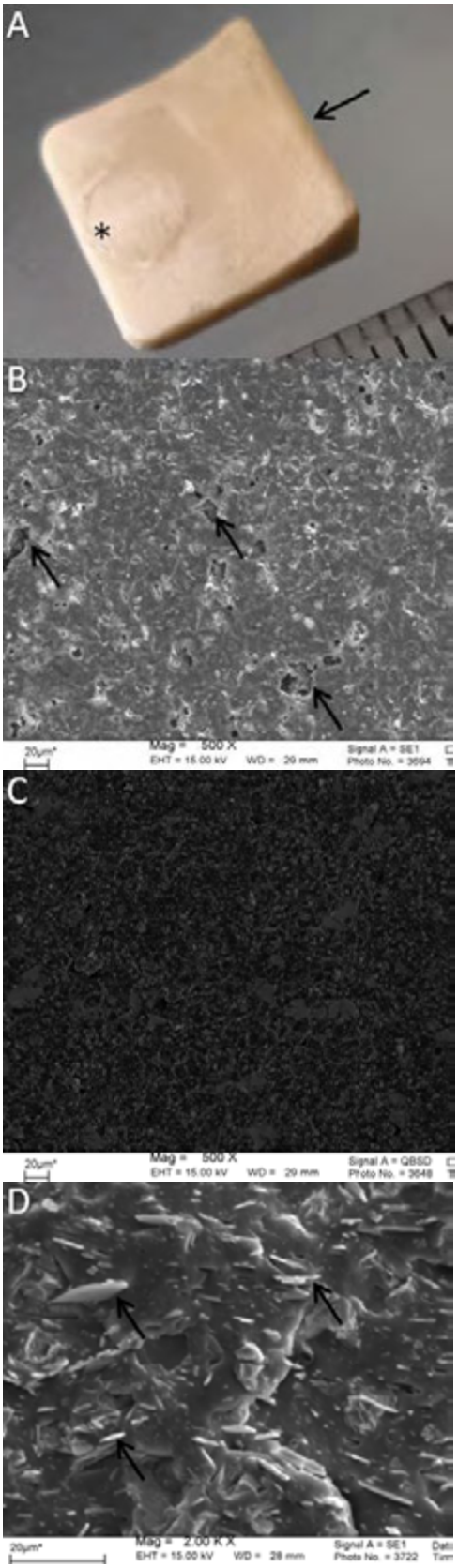

Figura 2. Membrana de PHB + HAP. A. Design da membrana desenvolvida com base nas características anatômicas da região periodontal, cuja extremidade superior (seta) é mais espessa do que a extremidade inferior (asterisco), formando uma concavidade. B e C. MEV da superfície da membrana de 50% HAP+PHB após fabricação. B. Técnica de elétrons secundários evidenciando superfície pouco complexa com poros não interconectados (setas). C. Técnica de elétrons retroespalhados demonstrando que os grânulos de HAP não estão expostos na superfície (ausência de grânulos brancos). D. Superfície de fratura de compósito 35% HAP+PHB. Cristais de HAP (setas indicam alguns deles) imersos na matriz do polímero PHB.

As superfícies das três membranas após a conformação com a broca odontológica (superfície c) apresentaram poros e estruturas lineares, sem diferenças significativas na distribuição, morfologia e tamanho destes elementos (Figura 3 e Tabela 1). Como estes elementos apresentaram tamanhos micrométricos, a topografia de superfície das membranas após o desgaste com broca odontológica é definida e denominada microtopográfica[7,8]. Essa característica favorece a regeneração de tecidos periodontais por diferentes ações: maior adesão da matriz de fibrina do coágulo sanguíneo, maior adesão e atividades plaquetárias e maior capacidade de osteocondução e adesão de osteoblastos[7,8,11,22,24]. Sabe-se que a matriz provisória formada pelo coágulo é o arcabouço para a formação de novos tecidos[1,4,]. Dessa forma, espera-se que tal topografia de superfície, mantenha o volume da matriz provisória por minimizar o processo de contração da matriz que normalmente ocorre durante a migração celular[7]. Assim, as células progenitoras poderiam ter um maior volume de arcabouço para migrarem e assim, regenerar o periodonto.

Por MEV com elétrons retroespalhados, pode-se observar também que os grânulos de HAP estavam expostos na superfície c (Figura 3 B, D e F). As membranas fabricadas com maior proporção de HAP em peso apresentaram área de HAP exposta nas superfícies significativamente maior ($p<0,01$): 10,6%±2,4 para membrana 3, 6,9%±0,9 para membrana 2 e 3,2%±0.6 para membrana 1. Diversos trabalhos abordam a bioatividade das cerâmicas como a HAP, definida pela formação de uma ligação química entre o biomaterial e o tecido ósseo, com consequente maior quantidade de tecidos regenerados[11,13,21]. Contudo, outros trabalhos demonstram que a adição de uma cerâmica a uma superfície aumenta a regeneração tecidual por conferir uma topografia de superfície mais complexa, independentemente de sua constituição química[7,8]. Por um destes mecanismos, ou talvez ambos, a adição de uma cerâmica a uma matriz

polimérica favorece a regeneração, formando uma interface direta entre o tecido ósseo e o biomaterial, determinando a importância de uma maior proporção de grânulos de HAP expostos na superfície das membranas[11,12,22].

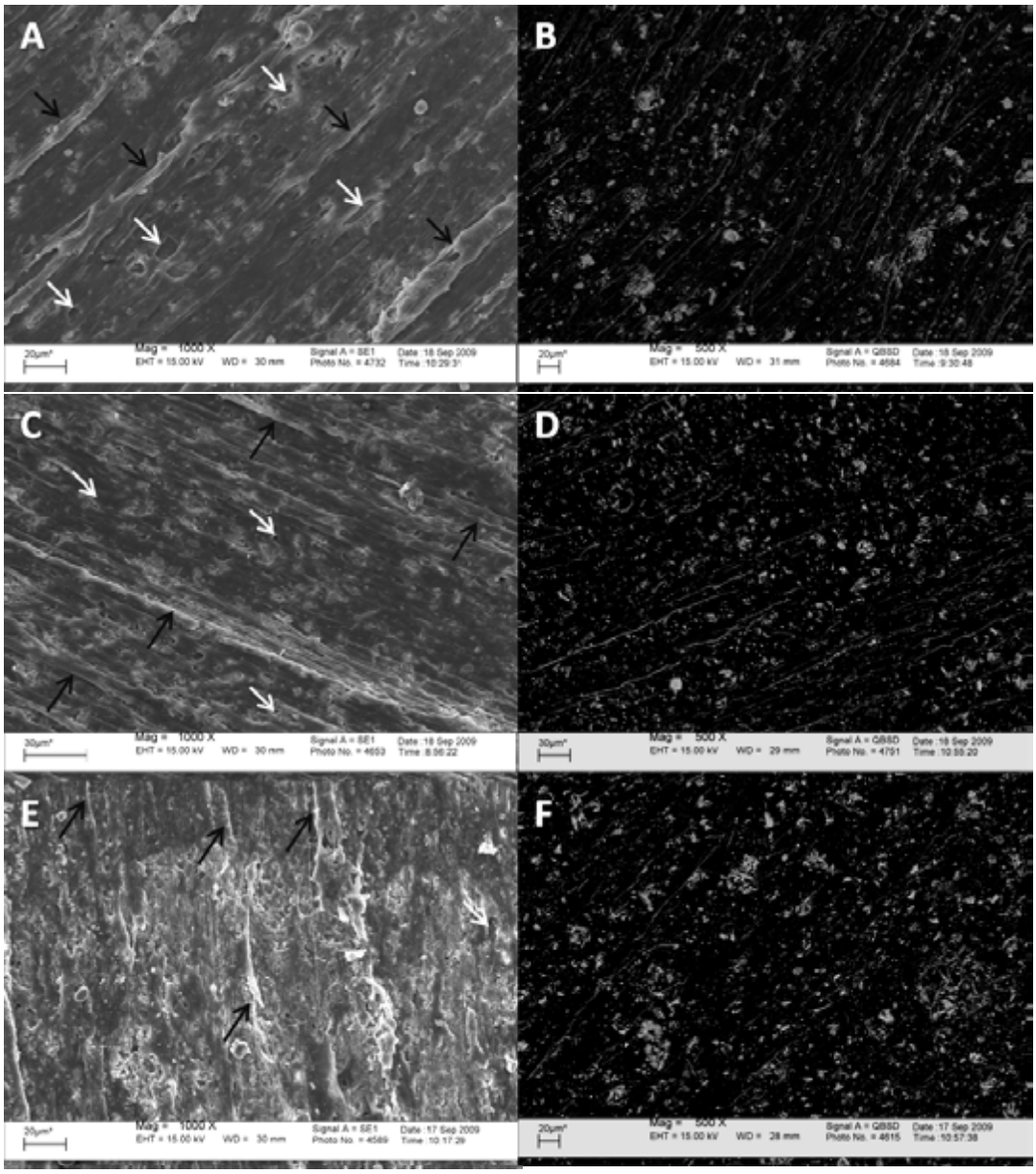

Figura 3. Imagens das superfícies dos compósitos de 25% (A, B), 35% (C, D) e 50% (E, F) de HAP+PHB após conformação com broca odontológica. A, C e E. MEV por elétrons secundários onde observam-se poros (setas brancas) e estruturas lineares (setas pretas) formando complexa microtopografia de superfície. B, D, F. MEV por elétrons retroespalhados, evidenciando a HAP exposta (pontos claros) nas superfícies dos compósitos em meio à matriz de PHB (matriz negra).

Tabela 1. Média e desvio padrão (em µm) do diâmetro dos poros e comprimento e largura dos elementos lineares na superfície das membranas após conformação com broca odontológica.

		Estruturas lineares (µm)	
	Poros (µm)	**comprimento**	**largura**
Membrana 1 (25% de HAP)	8,51 ± 3,50	73,35 ± 30,04	5,11 ± 2,47
Membrana 2 (35% de HAP)	6,61 ± 2,69	63,51 ± 27,45	3,55 ± 1,14
Membrana 3 (50% de HAP)	9,08 ± 4,11	75,98 ± 30,76	4,42 ± 2,28

A presença e proporção de HAP exposta nas superfícies das membranas influenciaram a topografia de forma com que essas superfícies puderam ser caracterizadas como microtopográficas. A figura 4 mostra os espectros de EDS para diferentes regiões observadas à MEV, algumas mais rugosas e complexas e outras mais lisas. Observou-se que as regiões rugosas continham altas concentrações de cálcio, fósforo e oxigênio, compatíveis com a HAP de fórmula $Ca_{10}(PO_4)_6(OH)_2$[11,22]. Já nas regiões lisas, altos picos de carbono foram observados, demonstrando que essas regiões eram constituídas pela matriz de PHB[11,22]. Essa diferença de topografia de superfície é evidente na figura 5, onde as regiões contendo HAP são mais complexas do que a lisa matriz de PHB. É importante notar que a matriz de PHB garantiu boa adesão aos grânulos de HAP, biomateriais que se mostraram assim compatíveis para a formação de compósitos. Ainda, o PHB foi encontrado em meio aos grânulos de HAP e não apenas em volta destes, provavelmente também pela boa afinidade do PHB com a HAP como também relatado em outros trabalhos[11,22].

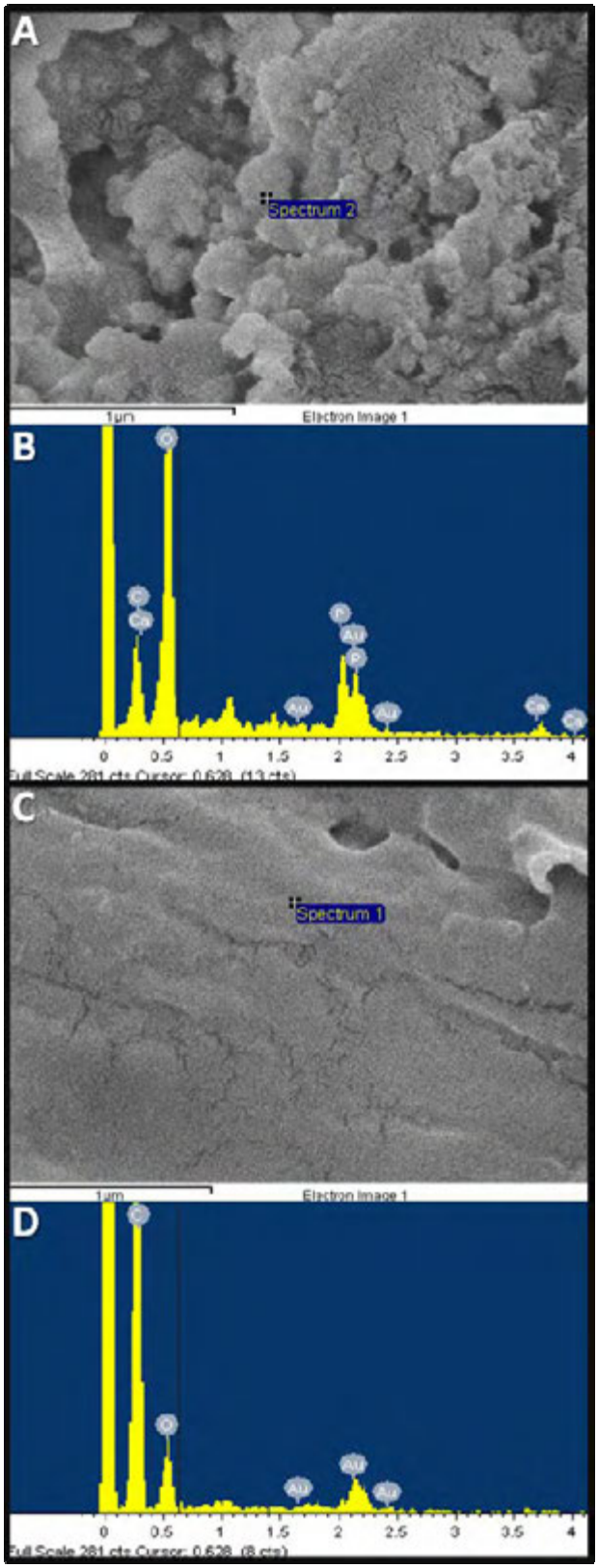

Figura 4. Espectrometria de energia dispersiva de amostras de compósito de HAP+PHB. A. Feixe concentrado na superfície rugosa da amostra (Spectrum 2), com composição química demonstrando ser este um grânulo de HAP (gráfico em B). C. Feixe concentrado em superfície lisa do compósito (Spectrum 1), com composição química compatível com polímero de origem natural (gráfico em D). (Au representa o ouro utilizado para recobrir o material para MEV).

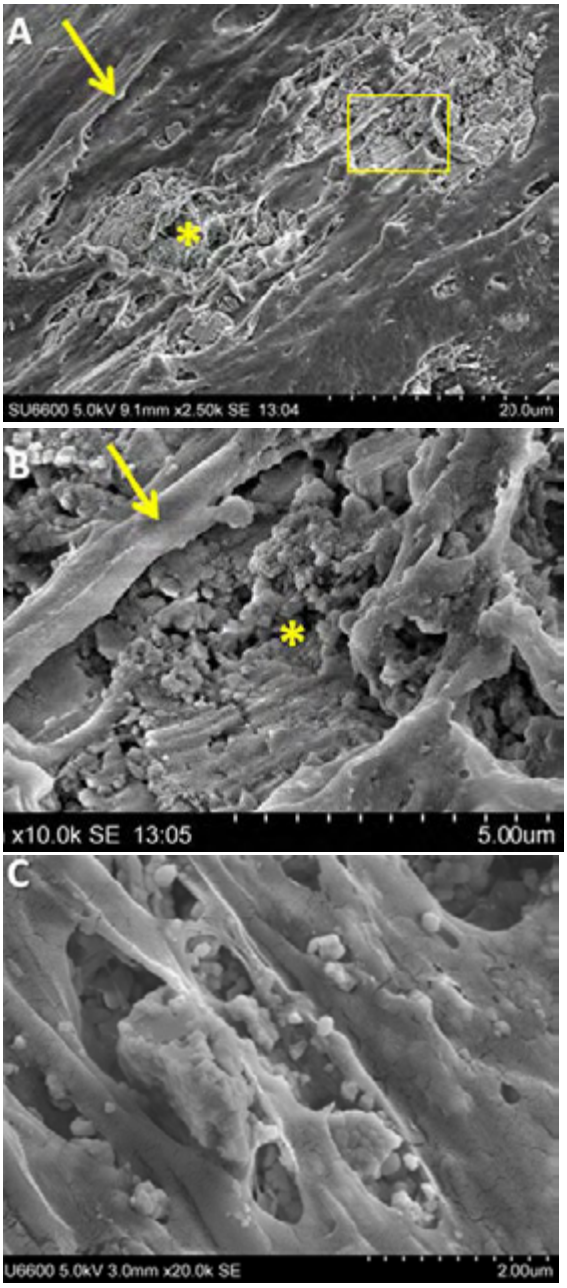

Figura 5. Fotos em MEV de superfícies dos compósitos de HAP+PHB. Compósito de 50% de HAP+PHB após desgaste com a broca odontológica. A. Observa-se a interação de grânulos rugosos de HAP (*) em meio à matriz lisa de PHB (seta) e a diferença na topografia de superfície dos dois biomateriais. B. Detalhe da área demarcada em A. Pode-se observar projeções do PHB (seta) em meio à superfície rugosa da HAP (*). C. Em maior aumento, observa-se a interação entre a matriz de PHB, lisa, e os grânulos de HAP.

Conclusões

Conclui-se que foi possível confeccionar membranas rígidas de compósitos de hidroxiapatita e polihidroxibutirato pelo método de moldagem por injeção. Estas membranas apresentaram alta cristalinidade e uma topografia de superfície com poros, estruturas lineares e grânulos de HAP expostos, caracterizada como microtopográfica, onde a hidroxiapatita influenciou positivamente a complexidade topográfica das

mesmas. Ainda, enfatiza-se a necessidade do desgaste da superfície das membranas com broca odontológica para expor os grânulos de hidroxiapatita, o que também contribui para uma maior complexidade topográfica.

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Capítulo III

Rigid resorbable membranes for periodontal regeneration in dogs

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Abstract

Background and objective: To favor periodontal regeneration, guided tissue regeneration (GTR) uses membranes to stabilize a defect area. However, most resorbable membranes collapse into the defect, impairing regeneration. Therefore, we developed resorbable but rigid membranes aiming at preventing such collapse and stabilizing the clot, and analyzed them in furcation defects.

Design: Experimental study

Methods: Procedures included periodontal disease induction, prophylaxis and GTR or open flap debridement alone. For GTR, the membranes were made of either 25% hydroxyapatite (HA) in polyhydroxybutyrate matrix (PHB) or 35% HA in PHB. Animals were clinically evaluated for gingival recession, clinical attachment level (CAL) and biopsies were collected at 60 and 120 days. Bone volume, trabeculae number, trabecular thickness and trabecular separation were quantified by micro-computed tomography, followed by qualitative histology.

Results: Membrane exposure was observed in both treated groups (25 and 35% HAP) usually starting on the 8th day after surgery, continuously progressing until 120 days. Mean CAL for all groups remained above normal values for dogs. Bone volumetric values were not significantly different. Partial formation of bone, cementum and periodontal ligament was observed in treated groups. An inflammatory infiltrate was observed in the dense connective tissue that partially filled the center of the treated defects with active osteoclasts on bone surface.

Conclusion: Although partial regeneration of the defect was observed, it was limited by wound contamination. Consequently, rigid resorbable membranes made of HA and PHB failed to improve the regeneration of class II furcation defects in dogs.

Introduction

Periodontal disease can be characterized by an inflammatory process of the periodontium caused by plaque accumulation, resulting on the destruction of alveolar bone, periodontal ligament and cementum.[1,2] As implied by accumulation, periodontal disease is a chronic condition directly influenced by age, where older animals tend to present more severe cases. Periodontal disease has become a very important issue in

small animals practice for different reasons: dogs are now reaching older ages, it is one of the most common diseases in these animals and it has local and systemic effects.[3,4] Locally, periodontium loss results, for example, on the formation of periodontal pockets (contaminated spaces between the inflamed gingiva and the exposed tooth), gingival recession, tooth mobility and ultimately, tooth loss.[1,5] As the periodontium becomes a continuous source of bacteria and pro-inflammatory mediators, periodontal disease has also been related to heart, kidney and liver disease.[6]

The treatment of periodontal disease comprises plaque and calculus removal by supra and sub-gingival scaling followed by root planing and tooth polishing, a sequence of procedures called prophylaxis. Other techniques such as gingivectomy and reverse bevel flap surgery are used to treat periodontal pockets greater than 6mm, while gingival grafts are used to increase gingival height where it is necessary.[2,5,7] Improvement in clinical parameters usually occur as an example, decrease in periodontal pockets depth (probing depth). However, it occurs by the formation of reparative connective tissues with the formation of a long junctional epithelium along the treated root.[8] Obviously, such reparative tissues do not restore the previous architecture of the periodontum and so, mechanical properties capable of supporting tooth functions are not re-established. Periodontal regeneration is defined by the formation of new cementum, periodontal ligament and bone, thus, prophylaxis is not a regenerative therapy.[8-10]

Studies on periodontal regeneration originated the theory that, with prophylaxis, epithelial and connective tissue cells from the gingiva would rapidly repopulate the defect area before cells from the surrounding alveolar bone and periodontal ligament. Thus, researchers focused on the development of physical barriers to prevent cells from the gingiva from occupying the defect.[9,11,12] This technique, guided tissue regeneration (GTR), showed better results both in clinical and histologic studies: greater gain in attachment level and decrease in probing depth than regular prophylaxis[12] with the formation of new supportive apparatus, i.e., cementum, periodontal ligament and bone also being demonstrated.[8-11,13]

At first, non-resorbable membranes were mostly used, best represented by the ones made of expanded polytetrafluoroethylene (ePTFE). Although pre-clinical and clinical studies showed good results, the need of a second surgery to remove the membrane has shifted the attention of researchers to the development of resorbable

membranes.[9,12] Such membranes are now being extensively analyzed in vitro and in pre-clinical studies, and many are already available for clinical application.[8,14-16] However, they are commonly softer than the non-resorbable ones, generating reports on its collapse into the defect.[17-19] In this case, little regeneration is achieved since the membrane occupies the area where tissue formation should be occurring. Associated to that, other works on GTR observed that space provision, i.e., the capacity of a membrane to stabilize the defect volume, is a determining aspect of the amount of regeneration achieved by such therapies.[20,21] Therefore, our group has developed a resorbable but rigid membrane to prevent such collapse into the defect, a space-providing device. This membrane is made by the association of two resorbable materials: the ceramic hydroxyapatite (HA) and the polymer polyhydroxybutyrate (PHB). The PHB matrix provides the membrane with its rigid stable form. HA particles were added in order to provide an inner topographically complex surface, an aspect known to favor fibrin attachment and platelet adhesion, favoring the regeneration.[22-24]

Materials and methods

The in vivo procedures were reviewed and approved by the Ethics Committee on Animal Research, Veterinary Department (Universidade Federal de Viçosa, Brazil). Ten healthy adult mongrel dogs, weighting about 10-15 kg and without periodontal disease were selected. The animals were fed soft dog food for the entire experiment. Three days prior to each surgical procedure, animals received 23.5 mg/kg oral spiramycin associated to 12.5 mg/kg dimetridazole a day (Spiraphar™, Virbac do Brasil, Brazil), medication that continued until the 3rd day after surgery.

The animals fasted for eight hours prior to anesthesia. Sedated with 0.1 mg/kg acepromazine IV, each animal was then anesthetized with 6 mg/kg propofol IV as an inducing agent and maintained with a mixture of oxygen and isoflurane. Chlorhexidine 0.12% was used to rinse the oral cavity of each dog pre and post-operative procedures.

Periodontal disease induction

Baseline clinical attachment level (CAL) was measured with a periodontal probe (PCP-UNC 15, Hu-Friedy, USA) from the cemento-enamel junction to the bottom of the gingival sulcus (or periodontal pocket), at the central area of the furcation of the

lower 3rd and 4th and upper 2nd left premolars. These teeth were selected as they are very similar in size, morphology and periodontal disease progression.[4,8,15] Also, standardized periapical radiographs were taken (Timex 70C, Gnatus, Brazil) prior to each of the procedures for a reference image of the furcation area.

Mucoperiosteal flaps were elevated to expose the buccal bone walls. A spherical diamond bur (FG1016, KG Sorensen, Brazil) was used to create class II furcation defects (deep furcation exposure preserving the lingual wall): 5mm apicocoronally (anatomical reference: cementum-enamel junction at the centre of the furcation), 5mm mesiodistally and 3mm buccolingually (anatomical reference: buccal aspect of the alveolar bone) (Figure 1A). Scaling was subsequently performed to remove periodontal ligament fibers and debris from root surfaces and the defects were completely filled with an impression material (Impregum Soft™, 3M do Brazil). The flaps were repositioned and closed with non-resorbable sutures (5-0 nylon suture, J&J Ethicon), and post-operative radiographs were taken.

Sutures were removed after 10 days and the impression material was kept in the defects for a total of 21 days. The material was removed under the same general anesthesia protocol and then a second CAL measurement was performed followed by prophylaxis, which comprised a surgical open flap procedure for scaling (Fig. 1B), root planing and irrigation with chlorhexidine 0.12%. After the flap was closed, a new set of radiographs was taken. Surgical wounds were rinsed with chlorhexidine 0.12% every 12 hours for two weeks.

Guided tissue regeneration (GTR)

Two weeks after prophylaxis, animals were anesthetized and new radiographs were taken. A surgical flap was raised once more and the defects were scaled to remove soft tissue. Each defect was measured in the apicocoronal, mesiodistal and buccolingual directions, dimensions further compared to the initial ones of 5 mm x 5 mm x 3 mm by one-way ANOVA. Using a round bur (FG1016, KG Sorensen, Brazil), notches were made on the surface of the roots to serve as future references of the apical limit of the defect.

The lower 3rd and 4th left premolars were assigned as the treated groups, receiving membranes made of 25% and 35% HA, respectively. Each HA+PHB membrane (approximately 1.0cm x 1.0cm x 1.5mm) was measured to cover the defect,

with its most coronal part immediately below the cementum-enamel junction. Next, the membrane was grinded with a round bur (FG1016, KG Sorensen, Brazil) to adjust its inner side to the contours of the alveolar bone at the measured site, with the margins extending approximately 3mm on mesial, distal and apical directions. The membrane was then placed covering the defect and fixed in place with a 1.5 mm x 6 mm screw (Ortovet, Brazil) positioned on the apical portion of the furcation area (Figure 1C). Finally, the flap was closed with non-resorbable sutures (5-0 nylon suture, J&J Ethicon). The same surgical procedure, including screw insertion, was repeated in the upper 2nd left premolar except for membrane placement, assigned as the control defect. Thus, each dog had two test groups and the control group (no membrane). Post-operative radiographs were taken immediately after the surgical procedure. Spiramycin (23.5 mg/kg/day) associated to dimetridazole (12.5 mg/kg/day) (Spiraphar™, Virbac do Brasil, Brazil) was administered until suture removal (10 days post-operative).

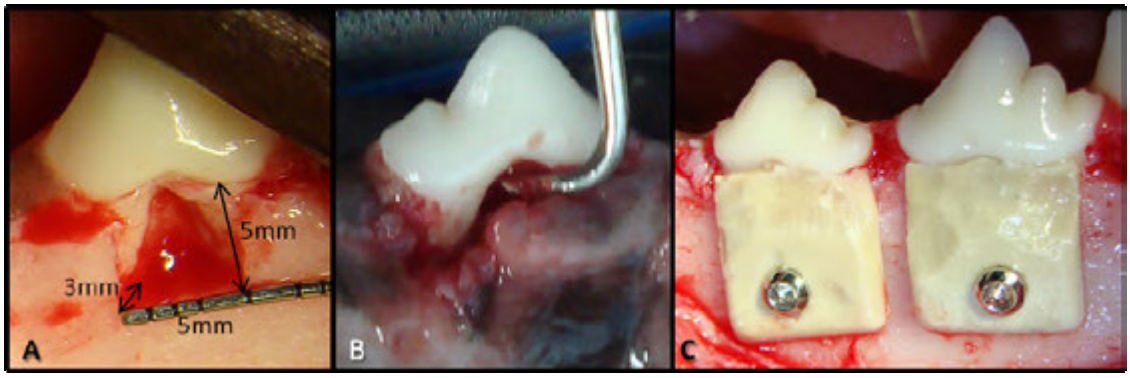


Figure 1. Sequence of in vivo procedures. A. The 5 mm x 5 mm x 3 mm class II furcation defect on the 4th right premolar, partially exposing its roots. After an impression material filled such defect for 21 days, and thus, inducing periodontal disease, scaling and root planing (B) was performed. Fourteen days later, the HA+PHB membranes were placed covering the defects of treated groups (C).

Animals were clinically evaluated on a daily basis for 14 days post-operative for dehiscence, gingival recession, inflammation, bleeding, edema and membrane exposure to the oral cavity. During these days, surgical wounds were rinsed with chlorhexidine 0.12% every 12 hours. By the end of this period, a tooth brushing prophylaxis routine was implemented every other day until the end of the observation period at 60 or 120 days.

CAL measurements, radiographs and topical application of chlorhexidine 0.12% were performed monthly. Five animals were euthanized by an overdose of general anesthetic at 60 days post-operative and the remaining at 120 days post-operative. Samples comprising the teeth of interest and surrounding alveolar bone were obtained and fixed in 10% formalin. The mean CAL of the control and HA+PHB membranes groups was compared in each time-point using one-way ANOVA, followed by Tukey test when necessary ($p<0.05$).

Micro-computed tomography (MicroCT)

Groups of three samples were stacked in a 30mm cylindrical holder and scanned using a micro-computed tomography system (MicroCT40, Scanco, Switzerland) at 85kV and 77μA. After image reconstruction, two-dimensional (2D) images representing axial cuts (10 μm thick) of the harvested samples were used to select the region of interest (ROI) for analysis. The defect's coronal limit corresponded to the cementum-enamel junction line. The apical limit comprised a plane 1mm coronally to each defect height, previously measured immediately before GTR, so that only new bone was included in the measurement. The root canals were used as anatomical reference points for the mesial, distal and lingual ROI limits. The buccal limit on the lower half of the defect was set at a 0.5 mm distance measured from the root buccal surface. For the upper half of the defect, the buccal ROI limit was set at the buccal root surface. These reference points were used to draw the defect area at different depths in the 2D images, which were morphed to form a 3D image representing our volume of interest. The quantification of bone formation (bone volume / total volume) as well as trabecular number, thickness and separation was possible by determining the gray-level distribution at specific thresholds for samples at 60 and 120 days post-operative. Control, 25% HA + PHB and 35% HA + PHB groups were compared using one-way ANOVA for each of these quantification parameters, followed by Tukey test when necessary ($p<0.05$).

Histology processing and analysis

One sample of each group at both time-points was decalcified in formic acid and sodium citrate, dehydrated and embedded in paraffin. Samples were sectioned with a thickness of 7μm. They were sectioned longitudinally, i.e., in a mesiodistal direction, in

order to observe the most coronal part of the furcation. Slides were stained with haematoxylin and eosin for light microscopy analysis followed by polarized light (Aristoplan microscope, Leitz, Germany).

The other four samples per group at both time-points were dehydrated in serial concentrations of ethanol (70, 95 and 100 v/v) and embedded in polymethyl methacrylate (Osteobed, Polysciences, USA) according to the manufacturer's protocol. Next, 15 µm-thick sections were obtained using the EXAKT cutting and grinding system (Exakt Technologies Inc., USA). One sample was sectioned in the buccolingual direction and another one is cross-section so that the membrane-tissue interface would be exposed in two different planes. The other two samples were sectioned in the mesiodistal direction for analysis of the furcation area. Sections were stained with haematoxylin and eosin for light microscopy analysis (Aristoplan, Leitz, Germany).

All samples were assessed for the tissues formed in the furcation defect area, guided by MicroCT images and measurements, with special attention to the periodontal support formed by bone, periodontal ligament and cementum.

Results

Periodontal disease induction

Before periodontal disease induction, all animals presented normal CAL measurements of less than 3mm. Periodontal pockets were found at prophylaxis, with mean CALs of 6.6 mm ± 2.54, 6.4 mm ± 2.31 and 7.36 mm ± 3.34 for the 2nd, 3rd and 4th premolars, respectively, all values of periodontal disease in dogs.[2,5] No significant differences between them were found (one-way ANOVA / p=0.82). Also, no significant difference (one-way ANOVA) was found between the size of the defects of the 2nd, 3rd and 4th premolars in all planes: mesiodistal (p=0.602), apicocoronal (p=0.937) and buccolingual (0.369).

GTR surgical and clinical evaluations

Grinding the membranes to the exact contour of the alveolar bone during surgery was a long lasting procedure that required the best of the surgeon's skills. Even so, completely occluding the defect at all sides (mesial, distal, coronal and apical) was not always accomplished. Healing occurred uneventfully with discrete bleeding and edema

on the first day after surgery in both groups. However, membrane exposure occurred associated to discrete gingival recession in all treated defects usually on the 8th day after surgery, except for one defect of the 25% HA+PHB which occurred on the 14th day. Such gingival recession progressed and the membrane exposure increased on the following time-points of 30, 60, 90 and 120 days after surgery (Figure 2). One 25% HA+PHB membrane broke on day 80 after surgery and three membranes of 35% HA+PHB broke on days 47, 58 and 76 after surgery; defects on which gingival recession was not observed on the following time-points. The mean CAL of the control group decreased over time, although it did not reach a mean value considered normal for dogs (3.8 mm ± 3.5). For both treated groups, mean CAL increased during the first 30 days after surgery, progressively decreasing on the following time-points to values of 7.0 mm ± 3.08 (25%) and 7.2 mm ± 4.3 (35%) at 120 days.

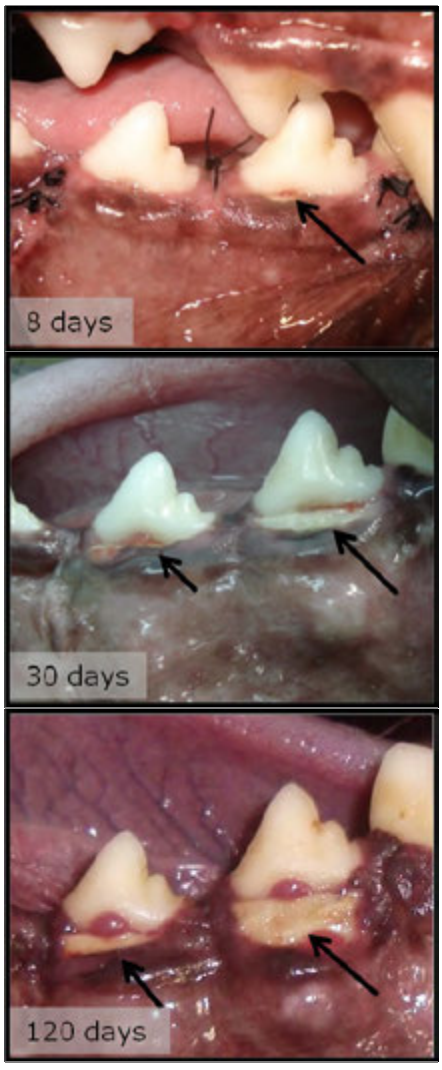


Figure 2. Discrete gingival recession with membrane exposure (arrows) was observed usually on the 8th day after surgery (A), progressing on the following time-points of 30 (B) and 120 (C) days.

MicroCT

For the control group, the extent of the alveolar bone was limited to the most lingual part of the defect (Figure 3A and B) on the buccolingual direction. Class III furcation defects (through-and-through defect, where all bone on the most coronal part of the furcation has been resorbed) were observed in three control samples of 120 days and in another three at 60 days (Figure 3A). Also six samples from 25% HA+PHB membrane presented class III furcation defects, three from each time-point. Of the 35% HA+PHB, one sample membrane exhibited class III furcation at 120 days. Bone tissue partially filled the furcation area of both treated groups, although not on the buccal area of the defect (Figure 4). Apically to the defect area, close to the screw, bone tissue was seen arising from the buccal surface of the alveolar bone towards the internal surface of the membrane, covering this surface and in direct contact with it (Figure 5).

MicroCT volumetric data showed no significant differences in bone volume between the three groups at both time-points, neither for trabeculae number, thickness and separation (*p* value ranged from 0.11 to 0.54).

Histological analysis

The defect area in control samples were mainly filled by a dense connective tissue (Figure 3C) at both time-points. At the interface with the root on the most coronal part of the defect, loose connective tissue was found. Cementum and periodontal ligament were observed along with alveolar bone in the few areas closer to the apical bottom of the defect where this last one could be found.

Partial regeneration of the periodontium was observed in treated groups, regardless of the percentage of HA in each membrane (25% or 35%). The central portions of the defects were filled by a dense connective tissue (Figure 6A) where a neutrophilic inflammatory infiltrate was found. Bone tissue was observed in the areas closer to the roots (Figure 6A). On the trabeculae surfaces at the interface with the dense connective tissue, many osteoclasts were found (Figure 6B), defining a pattern of bone resorption and fibrous tissue formation from the center of the defect towards the roots. A thin layer of cellular cementum covered the roots in the furcation (Figure 6B) from where collagen fibers emerged to the periodontal ligament. However, collagen fibers were loose, i.e., connected to the dense connective tissue or not attached to the alveolar bone, the two tissues that filled the furcation area (Figure 7). These fibers where best

seen under polarized light (Figure 7B). Buccolingual and cross-section views revealed that no periodontium formed on the buccal area of the defect, where an inflammatory infiltrate was also found in the dense connective tissue, only at the interface with the membranes (Figure 8). This inflammatory infiltrate was mostly neutrophilic, but a few macrophages were also found. No ankylosis or root resorption were observed.

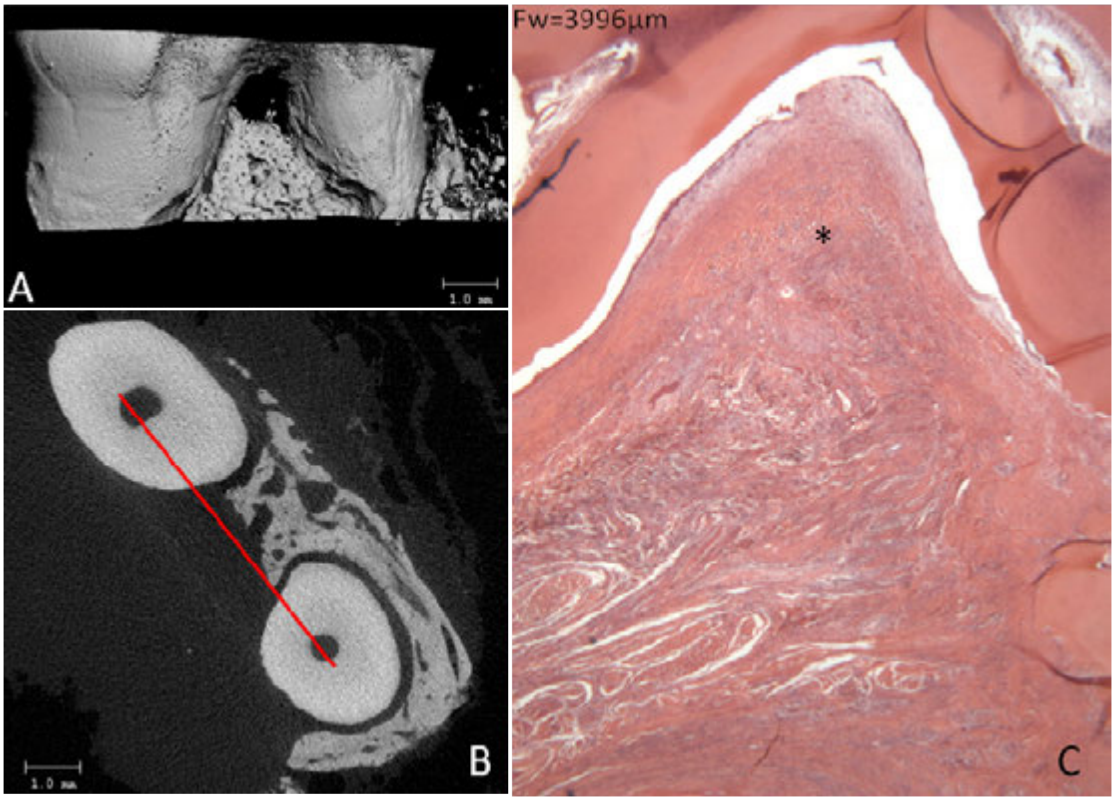


Figure 3. Images of a control sample at 120 days. Note the class III furcation defect (A) and the small extent of bone on the coronal (A) and buccal (B) parts of the furcation in MicroCT images. C. Photomicrograph of the same sample sectioned on a mesialdistal direction, obtained on the red line in B. A dense connective tissue (*) filled the defect in the central area. H&E. FW=Field width.

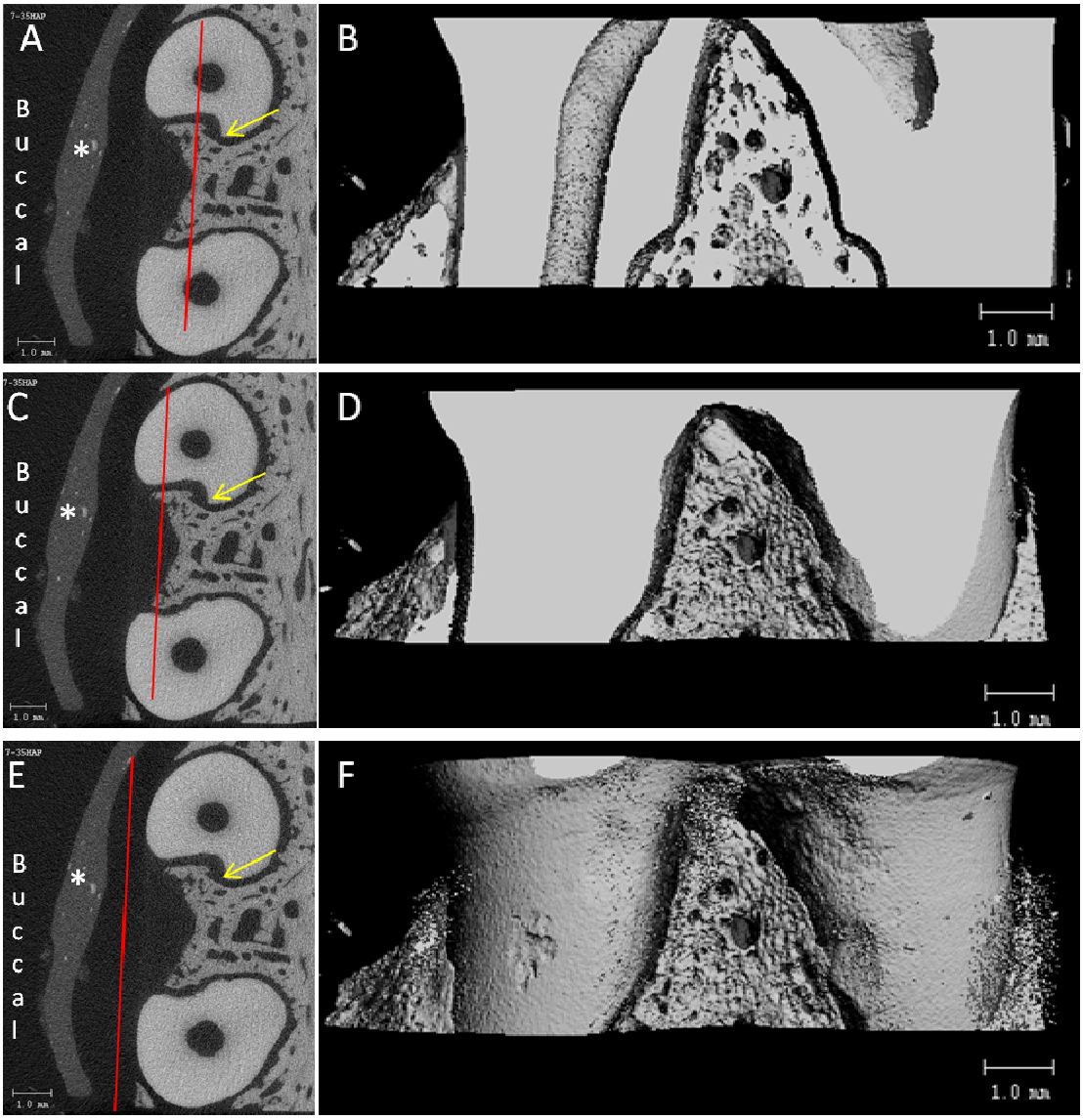


Figure 4. MicroCT images of the same 35% HA+PHB membrane sample at 60 days. A, C and D. Cross-sections as references (red lines) of the area from where the 3D images in B, D and F were obtained, respectively. Note that A is closer to the lingual wall (yellow arrows) and thus, more bone filled the defect (B). D and F are more distant to the lingual wall than A (compare red lines to the yellow arrows) and thus, no bone is sectioned (C, D, E and F). Also, C and E clearly show a greater bone resorption on the central area than on the borders closer to the roots.

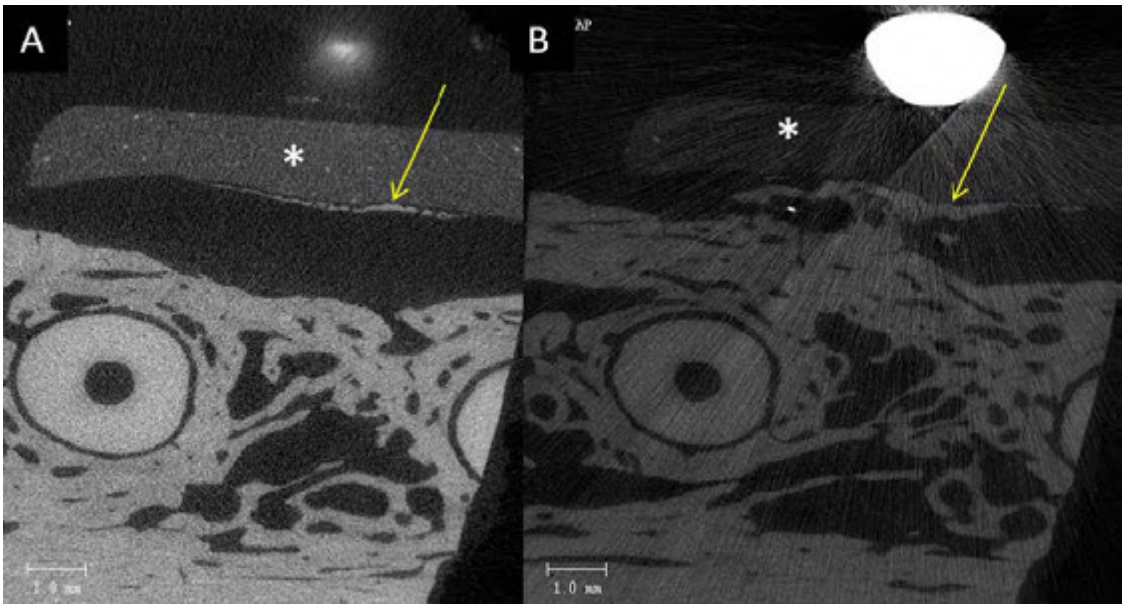


Figure 5. MicroCT images of a 25% HA+PHB membrane sample at 120 days. Bone tissue (arrow) arising from the buccal surface of the alveolar bone towards the internal surface of the membrane (*), growing on this surface and in direct contact with it.

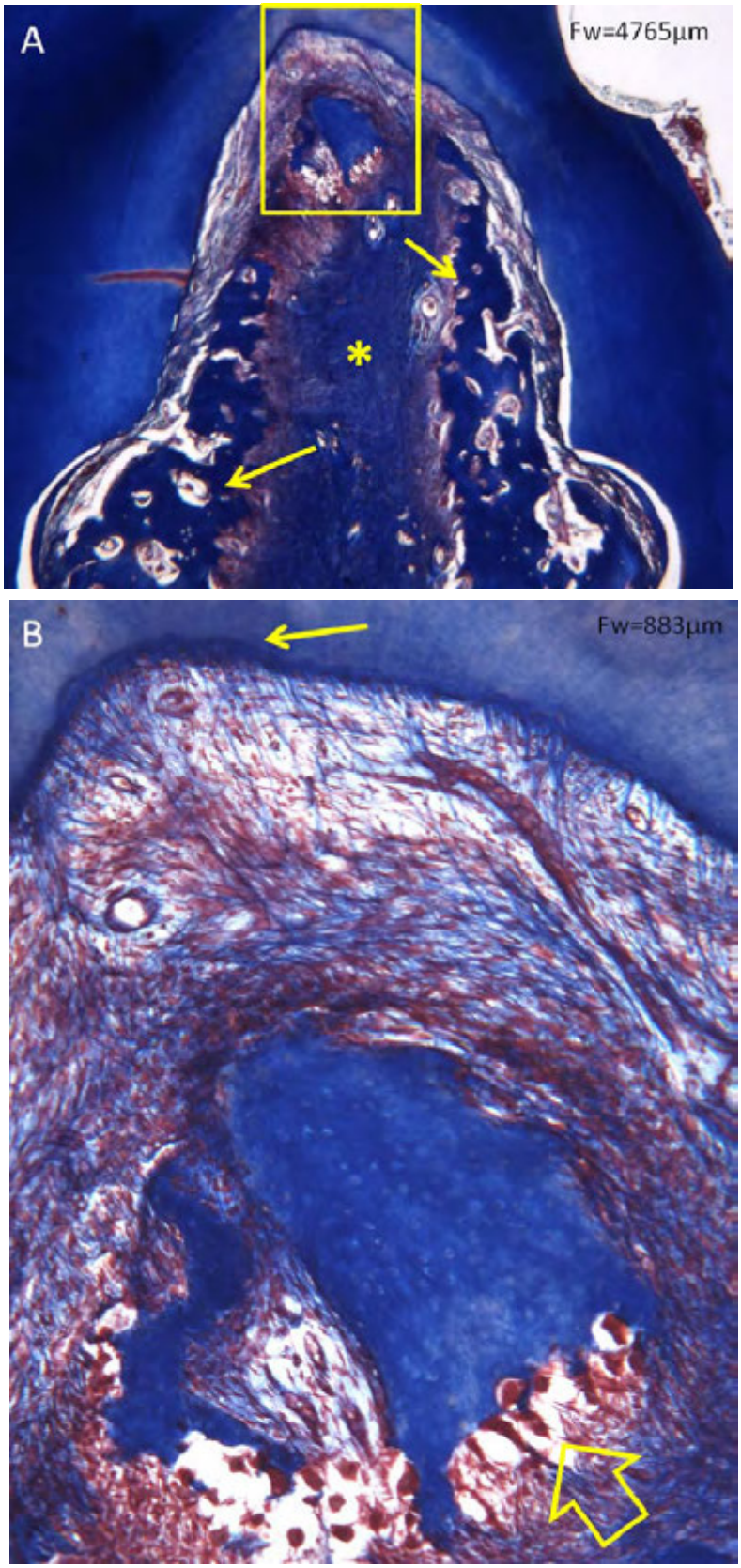


Figure 6. Photomicrographs of a 35%HA+PHB sample at 60 days. A. The central portions of the defects were filled by dense connective tissue (*). Bone tissue filled the areas closer to the roots (arrows). B. Enlargement of the most coronal part of the furcation marked in A. Osteoclasts (empty arrow) were found on the trabeculae surfaces at the interface with the dense connective tissue. A thin layer of cellular cementum covered the roots (arrow) from where collagen fibers emerged to the periodontal ligament. Photomicrographs taken at similar depths as figure 4C on a mesial-distal direction. Masson's trichrome. FW=Field width.

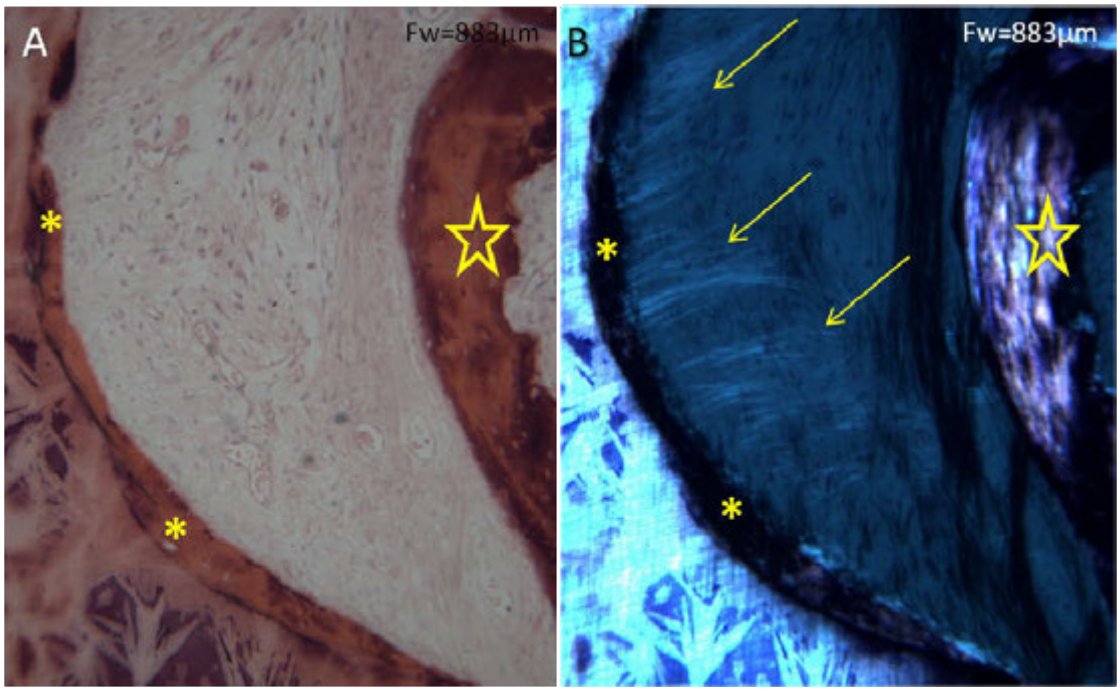
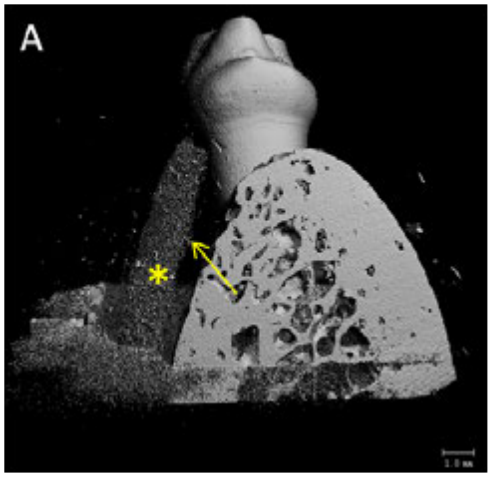
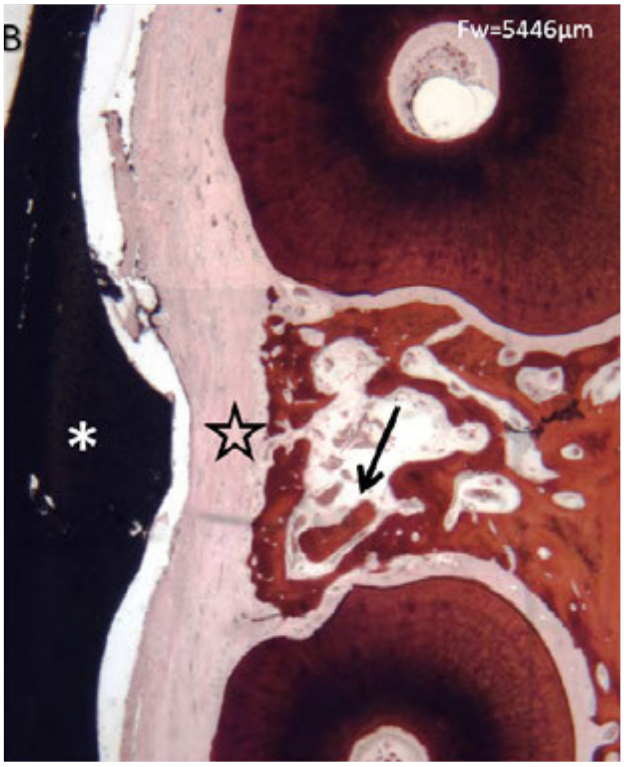


Figure 7. Notch area of a non-decalcified sample at the same area under light microscopy (A) and polarized light (B). Collagen fibers (arrows) were loose, i.e., they emerged from the cementum (*) but where not immersed in the alveolar bone (star). H&E. FW=Field width.





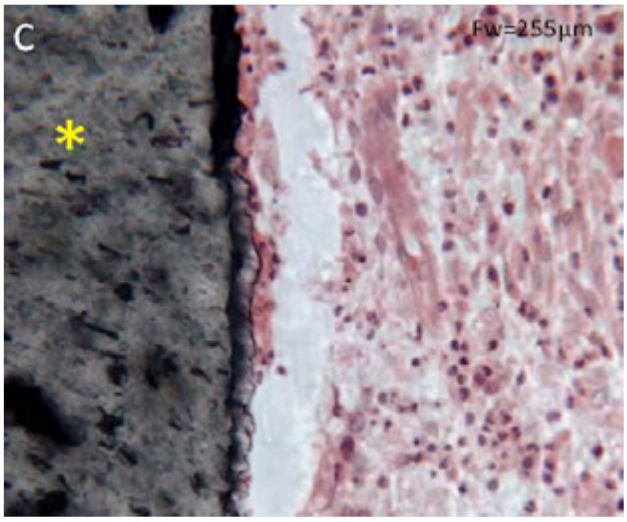


Figure 8. A. MicroCT 3D image of a 25% HA+PHB membrane group. The arrow shows the interface of the membrane (*) with the defect. B. Cross-section of a non-decalcified sample showing bone formation in the furcation (arrow) but connective tissue at the interface (star) with the membrane (*). C. Non-decalcified sample showing the inflammatory infiltrate in the connective tissue closer to the membrane (*). H&E. FW=Field width.

Discussion

In the present study, membranes of HA associated to PHB were analyzed on the regeneration of class II furcation defects. They were manufactured by injection molding, resulting on a rigid biomaterial that enabled the grinding procedure to contour the membranes to the alveolar bone using a common dental bur. Such rigid structure also sustained the application of a screw for membrane fixation. Before GTR procedure itself, periodontal defects were submitted to a periodontal disease induction process in

order to create chronic infected defects, since acute ones usually undergo regeneration regardless of treatment.[13,25]

After GTR, on a general perspective, some regeneration occurred with both membranes with a few samples at a clinically significant extent. Periodontal regeneration was proved by the analysis of different depths of the defect on a buccolingual direction both at MicroCT and histology, this last one being guided by the images and measurements of the first. Intersting observation is the presence of bone, periodontal ligament and cementum in the notch area, where tissues were removed to a greater extent towards the dentin. Indeed, many different materials have been shown to favor periodontal regeneration. Starting by graft materials such as autogenous bone grafts[25,26], anorganic bone matrix[9], and synthetic biomaterials like bioglass[27], all used to completely fill the defect.[28,29] Good results were found but root resorption and ankylosis are two major problems with these strategies.[8,9] Also, enamel matrix proteins[30] and platelet pellets[13] were studied on furcation defects with good results, although no complete regeneration was achieved. The most widely used strategy and commonly associated to the previous ones is GTR, the procedure that uses a membrane to cover and not fill the periodontal defect, placed just under the muco-periosteal flap.[8,18,20,30]

At first, membranes were thought to exclude cells of gingival and connective tissues by blocking their migration into the defect, thus, allowing the migration of progenitor cells from the remaining periodontal ligament and alveolar bone. As so, the membrane would select the cell types that would repopulate the defect, i.e., progenitors of osteoblasts, cementoblasts and periodontal ligament fibroblasts for the regeneration of the periodontium.[9,17,18,31] Development on periodontal regeneration strategies suggest now that the clot stability provided by membranes are just as important as excluding gingival tissue or, in fact, it is the real condition favoring periodontal regeneration in GTR.[8,16,20,32] Clot stability includes maintaining both the clot attachment to the roots and the clot volume. Therefore, progenitor cells could both (i) reach the roots where to lay down new cementum on the exposed dentin and (ii) have more volume of provisional matrix onto which they could establish, differentiate and produce new bone matrix.[20,30,31] This is where the design of our membrane was born: a rigid membrane that would maintain clot stability, a structure that could prevent the so common collapse into the defect of resorbable membranes made of collagen[8,19], poly-lactic acid [8,16] and PLGA[18], for example.

However, bone volumetric values of the treated groups were not different from those of the control. New alveolar bone, periodontal ligament and cementum were not observed in the entire defect, as they were not formed in the furcation areas closer to the buccal side. Important to note at this point is the inflammatory infiltrate observed at the interface with the inner sides of the membranes and the resorption of trabeculae clearly seen from the central area of the defect towards the roots. These results demonstrate the contamination of the membrane and the defect, which is already proven to negatively affect healing[20], despite the antibiotics used pre and post-operative. Data analysis, provides two possibilities (i) a limited regeneration and/or (ii) the resorption of part of the regenerated tissues. Defect contamination is known to impair regeneration by different mechanisms, including the inhibition of osteoblasts activity[33] while the resorption of the periodontium is a well known mechanism since it is the process of periodontal disease itself. Tissue destruction under contamination and in periodontal disease is a complex process. Briefly, the accumulation of plaque in the sub-gingival space leads to the accumulation of bacteria by-products.[1,34,35] This triggers many pathways of inflammation, like dysregulation of clotting and fibrinolytic pathways, the activation of matrix metalloproteinases, the production of cytokines and the recruitment and activation of polymorphonuclear leukocytes and osteoclasts, which all culminate with destruction of the cementum, periodontal ligament and alveolar bone.[33,35] One could imagine that the inflammatory infiltrate seen in contact with the membrane occurred as a result of a non-biocompatible material or an overwhelming degradation process. However, hits hypothesis was disconsidered because previous works have proven its biocompatibility and uneventful degradation.[36-39]

Two hypotheses can be explored on the process of membrane contamination. The first is that the membrane outer surface was not adhesive enough to the gingiva; consequently, it failed to prevent gingival recession which was followed by membrane exposure and contamination. A complex surface topography is an example of a strategy to improve gingival adhsesion to the outer surface of the membrane.[23,24] The second hypothesis is that microscopic spaces at the interface of the membrane with the defect allowed the contamination of the membrane and the defect on their most coronal part. In fact, this is supported by the observation of a progressive gingival recession starting from a non-exposed membrane on the first days after surgery as Figure 2 exemplifies. Thus, although the grinding technique is easy to be performed, perfect occlusion of such

morphologically variable defect cannot be easily achieved. It is suggested that the rigid HA+PHB composite could be better explored for bone plates and screws, since it is proven to have adequate mechanical properties and to be osteoconductive[37-40], like seen on Figure 5. For periodontal defects, the best approach would be membranes made of polymers that can be soften when heated to be molded to the defect contour, like PLGA[41], where the membrane could be perfectly fit to the various morphological contours of a defect and consequently, prevent wound contamination.

Therefore, rigid membranes made of either 25 or 35% HA in a PHB matrix did not improve the regeneration of class II furcation defects in dogs when compared to non treated sites, although partial regeneration of the defect was observed.

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Capítulo IV

Periodontal regeneration using a novel bilayered biomaterial

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Abstract

The regeneration of tissues affected by periodontal disease is a complex process since it encompasses the formation of bone, cementum and periodontal ligament. We developed a PLGA (polylactide-*co*-glycolide acid)+CaP bilayered biomaterial with a mechanically stable outer side and an inner topographically complex surface to favor tissue regeneration by providing a strong attachment to fibrin and a provisional matrix for progenitor cells migration. Comparing its use with only periodontal prophylaxis in the treatment of class II furcation defects in dogs, the experimental model included periodontal disease induction, prophylaxis and biomaterial implantation. Clinical evaluations, micro-computed tomography volumetric values, histology and backscattered electron imaging were used for data analysis. Healing occurred uneventfully and bone volumetric values were significantly greater in treated group. New cementum and periodontal ligament were seen along with the new bone only in treated group. Although periodontal regeneration has been reported, the advantages of employing our bilayered PLGA+CaP are twofold: it did not collapse into the defect and its inner side was able to maintain the blood clot throughout the buccal defect. Consequently, we demonstrated the regeneration of more buccal bone together with the periodontal ligament and cementum than has previously been reported with flexible and non-macroporous-sided traditional membranes.

1. Introduction

Periodontal disease or periodontitis is an infectious disease characterized by the destruction of tooth supporting tissues due to a severe inflammatory response elicited by bacteria harbored in tooth-retained plaque [1,2]. This is a worldwide health problem, reported to worsen with age, which can be associated with local and systemic conditions [1,3-5]. It is also the most common disease in dogs and has become a very important issue in veterinary medicine as these animals are now reaching older ages [6-7]. Interestingly, canine and human periodontal diseases share many aspects such as etiology [6,8,9], histological features which have been described since the 1970's [10], and the association with both cardiovascular [4,11,12] and renal [5,11,12] diseases.

Thus, diagnostic tools such as clinical attachment level and probing depth are also used in both human and veterinary dental fields [13].

Understanding the mechanisms of periodontal regeneration in humans and dogs has been the subject of numerous studies as the periodontium represents a unique environment requiring the coordinated formation of cementum, periodontal ligament (PDL), alveolar bone and gingiva [2,14-17]. Different treatment strategies for periodontal regeneration have been reported in the literature [18,19]. Scaling and root planing comprise the first line of treatment for periodontal defects [20-22]. This strategy, aimed at removing the causative agent (i.e. plaque, calculus) and smoothing the root surface for subsequent PDL attachment, fails to stabilize the clot and allows epithelial/connective tissue ingress to periodontal defects, resulting in tissue repair rather than regeneration. In humans, scaling and root planing is commonly used to prepare the periodontal environment for subsequent regenerative procedures [2,20]. In dogs, however, it represents the best and most conservative alternative to tooth extraction. Nevertheless, in veterinary practice, tooth loss is still very common either by extraction or secondary to advanced periodontal disease [21,22]. Overall, oronasal fistulas and pathologic jaw fractures are common outcomes of the bone resorption that follows tooth loss on the palatal aspect of the maxillary canine and the horizontal ramus of the mandible, respectively [21,23].

Despite the severe outcomes of periodontitis, regeneration of the periodontal tissue is not the main goal in the current veterinary practice. Possible reasons for this could be the high costs of the limited regenerative procedures available, or the fact that most regenerative strategies are still under research, which include the use of enamel matrix proteins [24], autologous or synthetic grafts [25-26] and guided tissue regeneration (GTR) [27].

GTR utilizes barrier membranes for defect site protection and clot stabilization while enabling progenitor cell recruitment and migration from the surrounding PDL and alveolar bone. These mesenchymal progenitors will in turn direct the regeneration of cementum (pre-cementoblasts), PDL (fibroblasts), and bone (pre-osteoblasts) [2,15]. Indeed, many different membranes have been used as GTR treatment in humans after experimental evaluations in dogs, including expanded polytetrafluoroethylene (ePTFE) [18,28], collagen [29-31], poly-l-lactic acid [32], lactic acid-glycolic acid copolymer [33] and polylactide acetyltributyl citrate [17]. Nevertheless, these materials are strictly

employed as physical barriers to protect the clot and prevent soft tissue ingress to periodontal defects [2]. Recently a new concept of membranes for GTR was introduced, where novel membranes are targeted at enhancing tissue regeneration by acting as osteoconductive scaffolds. Owen *et al*. [34] and Liao *et al*. [35] have developed membranes with topographically complex inner sides made of hydroxyapatite-collagen-PLGA and PLGA, respectively, and both with an outer smooth side. These studies demonstrated in vitro that the topographically complex surfaces favored cell migration.

Given the need for regenerative therapy of periodontal defects in both dogs and humans, we have developed a three-phase resorbable biomaterial which combines a polymer (polylactide-*co*-glycolide acid) with two phases of calcium phosphate (CaP) (OsteoScaf™) for GTR. This material is structurally stable and moldable, characteristics which favor adaptation to different defect morphologies and prevent membrane collapse. The inner side of this biomaterial was constructed with a topographically complex macroporous surface, previously shown to be osteoconductive [36]. It was designed with highly interconnected macroporosity and has an ability to wick up blood, which enhances platelet activation and fibrin matrix attachment within its architectural structure. The outer layer, on the other hand, was designed flat and intended to serve as space maintainer and clot stabilizing device. We, herein, sought to investigate the potential use of the PLGA+CaP bilayered biomaterial for the regeneration of periodontal defects in the dog, both as a human model and as a regenerative strategy for veterinary dentistry.

2. Materials and methods

2.1. Bilayered biomaterial fabrication

Each PLGA+CaP (polylactide-*co*-glycolide) bilayered biomaterial used in this study comprised (i) a flat outer layer, created by a Teflon / PLGA+CaP interface, and (ii) a 1mm thick rough macroporous inner layer. The fabrication followed a 2-step procedure. First, the inner side was prepared according to the protocol used for our macroporous composite scaffolds (OsteoScaf™), composed of 75/25 polylactide-*co*-glycolide (PLGA) and 2 phases of calcium phosphate (CaP) [36]. Briefly, CaP particles (27μm average size) were thoroughly mixed in dimethylsulfoxide-dissolved PLGA. The solution was dispersed onto a sugar mold [37] and allowed to solidify at -18°C. Next,

the solidified block was soaked in distilled water for 3 days for sugar leaching, in order to create a porous structure. Thin sheets of approximately 1.5cm x 1.5cm x 1mm were sectioned with a scalpel and then coated with a CaP layer by immersion in a modified SBF (simulated body fluid) solution at 37°C for 24 hours [36].

The second step consisted of the preparation of a PLGA+CaP solution which was poured onto a Teflon-coated aluminum foil. The previously CaP coated thin sheets were placed on this PLGA+CaP solution just prior to setting. A final bilayered sheet (inner layer: rough macroporous, outer layer: flat) was peeled off the teflon-coated aluminum foil and trimmed to detach the several membranes with individual sizes of 1.5cm x 1.5cm x 1mm.

2.2. Bilayered biomaterial surface analysis

Three membrane samples were coated by gold in an EMS550x sputter coater and analyzed by scanning electron microscopy (SEM; LEO 1430VP, England and Hitachi SU6600, Canada) at 15kV and 5kV. Ten pictures of both the inner and outer surfaces were taken at increasing magnifications (100, 2k and 18k) for analysis of pore size. Micro and macropores were identified by the superimposition of a 10 x10 grid on each micrograph taken at 2k and 100 times magnifications, respectively. Pore diameter was then attained using the Analysis 5 ™ software (Olympus, Japan).

2.3. In vivo experimental work

The in vivo procedures were reviewed and approved by the Ethics Committee on Animal Research, Veterinary Department (Universidade Federal de Viçosa, Brazil). Ten healthy adult (4 male and 6 female) mongrel dogs, weighting about 10-15kg and without periodontal disease were selected. The animals were fed soft dog chow for the entire experiment. Three days prior to each surgical procedure, animals received oral spiramycin (23.5mg/kg/day) associated to dimetridazole (12.5mg/kg/day) (Spiraphar™, Virbac do Brasil, Brazil) and the medication continued for a total of 6 days.

The animals fasted for 8 hours prior to anesthesia. First, sedation was obtained using acepromazine (0.1mg/kg intravenously [IV]). General anesthesia was induced with propofol (6 mg/kg IV) and maintained with a mixture of oxygen and isoflurane. Chlorhexidine 0.12% was used to rinse the oral cavity of each dog pre and post-operative procedures.

2.3.1. Periodontal disease induction

Baseline clinical attachment level (CAL) was measured with a periodontal probe (PCP-UNC 15, Hu-Friedy, USA) from the cemento-enamel junction (CEJ) to the bottom of the gingival sulcus (or periodontal pocket), at the central area of the furcation of the lower 4^{th} and upper 2^{nd} right premolars. These teeth were selected as they are very similar in size, morphology and periodontal disease progression [1,9,38]. Also, standardized periapical radiographs [6] were taken (Timex 70C, Gnatus, Brazil) prior to each procedure for a reference image of the furcation area.

Mucoperiosteal flaps were elevated to expose the buccal bone walls. A cylindrical diamond bur (FG1016, KG Sorensen, Brazil) was used to create class II furcation defects (exposing the roots on the buccal side but still keeping a lingual wall) with the following dimensions: 5mm apicocoronally (anatomical reference: CEJ at the centre of the furcation), 5mm mesiodistally and 3mm buccolingually (anatomical reference: buccal aspect of the alveolar bone) (Fig. 1A). Scaling was subsequently performed to remove periodontal ligament fibers and debris from root surfaces and the defects were completely filled with an impression material (Impregum Soft™, 3M do Brazil). The flaps were repositioned and closed with non-resorbable sutures (5-0 nylon suture, J&J Ethicon), and post-operative radiographs were taken.

Sutures were removed after 10 days and the impression material was kept in the defects for a total of 21 days. Immediately after the material was removed, a second CAL measurement was performed followed by prophylaxis, which comprised a surgical open flap procedure for scaling (Fig. 1B), root planning and irrigation with chlorhexidine 0.12%. After the flap was closed, a new set of radiographs was taken. Surgical wounds were rinsed with chlorhexidine 0.12% every 12 hours for 2 weeks.

2.3.2. Guided tissue regeneration (GTR)

Two weeks after prophylaxis, animals were anesthetized and new radiographs were taken. A surgical flap was raised once more and the defects were scaled to remove soft tissue. Each defect was measured in the apicocoronal, mesiodistal and buccolingual directions, dimensions further compared to the initial ones of 5mm x 5mm x 3mm using one-way ANOVA. Using a round bur (FG1016, KG Sorensen, Brazil), notches were made on the surface of the roots to serve as future reference for the apical limit of the defect.

The lower 4th right premolar was assigned as the treated group. The PLGA+CaP bilayered biomaterial (approximately 1.5cm x 1.5cm x 1mm) was immersed in sterile saline solution at approximately 40°C for 1 minute to soften. Next, the membrane was placed covering the defect (Fig 1C), with the margins extending approximately 3mm mesially, distally and apically, as suggested by Christgau *et al.* [2]. The membrane was then allowed to harden for a few minutes and stabilized with a 1.5mm x 6mm screw (Ortovet, Brazil) positioned on the apical portion of the furcation area (Fig 1C). Finally, the flap was closed with non-resorbable sutures (5-0 nylon suture, J&J Ethicon). The same surgical procedure (including screw insertion) was repeated in the control defect on the upper 2nd right premolar, except for membrane placement. Thus, each dog had both treated (PLGA+CaP bilayered biomaterial) and control (no biomaterial) groups. Post-operative radiographs were taken immediately after the surgical procedure. Spiramycin (23.5mg/kg/day) associated to dimetridazole (12.5mg/kg/day) (Spiraphar™, Virbac do Brasil, Brazil) was administered until suture removal.

Animals were clinically evaluated on a daily basis for 14 days post-operative for dehiscence, gingival recession, inflammation, bleeding, edema and membrane exposure to the oral cavity. During these days, surgical wounds were rinsed with chlorhexidine 0.12% every 12 hours. By the end of this period, a tooth brushing prophylaxis routine was implemented every other day until the end of the observation period (60 and 120 days).

CAL measurements, radiographs and prophylaxis were performed monthly. Five animals were euthanized by an overdose of general anesthetic at 60 days post-operative and the remaining at 120 days post-operative. Biopsies comprising the teeth of interest and surrounding alveolar bone were harvested and fixed in 10% formalin. The mean CAL of the control and PLGA+CaP membrane groups was compared in each time-point using one-way ANOVA, followed by Tukey test when necessary ($p<0.05$).

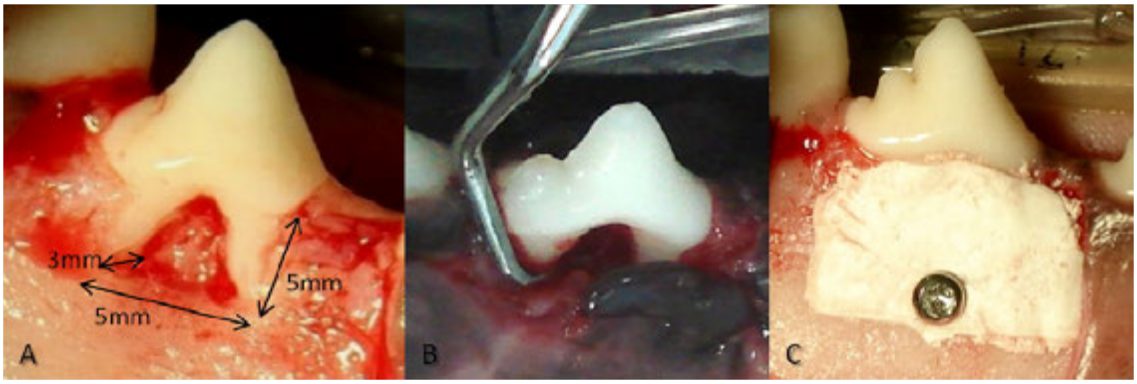

Figure 1. Sequence of in vivo procedures. The 5mm x 5mm x 3mm class II furcation defect, partially exposing the roots is shown in A. After an impression material filled such defect for 21 days, and thus, inducing periodontal disease, scaling and root planing (B) was performed. Fourteen days later, the PLGA+CaP bilayered biomaterial was placed covering the defect of the treated group (C).

2.4. Micro-computed tomography (MicroCT)

Groups of 3 samples were stacked in a 30mm cylindrical holder and scanned using a micro-computed tomography system (MicroCT40, Scanco, Switzerland) at 85kV and 77μA. After image reconstruction, two-dimensional (2D) images representing axial cuts (10μm thick) of the harvested samples were used to select the region of interest (ROI) for analysis. The defect's coronal limit (ROI top) corresponded to the CEJ line. The apical limit (ROI bottom) was comprised by a plane 1mm coronally to each defect height so that only new bone was included in the measurement. The root canals were used as anatomical reference points for the mesial, distal and lingual ROI limits. The buccal limit on the lower half of the defect was set at a 0.5mm distance measured from the root buccal surface. For the upper half of the defect the buccal ROI limit was set at the buccal root surface. These reference points were used to draw the defect area at different depths in the 2D images, which were morphed to form a 3D image representing our volume of interest. The quantification of bone formation (bone volume / total volume, or BV/TV) as well as trabecular number, thickness and separation was possible by determining the gray-level distribution at specific thresholds for samples at 60 and 120 days post-operative. Control and PLGA+CaP membrane groups were compared using one-way ANOVA for each of these quantification parameters, followed by Tukey test when necessary ($p<0.05$).

2.5. Histology processing and analysis

Two samples of each group at both time-points (60 and 120 days) were decalcified in formic acid and sodium citrate and embedded in paraffin. Samples were sectioned with a thickness of 7µm. One sample was sectioned longitudinally, in a mesiodistal direction, in order to observe the most coronal part of the furcation. The other sample was sectioned in cross-section, so that the tissue-membrane interface could be analyzed. Slides were stained with haematoxylin and eosin (H&E) for light microscopy qualitative analysis (Aristoplan microscope, Leitz, Germany).

The other 3 samples per group at both time-points were dehydrated in serial concentrations of ethanol (70, 95 and 100 v/v) and embedded in polymethyl methacrylate (Osteobed, Polysciences, USA) according to the manufacturer's protocol. Next, 30µm-thick longitudinal sections were obtained using the EXAKT cutting and grinding system (Exakt Technologies Inc., USA). One sample was cut in the buccolingual direction so that the membrane-tissue interface would be exposed. The other two samples were sectioned in the mesiodistal direction for analysis of the furcation area. The resulting slides were gold-coated for backscattered electron imaging (BSEI) of the membrane-tissue interface. After BSEI analysis, samples were further ground to 15µm thin sections and stained with H&E for light microscopy analysis (Aristoplan, Leitz, Germany).

Sectioning of the decalcified and non-decalcified samples were guided by reference points and linear measurements obtained from MicroCT images. All samples were assessed for the tissues formed in the furcation defect area, with special attention to the periodontal support formed by the connections between bone, PDL and cementum.

3. Results

3.1. Bilayered biomaterial surface analysis

Surface topography evaluation of the flat outer layer of the PLGA+CaP bilayered biomaterial (Fig 2A) on SEM showed few pores randomly distributed on a featureless surface. Of the few pores found, 61.67% were smaller than 50µm (22.02µm ± 11.46) and only 2.59% were greater than 200 µm (269.88µm ± 12.53). On the contrary, the rough porous side was characterized by the presence of interconnected

macropores (Fig 2B and 2C), where 66.17% were greater than 500µm (703.01µm ± 134.20). The surface of those interconnections were formed by globular particles and micropores (4.47µm ± 2.55) (Fig 2D) which, at greater magnifications, revealed to be covered by needle-shape structures randomly distributed forming nanopores (Fig 2E).

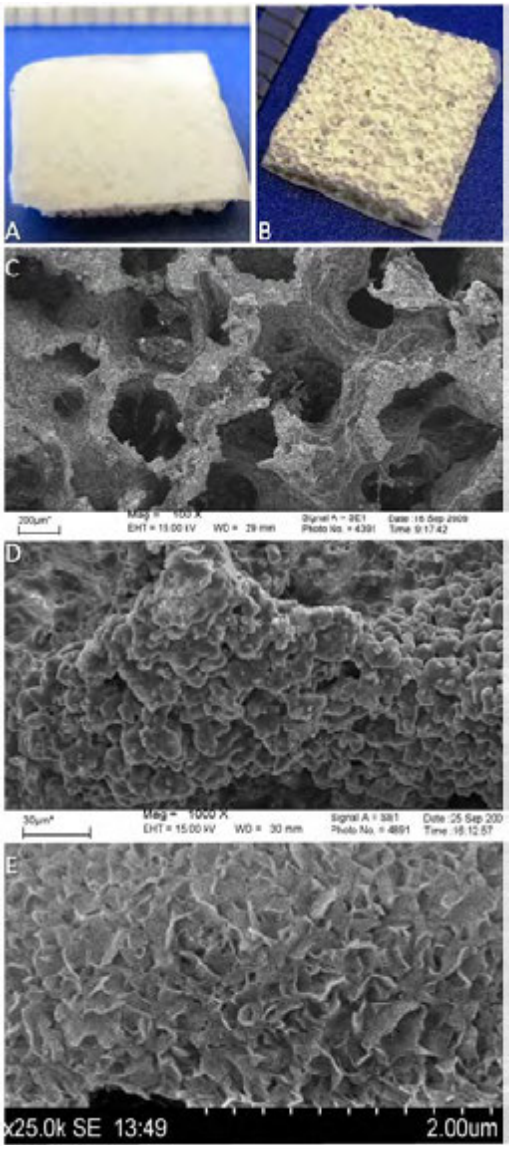

Figure 2. The flat (A) and the macroporous (B) surfaces of the bilayered biomaterial. The macro (C), micro (D) and nanopores (E) are seen under SEM on the macroporous side.

3.2. Periodontal disease induction

Before periodontal disease induction, all animals presented normal CAL measurements of less than 3mm. Periodontal pockets were found both at prophylaxis, with mean CALs of 7.7mm±2.76 for the 4th premolars and 7.3mm±3.78 for the 2nd premolars, and immediately before GTR, when the mean CAL was 6.8mm±1.75 for the 4th premolars and 6.6mm±2.54 for the 2nd premolars. No significant differences between

4^{th} and 2^{nd} premolars were found (one-way ANOVA). Also, no significant difference (one-way ANOVA) was found between the size of the defects of the 2^{nd} and 4^{th} premolars in all planes: mesiodistal (p=0.294), apicocoronal (p=0.695) and buccolingual (0.560).

3.3. GTR surgical and clinical evaluations

The bilayered PLGA+CaP biomaterial was easily moldable, adapting well to the contours of the defects and completely occluding it at all sides (mesial, distal, coronal and apical). Healing occurred as expected, with discrete bleeding and edema on the first day after surgery in both groups. During the first week after surgery, most of the defects, regardless of treatment, presented a discrete gingival recession that would spontaneously heal in one or two days. A discrete exposure of the membrane occurred in one animal 11 days after surgery, which healed in two days with no further interventions. No gingival recession was observed in PLGA+CaP group at 30, 60, 90 and 120 days after surgery while it occurred in four control defects on each of the first three time-points.

The mean CAL for the PLGA+CaP group decreased to a normal value on a greater rate than the controls, although no significant difference was found between time-points. For the PLGA+CaP group, the mean CAL of 6.8mm±1.75 measured immediately before GTR, a value resulted from the previous periodontal disease condition, had decreased to 3.9mm±1.91 at 30 days after GTR. At 60 days, mean CAL had reached a value considered normal for dogs (2.6mm±1.17), decreasing to 2.4mm±0.89 and 1.8mm±0.44 at 90 and 120 days, respectively. As for the mean CAL of the controls, the 6.6mm±2.54 CAL measured immediately before GTR decreased to 5.0mm±0.81 at 30 days. From 60 days forward, it remained slightly above normal values for dogs: 3.6mm±1.17, 3.6mm±1.34 at 90 days and 3.8mm±1.34 at 120 days.

3.4. MicroCT

MicroCT volumetric values showed more bone volume per total volume (original defect volume) in PLGA+CaP samples than in control ones at 60 ($p<0.01$) and 120 ($p<0.01$) days. Also trabeculae number (n) and thickness (t) of the PLGA+CaP group was significantly higher than control group at 60 [p(n)=0.022 / p(t)=0.018] and 120 [p(n)=0.011 / p(t)=0.014] days. The trabeculae separation for the PLGA+CaP group

was significantly shorter than control group also for both time-points (p 60 days=0.027, p 120 days= 0.018). Fig 3 summarizes these data which can also be related to the 2D (slices) and 3D images shown in Figs 4, 5, 6 and 7. When comparing the time-points in each group, trabeculae thickness of the samples obtained at 60 days was significantly different than the samples from 120 days in the PLGA+CaP group (p=0.006) as well as in the control group (p=0.032).

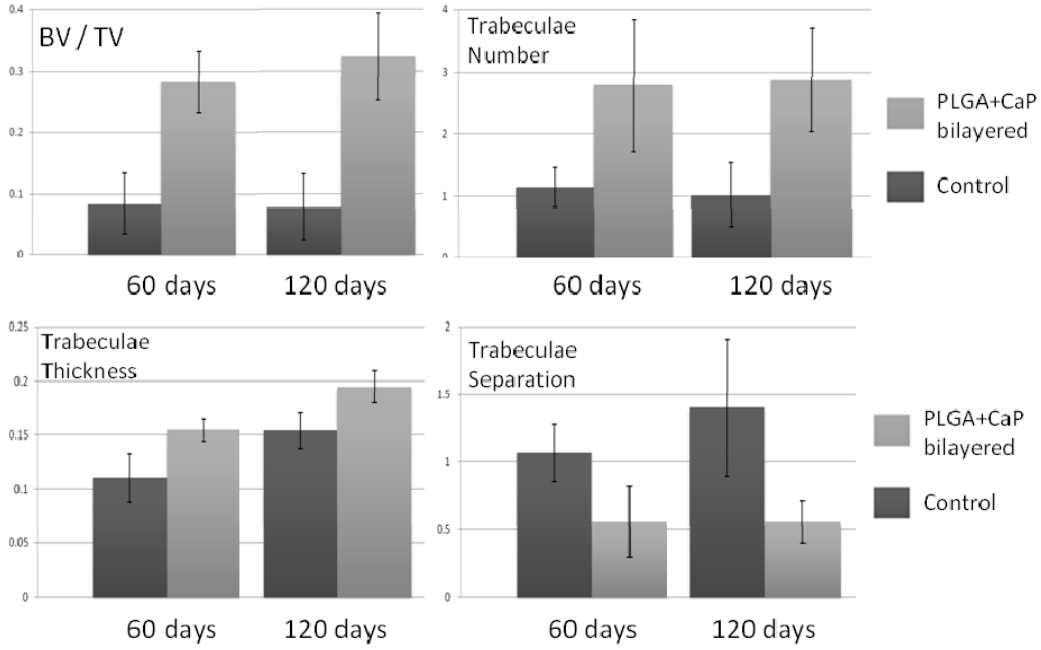

Figure 3. MicroCT volumetric values. Significant differences were found between PLGA+CaP and control group for all the volumetric data at both time-points (bone volume per total volume (BV/TV) and trabeculae number, thickness and separation.

On qualitative analysis of the images of control group it could be seen that the extent of the alveolar bone was limited to the most lingual part of the defect (Fig 4A, B) on the buccolingual direction. Class III furcation defects (through-and-through defect, with no lingual wall), were observed in 3 animals of 60 days and in another 3 animals at 120 days (Fig 4A). On the contrary, alveolar bone was observed up to the most coronal part of the furcation in all samples of the PLGA+CaP group (Figs 5A, 6A and 7A) and on the buccolingual direction, it reached the buccal part of the defect (Figs 6A, B and 7A, B, C).

3.5. Histological analysis

The defect area in control samples were mainly filled by a dense connective tissue (Fig 4C) at both time-points. At the interface with the root on the most coronal part of the defect, loose connective tissue was found (Fig 4D). Cementum and PDL were observed along with alveolar bone in the few areas closer to the apical bottom of the defect where this last one could be found.

In contrast, the periodontium had been formed in the defects of all samples of the PLGA+CaP group at both time-points. On the apical bottom of the defects, a reversal line outlined the interface between old and new alveolar bone, this later, mainly woven bone where some osteons could be found, mostly at 120 days. New cementum and PDL were always present along with the new alveolar bone from the apical bottom up to the most coronal part of the defect as well as from the lingual to the buccal area (Figs 5, 6 and 7). Trabeculae were densely covered by active osteoblasts and osteoid could be easily distinguished in the undecalcified samples (Fig 5F). Similarly, cementoid and highly active cementoblasts covered the new cementum, (Fig 5E), which was formed only by the cellular layer (Fig 7F) with no acellular one. In the cross-sections, the cellular cementum was found in continuity with the old cementum along the root surface (Fig 7F). Extrinsic collagen fibers emerged from the new cementum to a well organized PDL with parallel and perpendicular fibers and blood vessels. Under polarized microscopy, the birefringent collagen fibers were well characterized emerging from the new cementum, crossing the PDL space and inserting into new bone (Fig 7D and E). Remnants of the PLGA+CaP bilayered biomaterial were observed only in samples at 60 days (Fig 5B) immersed and in direct contact with the new bone (Fig 6C and D). No ankylosis or root resorption were observed.

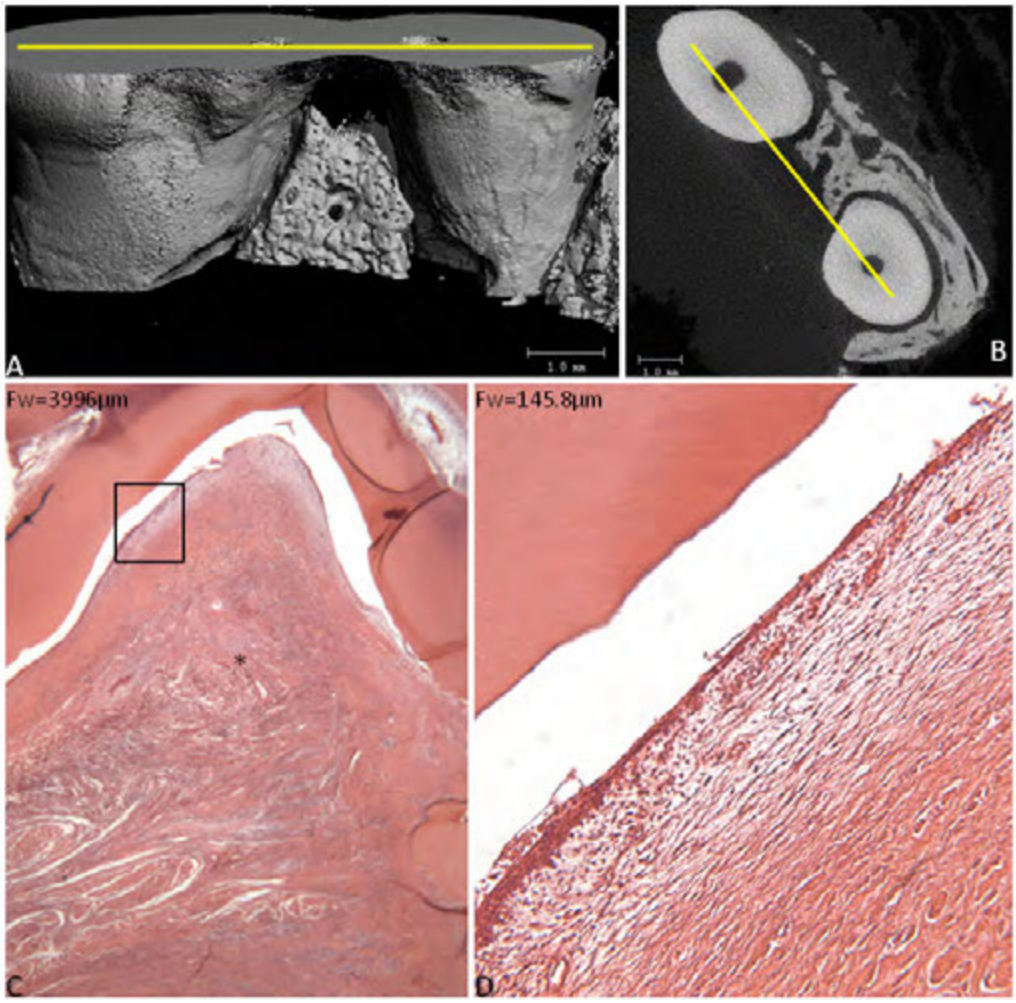

Figure 4. MicroCT images of a control sample at 120 days (top) and the corresponding photomicrographs. Note the small extent of bone to the coronal and buccal parts of the furcation (A and B). Yellow lines refer to the mesiodistal sections in C and D. A dense connective tissue filled the defect in the central area (C - *) and loose connective tissue was found on the interface with the roots (D – detail of the area marked in C).

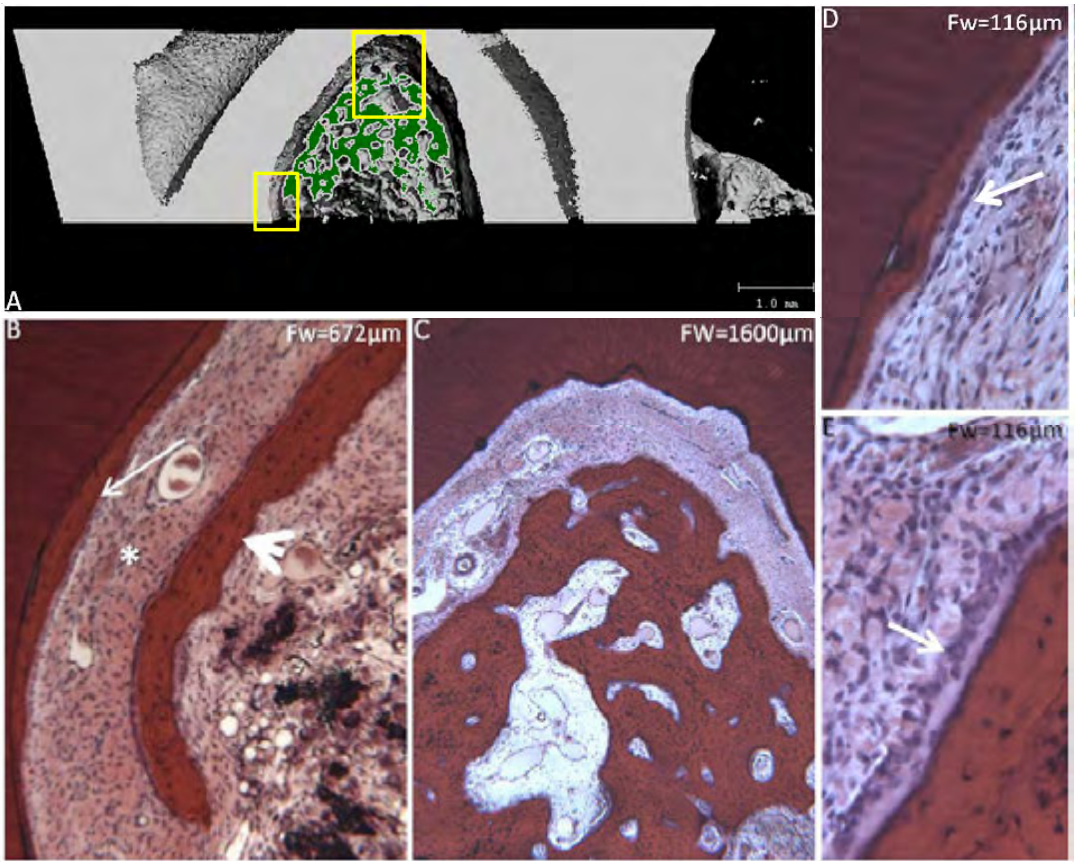

Figure 5. MicroCT image of a mesio-distal view (A) of a PLGA+CaP sample at 60 days and the corresponding photomicrographs (B, C, D and E). A. New alveolar bone fills the defect area, emphasized by the green color on the sectioned bone. C. Section obtained from the most coronal part of the furcation, marked in A. Note the alveolar bone up to the most coronal part of the furcation, with the newly formed periodontal ligament. B. The notch area marked in A on the bottom of the defect, where remnants of the PLGA+CaP biomaterial can be seen (empty arrows), as well as new bone (thick arrow head), periodontal ligament (*) and cementum (thin arrow). Cementoblasts and cementoid were seen in these areas (arrow in E) as well as osteoblasts and osteoid (arrow in D).

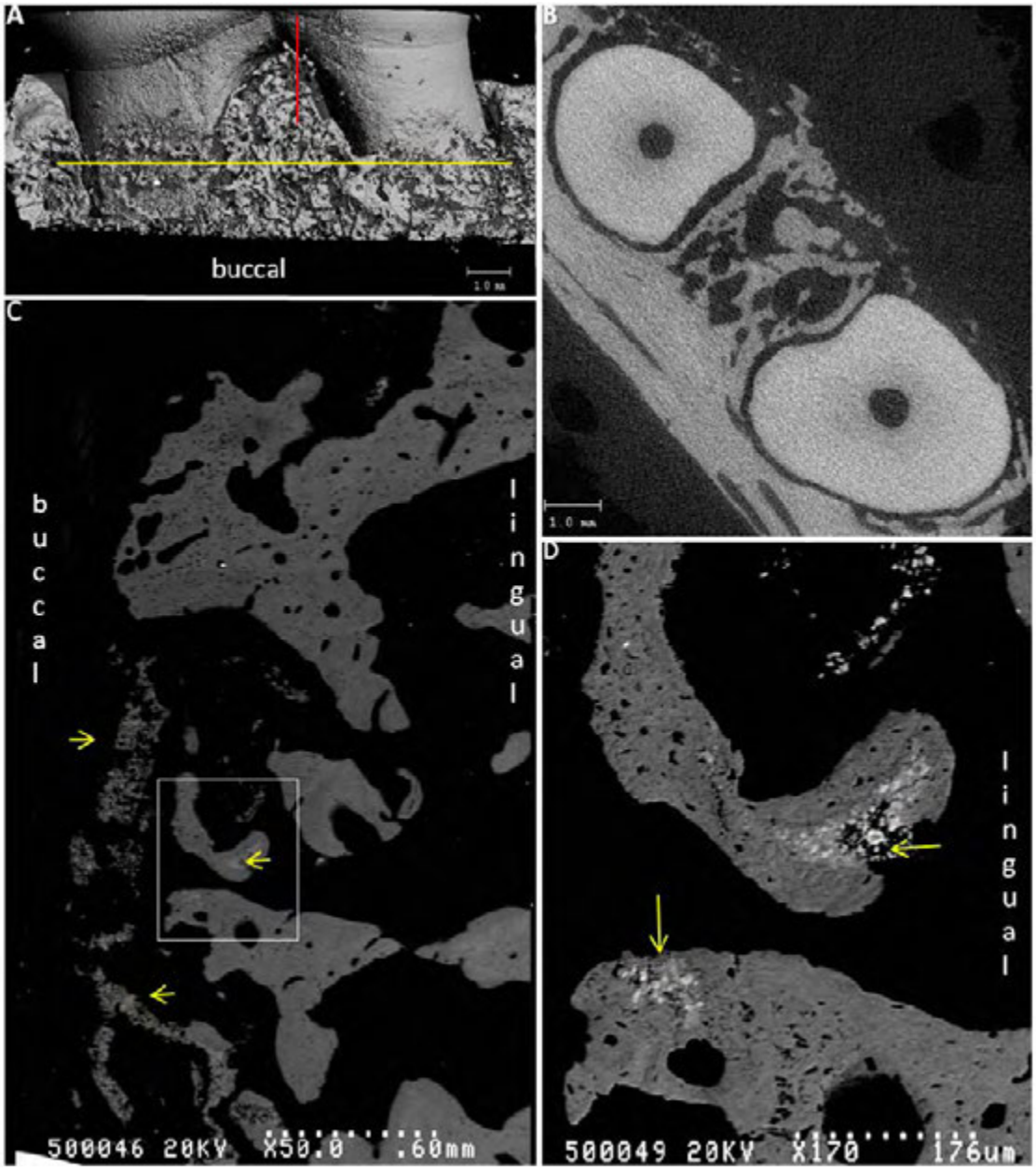

Figure 6. MicroCT (A and B) and SEM backscattered electron (C and D) images of a PLGA+CaP sample at 60 days. A. 3D image showing references of the images in B, C and D. Yellow line indicates the cross-section in B, where bone can be seen on the buccal side of the furcation. Red line indicates buccolingual sections on C and D. Remanents of the PLGA+CaP biomaterial can be seen on the buccal side, surrounded and in direct contact with new alveolar bone. D is an enlargement of the area marked in C.

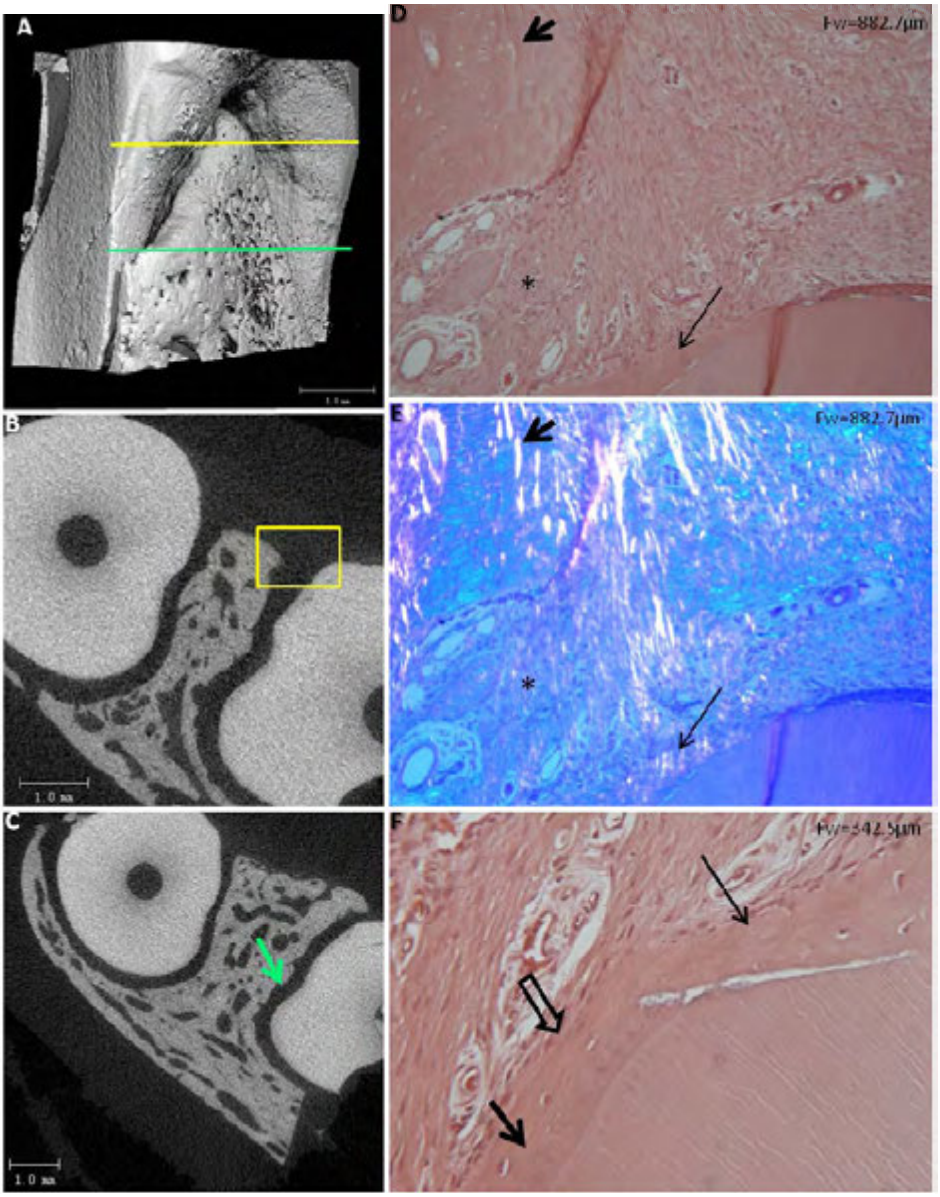

Figure 7. MicroCT images of a PLGA+CaP sample at 120 days (A, B and C). Alveolar bone extends to the coronal and the buccal parts of the furcation. Yellow and green lines mark the location of the cross-section in B and C, respectively. B. Location from where D and E were obtained. D. New cementum (thin arrows) and periodontal ligament (*) formed along with the new alveolar bone (large arrows). E. Same field as D under polarized light, where collagen fibers of the periodontal ligament insert perpendicularly in the new cementum, cross the periodontal ligament and also insert in the bone on the opposite side. F. Field taken from the area marked in C. New cellular cementum (thin arrow) continuous to the acellular (thick arrow) and cellular (empty arrow) layers of old cementum. (C. Same level as the control sample in Figure 4B).

4. Discussion

We here report the development of a PLGA+CaP bilayered biomaterial and its potential on periodontal regeneration, which comprises the formation of new alveolar bone, PDL and cementum [39,40]. Results clearly showed the regeneration of these three supportive tissues of the tooth in class II furcation defects on a greater extent when the biomaterial was applied.

Indeed, membranes made of different biomaterials such as ePTFE [2,28], collagen [2,29], polydioxanon [2] and polylactic acid (PLA) [2,41] have been reported to favor the regeneration of class II furcation defects. However, all these studies based their analyses and conclusions exclusively on the histomorphometry of mesiodistal sections. As already discussed by Christgau et al. [2], mesiodistal sections of a class II furcation defect can mistakenly originate from an area too close to the intact periodontal tissue on the lingual side, which can show more regeneration than sections taken from more buccal regions when quantified by histomorphometry. As follows, our work innovates in some aspects. The first is by showing histologically the difference on the regeneration between treated and control groups not only on mesiodistal sections but also on buccolingual and cross-section ones, hence, all possible planes of view. Furthermore, the linear measurements made on MicroCT images provided precise references to obtain histologic sections. Such references avoided mistakes on the location from where the sections where taken, especially mesiodistal ones, what made the analysis of the exact same area in PLGA+CaP and control groups a feasible task.

Another innovation is the quantification of the volume of the regenerated alveolar bone by MicroCT. To quantify bone regeneration, previous works have measured by histomorphometry the vertical extent of bone along the root surface, which is a 2D-estimate of the bone area regenerated. Micro CT, on the other hand, provides not an estimated area but the real bone volume regenerated. MicroCT data also comprises trabeculae number, thickness and separation, showing quantitatively a more mature bone in the PLGA+CaP samples than in control ones. Additionally, as PDL and cementum were always and only observed along with the new bone in every section (buccolingual, mesiodistal and cross-sections), a quantitative data of these tissues can be inferred from the volume of new bone: more PDL and cementum regenerated in the PLGA+CaP group than in the control one.

Up to now, to the extent of our knowledge, the PLGA+CaP bilayered biomaterial has shown the regeneration of more buccal bone than has been previously reported [2,30,32,33,42-44], what is probably a result of its design: the outer flat layer coupled to the macroporous inner layer. Such structure made it moldable to the tooth and mandible anatomies, but stiff enough to prevent a collapse into the defect. In fact, numerous reports on the collapse of resorbable membranes made of materials such as collagen [2,30], PLA [2,32] and PLGA [33] can be found; making it their major disadvantage today. By collapsing into the defect, the biomaterial occupies the space that should be available for the regeneration, impairing this process. Thus, the PLGA+CaP bilayered biomaterial can be entitle as a space-provision device, i.e., it presents stability over the defect providing a space for the regeneration to occur, which has a substantial influence on the amount of regeneration achieved in a defect [32,40,45].

Further on space-provision, in addition to not collapsing into the defect, the membrane must also sustain wound contraction and cell migration, parts of any repair process. Periodontal regeneration can only be attained after an intricate, overlapping sequence of events has occurred. Briefly, platelet activation will promote blood clot and fibrin matrix formation, which are followed by the formation and budding of new blood vessels (angiogenesis) through which progenitor cells migrate, directly influencing matrix contraction, until they reach a stable surface and start laying down matrix [2,46-48]. More specifically, the importance of the fibrin matrix and wound contraction was also discussed on periodontal regeneration [40]. Accordingly, the PLGA+CaP bilayered biomaterial was designed with a topographically complex inner surface (macropores and interconnections covered by micro and nanopores), to favor fibrin matrix attachment [49,50]. As the biomaterial is stiff and capable of maintaining its contour, it could in turn prevent or limit the contraction, providing a greater volume of provisional matrix trough which regeneration occurred. Additionally, the complex surface topography engenders greater platelet adhesion as well as osteoblast activity [34,35,49,50]. Upon signaling, progenitor cells for the regeneration process originate from the perivascular undifferentiated cells from vessels in the remaining PDL and alveolar bone as shown by McCulloch and colleagues [51,52] and also explored by our research group [53,54]. Hence, progenitor cells had both a greater volume of matrix through which they could migrate and the signaling for the migration to a more buccal

region, resulting on a greater regeneration of buccal bone in the furcation defect [2,30,32,33,42-44].

Different topographically complex membranes for GTR have been developed to favor periodontal regeneration, including a greater extent of bone on the buccal region. Their structure, degradation rate and influences on cell activity were analyzed in vitro, but are yet to be evaluated in periodontal defects [34,35,50,55-59]. In vivo works studied membranes with no complex topography in periodontal regeneration focusing on the buccal region, i.e., histologic sections on buccolingual views like the present work. Experimental works in dogs can be found [2,33,42,43,60], however, they cannot be directly compared to our work for different reasons. Three of them [42,43,60] evaluated acute defects which have been reported to spontaneously heal in some extent without GTR, what could have influenced the results [41,61]. Furthermore, acute defects are under an entirely different environment since they were not affected by periodontal disease which encompasses previous local infection and chronicity that, as an example, chemically affects the cementum [2,41]. From the studies using chronic defects, one did not analyze the furcation [33] and the other one described the healing sequence on furcation defects but no quantification of the regenerated tissues was made [2]. Overall, they all showed limited amount of regeneration on the buccal side.

Indeed, it is known that only a portion of the defect can be successfully regenerated by techniques and membranes available today [16]. Experimental works and clinical trials either clearly showed a lack of complete closure of furcation defects or failed to address their entire volume [28,41,62,63]. In the present study, although tissues inside the furcation regenerated and a greater amount of regeneration on the buccal region was achieved, not the entire buccal periodontium was formed, mainly lacking on the most coronal part of the defect. It is noteworthy that remnants of the biomaterial were found in samples at 60 but not at 120 days, showing that it was degraded or resorbed between these time-points. Based on the concepts of space-provision, prevention of wound contraction and cell migration already discussed, we hypothesize that if the PLGA+CaP bilayered biomaterial can remain more time in place, more buccal bone could be regenerated, as more cells could reach the buccal and coronal areas. This means a slower degradation rate of the PLGA+CaP bilayered biomaterial, which in fact, can be tuned across a wide range by modifying both the polymeric molecular weight and the co-polymer ratio [36,37]. Such modification raises

the possibility for the complete regeneration of a furcation defect to the extent of the previously healthy periodontium.

5. Conclusion

This study reports the development and pre-clinical analysis of the PLGA+CaP bilayered biomaterial that is resorbable, moldable to different anatomical morphologies, stiff to prevent a collapse into the defect and topographically complex, all favoring regeneration. With such biomaterial, periodontal regeneration of a class II furcation defect was achieved, which comprised the formation of new bone, periodontal ligament and cementum. To the best of our knowledge, we show the regeneration of more buccal bone than has been previously reported, being the first study addressing all views of a chronic periodontal defect.

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Anexo I: Normas “Pesquisa Veterinária Brasileira” - Capítulo I

Objetivo e política editorial

O objetivo da revista Pesquisa Veterinária Brasileira é contribuir, através da publicação dos resultados de pesquisa e sua disseminação, para a manutenção da saúde animal que depende, em grande parte, de conhecimentos sobre as medidas de profilaxia e controle veterinários. Com periodicidade mensal, a revista publica trabalhos originais e artigos de revisão de pesquisa no campo da patologia veterinária no seu sentido amplo, principalmente sobre doenças de importância econômica e de interesse para a saúde pública. Apesar de não serem aceitas comunicações ("Short comunications") sob forma de "Notas Científicas", não há limite mínimo do número de páginas do trabalho enviado, que deve porém conter pormenores suficientes sobre os experimentos ou a metodologia empregada no estudo. Os trabalhos, em 3 vias, escritos em português ou inglês, devem ser enviados, junto com disquete de arquivos (de preferência em Word 7.0), ao editor da revista Pesquisa Veterinária Brasileira, no endereço abaixo. Devem constituir-se de resultados ainda não publicados e não considerados para publicação em outra revista.

Apresentação de manuscritos

1. Os trabalhos devem ser oganizados, sempre que possível, em Título, *Abstract*, Resumo, Introdução, Material e Métodos, Resultados, Discussão, Conclusões (ou combinações destes três últimos), Agradecimentos e Referências:
a) o Título do artigo deve ser conciso e indicar o conteúdo do trabalho;
b) um *Abstract*, um resumo em inglês, deverá ser apresentado com os elementos constituintes observados nos artigos em português, publicados no último número da revista, ficando em branco apenas a paginação, e, no final, terá indicação dos *index terms*;
c) o Resumo deve apresentar, de forma direta e no passado, o que foi feito e estudado, dando os mais importantes resultados e conclusões; será seguida da indicação dos termos de indexação; nos trabalhos em inglês, Resumo e *Abstract* trocam de posição e de constituição (veja-se como exemplo sempre o último fascículo da revista);
d) a Introdução deve ser breve, com citação bibliográfica específica sem que a mesma assuma importância principal, e finalizar com a indicação do objetivo do trabalho;
e) em Material e Métodos devem ser reunidos os dados que permitam a repetição do trabalho por outros pesquisadores;
f) em Resultados deve ser feita a apresentação concisa dos dados obtidos; quadros devem ser preparados sem dados supérfluos, apresentando, sempre que indicado, médias de várias repetições; é conveniente, às vezes, expressar dados complexos por gráficos, ao invés de apresentá-los em quadros extensos;
g) na Discussão os resultados devem ser discutidos diante da literatura; não convém mencionar trabalhos em desenvolvimento ou planos futuros, de modo a evitar uma obrigação do autor e da revista de publicá-los;
h) as Conclusões devem basear-se somente nos resultados apresentados no trabalho;
i) os Agradecimentos devem ser sucintos e não devem aparecer no texto ou em notas de rodapé;
j) a lista de Referências, que só incluirá a bibliografia citada no trabalho e a que tenha servido como fonte para consulta indireta, deverá ser ordenada alfabeticamente pelo

sobrenome do primeiro autor, registrando os nomes de todos os autores, o título de cada publicação e, por extenso ou abreviado, o nome da revista ou obra, usando as normas da Associação Brasileira de Normas Técnicas - ABNT, *Style Manual for Biological Journals* (American Institute for Biological Sciences) e/ou *Bibliographic Guide for Editors and Authors* (American Chemical Society, Washington, D.C.).

2. Na elaboração do texto deverão ser atendidas as normas abaixo:
a) os trabalhos devem ser apresentados em uma só face do papel, em espaço duplo e com margens de, no mínimo, 2,5 cm; o texto será escrito corridamente; quadros serão feitos em folhas separadas, usando-se papel duplo ofício, se necessário, e anexados ao final do trabalho; as folhas, ordenadas em texto, legendas, quadros e figuras, serão numeradas seguidamente;
b) a redação dos trabalhos deve ser a mais concisa possível, com a linguagem, tanto quanto possível, no passado e impessoal; no texto, os sinais de chamada para notas de rodapé serão números arábicos colocados um pouco acima da linha de escrita, após a palavra ou frase que motivou a nota; essa numeração será contínua; as notas serão lançadas ao pé da página em que estiver o respectivo sinal de chamada; todos os quadros e todas as figuras serão mencionados no texto; estas remissões serão feitas pelos respectivos números e, sempre que possível, na ordem crescente destes; Resumo e *Abstract* serão escritos corridamente em um só parágrafo e não deverão conter citações bibliográficas;
c) no rodapé da primeira página deverá constar endereço profissional do(s) autor(es);
d) siglas e abreviações dos nomes de instituições, ao aparecerem pela primeira vez no trabalho, serão colocadas entre parênteses e precedidas do nome por extenso;
e) citações bibliográficas serão feitas pelo sistema "autor e ano"; trabalhos de dois autores serão citados pelos nomes de ambos, e de três ou mais, pelo nome do primeiro, seguido de "et al.", mais o ano; se dois trabalhos não se distinguirem por esses elementos, a diferenciação será feita pelo acréscimo de letras minúsculas ao ano, em ambos; todos os trabalhos citados terão suas referências completas incluídas na lista própria (Referências), inclusive os que tenham sido consultados indiretamente; no texto não se fará menção do trabalho que tenha servido somente como fonte; este esclarecimento será acrescentado apenas ao final das respectivas referências, na forma: "(Citado por Fulano 19...)"; a referência do trabalho que tenha servido de fonte será incluída na lista uma só vez; a menção de comunicação pessoal e de dados não publicados é feita, de preferência, no próprio texto, colocada em parênteses, com citação de nome(s) ou autor(es); nas citações de trabalhos colocados entre parênteses, não se usará vírgula entre o nome do autor e o ano, nem ponto-e-vírgula após cada ano; a separação entre trabalhos, nesse caso, se fará apenas por vírgulas, exemplo: (Flores & Houssay 1917, Roberts 1963a,b, Perreau et al. 1968, Hanson 1971);
f) a lista das referências deverá ser apresentada com o mínimo de pontuação e isenta do uso de caixa alta, sublinhando-se apenas os nomes científicos, e sempre em conformidade com o padrão adotado no último fascículo da revista, inclusive quanto à ordenação de seus vários elementos.

Anexo II: Normas “Polímeros: Ciência e Tecnologia” - Capítulo II

Objetivos

A revista “Polímeros: Ciência e Tecnologia” é editada trimestralmente pela Associação Brasileira de Polímeros - ABPol e tem o objetivo de divulgar trabalhos e atualidades de caráter científico, tecnológico e mercadológico da área de polímeros.

Tipos de trabalhos

Técnico-Científico: São trabalhos originais, com resultados inéditos que apresentam real avanço e significativa contribuição para a área de polímeros.

Conteúdo

A redação dos trabalhos deverá primar por clareza, brevidade e concisão. Os trabalhos deverão ser enviados em arquivos do programa “Word for Windows” com a extensão .DOC e necessariamente conter: Título, Resumo (de 100 a 200 palavras), Palavras-chave, Introdução, Experimental (se aplicável, podendo incluir Materiais, Métodos, etc), Resultados e Discussões, Conclusões, Agradecimentos (se aplicável), Referências Bibliográficas e *Title*, *Abstract*, *Keywords* (em Inglês).
Tabelas, figuras, gráficos e fotos - deverão ser enviados em arquivos separados e elaboradas levando-se em consideração que serão impressos no espaço de uma coluna (8 cm de largura) ou excepcionalmente de duas colunas (16,7 cm de largura). Caso se utilize algum programa para confecção de gráficos e figuras, o arquivo original deve ser enviado, especificando-se. No texto deverão ser sugeridos os locais onde devem aparecer, incluindo-se suas legendas. Os arquivos de imagens, desenhos e gráficos devem sempre que possível ser enviados em formato vetorial (CDR, EPS, AI, WMF etc), caso contrário os arquivos *bitmap* (TIF, BMP, JPG, PSD etc) devem ter boa definição (normalmente de 1000 a 2000 pixels de largura) tendo-se em mente que serão reproduzidos a partir desses originais. Os arquivos de fotografias devem ser em preto e branco, nítidas e em um dos formatos bitmap citados acima. No caso de micrografias, deve-se incluir a barra para referência dimensional. Todo o material ilustrativo deverá ter o arquivo correspondente nomeado de modo claro (por exemplo: figura-1a, figura-1b, quadro-1, tabela-1 etc).

Tamanho dos trabalhos

Todos os trabalhos encaminhados como artigos (independente da natureza) deverão ser apresentados numa extensão não excedendo a 20 laudas (incluindo figuras, tabelas e fotos). Os artigos de revisão, apesar de uma abordagem mais completa, não devem exceder 30 laudas; enquanto as comunicações, que são caracterizadas pela sua brevidade, não devem exceder 7 laudas.

Observação: Uma lauda corresponde a uma página de formato A4, com 25 linhas em espaçamento duplo, em fonte “Times New Roman”, tamanho 12. Cada figura, tabela ou foto equivale a uma lauda.

Referências bibliográficas

Referências Bibliográficas: As citações bibliográficas no texto devem aparecer entre colchetes, enumeradas na seqüência de aparecimento no texto e listadas no final do trabalho. As referências devem seguir o seguinte formato:

Artigos em Periódicos:
Cohen, D.; Siegmann, A. & Narkis, M. - Polym. Eng. Sci., 27, p.286 (1987).

Artigos em Anais:
Vesely, D. - "Microstructural Investigation of Polymer Blends", in: Anais do 4º Congresso Brasileiro de Polímeros, p.17, Salvador - BA, set/out (1997).

Livros/Capítulos de Livros:

- Tadmor, Z. & Gogos, C. G. - "Principles of Polymer Processing", John-Wiley, New York (1979).
- Hieber, C. A. - "Melt Viscosity Characterization and Its Application to Injection Molding", in: Injection and Compression Molding Fundamentals, cap.1, Avraam I. Isayev (ed.), Marcel Dekker Inc., New York (1987).

Teses:
Rabelo, D. - "Formação da Estrutura Porosa em Copolímeros à Base de Estireno e Divinilbenzeno", Tese de Doutorado, Universidade Federal do Rio de Janeiro, Brasil (1993).

Anexo III: Normas "Australian Veterinary Journal" - Capítulo III

Instructions for authors

Aims and scope
The *Australian Veterinary Journal* (AVJ) is the official journal of the Australian Veterinary Association. The AVJ aims to advance veterinary science by publishing and promoting high quality refereed scientific and clinical articles.

The AVJ publishes original articles, case reports, short contributions, clinical updates, diagnostic challenges, reviews and veterinary history articles. All articles are peer reviewed. The AVJ Peer review process operates under the guidelines of the World Association of Medical Editors (http://www.wame.org/).

Ethical considerations
Submission to the AVJ confirms that the protocol for the research project has been approved by a properly constituted Ethics Committee of the institution within which the work was undertaken and that, if applicable, it conforms to the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000). The AVJ retains the right to reject any manuscript on the basis of unethical conduct of either human or animal studies.
The handling and use of animals in experiments must conform to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. In cases likely to raise controversy, an appropriate reference in the article to approval by an animal experimentation ethics committee is recommended.

Manuscript style
The AVJ uses the Style Manual for Authors, Editors and Printers, Scientific Style and Format: The CBE Manual for Authors, Editors, and Publishers and recommends Strunk's classic book, for clarity of expression.

Do not use Enter at the end of lines within a paragraph. Do not underline anything.

Units
Use SI basic or derived units or declared units of the Australian metric system (e.g. ha, min, °C) where applicable. Write dates in the form 10 April 2002 and currency in the form A$33. Spell out single digit numbers that express a quantity (three sheep, five paddocks) but not if used with an SI or similar unit or its symbol (5 mL, 9 m, 7 weeks, 6°C) or as an identifier (group 4, farms 7 and 9). If you start a sentence with a number, spell it out. Type a space between a number and its unit symbol, except for °C and %. Use a comma as a thousands marker in numbers of more than four digits (e.g. 21,000).

Abbreviations
Abbreviations should be used only where they ease the reader's task by reducing repetition of long, technical terms. Use abbreviations only if the term is used three or more times. All abbreviations are to be listed in the abbreviations list and written out in full the first time they appear in the text, followed by the abbreviation in brackets. Exceptions are SI units and commonly used terms that can be understood from the

context, for example: IV, SC, IM, DNA, RNA, EDTA, IgA, IgG. These need not be written out in full or included in the abbreviations list.

Trade names
Mention the manufacturer and the essential information on drugs, reagents and equipment in parentheses within the text. Details on commonly used and well-known materials may not be necessary unless likely to influence the results.

Article format
Please refer to the specific instructions below for each type of article.

Title
The title should be concise, specific and informative, but should not make an assertive claim about the conclusions of the study. Avoid including geographical locations unless they are of epidemiological significance. Title should have only the initial letter capitalised.

Authors' names and addresses
Give initials and surnames in capitals without stops, following the convention of first name, then family name. Separate the authors' names with a comma, except the names of the last two authors, which are separated with 'and' in lower case letters. Include the addresses of the institutions at which the work was carried out. The submitting author will be the author for all correspondence. Include the submitting author's email address. The present address of the submitting author, if different from that where the work was carried out, should be supplied.

Headings
Do not indent headings or end headings with stops. Only the first letter is capitalised. Major headings are typed in bold on a separate line. First-order subheadings are typed on a separate line and italicised. Second-order subheadings are italicised and followed by a tab to separate them from the text, which follows on the same line. Do not number subheadings, paragraphs or itemised lists in the text.

Key words
Key words are used by indexes and electronic search engines, and should appear after the abstract. Use the heading 'Key words:' and then the key words separated by commas. Include up to six key words. Also enter the key words where prompted during the submission process. Key words are required for all papers.

Acknowledgments
Sources of funding and donations should be acknowledged. Authors should acknowledge only significant intellectual and technical contributions, and permission from those listed should be obtained before publication.

References
Use references judiciously. Cite only those publications that are essential for the understanding of the study.

Number text references consecutively with superscript Arabic numerals that follow any

punctuation marks, with no space in between. Construct the reference list in the same numerical sequence of the references in the text. References cited only in tables or in figure legends are numbered according to the first identification of the table or figure in the text. References to journals, books, conference proceedings, organisational papers, anonymous editorials, foreign language articles and internet websites, respectively, are written as follows:

1. Gibson KT, Hodge H, Whittem T. Inflammatory mediators in equine synovial fluid. Aust Vet J 1996;73:148–151.
2. Peterson ME, Randolph JF, Mooney CT. Endocrine diseases. In: Sherding RG, editor. The Cat: Diseases and Management. 2nd edn. Churchill Livingstone, New York, 1994:1403–1506.
3. Rhodes AP. Infectious bovine keratoconjunctivitis vaccination. In: Proceedings of the 23rd Seminar, Sheep and Beef Cattle Society, New Zealand Veterinary Association, June 1993.
4. Australian Veterinary Association. Tethering of sows and sow stalls. In: Greenwood PE, editor. Members' Directory and Policy Compendium. 1997:B5
5. Where do we stand on manpower? [editorial] Vet Rec 1995;137:1
6. Homberger FR. Mäusehepatitis-Virus. Schweiz Arch Tierheilkd 1996;138:183–188.
7. Council of Docked Breeds. The case for docking. http://www.cdb.org. 1992. Accessed 15 October 2001.

List all authors if there are five or fewer. When there are more than five authors, list only the first three and add 'et al'. Write titles of books, journals and other publications in italics. Do not underline or use bold letters.

The abbreviation of journals follows that of Serial sources for the BIOSIS previews database. A list of journal abbreviations can be found at
http://entnemdept.ifas.ufl.edu/all_journals.htm.

Journal abbreviations do not contain stops. Cite references to unpublished work only in the text, with a notation of (personal communication) or (unpublished). Please send a copy of any cited work that is included in the reference list as 'in press'. It is the authors' responsibility to check the accuracy of reference citations.

Tables

Tables should be self-contained and complement, but not duplicate, information contained in the text. Format tables with the table function in a word processor, such as MS Word, on a separate page with the legend typed above. Column headings should be brief, with units of measurement in parentheses. All abbreviations must be defined in footnotes to the table. Use superscript lower case letters to mark footnotes and superscript capital letters to mark statistical significance.

Number tables consecutively in the order they occur in the text, with Arabic numerals.

Figures

Include figures only if they are informative and necessary for the understanding of the text. Figures must be uploaded as separate files and not be embedded in the main text file. Each figure must be uploaded separately from other figures.

Line figures and graphs should be supplied in their original format, preferably as .xls or .eps files.

Photographs should be in sharp focus and cropped appropriately. They should be of sufficient clarity to enable identification of relevant features. Submit photographs as .tif

or .jpg files with a resolution of at least 300 dpi, and at least 8.6 cm in image width at that resolution. It is not possible to print images that are of insufficient resolution. Scale bars must be included on micrographs.
Digital manipulation of an image is acceptable only if it is done to enhance photographic density or to eliminate artefacts. Any digital manipulation must be mentioned in the figure legend. The author(s) must also state in the covering letter that the scientific content of the image has not been altered. The Editor may need to examine the original image.
Number figures consecutively in the order they occur in the text, with Arabic numerals.

Figure and table legends
Legends should be concise, but comprehensive. The figure or table and its legend must be understandable without reference to the text. Include definitions of any symbols used and define/explain all abbreviations and units of measurement.

Article types
Please look at a recent issue of the AVJ to see the elements of each article type. The Editors may change the categories of submitted articles at their discretion.

Original article
Maximum 6500 words including up to 40 references. Include a structured abstract of up to 250 words. The abstract's subdivision is up to the author, but should encompass the objective, design, procedure, results and conclusion. Type abstract subheadings in bold with only the first letter capitalised, separated by a tab from the text on the same line. The main headings, following an untitled introduction, are Materials and methods, Results, Discussion, Acknowledgments and References. The introduction should state the purpose of the study. The content of Materials and methods should enable others to reproduce the work. Present the findings in Results concisely and logically. Evaluate and interpret the findings in the Discussion, but do not present new data. If possible, write the main conclusions at the end of the Discussion. Headings may vary from standard if the variation makes the article more informative.

Conclusions
Conclusions should be justified by the results of the analysis. The null hypothesis (NH)
(i) Failure to reject the NH, that is, the finding that the effects tested are 'not significant', does not prove that the NH is true. Calculation of the CI for the effect may afford a basis for concluding that any effect is inconsequential.
(ii) Rejection of the NH, that is, the finding 'statistically significant', is prima facie evidence for the existence of the effect investigated, but bias as a possible reason for the difference must also be examined. The proposition that it is biologically unimportant should also be supported by argument from the CI. Discrepancies: Internal inconsistencies, for example, in the level of the end-measure between one part of the experiment and another, should be addressed in the discussion.

Anexo IV: Normas "Biomaterials" - Capítulo IV

Guide for Authors

Role of the Funding Source
You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. If the funding source(s) had no such involvement then this should be stated. Please see http://www.elsevier.com/funding.

Manuscript Preparation
General: Manuscripts must be word processed (preferably in Word format), double-spaced with wide margins and a font size of 12 or 10 pt. Files prepared in LaTEX are not supported. Figure captions should be in Arial font 9pt. Please check the current style of the journal, particularly the reference style (Vancouver), and avoid excessive layout styling as most formatting codes will be removed or replaced during the processing of your article. In addition, do not use options such as automatic word breaking, justified layout, double columns or automatic paragraph numbering (especially for numbered references). However do use bold face, italic, subscripts, superscripts etc. The corresponding author should be identified (include a Fax number and E-mail address). Full postal addresses must be given for all co-authors. The Editors reserve the right to adjust style to certain standards of uniformity. The preferred style is: Surname, Initials, Department, Institution, City/State, Postal Code, Country. Authors should retain copies of all versions of their manuscript submitted to the journal. Authors are especially requested to be vigilant over the submission of the correct version of the manuscript at the various stages of the editorial process.

English Language: Manuscripts should be proof-read and have English language errors corrected before submission as we may have to return papers due to poor language usage. Authors who require information about language editing and copyediting services pre- and post-submission please visit
http://www.elsevier.com/languagepolishing or contact authorsupport@elsevier.com for more information.

Paper Length: Authors are urged to write as concisely as possible.

Abstracts: All manuscripts are to be supplied with an Abstract of about 100-200 words in length.

Keywords: Authors must provide 4-6 keywords for indexing purposes.

Text: Follow this order when typing manuscripts:Title, Authors, Affiliations, Abstract, Keywords, Introduction, Materials and Methods, Results, Discussion, Conclusions, Acknowledgements, References, Appendix (where necessary), Figures, Figure Captions and Tables, Supplementary Data. The corresponding author should be identified with an asterisk and footnote. All other footnotes (except for table

footnotes) should be identified with superscript Arabic numbers. An abbreviated title for use as a running headline should also be supplied. Authors are requested to acknowledge funding sources for the work.

Units: The SI system should be used for all scientific and laboratory data; if, in certain instances, it is necessary to quote other units, these should be added in parentheses. Temperatures should be given in degrees Celsius. The unit 'billion' (109 in America, 1012 in Europe) is ambiguous and must not be used. If a large number of symbols are used it is helpful if authors submit a list of these symbols and their meanings.

References: All publications cited in the text should be presented in a list of references following the text of the manuscript. In the text refer to references by a number in square brackets on the line (e.g. Since Peterson[1]), and the full reference should be formatted according to the permutation of the Vancouver reference system with numbers in the text. It is suggested that authors refer to the following link for a comprehensive overview of the Vancouver reference styles: Vancouver Reference Styles

Examples of formatting follow:
1. Driessens FCM, Boltong MG, Bermudez O, Planell JA. Formulation and setting times of some calcium orthophosphate cements: a pilot study. J Mater Sci: Mater Med 1993;4:503-508.

2. Nancollas H. In vitro studies of calcium phosphate crystallisation. In: Mann S, Webb J, Williams RJP, editors. Biomineralization. Chemical and biochemical perspectives. New York: VCH, 1989. p. 157-182.

3. Brown W, Chow LC. Combinations of sparingly soluble calcium phosphates in slurries and paste as mineralizers and cements. US Patent No. 4612053, 1986.

N.B.: ?Et al? must be used after the first 6 authors have been named. *Biomaterials* does not use the publication month or day.

Online Sources: References to online sources should contain at a minimum the full URL and year the source was accessed. Furthermore, if known, the following information should be given: author names, dates, reference to a source publication. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.