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

Evelin Carine Alves Silva

Reação tecidual e potencial bioativo de cimentos endodônticos biocerâmicos em subcutâneo de ratos

Araraquara

2020

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Dissertação apresentada ao Programa de Pós-graduação em Odontologia - Área de Endodontia, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista - UNESP, para obtenção do Título de Mestre em Odontologia - Área de Endodontia.

Orientadora: Profª. Drª. Juliane Maria Guerreiro Tanomaru
Coorientador: Prof. Dr. Paulo Sérgio Cerri

Araraquara

2020

Silva, Evelin Carine Alves
Reação tecidual e potencial bioativo de cimentos endodônticos biocerâmicos em subcutâneo de ratos / Evelin Carine Alves Silva.-- Araraquara: [s.n.], 2020
67 f.; 30 cm.

Dissertação (Mestrado em Odontologia) – Universidade Estadual Paulista, Faculdade de Odontologia
Orientadora: Profa. Dra. Juliane Maria Guerreiro Tanomaru
Coorientador: Prof. Dr. Paulo Sérgio Cerri

1. Testes de materiais 2. Endodontia 3. Imuno-histoquímica I. Título

.

Ficha catalográfica elaborada pela Bibliotecária Marley C. Chiusoli Montagnoli, CRB/5646
Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara
Diretoria Técnica de Biblioteca e Documentação

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Comissão Julgadora

Dissertação para obtenção do grau de Mestre em Odontologia

Presidente e orientador: Profª. Drª. Juliane Maria Guerreiro Tanomaru

2º Examinador: Profº. Drº. Guilherme Ferreira da Silva

3º Examinador: Profª. Drª. Ticiana Sidorenko de Oliveira Capote

Araraquara, 19 de março de 2020

DADOS CURRICULARES

Evelin Carine Alves Silva

NASCIMENTO: 01 de maio de 1991-Porto Velho-Rondônia

FILIAÇÃO: Izabel Cristina Alves Pereira
Eduardo Romagna Pereira

2009-2013

Graduação em Odontologia pela Faculdade de Macapá-FAMA, Macapá-AP.

2014-2015

Especialização em Endodontia pela Faculdade Avantis-GOE, Macapá-AP.

2018-2020

Mestrado em Odontologia, área de concentração em Endodontia, pela Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Odontologia de Araraquara – FOAr/UNESP, Araraquara-SP.

ASSOCIAÇÕES SBPqO – Sociedade Brasileira de Pesquisa em Odontologia.

AGRADECIMENTOS

Um sonho sonhado sozinho é apenas um sonho, um sonho sonhado junto é realidade. Frase ensinada pela minha mãe há seis anos que me acompanha todos os dias e representa minha gratidão por mais essa fase concluída.

Agradeço ao meu Deus, autor da vida, que me possibilitou chegar até aqui, pela proteção diária, cuidado e todas as bênçãos incontáveis.

Ao meu amado esposo, companheiro e parceiro de vida. Obrigada pelo apoio incondicional, pelos sacrifícios, pela compreensão, ajuda e torcida em todos os momentos. A jornada se tornou mais leve com você ao meu lado.

À minha mãe (in memoriam) por toda educação, amor e dedicação devotada a mim ao longo dos meus 24 anos. Mesmo não estando presente, você é parte de mim e minha eterna saudade.

À minha irmã (minha eterna companheira) e ao meu padrasto pelo apoio, pelas orações, compreensão, torcida e amor de todos os dias. Se cheguei até aqui, é porque tive vocês durante o caminho.

À minha sogra por toda amizade, carinho, amor e torcida. Ao meu sogro e meus cunhados pelo apoio e por me ouvirem falar dia e noite sobre os meus ratos.

Aos meus amigos de Pós-Graduação que foram companhia, apoio e alegria. Obrigada Luiza, Mariana, Cristiane, Camila, Igma, Jéssica, Kénnia e Gissele.

Aos amigos da Histologia e Embriologia que me receberam como agregada com tanto carinho e foram companhias ao longo do dia. Obrigada Camila, Fabiane, Renata e Mateus. Em especial, Mateus e Camila, obrigada pelos ensinamentos e socorro.

Ao técnico Pedro Simões, muito obrigada por toda ajuda, todo ensinamento e paciência nas atividades do laboratório.

Ao Prof. Dr. Paulo Sérgio Cerri, obrigada por todos os ensinamentos, pela paciência, pelas correções e por me ensinar a desvendar a Histologia.

Ao Prof. Dr. Mário Tanomaru-Filho, pela oportunidade, pelos ensinamentos, correções, conselhos e apoio durante todo o decorrer do trabalho.

À Prof. Drª Juliane Maria Guerreiro-Tanomaru, minha orientadora, o meu muito obrigada pela confiança devotada em mim, pela carta de orientação concedida lá no início, por todas as cirurgias, ensinamentos, apoio, risadas e paciência. Sem sua orientação, nenhum resultado teria sido alcançado.

À CAPES:

O presente trabalho foi realizado com o apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de financiamento 001.

À

FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo (Processo nº 2017/14305-9) pelo apoio financeiro essencial para realização dessa pesquisa.

Silva ECA. Reação tecidual e potencial bioativo de cimentos endodônticos biocerâmicos em subcutâneo de ratos. [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2020.

RESUMO

Cimentos à base de silicatos de cálcio são desenvolvidos para obturação de canais radiculares. ***Publicação 1:*** Este estudo avaliou propriedades biológicas dos cimentos experimentais (CE-1 e CE-2) compostos por silicatos de cálcio e com adição de hipoclorito de cálcio (CE-2), em comparação ao AH Plus (AHP) e TotalFill BC Sealer (TBC). A reação tecidual provocada pelos materiais no subcutâneo de ratos foi avaliada por meio da implantação de tubos de polietileno nos períodos de 7, 15, 30 e 60 dias. Cortes foram corados com hematoxilina e eosina (HE) para análises morfológica e do número de células inflamatórias/mm^2(CI) e cortes foram utilizados para detecção de interleucina-6 (IL-6) e osteocalcina. Método de von Kossa foi realizado para identificação de depósitos de cálcio. Os dados foram submetidos aos testes ANOVA e Tukey, com significância de 5%. Aos 7 dias, CE-1, CE-2 e AHP apresentaram maior número de CI. AHP apresentou maior marcação para IL-6. Após 15 dias não houve diferença entre CE-2 e o grupo controle para IL-6. Aos 30 dias, AHP exibiu maior número de CI ($p<0,0001$) e CE-2 e o grupo controle os menores valores de CI e IL-6. Após 60 dias CE-2, TBC e controle apresentaram os menores valores ($p<0,05$). Os cimentos CE-1, CE-2 e TBC apresentaram estruturas positivas ao método von Kossa em todos os períodos observados e marcação positiva para osteocalcina. CE-2 apresentou quantidade de células positivas superior em todos os períodos quando comparado aos cimentos CE-1 e TBC ($p< 0.0001$). O cimento AH Plus e o grupo controle não exibiram marcação positiva em nenhum período analisado. Conclui-se que CE-1, CE-2 e TBC apresentam biocompatibilidade e potencial bioativo. ***Publicação 2:*** Bio-C Sealer e Sealer Plus BC são novos cimentos endodônticos biocerâmicos. O objetivo do estudo foi avaliar a reação tecidual e potencial bioativo dos cimentos Bio-C Sealer (BC) e Sealer Plus BC (SPBC) comparado ao AH Plus (AHP) em subcutâneo de ratos. A reação tecidual foi avaliada por meio da implantação de tubos de polietileno preenchidos com os materiais e vazios como grupo controle. Após 7, 15, 30 e 60 dias tubos e tecidos adjacentes foram removidos e realizada a contagem das células inflamatórias/mm^2(CI), imunomarcação de interleucina-6 (IL-6), osteocalcina (OC) e von Kossa para identificação de depósitos de cálcio. Os dados foram submetidos aos testes ANOVA e Tukey, com significância de 5%. Aos 7 dias, SPBC apresentou menor CI que BC ($p=0,0225$). AHP exibiu maior marcação para IL-6 ($p< 0.0001$). Após 15 dias, BC apresentou menor CI e IL-6 quando comparado aos demais materiais. Aos 30 dias, SPBC e AHP apresentaram maiores valores para CI ($p=0,0791$). Após 60 dias os cimentos à base de silicato de cálcio não apresentaram diferença estatística entre si ($p=0,8949$) tanto para CI quanto para IL-6, com valores inferiores ao AHP. Os materiais apresentaram estruturas positivas ao von Kossa. BC exibiu marcação para osteocalcina em todos os períodos. SPBC não exibiu marcação aos 7 dias, aos 60 dias foi inferior ao BC ($p= 0,076$). AH Plus e grupo controle não exibiram marcação para osteocalcina. Os cimentos Bio-C Sealer e Sealer Plus BC são biocompatíveis, e apresentam potencial bioativo.

Palavras Chaves: Teste de materiais. Endodontia. Imuno-histoquímica.

Silva, ECA. Tissue reaction and bioactive potential of subcutaneous bioceramic endodontic sealers in rats [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2019.

ABSTRACT

Calcium based silicate sealers are developed for filling root canals. ***Publication 1:*** This study evaluated biological properties of experimental sealers (CE-1 and CE-2) composed of calcium silicates and with the addition of calcium hypochlorite (CE-2), compared to AH Plus (AHP) and TotalFill BC Sealer (TBC). Methodology: The tissue reaction caused by the materials in the subcutaneous of rats was evaluated through the implantation of polyethylene tubes in the periods of 7, 15, 30 and 60 days. Cuts were stained with hematoxylin and eosin (HE) for morphological analysis and the number of inflammatory cells / mm2 (IC) and cuts were used to detect interleukin-6 (IL-6) and osteocalcin. Von Kossa's method was used to identify calcium deposits. The data were submitted to ANOVA and Tukey tests, with a significance of 5%. Results: At 7 days, CE-1, CE-2 and AHP had a higher number of IC. AHP showed higher marking for IL-6. After 15 days there was no difference between CE-2 and the control group for IL-6. At 30 days, AHP exhibited the highest number of IC ($p < 0.0001$), and CE-2 and the control group had the lowest IC and IL-6 values. After 60 days CE-2, TBC and control showed the lowest values ($p < 0.05$). The CE-1, CE-2 and TBC sealers presented positive structures to the von Kossa method in all observed periods and positive marking for osteocalcin. CE-2 showed a higher number of positive cells in all periods when compared to CE-1 and TBC sealers ($p < 0.0001$). The AH Plus sealer and the control group did not show positive marking in any of the analyzed period. Conclusions: It is concluded that CE-1, CE-2 and TBC have biocompatibility and bioactive potential. ***Publication 2***: Bio-C Sealer and Sealer Plus BC are new ready-to-use bioceramic endodontic sealers. The aim of the study was to evaluate the tissue reaction and bioactive potential of Bio-C Sealer (BC) and Sealer Plus BC (SPBC) sealers compared to AH Plus (AHP) in subcutaneous rats. Methods: The tissue reaction was evaluated by implanting polyethylene tubes filled with the materials and an empty one as a control group. After 7, 15, 30 and 60 days, adjacent tubes and tissues were removed and inflammatory cell counts / mm2 (IC), immunohistochemical reactions for interleukin-6 (IL-6), for osteocalcin (OC), and von Kossa technique to identify deposits of calcium were performed of calcium. The data were submitted to ANOVA and Tukey tests, with a significance of 5%. Results: At 7 days, SPBC presented a lower IC than BC ($p = 0.0225$). AHP exhibited greater marking for IL-6 ($p < 0.0001$). After 15 days, BC showed lower IC and IL-6-immunopositive cells when compared to other materials. At 30 days, SPBC and AHP showed higher values for IC ($p = 0.0791$). After 60 days, calcium silicate cements did not show statistical difference between themselves ($p = 0.8949$) for both IC and IL-6, with values lower than AHP. The materials showed positive structures to von Kossa. BC exhibited osteocalcin marking in all periods. SPBC showed no marking at 7 days, at 60 days it was lower than BC ($p = 0.076$). AH Plus and the control group did not exhibit osteocalcin marking. Conclusions: Bio-C Sealer and Sealer Plus BC sealers are biocompatible and have bioactive potential.

Keywords: Materials testing. Endodontics. Immunohistochemistry.

SUMÁRIO

1 INTRODUÇÃO

Cimentos à base de silicato de cálcio (SC) são utilizados por apresentarem biocompatibilidade[1], estabilidade dimensional, pH alcalino e potencial bioativo[2]. A bioatividade dos cimentos à base de silicato tricálcico ocorre em função do processo de hidratação do silicato de cálcio[3], resultando na capacidade de formação de hidroxiapatita[4-6].

O MTA (Mineral Trióxido Agregado) foi proposto para tratamento de perfurações, capeamento pulpar, obturação retrógrada, pulpotomia e tratamento de dentes com rizogênese incompleta[7]. A partir de modificações na composição, cimentos endodônticos de silicatos de cálcio foram desenvolvidos para obturação de canal radicular. O EndoSequence BC sealer é um exemplo de cimento endodôntico disponibilizado em seringa e pronto para uso, composto por silicato tricálcico, silicato dicálcico, hidróxido de cálcio, óxido de zircônio como agente radiopacificador e agentes espessantes, apresentando propriedade físicas e químicas de acordo com as normas ISO 6876/2001 e biocompatibilidade[8-9]. TotalFill BC Sealer (TBC; KFG FKG Dentaire AS, Suíça) é um cimento composto por silicatos de cálcio, hidróxido de cálcio, óxido de zircônio, óxido de tântalo e agentes espessantes, e apresenta citocompatibilidade[10].

Mais recentemente, o cimento à base de silicato de cálcio pronto para uso Bio-C Sealer (Angelus, Brasil) foi desenvolvido, apresentando pH alcalino e radiopacidade[10-11]. Outro cimento pronto para uso, Sealer Plus BC (MK Life, Brasil) é composto por silicato dicálcico, silicato tricálcico, entre outros componentes, e apresenta pH alcalino, liberação de íons de cálcio e adequados tempo de presa e radiopacidade[12].

Rodriguez-Lozano *et al.*[10] (2017) avaliaram a citotoxicidade de três cimentos endodônticos (TotalFill BC Sealer, AH Plus e MTA Fillapex) por meio da análise em células mesenquimais, e concluíram que o cimento TotalFill BC Sealer foi o mais citocompatível.

Zordan-Bronzel *et al.*[11] (2019) avaliaram propriedades físicas e químicas de um novo material à base de silicato de cálcio. O cimento Bio-C Sealer demonstrou tempo de presa, pH e radiopacidade adequados, apesar de alta solubilidade, porém, com baixa alteração volumétrica.

Considerando-se os componentes de cimentos à base de silicatos de cálcio, dois Cimentos experimentais (CE-1 e CE-2) foram desenvolvidos na Faculdade de Odontologia de Araraquara, compostos por silicato tricálcico, silicato dicálcico, fosfato de cálcio monobásico, hidróxido de cálcio, óxido de zircônio, tungstato de cálcio e polietilenoglicol 400 como veículo. Esse material experimental (CE-1) demonstrou citocompatibilidade, potencal bioativo por testes de atividade de fosfatase alcalina e de formação de nódulos mineralizados, além de ação antimicrobiana sobre *E. faecalis e C. albicans*, confirmando o potencial de uso no tratamento endodôntico[13]. Óxido de zircônio e tungstato de cálcio são radiopacificadores com adequadas propriedades físicas e biológicas[3,13]. O veículo Polietilenoglicol 400 tem sido usado na composição de medicações intracanal à base de hidróxido de cálcio[13]. Quando associado aos cimentos à base de SC, o polietilenoglicol melhora suas propriedades físico-químicas[8,13]. A adição de componentes antimicrobianos aos cimentos endodônticos pode contribuir para eliminar microorganismos que sobreviveram ao preparo biomecânico[14]. Com o objetivo de proporcionar eficiente atividade antimicrobiana outro cimento experimental foi desenvolvido a partir do CE-1, adicionando o antimicrobiano hipoclorito de cálcio a 2 %, apresentando eficácia contra *E. Faecalis,* e capacidade de dissolução de tecido orgânico e biocompatibilidade[15] .

Andrade *et al.*[16] (2018) estudaram em subcutâneo de ratos a resposta tecidual da associação de 80% de cimento de silicato tricálcico puro (TSC) e 20% de ZrO2, em comparação ao MTA Plus. Os autores concluiram que os dois materiais mostraram redução da reação inflamatória e de Interleucina-6 (IL-6), sugerindo que são biocompatíveis.

O AH Plus é um cimento endodôntico à base de resina epóxi com óxido de zircônio e tungstato de cálcio que possui maior radiopacidade do que o TotalFill BC Sealer[11.] Apesar da reação inicial promovida pelo AH Plus, a reação inflamatória diminui com o tempo sendo então considerado biocompatível, porém com base na tentativa de causar dano tecidual inicial, foi utilizado como controle positivo em estudos em ratos subcutâneos[8-10].

A biocompatibilidade e bioatividade podem ser avaliadas em tecido subcutâneo de ratos. Um material é considerado biocompatível quando sua resposta inflamatória apresenta redução com o decorrer do tempo[17-18]. A biocompatibilidade

de um cimento endodôntico depende diretamente de seus componentes, de seu tempo de presa e de sua solubilidade[19].

Fonseca *et al*.[19] (2016) avaliaram o processo inflamatório e expressão de IL-6 do MTA e Biodentine em tecido subcutâneo de rato e concluíram que estes materiais à base de silicato de cálcio apresentam biocompatibilidade. Silva *et al.*[20] *(*2017) avaliaram a influência da adição de óxido de zircônio e óxido de nióbio (radiopacificadores) em um cimento experimental à base de silicato de cálcio em tecido subcutâneo de rato e demonstraram biocompatibilidade para os materiais.

A relação entre interleucina-6 (IL-6) e a reação inflamatória são amplamente estudadas em subcutâneos de ratos com materiais a base de silicato de cálcio[4,18,19]. A IL-6 atua como mediadora da resposta do hospedeiro frente a uma agressão, podendo inclusive estar relacionada com a inflamação da polpa dentária[4,8].

A interleucina-6 é definida como citocina pró e anti-inflamatória, desenvolvida durante o processo inflamatório, sendo sintetizada em decorrência de estímulos como traumas e infecções por células como fibroblastos, macrófagos e neutrófilos[21].

Para avaliar a formação de tecido calcificado, o método von Kossa é utilizado baseado na avaliação da precipitação de cálcio sobre o material. A formação de tecidos mineralizados é comumente avaliada por imuno-histoquímica, sendo essa técnica utilizada para detectar proteínas como a osteocalcina, peptídeo este secretado pelos osteoblastos, durante a formação óssea[5].

Os estudos realizados de forma *in vivo* e *in vitro* são úteis na avaliação de novos materiais, pois orientam seus protocolos e indicações[22-24]. O estudo em tecido conjuntivo subcutâneo de ratos é uma metodologia controlada, recomendado pela *Fédération Dentaire International e International Standard-ISO*, como preliminares e indicativos para comparar o grau de irritabilidade dos materiais odontológicos[3,10]. A utilização de tubos de polietileno ocorre com o intuito de evitar a disseminação do material e simular o canal radicular [23].

Diante do exposto, tornam-se relevantes os estudos que avaliem as propriedades biológicas dos novos materiais de silicatos de cálcio.

2 PROPOSIÇÃO

2.1 Objetivo Geral: Este estudo teve como objetivo a avaliação das propriedades biológicas de dois cimentos experimentais e novos cimentos endodônticos biocerâmicos.

2.2 Objetivos Específicos:

- Avaliação dos cimentos experimentais compostos por silicato tricálcico, silicato dicálcico, fosfato de cálcio monobásico, hidróxido de cálcio, óxido de zircônio, tungstato de cálcio e polietilenoglicol (CE-1) e com adição de hipoclorito de cálcio (CE-2) em comparação com os cimentos TotalFill BC Sealer e AH Plus através do estudo em subcutâneo de ratos por meio das análises: Densidade numérica de células inflamatórias, espessura das cápsulas adjacentes aos implantes, reação imuno-histoquímica para detecção de IL-6, reação imuno-histoquímica para detecção de osteocalcina, reação e análise de von Kossa e análise sob luz polarizada.

- Avaliação das propriedades biológicas de dois novos cimentos biocerâmicos: Bio-C Sealer e Sealer Plus BC em comparação ao cimento AH Plus através de estudo *in vivo* por meio das seguintes análises: Densidade numérica de células inflamatórias, espessura das cápsulas adjacentes aos implantes, reação imuno-histoquímica para detecção de IL-6, reação imuno-histoquímica para detecção de osteocalcina, reação e análise de von Kossa e análise sob luz polarizada.

3 PUBLICAÇÕES

3.1 Publicação 1*

Evaluation of the biological properties of two experimental calcium silicate sealants: an in vivo study

Abstract

Aim This study evaluated the biological properties of experimental sealers (CE-1 and CE-2) composed of tricalcium silicate, dicalcium silicate, monobasic calcium phosphate, calcium hydroxide, zirconium oxide and calcium and polyethylene glycol tungstate and with addition of hypochlorite calcium (CE-2) compared to AH Plus (AHP) and TotalFill BC Sealer (TBC).

Methodology The tissue reaction caused by different materials in the subcutaneous tissue of rats was evaluated through the implantation of polyethylene tubes filled with the materials in the periods of 7, 15, 30 and 60 days. Cuts were stained with haematoxylin and eosin (HE) for morphological analysis and the number of inflammatory cells/mm^2 (IC) and cuts were used for immunohistochemical reaction for detection of interleukin-6 (IL-6) and osteocalcin. von Kossa's method was used to identify calcified structures in the capsule adjacent to the implants. The data was submitted to ANOVA and Tukey tests, with a significance of 5%.

Results At 7 days, CE-1, CE-2 and AHP had a higher number of IC. AHP showed higher marking for IL-6. After 15 days, regarding IL-6, there was no difference between CE-2 and the control group. At 30 days, AHP exhibited the highest number of IC ($p < 0.0001$) and CE-2 and the control group had the lowest IC and IL-6 values. After 60 days, all materials exhibited a decrease in IC. CE-2, TBC and control showed the lowest values ($p < 0.05$). CE-1 and TBC showed no difference for IL-6. CE-2 and control group were statistically similar. In this period, AHP had the highest number of IC and IL-6. ($p < 0.0001$). The CE-1, CE-2 TBC sealers showed positive structures to the von Kossa method in all observed periods and positive marking for osteocalcin. CE-2 showed a higher number of positive cells in all periods when compared to CE-1 and TBC sealers ($p < 0.0001$). The AH Plus sealer and the control group did not show positive marking in any analyzed period.

Conclusions It is concluded that the experimental sealer and its association with calcium hypochlorite, in addition to TotalFill BC Sealer, have biocompatibility and bioactive potential.

Keywords: Calcium silicate, biocompatibility, endodontics, immunohistochemistry

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* Artigo nas normas do periódico *International Endodontic Journal* para o qual pretende-se submeter.

INTRODUCTION

Calcium silicate root canal filling materials present physicochemical properties in accordance with ISO 6876/2001 and are biocompatible (Zhou *et al.* 2013, Zhou *et al.* 2015). Considering these advantages, two experimental sealers (CE-1 and CE-2) were developed and evaluated in this study, composed of tricalcium silicate, dicalcium silicate, monobasic calcium phosphate, calcium hydroxide, zirconium oxide and calcium tungstate, and polyethylene glycol 400 as a vehicle. It is well known that zirconium oxide and calcium tungstate are radiopacifiers, which do not interfere with physicochemical and biological properties (Khalil *et al.* 2016, Zordan-Bronzel *et al.* 2019). Polyethylene glycol has been used as a vehicle in the composition of intracanal medications based on calcium hydroxide due to its biocompatibility and low cost (Zordan-Bronzel *et al.* 2019). When associated with silicate calcium (SC)-based sealers, it improves the physical-chemical properties such as reduced setting time and increased sealer flow, resulting in greater calcium release during the initial period, promoting alkaline pH, favoring the repair process (Zhou *et al.* 2015, Zordan-Bronzel *et al.* 2019).

Previous research shows that the experimental sealer CE-1 demonstrated cytocompatibility, bioactive potential by alkaline phosphatase activity test and showed formation of mineralized nodules. Furthermore, presented antimicrobial activity on *E. faecalis* and *C. albicans*, (Zordan-Bronzel *et al.* 2019).

The addition of antimicrobial agents to endodontic sealers can contribute to the elimination of microorganisms that have survived to the root canal preparation (Mittag *et al*. 2012). In order to provide antimicrobial activity, another experimental sealer was developed similar to CE-1, adding 0.2-gram by weight of the calcium hypochlorite. Calcium hypochlorite is an alkaline white powder that is effective against *E. Faecalis* and demonstrates the ability to dissolve organic tissue (Bello *et al.* 2018)). Calcium hypochlorite can be used as an auxiliary medium, decreasing the microbial presence through the release of chlorine and alkaline potential (Almeida *et al.* 2014, Soligo *et al*. 2018, Bello *et al.* 2018).

TotalFill BC Sealer (TBC; KFG FKG Dentaire AS, Switzerland) is a ready-to-use calcium silicate based sealer, composed of calcium silicates, calcium hydroxide, zirconium oxide, tantalum oxide and thickening agents, which features adequate physicochemical properties (Tanomaru-Filho *et al.* 2017), cytocompatibility

(Rodríguez-Lozano *et al.* 2017), and potential for inducing mineralization and antimicrobial activity (Zordan-Bronzel *et al*. 2019). AH Plus is an endodontic sealer based on epoxy resin with zirconium oxide and calcium tungstate that has greater radiopacity than TotalFill BC Sealer (Candeiro *et al.* 2012, Tanomaru-Filho *et al*. 2017). Despite the initial reaction promoted by AH Plus, the inflammatory reaction decreases over time and, therefore, this sealer has been considered biocompatible (Cintra *et al*. 2017, Saraiva *et al.* 2018), however based on the attempt to cause tissue damage initial, it was used as a positive control in studies in rats subcutaneous. (Saraiva *et al.* 2018)

Although has already been demonstrated that CE-1 presents adequate physicochemical properties (Zordan-Bronzel *et al.* 2019), there is no studies about characteristics of CE-2.Thus, the aim of this study was to evaluate the tissue reaction and bioactive potential of these two experimental sealers in comparison with TotalFill BC and AH Plus after implantation in subcutaneous tissue of rats. The null hypothesis was that the different materials would not interfere in the intensity of the tissue reaction.

MATERIALS AND METHODS

Experimental procedure

The research protocol was approved by the Ethical Committee for Animal Research of the São Paulo (CEUA, Process number 35/2018). In the present study, forty adult male Holtzman rats (*Rattus norvegicus albinus*) were used and distributed in four groups (n = 6): sealers and control group (CG;empty polyethylene tubes). The materials (Table 1) were inserted in polyethylene tubes (10.0 mm length and a 1.6 mm diameter) and immediately implanted in dorsal subcutaneous sites. Four tubes were inserted per animal, one from each group, in the form of a quadrant rotation (ISO-10993-6, 2007). The animals were anesthetized with ketamine hydrochloride (80 mg/kg body weight, Virbac of Brasil Indústria e Comércio Ltda., São Paulo, SP, Brazil) and xylazine hydrochloride (4 mg/kg body weight, União Química). National Pharmaceutical S / A, São Paulo, SP, Brazil) administered intraperitoneally. The dorsal skin was shaved and disinfected with 5% iodine solution. After 7, 15, 30 and 60 days, the animals were sacrificed with anesthetic overdose, the implants with

adjacent tissues were removed, and the samples were processed for light microscopy.

Table 1 - Endodontic sealers used, manufacturer and proportions.

Sealers	Composition	Manufacturers	Proportion
Sealer Experimental (CE-1)	Powder: Tricalcium silicate °, dicalcium silicate °, monobasic calcium phosphate €, calcium hydroxide, zirconium oxide *, calcium tungstate *. Liquid: polyethylene glycol 400 *.	FOAr-UNESP, Araraquara, SP, Brasil	1g: 0,3g (powder / liquid)
Sealer Experimental with antimicrobial (CE-2)	Powder: Tricalcium silicate °, dicalcium silicate °, monobasic calcium phosphate €, calcium hydroxide, zirconium oxide *, calcium tungstate * and calcium hypochlorite. Liquid: polyethylene glycol 400 *.	FOAr-UNESP, Araraquara, SP, Brasil	1,0g: 0,3g: 0,2g HC (powder / liquid)
TotalFill BC (TBC)	Zirconium oxide, calcium silicates, calcium, monobasic phosphate, calcium hydroxide, filling and thickening agents	FKG Dentaire SA, La ChauxdeFonds, Switzerland	Ready to use
AH Plus (AHP)	*Paste A*: epoxy bisphenol-A resin and epoxy bisphenol-F, calcium tungstate (TC), zirconium oxide (Ozr), silica, iron oxide. *Paste B*: dibenzyldiamine, aminoadamantane, TC, OZr, silica, silicone.	Dentsply DeTrey GmbH, Konstanz, Alemanha	1g: 1g (folder / folder)

°Mineral Research Processing, Meyzieu, France; €Synth, Diadema, SP, Brasil; ᵖMerck, Darmstadt., Germany; *Sigma-Aldrich, St. Louis, MO, USA.

Histological procedures

The implants with the surrounding tissues were removed and immersed for 72 hours in a 4% formaldehyde solution buffered with 0.1 M sodium phosphate and pH adjusted to 7.2. After fixation, the specimens were dehydrated, diaphanized, immersed in liquid paraffin (60ºC) and embedded in paraffin. Longitudinal sections with 6 µm thickness were obtained. Non-serial sections were stained with

hematoxylin and eosin (HE) to estimate the number of inflammatory cells in the capsules and thickness of the capsules adjacent to the opening of the tubes.

Thickness of capsules

The thickness (in µm) of the capsules adjacent to the implanted tubes was measured. Using a camera (DP-71, Olympus - Japan) coupled to the light microscope (Olympus, BX-51, Japan), three images of non-serial sections stained with HE of each specimen were captured. The thickness of the capsules was estimated in the middle portion from its surface adjacent to the material to its limit with the adjacent tissues. After obtaining the values, the average value was calculated from the measurements obtained from the three sections for each specimen. This measurement was obtained in all specimens (n = six per group) and in all periods.

Numerical density of inflammatory cells

Quantitative analysis was performed on the capsules of all implants and for each specimen three non-serial HE-stained sections were used. The images of portions of the capsules, in their capsule/material interface, were captured using a DP-71 camera (Olympus, Tokyo, Japan) attached to the light microscope (BX51, Olympus). The number of inflammatory cells was estimated using the Olympus Image-Pro Express 6.0 program. In each captured image, the number of inflammatory cells in a standard area of 0.09 mm^2 of the capsule adjacent to the opening of the implanted tubes was computed (Viola *et al.* 2012, Silva *et al.* 2015).

Immunohistochemical detection of IL-6

For the detection of IL-6, the mouse anti-IL-6 antibody (Abcam, Cambridge Science, UK; code Ab 9324) was used. After dewaxing and hydration, the sections were immersed in 0.001 M sodium citrate buffer with pH 6.0 and subjected to microwave treatment. After cooling, the slides were washed in 0.01 M PBS buffer (pH 7.2) and then immersed in 5% aqueous hydrogen peroxide solution. The sections were washed again and then incubated with 2% bovine serum albumin (Sigma-

Aldrich Co., Saint Louis, Missouri, USA). Then, the sections were incubated overnight in a humidified chamber with anti-IL-6 antibody diluted at 1: 400. Subsequent to washes in 0.01 M PBS buffer, the sections were incubated detection of IL-6, the mouse anti-IL-6 antibody (Abcam, Cambridge Science, UK; code Ab 9324) was used. After washing, peroxidase activity was revealed by the 3,3'-diaminobenzidine chromogen (ImmPACTTM DAB Vector, Burlingame, CA, United States). The sections were counterstained with Carazzi's hematoxylin. As a negative control, the sections were incubated with non-immune serum instead of primary antibody in Labeled StreptAvidin-Biotin kit (Universal Dako LSAB, Dako Inc., Carpinteria, CA, USA; K0675) at room temperature. After washing, peroxidase activity was revealed by the 3,3'-diaminobenzidine chromogen (ImmPACTTM DAB Vector, Burlingame, CA, United States). The sections were counterstained with Carazzi's haematoxylin. As a negative control, the sections were incubated with non-immune serum instead of primary antibody. The number of IL-6 immunopositive cells was calculated with the aid of an image analysis program (Image-Pro Express 6.0, Olympus, Tokyo, Japan). Thus, the number of immunopositive cells/mm^2 of capsule was obtained for each implant.

Immunohistochemical detection of osteocalcin

After antigen recovery as described above, the slides were immersed in 5% aqueous hydrogen peroxide solution for endogenous peroxidase inactivation. After washing, the sections were incubated for 20 min in 2% bovine serum albumin (Sigma-Aldrich Co., Saint Louis, Missouri, USA) and then incubated with rabbit anti-osteocalcin antibody rat (1:200; Sigma-Aldrich Co., Saint Louis, Missouri, USA; code SAB1306277). After 16 hours, at 4ºC in a humidified chamber, the sections were incubated for 1 h in Labeled StreptAvidin-Biotin kit (Universal Dako LSAB, Dako Inc., Carpinteria, CA, USA; K0675) at room temperature. Subsequent to buffer washes, peroxidase activity was revealed by the 3,3'-diaminobenzidine chromogen (ImmPACTTM DAB Vector, Burlingame, CA, United States). The sections were counterstained with Carazzi's haematoxylin. As a negative control, sections were incubated with non-immune serum instead of primary antibody. Thus, the number of immunopositive cells/mm^2 of capsule was obtained for each specimen.

von Kossa reaction and analysis under polarized light

Sections of the capsules surrounding the implants were subjected to the von Kossa method, to detect calcium deposits. The sections were immersed in a 5% silver nitrate solution, for 1 hour, under the action of an incandescent lamp (100 Watts). The slides were washed in distilled water and, then, immersed in a 5% sodium hyposulfite solution. The sections were washed in distilled water and then stained with picrosirius-red and mounted in resinous medium (Permount®, Fisher Scientific, New Jersey, USA) (Viola *et al.* 2012, Silva *et al.* 2015).

Considering that calcite crystals exhibit birefringence when subjected to polarized light (Holland *et al.* 1999), sections close to those subjected to von Kossa were deparaffinized, dehydrated and mounted with Permount mounting media. The unstained sections were analyzed under a microscope light equipped with polarization filters (Olympus, BX51).

Statistical analysis

The statistical analysis was obtained with the aid of the GraphPad Prism 5 software (Jandel Scientific, Sausalito, CA, USA). The data was evaluated by the two-way ANOVA followed by the Tukey test. The level of significance considered was $p \leq 0.05$.

Results

Morphological description, IC number and capsule thickness

The sections stained with HE showed that the capsules of all groups exhibited inflammatory cells, mainly lymphocytes and macrophages, between profiles of blood vessels and the presence of fibroblasts and collagen fibers (Figs. 1A-1J and 2A-2J). Generally, the capsules in the periods of 7 and 15 days showed a predominance of inflammatory cells in relation to fibroblasts, with the exception of the CG group (Figs. 1A-1J). In the periods of 30 and 60 days, the capsules around the implants showed an evident reduction in the presence of inflammatory cells and a marked presence of fibroblasts and collagen fibers (Figs. 2A-2J). However, AHP capsules at 30 and 60 days still exhibited a marked presence of inflammatory cells

(Figs. 2D and 2I). In addition, in the TBC group, particularly at seven days, sealer particles dispersed by the capsules were observed; these particles were generally surrounded by giant cells (Fig. 1C).

As shown in Table 2, the number of ICs at 7 and 15 days was significantly higher ($p < 0.05$) in the capsules around all endodontic materials compared to the control group. At 7 days, significant differences were not found among the CE-1, CE-2 and AHP groups ($p > 0.05$). In the periods of 15 and 30 days, significant differences in the number of ICs were not observed between the CE-1 and TBC groups ($p > 0.05$). In these groups, the number of ICs was significantly lower compared to AHP ($p < 0.05$). At 30 and 60 days, the numerical density of ICs was significantly higher in CE-1 capsules compared to CE-2 ($p < 0.05$), while significant differences were not detected between the CE-2 and CG groups ($p > 0.05$). In all periods, the number of ICs in TBC capsules was significantly lower than in the AHP group ($p < 0.05$).

Regarding the thickness of the capsules (Table 2), all groups showed thicker capsules at 7 days. Over time, the thickness of all groups decreased, and at 60 days, the highest value was observed in AHP group. In the control group, no statistical difference was observed among the experimental periods. At 7 and 15 days, the values of capsule thickness of the CE-2 group were significantly higher than the other groups ($p < 0.0001$). In the 30-day period, no significant differences were detected in the thickness of the CE-1, CE-2 and AHP groups ($p > 0.05$), which showed significantly higher values than the TBC group ($p = 0.0006$). At 60 days, significant differences in capsule thickness were not detected among the CE-1, CE-2, TBC and CG groups while the AHP group showed a significantly higher value compared to the CE-1 ($p < 0.05$), CE- 2 ($p < 0.05$) and TBC ($p < 0.05$) groups.

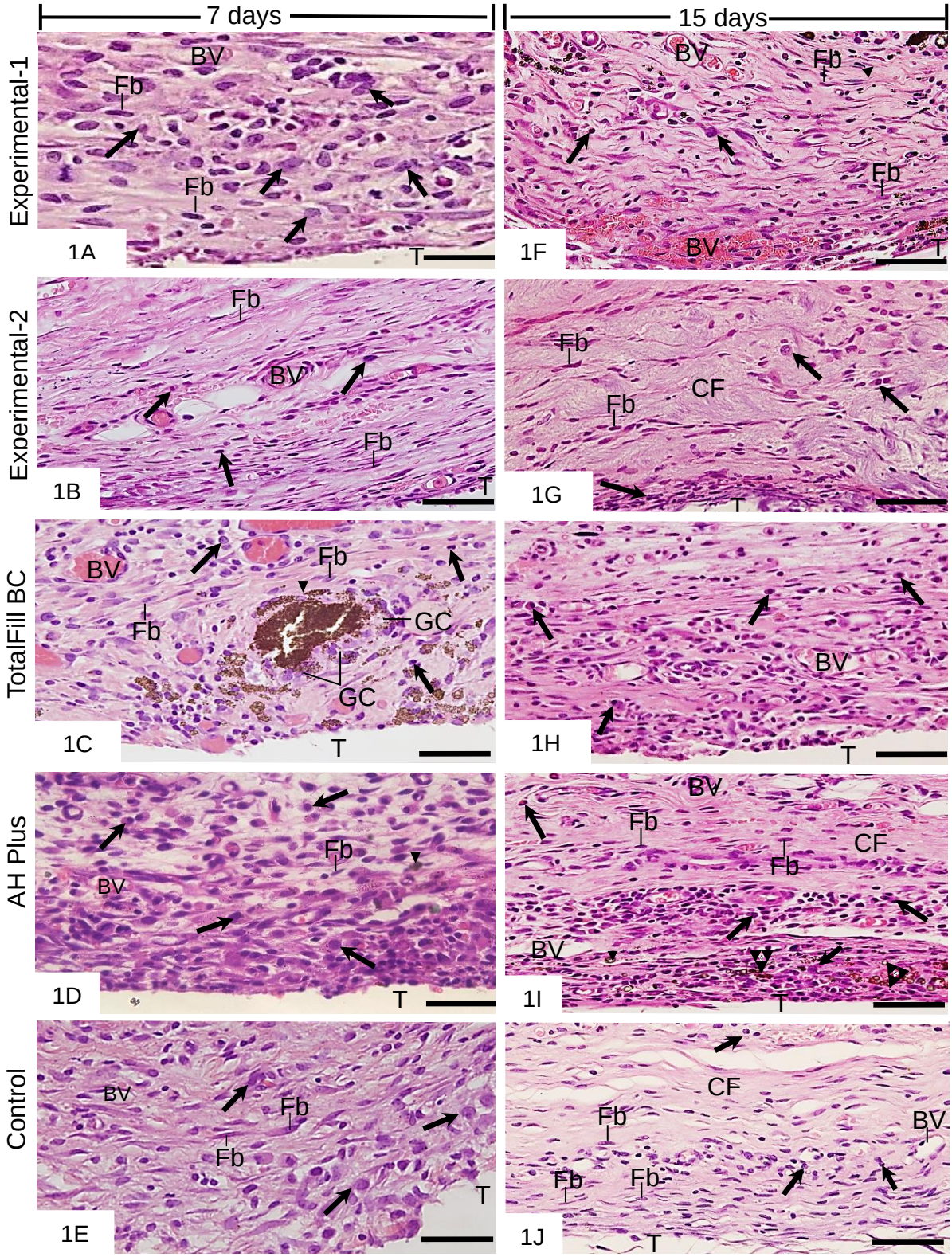

Figure 1: Photomicrographs showing portions of the capsules adjacent to the opening of the implanted tubes (T) in the subcutaneous tissue, at 7 (Figs. 1A-1E) and 15 (Figs. 1F-1J) days. The capsules show numerous inflammatory cells (arrows), mainly lymphocytes, plasma cells and macrophages, among few fibroblasts (Fb). Note that the capsules adjacent to the AHP (Figs. 1D and 1I) have an apparent predominance of inflammatory cells (arrows) compared to the other groups. BV, blood vessels; arrowhead, particles of material; GC, giant cells; CF, collagen fibers. Bars: 18 µm

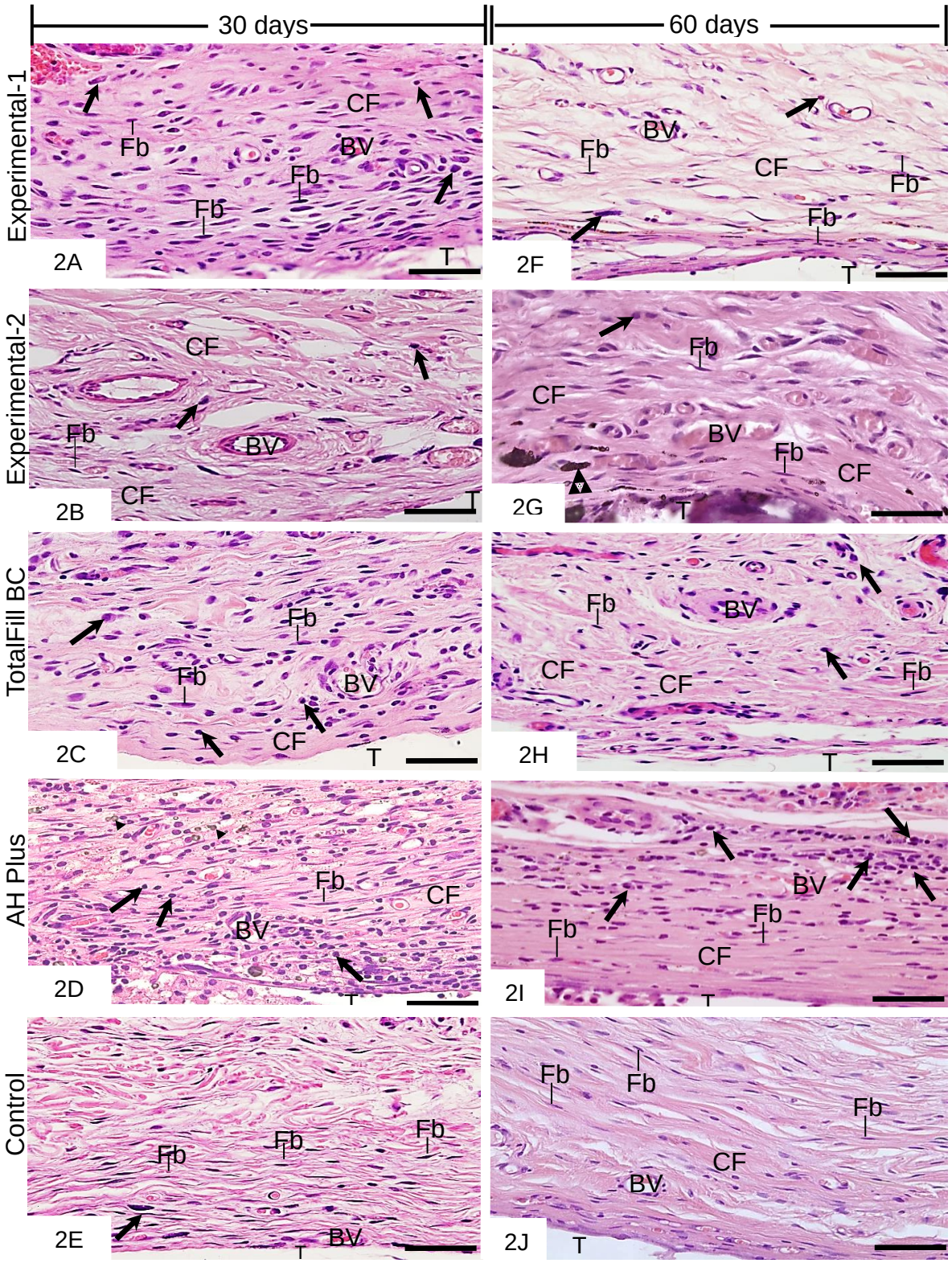

Figure 2: Photomicrographs showing portions of the capsules adjacent to the opening of the implanted tubes (T) in the subcutaneous tissue, at 30 (Figs. 2A-2E) and 60 (Figs. 2F-2J) days. **Figs. 2A-2E**: the capsules exhibit a predominance of fibroblasts (Fb) over inflammatory cells (arrows), except for AHP (Fig. 2D). In this group, several inflammatory cells (arrows) are still observed. **Figs. 2F-2J:** the capsules have typical fibroblasts (Fb) between bundles of collagen fibers (CF); few inflammatory cells (arrows) are seen in the capsules. Note that in the AHP group (Fig. 2I) there are accumulations of inflammatory cells (arrows), mainly, close to the blood vessel (BV). Bv, blood vessels; arrowhead, particles of material. Bars: 18 µm.

Immunohistochemical detection of IL-6

Capsules from all groups exhibited cells with IL-6 positive cytoplasm (in brown). At 7 days, numerous positive cells (inflammatory cells and fibroblasts) were observed in the capsules mainly from the CE-1, TBC and AHP groups; capsules in the CE-2 and CG groups exhibited poor IL-6 labeling (Figs. 3A-3J). An evident reduction in the immunoexpression of IL-6 was observed in the capsules of all groups at 60 days (Figs. 3F-3J).

According to Table 2, the highest values of IL-6-immunolabelled cells were observed in the capsules of all groups at 7 days. In the 7-day period, the number of immunostained cells was significantly lower in the CE-2 groups compared to the CE-1 ($p < 0.0001$) and AHP ($p < 0.0001$) groups. At 15, 30 and 60 days, immunoexpression was significantly lower in the CE-2 capsules than in the CE-1 groups ($p <0.05$) and TBF ($p >0.05$). In these periods, no significant difference was found between the CE-2 and CG groups ($p >0.05$). In all periods, the number of immunopositive cells was significantly higher in the capsules of the AHP group.

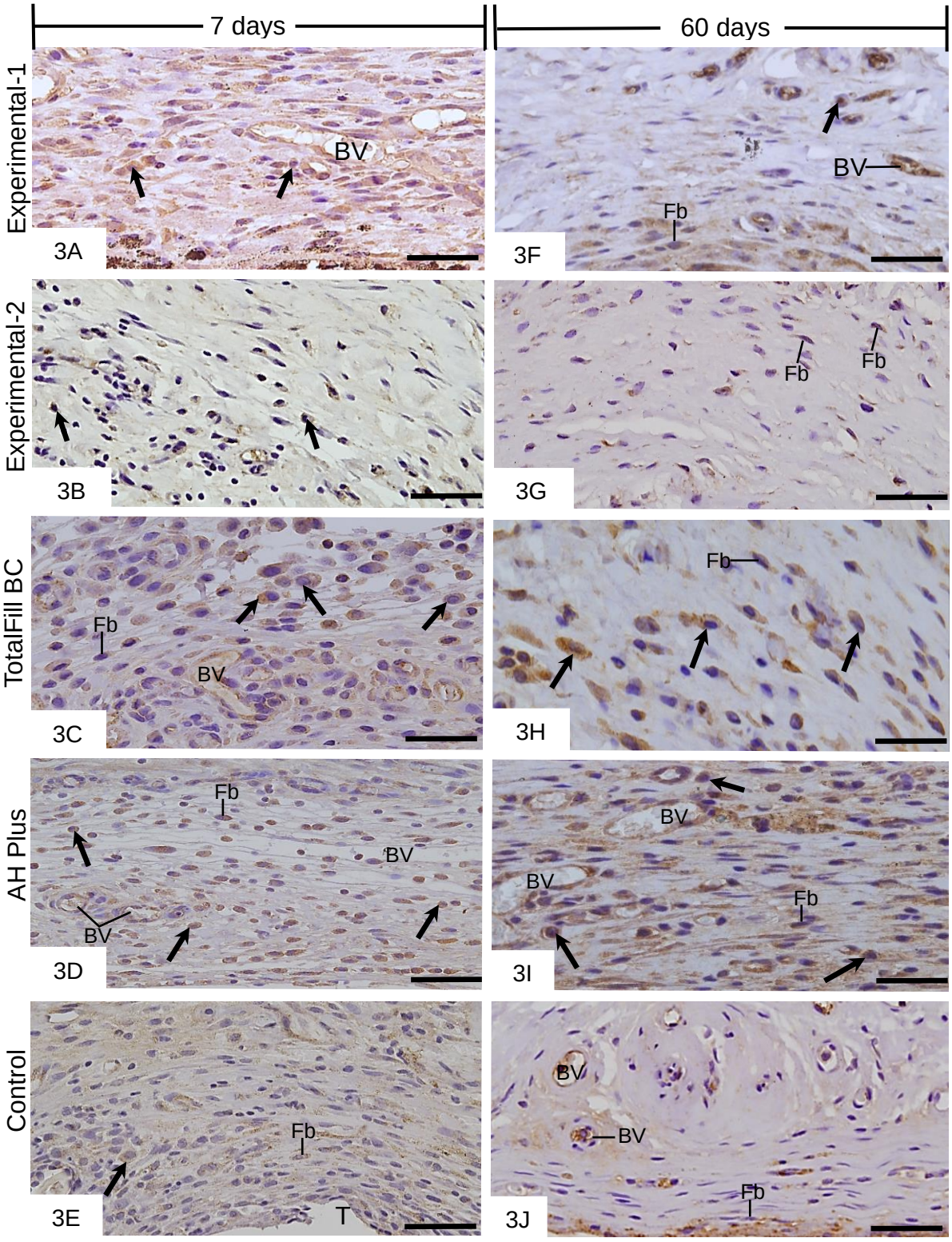

Figure 3: Photomicrographs showing portions of the capsules adjacent to the opening of the tubes implanted in the subcutaneous tissue for 7 (Figs. 3A-3E) and 60 (Figs. 3J-3H) days. The sections were subjected to immunohistochemistry to detect IL-6 (in brown) and counterstained with hematoxylin. **Figs. 3A-3E** - the capsules contain several immunopositive inflammatory cells (arrows) and few fibroblasts (Fb). **Figs. 3J-3H** - inflammatory cells (arrows) and immune-positive fibroblasts (Fb) are present in the capsules after 60 days. Note a weak immunoexpression in the capsules of the control group (Figs. 3E and 3J); at 60 days, the positivity is present in vascular cells and some close to the surface of the capsules. Bv, blood vessels. Bars: 18 µm.

Immunohistochemical detection of osteocalcin

The analysis of the sections showed a differential pattern in the immunopositivity for osteocalcin (in brown) according to the groups. The capsules of the CE-1, CE-2 and TBC groups exhibited some cells with positive cytoplasm. In contrast, immunopositivity to OC was not observed in the capsules of the specimens AHP and CG (Figs. 4A-4J).

Quantitative analysis (Table 2) showed that, in all periods, the number of OC-positive cells was significantly higher in the CE-2 capsules compared to the CE-1 groups (7 and 15 days: $p < 0.0001$; 30 days : $p = 0.2097$; 60 days: $p < 0.0001$) and TBC (7 and 15 days: $p < 0.0001$; 30 days: $p = 0.0195$; 60 days: $p < 0.0001$). In all periods, no significant differences were detected between the CE-1 and TBC groups (7 days: $p = 0.9997$; 15 days: $p = 0.9768$; 30 days: $p = 0.8647$; 60 days: $p = 0.9997$). In the CE-1 and TBC groups, a significant increase in the number of immunopositivity was seen in the period of 30 days compared to the 15 days ($p = 0.0195$). From 30 to 60 days, no significant differences were detected in both groups. Significant differences were not detected in the CE-2 group over time ($p = 0.9768$).

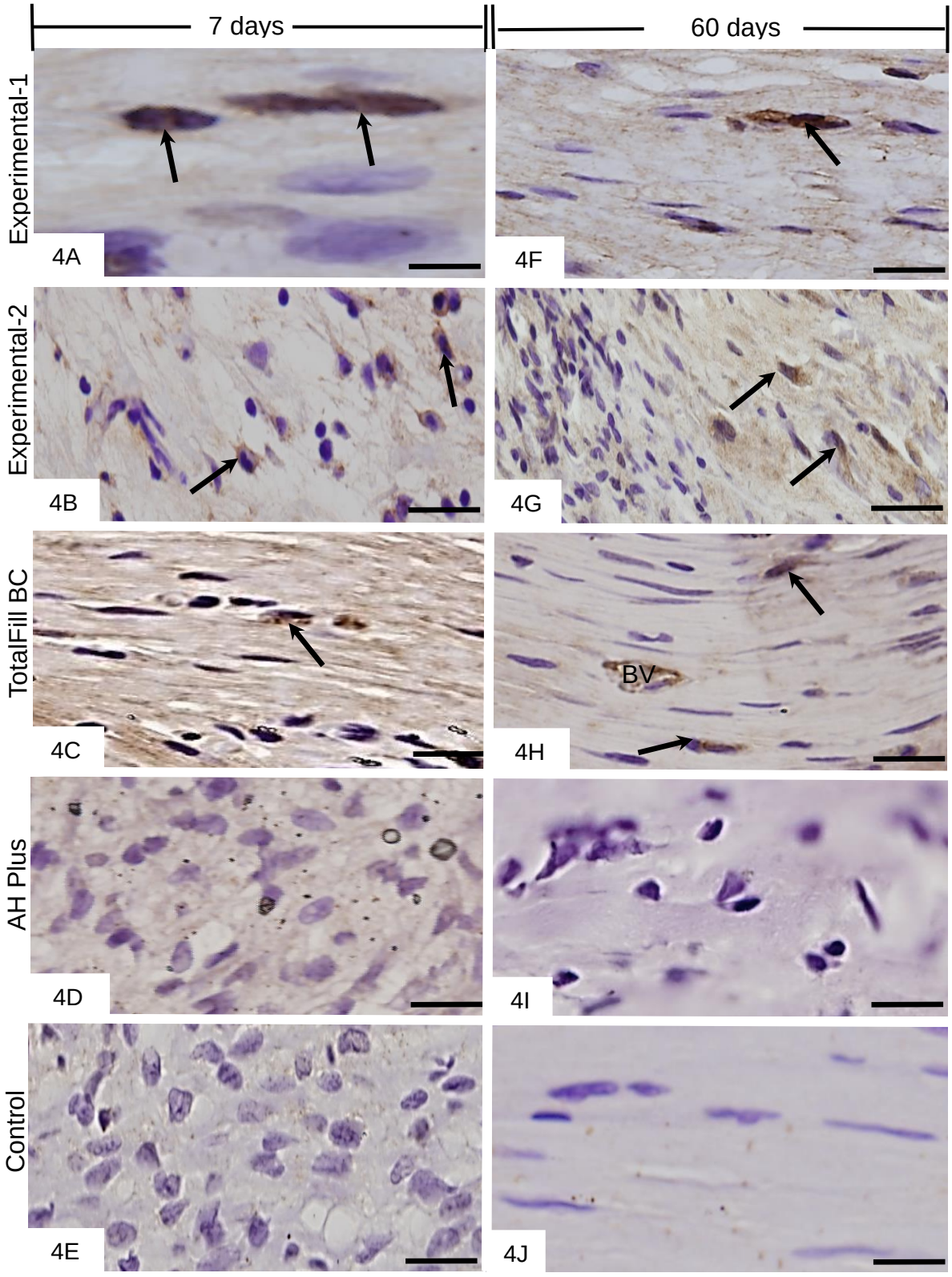

Figure 4: Photomicrographs showing portions of the capsules adjacent to the opening of the tubes implanted in the subcutaneous tissue for 7 (Figs. 4A-4E) and 60 (Figs. 4F-4J) days. The sections were subjected to immunohistochemistry to detect osteocalcin (brown) and counterstained with haematoxylin. The capsules of the Experimental-1, Experimental-2 and TotalFill BC groups contain immunopositive fusiform / elliptical cells (arrows), while the capsules of the AH Plus and control groups do not show immunoreactivity (Fig. 4D, 4E, 4I and 4J). BV, blood vessel. Bars: 18 µm.

Table 2 - Capsule thickness (µm), number of inflammatory cells (IC) per mm^2, number of IL-6 immunopositive cells and number of osteocalcin-positive (OC) cells per mm^2. Groups: Experimental (CE-1 and CE-2), TotalFill BC (TBC), AH Plus (AHP) and Control (CG) after 7, 15, 30 and 60 days. Mean (standard deviation).

		CE-1	CE-2	TBC	AHP	CG
7 days	**Thickness**	257 ± 26$^{a;1}$	487 ± 26$^{b;1}$	259 ± 24$^{a;1}$	339 ± 65$^{c;1}$	149 ± 86$^{d;1}$
	IC	775 ± 57$^{a;1}$	716 ± 46$^{a;1}$	583 ± 65$^{b;1}$	787 ± 73$^{a;1}$	251 ± 35$^{c;1}$
	IL-6	634 ± 6$^{a;1}$	364 ± 6$^{b;1}$	447 ± 4$^{b;1}$	762 ± 6$^{c;1}$	212 ± 5$^{d;1}$
	OC	20 ± 3$^{a;1}$	66 ± 9$^{b;1}$	12 ± 3$^{a;1}$	-	-
15 days	**Thickness**	230 ± 28$^{a;1}$	382 ± 31$^{b;1}$	167 ± 21$^{a;2}$	232 ± 20$^{a;2}$	182 ± 11$^{a;1}$
	IC	351 ± 36$^{a;2}$	577 ± 27$^{b;2}$	340 ± 80$^{a;2}$	632 ± 94$^{b;2}$	235 ± 15$^{c;1}$
	IL-6	370 ± 5$^{a;2}$	227 ± 20$^{b;2}$	331 ± 4$^{a;2}$	520 ± 6$^{c;2}$	171 ± 4$^{b;2}$
	OC	18 ± 3$^{a;1}$	79 ± 11$^{b;1}$	13 ± 3$^{a;1}$	-	-
30 days	**Thickness**	221 ± 23$^{a;1}$	216 ± 11$^{a;2}$	159 ± 24$^{b;2}$	223 ± 24$^{a;2}$	107 ± 04^{b,1}
	IC	321 ± 67$^{a;2}$	255 ± 27$^{b;3}$	290 ± 37$^{a;2}$	443 ± 39$^{c;3}$	176 ± 25^{b,2}
	IL-6	268 ± 4$^{a;3}$	170 ± 1$^{b;2}$	223 ± 3^{a,3}	451 ± 6$^{c;3}$	136 ± 2^{b,2}
	OC	42 ± 8$^{a;2}$	62 ± 5$^{b;1}$	33 ± 6$^{a;2}$	-	-
60 days	**Thickness**	162 ± 29$^{a;2}$	146 ± 6$^{a;3}$	176 ± 88$^{a;2}$	191 ± 43$^{b;3}$	104 ± 20^{a,1}
	IC	233 ± 40$^{a;3}$	95 ± 7$^{b;4}$	129 ± 32$^{b;3}$	321 ± 90$^{c;4}$	68 ± 11^{b,3}
	IL-6	210 ± 5$^{a;3}$	99 ± 9$^{b;3}$	160 ± 4^{a,3}	275 ± 7$^{c;4}$	81 ± 2^{b,3}
	OC	33 ± 7$^{a;2}$	81 ± 6$^{b;1}$	35 ± 5$^{a;2}$	-	-

Comparison between groups in the same period is indicated by superscript letters on the line.
Same letters = no statistically significant difference.
The comparison between periods in the same group is indicated by superscript numbers in the columns; same numbers = no statistically significant difference.
Tukey's test ($p \leq 0.05$)

von Kossa reaction and analysis under polarized light

The CE-1, CE-2, TBC and AHP cements showed black / brown structures, positive for the von Kossa method in all observed periods, dispersed between the collagen fibers and on the surface of the capsules adjacent to the materials (Figs.

5A-5C and 5I-5K). AH Plus showed small positive particles dispersed between the tissue components (Figs. 5D and 5L).

The non-colored sections analyzed under the microscope with polarized light revealed birefringent structures in regions compatible with the results obtained with von Kossa (Figs. 5E-5H and 5M-5P). Positive and birefringent structures were not found in the capsules of the control group (data not shown).

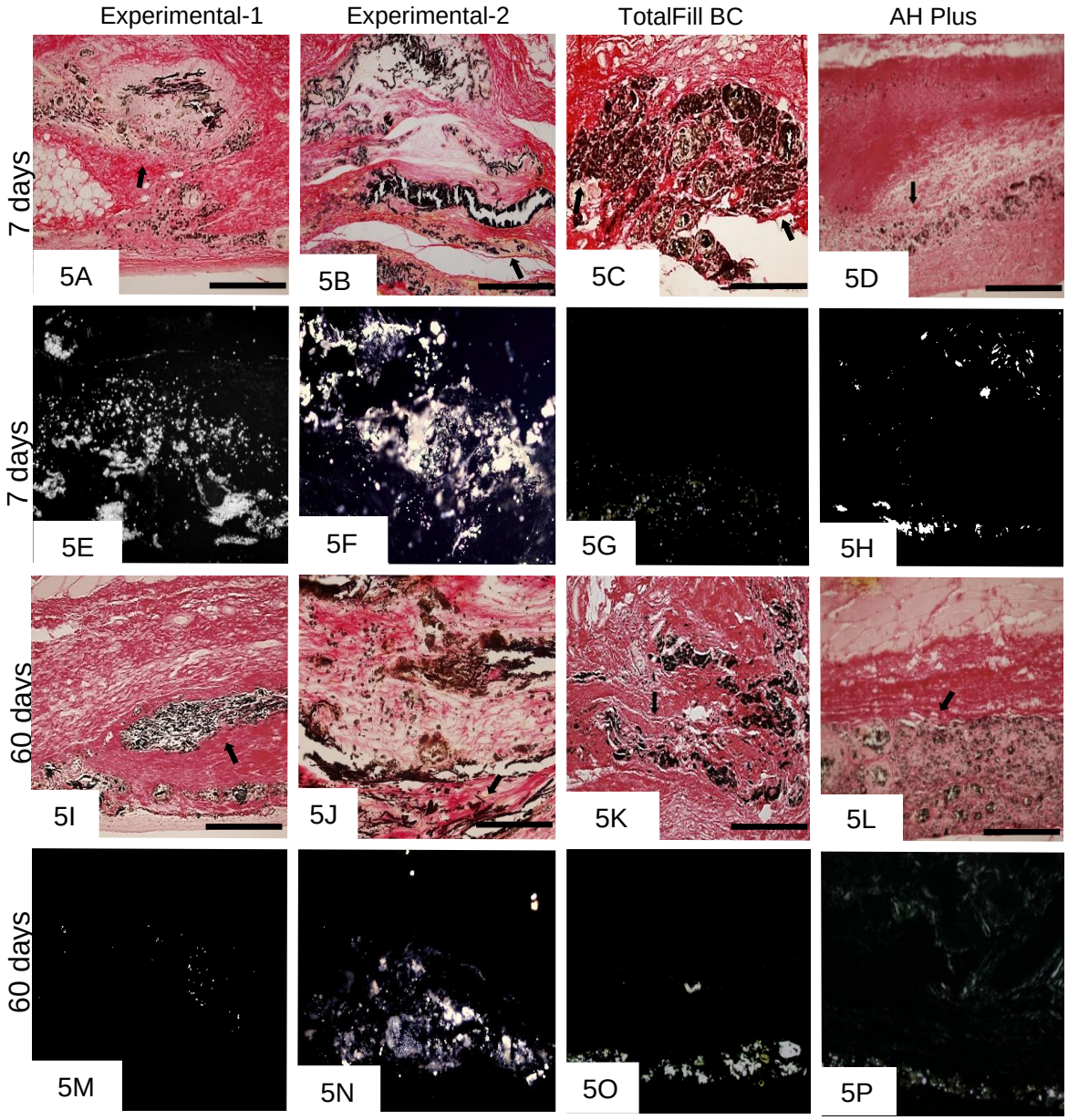

Figure 5: Photomicrographs showing portions of capsules adjacent to the opening of the tubes implanted in the subcutaneous connective tissue after 7 (Fig. 5A-5H) and 60 (Fig. 5I-5P) days. Figs. 5A-5D and 5I and 5L - sections were submitted to Kossa reaction and counterstained with picrosirius red. The capsules of the Experimental-1, Experimental-2, TotalFill BC and AH Plus groups exhibit positive structures to von Kossa method (black / brown). Figs. 5E-5H and 5M-5P - uncolored sections analyzed with a polarization microscope. Birefringent deposits are present in the capsules adjacent to the materials. Bars: 36 µm.

Discussion

The null hypothesis was rejected, as the experimental sealers (CE-1 and CE-2) promoted less inflammation when compared to the AH Plus sealant and had bioactive potential through osteocalcin marking, which does not occur for AH Plus sealer.

At 7 days, TotalFill BC (TBC; KFG FKG Dentaire AS, Switzerland) had fewer inflammatory cells than other materials, followed by experimental sealants. The initial inflammatory response can be justified by the alkaline pH resulting from the composition based on calcium silicates and formation of calcium hydroxide, promoting the recruitment of inflammatory cells (Vosoughhosseini *et al.* 2008, Silva *et al*. 2015, Andrade *et al.* 2018). CE-2 induced the formation of thicker capsules compared to CE-1, justified by the high alkalinity provided by the composition of calcium silicates and association with calcium-based antimicrobials (Gomes-Filho *et al.* 2009, Viola *et al.* 2012).

The TBC sealer showed greater particle leakage to the surrounding tissues, due to its high solubility and flow (Tanomaru-Filho *et al.* 2017). AH Plus sealer, based on epoxy resin demonstrated an inflammatory reaction with a thick capsule and a greater number of inflammatory cells, which may be related to its resin-based composition (Cintra *et al*. 2017, Andrade *et al.* 2018), since the inflammatory process is a result of the response to the elements of the materials that stimulate the production and release of cytokines (Saraiva *et al.* 2018, Fonseca *et al.* 2019). The control had the lowest number of inflammatory cells, and the inflammatory cells presented were justified by the trauma of the surgical procedures (Viola *et al.* 2012).

At 15 and 30 days, the TBC and CE-1 sealants showed fewer cells positive for IC and IL-6 when compared to AH Plus. The CE-2 and AH Plus at 15 days showed higher IC. However, compared to IL-6, CE-2 showed values significantly similar to the control group. The introduction of components in tricalcium silicate sealants, such as the antimicrobial component, can directly influence its properties, altering the induced tissue reaction and the bioactive potential (Koutroulis *et al.* 2019). All materials showed a gradual and significant reduction in the number of inflammatory cells and IL-6 over the periods, a cytokine closely associated with the intensity of the inflammatory reaction induced by endodontic materials implanted in the subcutaneous tissue (Silva *et al*. 2015, Fonseca *et al.* 2016, Saraiva *et al.* 2018).

Regarding immunostaining for IL-6, there were no differences between TBC and CE-1, with AHP showing the highest values, in all periods. The relationship between interleukin-6 (IL-6) and the inflammatory reaction is widely studied in subcutaneous mice as a mediator of the host's response to an aggression. The decrease in IL-6 is associated with a reduction in the inflammatory process in calcium silicate capsules in the tissues of rats (Silva *et al.* 2015, Fonseca *et al.* 2016, Andrade *et al.* 2018, Saraiva *et al.* 2018). At 60 days, there was a reduction in the number of inflammatory cells and capsule thickness in all groups. The CE-2 sealer showed the lowest number of inflammatory cells among the bundles of collagen fibers, with a greater amount of fibroblasts, and a more organized tissue, showing that the addition of antimicrobials favored the biological response. Blattes *et al*. (2017) demonstrated that calcium hypochlorite induced adequate migration and cell viability. Among the groups with materials, the capsules around the CE-2 and the TBC had the lowest incidence of inflammatory cells, being higher only in the control group. On the other hand, the inflammatory infiltrate in the AHP capsules was significantly higher compared to CE-2 and CG. Rodriguez-Lozano *et al.* (2017) concluded that TotalFill BC Sealer has less cytotoxicity than AH Plus. The CE-1 sealer showed a lower number of inflammatory cells when compared to AH Plus, showing an organized tissue with collagen fibers and fibroblasts. The presence of fibroblasts in the 60-day period indicates a reduction in the inflammatory process and progress in repair (Silva *et al.,* 2017, Fonseca *et al.* 2019). Zordan-Bronzel *et al.* (2019) demonstrated that the experimental sealer used in this work was cytocompatible with potential for use as a filling sealer.

AH Plus had the highest number of inflammatory cells and IL-6 immunopositive cells in all periods. However, the number of these cells decreased significantly over the periods. Cintra *et al*. (2017) carried out a subcutaneous study of rats comparing the inflammatory reaction promoted by EndoFill, AH Plus and Sealer Plus sealers, concluding that EndoFill sealer induced severe inflammation, and AH Plus sealer was considered biocompatible. Zhou *et al.* (2015) reported that the cytotoxic effect of AH Plus immediately after manipulation was classified as moderate to severe, resulting from the presence of amines used to accelerate polymerization. Saraiva *et al.* (2018) in a study with subcutaneous rats concluded that AH Plus sealer induced greater IL-6 marking when compared to MTA Plus and MTA Fillapex sealers.

Regarding the thickness of the capsules, there was no statistical difference at 60 days between CE-1, CE-2, TBC and the control group that showed the lowest values, being justified by the study by Peng *et al*. 2011 who concluded that the tricalcium silicate component induces cell proliferation.

The bioactive potential of the sealers was evaluated by the von Kossa histochemical technique and by the immunohistochemical reaction of osteocalcin being one of the non-collagen proteins with the ability to bind to the calcium that participates in the beginning of the mineralization process. Osteocalcin has been used as a marker for mature osteoblast (Viola *et al.* 2012). A bioactive material is one that promotes biological response at the material interface, promoting the formation of regeneration tissue, through the development of the formation of calcium phosphate crystals and the formation of an apatite layer (Niu *et al.* 2014). Calcium silicate-based sealers have the property of releasing calcium that favors mineralization through the differentiation of cells belonging to dental pulp (Koutroulis *et al*. 2019).

The CE-1, CE-2, TBC and AHP sealers presented irregular structures with calcium deposits in the capsules adjacent to the tubes. Zordan-Bronzel *et al.* (2019) demonstrated that TBC and CE-1 showed greater formation of mineralized nodules, which can be justified by the presence of calcium silicates that act as an inducer of cell differentiation and proliferation (Peng *et al.* 2011, Mestieri *et al.* 2017, Zordan-Bronzel *et al.* 2019). The analysis under polarized light of the capsules of these sealers showed birefringent structures, confirming the results obtained by the von Kossa method. AHP exhibited birefringent structures in greater quantity in the period of 7 days when compared to the period of 60 days, in agreement with the study by Candeiro *et al.* (2012), who concluded that AH Plus sealer released less calcium when compared to bioceramic sealer. Borges *et al.* (2012) reported that AH Plus sealers releases significantly lower calcium ion values when compared to bioceramic sealers. The CE-1, CE-2 and TBC sealers showed positive immunostaining for osteocalcin in all periods, showing higher values in the periods of 30 and 60 days, pointing to the bioactive potential of these sealants and, consequently, showing potential for tissue repair. The CE-1 and TBC sealers did not present statistical difference between them, compatible with the study by Koutroulis *et al.* (2019) who concluded that the materials Biodentine and TotalFill BC show greater release of calcium ions over time. CE-2 showed higher values in all periods analyzed, which

can be justified by its composition that joins calcium-based components with calcium-based antimicrobial, stimulating tissue repair. There is evidence that calcium silicate sealers in the hydration process, when in contact with tissue fluids, interact with the existing phosphates producing hydroxyapatite (Niu *et al.* 2014).

In the present study, experimental sealers showed a significant reduction in the inflammatory process, since it was observed a significant reduction in the number of inflammatory cells and in the immunoexpression of IL-6 in the capsules, the Experimental Sealer-2 exhibiting the smallest chemical process, being similar statistically to the control group at 60 days. In addition, the calcium structures located next to the capsules and marked with immunoglobulin for osteocalcin exhibited the bioactive potential of CE-1 and CE-2, with the sealer experiment-2 with the addition of antimicrobials or with the greatest bioactive potential, the substances and potential for its use as filling sealer.

Conclusion

In the present study, calcium silicate-based sealants demonstrated biocompatibility and bioactive potential. CE-2, showing greater biocompatibility and greater bioactive potential, can be considered for clinical use as filling materials for the root canal.

Acknowledgements

The authors thank Mr. Pedro Sérgio Simões for kind help and technical assistance. We thank CAPES (Brazil) for the fellowship to Evelin Carine Alves Silva (CAPES code: 001). This study was supported by FAPESP (Brazil) (Process nº 2017/14305-9).

Conflict of interest

The authors deny any conflict to interests to this study

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3.2 Publicação 2*

Biocompatibility and bioactive potential of new calcium silicate-based endodontic sealers: Bio-C Sealer and Sealer Plus BC

ABSTRACT

Introduction: Bio-C Sealer (BC) and Sealer Plus BC (SPBC) are new bioceramic endodontic sealers. The aim of the study was to evaluate the tissue reaction and bioactive potential of BC and SPBC sealers in comparison to AH Plus (AHP) sealers in subcutaneous of rats. **Methods**: The tissue reaction was evaluated by implanting polyethylene tubes filled with materials and empty as a control group. After 7, 15, 30 and 60 days, adjacent tubes and tissues were removed and inflammatory cell counts / mm^2 (IC), immunostaining interleukin-6 (IL-6), osteocalcin (OC) and von Kossa to identify deposits of calcium were performed. Data were submitted to ANOVA and Tukey tests, with a significance of 5%. **Results**: At 7 days, SPBC had a lower IC than BC ($p = 0.0225$). AHP exhibited greater labeling for IL-6 ($p < 0.0001$). After 15 days, BC showed lower IC and IL-6 when compared to other materials. At 30 days, SPBC and AHP showed higher values for IC ($p = 0.0791$). After 60 days, calcium silicate sealers did not show statistical difference between themselves ($p = 0.8949$) for both IC and IL-6, with values lower than AHP. The materials showed positive structures to von Kossa. BC exhibited osteocalcin marking in all periods. SPBC showed no marking at 7 days, at 60 days it was lower than BC ($p = 0.076$). AH Plus and the control group did not exhibit osteocalcin marking. **Conclusions**: Bio-C Sealer and Sealer Plus BC sealers are biocompatible and have bioactive potential.

Keywords
Calcium silicate, biocompatibility, endodontics.

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* Artigo nas normas do periódico *Journal of Endodontics* para o qual pretende-se submeter.

INTRODUCTION

Calcium silicate-based sealers were developed to improve properties and reduce the possibility of presence of heavy metals for Mineral Trioxide Aggregate (MTA) based materials[1]. EndoSequence BC and TotalFill BC Sealer (KFG FKG Dentaire AS, Switzerland) are endodontic sealers composed of calcium silicates, zirconium oxide, monobasic calcium phosphate, alcohol hydrogen, available in syringes. These materials have biocompatibility, release calcium ions, present high pH, dimensional stability and radiopacity[2,3,4]. Tricalcium silicate with zirconium oxide in the subcutaneous tissue of rats showed high number of fibroblasts and higher collagen content when used in MTA[5.]

New endodontic sealers based on calcium silicates and ready have been developed. Among them, the Bio-C Sealer (Angelus, Brazil) is composed of calcium silicates, calcium aluminum, calcium oxide, zirconium oxide, iron oxide, silicon dioxide and dispersing agent. Compared to TotalFill BC and AH Plus, Bio-C Sealer exhibits the shortest setting time, alkaline pH and highest solubility with low dimensional change[6]. When compared to Bio-C Repair in a study using mesenchymal cells of the periodontal ligament, the Bio-C Sealer presents the lowest cell viability in all the tested dilutions, however, it was better than AH Plus[7]. The Sealer Plus BC (SPBC; MK Life, Brazil) is composed of calcium disilicate, nanoparticulate calcium trisilicate and zirconium oxide. This sealer has an alkaline pH, releases calcium ions and adequate setting time and radiopacity. When compared to AH Plus (Dentsply De Trey Gmbh, Konstanz, Germany), Sealer Plus BC showed greater solubility, less radiopacity and higher pH[8]. When compared to MTA Fillapex and AH Plus, Sealer Plus BC showed greater biocompatibility in the subcutaneous of rats, being similar to the control in the period of 30 days[9].

In view of the new calcium silicate endodontic sealants ready for use, the objective of this study was to evaluate the biocompatibility and bioactive potential of these two new sealants, comparing them to AH Plus as a positive control. The null hypothesis is that the difference between the compositions of the materials does not interfere with the reaction induced in the subcutaneous tissue of rats.

MATERIALS AND METHODS

The materials used in this study, their manufacturers, compositions and proportions are included in Table 1.

Experimental procedure

The research protocol was approved by the Ethical Committee for Animal Research of the São Paulo (CEUA 35/2018). Forty adult male Holtzman rats (*Rattus norvegicus albinus*) were used and distributed in four groups (n = 6): sealers and control group. The materials were inserted in the polyethylene tubes and placed into dorsal subcutaneous sites. Four tubes were inserted per animal, one from each group. The animals were anesthetized with ketamine hydrochloride (80 mg/kg body weight, Virbac do Brasil Indústria e Comércio Ltda., São Paulo, SP, Brazil) and xylazine hydrochloride (8 mg/kg body weight, União Química, São Paulo, SP, Brazil) administered intraperitoneally. After 7, 15, 30 and 60 days, the animals were sacrificed with anesthetic overdose, the implants with adjacent tissues were removed.

Table 1 - Endodontic sealers used

Sealers	Composition	Manufacturers	Proportion
Bio C- Sealer (BC)	Calcium silicates, calcium aluminate, calcium oxide, zirconium oxide, iron oxide, silicon dioxide, dispersing agente	Angelus,Londrina,Brasil	Ready to use
Sealer Plus BC (SPBC)	Calcium disilicate, nanoparticulate calcium trisilicate, zirconium oxide	MK Life, Porto Alegre, Brasol.	Ready to use
AH Plus (AHP)	*Paste A*: epoxy bisphenol-A resin and epoxy bisphenol-F, calcium tungstate (TC), zirconium oxide (Ozr), silica, iron oxide. *Paste B*: dibenzyl-diamine, aminoadamantane, TC, OZr, silica, silicone.	Dentsply DeTrey GmbH, Konstanz, Alemanha	1g: 1g (folder / folder)

Histological procedures

The implants with the surrounding tissues were removed and immersed for 72 hours in a 4% formaldehyde buffered solution at pH 7.2. After fixation, the specimens were dehydrated, diaphanized, immersed in liquid paraffin (60ºC) and embedded in paraffin. Longitudinal sections with 6 μm thickness were obtained. Non-serial sections were stained with hematoxylin and eosin (HE) to estimate the number of

inflammatory cells in the capsules and thickness of the capsules adjacent to the opening of the tubes.

Numerical density of inflammatory cells

Quantitative analysis was performed on the capsules of all implants and for each specimen three non-serial HE-stained sections were used. The images of portions of the capsules, in their capsule/material interface, were captured using a camera attached to the light microscope. In each captured image, the number of inflammatory cells in a standard area of 0.09 mm^2 of the capsule adjacent to the opening of the implanted tubes was computed (Andrade *et al.* 2018[10], Viola *et al.* 2012[11], Silva *et al.* 2015[12]).

Thickness of capsules

The thickness (in μm) of the capsules adjacent to the implanted tubes was measured. Three images of non-serial sections stained with HE of each specimen were captured. The thickness of the capsules was estimated in the middle portion from its surface adjacent to the material to its limit with the adjacent tissues. After obtaining the values, the average value was calculated from the measurements obtained from the three sections for each specimen.

Immunohistochemical detection of IL-6

For the detection of IL-6, the sections were incubated overnight in a humidified chamber with mouse anti-IL-6 antibody (1:400 Abcam, Cambridge Science, UK; code Ab 9324,). Subsequently, the sections were incubated in Labeled StreptAvidin-Biotin kit (Universal Dako LSAB, Dako Inc., Carpinteria, CA, USA; K0675). After washing, peroxidase activity was revealed by the 3,3'-diaminobenzidine chromogen (ImmPACTTM DAB Vector, Burlingame, CA, United States) and the sections were counterstained with Carazzi's hematoxylin.

Immunohistochemical detection of osteocalcin

The sections were incubated with rabbit anti-osteocalcin antibody (1:200; Sigma-Aldrich Co., Saint Louis, Missouri, USA; code SAB1306277). After 16 hours,

in a humidified chamber, the sections were incubated in Labeled StreptAvidin-Biotin kit (Universal Dako LSAB, Dako Inc., Carpinteria, CA, USA; K0675). Subsequent to buffer washes, peroxidase activity was revealed by the 3,3'-diaminobenzidine chromogen (ImmPACTTM DAB Vector, Burlingame, CA, United States) and the counterstaining was performed with Carazzi's hematoxylin.

von Kossa reaction and analysis under polarized light

The sections were immersed in a 5% silver nitrate solution for 1 hour and, subsequently, the sections were immersed in a 5% sodium hyposulfite solution. The sections were stained with picrosirius-red and mounted in resinous medium (Permount®, Fisher Scientific, New Jersey, USA) (Viola *et al.* 2012[11], Silva *et al.* 2015[12]).The unstained sections close to those subjected to von Kossa were analyzed under a light microscope equipped with polarization filters (Olympus, BX51).

Statistical analysis

The statistical analysis was obtained with the aid of the GraphPad Prism 5 software (Jandel Scientific, Sausalito, CA, USA). The data were evaluated by the two-way ANOVA followed by the Tukey test. The level of significance considered was $p \leq 0.05$

RESULTS

Numerical density of inflammatory cells

At 7 days SPBC showed lower values than BC ($p = 0.0225$) (Fig. 1A and 1B; Table 2). After 15 days, BC showed a lower IC value than SPBC and AHP (Table 2; Fig 1E, 1F and 1G). At 30 days, there was no statistical difference between SPBC and AHP in the number of IC ($p = 0.0791$) (1J and 1K). In the 60-day period, BC and SPBC showed no statistical difference between them ($p = 0.8949$). AHP presented the highest values when compared to other groups ($p < 0.0001$) (Fig. 1K and Table 2).

Thickness of the capsules adjacent to the implants

The capsules exhibited a moderate inflammatory reaction. At 7 days BC and SPBC showed no statistical difference between them ($p = 0.9962$). At 15 and 30 days there was no statistical difference between the BC and AHP groups ($p = 0.5075$), SPBC exhibited the highest values. After 60 days, all materials showed a reduction in the thickness of the capsules, with no statistical difference between them (Table 2).

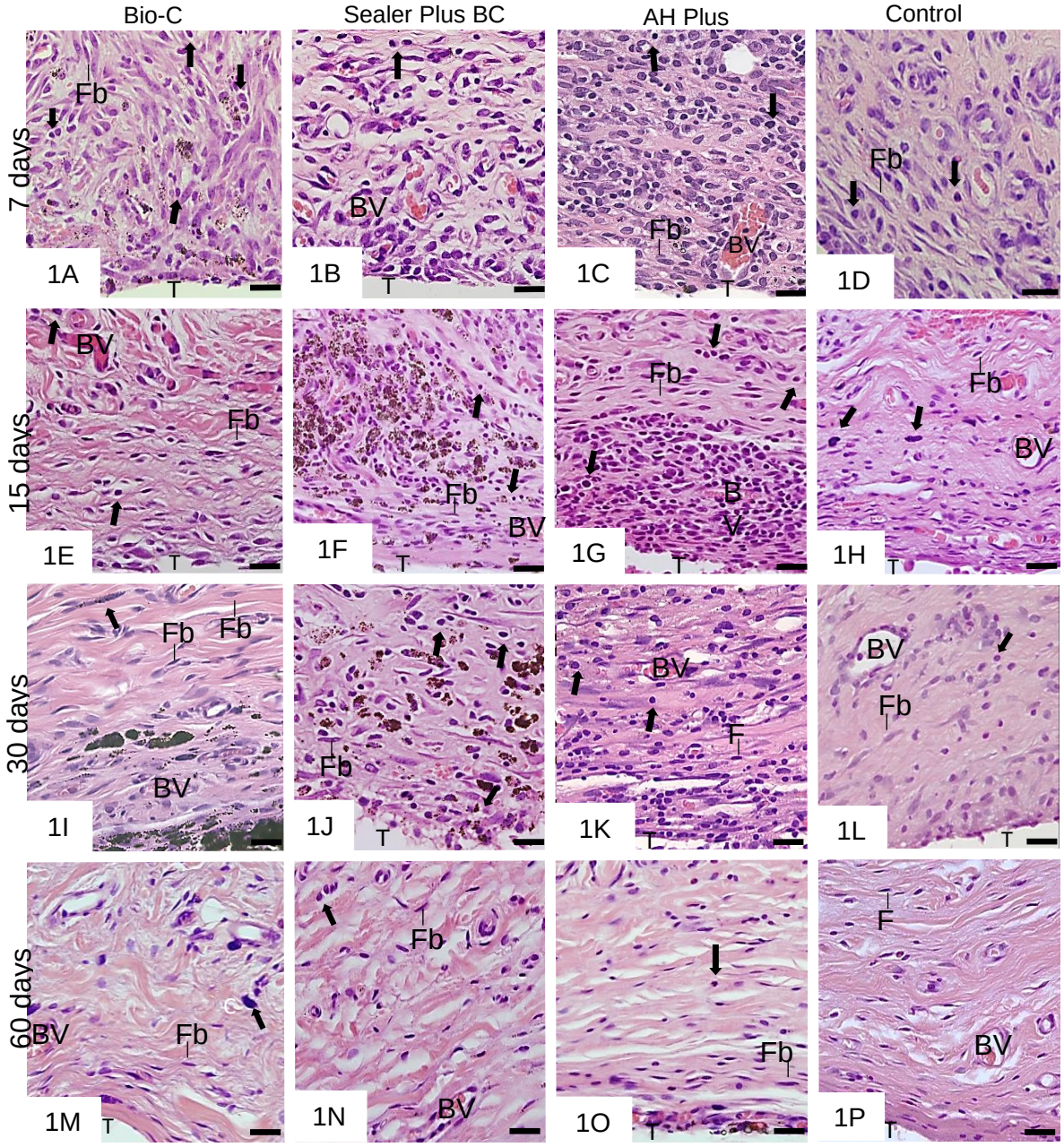

Figure 1: Photomicrographs of the sections showing portions of the capsules adjacent to the opening of the implanted tubes (T) after 7, 15, 30 and 60 days of implantation. The capsules show numerous inflammatory cells, mainly lymphocytes, plasma cells and macrophages. In the capsules of the control group (Fig. 1D), the inflammatory cells (arrows) have a lower amount, with an evident presence of fibroblasts (Fb). The capsules of the AH Plus and BC group show a greater number of inflammatory cells (Fig. 1A and 1C). Figures 1E to 1H present capsules after 15 days with a reduction in the total number of cells. The AH Plus group exhibited a greater number of cells (Fig 1G). At 30 days, the capsules of AH Plus (Fig. 1K) and SPBC (1J) exhibit a greater number of inflammatory cells, compared to the other groups, while BC (Fig. 1I) already have a lower amount of inflammatory cells. The control group (Fig. 1L) has fibroblasts arranged between the collagen fibers. Figs 1M-1P - show capsules after 60 days. The capsules of groups BC (Fig. 1M) and SPBC (Fig. 1N) exhibit mainly fibroblasts (Fb) located between bundles of collagen fibers. The AH Plus sealer capsule (Fig. 1O) has more inflammatory cells compared to the other groups. Fb, fibroblasts; BV, blood vessels. Bars: 18 µm.

Immunohistochemical detection of IL-6

At 7 days, the number of IL-6-immunostained inflammatory cells (Table 2) was significantly higher compared to the other periods ($p < 0.0001$). AHP exhibited the highest values ($p < 0.0001$) (Fig 2C). At 15 and 30 days, BC exhibited less immunolabelling when compared with SPBC ($p = 0.0054$ and $p = 0.0187$, respectively). At 60 days, BC, SPBC and control showed the lowest values (Figs 2I, 2J and 2L), with no difference between BC and SPBC ($p = 0.3867$). The AHP presented the highest values in all the periods ($p < 0.0001$).

Immunohistochemical detection of osteocalcin

According to Table 2, only the BC group exhibited immunolabelling for osteocalcin at 7 days (Fig.2E). At 15 and 30 days, BC and SPBC groups had immunopositive cells. At 60 days, BC showed a higher number of immunolabelled cells compared to SPBC ($p = 0.0076$; Fig 2M and 2N). At all periods, the AH Plus sealer and the control group did not show immunolabelling (Fig 2G, 2H, 2O and 2P).

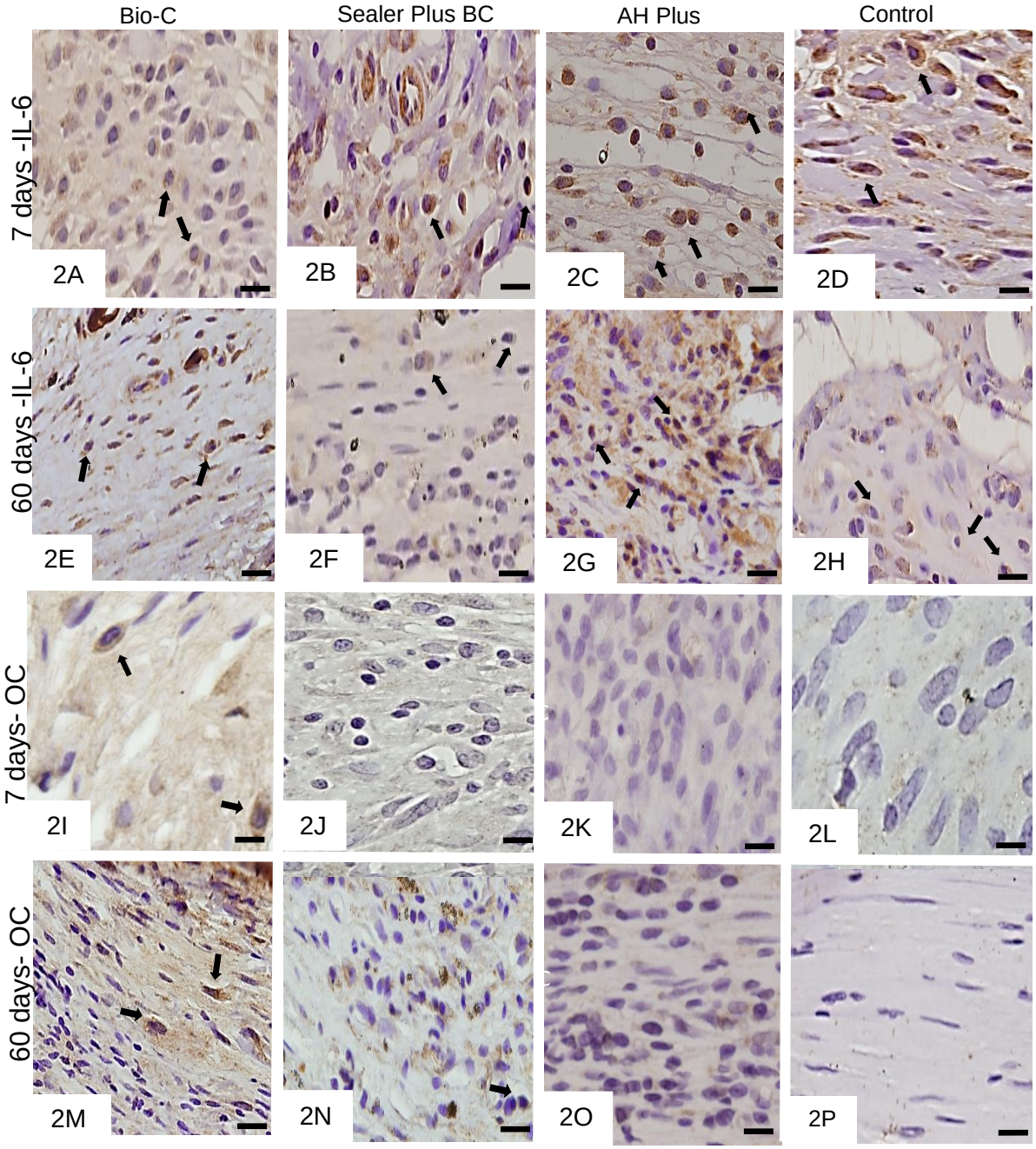

Figure 2: Photomicrographs showing portions of the capsules adjacent to the opening of the subcutaneous implanted tubes for 7 (Figs. 2A-2D) 60 (2E-2H) IL-6 and 7 days (Figs. 2I-2L) 60 (2M-2P) OC. The sections were subjected to immunohistochemistry to detect IL-6 (brown) and OC and counterstained with hematoxylin. The capsules contain several IL-6 immunopositive cells, inflammatory cells (arrows), in the period of 7 days, at 60 days reduced marking is observed in all groups. AH Plus (Fig. 2K) with greater quantity. At 7 days, only Bio-C showed marking for OC, at 60 days Bio-C and Sealer Plus BC showed marking for OC. Bars: 18 µm.

Table 2 - Capsule thickness (µm), number of inflammatory cells (IC) per mm^2 and number of immunopositive cells for IL-6 and osteocalcin per mm^2.Bio-C (BC), Sealer Plus BC (SPBC), AH Plus (AHP) and Control (CG) after 7, 15, 30 and 60 days

		BC	SPBC	AHP	CG
7 days	**thickness**	285±79$^{b;1}$	270±97$^{b;1}$	343±62$^{a;1}$	152±72$^{c;1}$
	IC	775±26$^{a;1}$	690±40$^{b;1}$	865 ± 63$^{a;1}$	239±32$^{c;1}$
	IL-6	549±07$^{b;1}$	451±05$^{c;1}$	782 ± 08$^{a;1}$	210 ± 06$^{d;1}$
	OC	18 ± 05$^{a;1}$	0 ± 0$^{b;1}$	-	-
15 days	**thickness**	252±59$^{b;1}$	309±142$^{a;1}$	242±18$^{b;1}$	184±09$^{b;1}$
	IC	346±49$^{c;2}$	480±48$^{b;2}$	681±64$^{a;2}$	232±13$^{d;1}$
	IL-6	253±04$^{b;2}$	357±04$^{c;2}$	531 ± 06$^{a;2}$	169 ± 03$^{d;2}$
	OC	31 ± 06$^{a;2}$	11 ± 04$^{b;2}$	-	-
30 days	**thickness**	232±42$^{b;1}$	333±160$^{a;2}$	221±22$^{b;2}$	109±06^{c,1}
	IC	362±27$^{b;3}$	440±17$^{a;2}$	468 ± 41$^{a;3}$	173±22^{c,2}
	IL-6	201±02$^{c;2}$	292±04^{b,3}	454 ± 04$^{a;2}$	132 ± 04^{d,2}
	OC	20 ± 05$^{a;1}$	11 ± 07$^{a;2}$	-	-
60days	**thickness**	172±60$^{a;2}$	178±36$^{a;3}$	194±41$^{a;2}$	111±35^{a,1}
	IC	233 ± 40$^{b;3}$	129 ± 32$^{b;3}$	352 ± 84$^{a;4}$	66 ± 12^{c,3}
	IL-6	146±03$^{b;3}$	193±03^{b,3}	279 ± 06$^{a;3}$	79 ± 02^{c,3}
	OC	46 ± 09$^{a;3}$	14 ± 03$^{b;2}$	-	-

Mean (standard deviation).
The comparison between groups in the same period is indicated by superscript letters on the line. Same letters = no statistically significant difference.
The comparison between intervals in the same group is indicated by numbers superscript in the columns; same numbers = no statistically significant difference.
Tukey's test ($p \leq 0.05$)

von Kossa reaction and analysis under polarized light

BC, SPBC and AHP presented von Kossa-positive structures in all periods (Fig 3A, 3B, 3C, 3G, 3H and 3I). The control group did not exhibit positive structures. At all periods, BC and SPBC showed birefringent structures spread by the adjacent tissues, while in the AH Plus specimens presented structures located only on the capsule surface. The control group did not exhibit birefringent structures.

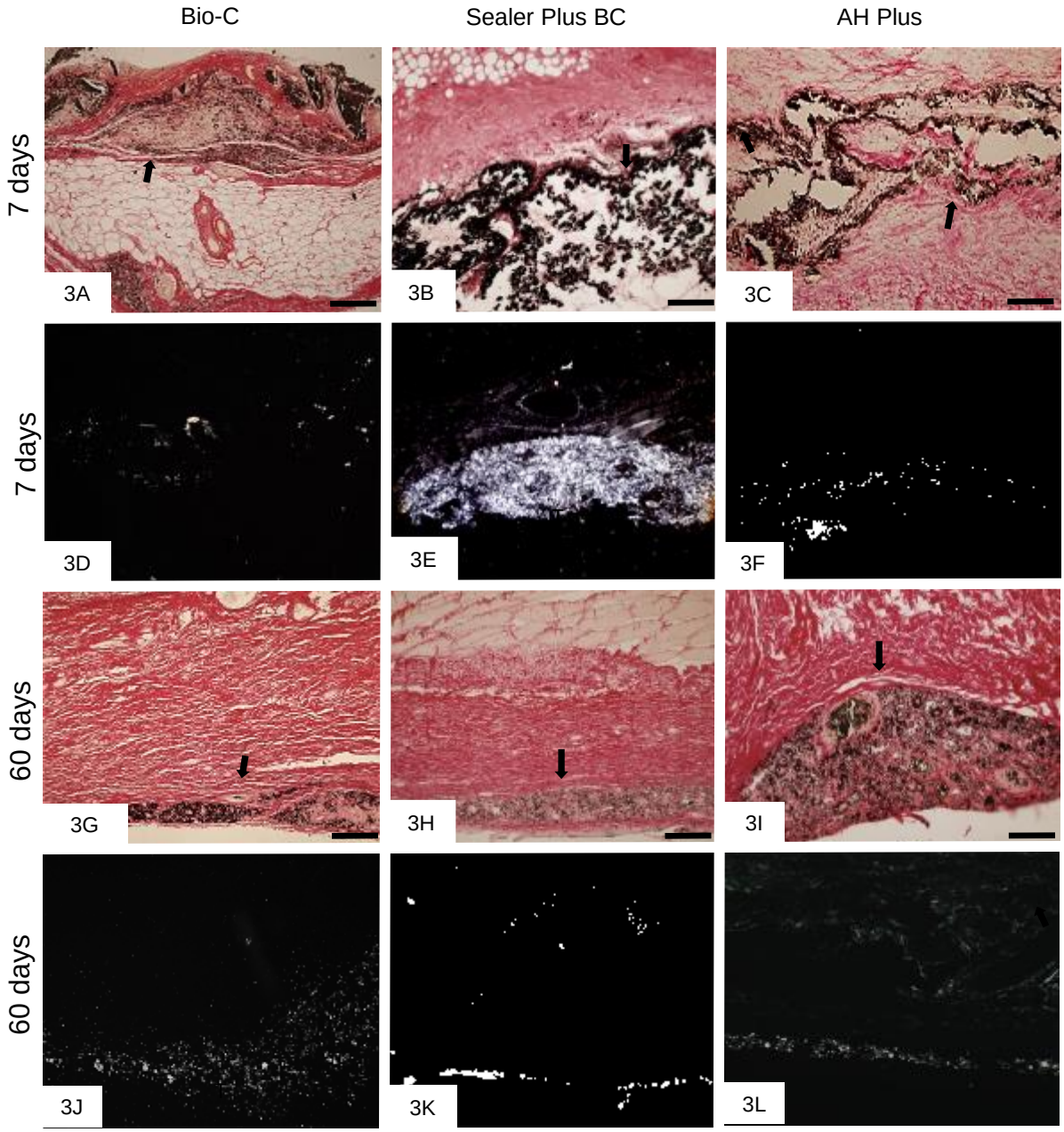

Figure 3: Photomicrographs of sections showing portions of capsule adjacent to the opening of the tubes implanted in the subcutaneous tissue submitted to the von Kossa reaction after 7 and 60 days. The BC (3A and 3G), SPBC (3B and 3H), AH Plus (3C and 3I) capsules exhibit positive von Kossa structures (black / brown) and 3D, 3E, 3F, 3J, 3K and 3L compatible birefringent structures. Positive structures for calcium precipitation. Von Kossa and Picrosirius-red.

DISCUSSION

The present study demonstrated that the Bio-C Sealer and Sealer Plus BC showed lower inflammatory infiltrated than the AH Plus, rejecting the null hypothesis and confirming that tricalcium silicate sealers have adequate biological properties[13,14]. At 7 days, all materials showed a moderate inflammatory reaction, with the presence of plasma cells, neutrophils, macrophages and giant cells close to the sealer particles. The control had the lowest number of inflammatory cells and the least thick capsule, followed by SPBC sealer, which is in agreement with the study by Benetti *et al.* (2019)[9] who report that the initial inflammation of the material can be justified by the presence of calcium hydroxide in its composition, which can promote necrosis in the tissue due to its alkalinity. The Bio-C Sealer and AH Plus sealers were similar, with a higher number of inflammatory cells in the initial period compared to the other materials, possibly due to presence of hydroxyl ions that act in the alkalinization and formation of the initial necrotic layer[13]. An *in vitro* study with experimental calcium silicate-based sealer showed cytocompatibility, bioactive potential through the formation of mineralized nodules[16].

The alkalinity of the Bio-C Sealer contributes to the osteogenic potential, a tissue repair mechanism [10,11,12,15]. For AH Plus sealer, the presence of a greater number of inflammatory cells may be explained by its composition that contains resin, an irritating potential released by the material[9]. Regarding the immunolabeling for IL-6, which mediated the host's response induced by materials implanted in the subcutaneous connective tissue, the BC and SPBC sealers did not show significant difference.

Within 15 days, a significant reduction in the number of inflammatory cells and IL-6 was observed in BC sealer, showing a reduction in the inflammatory reaction. This reduction may be related to the presence of tricalcium silicate, which promotes cell proliferation and viability in periodontal stem cells exposed to the material for a period of 21 days while the AH Plus sealer induced reduction in the cell viability[18]. Thus, BC and TotalFill BC showed the best results in an *in vitro* study with cells of the periodontal ligament[17]. Here, SPBC and AHP had the highest IC values at 30 days. Rodriguez-Lozano *et al.* (2017)[18] demonstrated that TotalFill BC Sealer in cells of mesenchymal origin shows less cytotoxicity than AH Plus[18].

In the present study, all materials exhibited a reduction in inflammatory cells and capsule thickness over time. At 60 days, Bio-C Sealer and Sealer Plus BC showed no significant difference between them, with values lower than AH Plus. Moreover, the capsules showed few inflammatory cells among the collagen fibers accomapined by significant reduction in the immunoexpression of IL-6, indicating the biocompatibility of Bio-C Sealer and Sealer Plus BC. These results are related to the composition of bioceramics with tricalcium and dicalcium silicate, zirconium oxide[8], which have adequate biological properties[5,16,17]. Benetti *et al.* (2019)[9] report that Sealer Plus BC implanted in rat subcutaneous presents biocompatibility. EndoSequence Repair, a repair material based on calcium silicate, showed greater irritability when compared to MTA, especially after 7 and 15 days of implantation in the rat subcutaneous[19]. The combination of zirconium oxide and niobium with calcium silicate sealer shows a capsule with a large amount of collagen and reduced inflammatory process[5,12]. The presence of fibroblasts in the capsules in parallel with reduction in the inflammatory reaction after 60 days, is indicative of tissue repair.

AH Plus sealer showed a reduction in the number of inflammatory cells at 60 days, but maintaining a higher number than other materials. When analyzed in the subcutaneous tissue of rats, in the periods of 7, 15, 30, and 60 days, it was found that the AH Plus sealer presented a greater quantity of IL-6-immunostained cells when compared to the MTA sealer, attributed to its base composition of resin[11]. Bioceramic sealers when hydrated form calcium hydroxide and interact with tissue fluids and calcium phosphates thus providing hydroxyapatite formation [20,21.]

The bioactivity of a material can promote a biological response at its interface, creating an environment compatible with osteogenesis[20,21,22].The formation of the organic matrix of mineralized tissues can be evaluated by immunohistochemistry to detect proteins such as osteocalcin, secreted by osteoblasts, identified as marker of mature osteoblasts[9] and by the von Kossa histochemical technique used for detection of calcium precipitates. The Bio-C Sealer and Sealer Plus BC sealers presented irregular structures von Kossa-postive, at 7 days. In the capsules of SPBC sealer did not show immunolabelling for osteocalcin while the capsules of BC sealer showed immunoreactivity in all periods, being justified by its composition based on calcium silicates that promotes the formation of hydroxyapatite during its hydration process[7]. López-Gárcia *et al*.(2019)[7] reported that the Bio-C Sealer presented a higher calcium ions release than Bio-C Repair, and that the both materials stimulate

the migration of cells in the healing process. AH Plus exhibited von Kossa positivity, but did not exhibit positivity for osteocalcin in none of the evaluated periods, in agreement with the study by Carvalho *et al.*(2017)[23]. Calcium silicate sealers have the property of releasing calcium that favors mineralization through the differentiation of cells belonging to dental pulp[13].

In order to identify amorphous calcite deposits, the birefringence analysis was performed, which proved the findings obtained in the von Kossa assay. The reaction of calcium ions with carbon dioxide leads to the formation of calcite crystals, birefringent structures that later they will lead to the formation of calcified structures[24,25.] Calcium silicate-based sealers showed birefringent structures in all analyzed periods. At 60 days, scarce birefringent structures were found in the capsules around AHP, which shows low calcium release in the initial period, with less release at 60 days[26,27,28,29,30].

Conclusion

Bio-C Sealer and Sealer Plus BC are biocompatible materials with reduced inflammatory process over time. In addition, these sealers had bioactive potential.

Acknowledgements

We thank CAPES (Brazil) for the fellowship to Evelin Carine Alves Silva (CAPES code: 001).

The authors deny any conflicts of interest related to this study.

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4 DISCUSSÃO

A presente pesquisa avaliou a biocompatibilidade e potencial bioativo de cimentos obturadores à base de silicato de cálcio em comparação ao cimento AH Plus, que apresenta boas propriedades físicas, químicas e biocompatibilidade.

Para o estudo das propriedades biológicas, foram avaliados, em relação a biocompatibilidade a densidade de células inflamatórias, a espessura das cápsulas adjacentes aos tubos implantados e o número de células imunopositivas para IL-6. Para análise do potencial bioativo, foi avaliado o número de células positivas para osteocalcina, a presença de estruturas positivas ao método de von Kossa e a presença de estruturas birrefrigentes. Todos os testes foram avaliados nos períodos de 7, 15, 30 e 60 dias.

Um material biocompatível é aquele que apresenta redução do processo inflamatório no decorrer do tempo[17-20]. Os cimentos Experimental-1, Experimental-2 TotalFill BC, Bio-C e Sealer Plus BC apresentaram redução no número de células inflamatórias no decorrer dos períodos. Aos 60 dias, as cápsulas adjacentes aos tubos implantados se apresentaram similares às cápsulas do grupo controle. Em relação a densidade de células inflamatórias, os cimentos Experimental-2, TotalFill BC e Sealer Plus BC apresentaram valores similares ao do grupo controle sendo inferior aos outros grupos, resultados que estão de acordo com Rodriguez-Lozano *et al.*[10] (2017), que conclui que o cimento TBC não é citotóxico e Benetti *et al.*[12] (2019) que relatou que o material SPBC apresentou biocompatibilidade, seguidos pelos grupos dos cimentos Experimental-1 e Bio-C, que apresentaram valores inferiores ao cimento AH Plus.

Os leucócitos são as células que produzem e liberam citocinas que agem na resposta do sistema imune. A interleucina-6, citocina que atua como uma pró-inflamatória e uma miocina anti-inflamatória, é produzida por neutrófilos, macrófagos, monócitos, células endoteliais, fibroblastos e outras células em resposta a micro-organismos[16,19,20,24]. Sendo assim, a análise de marcação da IL-6 permite avaliar o grau do processo inflamatório. Aos 60 dias, os cimentos CE-1, CE-2, TBC e BC apresentaram valores de imunomarcação positiva inferiores quando comparados aos outros materiais.

Os cimentos obturadores à base de silicato de cálcio são considerados materiais bioativos pois favorecem a formação de hidroxiapatita por meio do processo de hidratação dos silicatos e liberam íons de cálcio, apresentando pH alcalino e papel na indução de mineralização[2,22,25-27]. O material bioativo é aquele que interage quimicamente com os tecidos estimulando reparação por tecido mineralizado[28]. Todos os cimentos estudados apresentaram estruturas irregulares contendo cálcio. Os cimentos CE-1, CE-2, TBC, BC e SPBC apresentaram estruturas em todos os períodos analisados, localizadas nas cápsulas e no decorrer do tecido. Já o cimento AH Plus apresentou estruturas principalmente no período de 7 dias, e positividade reduzida aos 60 dias, sendo essas estruturas localizadas apenas nas cápsulas, o que é confirmado pelos estudos de Candeiro *et al.*[1](2012) que mostra pequena quantidade de íons cálcio sendo liberada pelo cimento à base de resina epóxi aos 60 dias. Os resultados foram confirmados pela técnica de birrefringência, que detectam calcita amorfa. Os cimentos CE-1, CE-2 e TBC, BC apresentaram imunomarcação positiva para a osteocalcina em todos os períodos analisados, apresentando valores superiores nos períodos de 30 e 60 dias, exibindo potencial para reparação tecidual. O cimento SPBC não apresentou positividade no período de 7 dias. Os cimentos CE-1, TBC, BC e SPBC não apresentaram diferença estatística entre si aos 60 dias, CE-2 apresentou valores superiores aos 60 dias, podendo ser justificado pela sua composição que une componentes à base de cálcio com antimicrobiano à base de cálcio, estimulando o processo de reparação. O cimento AH Plus não exibiu positividade para osteocalcina em nenhum dos períodos avaliados, sendo justificado pelo estudo de Carvalho *et al*.[30] (2017) que relata que a bioatividade não é esperada para o AH Plus. Materiais à base de silicato de cálcio apresentam como propriedade a liberação de cálcio, favorecendo a mineralização[25].

5 CONCLUSÃO

Os cimentos Experimental-1, Experimental-2 TotalFill BC, Bio- C Sealer e Sealer Plus BC apresentaram redução significante de células inflamatórias aos 60 dias, sendo considerados materiais biocompatíveis. Os materiais apresentam potencial bioativo, exibindo estruturas com presença de cálcio, calcita amorfa e positividade para osteocalcina.

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APÊNDICE A – METODOLOGIA DETALHADA DA PUBLICAÇÃO 1 e 2

Animais

Foram utilizados 80 ratos adultos Holtzman (*Rattus norvegicus albinus*) que foram mantidos em gaiolas de aço inoxidável forradas com maravalha. A maravalha foi trocada no mínimo três vezes por semana. A limpeza das gaiolas e bebedouros também foi realizada. Os ratos ficaram sob luz de 12 em 12 horas de escuro ciclo, com temperatura (23 ± 2ºC) e umidade (55 ± 10%) controladas. Além de água e alimento fornecidos *ad Libitum*, a fim de evitar desconforto dos animais. Os ratos foram distribuídos equitativamente em grupos. Subprojeto1: Experimental-1, Experimental-2, Totalfill BC Sealer, AH Plus e um grupo controle**;** Subprojeto 2**:** Bio-C Sealer, Sealer Plus BC, AH Plus e um grupo controle**.** Os animais foram anestesiados com 80 mg/kg de ketamina e 4 mg/kg de xilazina que foi administrado por via intraperitoneal. A pele dorsal foi rapada e desinfectada com solução de iodo a 5%. Foi realizada uma incisão com lâmina de bisturi. Posteriormente, os tubos de polietileno, de aproximadamente 10 mm de comprimento e 1,6 mm de diâmetro, foram preenchidos com os materiais e vazios para o grupo controle e implantados no tecido subcutâneo, sendo inseridos quatro em cada animal, em forma de rodízio de quadrante.O local da pele incisada foi suturado com pontos simples. Após 7, 15, 30 e 60 dias de implantação, os animais foram eutanasiados com sobredose anestésica e os implantes com os tecidos adjacentes foram removidos, imersos em formaldeído a 4% pelo período de 48 horas.

Figura A1- Animal antes e após o procedimento cirúrgico.

Fonte: Arquivo pessoal do autor.

Figura A2- Tubo de Polietileno Implantando.

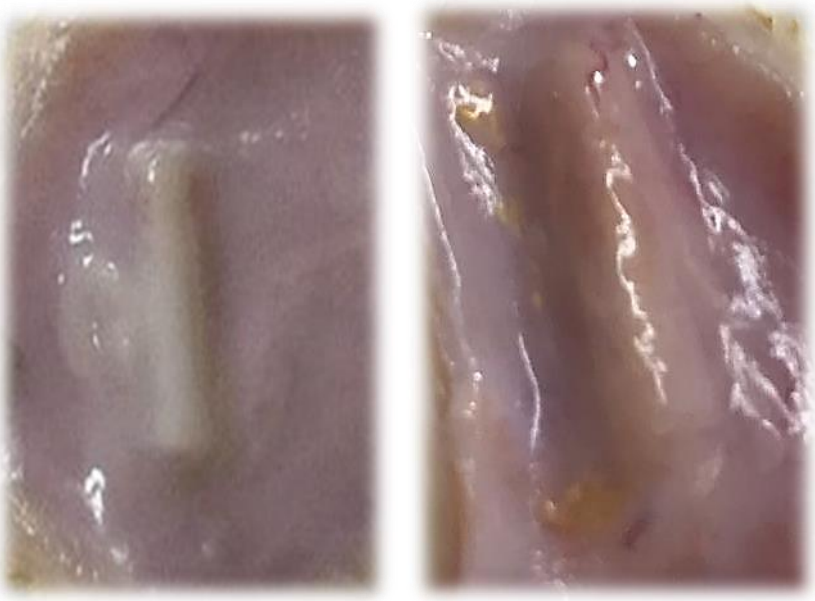
Fonte: Arquivo pessoal do autor

Processamento histológico

Os tecidos circunjacentes aos tubos de polietileno implantados foram removidos e colocados na solução de formaldeído a 4% tamponado com fosfato de sódio 0,1 M com pH 7,2, durante 48 horas. Após fixação, as peças foram desidratadas, diafanizadas, embebidas em parafina líquida (60ºC) e incluídas em

parafina. Com um micrótomo rotativo e navalhas descartáveis de aço inoxidável foram obtidos cortes com 6 µm de espessura. Alguns cortes foram corados com hematoxilina e eosina (HE) para a análise morfológica e estimativa do número de células inflamatórias nas cápsulas. Em lâminas previamente tratadas com silano a 4%, cortes foram aderidos para realização da reação imuno-histoquímica para detecção de interleucina-6 (IL-6) e da osteocalcina.

Figura A3- Tecido removido do subcutâneo do animal



Fonte: Arquivo pessoal do autor

Espessura das cápsulas

A espessura (em µm) das cápsulas adjacentes aos tubos implantados foi mensurada. Usando uma câmera (DP-71, Olympus – Japão) acoplada ao microscópio de luz (Olympus, BX-51, Japão) foram capturadas três imagens de cortes não seriados corados com HE de cada espécime. A espessura das cápsulas foi estimada na porção média a partir de sua superfície adjacente ao material até o seu limite com os tecidos adjacentes. Após a obtenção dos valores, foi calculado o valor médio a partir das medidas obtidas dos três cortes para cada espécime. Esta mensuração foi obtida em todos os espécimes (n=6 por grupo) e em todos os períodos.

Reação imuno-histoquímica para detecção de IL-6

Para a detecção de IL-6, o anticorpo primário anti-IL-6 utilizado foi o produzido em camundongo (Abcam, cód.Ab 9324, Cambridge Science, UK). Após desparafinização e hidratação, os cortes foram imersos em tampão citrato de sódio 0,001 M com pH 6,0 e submetidos ao tratamento com microondas para recuperação antigênica por 30 minutos. Após o resfriamento, as lâminas foram lavadas em tampão PBS 0,01 M (pH 7,2) e, em seguida, imersas em solução aquosa de peróxido de hidrogênio a 5% por 30 minutos. Os cortes foram lavados novamente e, então, incubados com albumina do soro bovino a 2% (Sigma-Aldrich Co., Saint Louis, Missouri, USA). Em seguida, os cortes foram incubados *overnight* em câmara úmida com anticorpo primário anti-IL-6 na diluição 1:400. Subsequentemente às lavagens em tampão PBS 0,01 M os cortes foram incubados em anticorpo secundário kit Labeled StreptAvidin-Biotin (Universal Dako LSAB, Dako Inc., Carpinteria, CA, EUA; K0675) por 60 minutos. Subsequentemente às lavagens em tampão, a atividade da peroxidase foi revelada pelo cromógeno 3,3'-diaminobenzidina (ImmPACTTM DAB Vector, Burlingame, CA, Estados Unidos). Os cortes foram contracorados com hematoxilina de Carazzi. Como controle negativo, na incubação com o anticorpo primário os cortes foram incubados com soro não imune. O número de células imunopositivas a IL-6 foi calculado com auxílio de um programa de análise de imagens (Image-Pro Express 6.0, Olympus, Tóquio, Japão). Assim, o número de células imunopositivas/mm^2 de cápsula foi obtido para cada implante.

Reação Imuno-histoquímica para detecção de osteocalcina

Após desparafinização e hidratação, os cortes foram imersos em tampão citrato de sódio 0,001 M com pH 6,0 e submetidos ao tratamento com microondas por 15 minutos a 96-98ºC. Após o resfriamento, as lâminas foram lavadas em tampão PBS 0,01 M (pH 7,2) e, em seguida, imersas em solução aquosa de peróxido de hidrogênio a 5%. Os cortes foram lavados novamente e, então, incubados com albumina do soro bovino a 2% (Sigma-Aldrich Co., Saint Louis, Missouri, USA) e incubadas com anticorpo anti-osteocalcina de rato de coelho (1:

200; Sigma-Aldrich Co., Saint Louis, Missouri, EUA). Após 16 horas, a 4ºC em câmara umidificada, as seções foram incubadas por 1 h em kit Labeled StreptAvidin-Biotin (Universal Dako LSAB, Dako Inc., Carpinteria, CA, EUA; K0675) à temperatura ambiente. Após as lavagens com tampão, a atividade da peroxidase foi revelada pelo cromogênio da 3,3'-diaminobenzidina (ImmPACTTM DAB Vector, Burlingame, CA, Estados Unidos). As seções foram contrastadas com a hematoxilina de Carazzi. Como controle negativo, as seções foram incubadas com soro não imune em vez de anticorpo primário. Assim, o número de células imunopositivas / mm^2 de cápsula foi obtido para cada amostra.

Técnica de von Kossa

Cortes dos fragmentos dos implantes no subcutâneo foram submetidos à técnica de von Kossa, para detectar precipitação de fosfato no tecido adjacente ao material. Após desparafinização e hidratação, os cortes foram imersos na solução de Nitrato de Prata a 5%, durante 1 hora, sob a ação de uma lâmpada incandescente (100 Watts). As lâminas foram lavadas em água destilada por 3 min e em seguida, imersas em solução de Hipossulfito de Sódio a 5% por 5 min. Em seguida, os cortes foram novamente lavados em água destilada por 5 min e então corados pelo Picrosirius e montados em meio resinoso.

Análise de sob luz polarizada

Considerando que os cristais de calcita exibem birrefringência quando submetidos à luz polarizada, cortes próximos àqueles submetidos ao von Kossa foram desparafinizados, desidratados e montados. Os cortes sem coloração foram analisados ao microscópio de luz equipado com filtros de polarização (Olympus, BX51).

Análise estatística

A análise estatística foi obtida com o auxílio do programa GraphPad Prism 5 software (Jandel Scientific, Sausalito, CA, USA). Os dados foram avaliados pelo two-

way ANOVA seguido pelo teste de Tukey. O nível de significância considerado foi p ≤0,05.

ANEXO A – CERTIFICADO COMITÊ DE ÉTICA

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

CERTIFICADO

Certificamos que a proposta intitulada ***"REAÇÃO TECIDUAL E BIOATIVIDADE DE CIMENTOS ENDODÔNTICOS BIOCERÂMICOS EM SUBCUTÂNEO DE RATOS"***, registrada com o nº 35/2018, sob a responsabilidade do(a) **Prof(a). Dr(a). Juliane Maria Guerreiro Tanomaru** – 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 14/12/2018.

Finalidade	( ) Ensino (**X**) Pesquisa Científica
Vigência do Projeto	Abril/2020
Espécie/linhagem	Rato – Holtzman
Nº de animais	80
Peso/Idade	220 g/ adultos jovens
Sexo	Macho
Origem	Biotério Central da Faculdade de Odontologia de Araraquara – UNESP

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

Comissão de Ética no Uso de Animais - CEUA
Rua Humaitá nº 1 680 – Centro – CEP 14801-903 – Caixa Postal nº 331 - ARARAQUARA – SP
5º andar – fone (16) 3301-6459 / fax (16) 3301-6433 / e-mail: ceua@foar.unesp.br - home page: http://www.foar.unesp.br

Não autorizo publicação deste trabalho pelo prazo de dois anos.

Araraquara, 25 de março de 2020.

Evelin Carine Alves Silva