



UNESP - Universidade Estadual Paulista
“Júlio de Mesquita Filho”
Faculdade de Odontologia de Araraquara



Mateus Machado Delfino

**Parâmetros hepáticos, biocompatibilidade e bioatividade do Bio-C Pulpo e
MTA Repair HP**

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MTA Repair HP**

Tese apresentada à Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara como requisito para obtenção do título de Doutor em Odontologia, na Área de Endodontia

Orientador: Prof. Dr. Paulo Sérgio Cerri

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MTA Repair HP**

Comissão julgadora

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À CAPES:

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RESUMO

Objetivo: avaliar a resposta do tecido conjuntivo promovida pelo Bio-C Pulpo, MTA Repair HP (MTA-HP) em comparação ao MTA Branco (MTA-B), usado como padrão ouro. Além disso, foi avaliado se esses materiais causam alterações hepáticas. **Metodologia:** tubos de polietileno preenchidos com Bio-C Pulpo, MTA-HP e MTA-B e tubos vazios (grupo controle, GC) foram implantados no tecido conjuntivo do subcutâneo de 80 ratos por 7, 15, 30 e 60 dias. O escore de reação inflamatória (ERI), espessura da cápsula, a quantidade de colágeno, o número de células inflamatórias (CI) e fibroblastos (FB), reações imuno-histoquímicas para detecção de interleucina-6 (IL-6), fosfatase alcalina (ALP), fator de crescimento de fibroblastos-1 (FGF-1), Ki-67 e IL-10 foram avaliados. A reação de von Kossa seguida da detecção de ALP, também foi realizada. Aos 60 dias, os níveis séricos de transaminase glutâmico-oxaloacética (TGO) e transaminase glutâmico-pirúvica (TGP) foram medidos e fragmentos do fígado foram coletados para análises histológicas e morfométricas; os dados foram avaliados pelo ANOVA de uma via seguida pelo pós-teste de Sidak. Os dados de biocompatibilidade e bioatividade foram submetidos à análise ANOVA two-way seguida do teste post-hoc de Tukey, exceto os dados referentes ao ERI. Os dados do ERI foram submetidos ao teste não paramétrico ANOVA de Kruskal-Wallis seguido pelo teste de Dunn ($p \leq 0,05$). **Resultados:** nenhuma diferença significativa foi detectada nas concentrações séricas de TGO e TGP e no número de hepatócitos entre as amostras experimentais e GC. Embora Bio-C Pulpo tenha apresentado o maior número de CI e células imunomarcadas à IL-6 ($p < 0,0001$) em todos os períodos, não foi observada diferença significativa no ERI entre os materiais biocerâmicos, exceto aos 60 dias. Nesse período, o MTA-B apresentou ERI menor. Todos os grupos mostraram uma redução significativa na espessura da cápsula e no número de CI e de células IL-6-imunopositivas com o decorrer do tempo. Os espécimes de Bio-C Pulpo, MTA-HP e MTA-B mostraram maior imunoexpressão de ALP do que o GC ($p < 0,0001$), em todos os períodos. Estruturas von Kossa-positivas e birrefringentes foram observadas nas cápsulas ao redor dos materiais. Células imunomarcadas à ALP foram observadas adjacente às estruturas von Kossa-positivas. Em relação ao número de FB, não foram detectadas diferenças significantes entre os grupos Bio-C Pulpo, MTA-HP e MTA-B ($p > 0,05$), aos 7 dias. As cápsulas de todos os grupos exibiram um aumento significativo no número de FB e na quantidade de colágeno ao longo do tempo. De 7 a 60 dias, uma redução significativa na imunoexpressão do FGF-1 e do Ki-67 foi observada em todas as amostras. Aos 7 e 15 dias, a maior imunoexpressão para Ki-67 foi encontrada nas amostras Bio-C Pulpo, enquanto, após 30 e 60 dias, nenhuma diferença significativa foi observada entre os materiais biocerâmicos. De 7 para 60 dias, um aumento significativo na imunomarcagem de IL-10 foi encontrado em todos os grupos. Aos 60 dias, as cápsulas ao redor dos materiais apresentaram um maior número de células marcadas com IL-10 do que nas amostras do GC ($p < 0,0001$). **Conclusões:** os materiais Bio-C Pulpo, MTA-HP e MTA-B não causaram alterações morfológicas no fígado e nos níveis séricos de TGO e TGP. O Bio-C Pulpo e o MTA-HP são biocompatíveis e apresentam potencial bioativo semelhante ao MTA-B. Os materiais biocerâmicos estimulam a secreção de IL-10, que deve exercer uma atividade anti-inflamatória nas cápsulas ao redor dos implantes permitindo a formação de cápsulas ricas em colágeno.

Palavras chave: Teste de materiais. Imuno-histoquímica. Fígado.

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ABSTRACT

Aim To evaluate the connective tissue response promoted by Bio-C Pulpo, MTA Repair HP (MTA-HP) compared to White MTA (WMTA), used as the gold standard. In addition, it was evaluated whether these materials cause liver changes. **Methodology** Inflammatory reaction score (IRS), capsule thickness, amount of collagen, number of inflammatory cells (IC) and fibroblasts (FB), immunohistochemical reactions for detection of interleukin-6 (IL-6), alkaline phosphatase (ALP), fibroblast growth factor-1 (FGF-1), Ki-67 and IL-10 were evaluated. The von Kossa reaction followed by the detection of ALP was also performed. At 60 days, serum levels of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were measured and liver fragments were collected for histological and morphometric analyses; data were evaluated by one-way ANOVA followed by Sidak post-test. The biocompatibility and bioactivity data were submitted to two-way ANOVA analysis followed by Tukey's post-hoc test, except for the data referring to the ERI. ERI data were submitted to the non-parametric Kruskal-Wallis ANOVA test followed by the Dunn's test ($p \leq 0.05$). **Results** No significant difference was detected in serum GOT and GPT concentrations and in the number of hepatocytes among the experimental and CG samples. Although Bio-C Pulpo had the highest IC and IL-6-immunolabelled cells ($p < 0.0001$) in all periods, no significant difference was observed in the IRS among the bioceramic materials, except at 60 days. In this period, the WMTA showed lower IRS. All groups showed a significant reduction in the capsule thickness and in the number of IC and IL-6-immunolabelled cells over time. Bio-C Pulpo, MTA-HP and WMTA specimens showed greater immunoexpression of ALP than CG ($p < 0.0001$). In all periods, von Kossa-positive and birefringent structures were observed in the capsules around the materials. ALP-immunolabelled cells were also seen near to von Kossa-positive structures. Regarding the number of FB, significant differences were not detected among Bio-C Pulpo, MTA-HP and WMTA groups ($p > 0.05$), at 7 days. The capsules of all groups exhibited a significant increase in the number of FB and in the amount of collagen over time. From 7 to 60 days, a significant reduction in FGF-1 and Ki-67 immunoexpression was observed in all samples. At 7 and 15 days, the highest immunoexpression for Ki-67 was found in the Bio-C samples, while after 30 and 60 days, no significant difference was observed between the bioceramic materials. From 7 to 60 days, a significant increase in IL-10 immunostaining was found in all groups. At 60 days, the capsules around the materials had a higher number of IL-10-immunolabelled cells than in CG samples ($p < 0.0001$). **Conclusions** Bio-C Pulpo, MTA-HP and WMTA materials did not cause morphological changes in the liver as well as no significant alteration in the serum GOT and GPT levels. Despite changes made in Bio-C Pulpo and MTA-HP to improve their physicochemical properties, these bioceramic materials are biocompatible and exhibit bioactive potential similar to the WMTA. The bioceramic materials stimulate the IL-10 secretion, which may exert an anti-inflammatory activity in the capsules around the implants allowing the formation of collagen-rich capsules.

Keywords: Material testing. Immunohistochemistry. Liver.

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1 INTRODUÇÃO

O Mineral Trióxido Agregado (MTA), material à base de silicato tricálcico foi introduzido na Universidade de Loma Linda, em 1993¹⁻³. Este material foi desenvolvido para selar comunicações entre o sistema de canais radiculares e o periodonto⁴ e também em cirurgia parodontônica com obturação retrógrada³⁻¹¹. O MTA tem uma história de sucesso, com uma ampla variedade de aplicações clínicas¹², utilizado também em capeamento pulpar direto e em *plugs* apicais de dentes com rizogênese incompleta⁵⁻¹³.

O MTA é composto de cimento Portland (80%) e óxido de bismuto (20%). Como consequência, a principal fase constituinte e mais reativa do MTA é o silicato tricálcico compreendendo cerca de 68% do cimento Portland¹⁴, aluminato tricálcico, óxido tricálcico e óxido de silicato¹. Partículas de outros óxidos minerais em menores quantidades também estão presentes, o que contribui para as propriedades físico-químicas ideais. O tamanho das partículas, relação líquido-pó e a presença de bolhas de ar incorporadas durante a espatulação também interferem nas propriedades físicas do material⁴. Este material apresenta pH em torno de 12,5, excelentes propriedades de vedação e adaptação marginal² e devido sua natureza hidrofílica, não requer sua utilização em campo seco^{4,15}.

Materiais biocompatíveis, capazes de induzir a formação de tecido mineralizado, apresentam diversas aplicações clínicas^{5,7,8,16}, sendo essenciais para a recuperação e o prognóstico da periodontite apical, especialmente em casos de perda óssea¹⁷. Cimentos reparadores devem apresentar propriedades físico-químicas e biológicas ideais, além de apresentarem potencial de osteoindução^{16,17}. O MTA tem sido considerado o material mais eficaz no reparo tecidual, devido à sua boa capacidade de selamento, biocompatibilidade e bioatividade⁵⁻⁸.

O MTA apresenta excelente biocompatibilidade¹⁴ e capacidade para induzir a formação de tecido mineralizado^{14,18,19}. O silicato tricálcico apresenta bioatividade¹⁷, reage na presença de fluídos fisiológicos, produzindo hidroxiapatita. A capacidade de formação de apatita é reforçada com a adição do fluoreto de cálcio¹⁴. As excelentes propriedades biológicas do MTA são atribuídas ao seu pH alcalino e a sua biointeratividade, capacidade de liberar íons cálcio e manter o pH elevado, induzindo a formação de tecido mineralizado^{17,18}.

No entanto, as composições tradicionais do MTA apresentam algumas desvantagens^{8,20}, por isso, as propriedades físico-químicas do MTA têm sido amplamente investigadas, devido seu tempo de presa prolongado, descoloração do dente e da gengiva marginal, consistência arenosa e dificuldades no manuseio¹⁷⁻²³. Uma dessas desvantagens é o difícil manuseio, pois, o pó é fornecido para aglutinação em água destilada, resultando em um material de consistência arenosa⁸, o que dificulta a inserção do MTA⁸, acentuada em procedimentos cirúrgicos, como obturação retrógrada e no preenchimento de perfurações^{5,20}. Modificações na formulação original do MTA tem sido sugerida para melhorar suas características^{18,19,24,25}. Alterações químicas pequenas ou até mesmo a substituição do agente radiopacificador pode modificar o comportamento físico-químico e biológico desses materiais^{16,22,24,25}. A busca por novos materiais baseados no cimento Portland, que possam superar as características indesejáveis do MTA, visa desenvolver um cimento biocompatível com propriedades físico-químicas ideais²¹. O grande interesse neste material é a biocompatibilidade, biointeratividade e bioatividade^{5,17}.

A Associação Americana de Endodontia (AAE) recomenda que o uso de um novo material seja baseado em estudos biológicos e clínicos²⁶. Os materiais endodônticos, antes do seu uso clínico, devem ter suas propriedades físico-químicas e biológicas avaliadas por estudos *in vitro* e *in vivo*, evitando assim, efeitos adversos²⁷. Os materiais que permanecerão em íntimo contato com tecidos pulpare ou periapicais, devem apresentar baixa citotoxicidade, biocompatibilidade²⁷, e potencial para induzir a formação de uma barreira mineralizada^{5,28}.

MTA com modificações na sua formulação têm sido introduzidos no mercado^{5,16}. O MTA Branco (Angelus Indústria de produtos Odontológicos S/A, Londrina – PR – Brasil) surgiu como uma nova formulação em relação ao MTA Angelus (pó cinza), foi lançado no mercado com a proposta de evitar a descoloração coronária causada pelo MTA cinza²⁹. O MTA Branco apresenta em sua composição silicato tricálcico, silicato dicálcico, aluminato tricálcico, óxido de cálcio, óxido de bismuto, sendo que o líquido é constituído de água destilada³⁰. O MTA Branco proporciona pH em torno de 7.8²¹ a 9¹⁶, liberação de cálcio em 0,24 mg/L, solubilidade entre 2,2%²² e 2,4%²¹ e tempo de presa de 48 minutos¹⁸. Este MTA é indicado para o tratamento de perfurações radiculares e de furca, reabsorção radicular, capeamento pulpar, pulpotomia, apicificação e *plugs* apicais³⁰. A formulação tradicional do MTA apresenta algumas desvantagens, como dificuldades de manuseio, consistência

granular e longo tempo de presa. Além disso, o uso de óxido de bismuto como agente radiopacificador promove a descoloração do dente e da gengiva marginal e afeta as propriedades físico-químicas desse material^{10,11,22,29}. Diante das desvantagens do óxido de bismuto e de acordo com o fabricante, o radiopacificador óxido de bismuto foi substituído por tungstato de cálcio na nova formulação do MTA Branco.

Na busca pela melhoria do MTA, uma nova formulação - o MTA Repair HP (Angelus Indústria de produtos Odontológicos S/A, Londrina – PR – Brasil) - foi lançado na forma de um material biocerâmico de alta plasticidade^{8,31}, visando manter as propriedades biológicas do MTA, aprimorando as propriedades físico-químicas²², proporcionando um melhor manuseio e inserção do material^{8,31}. De acordo com o fabricante, este novo material possui alta plasticidade e melhores propriedades físico-químicas em comparação com o MTA Branco^{5,9,16,20,22,32}. A principal diferença em relação ao seu antecessor é adição de um plastificante orgânico à água destilada²². O MTA Repair HP é constituído por silicato tricálcico, silicato dicálcico, aluminato tricálcico, óxido de cálcio, tungstato de cálcio e o líquido contém água destilada, além de um agente plastificante^{5,8,16,20,31,32}; assim, o seu líquido proporciona maior plasticidade, melhorando o manuseio e inserção do material no local de reparo. Após o manuseio, a consistência obtida deste cimento é semelhante a uma massa de modelar, não arenosa⁵. O MTA Repair HP apresenta radiopacidade de 4.50 mm/Al, solubilidade de 8%, pH em torno de 9, liberação de cálcio de 14 mg/L e tempo de presa final de aproximadamente 85 minutos¹⁶. As indicações são as mesmas do MTA Branco⁵. O extrato de MTA Repair HP apresentou baixa citotoxicidade quando adicionado a cultura primária de osteoblastos, culminando com baixo índice de células em apoptose ou necrose. Em subcutâneo de ratos, este cimento induziu uma moderada reação inflamatória após 30 dias^{5,8,22,31}.

Bio-C Pulpo (Angelus Indústria de produtos Odontológicos S/A, Londrina – PR – Brasil) é novo material da Angelus, que ainda não está sendo comercializado no mercado. O material foi enviado pela empresa ao departamento de Odontologia Restauradora para que fossem feitas as análises físico-químicas e de biocompatibilidade do produto. Este material é constituído de silicato de cálcio (50-65%), aluminato de cálcio (1-4%), hidróxido de cálcio (0,5-2%), óxido de zircônio (20-30%), fluoreto de cálcio (1-4%), dióxido de silício (2-6%), óxido de ferro (0,05-1%)³³, enquanto que o líquido é à base de água destilada, adicionado agente plastificante,

cloreto de cálcio e metilparabeno³³⁻³⁶. O Bio-C Pulpo é indicado para pulpotomia de dentes decíduos e permanentes, forramento de preparo cavitário/base restauração e para tratamento restaurador atraumático, técnica A.R.T. O Bio-C Pulpo apresenta tempo de trabalho entre 4 a 6 minutos, presa final de 12 minutos, pH em torno de 12,5 e radiopacidade de 9 mm/Al, de acordo com a norma ISO 9917^{33,34,36,37}.

A resposta biológica pode ser investigada por meio da avaliação histopatológica da cápsula que se forma em resposta aos materiais, por meio de análises morfológica e quantitativa do tecido circundante ao implante³⁸⁻⁴⁰. O implante no subcutâneo é um método recomendado pela ISO 10993⁴¹ para avaliar a biocompatibilidade de materiais dentários e, portanto, é amplamente utilizado^{19,25,38,39,40,42-46}. O uso do tubo de polietileno previne a difusão do material, além de simular a situação de preenchimento de canal radicular^{42,43}. Considerando que a resposta tecidual aos materiais compreende uma cascata de eventos celulares e moleculares, a detecção imuno-histoquímica de citocinas inflamatórias e fatores de crescimento têm sido utilizada para melhor caracterizar a resposta tecidual^{24,25,38,45-47}. Dentre os diversos fatores e citocinas envolvidas neste processo, podemos citar a interleucina-6 (IL6)^{25,40,45,48}, uma citocina multifuncional que desempenha um papel importante na regulação da resposta imunitária, hematopoese, oncogênese e no processo inflamatório⁴⁹⁻⁵¹.

A IL-6 tem sido associada com a instalação do processo inflamatório em resposta ao implante de materiais endodônticos no tecido subcutâneo^{24,25,38,40,45,47,52} bem como na lesão periodontal⁵³. Frente a uma lesão tecidual, a IL-6 é uma das interleucinas que participa da resposta mediada pelo hospedeiro, participando na patogênese de várias doenças inflamatórias⁵³⁻⁵⁵. Esta citocina é produzida por neutrófilos, monócitos, células endoteliais, fibroblastos e outras células em resposta a micro-organismos⁵³, às substâncias liberadas por materiais odontológicos^{24,25,38,45,56} e também à estimulação por outras citocinas, como a interleucina-1 (IL-1) e fatores de necrose tumoral alfa (TNF- α)^{57,58}. A IL-6 possui várias propriedades pró-inflamatórias, tais como a produção de quimosinas e moléculas de adesão aos linfócitos, indução de proteínas de fase aguda em células de fígado e aumento de neutrófilos no sangue^{57,58}.

A síntese de citocinas é complexa, sua expressão e efeitos são coordenados por muitos fatores, incluindo outras células e mediadores químicos⁵⁹. A reação tecidual observada é consequência da resposta do hospedeiro às substâncias liberadas pelos materiais. Portanto, as células hospedeiras (inflamatórias e

residentes) produzem e liberam várias citocinas e fatores de crescimento que desempenham um papel específico na complexa cascata de eventos celulares envolvidos na resposta tecidual⁴⁵.

A acentuada imunoexpressão de IL-6 nas cápsulas, em períodos iniciais, é concomitante a incidência de linfócitos, macrófagos e plasmócitos, indicando que esta interleucina participa na instalação e progresso da resposta imuno-inflamatória promovida pelos implantes no subcutâneo^{24,25,38,40,45}. A redução da reação inflamatória ocorre concomitantemente à redução da imunoexpressão de IL-6 nas cápsulas adjacentes aos materiais^{38,45,56} e presença marcante de fibroblastos e fibras colágenas^{38,40,45}. Assim, a presença desta citocina em cápsulas formadas em resposta aos materiais odontológicos implantados no tecido conjuntivo do subcutâneo parece estar associada à instalação da reação inflamatória. No entanto, com o decorrer do tempo os materiais biocompatíveis mostram uma redução na positividade desta citocina, a qual tem sido associada à remodelação das cápsulas, ou seja, a proliferação de fibroblastos e, subsequentemente, a formação de colágeno em substituição ao processo inflamatório inicialmente presente^{38,45,46}.

Em contraposição à função pró-inflamatória da IL-6 exercida nas cápsulas formadas em resposta aos biomateriais, a IL-10 apresenta uma marcante função anti-inflamatória. Está bem estabelecido que a IL-10 inibe a síntese e liberação de diversas citocinas pró-inflamatórias, dentre elas a IL-1, IL-6 e IL-8^{60,61}. Há evidência de que o MTA induz as células ósseas a expressarem IL-4 e IL-10⁶². Considerando que a IL-10 é uma citocina que inibe a IL-6 e participa no processo de reparação dos tecidos^{63,64}, a detecção da IL-10 deve fornecer resultados que reforçam à regressão da reação inflamatória e a formação de uma cápsula fibrosa, ou seja, suportam a ideia que estes materiais possam ser biocompatíveis. Portanto, a detecção da IL-10 possibilitará uma melhor compreensão dos mecanismos celulares e moleculares envolvidos na instalação e regressão da reação inflamatória induzida pelos biomateriais.

No momento, há um claro entendimento de que a resposta do hospedeiro ao material escolhido tem papel importante no resultado da terapia endodôntica, pois as células produzem e liberam fatores de crescimento, citocinas e quimiocinas, que controlam o processo de cicatrização do tecido. Dentre vários fatores, o fator de crescimento de fibroblastos-1 (FGF-1) participa da remodelação tecidual, promovendo a proliferação e migração de fibroblastos⁴⁶. Esse fator tem papel fundamental no repara tecidual devido às suas ações mitogênicas e angiogênicas⁴⁷. Assim, a

reparação dos tecidos depende da vascularização e proliferação celular, particularmente, das células responsáveis pela produção dos componentes estruturais da matriz extracelular do tecido. Um marcador amplamente utilizado para identificar células em proliferação é o Ki-67⁴⁷. O Ki-67 é uma proteína do córtex nuclear não histona expressa durante as fases G1, S, G2 e M do ciclo celular, mas não em células em estado quiescente⁴⁷.

Além dos biomateriais serem biocompatíveis, devem exibir potencial bioativo. Os cimentos de silicato de cálcio possuem comportamento bioativo, sendo biointerativos, capazes de liberarem íons para nucleação dos depósitos de apatita na superfície em um curto período de indução, sendo fundamentais para induzir a remineralização^{65,66}. Estes cimentos são capazes de liberar íons biologicamente relevantes (Ca^{+2} e OH^-), atuando em diversas células envolvidas no processo de mineralização, dentre elas os osteoblastos e os cementoblastos, sendo considerados, portanto osteoindutores. Os cimentos que contém silicato de cálcio apresentam pH alcalino em contato com os fluidos tissulares, com capacidade de nuclear depósitos de cálcio^{9,22,66}. Os íons de cálcio podem então reagir com o dióxido de carbono nos tecidos para formar granulações de carbonato de cálcio, observadas como cristais de calcita birrefringentes à luz polarizada^{5,8,40,67} e estruturas positivas (marcadas em preto) ao método de von Kossa^{19,25,40}. Estes cristais podem servir como locais de nucleação, estimulando a deposição de tecidos mineralizados⁶⁷. O processo de biomineralização é complexo e depende de diversos eventos celulares e enzimáticos. Dentre as enzimas envolvidas neste processo, a fosfatase alcalina (ALP) desempenha papel fundamental desde que o seu pH alcalino favorece à precipitação de fosfato de cálcio sobre a matriz orgânica óssea⁶⁸. Assim sendo, a presença de ALP tem sido associada à formação dos tecidos, particularmente, os tecidos mineralizados⁶⁹. A ALP é considerada um marcador bioquímico de formação óssea que pode ser usado para avaliar a atividade osteoblástica^{7,13}. Além de osteoblastos, os cementoblastos e os fibroblastos do ligamento periodontal expressam positividade à ALP^{7,13}. Dessa maneira, a ALP é usada, principalmente em estudos in vitro, para avaliar a bioatividade de materiais endodônticos^{7,13}.

1.1 Alterações Sistêmicas

Considerando que os materiais odontológicos apresentam solubilidade e, portanto, liberam substâncias, estas por meio da corrente sanguínea podem atingir órgãos intimamente associados a manutenção do metabolismo do organismo, dentre eles o fígado, um órgão vital interposto à corrente sanguínea. O fígado está envolvido em diversas funções do corpo, sendo o principal local para biotransformação e remoção de macromoléculas da corrente sanguínea⁷¹, com a participação dos hepatócitos e das células de Kupffer⁷². As células de Kupffer liberam citocinas como interleucina 1 (IL-1) e (IL-6), fator de necrose tumoral α (TNF- α), fator de crescimento transformante β (TGF- β) e interferon γ (INF- γ) e sua principal função é remover partículas advindas pelo sistema porta hepático⁷³. A resposta destas células frente a patógenos pode contribuir para lesões hepáticas, lesionando os hepatócitos^{71,72,74}.

A ISO 10993-1 recomenda avaliar a toxicidade sistêmica de materiais que entram em contato com sangue⁷⁵. Estudos anteriores mostram o efeito sistêmico causado por vários materiais Odontológicos, evidenciando o efeito adverso em diferentes órgãos do corpo⁷⁶⁻⁷⁸. Há relatos de que alguns materiais induziram no fígado o acúmulo de infiltrado inflamatório, congestão vascular⁷⁹, presença de linfócitos interlobulares, aumento nas células de Kupffer ao redor do espaço porta^{80,81}, apoptose e vacuolização de hepatócitos⁸⁰. Outro efeito sistêmico adverso é a produção de citocinas, como IL-1 e IL-6, no tecido subcutâneo⁸², que podem por meio da circulação sanguínea alcançar o fígado causando reação inflamatória⁸¹. Estas alterações patológicas são indicativos de uma lesão hepatocelular no fígado⁸⁰ e, portanto, a análise morfológica e bioquímica do fígado é um indicativo de toxicidade.

Ensaio pré-clínicos de toxicidade hepática devem incluir biomarcadores mensuráveis a partir de amostras obtidas em procedimentos não invasivos, como a coleta de sangue⁸³. Os testes bioquímicos para assegurar a função hepática incluem a mensuração dos níveis séricos das enzimas transaminase glutâmico-oxalacética (TGO) e transaminase glutâmico-pirúvica (TGP)^{71,81,83-85}.

Considerando que os cimentos MTA Branco e MTA Repair HP são relativamente recentes no mercado e, conseqüentemente, ainda pouco se sabe sobre a sua biocompatibilidade e o Bio-C Pulpo sendo um novo produto, no qual há escassos relatos na literatura a respeito de sua biocompatibilidade, o nosso propósito foi avaliar os parâmetros morfológicos e bioquímicos hepáticos e a reação tecidual promovida

no subcutâneo por estes cimentos. O MTA Branco foi usado como parâmetro (controle positivo) desde que já está bem estabelecido na literatura que este material apresenta biocompatibilidade e bioatividade^{21,25,29}.

2 PROPOSIÇÃO

O objetivo foi analisar a reação tecidual promovida pelos cimentos Bio-C Pulpo e MTA Repair HP implantados no subcutâneo de ratos, a fim de analisar possíveis alterações morfológicas e bioquímicas do fígado bem como avaliar a reação tecidual promovida pelos cimentos no subcutâneo, com enfoque na biocompatibilidade e potencial bioativo destes materiais. A resposta biológica destes materiais foi comparada a do MTA Branco, usado como padrão ouro. Os nossos objetivos específicos foram:

Publicação 1:

Avaliar se Bio-C Pulpo e MTA-HP implantados em tecidos subcutâneos induzem alterações estruturais no fígado, bem como na transaminase glutâmico-oxaloacética sérica (TGO) e níveis de transaminase glutâmico-pirúvica (TGP).

Avaliar se os materiais Bio-C Pulpo e MTA-HP são biocompatíveis e se apresentam um potencial bioativo.

Publicação 2:

O objetivo deste estudo foi avaliar se as alterações realizadas no Bio-C Pulpo e no MTA-HP interferem na reparação do tecido conjuntivo após implantação no subcutâneo de ratos.

3 PUBLICAÇÕES

O presente trabalho resultou em dois artigos. O artigo **1** intitulado: Comparison of Bio-C Pulpo and MTA Repair HP with White MTA: effect on liver parameters and evaluation of biocompatibility and bioactivity, foi publicado no “*International Endodontic Journal*”.

Delfino MM, de Abreu Jampani JL, Lopes CS, Guerreiro-Tanomaru JM, Tanomaru-Filho M, Sasso-Cerri E, Cerri PS. Comparison of Bio-C Pulpo and MTA Repair HP with White MTA: effect on liver parameters and evaluation of biocompatibility and bioactivity in rats. Int Endod J. 2021 Sep;54(9):1597-1613. doi: 10.1111/iej.13567. Epub 2021 Jul 12. PMID: 33999424.

O artigo 2 intitulado: The participation of fibroblast growth factor-1 and interleukin-10 in the connective tissue repair following subcutaneous implantation of bioceramic materials in rats, será submetido a publicação no “International Endodontic Journal”.

3.1 Publicação 1*

Comparison of Bio-C Pulpo and MTA Repair HP with White MTA: effect on liver parameters and evaluation of biocompatibility and bioactivity

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Running title: Bioceramic materials: tissue response

Key Words: Biocompatibility; Bioactivity; Inflammatory infiltrate; Immunohistochemistry; Interleukin-6; Systemic effect; Liver.

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Abstract

Aim To evaluate the connective tissue response promoted by Bio-C Pulpo, MTA Repair HP (MTA-HP) and White MTA (WMTA) and whether these materials cause liver changes. **Methodology** Polyethylene tubes filled with Bio-C Pulpo, MTA-HP and WMTA, and empty tubes (control group, CG) were implanted into 80 rats subcutaneous tissues for 7, 15, 30 and 60 days. Inflammatory reaction score (IRS), capsule thickness, and number of inflammatory cells (IC), von Kossa reaction, interleukin-6 (IL-6) and alkaline phosphatase (ALP) immunohistochemistry reactions were carried out. Combined methods, von Kossa reaction followed by immunohistochemistry for detection of ALP, were also performed. At 60 days, the serum glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) levels were measured and liver fragments were collected for histological analysis; the data were assessed by One-Way ANOVA analysis followed by Sidak's post-test. The biocompatibility and bioactivity data were subjected to the two-way ANOVA analysis followed by Tukey post-hoc test, except the data concerning to IRS. The IRS data were subjected to the Kruskal-Wallis ANOVA non parametric test followed by Dunn's test ($p \leq 0.05$). **Results** No significant difference was detected in serum GOT and GPT concentrations and in the number of hepatocytes among the experimental and CG samples. Although Bio-C Pulpo had the highest IC and IL-6-immunolabelled cells ($p < 0.0001$) in all periods, no significant difference was observed in the IRS among the bioceramic materials, except at 60 days. In this period, the WMTA showed lower IRS. All groups showed a significant reduction in the capsule thickness and in the number of IC and IL-6-immunolabelled cells over time. Bio-C Pulpo, MTA-HP and WMTA specimens showed greater immunoexpression of ALP than CG ($p < 0.0001$). In all periods, von Kossa-positive and birefringent structures were observed in the capsules around the materials. ALP-immunolabelled cells were also seen near to von Kossa-positive structures. **Conclusions** Bio-C Pulpo, MTA-HP and WMTA materials did not cause morphological changes in the liver as well as no significant alteration in the serum GOT and GPT levels. Moreover, these bioceramic materials are biocompatible and exhibit bioactive potential. However, the Bio-C Pulpo induces higher inflammatory reaction than MTA-HP and WMTA.

Keywords: Biocompatibility; Bioactivity; Inflammatory infiltrate; Immunohistochemistry; Interleukin-6; Systemic effect; Liver.

Introduction

Mineral trioxide aggregate (MTA) is considered as gold standard material for several clinical procedures, due its physicochemical and biological properties (Duarte *et al.* 2012, Cintra *et al.* 2017, Tanomaru-Filho *et al.* 2017, Lee *et al.* 2018, Benetti *et al.* 2019, ElReash *et al.* 2019, Ferreira *et al.* 2019). The traditional MTA formulation presents some drawbacks such as handling, granular consistency and long setting-time. Furthermore, the use of bismuth oxide promotes discoloration and affects the physicochemical properties (Marciano *et al.* 2017, De Souza *et al.* 2018, Ferreira *et al.* 2019, ElReash *et al.* 2019). According to the manufacturer, the bismuth oxide was replaced by calcium tungstate in White MTA Angelus (WMTA, Angelus, Londrina, PR, Brazil).

MTA Repair HP (MTA-HP, MTA “High Plasticity”, Angelus, Londrina, PR, Brazil) was developed as a reparative material with high plasticity aiming to maintain the biological properties of the MTA, improving its chemical and physical properties (Tomás-Catalá *et al.* 2018, Benetti *et al.* 2019, ElReash *et al.* 2019). An organic plasticizer was added to the distilled water to improve the plasticity of the material (Silva *et al.* 2016, Guimarães *et al.* 2018, Tomás-Catalá *et al.* 2018, Benetti *et al.* 2019, Ferreira *et al.* 2019, ElReash *et al.* 2019). The MTA-HP showed a greater resistance to dislodgment than WMTA (Silva *et al.* 2016), but in regards to setting time, solubility and water absorption significant differences were not found between this repair cement with WMTA (Ferreira *et al.* 2019). However, the addition of the organic plasticizer provided to the MTA-HP greater flow ability than WMTA, favoring its handling and insertion (Ferreira *et al.* 2019). Currently, both materials (WMTA and MTA-HP) contain calcium tungstate as radiopacifying agent (Tomás-Catalá *et al.* 2018, Benetti *et al.* 2019, Ferreira *et al.* 2019, ElReash *et al.* 2019) to avoid teeth discoloration (De Souza *et al.* 2018, ElReash *et al.* 2019).

Recently, a new bioceramic material was developed, Bio-C Pulpo (Bio-C Pulpo, Angelus, Londrina, PR, Brazil). Its main clinical indication is pulpotomy, but the manufacturer also indicates the material for protection of the dentine-pulp complex, traumatic restorative treatment, and as a cavity liner. Bio-C Pulpo (Angelus, Londrina, Brazil) is composed of powder containing calcium silicate [(tricalcium silicate (Ca_3SiO_5) and dicalcium silicate (Ca_2SiO_4)], calcium aluminate, calcium hydroxide, zirconium oxide, calcium fluoride, silicon dioxide, and iron oxide. The powder of Bio-C Pulpo is

dissolved in the liquid, which contains distilled water, plasticizing material, calcium chloride and methylparaben (Cosme-Silva *et al.* 2019, Koutroulis *et al.* 2019). According to the manufacturer, the plasticizing material provides a higher plasticity, improving handling, which may facilitate use in clinical procedures. Moreover, the manufacturer also claims that unlike other MTA cements, which use water and have a coarse sandy consistency, Bio-C Pulpo exhibits characteristic of 'modelling putty' after mixing (Cosme-Silva *et al.* 2019).

Considering that repair and root-end filling cements have a close contact with connective tissue, these materials should be biocompatible and stimulate the tissue repair (Saraiva *et al.* 2018, Ferreira *et al.* 2019). The subcutaneous implantation is widely used to evaluate the connective tissue reaction induced by dental materials (Saraiva *et al.* 2018, Ferreira *et al.* 2019, Delfino *et al.* 2020). Since that these materials release some heavy metals which can reach the bloodstream, the ISO 10993-1 also recommends assessing the systemic toxicity of materials (International Organization for standardization, 2006). The response to pathogens and irritant substances can promote changes in the liver histoarchitecture comprising its functional activity (Liu *et al.* 2012, Dixon *et al.* 2013, Peter *et al.* 2017). Studies have reported systemic effects caused by dental materials, showing the adverse effect on different organs of the body (Bakopoulou *et al.* 2009, Khalil & Eid 2013, Garcia *et al.* 2017). Grey ProRoot MTA (Dentsply, Tulsa Dental, Tulsa, OK, USA) and DiaRoot BioAggregate (Diadent Group International, Burnaby, BC, Canada), bioceramic-based root repair materials, implanted into subcutaneous induced an inflammatory infiltrate in the liver and an increase in the number of Kupffer cells around the portal space (Khalil & Eid 2013). Morphological changes in the liver and kidney were also reported as systemic effect promoted by MTA implanted into dorsal subcutaneous of rats (Garcia *et al.* 2017). A significant increase in the plasma aluminium levels was found in the rats that had MTA and MTA-Fillapex placed in the dental socket (Demirkaya *et al.* 2016). Alterations in the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were also induced by Grey ProRoot MTA (Khalil & Eid 2013) and MTA (Garcia *et al.* 2017) indicating that these cements may interfere in the hepatic functional activity.

It is known that the plasticizer added in the Bio-C Pulpo and MTA-HP to improve plasticity and optimize clinical treatment time can alter the biological response (Lima *et al.* 2009). Although the manufacturer does not inform the components of the

plasticizer present in the Bio-C Pulpo and MTA-HP, plasticizers generally contain resins. The resins are able to release compounds, which inhibit the cell proliferation and viability, disrupt the cell integrity, decrease the enzyme activity and can induce cell death. Thus, the substances released by resins has been associated with a variety of local and systemic adverse reactions (Bakopoulou *et al.* 2009).

Considering the addition of plasticizers in these new bioceramic materials, the purpose of this study was to evaluate whether Bio-C Pulpo and MTA-HP implanted into subcutaneous tissues induce structural changes in the liver as well as in the serum glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) levels. Moreover, the tissue reaction induced by these bioceramic materials implanted in the subcutaneous connective tissue was compared with WMTA at different time points. A possible bioactivity of these materials was also investigated by von Kossa method, presence of birefringent structures and immunohistochemistry reaction for detection of alkaline phosphatase (ALP). The null hypothesis was that the new bioceramic-based materials, Bio-C Pulpo and MTA-HP, would not improve the biological properties in comparison with WMTA.

Material and methods

Animal procedures

This research was approved by the Ethical Committee for Animal Research of Araraquara Dental School (CEUA 02/2019; São Paulo State University - UNESP, Brazil).

Eighty Holtzman adult male rats (*Rattus norvegicus albinus*) were maintained in polyethylene cages under 12h light/12h dark cycle at controlled temperature ($23\pm 2^{\circ}\text{C}$) and humidity ($55\pm 10\%$), with water and food provided *ad libitum*. The animals were distributed into four groups containing 20 animals each: CG (control group, empty polyethylene tubes), Bio-C Pulpo (Bio-C Pulpo, Ângelus, Londrina, Paraná, Brazil), MTA-HP (MTA Repair HP, Ângelus, Londrina, Paraná, Brazil) and WMTA (White MTA, Ângelus, Londrina, Paraná, Brazil). The WMTA was used to assess whether the changes in the MTA-HP and Bio-C Pulpo could induce undesirable local and systemic reactions, since the WMTA is the predecessor of these new materials. Sample size was estimated using previous studies (da Fonseca *et al.* 2016, Delfino *et al.* 2020) for the detection of a 50% difference among experimental groups and CG, considering a 20% variability and 90% test power and an alpha error of 0.05 to recognize a significant difference (Singh & Masuku 2013). Thus, a sample of 5 rats per group in each time point was required, totalling twenty animals per group.

The chemical composition of materials and the powder:liquid ratio are described in the Table 1. The manipulation of materials was made inside a laminar flux chamber under aseptic conditions and according to manufacturer's recommendations. Bio-C Pulpo was manipulated using 1 dose of powder per 3 drops of liquid during 60 seconds, when the material reaches a condensable mass consistency. MTA Repair HP was manipulated using a ratio of 0.085g per 2 drops of vehicle until complete homogenization (approximately 40 seconds), obtaining a cement similar a modeling clay. Regarding White MTA, it was prepared using a powder:liquid mixing ratio of a 1 g:0.33 mL; after 30 seconds of handling, the material exhibited a sandy consistency. The polyethylene tubes (Embramed Indústria Comércio, São Paulo, SP, Brazil) with 10.0 mm length and 1.6 mm diameter, previously sterilized with ethylene oxide, were filled with the materials and implanted into the subcutaneous tissue.

The animals were anaesthetized with an intraperitoneal injection of ketamine hydrochloride (80 mg/kg of body weight) combined with xylazine hydrochloride (8

mg/kg of body weight). After shaved and disinfection with 5% iodine solution, a 2.0 cm-long incision was made in a head-to-tail orientation using a n° 15 scalpel (Fibra Cirúrgica, Joinville, SC, Brazil), and the polyethylene tube was placed into the subcutaneous pocket. On the 7th, 15th, 30th and 60th day, the animals were killed and implants surrounded by connective tissue were removed. At 60 days, the animals were anesthetized and the blood samples, liver and implants were collected from animals. The morphological and quantitative analyses were performed using an Olympus microscope (BX-51, Olympus, Tokyo, Japan) and an image analysis system (Image-Pro Express 6.0, Olympus). The quantitative analyses (liver and capsules surrounding the implants) were performed by one calibrated and blinded examiner. The examiner was previously calibrated by an experienced researcher with a Cohen's kappa coefficient value agreement exceeded 0.82. Each quantitative analysis was carried out twice, with intervals of at least 4 weeks.

- Serum hepatic enzymes level *and morphological analyses of liver*

To evaluate whether repair materials cause systemic changes, the morphological and enzymatic analyses of liver were performed at 60 days. In this period, the animals were anesthetized and blood samples were collected via cardiac puncture with BD Vacutainer® Blood Collection Tubes (SSTII Plus, BD Biosciences) for serum enzymatic assays of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT). Liver fragments were also collected for histological analyses. This time point (60 days) was chosen because the tissue remains in contact with the materials for a long period, allowing to evaluate if the material presents toxicity and causes systemic changes.

To measure the serum GOT and GPT concentration, the blood samples collected from animals from all groups were immediately stored on ice. Subsequently, the blood samples were centrifuged at 3500g for 10 min and the serum was stored at -20° C in the first 24 hours and, thereafter, the serum samples were placed on freezer at -80° C. The serum GOT was measured by AST (aspartate aminotransferase) and serum GPT concentration was obtained by ALT (alanine Aminotransferase) by chemiluminescence immunoassay kits (Beckman Coulter, CAR, USA). Serum GOT and GPT concentration were measured using a multiparametric automatic analyzer (Cobas ÍNTEGRA® 400 Plus; Roche Diagnóstica Brasil Ltda., São Paulo, SP, Brazil).

The analyses were performed at São Lucas Clinical and Microbiological Laboratory (Araraquara, SP, Brazil).

The liver fragments were immediately immersed in 4% formaldehyde (freshly prepared from paraformaldehyde) buffered in 0.01M sodium phosphate at pH 7.2. After 48 hours, the liver fragments were dehydrated, treated with xylene and embedded in paraffin.

In each animal, two liver fragments were analyzed. Five slides randomly chosen containing two semi-serial sections (the smallest distance between the sections was 150 μm) of each fragment were stained with haematoxylin and eosin (HE), and analyzed under light microscope. In the morphological analyzes, the following parameters were considered: portal triad, portal space, hepatic sinusoids and hepatocytes. For quantitative analysis of the number of hepatocytes near to the portal triad, sixteen standardized fields (0.09 mm^2) were captured at x40 magnification, totalling a total area of 1.44 mm^2 per fragment. Considering that in each animal two fragments of liver were analyzed, the number of hepatocytes was estimated in a standardized area of 2.88 mm^2 per animal. Thus, the numerical density of hepatocytes was obtained dividing the total number of hepatocytes estimated in each animal by the total area.

- Analyses of implants in the subcutaneous

The analyses of tissue reaction of implants in the subcutaneous were performed in all groups at 7, 15, 30, and 60 days. The animals were euthanized with overdose of ketamine and xylazine and, the implanted tubes with adjacent tissues were removed, fixed for 48 hours in 4% formaldehyde buffered at pH 7.2, and the specimens were processed for paraffin embedding. From each specimen, forty longitudinal sections (6- μm thick), containing entire polyethylene tube surrounded by tissues, were obtained. Five non-serial sections (the shortest distance between the sections was 100 μm) randomly chosen were stained with HE for morphological description, analysis of numerical density of inflammatory cells and measurement of capsule thickness. In each specimen, two non-serial sections were randomly chosen and were subjected to immunohistochemistry reactions for detection of interleukin-6 (IL-6) and alkaline phosphatase. The von Kossa reaction was also carried out to evaluate the presence of calcium deposits in the capsules. The von Kossa reaction, used to evaluate the

presence of calcium deposits in the capsules, was performed in two non-serial sections. Moreover, von Kossa method followed by immunohistochemistry for detection of alkaline phosphatase (ALP) was also carried out.

Biocompatibility analysis

- Capsule thickness measurement

The capsule thickness was estimated using the images captured at x65 magnification. For each specimen, three HE-stained non-serial sections (the smallest distance between the sections was 100 μm) were used, totalling 15 sections per group in each period. Using an image analysis system (Image-Pro Express 6.0, Olympus), the thickness measurement was made from the capsule surface to the adjacent tissues (Saraiva *et al.* 2018). Thus, the mean value of capsule thickness/specimen and per group was calculated. The measurement of the capsule thickness was performed by one calibrated and blinded examiner.

- Numerical density of inflammatory cells

The number of inflammatory cells was obtained in the capsules of all the animals. In each specimen, images were captured from three HE-stained non-serial sections (the smallest distance between the sections was 100 μm) using a light microscope at x695 magnification (Silva *et al.* 2015, da Fonseca *et al.* 2016, Delfino *et al.* 2020). Thus, the number of cells was obtained from a standardized field (0.09 mm^2) of the capsule in close juxtaposition to the opening of implanted tube. The inflammatory cells (neutrophils, lymphocytes, plasma cells and macrophages) were recognized by the rounded shape or kidney-shaped nucleus (macrophages), round cells exhibiting dense nucleus (lymphocytes), ovoid cells with eccentrically located nucleus (plasma cells) and rounded cells with multilobulated nucleus (neutrophils). Thus, the number of inflammatory cells per millimetre square of capsule was obtained dividing the total number of inflammatory cells by the total standardized field of capsule (da Fonseca *et al.* 2016, Delfino *et al.* 2020, Hoshino *et al.* 2020).

- Inflammatory reaction score

The inflammatory reaction in the capsules was scored according to the number of inflammatory cells in each field (Yaltirik *et al.* 2004, Cintra *et al.* 2017, Delfino *et al.* 2020). The score was given to each implant from three non-serial HE-stained sections evaluated by one blinded and calibrated examiner. The inflammation reaction score was obtained in the capsules as follows: 0, without inflammatory infiltrate (none or scarce inflammatory cells); 1, mild inflammatory reaction (capsule containing until 25 inflammatory cells/field); 2, moderate inflammatory reaction (capsule containing from 26 until 125 inflammatory cells/field); 3, severe/intense inflammatory reaction (capsule containing over 125 inflammatory cells/field). Thus, the score of each specimen was attributed from the data obtained in the numerical density of inflammatory cells (described above). The analysis was performed in five animals per group and the mean value of each group was calculated.

-Immunohistochemical detection of IL-6

To unmask antigenic sites, deparaffinized sections were immersed in 0.001 M sodium citrate buffer at pH 6.0 and submitted to microwave oven treatment for 30 minutes at 96-98°C. After cooling, the slides were immersed for 20 min in 5% hydrogen peroxide to inactivate the endogenous peroxidase and, subsequently, the sections were incubated at room temperature with 2% bovine serum albumin (BSA; Sigma-Aldrich Co., St. Louis, MO, USA). Sections were then incubated at 4°C overnight in the mouse monoclonal anti-IL-6 antibody (Abcam Inc., Cambridge, MA, USA; code: ab9324) diluted at 1:400. After washing, the sections were incubated for 1 hour at room temperature with labelled polymer-HRP (EnVision + Dual Link System-HRP, Dako Inc., Carpinteria, CA, USA; K4061). The peroxidase activity was revealed by 3,3'-diaminobenzidine (DAB Substrate, Vector Laboratories, Burlingame, CA, USA) for 3 min. After washing in tap water, the sections were counterstained with haematoxylin. In the negative controls, the step of incubation in primary antibody was replaced by incubation in mouse non-immune serum.

The numerical density of IL-6-immunolabelled cells (brown-yellow colour) was performed according methodology previously described (Silva *et al.* 2015, Saraiva *et al.* 2018, Delfino *et al.* 2020). Briefly, in each section the number of IL-6-immunostained (brown-yellow colour) was counted in a standardized field (0.09 mm²) at x695

magnification. The number of IL-6-immunolabelled cells per mm² was obtained for each animal (n=5 per group/period).

Bioactivity evaluation

-Immunohistochemical detection of alkaline phosphatase (ALP)

To unmask antigenic sites, the slides were immersed in 0.001 M sodium citrate buffer (pH 6.0) and kept at 96-98°C for 30 minutes. After cooling, the endogenous peroxidase was blocked and the sections were incubated with 5% BSA (Sigma-Aldrich Co., St. Louis, MO, USA). Then, the sections were incubated in a humidified chamber at 4°C for 16-18h with rabbit anti-alkaline phosphatase antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; code: sc30203) diluted at 1:400. After washing, the sections were incubated at room temperature with Super Enhancer™ reagent for 30 min, washed in buffer and incubated with anti-mouse and anti-rabbit secondary antibody conjugated to the Polymer-HRP reagent (Super Sensitive™ IHC Detection Systems, BioGenex Laboratories, Fremont, CA, USA) for 30 min. at room temperature. Subsequently, the immunoreaction was revealed by 3,3'-diaminobenzidine (DAB Substrate, Vector Laboratories, Burlingame, CA, USA) for 3 min. The counterstaining was performed with haematoxylin. As negative controls, sections were incubated in non-immune serum instead of primary antibody. The number of ALP-immunolabelled cells (brown-yellow colour) was counted in a standardized field (0.09 mm²) at x695 magnification using an image analysis system (Image-Pro Express Olympus). The number of ALP-immunolabelled cells per mm² was estimated in all animals in each period (n=5 per group/period).

- von Kossa histochemical reaction and analysis under polarized light

Non-serial sections of all specimens were submitted to the von Kossa method for detection of calcium salts structures (Meloan & Puchtler 1985) in the capsules (brown/black colour). After dewaxing, the slides were immersed for 1 hour in 5% silver nitrate solution, washed in distilled water and the sections were treated with 5% sodium thiosulfate for 5 min. The sections were washed in distilled water, stained with picrosirius-red method and analyzed under light microscope. In the negative control, the sections were incubated in the medium without silver nitrate. Unstained sections

were also analyzed under polarized illumination to evaluate the presence of birefringent structures (Holland *et al.* 1999, Cintra *et al.* 2017, Bueno *et al.* 2019, Benetti *et al.* 2019, Delfino *et al.* 2020, Hoshino *et al.* 2020).

von Kossa method combined with immunohistochemical detection of ALP

Deparaffinized sections were firstly subjected to the von Kossa histochemical method and, subsequently, the sections were submitted to the immunohistochemistry for the detection of ALP, as described by Hoshino *et al.* (2020). Briefly, after von Kossa reaction, the sections were immersed in sodium citrate buffer pH 6.0 and heated during 20 min. at 96-98° C for antigen retrieval. After blocking endogenous peroxidase, the sections were incubated with 2% bovine-serum-albumin (Sigma-Aldrich Chemie) during 30 min. Subsequently, the sections were incubated overnight with rabbit anti-alkaline phosphatase antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:400, at 4° C. After the washings, the sections were incubated with *Super Sensitive™ IHC Detection Systems* (BioGenex Laboratories, Fremont, CA, USA) and the immunoreaction was revealed by 3,3'-diaminobenzidine (Dako Inc., Carpinteria, CA, USA) for 3 min. The sections were counterstained with haematoxylin. A non-immune serum, instead of primary antibody, was used as negative control.

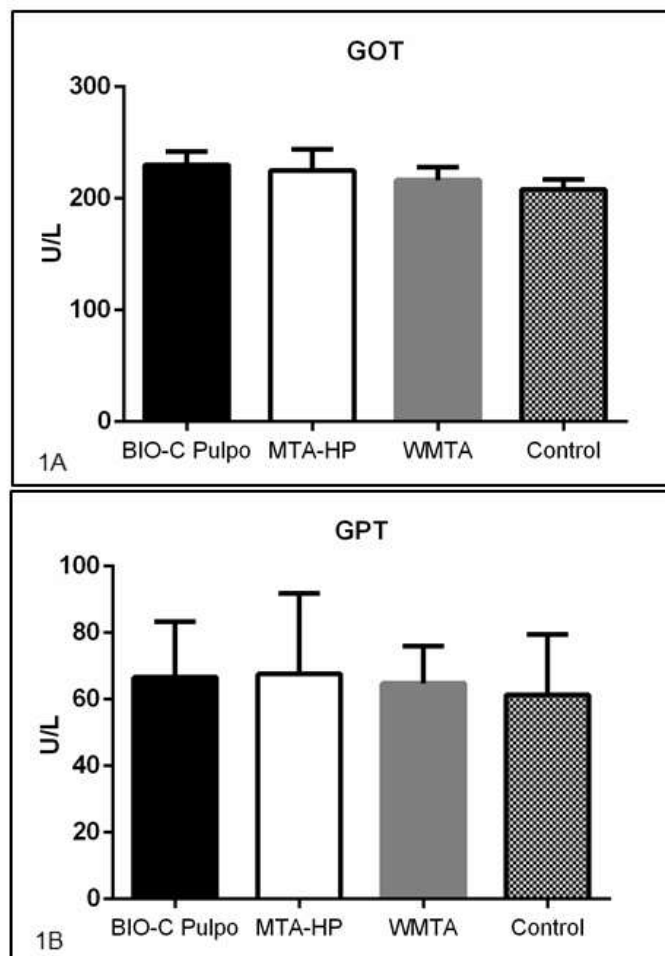
Statistical analysis

The serum GOT and GPT levels, and numerical density of hepatocytes were assessed by One-Way ANOVA analysis followed by the Sidak's post-hoc test. The data concerning to biocompatibility and bioactive potential were evaluated by two-way ANOVA analysis followed by the Tukey post-hoc test (GraphPad Prism 5.0 software, San Diego, CA, USA) at significance level of $p \leq 0.05$. To evaluate the differences in the inflammatory reaction score data, the Kruskal-Wallis ANOVA non parametric test was used. Dunn's test was used for pairwise comparisons at 5% significance.

Results

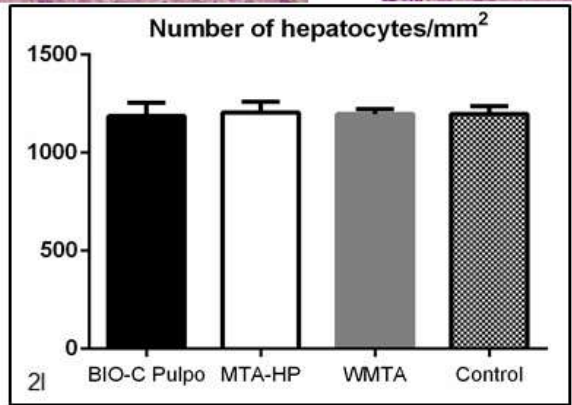
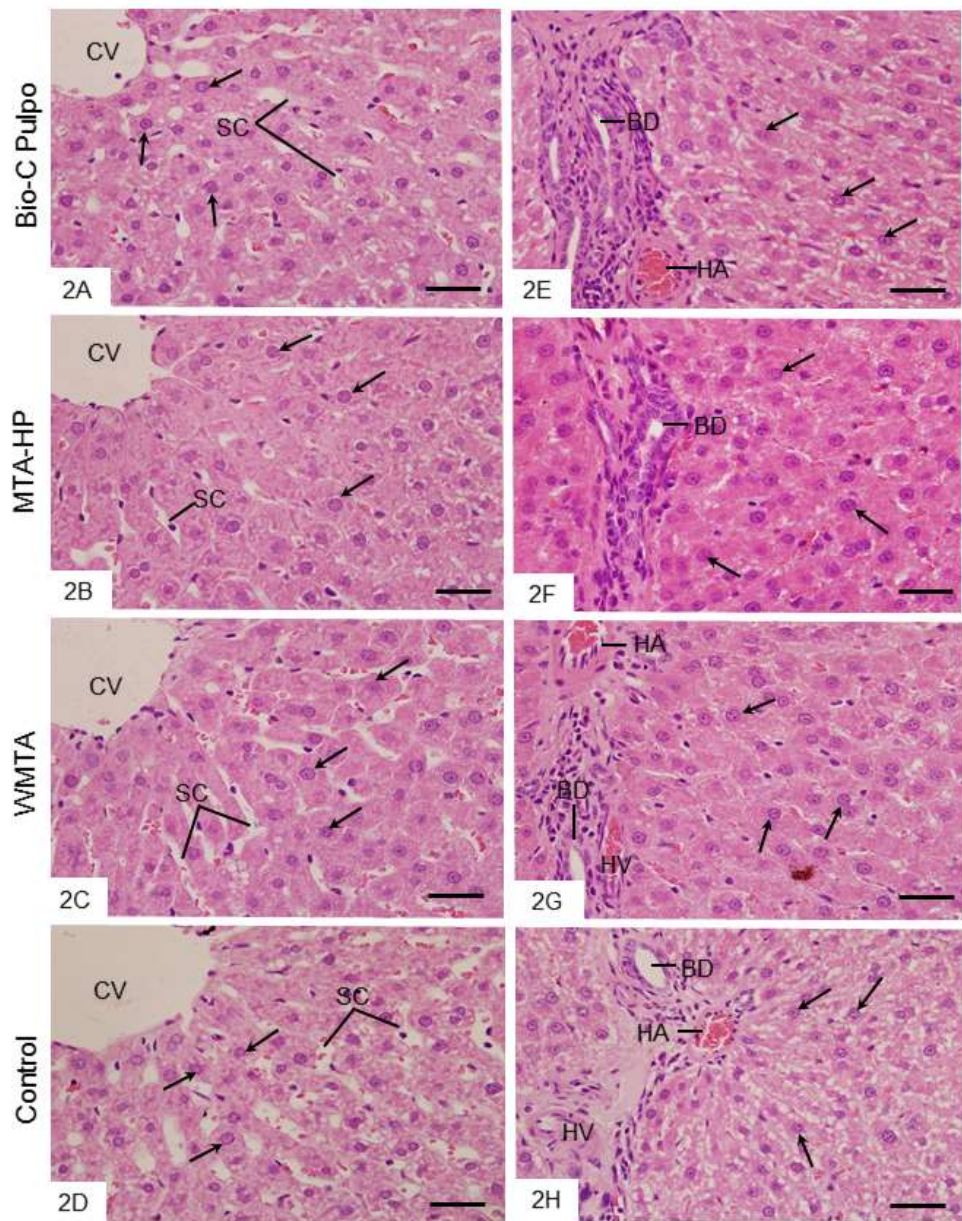
- Serum hepatic enzymes level and morphological analyses of liver

As shown in the Figs. 1A and 1B, no significant differences were detected in serum GOT and GPT concentrations among the experimental groups and CG specimens ($p > 0.05$). The histological analysis of liver fragments of all groups revealed large hepatocytes containing one or two nuclei with evident nucleoli; the round nuclei were subtly stained by haematoxylin. The hepatocytes were arranged in cord cells, which irradiated from central-lobular vein to the periphery of the hepatic lobule and an intricate network of sinusoids capillaries were present between these plate hepatocytes (Figs. 2A-2D). Moreover, the portal triad contained the typical components with normal morphological features (Figs. 2E-2H). Significant differences in the number of hepatocytes were not observed among the animals that had the repair materials implanted and CG animals (Fig. 2I).



Figures 1A-1B – The serum levels of glutamic-oxaloacetic transaminase (GOT - Fig. 1A) and glutamic-pyruvic transaminase (GPT – Fig. 1B) in Bio-C Pulpo, MTA-HP, WMTA and Control groups at 60 days. In rats with endodontic materials, the GOT and GPT levels were similar to Control rats ($p > 0.05$).

Values expressed as mean $\pm$ SD. One-way ANOVA followed by Sidak's test.

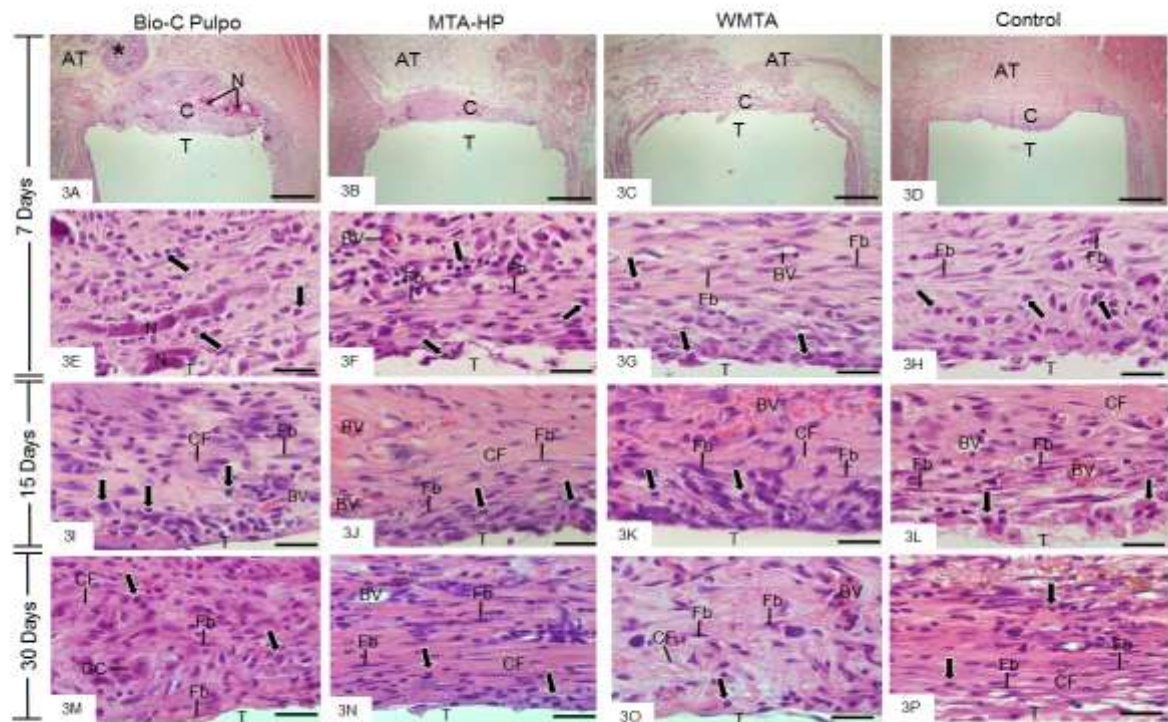


Figures 2A-2H - Light micrographs of liver sections stained with HE. In 2A-2D - hepatocytes with round-shaped nuclei (arrows) are observed near to the central vein (CV) of a hepatic lobule. Sinusoids capillaries (SC) are situated between the strips of hepatocytes. Bars: 50 μ m. In 2E-2H - sections showing portions of liver near to the portal space with bile duct (BD), hepatic portal vein (HV) and hepatic artery (HA). Large hepatocytes with round/spherical nucleus (arrows) are in close juxtaposition forming strands of cells. Bars: 30 μ m. **Figure 2I** – Numerical density of hepatocytes in the region near to the portal space in Bio-C Pulpo, MTA-HP, WMTA and Control groups at 60 days. No significant difference is observed among the groups. Values expressed as mean $\pm$ SD. One-way ANOVA followed by Tukey's test.

Biocompatibility analysis of subcutaneous implants

- *Morphological findings*

At 7 days, the reaction provoked by implants was restricted to well-defined capsules, with exception in Bio-C Pulpo specimens (Figs. 3A-3D). In these specimens, dense inflammatory infiltrate was also seen in the adjacent tissues. Moreover, necrosis areas were present in the capsules around the Bio-C Pulpo (Figs. 3A and 3E). Higher magnification revealed that the capsules around all implants contained several inflammatory cells, mainly mononuclear cells (macrophages, lymphocytes and plasma cells), in the periods of 7 and 15 days (Figs. 3E-3L). However, a gradual rearrangement of cellular content was evident in the capsules over time. At 30 days, a marked presence of fibroblasts between bundles of collagen fibres was noticed in all groups (Figs. 3M-3P). At 60 days, thin capsules surrounded the implants and inflammatory infiltrate areas were not found in the adjacent tissues (Figs. 4A-4E). In this period, some inflammatory cells were present among fibroblasts and bundles of collagen fibres (Figs. 4E-4H).



Figures 3A-3P - Light micrographs showing portions of capsule from sections stained with HE. Bars: 500 µm. **Figs. 3A-3D** – general view of capsules around the opening of the polyethylene tubes after 7 days of implantation in the subcutaneous tissues. In the Bio-C Pulpo (**Fig. 3A**), the thick capsule (C) adjacent to the material exhibits necrosis areas (N); dense inflammatory infiltrated (asterisk) is also observed in the adjacent tissue (AT). In other groups, the inflammatory reaction is present in the capsules only (**Figs. 3B-3D**). **Figs. 3E-3H** – high magnifications of Figs. 3A-3D show several inflammatory cells (arrows). In the Bio-C Pulpo (**Fig. 3E**), necrosis areas are also observed (N). **Figs. 3I-3L** (15 days) and **Figs. 3M-3P** (30 days) – inflammatory cells (arrows) and fibroblasts (Fb) are dispersed between collagen fibres (FC). BV, blood vessel; GC, giant cell; T, space of polyethylene tubes; AT, adjacent tissues. Bars: 30 µm.

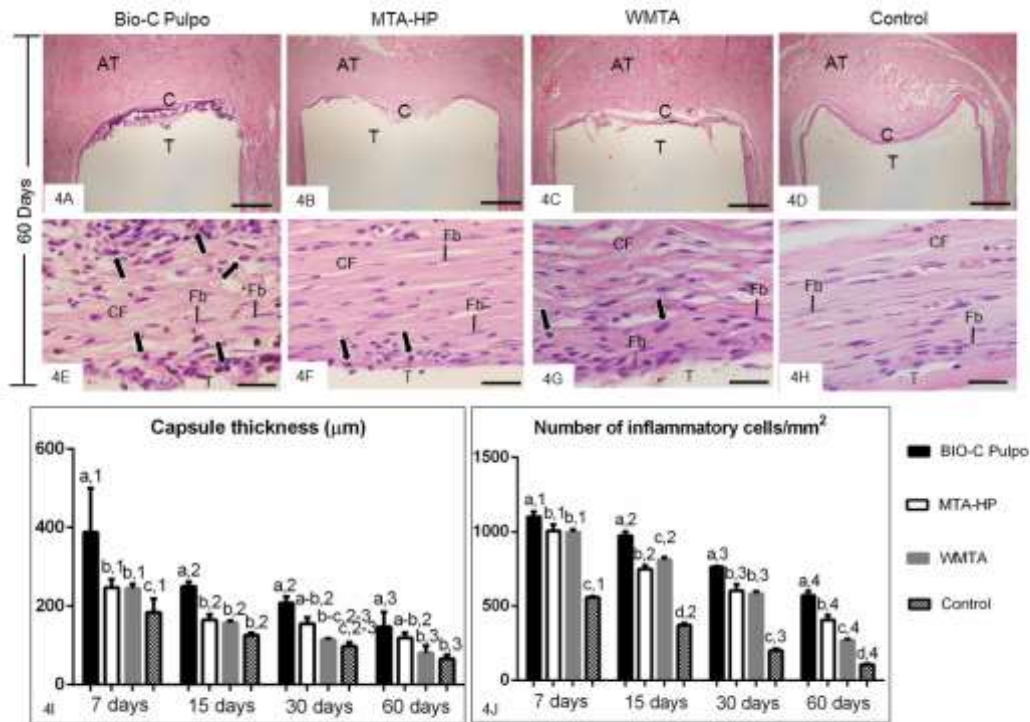
- Capsule thickness measurement

At 7 days, the thickness of the capsules varied from 387 µm (Bio-C Pulpo) to 183 µm (CG) and these values reduced significantly in all groups over time (**Fig. 4I**). At 7 and 15 days, the capsules of Bio-C Pulpo were significantly thicker than in other groups whereas no significant difference was observed between MTA-HP and WMTA (7 days: $p = 0,9996$; 15 days: $p = 0.9896$). After 15 days, significant differences were

also observed between MTA Repair and CG ($p = 0.2734$) and WMTA and CG ($p = 0.4537$) specimens. At 30 and 60 days, significant differences were not observed between Bio-C Pulpo and MTA-HP (30 days: $p = 0.0698$; 60 days: $p = 0.5434$), and between MTA-HP and WMTA (30 days: $p = 0.2253$; 60 days: $p = 0.2647$). At 60 days, there was no significant difference between MTA-HP and CG ($p = 0.0596$) and WMTA and CG ($p = 0.8865$). Although, the capsules of Bio-C Pulpo were significantly thicker than CG at 60 days, the thickness of these capsules was around $146 \mu\text{m}$.

- Numerical density of inflammatory cells

As shown in the Figure 4J, the number of inflammatory cells was significantly reduced in the CG specimens in comparison with other groups at all time points ($p < 0.0001$). In contrast, the Bio-C Pulpo had the highest values of inflammatory cells ($p < 0.0001$) in all periods. Although significant differences were not observed between MTA-HP and WMTA at 7 and 30 days, the number of inflammatory cells was significantly greater in the MTA-HP than WMTA at 60 days ($p < 0.0001$). The number of inflammatory cells reduced significantly in the capsules of all groups over time.



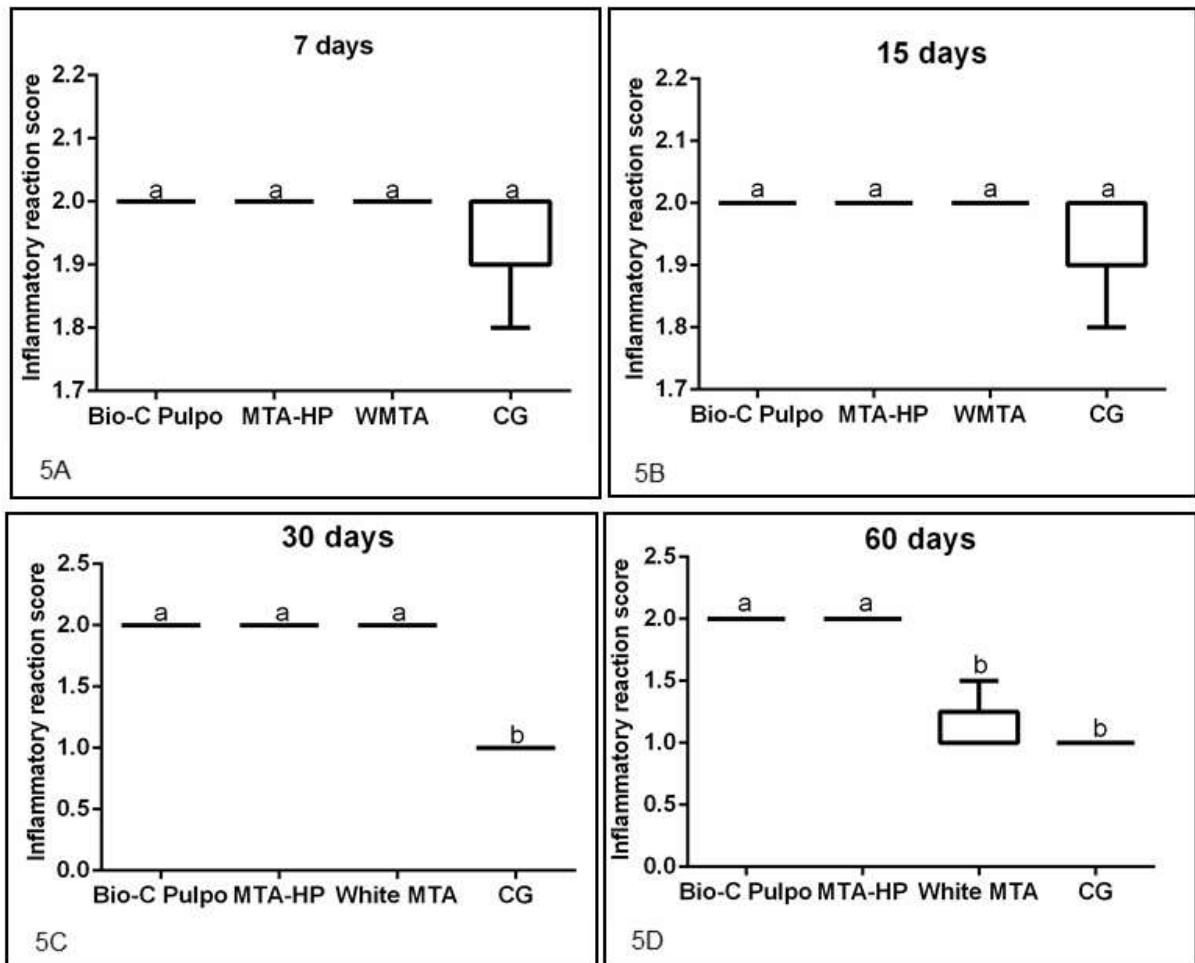
Figures 4A-4H – Light micrographs showing portions of capsule from sections stained with HE. Bars: 500 μm. **Figs. 4A-4D** – general view of capsules around the opening of the polyethylene tubes after 60 days of implantation in the subcutaneous tissues. The thin capsules are well-defined (C). The inflammatory infiltrated is absent in the adjacent tissues (AT). **Figs. 4E-4H** – at high magnification, the capsules show typical bundles of collagen fibres (CF) arranged among fibroblasts (Fb) and inflammatory cells (arrows); marked presence of inflammatory cells (arrows) is still seen in specific areas of capsules around the Bio-C Pulpo (Fig. 4E). T, space of polyethylene tubes. Bars: 30 μm.

Figs. 4I and 4J – Graphics showing the data (mean±standard deviation) of thickness of capsules (Fig. 4I) and numerical density of inflammatory cells (Fig. 4J) of Bio-C Pulpo, MTA-HP, WMTA and Control groups at 7, 15, 30 and 60 days. The superscript letters represent the comparison among groups at each time point; different letters indicate significant difference. Superscript number indicates the analysis of each group over time; different superscript numbers indicate significant differences among the periods. Tukey's test ($p \leq 0.05$).

- Inflammatory reaction score

As shown in the Figures 5A-5D, a moderate inflammatory infiltrate was present in the Bio C Pulpo and MTA-HP specimens, at all time points. At 7 (Fig. 5A), 15 (Fig. 5B) and 30 (Fig. 5C) days, no significant difference was found among the bioceramic materials. At 60 days (Fig. 5D), the inflammatory reaction score in WMTA specimens was significantly reduced in comparison with Bio C Pulp and MTA-HP, but no significant difference was observed between Bio C Pulpo and MTA-HP. In the CG, all

specimens exhibited a moderate inflammatory reaction at 7 and 15 days (Figs. 5A and 5B). At 30 and 60 days (Figs. 5C and 5D), the capsules of CG showed a mild inflammatory reaction. At 30 days, the degree of inflammatory reaction in the CG specimens was significantly lower than in other groups while, at 60 days, significant difference was not observed between CG and WMTA ($p < 0.05$).

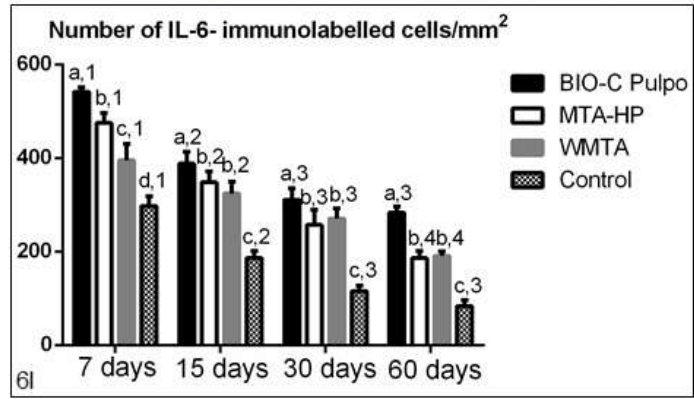
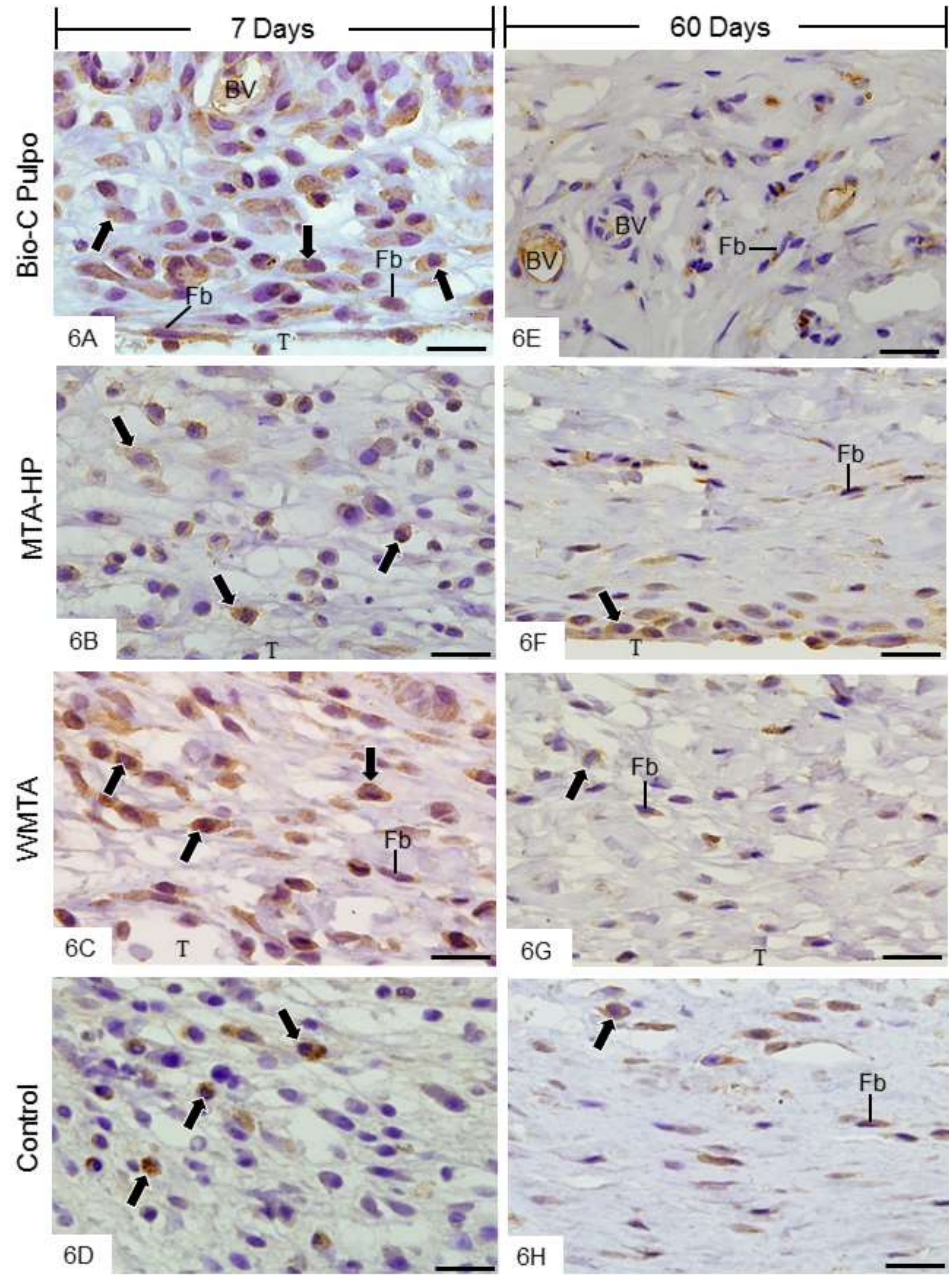


Figures 5A-5D – Graphics showing the inflammatory reaction score of Bio-C Pulpo, MTA-HP, WMTA and Control groups at 7 (Fig. 5A), 15 (Fig. 5B), 30 (Fig. 5C) and 60 (Fig. 5D) days. Superscript letters represent the comparison among groups; different letters indicate significant difference among groups. Kruskal-Wallis Anova followed by Dunn's test ($p \leq 0.05$).

- Immunoexpression of IL-6

In the sections submitted to immunohistochemistry for IL-6 detection, cytoplasmic immunolabelling (brown/yellow colour) was seen in the inflammatory cells and fibroblasts in the capsules of animals from all the groups. At 7 days, the capsules of all the groups exhibited an evident immunoexpression (Figs. 6A-6D), which gradually reduced over time (Figs. 6E-6H). Usually, on 60th day, the capsules contained few immunolabelled fibroblasts and inflammatory cells (Figs. 6E-6H). Immunostained cells were not observed in the sections incubated with non-immune serum instead primary antibody (data not illustrated).

The quantitative analysis (Fig. 6I) revealed that, in all periods, the number of IL-6-immunolabelled cells was significantly greater in the capsules of Bio-C Pulpo than in other groups ($p < 0.0001$). Although at 7 days, the number of immunolabelled cells was significantly greater in the MTA-HP than WMTA ($p < 0.0001$), no significant difference was found in the other periods. In all periods, the Control group exhibited a reduced number of immunolabelled cells in comparison with Bio-C Pulpo, MTA-HP and WMTA ($p < 0.0001$). At 60 days, the value in the Bio-C Pulpo was about 3.4 folds higher than CG specimens, while in the MTA-HP and WMTA was approximately of 2.3 folds. From 7 to 60 days, a significant reduction in the immunoexpression was observed in the capsules of all groups ($p < 0.0001$).



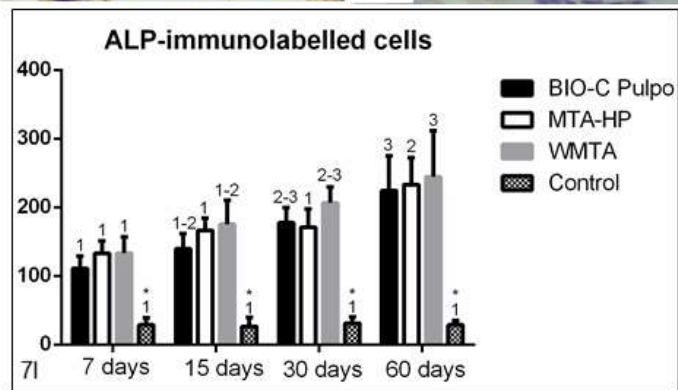
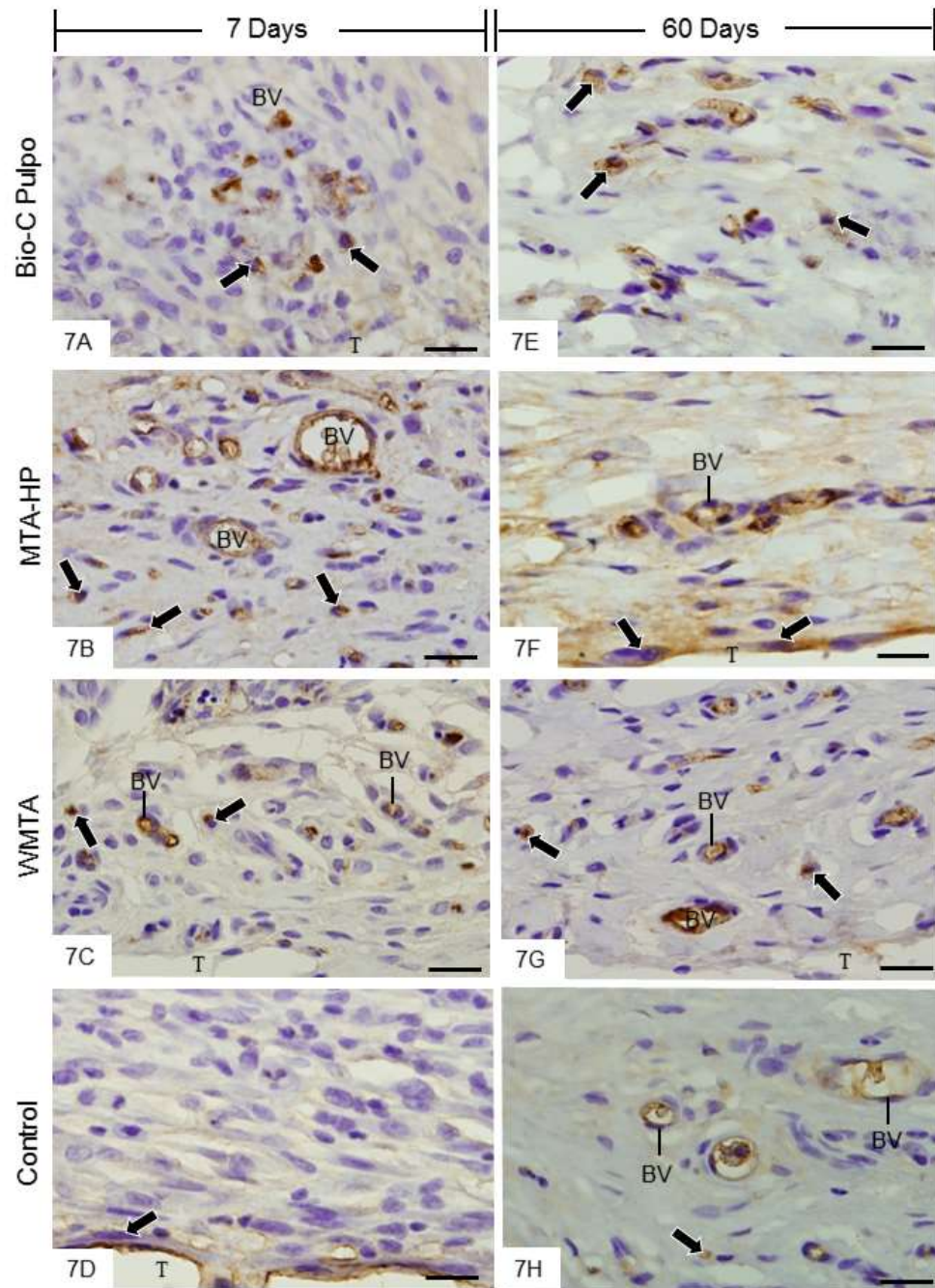
Figures 6A-6H - Light micrographs showing portions of capsule from sections subjected to immunohistochemistry for detection of IL-6 and counterstained with haematoxylin. At 7 days (Figs. 6A-6D), various inflammatory cells (arrows) exhibiting immunolabelling in their cytoplasm (brown-yellow colour) are present in the capsules of all groups. At 60 days (Figs. 6E-6H), the capsules of all groups show an evident reduction in the immunoexpression. Inflammatory cells (arrows) and some fibroblasts (Fb) exhibit weak immunolabelling. BV, blood vessel; T, space of polyethylene tubes. Bars: 40 μ m.

Figure 6I – Numerical density of IL-6-immunostained cells (mean $\pm$ standard deviation) in Bio-C Pulpo, MTA-HP, WMTA and Control groups at 7, 15, 30 and 60 days. Superscript letters indicate the comparison among the groups; superscript different letters indicate significant difference. Superscript numbers indicate the analysis of each group over time; different numbers indicate significant difference. Tukey's test ($p\leq 0.05$).

Bioactive potential***-Alkaline phosphatase (ALP)***

Although ALP-immunolabelled cells were seen in the capsules of all groups (Figs. 7A-7H), few immunostained cells were present in the CG specimens (Figs. 7D and 7H). In these specimens, weak immunolabelling was often observed in the cells situated in the surface of capsules and in the vascular cells (Figs. 7D and 7H). In contrast, various immunolabelled cells were observed dispersed throughout the capsules around repair materials at 7 (Figs. 7A-7D) and 60 (Figs. 7E-7H) days.

According to Figure 7I, the number of ALP-immunolabelled cells was significantly greater in Bio-C Pulpo, MTA-HP and WMTA groups ($p < 0.0001$) than in CG ($p < 0.0001$), at all time points. In all periods, no significant difference was detected among Bio-C Pulpo, MTA-HP and WMTA groups. From 7 to 60 days, a significant increased in the immunoexpression was observed in the Bio-C Pulpo, MTA-HP and WMTA groups ($p < 0.0001$). In contrast, significant differences in the number of ALP-immunolabelled cells were not found in the CG specimens over time.



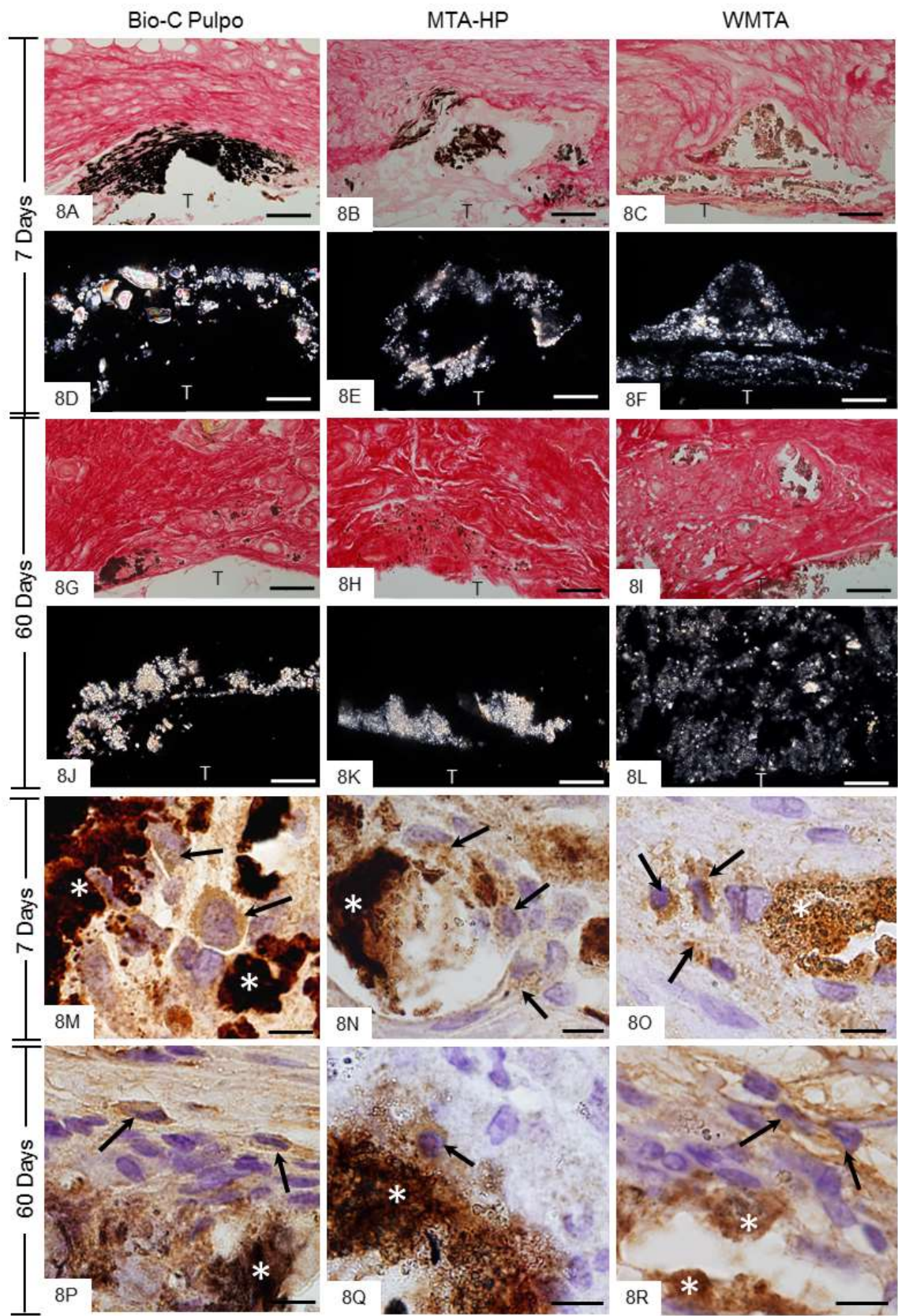
Figures 7A-7H - Light micrographs showing portions of capsule from sections subjected to immunohistochemistry for detection of alkaline phosphatase (brown-yellow colour) and counterstained with haematoxylin. **Figs. 7A-7H** – various round/ovoid and elliptical immunolabelled cells (arrows) are seen in the capsules around biomaterials (Figs. 7A-7C and 7E-7G). In the CG (Figs. 7D and 7H), weak immunolabelling is observed in the scarce cells (arrows). Note that endothelial cells show immunoreactivity to ALP. BV, blood vessel; T, space of polyethylene tubes. Bars: 40 μ m. **Figure 7I** – Numerical density of ALP-immunostained cells (mean $\pm$ standard deviation) in Bio-C Pulpo, MTA-HP, WMTA and Control groups at 7, 15, 30 and 60 days. Superscript asterisk indicates the comparison among the groups. Superscript numbers indicate the analysis of each group over time; different numbers indicate significant difference. Tukey's test ($p \leq 0.05$).

- *von Kossa histochemical reaction and analysis under polarized illumination*

Bio-C Pulpo, MTA-HP and WMTA specimens exhibited von Kossa-positive structures in the capsules at 7 (Figs. 8A-8C) and 60 days (Figs. 8H-8I). In contrast, the capsules of CG did not show positivity to the von Kossa reaction. No reaction was also found in the sections incubated in the free-silver nitrate medium, used as negative control (data not shown). The unstained sections analyzed under polarized light showed birefringent deposits in the capsules adjacent to the Bio-C Pulpo, MTA-HP and WMTA specimens (Figs. 8D-8F and 8J-8L) while no birefringent structure was seen in the CG specimens (data no shown).

- *von Kossa method combined with immunohistochemical detection of ALP*

The sections subjected to the von Kossa method followed by immunohistochemistry for ALP detection revealed immunolabelling in the cytoplasm of elongated/fusiform and round/ovoid cells situated near to the von Kossa-positive structures in the Bio-C Pulpo, MTA-HP and WMTA specimens (Figs. 8M-8R). ALP-immunolabelled cells were not seen in the negative control sections (data not shown).



Figures 8A-8L – Light micrographs of portions of capsule from sections submitted to the von Kossa reaction and counterstained with picrosirius (Figs. 8A-8C and 8G-8I) and unstained sections analyzed under polarized light (Figs. 8D-8F and 8J-8L). At 7 days, von Kossa-positive deposits (in black colour) are seen in the capsules of Bio-C Pulpo (Fig. 8A), MTA-HP (Fig. 8B) and WMTA (Fig. 8C) groups. The capsules from unstained sections show birefringent structures around these materials (Figs. 8D-8F). After 60 days, von Kossa-positive deposits (Figs. 8G-8I) and birefringence (Figs. 8J-8L) are present in the capsules around the Bio-C Pulpo, MTA-HP and WMTA specimens. T, space of polyethylene tubes. Bars: 20 μ m. **Figures 8M-8R** – Light micrographs of portions of capsule from sections submitted to the von Kossa reaction followed by immunohistochemistry for detection of alkaline phosphatase and counterstained with haematoxylin. ALP-positive immunolabelling is observed in the elongated and round/ovoid cells (arrows) situated near to the von Kossa-positive structures (asterisks). Bars: 10 μ m.

Discussion

This is the first study that evaluated the morphological features of liver and serum GOT and GPT levels in rats with Bio-C Pulpo, MTA-HP, and WMTA implanted in the subcutaneous tissues. Moreover, the biocompatibility and bioactive potential of Bio-C Pulpo and MTA-HP in comparison with WMTA were also investigated.

The liver functional state can be determined by estimating the biochemical parameters, such as GOT and GPT. Thus, high levels of these enzymes are detected in bloodstream when the liver is damaged or diseased (Mahmud *et al.* 2012, Wardani *et al.* 2017). In the present study, the materials were not able to induce changes in the serum GOT and GPT levels. Moreover, no histopathological changes were observed in the liver sections. Few studies have focused on the systemic changes promoted by dental materials. Although MTA (Angelus) placed in the incisor extracted sockets caused an increase in the levels of aluminum in the plasma, no significant alteration in the aluminum concentration was detected in the liver samples of rats (Demirkaya *et al.* 2016). In contrast, a significant increase in the serum levels of alanine aminotransferase and aspartate aminotransferase associated with an inflammatory reaction in the liver samples were noticed in rats after 30 days of Grey ProRoot MTA implantation in the subcutaneous (Khalil & Eid 2013). Here, the WMTA contains the calcium tungstate as radiopacifier agent, in place of bismuth oxide. Several studies have reported that bismuth oxide inhibits cell proliferation¹, Min *et al.* 2007, Kim *et al.* 2008) and presents toxicity when added to human pulp cell cultures (Min *et al.* 2007). In the present study, we demonstrated, for the first time, that calcium silicate-based materials (Bio-C Pulpo, MTA-HP and White MTA) do not induce toxicity following a long period of time.

The morphological and morphometrical analyses of connective tissue reaction promoted by materials revealed significant reduction in the inflammatory infiltrate in all groups over time. The analysis of inflammatory reaction score revealed that the bioceramic materials induced initially a moderate inflammatory reaction similarly to that observed in the CG samples. Several studies have demonstrated that materials containing calcium silicate provide an alkaline pH to the microenvironment, inducing leukocytes recruitment from the blood vessels (Silva *et al.* 2015, Ferreira *et al.* 2019, Hoshino *et al.* 2020). Inflammatory and resident cells secrete several cytokines and growth factors, which modulate the immune and inflammatory responses (Saraiva *et al.* 2018, da Fonseca *et al.* 2019, Delfino *et al.* 2020). Among the cytokines, interleukin-

6 (IL-6) has been associated with the onset of the inflammatory reaction (de Oliveira *et al.* 2017, Saraiva *et al.* 2018, Delfino *et al.* 2020) and has been considered a parameter in the biocompatibility analysis (Delfino *et al.* 2020). In the present study, a reduction in the number of IL-6-immunostained cells was also observed in all groups over time. The thickness of capsules indicates the extent of the inflammatory reaction around the materials implanted in the connective tissue; thus, the significant reduction in the thickness of capsules verified from 7 to 60 days reinforces the idea that the inflammatory reaction was gradually decreased. Here, all groups showed capsules measuring below 150 μm , therefore, the presence of thin capsules on 60th day after implantation in the subcutaneous tissues (Yaltirik *et al.* 2004), indicates that the materials are biocompatible (Saraiva *et al.* 2018, Delfino *et al.* 2020).

Our findings clearly showed a variability in the degree of tissue damage induced by different materials in the connective tissue. Bio-C Pulpo induced the recruitment of greater number of inflammatory cells and higher immunoexpression of IL-6 than MTA-HP and WMTA in all periods. At 7 days, the capsules around Bio-C Pulpo were about 2-fold thicker than in MTA-HP and WMTA specimens, indicating that Bio-C Pulpo induced intense damage to the connective tissue. This differential response may be due to differences in the chemical composition of these materials. Although Bio-C Pulpo contains calcium silicate, the addition of calcium hydroxide may explain, at least in part, the higher tissue injury caused by this material in comparison to the MTA-HP and WMTA. It is known that calcium hydroxide exerts an irritant effect on tissues, promoting the extensive necrosis areas when in direct contact with tissues (Hoshino *et al.* 2020, Silva *et al.* 2020). The Bio-C Pulpo provides a pH between 12 and 13 (Koutroulis *et al.* 2019) while the pH provided by MTA-HP is 9 (Guimarães *et al.* 2018). Thus, the elevated pH may be responsible for high incidence of inflammatory cells and IL-6 immunoexpression verified around Bio-C Pulpo specimens at initial periods. The high pH of Bio-C Pulpo was associated with the cytotoxicity caused by this material when added to primary human dental pulp cells (Koutroulis *et al.* 2019). The high incidence of inflammatory cells observed at 7 days in the Bio-C Pulpo specimens did not reflect on the inflammatory reaction score. In fact, although the numerical density of inflammatory cells was different among Bio-C Pulpo, MTA-HP and WMTA, the score represents a classification of the inflammatory infiltrate intensity based on a relatively wide range of inflammatory cells. Thus, significant differences in the inflammatory reaction score between Bio-C Pulpo and MTA-HP were not observed at all time points.

Besides calcium silicate and calcium hydroxide in the powder, the liquid of Bio-C Pulpo contains methylparaben and a plasticizing material, containing distilled water, plasticizer and calcium chloride (Lima *et al.* 2020). There is evidence that methylparaben is noncarcinogenic and, therefore, it is widely used in the pharmaceutical products as an antimicrobial preservative (Soni *et al.* 2002). The addition of plasticizer to the material generally improves its handling and reduces its setting time. In fact, it has been reported that initial setting time of WMTA is 22 min while of MTA-HP is around 17 min (Ferreira *et al.* 2019) and of Bio-C Pulpo vary from 7 min (Koutroulis *et al.* 2019) to 12 min (Lima *et al.* 2020). However, it is important to emphasize that the proportion of the powder components and particles size also interfere in the biological response as well as in the chemical and physical properties of the materials (Silva *et al.* 2015, Cosme-Silva *et al.* 2019, Ferreira *et al.* 2019). Our results showed that the irritant potential of Bio-C Pulpo decreased over time since a significant reduction in the number of IL-6-immunostained cells was associated with the formation of collagen fibres in the thin capsules on 60th day.

The similar findings obtained between MTA-HP and WMTA indicate that the changes observed in the MTA-HP did not alter the biocompatibility of the tricalcium silicate-based material. No significant difference in the biological response was observed following the addition of propylene glycol, a plasticizer agent, to the MTA (Ferreira *et al.* 2019). Moreover, the calcium tungstate, used as a radiopacifier in MTA-HP, has shown adequate cytocompatibility in contact with periodontal and osteogenic cells (Gomes-Cornélio *et al.* 2011). Thus, bioceramic materials presented good tolerance in the connective tissues compared to the control group. The biocompatibility of MTA-HP and WMTA may be related to their chemical and/or physical properties, such as low solubility and short setting time (Tanomaru-Filho *et al.* 2017).

In addition to biocompatibility, the materials used in the pulpotomy and as repair cement may stimulate the production of hard tissue. The tricalcium silicate-based cements in contact with the tissue fluids produce calcium silicate hydrate and calcium hydroxide (Tanomaru-Filho *et al.* 2017, Collado-González *et al.* 2019), which after dissociation, releases calcium and hydroxyl ions (Cintra *et al.* 2017, Bueno *et al.* 2019, Benetti *et al.* 2019). Although there is evidence that during Bio-C Pulpo hydration no crystalline calcium hydroxide is formed, this material releases great amount of calcium ions (Koutroulis *et al.* 2019). Here, von Kossa-positive structures were observed in Bio-C Pulpo, MTA-HP and WMTA specimens, at all-time points, indicating the

presence of calcite precipitate in the capsules (Holland *et al.* 1999, Cintra *et al.* 2017, Benetti *et al.* 2019, Bueno *et al.* 2019, Delfino *et al.* 2020, Hoshino *et al.* 2020). No von-Kossa-positive deposits were detected in the CG specimens, confirming that bioceramic materials may favour the formation of calcium deposits (Benetti *et al.* 2019, Bueno *et al.* 2019, Delfino *et al.* 2020). Moreover, the analysis under polarized light revealed the presence of birefringent structures in the capsules around Bio-C Pulpo, MTA-HP and WMTA. Since these birefringent structures may be calcite crystals (Holland *et al.* 1999, Cintra *et al.* 2017, Benetti *et al.* 2019, Bueno *et al.* 2019, Delfino *et al.* 2020, Hoshino *et al.* 2020), it seems that these bioceramic materials present a bioactive potential.

An *in vivo* study reported immunostained cells for osteocalcin and osteopontin, glycoproteins used as markers of hard tissue producing-cells, in the capsules around the Bio-C Pulpo implanted in the subcutaneous tissues (Cosme-Silva *et al.* 2019). Another marker of hard tissue producing-cells, is the alkaline phosphatase (ALP), an enzyme, at a pH optimum of 9.2, provides inorganic phosphate to form hydroxyapatite, participating in tissue mineralization (Bellows *et al.* 1991, Daltoé *et al.* 2016, Zordan-Bronzel *et al.* 2019). Although ALP activity was observed in the capsules of all groups, a greater immunoexpression was detected around bioceramic materials than in CG specimens, indicating that these materials may stimulate the production of this enzyme by the surrounding connective cells. It is important to emphasize that an intense immunoexpression of ALP was observed in the capsules of bioceramic materials over time. Moreover, ALP-immunolabelled cells next to von Kossa-positive structures were observed following the combined method (von Kossa/ALP reaction), suggesting that these calcium silicate-based materials may maintain the calcium release for a prolonged period after setting time, ensuring their bioactive potential. However, further studies are necessary to clarify if these bioceramic materials, particularly the Bio-C Pulpo, induce the formation of mineralized tissues.

Conclusion

Bio-C Pulpo, MTA-HP and WMTA showed no harmful effect on liver structural integrity and serum GOT and GPT levels, indicating that these materials do not promote systemic changes. Despite changes made in Bio-C Pulpo and MTA-HP to improve their physicochemical properties, these bioceramic materials are biocompatible and exhibit bioactive potential similar to the WMTA.

Conflict of interest

The authors deny any conflict of interests to this study.

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Table 1- Chemical composition and powder:liquid ratio of bioceramic materials

Materials/ Manufacturer	Powder	Liquid	Powder:liquid ratio
Bio-C Pulpo Angelus, Londrina, PR, Brazil	Calcium silicate (50-65%), calcium aluminate (1-4%), calcium hydroxide (0.5-2%), zirconium oxide (20-30%), calcium fluoride (1-4%), silicon dioxide (2-6%), iron oxide (0.05-1%)	Distilled water, plasticizer, calcium chloride, methylparaben	1 dose of powder for 3 drops of liquid.
MTA Repair HP Angelus, Londrina, PR, Brazil	Tricalcium silicate (45-55%), dicalcium silicate (10-15%), tricalcium aluminate (5-12%), calcium oxide (1-5%), calcium tungstate (20-30%)	Distilled water and plasticizer	Dispense the contents of one package (0.085g) and 2 drops of liquid.
White MTA Angelus, Londrina, PR, Brazil	Tricalcium silicate (45-55%), dicalcium silicate (10-15%), tricalcium aluminate (5-12%), calcium oxide (1-5%), calcium tungstate (20-30%)	Distilled water	1 g. of powder and 0.33 mL of distilled water.

3.2 Publicação 2*

The participation of fibroblast growth factor-1 and interleukin-10 in the connective tissue repair following subcutaneous implantation of bioceramic materials in rats

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Running title: Tissue repair induced by bioceramic materials

Key Words: Bioceramic materials; Immunohistochemistry; Cell proliferation; Ki-67; Interleukin-10; Collagen

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Abstract

Aim To evaluate whether Bio-C Pulpo (Bio-C) and MTA Repair HP (MTA-HP) promote the connective tissue repair.

Methodology Polyethylene tubes were filled with Bio-C Pulpo, MTA-HP and White MTA (WMTA), used as gold standard; empty tubes were used as control group (CG). The polyethylene tubes were implanted into subcutaneous tissues of Holtzman rats for 7, 15, 30 and 60 days. Number of fibroblasts (FB), Ki-67-, fibroblast growth factor 1- (FGF-1) and interleukin-10-immunolabelled cells (IL-10) were quantified. The amount of collagen was measured in the picrosirius red-stained sections. The data were subjected to two-way ANOVA followed by Tukey's test ($P \leq 0.05$).

Results At 7 days, significant differences in the number of FB were not detected among Bio-C Pulpo, MTA-HP and WMTA groups ($p > 0.05$). The capsules of all groups exhibited a significant increase in the number of FB and in the amount of collagen over time. From 7 to 60 days, a significant reduction in the number of FGF-1 and Ki-67-immunolabelled cells was seen in the capsules of all specimens. In all periods, no significant difference in the number of FGF-1 immunofluorescent cells was detected between Bio-C Pulpo and CG specimens. At 60 days, significant differences in the immunoexpression of FGF-1 were not observed among the groups. At 7 and 15 days, the highest immunoexpression for Ki-67 was found in Bio-C specimens while, after 30 and 60 days, no significant difference was observed among the bioceramic materials. At 7 days, few IL-10 immunolabelled cells were present in the capsules whereas, at 60 days, a significant increase in the IL-10-immunostaining was found in all groups. At 60 days, the Bio-C Pulpo, MTA-HP and WMTA groups showed a greater number of IL-10-immunolabelled cells than in CG specimens ($p < 0.0001$).

Conclusions Bio-C Pulpo, MTA-HP and WMTA stimulate the fibroblast proliferation leading to the formation of collagen-rich capsules. FGF-1 seems to control the fibroblast proliferation while IL-10 may mediate the remodelling of capsules around Bio-C Pulpo, MTA-HP and WMTA bioceramic materials.

Introduction

Mineral trioxide aggregate (MTA) is considered the gold standard for pulpotomies, root perforations and root-end filling among other clinical applications due to its physicochemical and biological properties (Ferreira *et al.* 2019, Benetti *et al.* 2019, Cosme-Silva *et al.* 2019, Cosme-Silva *et al.* 2020, Lima *et al.* 2020, Barbosa *et al.* 2021). Biological properties of calcium silicate-based cements, such as MTA, are related to the release of calcium ions, since calcium stimulates the proliferation of cells (Silva *et al.* 2015, Cosme-Silva *et al.* 2020), induces hard tissue formation and promotes tissue neoformation when placed in contact with dental pulp (Smaïl-Faugeron *et al.* 2018, Silva *et al.* 2019, Lima *et al.* 2020) and periodontal ligament (da Silva *et al.* 2011, da Fonseca *et al.* 2019a). However, MTA formulation presents some drawbacks such as handling difficulties, granular consistency and prolonged setting-time (Cosme-Silva *et al.* 2019, Cosme-Silva *et al.* 2020, Lima *et al.* 2020, Hoshino *et al.* 2021). Recently, the calcium tungstate replaced the bismuth oxide, a radiopacifying agent of MTA. This change in the radiopacifying agent was made since several studies reported that bismuth oxide is responsible for discoloration of the tooth and marginal gingiva and affects some physicochemical properties of MTA material (Marciano *et al.* 2017, De Souza *et al.* 2018, Ferreira *et al.* 2019, Lima *et al.* 2020). The change in the formulation of white MTA maintained the biocompatibility and bioactive potential of this bioceramic material (Delfino *et al.* 2021).

The Angelus launched MTA Repair HP (MTA-HP, MTA “High Plasticity”, Angelus, Londrina, PR, Brazil), a reparative material with high plasticity (Benetti *et al.* 2019, ElReash *et al.* 2019). In the MTA Repair HP, an organic plasticizer was added to the distilled water to improve the plasticity of the material (Benetti *et al.* 2019, Ferreira *et al.* 2019, ElReash *et al.* 2019) and, consequently, to facility its insertion into tooth cavities (Cosme-Silva *et al.* 2020, Lima *et al.* 2020).

New bioceramic material Bio-C Pulpo (Bio-C Pulpo, Angelus, Londrina, PR, Brazil) was developed for pulpotomy and has good radiopacity (Lima *et al.* 2020). The main clinical indication of Bio-C pulpo is for pulpotomy, but the manufacturer also indicates this material for protection of the dentine-pulp complex and as a cavity liner. The Bio-C Pulpo (Angelus, Londrina, Brazil) is composed of powder containing calcium silicate [(tricalcium silicate (Ca_3SiO_5) and dicalcium silicate (Ca_2SiO_4)], calcium aluminate, calcium hydroxide, zirconium oxide, calcium fluoride, silicon dioxide, and iron oxide. The liquid of Bio-C contains distilled water, plasticizer, calcium chloride

(catalyst) (Lima *et al.* 2020), and methylparaben (Cosme-Silva *et al.* 2019, Koutroulis *et al.* 2019, Cosme-Silva *et al.* 2020, Lima *et al.* 2020). Bio-C Pulpo induced a tissue response similar to the MTA HP Repair and white MTA, exhibiting biocompatibility and bioactive potential after implantation into connective tissue of rat subcutaneous. Furthermore, no structural changes were observed in the liver of animals that received the implants, suggesting that the addition of plasticizers to the liquid improve the consistency of the materials maintained the desired biological properties of a bioceramic material (Delfino *et al.* 2021).

Due to the close contact between repair cements and periodontal and pulp tissues, it is necessary to understand the cellular events caused by biomaterials in connective tissues (da Fonseca *et al.* 2019a and 2019b, Barbosa *et al.* 2021). The initial tissue response induced by biomaterials is the recruitment of inflammatory cells that release mediators, such as cytokines and growth factors, which mediate the cellular response (da Fonseca *et al.* 2019a, Hoshino *et al.* 2021, Delfino *et al.* 2020 and 2021). From a biomaterial, it is expected that proinflammatory cytokines are released for a short time and, then, the immune cells can produce anti-inflammatory cytokines and factors that stimulate the proliferation of resident cells capable of producing the structural components of the tissue. Among several factors involved in connective tissue repair, fibroblast growth factor-1 (FGF-1) plays a mitogenic role, stimulating fibroblast proliferation and collagen formation (Silva *et al.* 2017, da Fonseca *et al.* 2019b). Ki-67 is a non-histone nuclear cortex protein expressed during the G1, S, G2, and M phases of the cell cycle, but not in quiescent state cells (Penault-Llorca & Radosevich-Robin 2017, da Fonseca *et al.* 2019b). Thus, the Ki-67 is widely used to identify proliferating cells (Sun & Kaufman 2018). Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine that plays a crucial, and often essential, role in preventing inflammatory and autoimmune pathologies (Iyer & Chang 2012, Saxena *et al.* 2015). IL-10 has a suppressive effect on both the innate and adaptive response of the immune system, playing a role in maintaining tissue and organs homeostasis (Neumann *et al.* 2019).

The aim of this study was to evaluate whether changes made in the Bio-C Pulpo and MTA-HP interfere in the connective tissue repair after implantation into subcutaneous of rats. For this purpose, the number of fibroblasts and the amount of collagen in the capsules were computed after 7, 15, 30 and 60 days of implantation. Moreover, immunohistochemistry reactions for detection of IL-10, FGF-1 and Ki-67

were carried out. The null hypothesis was that the new bioceramic-based materials, Bio-C Pulpo and MTA-HP, would not induce the connective tissue repair when compared with WMTA.

Materials and methods

Animal procedures

This research was approved (protocol # 02/2019) by the Ethical Committee for Animal Research of Araraquara Dental School (São Paulo State University - UNESP, Brazil) in compliance with Brazilian national law on animal use. Animal care was described according to Preferred Reporting Items for Animal Studies in Endodontology (PRIASE) 2021 guidelines (Nagendrababu *et al.* 2021).

Eighty adult male Holtzman rats (*Rattus norvegicus albinus*), with an average weight of 250 g, were maintained in polyethylene cages under 12h light/12h dark cycle at controlled temperature ($23\pm 2^{\circ}\text{C}$) and humidity ($55\pm 10\%$), with water and food provided *ad libitum*. The animals were housed in polypropylene cages (40 cm $\times$ 30 cm $\times$ 15 cm) filled with a layer of white pine shavings; in each cage were maintained four rats. The animals were randomly allocated into four groups (20 animals per group): CG (control group, empty polyethylene tubes), Bio-C Pulpo (Bio-C Pulpo, Ângelus, Londrina, Paraná, Brazil), MTA-HP (MTA Repair HP, Ângelus, Londrina, Paraná, Brazil) and WMTA (White MTA, Ângelus, Londrina, Paraná, Brazil). Sample size was estimated based in studies that also evaluated the biomaterial effects on connective tissue repair (Silva *et al.* 2017, da Fonseca *et al.* 2019b). The sample size was calculated considering a 50% difference among experimental groups and CG, 20% variability and 90% test power and an alpha error of 0.05 to recognize a significant difference (Singh & Masuku 2013). In each period, a sample of 5 rats per group was required, totalling twenty animals per group.

The materials were mixed according to manufacturer's recommendations under aseptic conditions. The Bio-C Pulpo was mixed in the ratio of 1 dose of powder for 3 drops of liquid. Sixty seconds after mixing, the cement had a condensable mass consistency. MTA-HP was prepared using one package containing the powder (0.085g) and 2 drops of liquid. This material was spatulated for 40 seconds until complete homogenization, obtaining a cement similar to a modeling clay. WMTA was

manipulated using powder:liquid mixing ratio of 1 g:0.33 mL for 30 seconds obtaining a cement of sandy consistency.

After mixing, each material was inserted into polyethylene tubes (Embramed Indústria Comércio, São Paulo, SP, Brazil) with 10.0 mm length and 1.6 mm diameter, previously sterilized with ethylene oxide. The animals were anaesthetized with an intraperitoneal injection containing 0.08 mL of 10% ketamine hydrochloride and 0.04 mL of 2% xylazine hydrochloride per 100 g of body weight. After shaved and disinfection with 5% iodine solution, a 2.0 cm-long incision was made in a head-to-tail orientation using a nº 15 scalpel (Fibra Cirúrgica, Joinville, SC, Brazil), and the polyethylene tube was placed into the subcutaneous pocket. On the 7th, 15th, 30th and 60th day, the animals were sacrificed with overdose of ketamine and xylazine, and the implants with adjacent tissues were removed. During all experiment, the researchers monitored the animals to minimize the risk of suffering and provide their good welfare.

The morphological and quantitative analyses were performed using an Olympus microscope (BX-51, Olympus, Tokyo, Japan) and an image analysis system (Image-Pro Express 6.0, Olympus). The morphometric data were performed by one calibrated and blinded examiner.

Histological procedures

The specimens were fixed for 48 hours in 4% formaldehyde (prepared from paraformaldehyde freshly prepared) in 0.1 M sodium phosphate buffer at pH 7.2. After fixation, the specimens were dehydrated in graded concentrations of ethanol, treated in xylene and embedded in paraffin for 4 hours at 60° C. Subsequently, the specimens were embedded in paraffin and longitudinal sections with 6-µm thickness were obtained. In each specimen, five slides with two non-serial sections were stained with Masson's trichrome for morphological description; the number of fibroblasts in the capsules was also computed. The content of collagen in the capsules was measured in picro-sirius red-stained sections. Non-serial sections adhered to sialanized slides were submitted to the immunohistochemistry reactions for detection of interleukin-10 (IL-10) and Ki-67.

- Numerical density of fibroblasts

The number of fibroblasts was estimated in the capsules of all the animals. In each animal, three non-serial sections stained with Masson trichrome were used. Using a digital camera (DP-71, Olympus, Tokyo, Japan) attached to a light microscope (Olympus BX-51) at x695 magnification, a standardized field (0.09 mm²) in the central portion of the capsule in close juxtaposition to the opening of implanted tube was captured. In each field, the number of fibroblasts was computed using an image analysis system (Image-Pro Express 6.0, Olympus). The fibroblasts were identified by elongated/fusiform morphology. Thus, the number of fibroblasts per millimetre square of capsule was obtained dividing the total number of fibroblasts by the total standardized field of capsule, as previously described (Silva *et al.* 2017, Saraiva *et al.* 2018, da Fonseca *et al.* 2019b).

Immunofluorescence for detection of FGF-1

The sections were dewaxed in heated xylene, hydrated and the slides were immersed in 0.001 M sodium citrate buffer pH 6.0 and placed into a microwave oven at 98-100°C for the antigen retrieval. After a cooling-off period, the slides were washed in 0.01 M sodium phosphate buffer (PBS) at pH 7.2 and the sections were incubated for 20 min with with 2% bovine-serum-albumin (BSA; Sigma-Aldrich Co., St. Louis, MO, USA) at room temperature. Afterwards, the sections were incubated in a humidified chamber at 4°C with mouse monoclonal anti-FGF-1 antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA; code: sc55520) for 16-18h. The sections were washed in PBS and incubated in the dark with Alexa Fluor®488 anti-mouse IgG antibody (1:1000; Molecular Probes® by Life Technologies, Calrsbad, USA) for 1 hour at room temperature. After washing in PBS, nuclear staining was performed with DAPI (1:500, Molecular Probes by Life Technologies; CA, USA) for 5 min in the dark at the room temperature and the slides were mounted with Fluoromount® mounting medium (Dako Inc., Carpinteria, CA, USA). As negative control, the sections were incubated with non-immune serum in place of primary antibody. The sections were analyzed using a fluorescent microscope DM4000 B LED (Leica, Wetzlar, Germany) with a DFC 550 Camera (Leica, Germany) and the Leica Application Suite software (LAS 4.3, Leica, Germany).

From two non-serial sections per specimen were captured at x40 magnification. In the standardized field (0.146 mm²), the number of cells exhibiting FGF-immunofluorescence was counted (Delfino *et al.* 2020). In all specimens, the number of FGF-1-immunolabelled cells per mm² was calculated in all time points (n=5 per group/period).

-Immunohistochemical detection of IL-10 and Ki-67

Sections adhered to silanized slides were subjected to immunohistochemistry for the detection of Ki-67 and IL-10. To unmask antigenic sites, deparaffinized sections were immersed in 0.001 M sodium citrate buffer at pH 6.0 and submitted to microwave oven treatment for 30 minutes at 96-98°C. After cooling, the endogenous peroxidase was blocked with 5% hydrogen peroxide and the sections were incubated with 2% bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO, USA) during 20 minutes. Subsequently, the sections were incubated overnight in a humidified chamber at 4°C in the following primary antibodies: 1) mouse monoclonal anti-IL-10 antibody (Santa Cruz Biotechnology, Inc. CA, USA, code sc-8438), diluted 1:100 or rabbit monoclonal anti-Ki-67 antibody (Spring Bioscience Corp., Pleasanton, CA, USA, code ab-16667) diluted at 1:100. After washing in Tris-HCl buffer, the immunoreaction was amplified using a Labelled StreptAvidin-Biotin Kit (Universal Dako LSAB, Dako Inc., Carpinteria, CA, USA). Peroxidase activity was revealed by 3,3'-diaminobenzidine (ImmPACT™ DAB Substrate, Vector Laboratories, Burlingame, CA, USA) for 3 min. After washing in tap water, the sections were counterstained with haematoxylin. For the sections used as negative controls, the step of incubation in primary antibodies was replaced by incubation in mouse or rabbit non-immune serum.

The number of Ki-67- and IL-10-immunolabelled cells (brown-yellow color) was counted in a standardized field (0.009 mm²) at x695 magnification using an image analysis system (Image-Pro Express Olympus). The number of Ki-67- and IL-10-immunolabelled cells per mm² was obtained for each animal (n=5 per group/period).

- Content of collagen in the capsules

In each specimen, three non-serial sections were stained with 0.1% picrosirius-red solution and analyzed under polarized light (BX51, Olympus, Tokyo, Japan) and the amount the birefringent collagen was measured (Silva *et al.* 2017, Saraiva *et al.* 2018, Hoshino *et al.* 2020). In each section, a standardized area (0.09mm²) of the

capsule was obtained at x695 magnification. Thus, a total area of 0.27mm² was evaluated per implant. The amount of birefringent collagen was computed using an image analysis software (ImageJ®, <http://rsbweb.nih.gov/ij/>). To estimate the birefringent collagen was considered in the histogram the following hue definitions: red/orange (2-23 and 230-256), yellow (39-51) and green (52-128), as described by Koshimizu *et al.* (2013) and de Pizzol-Júnior *et al.* (2018). Thus, the amount of birefringent collagen was calculated and expressed as percentage of the number of pixels occupied by collagen from total pixels of the image (Silva *et al.* 2017, de Pizzol-Júnior *et al.* 2018, Hoshino *et al.* 2021).

Statistical analysis

Differences among the groups in each period and the differences of each group over time were evaluated by two-way ANOVA analysis followed by the Tukey post-test (GraphPad Prism 5.0 software, San Diego, CA, USA) at significance level of $p \leq 0.05$. All data were presented as mean and standard deviation.

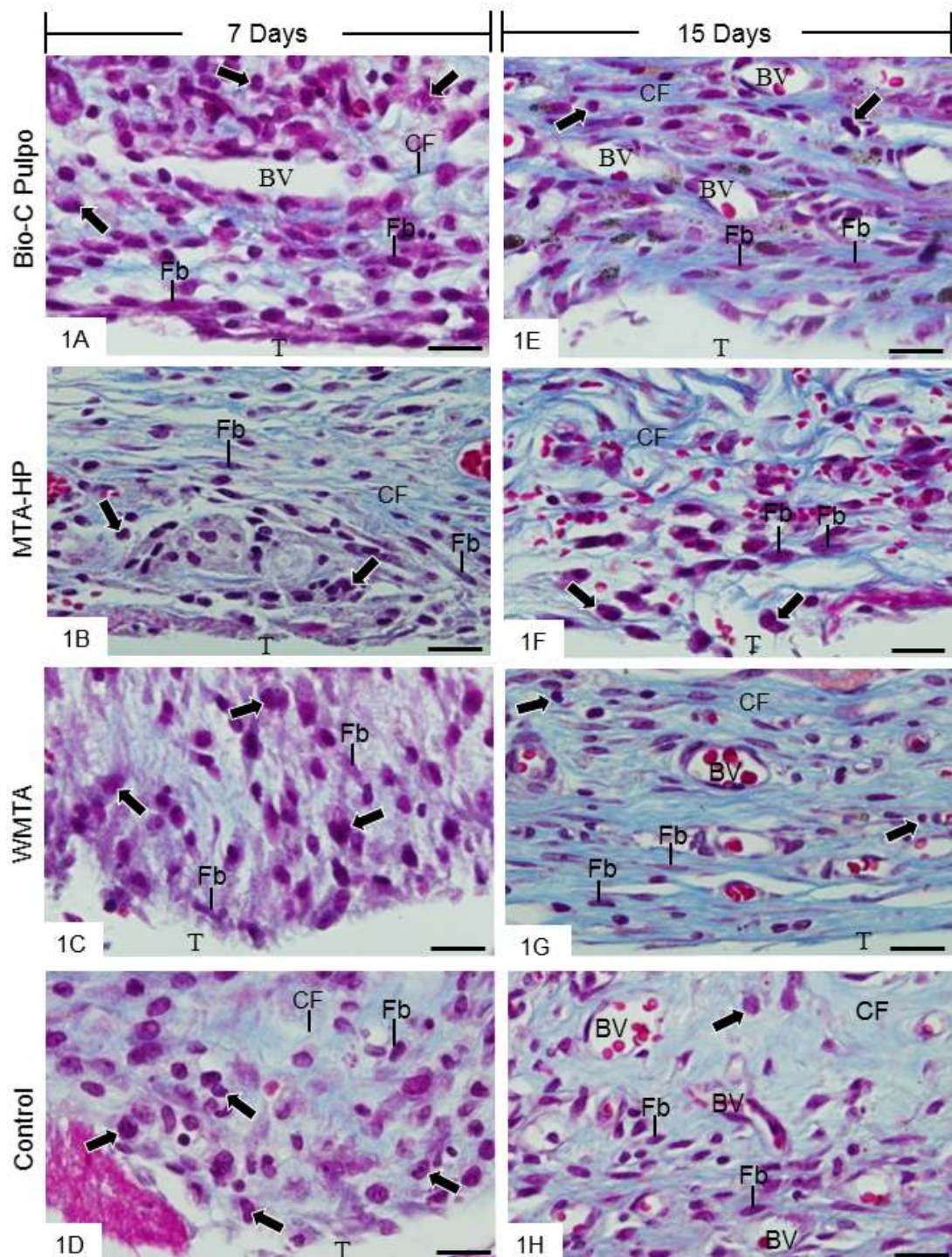
Results

- Histological features and numerical density of fibroblasts

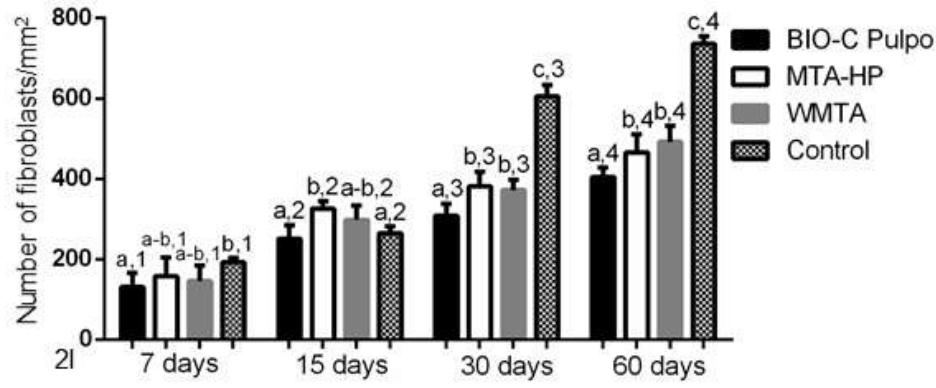
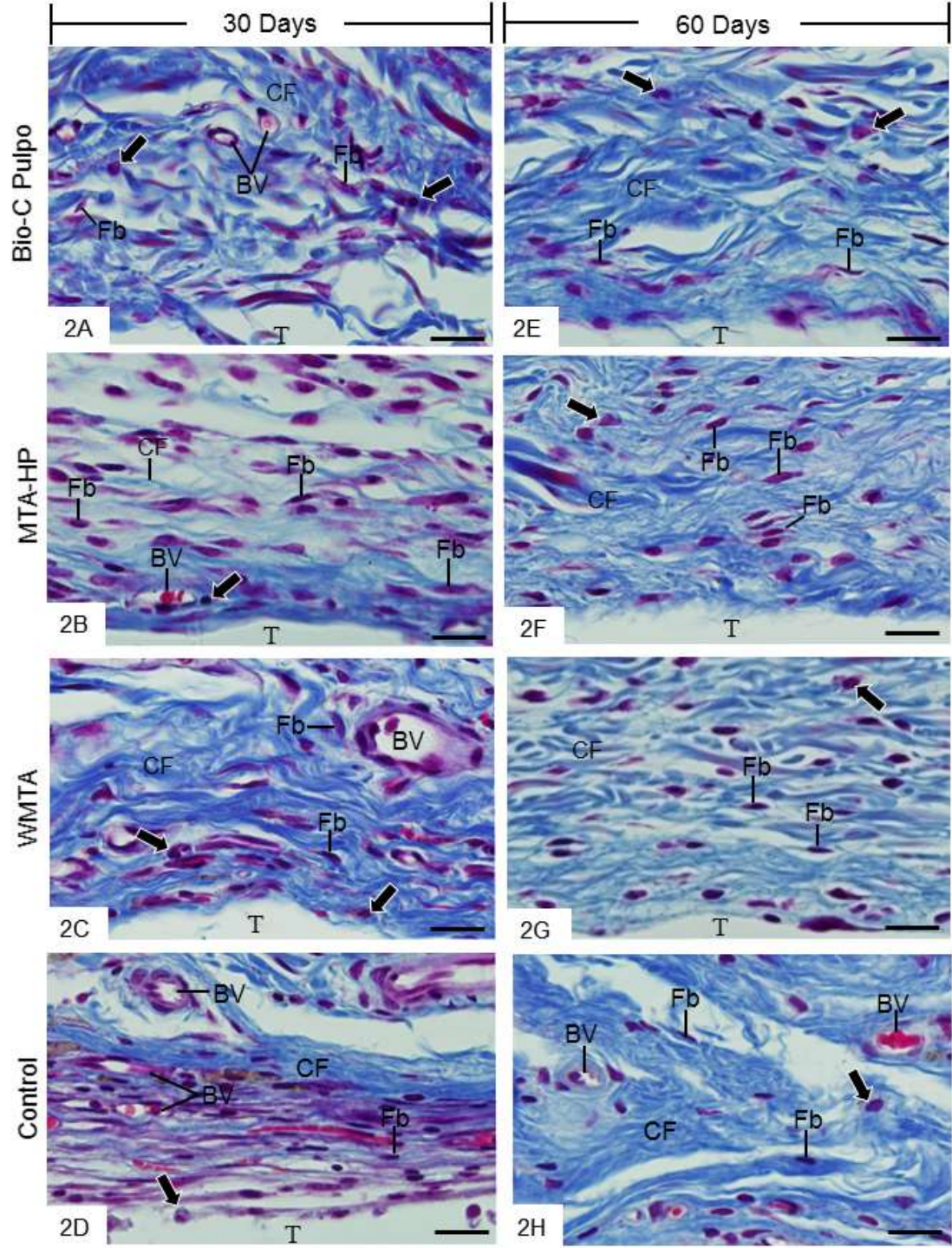
The analysis of sections stained with Masson's trichrome showed evident changes in the cellular population and extracellular matrix components from capsules around implants over time. After 7 days, the capsules contained mainly inflammatory cells among few fibroblasts, identified by the fusiform/elongated shape, which were surrounded by scarce fibrous components (Figs. 1A-1D). At 15 days, thin collagen fibres (in blue colour) and several blood vessels profiles were seen in all groups (Fig. 1E-1H). At 30 and 60 days, the capsules showed a markedly presence of fibroblasts and bundles of collagen fibres, stained in blue (Figs. 2A-2H). At 60 days (Figs. 2E-2H), the capsules exhibited mainly fibroblasts intermingled with an enhanced content of thick collagen fibres bundles compared to those at 7 days (Figs. 1A-1D).

Quantitative analysis (Fig. 2I) showed that the lowest values of fibroblasts were found in the capsules of all groups at 7 days. In all groups, a significant and gradual increase in the number of fibroblasts was observed over time. At 7 days, no significant difference was detected among Bio-C Pulpo, MTA-HP and WMTA as well as among MTA-HP, WMTA and CG specimens. However, the number of fibroblasts was

significantly lower in the Bio-C Pulpo than in CG specimens ($p=0.0165$). After 15 days, significant differences in the number of fibroblasts were not observed among Bio-C Pulpo, WMTA and CG specimens. At 30 and 60 days, the numerical density of fibroblasts was significantly lower in the Bio-C Pulpo specimens in comparison with other groups, while no significant difference was found between the MTA-HP and WMTA specimens ($p\geq 0.571$). On 30th and 60th days, the greatest values of fibroblasts were observed in the capsules of CG specimens ($p<0.0001$). In all periods, no significant difference was detected between the MTA-HP and WMTA groups.



Figures 1A-1H - Light micrographs showing portions of capsule from sections stained with Masson's trichrome. Bars: 40 μm. **Figs. 1A-1D** - general view of capsules around the opening of the polyethylene tubes after 7 days and 15 days (**Figs. 1E-1H**) of implantation in the subcutaneous tissues. **Figs. 1A-1D** - capsules adjacent to the opening of the polyethylene tubes (T) at 7 days. Note the evident presence of inflammatory cells (arrows) and few fibroblasts (Fb) and, scarce and delicate collagen fibers (CF), in blue colour, along the entire length of the capsules. **Figs. 1E-1H** - capsules adjacent to the opening of the polyethylene tubes (T) at 15 days. The capsules exhibit a large amount of collagen fibers (CF) among several fibroblasts (Fb); inflammatory cells (arrows) are dispersed by the capsules. BV, blood vessel. Bars: 40 μm.



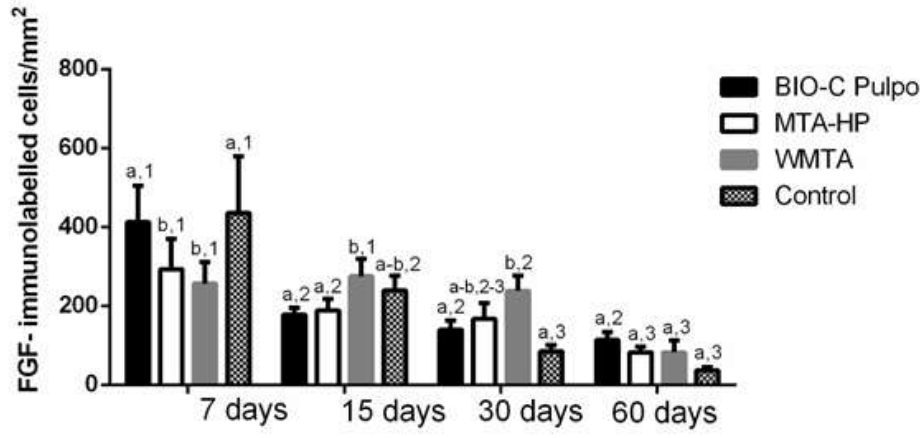
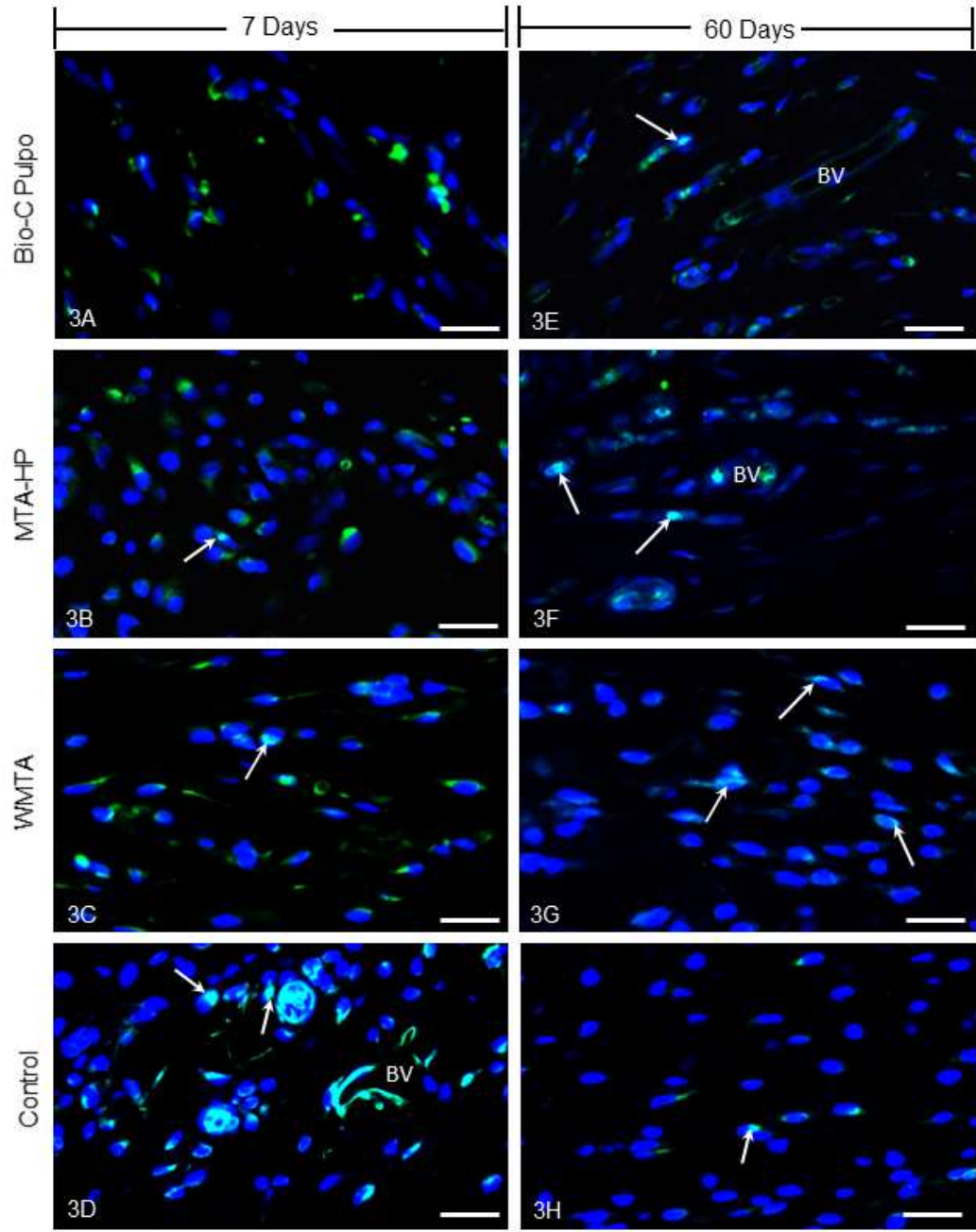
Figs. 2A-2H - Light micrographs showing portions of capsule adjacent to the opening of the polyethylene tubes (T) at 30 (Figs. 2A-2D) and 60 days (Figs. 2E-2H). The capsules exhibit a large amount of collagen fibers (CF) among several fibroblasts (Fb); inflammatory cells (arrows) are dispersed by the capsules. BV, blood vessel. Bars: 40 μ m.

Figure 2I – Numerical density of fibroblasts (mean $\pm$ standard deviation) in Bio-C Pulpo, MTA-HP, WMTA and CG groups at 7, 15, 30 and 60 days. Superscript letters indicate the comparison among the groups; superscript different letters indicate significant difference. Superscript numbers indicate the analysis of each group over time; different numbers indicate significant difference. Tukey's test ($p\leq 0.05$).

- Immunofluorescence for detection of FGF-1

FGF-1 immunofluorescence (green colour) was observed in the cytoplasm and/or nuclei of several cells in the capsules of all groups at 7 days (Figs. 3A-3D). In contrast, few cells showed FGF-1 immunoreactivity in the connective tissue around all groups after 60 days of implantation (Figs. 3E-3H). Immunolabelled cells were not seen in negative controls (data not shown).

The quantitative analysis (Fig. 3I) revealed a significant reduction in the number of FGF-1-immunolabelled cells from 7 to 60 days ($p < 0.0001$), in all specimens. In all periods, no significant difference was detected between Bio-C Pulpo and CG specimens ($p \geq 0.128$) as well as between MTA-HP and WMTA ($p \geq 0.071$). At 60 days, significant differences were not found among the groups ($p \geq 0.128$).



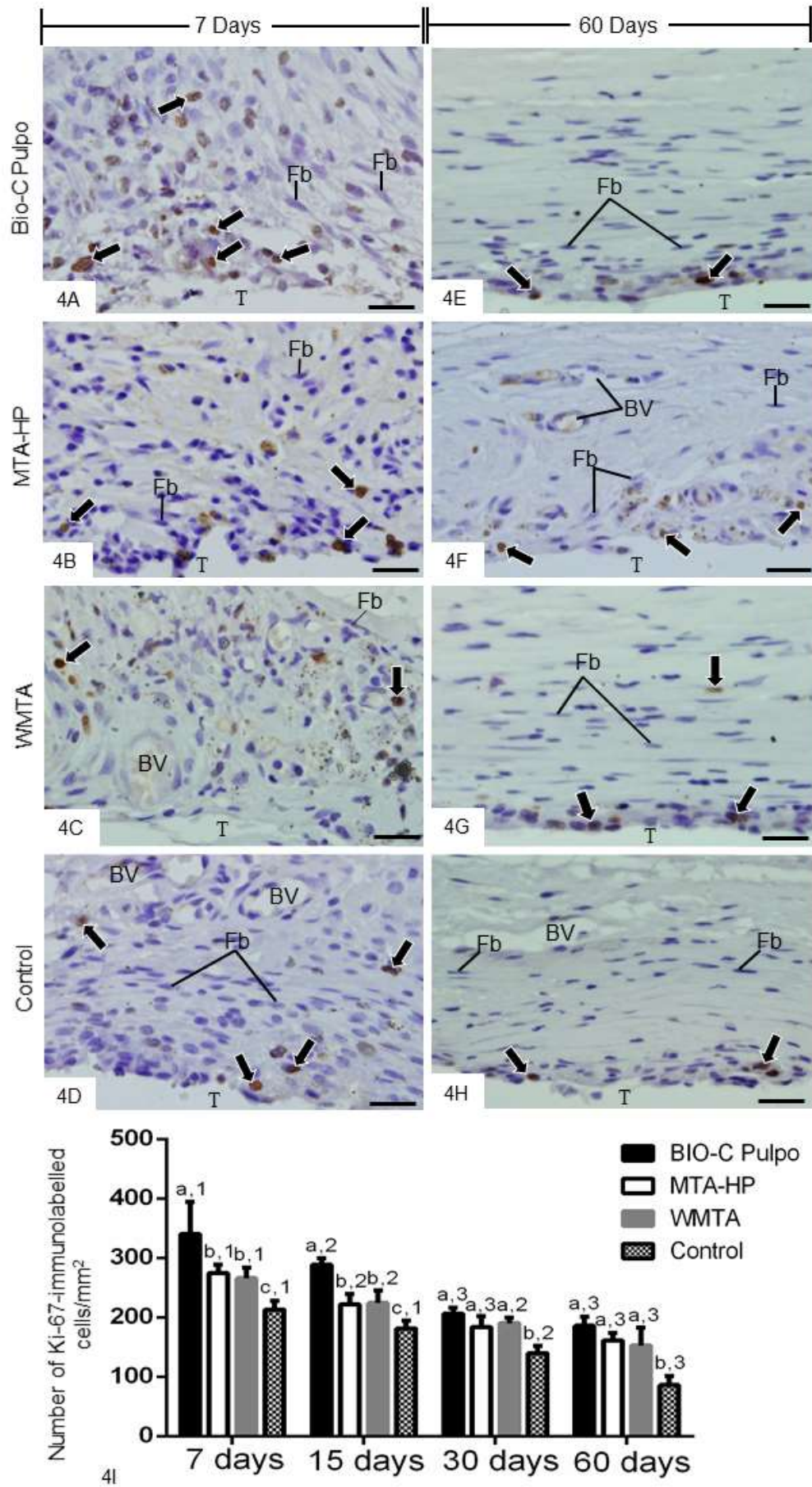
Figures 3A-3H - Light micrographs of sections showing portions of capsule subjected to immunofluorescence reaction for detection of FGF-1 (green colour) and nuclear staining with DAPI (blue colour). Cells with immunofluorescence in their cytoplasm (green colour) and other cells exhibiting FGF-1-immunolabelling in their nucleus (blue + green combined colours; arrows) are observed in the capsules. BV, blood vessel. Bars: 40 μ m.

Figure 3I – Numerical density of FGF-1-immunolabelled cells (mean $\pm$ standard deviation) in Bio-C Pulpo, MTA-HP, WMTA and CG groups at 7, 15, 30 and 60 days. Superscript letters indicate the comparison among the groups; superscript different letters indicate significant difference. Superscript numbers indicate the analysis of each group over time; different numbers indicate significant difference. Tukey's test ($p\leq 0.05$).

- Immunoexpression for Ki-67

At 7 days (Figs. 4A-4D), sections of all groups exhibited Ki-67 immunolabelling in some cells with ovoid/elliptical nucleus (brown-yellow colour). In all groups, an evident reduction in the immunoexpression of Ki-67 was observed in the capsules at 60 days (Figs. 4E-4H). In the negative controls, there was no immunolabelling in the capsules (data not shown).

As shown in 4I, the number of Ki-67-immunolabelled cells was significantly greater in the capsules of Bio-C Pulpo, MTA-HP and WMTA than in CG specimens in all time points. At 7 and 15 days, the highest values of Ki-67-immunolabelled cells were observed in the capsules of Bio-C Pulpo ($p < 0.001$) while no significant difference was found between MTA-HP and WMTA ($p > 0.05$). After 30 and 60 days, no significant difference in the immunoexpression of Ki-67 among Bio-C Pulpo, MTA-HP and WMTA groups was seen. A significant reduction in the number of immunolabelled cells was verified in all groups over time.



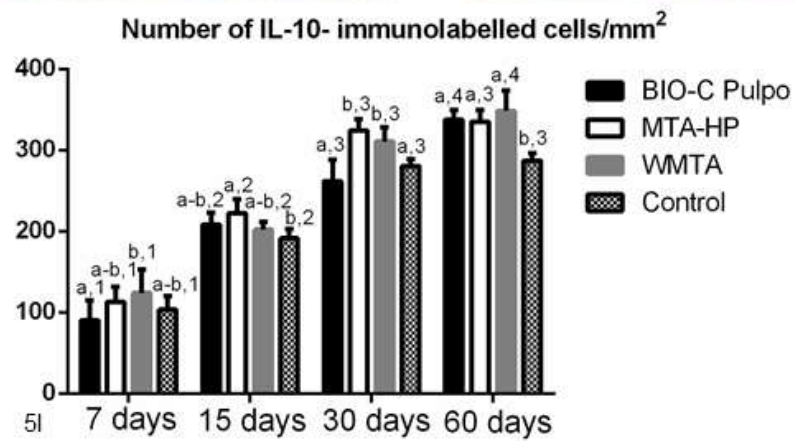
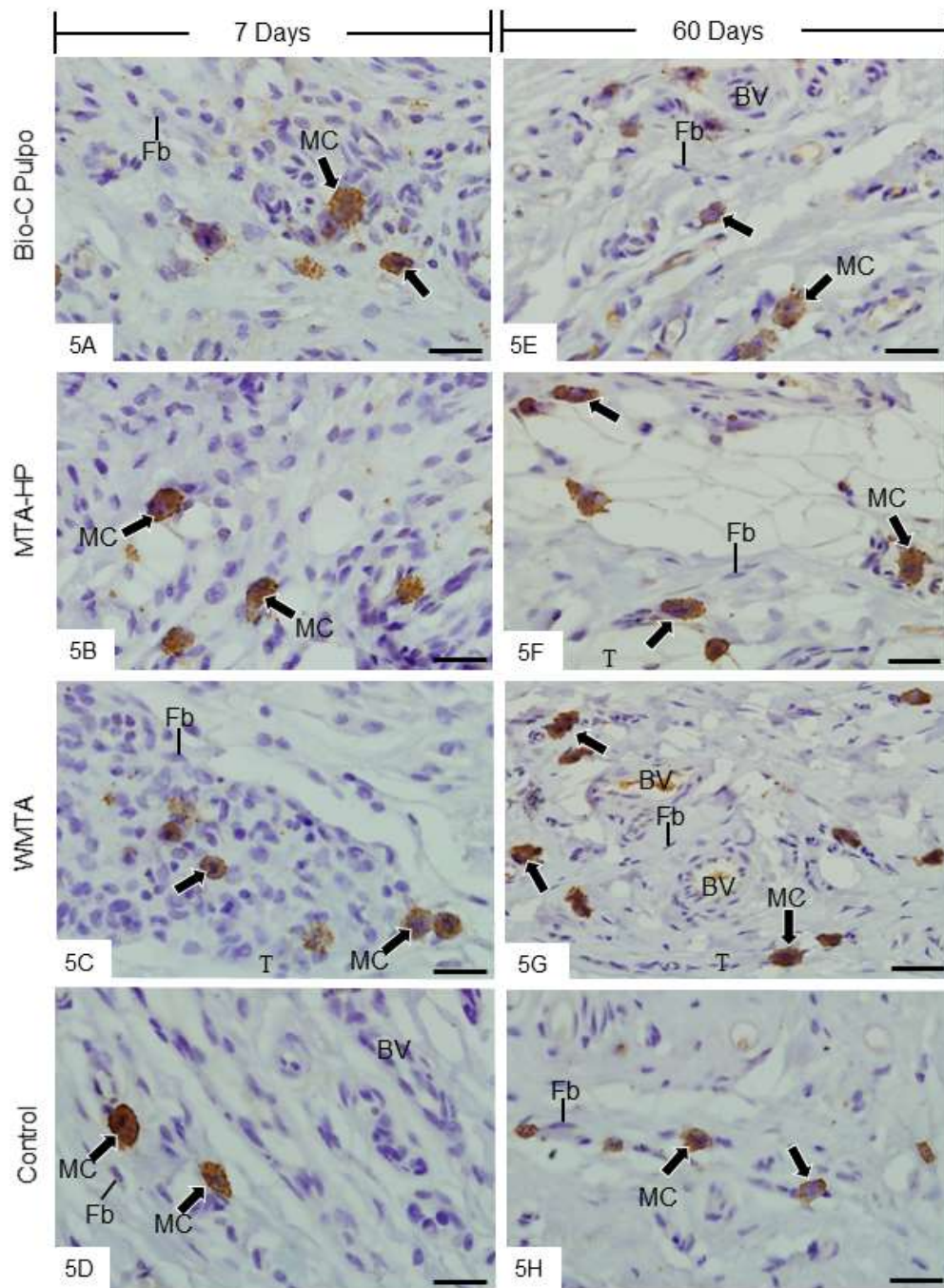
Figures 4A-4H - Light micrographs of sections showing portions of capsule after 7 days (**Figs. 4A-4D**) and 60 days (**Figs. 4E-4H**) of implantation in the subcutaneous tissues. The sections were subjected to immunohistochemistry for detection of Ki-67 and counterstained by haematoxylin. Immunolabelled cells (arrows); space of polyethylene tubes (T); fibroblasts (Fb). Bars: 40 μ m.

Figure 4I – Numerical density of Ki-67-immunostained cells (mean $\pm$ standard deviation) in Bio-C Pulpo, MTA-HP, WMTA and CG groups at 7, 15, 30 and 60 days. Superscript letters indicate the comparison among the groups; superscript different letters indicate significant difference. Superscript numbers indicate the analysis of each group over time; different numbers indicate significant difference. Tukey's test ($p\leq 0.05$).

- Immunoexpression of IL-10

Sections subjected to immunohistochemistry for detection of IL-10 showed positive immunolabelling in the cytoplasm (brown/yellow colour) of some cells, mainly mast cells, in the capsules around all implants (Figs. 5A-5H). In all groups, few immunolabelled cells were present in the capsules at 7 days whereas, at 60 days, an enhanced immunoexpression was found (Figs. 5E-5H). In the sections used as negative control, no immunolabelled cell was seen (data not shown).

The quantitative analysis (Fig. 5I) revealed that the lowest values of IL-10-immunolabelled cells were observed in all groups at 7 days. At 7 days, the number of IL-10-immunolabelled cells in the Bio-C Pulpo specimens was significantly lower than in WMTA specimens ($p=0.0235$) while no significant difference between Bio-C Pulpo and MTA-HP, and Bio-C Pulpo with CG specimens was verified. On 15th day, the capsules around MTA-HP had greater number of immunolabelled cells than in CG specimens ($p=0.0443$) whereas there was no significant difference among other groups. After 30 days, the immunoexpression was significantly greater in the MTA-HP and WMTA than in Bio-C Pulpo ($p<0.001$ and $p=0.0003$, respectively) and CG specimens ($p=0.0016$ and $p=0.0463$, respectively). In this period, no significant difference between MTA-HP and WMTA as well as Bio-C Pulpo and CG was found. At 60 days, the number of immunolabelled cells was significantly greater in Bio-C Pulpo, MTA-HP and WMTA groups than in CG specimens ($p<0.0001$). Moreover, no significant difference was verified among Bio-C Pulpo, MTA-HP and WMTA groups at 60 days. In all groups, a significant increase in the immunoexpression of IL-10 was observed in the capsules over time.

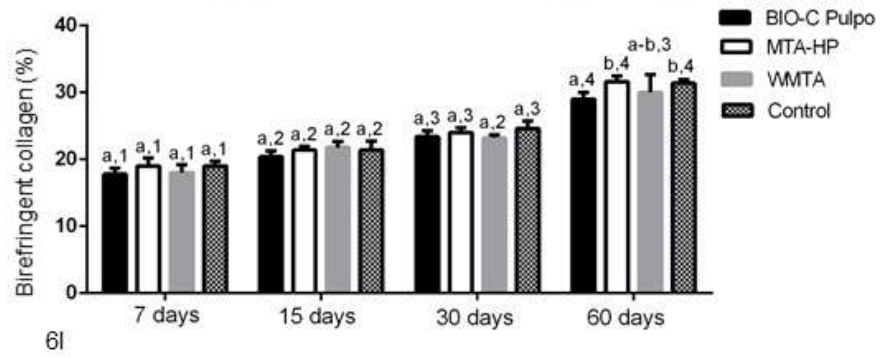
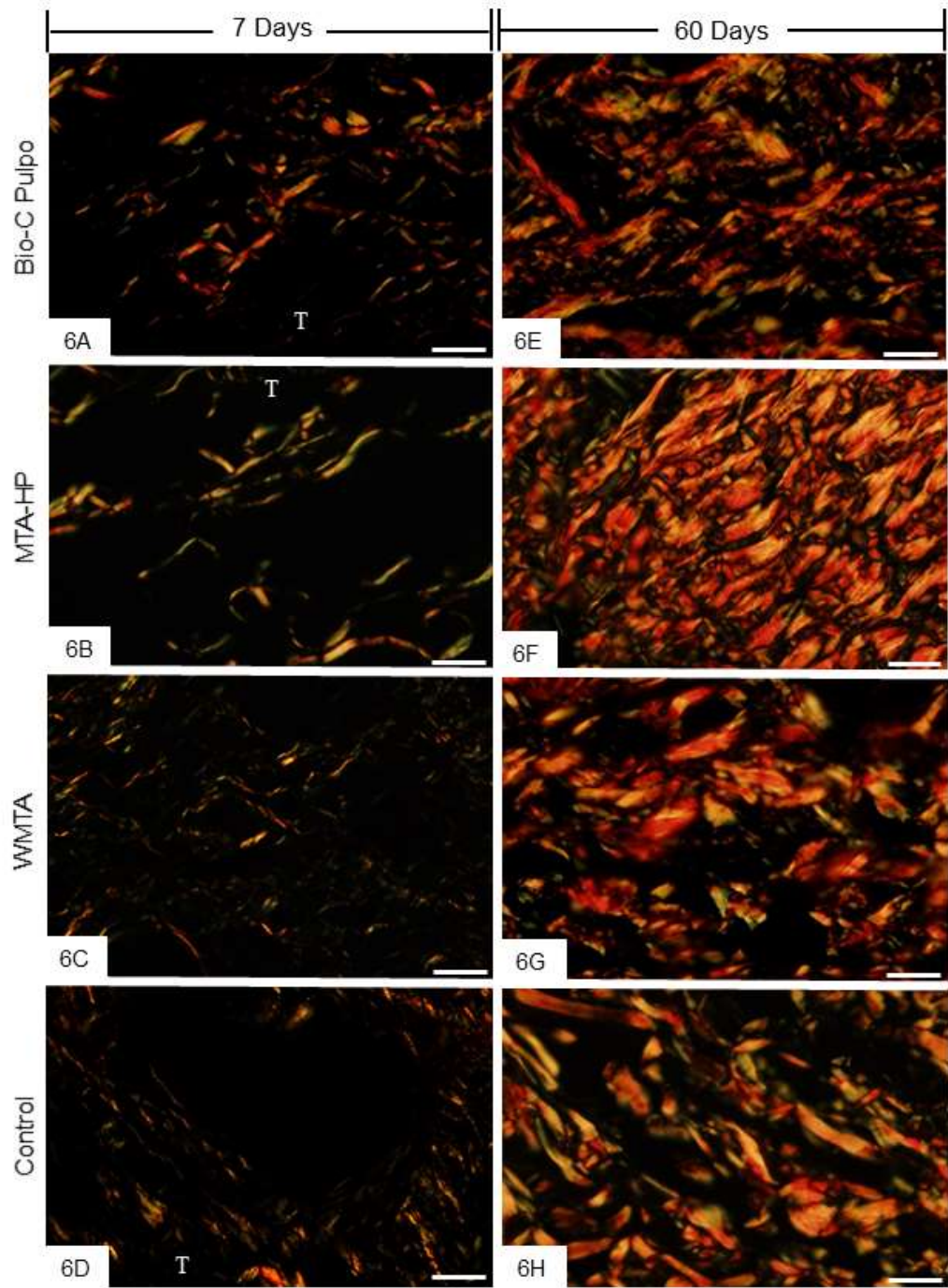


Figures 5A-5H - Light micrographs of sections showing portions of capsule at 7 days (**Figs. 5A-5D**) and 60 days (**Figs. 5E-5H**). The sections were subjected to immunohistochemistry for detection of IL-10 and counterstained by haematoxylin. IL-10-immunolabelled cells (arrows); MC, mast cells; space of polyethylene tubes (T); fibroblasts (Fb). Bars: 40 μ m.

Figure 5I – Numerical density of IL-10-immunostained cells (mean $\pm$ standard deviation) in Bio-C Pulpo, MTA-HP, WMTA and CG groups at 7, 15, 30 and 60 days. Superscript letters indicate the comparison among the groups; superscript different letters indicate significant difference. Superscript numbers indicate the analysis of each group over time; different numbers indicate significant difference. Tukey's test ($p\leq 0.05$).

- Content of collagen in the capsules

The sections submitted to the picrosirius-red and analyzed under a polarized light microscope showed that the capsules contained few birefringent collagen fibres at 7 days (Figs. 6A-6D). In contrast, an enhanced birefringence was observed in all the groups after 60 days (Figs. 6E-6H). According to the quantitative analysis (Fig. 6I), significant differences were not detected among the groups at 7, 15 and 30 days. At 60 days, the amount of birefringent collagen was significantly lower in Bio-C Pulpo than MTA-HP ($p=0.0024$) and CG ($p=0.0059$). However, no significant difference was detected between Bio-C Pulpo and WMTA ($p=0.4887$), and among MTA-HP, WMTA and CG specimens ($p>0.05$). In all groups, a significant increase in the content of birefringent collagen was verified in the capsules over time.



Figures 6A-6H - The sections were stained by picosirius red and analyzed under polarized light microscope. **Figs. 6A-6D** - At 7 days, few birefringent collagen fibers (in red, yellow, green colours) are seen in the capsules. **Figs. 6E-6H** - At 60 days, the capsules exhibit thick birefringent bundles of collagen fibers. T, space of polyethylene tubes. Bars: 40 μ m.

Figure 6I – Percentage of collagen (mean $\pm$ standard deviation) in Bio-C Pulpo, MTA-HP, WMTA and CG groups at 7, 15, 30 and 60 days. Superscript letters indicate the comparison among the groups; superscript different letters indicate significant difference. Superscript numbers indicate the analysis of each group over time; different numbers indicate significant difference. Tukey's test ($p\leq 0.05$).

Discussion

This study evaluated the tissue response induced by Bio-C Pulpo and MTA-HP compared to WMTA focusing on tissue repair. Besides the biocompatibility, a biomaterial may also allow the tissue repair, allowing the fibroblast proliferation and collagen formation (Silva *et al.* 2017, Saraiva *et al.* 2018, da Fonseca *et al.* 2019b, Delfino *et al.* 2020).

Repair materials may induce cell proliferation and differentiation and, consequently, the formation of extracellular matrix components. Here, the capsules around the specimens after 7 days of implantation showed high immunoexpression of FGF-1, a mitogenic and angiogenic factor (Uriel *et al.* 2006, Moya *et al.* 2010). The acidic (FGF-1) and basic (FGF-2) fibroblast growth factors released by macrophages and damaged endothelial cells stimulate the endothelial cell proliferation culminating in the formation of new blood vessels in the injured area. Thus, this factor may participate in the establishment of granulation tissue, characterized by marked increase in the blood vessels. Otherwise, FGF is also a mitogenic factor that stimulates fibroblast proliferation and migration (Tsirogianni *et al.* 2006). Our findings indicate that FGF-1 may exert a mitogenic role in response to the implants into subcutaneous since the highest values of ki-67-immunolabelled cells were also found in the capsules of all specimens at 7 days. It is well known that Ki-67 is a marker of cell proliferation (Penault-Llorca & Radosevich-Robin 2017, Fonseca *et al.* 2019b) and, therefore, the incidence of this protein reflects in the increase of fibroblasts, as observed in the specimens over time. It is important to emphasize that, in all periods, the capsules around the materials implanted contained a greater Ki-67 immunoexpression than in the CG specimens, indicating that bioceramic materials may induce cell proliferation.

In fact, the significant increase in the number of fibroblasts detected in the capsules around the all specimens over time, point to proliferative activity of resident fibroblasts and mesenchymal cells from connective tissue of subcutaneous. Here, the significant increase in the number of fibroblasts was accompanied by increase in the content of collagen observed in Masson' trichrome and under polarized illumination in the Bio-C Pulpo, MTA-HP and WMTA samples as well as around the CG specimens, indicating that the bioceramic materials provide favorable conditions for remodelling process and tissue repair. The accentuated Ki-67 immunoexpression accompanied with an increase in the number of fibroblasts in the capsules around WMTA and Biodentine was also associated with regression of inflammatory reaction initially

induced by materials and formation of fibrous capsule (da Fonseca *et al.* 2019b). In the present study, the reduction in the number of FGF-1 and Ki-67-immunolabelled cells was accompanied by an increase in the number of fibroblasts and in the amount of collagen in the capsules of all the groups over time. These findings indicate that after the proliferative activity, there is a balance in the fibroblast population and, consequently, this activity reduces to reach a physiological index. Our results reinforce this idea since a significant reduction in the Ki-67 index was observed in all groups, including the CG, over time.

Furthermore, the accentuated immunoexpression of IL-10 in the capsules around Bio-C Pulpo, MTA-HP and WMTA supports the concept that bioceramic materials promote the connective tissue repair, since IL-10 has potent anti-inflammatory effects and exerts a control in the inflammatory and immune responses (Moore *et al.* 2001, Iyer & Cheng 2012). This cytokine inhibits the nuclear factor-kappa B (NF- κ B), resulting in the reduction of pro-inflammatory cytokines such as interleukin-1 beta (IL-1 β), IL-6 and tumor necrosis factor α (Martinho *et al.* 2014) and, consequently, lead to a decrease in the inflammatory infiltrate. Several cells including macrophages, T and B cells and mast cells (Moore *et al.* 2001) produce the IL-10. Here, a marked immunostaining in mast cells was observed in the capsules of all groups. Among the actions of IL-10, this interleukin seems to modulate the production of inflammatory cytokines by mast cells via IL-10 receptor (Blank *et al.* 2014, Mukai *et al.* 2018), exerting an essential role in the tissue repair and remodelling (Blank *et al.* 2014, Mukai *et al.* 2018). High incidence of mast cells in capsules around Biodentine was associated with proliferation of fibroblasts and collagen formation, pointing to the participation of mast cells in the remodelling of connective tissue (da Fonseca *et al.* 2019b). Considering that, in all time points, the immunoexpression of IL-10 was significantly greater in the capsules around the bioceramic materials than in CG specimens, it is conceivable to suggest that these materials may stimulate the IL-10 production by connective tissue cells, favoring the regression of inflammatory infiltrate and connective tissue repair.

The main difference in the composition of the MTA-HP in relation to the WMTA is the change in the liquid, where a plasticizer was added in the distilled water to facilitate the manipulation and insertion of the material. In relation to Bio-C Pulpo, this new material has a different composition compared with MTA, containing zirconium oxide, calcium fluoride, silicone dioxide, iron oxide and, in the liquid, the presence of

calcium chloride and methylparaben, in addition to water distilled and the plasticizer. There is evidence that Bio-C Pulpo is a biocompatible material and has bioactive potential (Delfino *et al.* 2021). In the present study, the immunohistochemical findings indicate that Bio-C Pulpo enables the release of FGF-1 and IL-10, involved in the complex cascade of molecular events of tissue repair, in a manner similar to that observed in the CG specimens.

Thus, the significant increase in the immunoexpression of IL-10 and in the number of fibroblasts associated with accentuated content of collagen fibres in the capsules indicate that these bioceramic materials lead to a complex cascade of cellular events, which allow the proliferation of cells and formation of matrix extracellular component involved in the repair connective tissue. These findings, therefore, indicate that the changes made in the Bio-C Pulpo and MTA-HP in comparison to predecessor WMTA did not interfere in the suitable biological properties provide by tricalcium silicate-based material.

Conclusion

Bio-C Pulpo, MTA-HP and WMTA induced the fibroblast proliferation and formation of collagen-rich capsules. These bioceramic materials stimulate the IL-10 secretion, which may exert an anti-inflammatory activity in the capsules around the implants in the subcutaneous. Thus, our results taken together indicate that Bio-C Pulpo and MTA-HP, similarly to the WMTA, allow the formation of collagen-rich capsules indicating that these bioceramic materials induce the connective tissue repair.

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Conflict of interest

The authors deny any conflict of interests to this study.

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4 CONCLUSÃO

- Bio-C Pulpo, MTA Repair HP e MTA Branco não apresentaram efeito prejudicial na integridade estrutural do fígado e nos níveis séricos de TGO e TGP, sugerindo que esses materiais não promovem alterações sistêmicas.
- A redução significativa do processo inflamatório acompanhada pela formação de fibras colágenas nas cápsulas do Bio-C Pulpo, MTA-HP e MTA-B indica que esses cimentos são biocompatíveis.
- A redução do número de células imunopositivas à IL-6 e o aumento do número de células imunopositivas à IL-10 indica que estes cimentos permitem a formação de delgadas cápsulas fibrosas. Assim, estes cimentos devem possibilitar a regeneração e/ou reparação dos tecidos conjuntivos.
- A presença de células imunopositivas à fosfatase alcalina e a presença de cristais de calcita, indicam que os materiais apresentam potencial bioativo.

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¹ * De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: <http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf>

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APÊNDICE A

Publicação 1

Material e Método

- Animais

O protocolo da presente pesquisa foi analisado e aprovado pelo Comitê de Ética no Uso de Animais (CEUA) da Faculdade de Odontologia de Araraquara – FOAr/UNESP (Processo nº 02/2019 – ANEXO A).

Foram utilizados 80 ratos Holtzman (*Rattus norvegicus albinus*) adultos jovens, machos, pesando em média 220 gramas, provenientes do biotério de Criação da Faculdade de Odontologia do Campus de Araraquara (FOAr-UNESP). Os animais foram mantidos no Biotério do Subsolo do Prédio de Biomorfologias da FOAr, com temperatura ($23\pm 2^{\circ}\text{C}$) e umidade ($55\pm 10\%$) controladas e fotoperíodo de 12 horas. Os animais foram mantidos em gaiolas de polietileno forradas com maravalha e receberam ração granulada (Nutrilabor; Guabi, Campinas, SP, Brasil) e água *ad libitum*. A maravalha das gaiolas foi trocada, no mínimo, três vezes por semana; a limpeza das gaiolas e bebedouro também foi feita, a fim de manter a higiene do seu ambiente, evitando dessa forma desconforto aos animais.

Os animais foram distribuídos equitativamente em quatro grupos (20 animais de cada grupo), de acordo com os materiais implantados no subcutâneo: Bio-C (Grupo Bio-C Pulpo; Angelus, Londrina, PR, Brasil; Figura. 1^a), MTA-HP (Grupo MTA Repair HP; Angelus, Londrina, PR, Brasil; Figura. 1B), MTA-B (Grupo MTA Branco; Angelus, Londrina, PR, Brasil; Figura. 1C) e GC (Grupo Controle – tubo de polietileno vazio). A composição e procedência dos materiais, bem como a manipulação dos materiais estão descritas no Quadro A1.

Quadro A1 – Nome do material e procedência comercial, composição e forma de manipulação dos materiais

MATERIAL/ PROCEDÊNCIA	COMPOSIÇÃO		FORMA DE MANIPULAÇÃO
Bio-C Pulpo – Angelus Indústria de produtos Odontológicos S/A, Londrina – PR – Brasil	Pó: Silicato de cálcio, aluminato de cálcio, hidróxido de cálcio, óxido de zircônio, fluoreto de cálcio, dióxido de silício, óxido de ferro	Líquido: Água destilada, plastificante, cloreto de cálcio, metilparaben o	1 dose de pó para 3 gotas do líquido. Espatular por 60 segundos, atingindo uma consistência de massa condensável
MTA Repair HP – Angelus Indústria de produtos Odontológicos S/A, Londrina – PR – Brasil	Pó: Silicato tricálcico, silicato dicálcico, aluminato tricálcico, óxido de cálcio, tungstato de cálcio	Líquido: Água destilada e plastificante	Dispensar o conteúdo de uma embalagem (0,085g) e 2 gotas do líquido. Espatular por 40 segundos até a completa homogeneização, obtendo um cimento semelhante a uma massa de modelar
MTA Branco – Angelus Indústria de produtos Odontológicos S/A, Londrina – PR – Brasil	Pó: Silicato tricálcico, silicato dicálcico, aluminato tricálcico, óxido de cálcio, tungstato de cálcio	Líquido: Água	Dispensar um sachê de MTA (ou de uma pá dosadora) e 1 gota de água destilada. Espatular por 30 segundos, obtendo um cimento de consistência arenosa

Fonte: Elaboração própria.

Figura A1 – Ilustração dos cimentos biocerâmicos avaliados



Fotos dos cimentos que foram manipulados e implantados no subcutâneo dorsal dos ratos. **Figura 1A** – Bio-C Pulpo (Angelus, Londrina, Brasil). **Figura 1B** – MTA Repair HP (Angelus, Londrina, Brasil). **Figura 1C** – MTA Branco (Angelus, Londrina, Brasil). Fonte elaboração: própria.

- Procedimentos cirúrgicos

Os ratos foram anestesiados com 0,08 ml/100 g de peso corporal de Cloridrato de Cetamina a 10% (Virbac do Brasil Indústria e Comércio Ltda., São Paulo, SP, Brasil), associada a 0,04 ml/100 g de peso corporal de Cloridrato de Xilazina a 2% (União Química Farmacêutica Nacional S/A, São Paulo, SP, Brasil), administrada com seringa e agulha de insulina por via intraperitoneal (Figura. 2A).

Os materiais foram manipulados e inseridos em tubos de polietileno (Embramed Indústria e Comércio Ltda., São Paulo, SP, Brasil) medindo 10 mm de comprimento x 1,5 mm de diâmetro, previamente esterilizados em óxido de etileno¹⁻⁵. Após a tricotomia e assepsia com solução de iodo a 5% da região dorsal do animal, realizou-se uma incisão de 2 cm com uma lâmina de bisturi nº 15, orientando-se na direção craniocaudal (Figuras. 2B e 2C). O tecido foi divulsionado e, posteriormente, os tubos preenchidos com um dos materiais (Bio-C Pulpo; MTA-HP e MTA Branco) e vazios (grupo controle) foram colocados na bolsa criada no subcutâneo dorsal (Figura. 2D). Em cada animal foram implantados 2 tubos do mesmo grupo. Foram realizados 10 implantes para cada material por período experimental. Portanto, a colocação de 2 implantes (do mesmo grupo) em cada animal teve como objetivo obter uma amostra mínima (de 5 implantes/grupo em cada período) sem o aumento no número de animais.

Após a colocação dos tubos de polietileno na bolsa criada no subcutâneo dorsal, o local da incisão foi suturado em pontos simples utilizando fio de seda (Seda

4-0, ETHICON, São José dos Campos, SP-Brasil) (Figura. 2E). Os animais foram, então, colocados em gaiolas, devidamente identificados de acordo com o grupo e o período experimental, sendo observados até a recuperação da anestesia. Para minimizar o desconforto pós-operatório dos animais, foi administrada dipirona sódica, na dosagem de 0,03 mg para cada 100 g de peso corporal nos três primeiros dias após a cirurgia. Os animais foram observados diariamente para a verificar a ingestão de ração e água e o seu comportamento (irritabilidade, postura nas gaiolas dentre outros sinais).

Decorridos os períodos de 7, 15 e 30 dias, os animais foram eutanasiados com sobredose de Cloridrato de Cetamina (Virbac do Brasil Indústria e Comércio Ltda., São Paulo, SP, Brasil) e Cloridrato de Xilazina (União Química Farmacêutica Nacional S/A, São Paulo, SP, Brasil) para a remoção dos implantes com os tecidos circundantes. Os animais do período de 60 dias, foram anestesiados com 0,08 ml/100 g de peso corporal de Cloridrato de Cetamina a 10% (Virbac do Brasil Indústria e Comércio Ltda., São Paulo, SP, Brasil), associada a 0,04 ml/100 g de peso corporal de Cloridrato de Xilazina a 2% (União Química Farmacêutica Nacional S/A, São Paulo, SP, Brasil). Subsequentemente, foi realizada uma incisão no abdome, divulsão da cavidade abdominal até a cavidade torácica e exposição do coração. O sangue foi coletado por punção cardíaca do ventrículo esquerdo com tubo a vácuo (Vacuette, Z soro ativador de coágulo gel; Greiner Bio-One, Americana, SP-Brasil) acoplado a uma agulha múltipla (MedMúltipla, 25x8, 21Gx1; Med Goldman, Manaus, AM-Brasil) conectada a um adaptador BD Vacutainer (Becton Dickinson, Curitiba, PR-Brasil), como ilustrado na Figura 2F. O sangue foi coletado (aproximadamente 5 ml) e mantido na bancada por quinze minutos, antes da centrifugação. Após o sacrifício, fragmentos do fígado (Figura. 2G), 2 fragmentos de lóbulos hepáticos de cada animal, e, subsequentemente, os implantes com os tecidos circundantes foram removidos (Figuras. 2H-2J).

Figura A2 – Sequência dos procedimentos cirúrgicos

Sequência cirúrgica de inserção e remoção dos implantes no subcutâneo e punção do sangue. **Figura 2^a**- administração da anestesia por via intraperitoneal, após contenção do animal. **Figuras 2B – 2C** – Após a anestesia, realizou-se tricotomia (**Figura. 2B**) e com um bisturi foi realizada a incisão no sentido crânio caudal. **Figura D** – Divulsão dos tecidos com uma tesoura de ponto romba e inserção dos tubos. **Figura 2E** – Sutura com fio de seda. As setas indicam o local dos tubos no subcutâneo. **Figuras 2F – 2G** – Após anestesia, tricotomia, antisepsia com solução de iodo a 5%, com uma tesoura realizou-se a incisão no tórax para localização do coração. **Figura 2F** – Punção cardíaca para coleta do sangue. **Figura 2G** – Localização e remoção de um fragmento do fígado para o processamento histológico. **Figuras 2H – 2J** – incisão da pele para localização e remoção do implante e tecidos adjacentes para o processamento histológico. Em **2I**, a seta indica o tubo de polietileno envolvido pela cápsula. A **Figura 2J** ilustra a porção da pele removida contendo o implante no subcutâneo.

Fonte: Elaboração própria.

Processamento do sangue

Após a coleta, os tubos contendo o sangue de cada animal foram mantidos em temperatura ambiente por quinze minutos para formação do coágulo. Em seguida, os tubos foram centrifugados (Excelsa, Guarulhos, SP-Brasil) a 3500 rpm durante 10 minutos para a separação do soro. O soro foi reservado em *eppendorf* e imediatamente armazenadas em gelo, etiquetados e mantido à -20° C nas primeiras 24 horas e após este período, as amostras foram armazenadas à -80° C, até o momento de encaminhamento para análise laboratorial dos níveis séricos de transaminase glutâmico-oxalacética (TGO) e transaminase glutâmico-pirúvica (TGP). A concentração sérica de TGO e TGP foi medida em analisador automático multiparamétrico (Cobas ÍNTEGRA® 400 Plus; Roche Diagnóstica Brasil Ltda., São Paulo, SP, Brasil).

Processamento para microscopia de luz

Os fragmentos dos lóbulos hepáticos e os implantes envolvidos pelos tecidos adjacentes foram fixados em solução de formaldeído a 4% (preparado a partir do paraformaldeído) tamponado com fosfato de sódio 0,1 M com Ph 7,2, durante 48 horas. Após a fixação, as peças foram desidratadas, diafanizadas, embebidas em parafina líquida (60°C) e incluídas em parafina. Os espécimes foram incluídos de maneira a obter cortes longitudinais em relação ao longo eixo dos tubos de polietileno. Com um micrótomo (Leica, modelo HM 325) foram obtidos, a partir dos blocos, cortes com 6 µm de espessura dos lóbulos hepáticos, cortados, no mínimo, 2 fragmentos de lóbulos hepáticos de cada animal. Em relação aos implantes no subcutâneo, foram obtidos cortes longitudinais que foram aderidos a lâminas de vidro e corados com H&E (Hematoxilina de Carazzi e Eosina), von Kossa e cortes não corados foram analisados ao microscópio de polarização. Cortes das cápsulas envolvendo os implantes foram aderidas as lâminas de vidro silanizadas e submetidos as reações imuno-histoquímicas [interleucina-6 (IL-6), interleucina-10 (IL-10), Ki-67, FGF-1 e fosfatase alcalina (ALP)]. Alguns cortes foram submetidos ao método histoquímica de von Kossa e, subsequentemente, submetidos à detecção imuno-histoquímica da ALP.

Para descrição das alterações morfológicas e captura das imagens, foi utilizado o microscópio Olympus (modelo BX-51, Japão) com uma câmera digital acoplada (câmera de captura DP-71, Olympus).

Métodos de Análise

- Análise sistêmica

Análise bioquímica

A partir do soro, em um analisador automático multiparamétrico (Cobas Íntegra, São Paulo, SP-Brasil) foi realizada a quantificação (Laboratório São Lucas, Araraquara, SP-Brasil) dos níveis séricos de transaminase glutâmico-oxalacética (TGO) e transaminase glutâmico-pirúvica (TGP) de cada animal. A taxa de oxidação da nicotinamida adenina dinucleotídeo reduzido (NADH) é diretamente proporcional à atividade catalítica de TGO e TGP e é determinada pela medida da diminuição da absorbância a 320nm. Os resultados da reação para TGO e TGP foram automaticamente convertidos de $\mu\text{kat/L}$ para U/L. A média dos valores dos cinco animais do grupo controle (GC) constituíram os valores de referência de cada parâmetro.

- Análise morfológica e morfométrica da estrutura do fígado

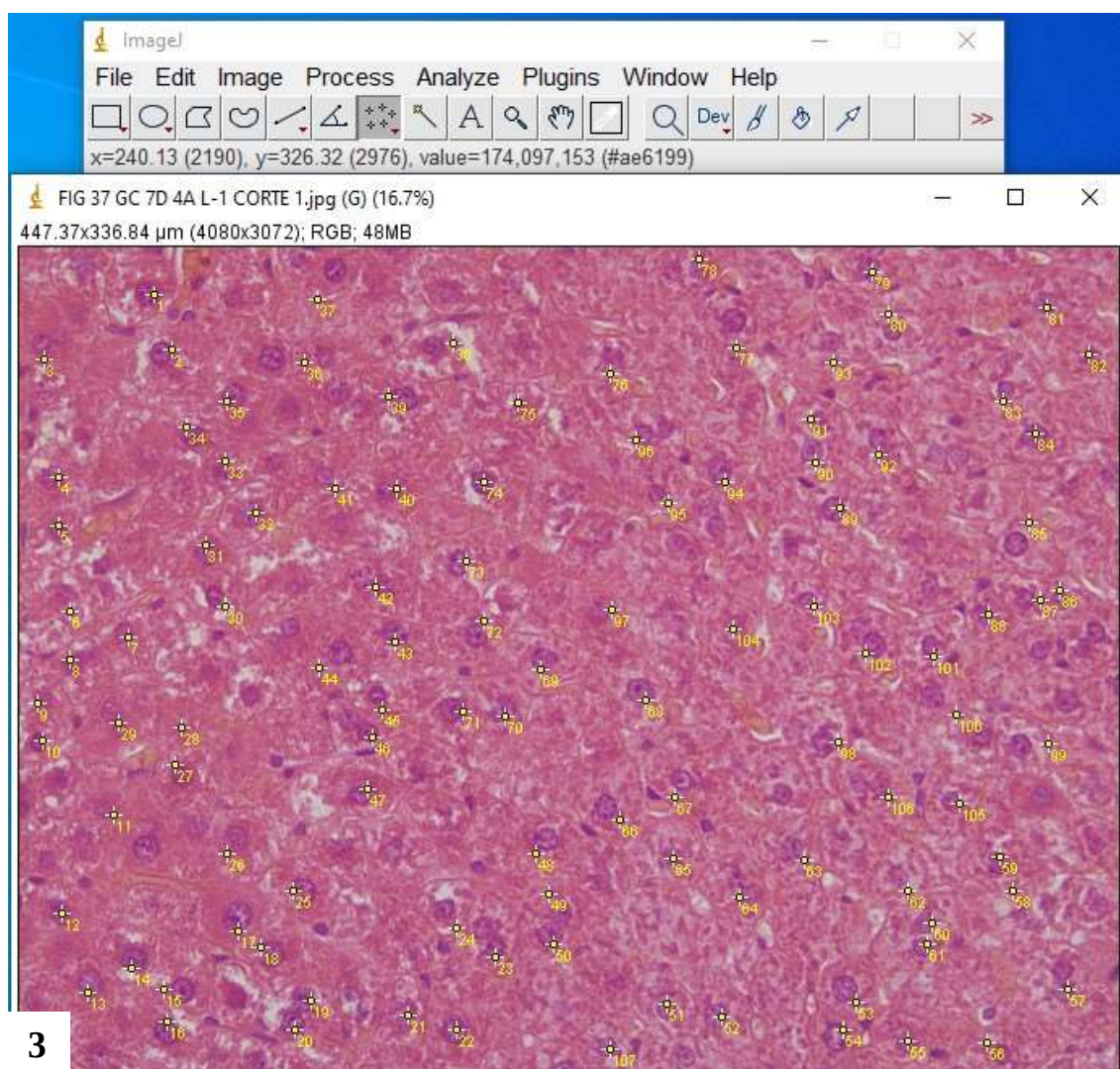
A partir dos cortes corados com HE, foi realizada a descrição morfológica dos fragmentos de lóbulos hepáticos, avaliando as possíveis alterações sistêmicas induzidas pelos cimentos. Foi considerado nesta análise, a integridade estrutural do parênquima e estroma hepático, com atenção também na integridade dos componentes do espaço porta. Foi avaliado a presença de células inflamatórias no estroma, núcleos (se há condensação da cromatina) e presença de vacúolos, pigmentos outras alterações no citoplasma dos hepatócitos.

Densidade numérica de hepatócitos

A fim de avaliar se os materiais biocerâmicos induzem alterações no fígado, foi realizada a análise da densidade numérica de hepatócitos. Esta avaliação foi realizada em 3 cortes não seriados corados com HE, mantendo-se um intervalo mínimo de 100µm entre os cortes. Esta análise quantitativa foi realizada em 3 cortes de cada fragmento dos lóbulos hepáticos, sendo que em cada corte foram capturados dois campos próximos ao espaço porta. Esta análise foi realizada nos cinco animais de cada grupo após 60 dias aos implantes.

Com o auxílio de uma câmera (DP-71, Olympus, Japão) acoplada ao microscópio de luz (Olympus, modelo BX-51), em aumento de 20x, imagens do fragmento do lóbulo hepático foram capturadas. A fim de padronização, os campos foram capturados na região adjacente ao espaço porta (tríade portal). Posteriormente, com um programa de análise de imagens (ImageJ) foi computado o número de núcleos de hepatócitos (Figura. 3). Dessa maneira foi obtido a densidade numérica de hepatócitos dividindo-se o número total de núcleos de hepatócitos pela área total (1,44 mm²).

Figura A3 – Imagem ilustrativa do método quantitativo de hepatócitos.



“Print screen” de uma fotomicrografia mostrando uma porção do corte de fígado capturada com a câmera DP-71 acoplada ao microscópio Olympus. Com o auxílio do programa de análise de imagens (ImageJ), a fotomicrografia foi aberta e foi realizada a contagem dos núcleos de hepatócitos. Note que à medida que o examinador computa o núcleo do hepatócito, este é numerado (contabilizado). Coloração HE.

Fonte: Elaboração própria.

- Reação tecidual dos implantes no subcutâneo

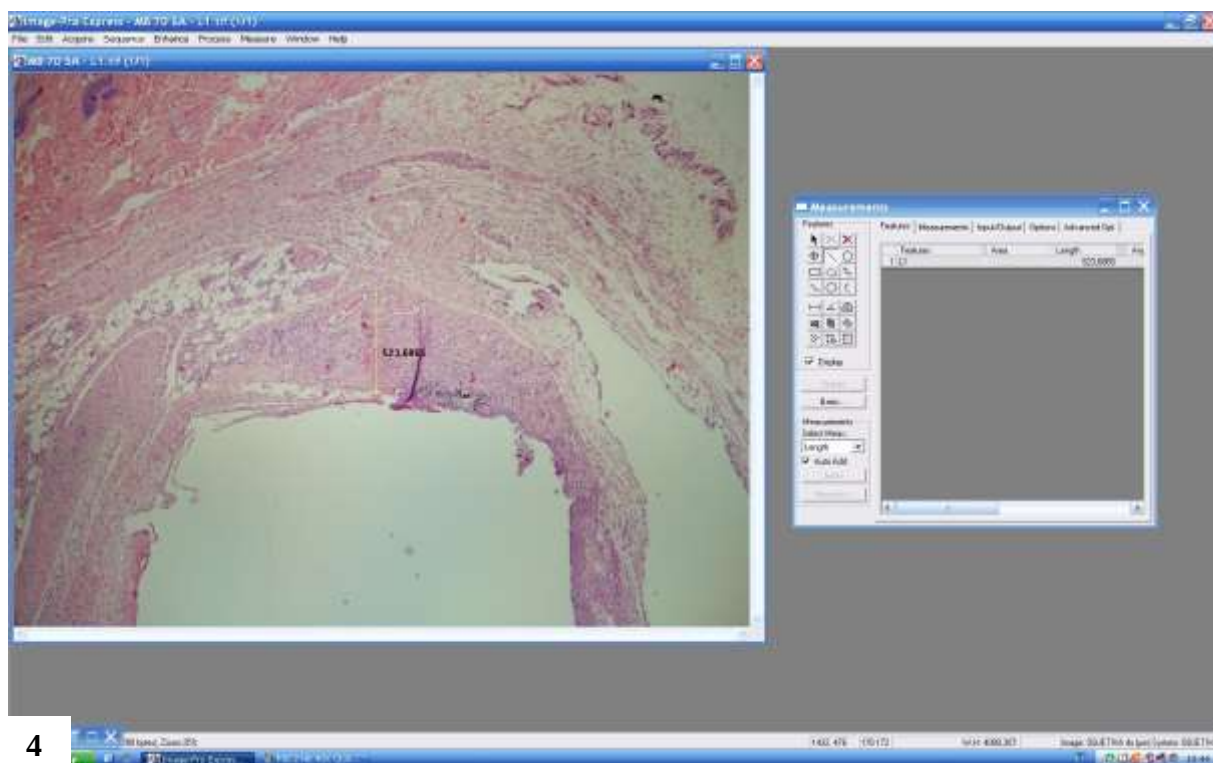
Análise morfológica

A partir dos cortes corados com H&E, foi realizada a descrição morfológica das cápsulas adjacentes aos implantes, ou seja, na abertura dos tubos, avaliando a reação tecidual induzida pelos materiais reparadores. Consideramos nesta análise, a presença ou não do processo inflamatório, bem como a sua extensão; foi considerado também o tipo de células inflamatórias, presença de células gigantes multinucleadas, vasos sanguíneos, fibroblastos e fibras colágenas.

Espessura das cápsulas adjacentes aos implantes

A extensão da reação inflamatória promovida pelos materiais foi avaliada por meio da mensuração da espessura (em μm) das cápsulas adjacentes às extremidades dos tubos implantados. Obteve-se a mensuração a partir de 3 cortes não seriados (intervalo mínimo de 100 μm) de cada espécime, em todos os grupos e períodos ($n=5/\text{grupo}$ em cada período) por um único avaliador calibrado e “cego” ao experimento. A estimativa da espessura das cápsulas foi realizada conforme metodologia padronizada^{2,4,5}. Resumidamente, com a objetiva de 4x (aumento final de 65x) foram capturadas imagens da porção central das cápsulas. Posteriormente, a imagem capturada foi “aberta” no programa de análise de imagens (Image-Pro Express 6.0, Olympus – Japão); usando o programa foi traçada uma linha perpendicular desde à superfície da cápsula até o seu limite com os tecidos adjacentes (Figura. 4). O programa forneceu a medida em micrômetros (μm). O valor da espessura da cápsula em cada amostra foi obtido a partir do valor médio adquirido nas medidas dos três cortes/implante. Ao final, foi calculada a média para cada grupo em cada período. As cápsulas são classificadas como finas quando apresentaram espessura igual ou menor que 150 μm enquanto àquelas com valores superiores são consideradas cápsulas espessas⁶.

Figura A4- Imagem ilustrativa da mensuração da espessura das cápsulas



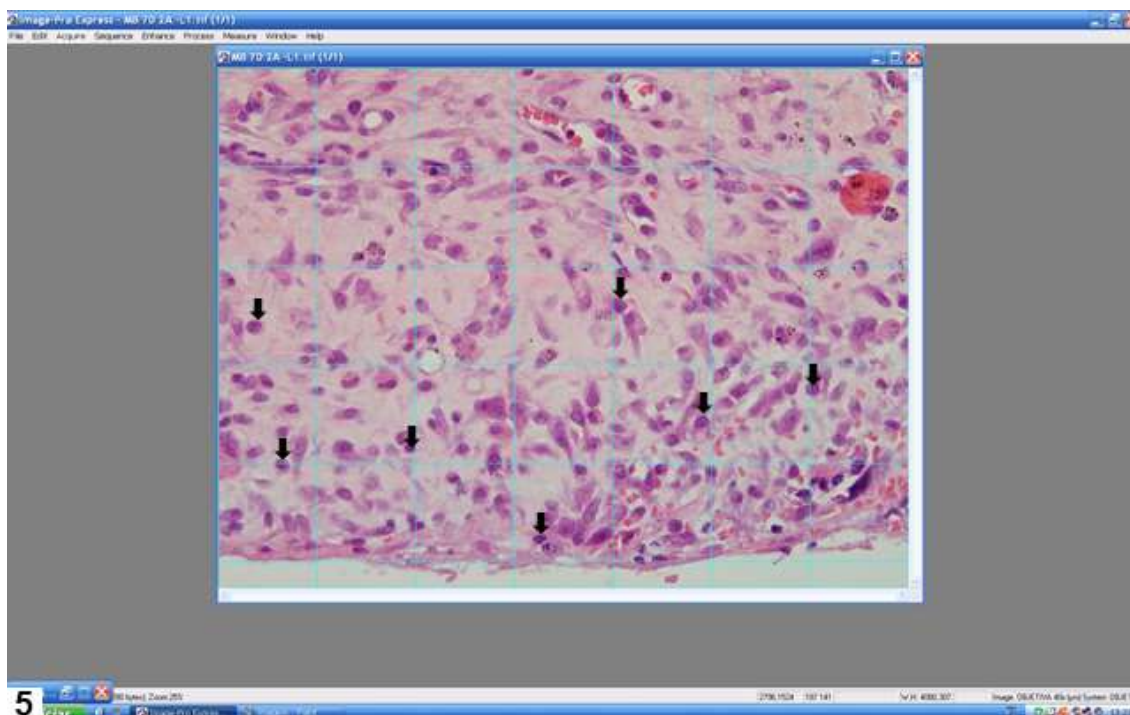
“Print screen” de uma fotomicrografia mostrando uma porção da cápsula e parte dos tecidos adjacentes. Com o auxílio do programa de análise de imagens (Image-Pro Express, Olympus), a fotomicrografia é aberta, realizada a calibração do sistema e, subsequentemente, com uso da ferramenta “measurement”, é traçada uma linha a partir da superfície da cápsula até o limite com os tecidos adjacentes; o programa fornece a extensão da linha traçada que representa a espessura da cápsula (em μm). Coloração HE.

Fonte: Elaboração própria.

Densidade numérica de células inflamatórias

A intensidade da reação inflamatória promovida pelos materiais foi avaliada por meio da obtenção do número de células inflamatórias por mm^2 nas cápsulas adjacentes aos implantes nos diferentes períodos. O número destas células foi estimado a partir de três cortes de cada implante, manteve-se um intervalo mínimo de 100 μm entre os cortes. Com auxílio de uma câmera (DP-71, Olympus – Japão) acoplada ao microscópio de luz (Olympus, modelo BX-51), usando a objetiva de 40x (aumento final de 695x), imagens das cápsulas foram capturadas. As células inflamatórias foram identificadas considerando as características morfológicas tais como células com formato arredondado/ovoide, com núcleo lobulado (neutrófilos e eosinófilos), núcleo arredondado e fortemente basófilo (linfócitos) e núcleos irregulares e volumosos (macrófagos). Posteriormente, com um programa de análise de imagens (Image-Pro Express 6.0, Olympus – Japão), foi realizada a contagem de células inflamatórias (Figura. 5), em cada corte, em uma área teste de aproximadamente 0,09 mm^2 da cápsula. O valor total de células inflamatórias em cada espécime (obtido a partir das áreas dos três cortes/implante) foi dividido pela área total (0,27 mm^2) obtendo, assim, um valor de células inflamatórias/ mm^2 . Ao final, foi calculada a média para cada grupo em cada período. A análise foi realizada por um examinador calibrado e “cego” aos grupos e períodos.

Figura A5- Imagem ilustrativa do método quantitativo.



“Print screen” de uma fotomicrografia mostrando uma porção da cápsula. A imagem, previamente capturada, é aberta no programa de análise de imagens (Image-Pro Express, Olympus). O examinador com o programa marca as células identificadas como células inflamatórias (setas). Note que, após calibração, uma grade reticular contendo 30 intersecções/pontos (sistema teste) é sobreposta à imagem; esta grade é usada somente para auxiliar na contagem, serve como uma “guia” durante a contagem das células inflamatórias. Coloração HE.
Fonte: Elaboração própria.

- Escore da reação inflamatória

O escore da intensidade da reação inflamatória foi mensurado de acordo com o número de células inflamatórias em cada campo (área) da cápsula^{4,6,7}, em aumento de 40x. O escore foi atribuído a cada implante a partir de três cortes não seriados corados com HE, examinados por um avaliador calibrado e “cego” ao experimento. O escore da reação inflamatória foi atribuído de acordo com a seguinte classificação⁶: 0, sem infiltrado inflamatório (nenhuma ou células inflamatórias escassas); 1, reação inflamatória leve (cápsulas contendo até 25 células inflamatórias/campo); 2, reação inflamatória moderada (cápsula contendo de 26 a 125 células inflamatórias/campo); 3, reação inflamatória severa/intensa (cápsula contendo mais de 125 células inflamatórias/campo). A análise foi realizada em cinco animais por grupo e a média de cada grupo foi calculada.

- Reação imuno-histoquímica para detecção de IL-6

A interleucina-6 (IL-6) foi detectada utilizando o anticorpo primário anti-IL-6 produzido em coelho (anti-IL-6, Abcam Inc., Cambridge, MA, USA; código ab-9324). Após desparafinização e hidratação, os cortes foram imersos em tampão citrato de sódio 0,001M (Ph 6,0) e submetidos ao tratamento com micro-ondas, durante 30 minutos a temperatura de 94-98°C. Após o resfriamento e inativação da peroxidase endógena com peróxido de hidrogênio a 5%, os cortes foram incubados com o anticorpo primário anti-IL-6 (titulação 1:200) em câmara úmida a 4° C durante 16-18 horas. Após a lavagem em tampão Tris-HCl, os cortes foram incubados com polímeros conjugados com anticorpos secundários Envision (Envision™ + Dual Link System-HRP, Dako Inc., Carpinteria, CA, USA) por 40 minutos, a temperatura ambiente. Após a lavagem em tampão Tris-HCl, os cortes foram lavados com tampão Tris-HCl e a atividade da peroxidase foi revelada com solução de 3,3-diaminobenzidina (Betazoid DAB Chromogen Kit – Dako Inc., Carpinteria, CA, EUA) durante 3-5 minutos. Após a lavagem em água corrente e água destilada, os cortes foram contracorados com hematoxilina de Carazzi. No controle negativo, o anticorpo primário foi substituído por soro não imune.

- Densidade numérica de células IL-6-imunomarcadas

A fim de verificar se houve diferenças entre os grupos em cada período bem como ao longo do tempo, foi realizada a quantificação de células imunopositivas a IL-6 (em castanho). Com auxílio de uma câmera (DP-71, Olympus – Japão) acoplada ao microscópio de luz (Olympus, modelo BX-51), em aumento de 40x, imagens da cápsula adjacente aos implantes foram capturadas.

Posteriormente, com um programa de análise de imagens (Image-Pro Express 6.0, Olympus – Japão), as células positivas à IL-6- (em castanho) em cada área teste de aproximadamente 0,09mm² da cápsula, foram computadas obtendo-se, assim, um valor de células imunopositivas. A estimativa de células imunopositivas foi realizada nos 5 animais (5 implantes) de cada grupo por período. Assim, o número de células imunopositivas/mm² da cápsula foi obtido para cada animal e posteriormente, foi calculado a média para cada grupo em cada período.

- Potencial bioativo dos materiais

- Reação imuno-histoquímica para detecção de ALP

Para realizar a recuperação antigênica, as lâminas foram imersas em tampão citrato de sódio 0,001 M (Ph 6,0) e mantidas a 96-98 ° C por 30 minutos. Após o resfriamento, a peroxidase endógena foi bloqueada e os cortes foram incubados com 5% de BSA (Sigma-Aldrich Co., St. Louis, MO, EUA). Em seguida, os cortes foram incubados em câmara umidificada a 4°C por 16-18h com anticorpo anti-fosfatase alcalina produzido em coelho (Santa Cruz, CA, EUA; código: sc30203) diluição a 1:400. Após a lavagem, os cortes foram incubados à temperatura ambiente com reagente Super Enhancer™ por 30 minutos, lavadas em tampão e incubadas com anticorpo secundário anti-camundongo e anti-coelho conjugado ao reagente Polímero-HRP (Super Sensitive™ IHC Detection Systems, BioGenex Laboratories Fremont, CA, EUA) por 30 minutos em temperatura ambiente. Posteriormente, a imunorreação foi revelada por 3,3'-diaminobenzidina (substrato DAB, Vector Laboratories, Burlingame, CA, EUA) por 3 min. Os cortes foram contra corados com hematoxilina. Como controles negativos, as seções foram incubadas em soro não imune, substituindo a incubação em anticorpo primário.

O número de células ALP-imunomarcadas (em castanho) foi contado em um campo padronizado (0,09 mm²) a partir de imagens capturadas com a objetiva de 40x (ampliação final de 695x) usando um sistema de análise de imagem (Image-Pro Express Olympus). O número de células imunomarcadas à ALP por mm² foi estimado em todos os animais em cada período (n = 5 por grupo/período).

- Reação histoquímica de von Kossa

O método de von Kossa^{8,9} foi utilizado para avaliar a presença de precipitados de cálcio nas cápsulas adjacentes às extremidades dos tubos implantados. Este método foi realizado nos espécimes de todos os grupos e períodos. Os cortes desparafinizados foram incubados em solução aquosa contendo 5% de nitrato de prata, sob a ação de uma lâmpada incandescente (100 watts), durante 1 hora. Após a incubação, os cortes foram lavados rapidamente em água destilada e, subsequentemente, os cortes foram imersos em 5% de hipossulfito de sódio por 5 minutos. Em seguida, os cortes foram lavados rapidamente em água corrente e durante 5 minutos em água destilada. Após a lavagem, os cortes foram imersos na solução de picrossirius-red durante 1 hora. Subsequentemente, os cortes foram lavados, desidratados e montados em meio resinoso (Permunt[®], Fisher Scientific, New Jersey, USA) para análise ao microscópio de luz.

- Análise de birrefringência

Considerando os cristais de calcita exibem birrefringência quando submetido à luz polarizada¹⁰⁻¹², cortes foram desparafinizados e montados e montados com Permunt. Os cortes sem coloração (não corados) foram analisados sob luz polarizada (Microscópio de polarização, Olympus, BX51).

- Reação histoquímica de von Kossa seguida pela detecção de ALP

Com o propósito de tentar estabelecer uma correlação entre a presença de cálcio e/ou depósitos de cálcio amorfo nas cápsulas, possivelmente depositadas em decorrência da indução dos materiais, alguns cortes foram submetidos ao método de von Kossa e, subsequentemente, foram submetidos ao método imuno-histoquímico

para detecção da fosfatase alcalina, desde que esta enzima é considerada como um marcador de células que secretam os tecidos mineralizados e, portanto, um indicativo de bioatividade de materiais endodônticos^{5,13,14}. Os cortes foram inicialmente submetidos ao von Kossa e, subsequentemente, às etapas para detecção imuno-histoquímica da fosfatase alcalina. Após o tratamento em tampão citrato de sódio 0,001M (Ph 6,0) no micro-ondas, a peroxidase endógena foi inativada com peróxido de hidrogênio a 5% e os cortes foram incubados com o anticorpo primário anti-fosfatase alcalina produzido em coelho (Santa Cruz Biotechnology, USA, titulação 1:400; código ab-9324) em câmara úmida a 4° C durante 16 horas. Após a lavagem em tampão Tris-HCl, os cortes foram incubados com polímeros conjugados com anticorpos secundários Envision (Envision™ + Dual Link System-HRP, Dako Inc., Carpinteria, CA, USA) durante 40 minutos à temperatura ambiente. Subsequentemente, os cortes foram lavados com tampão Tris-HCl, a atividade da peroxidase foi revelada com solução de 3,3-diaminobenzidina (Betazoid DAB Chromogen Kit – Dako Inc., Carpinteria, CA, EUA) durante 3-5 minutos. Após a lavagem em água corrente e água destilada, os cortes foram contracolorados com hematoxilina de Carazzi. No controle negativo, o anticorpo primário foi substituído por soro não imune ao invés do anticorpo primário.

Análise Estatística

Os níveis séricos de TGO e TGP e a densidade numérica dos hepatócitos foram avaliados pela análise One-Way ANOVA seguida pelo pós teste de Sidak. Os dados relativos à biocompatibilidade e potencial bioativo foram avaliados pela análise ANOVA two-way seguida pelo teste de Tukey (software GraphPad Prism 5.0, San Diego, CA, EUA) com nível de significância de $p \leq 0,05$. Os dados do escore da reação inflamatória foram submetidos ao teste não paramétrico ANOVA Kruskal-Wallis seguido pelo teste Dunn, sendo o nível para significância de $p \leq 0,05$.

APÊNDICE B

Publicação 2

Material e Método

- Animais

O protocolo da presente pesquisa foi analisado e aprovado pelo Comitê de Ética no Uso de Animais (CEUA) da Faculdade de Odontologia de Araraquara – FOAr/UNESP (Processo nº 02/2019 – ANEXO A).

Foram utilizados 80 ratos Holtzman (*Rattus norvegicus albinus*) adultos jovens, machos, pesando em média 220 gramas, provenientes do biotério de Criação da Faculdade de Odontologia do Campus de Araraquara (UNESP). Os animais foram mantidos no Biotério do Subsolo do Prédio de Biomorfológicas da Faculdade de Odontologia de Araraquara (UNESP), com temperatura ($23\pm 2^{\circ}\text{C}$) e umidade ($55\pm 10\%$) controladas e fotoperíodo de 12 horas. Os animais foram mantidos em gaiolas de polietileno forradas com maravalha e receberam ração granulada (Nutrilabor; Guabi, Campinas, SP, Brasil) e água *ad libitum*. A maravalha das gaiolas foi trocada, no mínimo, três vezes por semana; a limpeza das gaiolas e bebedouro também foi feita, a fim de manter a higiene do seu ambiente, evitando dessa forma que houvesse qualquer desconforto aos animais.

Os animais foram distribuídos equitativamente em quatro grupos, de acordo com os materiais implantados no subcutâneo: Bio-C (Grupo Bio-C Pulpo), MTA-HP (Grupo MTA Repair HP), MTA-B (Grupo MTA Branco) e GC (Grupo Controle – tubo vazio). A composição e procedência dos materiais, bem como a manipulação dos materiais estão descritas no Quadro A1.

Os procedimentos cirúrgicos foram descritos na Publicação 1. Portanto, aqui serão somente descritas as Metodologias de Análise usadas para o Artigo 2.

Processamento para microscopia de luz

Os implantes envolvidos pelos tecidos adjacentes foram fixados em solução de formaldeído a 4% (preparado a partir do paraformaldeído) tamponado com fosfato de sódio 0,1 M com Ph 7,2, durante 48 horas. Após a fixação, as peças foram desidratadas, diafanizadas, embebidas em parafina líquida (60°C) e incluídas em

parafina. Os espécimes foram incluídos de maneira a obter cortes longitudinais em relação ao longo eixo dos tubos de polietileno. Com um micrótomo (Leica, modelo HM 325) foram obtidos, a partir dos blocos, cortes com 6 μm de espessura das cápsulas de cada animal. Em relação aos implantes no subcutâneo, foram obtidos cortes longitudinais que foram aderidos a lâminas de vidro e corados com tricrômico de Masson e picrosirius-red. Cortes das cápsulas envolvendo os implantes foram aderidas as lâminas de vidro silanizadas e submetidos as reações imuno-histoquímicas.

A remodelação das cápsulas em resposta aos implantes, ou seja, a regressão do processo inflamatório e a formação de um tecido conjuntivo contendo fibroblastos e fibras colágenas, foi avaliada por meio da quantificação de fibroblastos nas cápsulas, Ki-67 – um marcador de proliferação celular, interleucina-10 (IL-10), citocina anti-inflamatória, FGF-1 (fator de crescimento de fibroblastos) e estimativa do conteúdo de colágeno.

Métodos de Análise

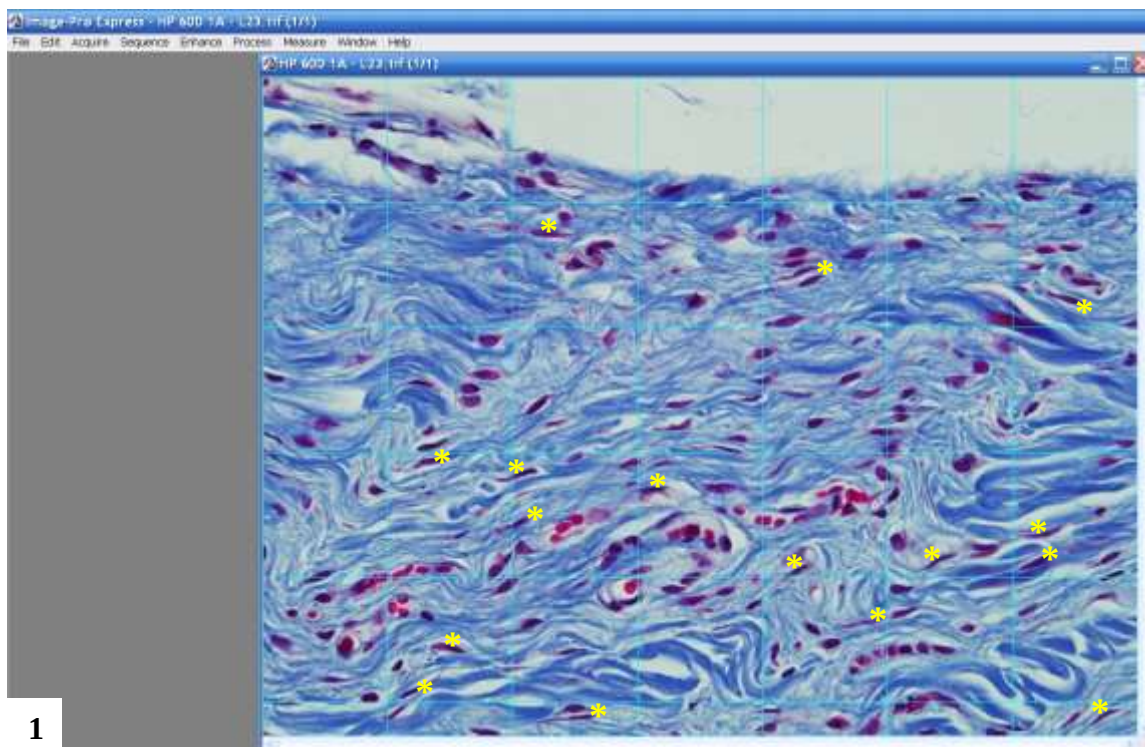
- Análise da reparação das cápsulas

Densidade numérica de fibroblastos

O número de fibroblastos por mm^2 nas cápsulas adjacentes aos implantes foi obtido a partir de cortes corados com tricrômico de Masson (Figura B1). O número destas células foi estimado a partir de três cortes não seriados de cada implante, manteve-se um intervalo mínimo de 100 μm entre os cortes. Com auxílio de uma câmera (DP-71, Olympus – Japão) acoplada ao microscópio de luz (Olympus, modelo BX-51), usando a objetiva de 40x (aumento final de 695x), imagens do tecido adjacente aos materiais implantados foram capturadas. Posteriormente, com um programa de análise de imagens (Image-Pro Express 6.0, Olympus – Japão), foi realizada a contagem dos fibroblastos, em cada corte, em uma área teste de aproximadamente 0,09 mm^2 da cápsula (Figura B1). Os fibroblastos foram identificados de acordo com sua morfologia, ou seja, considerando o seu formato fusiforme ou elíptico. O valor total de fibroblastos em cada espécime (obtido a partir das áreas dos três cortes/implante) foi dividido pela área total (0,27 mm^2) obtivemos, assim, um valor de fibroblastos/ mm^2 . Ao final, foi calculada a média para cada grupo

em cada período. A análise foi realizada por um examinador calibrado e “cego” aos grupos e períodos.

Figura B1- Imagem ilustrativa do método quantitativo.



“Print screen” de uma fotomicrografia mostrando uma porção da cápsula capturada usando a objetiva de 40x. A imagem foi capturada com a câmera DP-71 acoplada ao microscópio Olympus. Com o auxílio do programa de análise de imagens (Image-Pro Express, Olympus), a fotomicrografia foi aberta e, após calibração, uma grade reticular contendo 30 intersecções/pontos (sistema teste) foi sobreposta à imagem; esta grade foi usada somente para auxiliar como uma “guia” durante a contagem de fibroblastos. Coloração Tricrômico de Masson. Fonte: Elaboração própria.

- Imunofluorescência para detecção de FGF-1

Os cortes foram desparafinizados em xileno aquecido, hidratados e as lâminas imersas em tampão citrato de sódio 0,001 M Ph 6,0 e colocadas em forno de micro-ondas a 98-100° C para a recuperação antigênica. Após um período de resfriamento, as lâminas foram lavadas em tampão de fosfato de sódio 0,01 M (PBS) a Ph 7,2 e os cortes foram incubados por 20 min com 2% de albumina de soro bovino (BSA; Sigma-Aldrich Co., St. Louis, MO, EUA) à temperatura ambiente. Em seguida, os cortes foram incubados em câmara umidificada a 4°C com anticorpo monoclonal de camundongo anti-FGF-1 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, EUA; código: sc55520) por 16-18h. Os cortes foram lavados em PBS e incubados no escuro com anticorpo anti-IgG de camundongo Alexa Fluor®488 (1:1000; Molecular Probes® por Life Technologies, Calrsbad, EUA) por 1 hora em temperatura ambiente. Após a lavagem em PBS, a coloração nuclear foi realizada com DAPI (1:500, Molecular Probes by Life Technologies; CA, EUA) por 5 min no escuro em temperatura ambiente e as lâminas foram montadas com meio de montagem Fluoromount® (Dako Inc., Carpinteria, CA, EUA). Como controle negativo, os cortes foram incubados com soro não imune em substituição ao anticorpo primário. Os cortes foram analisados em microscópio de fluorescência DM4000 B LED (Leica, Wetzlar, Alemanha) com câmera DFC 550 (Leica, Alemanha) e software Leica Application Suite (LAS 4.3, Leica, Alemanha). Dois cortes não seriados por espécime foram capturados com ampliação de 40x. No campo padronizado (0,146mm²), o número de células exibindo imunopositividade ao FGF (fluorescência em verde) foi computado. Em todas as amostras, o número de células imunomarcadas com FGF-1 por mm² foi calculado em todos os períodos (n = 5 por grupo / período).

- Reação imuno-histoquímica para detecção de IL-10

Com o decorrer do tempo, é desejável que um material biocompatível favoreça um aumento dos fatores associados à síntese de proteínas associadas a reorganização estrutural dos tecidos. Dentre estas citocinas, a IL-10 tem uma participação ativa na reparação dos tecidos¹⁵. A interleucina-10 (IL-10) foi detectada utilizando o anticorpo primário anti-IL-10 produzido em camundongo (anti-IL-10, Santa Cruz Biotechnology, USA; sc-8438), com protocolo semelhante ao descrito no item “Reação imuno-histoquímica para detecção de IL-6”, sendo modificado apenas a titulação do anticorpo primário anti-IL-10 (titulação 1:100).

A fim de verificar se houve diferenças entre os grupos em cada período bem como ao longo do tempo, foi realizada a quantificação de células imunopositivas a IL-10 (em castanho). Com auxílio de uma câmera (DP-71, Olympus – Japão) acoplada ao microscópio de luz (Olympus, modelo BX-51), em aumento de 40x, imagens da cápsula adjacente aos implantes foram capturadas.

Posteriormente, com um programa de análise de imagens (Image-Pro Express 6.0, Olympus – Japão), as células positivas à IL-10- (em castanho) em cada área teste de aproximadamente 0,09mm² da cápsula, foram computadas obtendo-se, assim, um valor de células imunopositivas. A estimativa de células imunopositivas foi realizada nos 5 animais (5 implantes) de cada grupo por período. Assim, o número de células imunopositivas/mm² da cápsula foi obtido para cada animal e posteriormente, foi calculado a média para cada grupo em cada período.

- Reação imuno-histoquímica para detecção Ki-67

Cortes aderidos em lâminas silanizadas foram submetidos à imuno-histoquímica para detecção do Ki-67. Para recuperação dos sítios antigênicos, os cortes desparafinizados foram imersos em tampão citrato de sódio 0,001 M em Ph 6,0 e submetidos a tratamento em forno de micro-ondas por 30 minutos a 96-98°C. Após o resfriamento, a peroxidase endógena foi bloqueada com peróxido de hidrogênio a 5% em solução aquosa por 30 minutos à temperatura ambiente; foi realizada uma troca após 15 minutos. Os cortes foram lavados em tampão Tris-HCl 0,1M (Ph 7,2),

realizando-se 4 trocas de 5 minutos cada e, subsequentemente, os cortes foram incubados com albumina de soro bovino a 2% (Sigma-Aldrich Co., St. Louis, MO, EUA) durante 30 minutos à temperatura ambiente. Posteriormente, os cortes foram incubados durante 16 a 18 horas em uma câmara umidificada a 4°C no anticorpo monoclonal de coelho anti-Ki-67 (Spring Bioscience Corp., Pleasanton, CA, EUA, código ab-16667) diluído em 1:100. Após lavagem em tampão Tris-HCl, a imunorreação foi amplificada usando um Kit StreptAvidin-Biotin (Universal Dako LSAB, Dako Inc., Carpinteria, CA, EUA). Os cortes foram incubados no polímero deste Kit durante 1 hora à temperatura ambiente. A atividade da peroxidase foi revelada por 3,3'-diaminobenzidina (ImmPACT™ DAB Substrate, Vector Laboratories, Burlingame, CA, EUA) por 3 min. Após lavagem em água corrente, os cortes foram contrastados com hematoxilina. Os cortes usados como controle negativo, foram incubados em soro não-imune de coelho em substituição à incubação no anticorpo primário. O número de células imunomarcadas com Ki-67- (em castanho foi contada em um campo padronizado (0,09 mm²) capturado com a objetiva de 40x, fornecendo um aumento final de 695x. Esta quantificação foi realizada com auxílio do sistema de análise de imagem (Image-Pro Express Olympus). O número de células imunomarcadas com Ki-67 por mm² foi obtido para cada animal (n=5 por grupo/período).

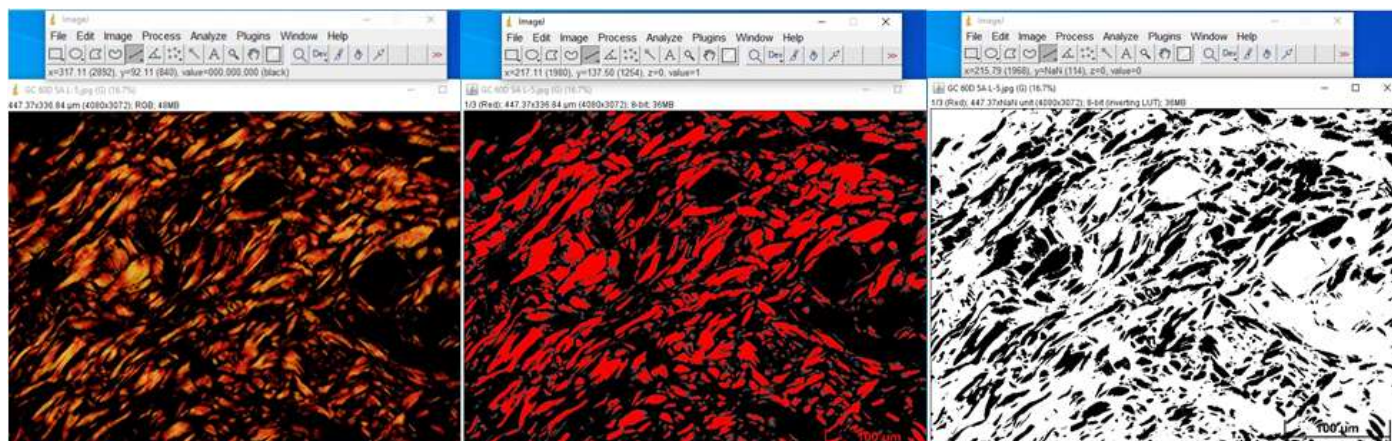
- Estimativa do conteúdo de colágeno birrefringente

Embora que os materiais biocompatíveis, inicialmente, induzam um processo inflamatório, com o decorrer do tempo os biomateriais devem permitir uma involução deste processo com uma concomitante proliferação de fibroblastos e formação de colágeno^{2,5,16}. Dessa maneira, o conteúdo de colágeno foi quantificado nas cápsulas. O conteúdo de colágeno nas cápsulas foi estimado a partir de cortes corados com solução a 0,1% de picrosirius-red e analisados com filtros de polarização acoplados ao microscópio de luz (BX-51, Olympus). Foram utilizados 3 cortes, com intervalo mínimo de 100 µm, por implante de todos grupos e períodos. Em cada corte, uma imagem da cápsula foi capturada com auxílio de uma câmera digital (DP-71, Olympus, Tóquio, Japão) e objetiva de 40x; esta imagem com área de aproximadamente 0,09 mm², usada em todos os cortes, foi considerada a área padrão. Dessa maneira, o

colágeno foi quantificado numa área de 0,27 mm² de cada implante. Todas as imagens foram capturadas com parâmetros de intensidade de luz, abertura do diafragma do condensador, altura do condensador, objetiva, angulação do polarizador e brilho da imagem padronizados.

A quantidade de colágeno foi estimada usando a metodologia previamente descrita^{16,17}. As imagens capturadas foram “abertas” no processador de imagens “ImageJ” (ImageJ, National Institutes of Health, Bethesda, MD, EUA), as cores proporcionadas pela birrefringência das imagens foram isoladas utilizando-se o histograma de matiz disponível na guia “Threshold Colour”. As imagens foram convertidas para preto e branco, de modo que cada pixel não preto se torna branco e cada pixel preto permanecerá preto. Em seguida, a porcentagem ocupada pelos matizes (vermelho/alaranjado, amarelo e verde) foi calculada a partir do número de pixels em cada faixa de matiz. Para esta mensuração, o histograma de matiz foi selecionado para cada comprimento de onda, ou seja, vermelho/alaranjado (matizes 2 a 38 e 230 a 256), amarelo (matiz 39 a 51) e verde (matiz 52 a 128); o número de pixels de um comprimento de onda foi determinado em porcentagem a partir do número total de pixels da imagem. O valor total de colágeno (em porcentagem) foi calculado a partir da soma dos três 3 matizes obtidos nos 3 cortes de cada implante.

Figura B2 – Imagem ilustrativa da estimativa de colágeno birrefringente.



“Print screen” de uma fotomicrografia mostrando uma porção da cápsula capturada usando a objetiva de 40x. A imagem foi capturada com a câmera DP-71 acoplada ao microscópio Olympus. Com o auxílio do programa de análise de imagens (Image-J), a fotomicrografia foi aberta e, após calibração a porcentagem ocupada pelos matizes (vermelho/alaranjado, amarelo e verde) foi calculada a partir do número de pixels em cada faixa de matiz. Coloração Picrosirius-red.
Fonte: Elaboração própria.

Análise Estatística

Os dados foram avaliados pela análise ANOVA two-way seguida pelo teste de Tukey (software GraphPad Prism 5.0, San Diego, CA, EUA) com nível de significância de $p \leq 0,05$. Todos os dados foram apresentados com média e desvio padrão.

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² * De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: <http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf>

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ANEXO A – AUTORIZAÇÃO DO COMITÊ DE ÉTICA

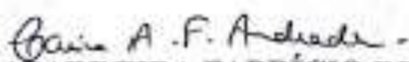


UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araraquara
FACULDADE DE ODONTOLOGIA

**CERTIFICADO**

Certificamos que a proposta intitulada ***"AVALIAÇÃO DA REAÇÃO TECIDUAL PROMOVIDA PELO BIO-C PULPO E MTA REPAIR HP NO SUBCUTÂNEO E DA TOXICIDADE HEPÁTICA"***, registrada com o nº 02/2019, sob a responsabilidade do(a) **Prof(a). Dr(a). Paulo Sérgio Cerri** – que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA FACULDADE DE ODONTOLOGIA DE ARARAQUARA** em reunião de 21/02/2019.

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização	Março/2022
Espécie/linhagem/raça	Rato – Holtzman (<i>Rattus norvegicus albinus</i>)
Nº de animais	80
Peso/Idade	220g – adultos jovens
Sexo	Macho
Origem	Biotério Central da Faculdade de Odontologia de Araraquara – UNESP


Profa. Dra. CARINA APARECIDA FABRÍCIO DE ANDRADE
 Coordenadora da CEUA

ANEXO B – AUTORIZAÇÃO DE REPRODUÇÃO DA PUBLICAÇÃO 1

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Feb 22, 2022

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Mateus Machado Delfino