UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

GABRIELA CARDOSO FERREIRA

PROPRIEDADES FÍSICO-QUÍMICAS E BIOLÓGICAS DE CIMENTOS ENDODÔNTICOS A BASE DE SILICATO DE CÁLCIO E A BASE DE RESINA EPÓXI: ESTUDO *IN VITRO* E *IN VIVO*.

PORTO ALEGRE
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Orientador (a): Profª. Drª. Fabiana Soares Grecca

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“É preciso estudar muito
para saber um pouco.”

Montesquieu

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RESUMO

Este trabalho teve como objetivo avaliar *in vitro* e *in vivo* as propriedades físico-químicas e biológicas de cimentos obturadores a base de resina epóxi, AH Plus (AHP) e Sealer Plus (SP) e a base de silicato de cálcio, MTA Fillapex (MF) e Sealer Plus BC (BC). Para os testes físico-químicos, os cimentos foram submetidos às análises de radiopacidade, espessura de película, escoamento, de acordo com a ISO 6876/2012, tempo de presa de acordo com a ASTM C266-08, pH, e composição elemental dos cimentos por meio da espectroscopia de energia dispersiva (EDX). Para avaliar a citotoxicidade, proliferação celular e bioatividade, células da papila apical humana (APCs) foram expostas aos extratos dos cimentos a 10% e submetidas aos ensaios de viabilidade brometo de 3-(4,5-dimetiltiazólio)-2,5-difenil tetrazólio (MTT) e sulfurodamina B (SRB); ensaio *scratch*; atividade da enzima fosfatase alcalina (ALP) e deposição de nódulos mineralizados pelo corante vermelho de Alizarina (ALZ), respectivamente. Os resultados foram submetidos aos testes estatísticos apropriados com nível de significância de 95%. O cimento BC apresentou os maiores valores de pH em todos períodos comparado aos outros cimentos ($p<.05$), exceto no período de 28 dias comparado ao MF ($p>.05$). Todos os cimentos testados apresentaram radiopacidade maior do que 3 mm Al, AHP mostrou a maior radiopacidade (8.3 mm Al) ($p<.05$). MF apresentou maior espessura de película ($p<.05$). Não houve diferença estatística para escoamento ($p>.05$). AHP mostrou o maior tempo de presa inicial ($p<.05$) e o MF, após 7 dias, não apresentou tempo de presa final. Análise de EDX mostrou picos de cálcio (Ca) para o MF e o BC. SP não apresentou Ca e zircônia (Zr), citados na composição do fabricante. MF apresentou Zr, não citado na composição do fabricante. No MTT, em 24 horas, o BC foi similar ($p>.05$) a todos grupos. No SRB, o BC obteve a maior viabilidade celular ($p<.05$). Na análise *scratch*, após 48h, MF, SP, BC e o controle promoveram migração celular e o fechamento da ferida. Após 72h, o AHP e SP não fecharam a área. No ensaio ALP 24h, o SP e o MF exibiram menores valores comparados ao controle ($p<.05$). No ALZ, o BC obteve a maior deposição de nódulos mineralizados ($p<.05$). *In vivo*, nenhum material mostrou diferença significante para infiltrado inflamatório comparado ao grupo controle em 7 dias. Após 90 dias, MF mostrou maior reação inflamatória ($p<.05$). O MF apresentou eosinófilos aos 7 dias ($p<.05$). Houve formação de condensação fibrosa aos 30 e 90 dias ($p>.05$). Em 30 e 90 dias, o MF e o BC apresentaram os maiores escores para macrófagos ($p>.05$). Concluiu-se que os cimentos testados apresentaram propriedades físico-químicas de acordo com a ISO 6876/2012 e a ASTM C266-08, exceto o cimento MF para o tempo de presa. BC, MF e SP apresentaram viabilidade celular enquanto o AHP uma leve citotoxicidade e consequente morte celular no *scratch*. Todos materiais obtiveram depósitos de cálcio e fostato e atividade da enzima fosfatase alcalina, entretanto, o BC apresentou resultados superiores para a produção de nódulos mineralizados. Apesar de ser citocompatível, os cimentos à base de silicato de cálcio mostraram maior atividade de macrófagos ao longo do tempo, principalmente, o MF, o qual também apresentou células gigantes e eosinófilos apresentando uma biocompatibilidade moderada.

Palavras-chave: endodontia, cimento de silicato, teste de materiais, cimento obturador endodôntico, propriedades físicas e químicas, citotoxicidade, histocompatibilidade.

ABSTRACT

This study aimed to evaluate *in vitro* and *in vivo* physicochemical and biological properties of resin-based, AH Plus (AHP) and Sealer Plus (SP) and calcium silicate-based sealers, MTA Fillapex (MF) and Sealer Plus BC (BC). For physical-chemical tests, the sealers were submitted to the analyzes of radiopacity, film thickness and flow according to ISO 6876/2012, setting time according to ASTM C266-08, pH, and sealers' elemental composition by dispersive energy spectroscopy (EDX). To evaluate cytotoxicity, cell proliferation and bioactivity, apical papilla cells (APCs) were exposed to the 10% sealers' extracts and submitted to the viability assays 3- (4,5-dimethylthiazolium bromide) -2,5 diphenyl tetrazolium (MTT) and sulfurodamine B (SRB); scratch assay; alkaline phosphatase enzyme activity (ALP) and deposition of mineralized nodules by Alizarin red staining (ALZ), respectively. The results were submitted to appropriate statistical tests with a significance level of 95%. The BC showed the highest pH values in all periods compared to the other sealers ($p<.05$), except in the 28d period compared to the MF ($p>.05$). All sealers tested presented radiopacity greater than 3 mm Al and AHP showed the highest radiopacity (8.3 mm Al) ($p<.05$). MF presented higher film thickness ($p<.05$). There was no statistical difference for flow ($p>.05$). AHP showed the highest initial setting time ($p<.05$) and the MF, after 7 days, did not present final setting. EDX analysis showed calcium peaks (Ca) for MF and BC. SP showed no presence of Ca and zirconia (Zr), exhibited in the manufacturer composition. MF presented Zr, not mentioned in the manufacturer's composition. At MTT, in 24 hours, BC was similar ($p>.05$) to all groups. At SRB, BC presented the highest cell viability ($p<.05$). In the scratch analysis, after 48h, MF, SP, BC and the control promoted cell migration and wound closure. After 72 hours, AHP and SP did not close the area. At ALP 24h, SP and MF presented lower values than control ($p<.05$). At ALZ, BC obtained the highest deposition of mineralized nodules ($p<.05$). *In vivo*, no material showed significant difference for inflammatory infiltrate compared to control in 7 days. After 90 days, MF showed a higher inflammatory reaction ($p<.05$). MF presented eosinophils at 7 days ($p<.05$). There was formation of fibrous condensation at 30 and 90 days ($p>.05$). At 30 and 90 days, MF and BC had the highest scores for macrophages ($p>.05$). It was concluded that the sealers tested had physical-chemical properties according to ISO 6876/2012 and ASTM C266-08, except the MF for setting time. BC, MF and SP presented cellular viability while AHP showed a slight cytotoxicity and consequent cell death at scratch. All materials obtained deposits of calcium and phosphate and alkaline phosphatase enzyme activity, however, the BC presented superior results to produce mineralized nodules. Despite being cytocompatible, calcium silicate sealers showed higher macrophage activity over time, especially MF, which also presented giant cells and eosinophils with moderate biocompatibility.

Key words: endodontics, canals sealer, calcium silicate, materials testing, cytotoxicity, bioactivity, biocompatibility, cell migration.

SUMÁRIO

ANTECEDENTES E JUSTIFICATIVA

O sucesso do tratamento endodôntico depende da limpeza, modelagem e obturação do canal radicular (HARGREAVES; COHEN, 2011). Na obturação, cimentos são associados à guta-percha para que atuem como agentes ligantes entre o material sólido e a dentina do canal radicular (MICHAUD et al., 2008; PRULLAGE et al., 2016), fazendo com que haja selamento da região do forame apical e, o preenchimento das irregularidades e das variações presentes no sistema de canais radiculares (ØRSTAVIK, 2005; VERSIANI et al., 2006; RESENDE et al., 2009). Essas variações, por sua vez, apresentam-se como áreas de difícil acesso, como deltas apicais, canais acessórios, ramificações e espaços em que a guta-percha é incapaz de alcançar, reforçando assim, a importância desempenhada pelo cimento obturador (KONTAKIOTIS; TZANETAKIS; LOIZIDES, 2007).

Pode-se dividir os cimentos em categorias a partir da base de sua composição principal e, os disponíveis no mercado, são classificados de acordo com a sua composição química, sendo eles: cimentos à base de óxido de zinco e eugenol, os que contém hidróxido de cálcio, à base de ionômero de vidro, à base de resina metacrilato, à base de resina epóxi e à base de silicato de cálcio (AL-HADDAD, 2016).

Para serem comercializados, cimentos endodônticos deveriam apresentar requisitos físico-químicos e biológicos estabelecidos pela *International Organization for Standardization* (ISO) ou pela *American National Standard/American Dental Association* (ANSI/ADA). Apesar de existir grande variedade de cimentos obturadores, nenhum material ainda cumpriu todos os requisitos exigidos (CARVALHO-JUNIOR et al., 2003; VERSIANI et al., 2006).

Segundo DE-DEUS et al., 2009, um cimento deve apresentar adequadas propriedades físico-químicas e, concomitantemente, ser biocompatível, pois ele entra em contato direto com o tecido periapical e/ou tecidos perirradiculares através do forame e delta apical (ØRSTAVIK, 2005). Assim, diversos testes *in vitro* e *in vivo,* se fazem necessários anteriormente ao uso em humanos (HAUMAN; LOVE, 2003).

Um material é biocompatível quando ele entra em contato com o tecido e não desencadeia uma reação adversa, como toxicidade, irritação, inflamação,

alergia ou carcinogenicidade (SUN; WATAHA; HANKS, 1997). Silveira e colaboradores (2011) definiram como biocompatível uma resposta inflamatória induzida por um agente externo ao tecido conjuntivo e que se torna insignificante ao longo do tempo.

Um método muito utilizado para determinar biocompatibilidade é a implantação de materiais endodônticos em tubos no tecido conjuntivo do dorso de ratos (MITTAL; CHANDRA; CHANDRA, 1995; SILVEIRA et al., 2011), devido à semelhança do genoma de ratos e humanos (KOLA, 2004). Dessa maneira, a capacidade de gerar efeito inflamatório pode ser determinada através de análises histopatológicas de tecidos que envolvem os implantes contendo os materiais inseridos nesses animais (ZMENER; GUGLIELMOTTI; CABRINI, 1988; ECONOMIDES et al. 1995; SILVEIRA et al., 2011).

Através de estudos *in vitro*, com cultura celular, é possível explanar sobre os mecanismos que envolvem as diferentes respostas biológicas dos materiais (CALLADO-GONZÁLEZ et al., 2017).

Além disso, o cimento obturador deve ser bacteriostático, dimensionalmente estável, prover selamento lateral e apical, ser radiopaco e passível de remoção, ter bom escoamento, não manchar a estrutura dentária e ser insolúvel aos fluidos teciduais (GROSSMAN, 1976; FOROUGH et al., 2014).

Dentre as propriedades físico-químicas desejadas, a radiopacidade permite a distinção entre o material e as estruturas anatômicas dentais e adjacentes, ademais é relevante para a avaliação da qualidade do preparo químico-mecânico e da obturação (MCCOMB; SMITH 1976; BODRUMLU; SUMER; GUNGOR, 2007; BORGES et al., 2011; COLLARES et al., 2013). Para que ela seja mensurada e avaliada, a ISO 6876 (2012) e a especificação n. 57 da ANSI/ADA (2000) estandardizaram a radiopacidade com um grau mínimo de 3 mm de espessura quando comparado a uma escala padronizada de alumínio para que o material seja utilizado clinicamente.

A espessura de película adequada é outra propriedade que o cimento obturador deve possuir, define-se como uma fina camada de cimento entre a guta-percha e as paredes dentinárias com objetivo de preencher pequenos espaços e prevenindo a passagem de fluidos através do sistema de canais radiculares (COLLARES et al., 2013). Outrossim, permite que o cimento penetre nos túbulos dentinários e canais acessórios. Uma espessura de película alta não

é desejável por interferir na adaptação dos cones de guta-percha durante o procedimento de obturação (ØRSTAVIK, 1982). A ISO 6876 (2012) e a ANSI/ADA n°57 definiram que a espessura de película adequada não deva ultrapassar 50 µm.

Com relação ao escoamento, esse deve ser suficiente para que todas as superfícies internas do conduto radicular sejam atingidas, penetrando em espaços vazios e aderindo-se entre a guta-percha e as paredes do canal, favorecendo a formação de uma obturação maciça e tridimensional (CAICEDO; VON FRAUHOFER, 1988; LOPES; SIQUEIRA JR, 2015). Todavia, um alto escoamento permite a extrusão de material via forame apical, o que pode gerar injúria aos tecidos periapicais (DUARTE et al., 2010). A ISO 6876 (2012) e a ANSI/ADA n°57 determinam como desejável um escoamento mínimo de ≥ 20 mm e ≥ 25 mm, respectivamente.

O pH apresentado pelos cimentos é uma propriedade que pode afetar diretamente no processo de cicatrização, em razão de que ele está diretamente relacionado com os efeitos microbianos e a deposição de tecido mineralizado (MCHUGH et al., 2004; STUART et al., 2006; OKABE et al., 2006; DESAI; CHANDLER, 2009). Cimentos que apresentam pH alcalino são capazes de contribuir para a deposição de tecido duro através da ativação da fosfatase alcalina, enzima envolvida estritamente no processo de mineralização (STOCK, 1985). Por outro lado, o pH alcalino tem um efeito destrutivo sobre as membranas e estruturas proteicas de bactérias, sendo favorável, visto que muitas delas podem permanecer nas ramificações do sistema de canais radiculares após o preparo químico-mecânico e medicação intracanal (GOMES et al., 2004).

O tempo de presa de um cimento é outro parâmetro a ser considerado, pois, a partir dele, é possível calcular o intervalo de tempo disponível para a obturação dos canais radiculares. Essa propriedade pode sofrer interferência de fatores como temperatura, relação entre pó e líquido, granulometria, meio ambiente e pH. Esse tempo não deve ser longo para não haver a deterioração do cimento, favorecendo a penetração de agentes irritantes e a liberação de possíveis subprodutos tóxicos, e também não deve ser curto, para que o tratamento endodôntico seja finalizado adequadamente (ALLAN; WALTON; SHAFFER, 2001).

Materiais que pleiteiam melhores desempenhos físico-químicos e biológicos são continuamente apresentados. Dentre eles, novos cimentos a base de resina epóxi e a base de silicato de cálcio têm sido propostos.

O cimento AH Plus (Dentsply, DeTrey GmbH, Konstanz, Alemanha), é um cimento à base de resina epóxi formado da mistura de uma pasta base e pasta catalisadora. A pasta base é composta por bisphenol A, óxido de ferro, sílica, óxido de zircônia e tungstato de cálcio. A pasta catalisadora é composta de Dibenzil-5-oxanonane-diamina-1,9 e amina adamantada (LEE et al., 2017). Ele é considerado “padrão-ouro” e utilizado com frequência como material de comparação na pesquisa endodôntica (SILVA et al, 2017), devido às boas propriedades físico-químicas, antimicrobianas e biológicas (DE ALMEIDA; LEONARDO; TANOMARU-FILHO, 2000; ZHOU et al., 2013; LEONARDO et al, 1999; SALEH et al, 2004). Neste cimento, a pasta epóxi e a de poliaminas são misturados durante a sua manipulação e cada grupo amina pode reagir com um grupo epóxi para formar uma ligação covalente (SILVA et al., 2017). O polímero resultante é rígido e forte o que pode explicar sua baixa solubilidade e alta estabilidade dimensional (VERSIANI et al. 2006, RESENDE et al. 2009). Apesar de ser considerado padrão-ouro, na presença de umidade, esse cimento não se veda eficientemente (ROGGENDORF et al., 2007), não possui propriedades bioativas e tampouco possui potencial osteogênico (KIM et al., 2013; BORGES et al., 2012).

O cimento Sealer Plus (MKLife - Medical and Dental Products, Brasil), lançado no mercado recentemente, é também um cimento à base de resina epóxi e segundo o fabricante, possui uma viscosidade satisfatória, que penetra e sela os canais laterais e baixa contração após a presa evitando espaços entre o cimento e a parede do canal. É composto a partir da mistura de duas pastas, a pasta base contém: Bisfenol A-coepiclorohidrina, Bisfenol F resina epóxi, óxido de zircônia, silicone e siloxanos, óxido de ferro e hidróxido de cálcio. A pasta catalisadora contém: hexametiletilenotetramina, óxido de zircônio, silicone e siloxanos, hidróxido de cálcio e tungstato de cálcio.

Segundo Vertuan et al. (2018), o Sealer Plus apresenta propriedades físico-químicas que estão de acordo com a ANSI / ADA (nº 57) e ISO 6876, sendo elas, solubilidade, radiopacidade e pH. Cintra e colaboradores (2017), compararam a citotoxicidade e a biocompatibilidade do cimento Sealer Plus com

o AH Plus, Endofill e SimpliSeal e obtiveram resultados favoráveis ao Sealer Plus. Esse promoveu maior viabilidade celular em fibroblastos para quase todos os períodos e diluições analisados. Além disso, foi histologicamente mais biocompatível em tecido subcutâneo de ratos.

Cimentos biocerâmicos ou a base de silicato de cálcio geram a expectativa de ser uma alternativa eficaz na obturação dos canais radiculares por apresentarem biocompatibilidade e bioatividade, manterem o pH elevado durante o uso e apresentarem capacidade de selamento (PATIL et al., 2017). Este resultado é atribuído à presença de fosfato de cálcio que estimula o processo osteogênico, formando hidróxido de cálcio durante a sua reação de hidratação, que por sua vez, é capaz de interagir com as células do tecido resultando na formação de cristais de hidroxiapatita e carbonatoapatita, caracterizando também sua bioatividade (PRATTI; GANDOLFI, 2015; COSTA et al., 2016). Segundo resultados de estudos, cimentos biocerâmicos tem o potencial de promover a regeneração óssea quando são involuntariamente extruídos através do forame apical durante a obturação do canal radicular ou reparação das perfurações radiculares (BAE et al., 2010).

O MTA Fillapex® (Angelus, Londrina, Parana) é um cimento obturador composto de silicato de cálcio e resina salicilato. É apresentado na forma pasta/pasta sendo composto de resina de salicilato, resina natural, tungstato de cálcio, nanopartículas de sílica e pigmentos, além do silicato de cálcio. Ferreira et al. (2013) demonstraram que este cimento dispõe de propriedades físico-químicas adequadas, como radiopacidade, escoamento satisfatórios e o pH alcalino. Contudo, Faraoni et al. (2013) mostraram que o MTA Fillapex apresentou tempo de presa inicial maior do que o descrito pelo fabricante. Resultados relacionados à resposta biológica do MTA Fillapex são conflitantes. Quando recém misturado, este material apresentou alta citotoxicidade e genotoxicidade (BIN et al., 2012). Quando este cimento foi implantado em tecido subcutâneo de ratos, permaneceu tóxico mesmo após 90 dias (ZMENER et al., 2012).

Silva, Santos e Zaia (2013) verificaram que o MTA Fillapex apresentou um efeito citotóxico grave nas células de fibroblastos no período imediato. Além disso, esse efeito não diminuiu com o tempo. O nível de citotoxicidade permaneceu moderado mesmo cinco semanas após a mistura. Todavia,

SALLES et al. (2012) observaram que, apesar de apresentar efeitos tóxicos iniciais, a citotoxicidade do MTA Fillapex diminui e o cimento apresentou bioatividade adequada estimulando locais de nucleação para a formação de cristais de apatita em cultura de células de osteoblastos humanos.

A procura por um material ideal é constante e motiva o estudo das propriedades dos materiais já existentes e a pesquisa pelo desenvolvimento de novos. A MKLife - Medical and Dental Products lançou o cimento biocerâmico Sealer Plus BC que contém silicato de cálcio, óxido de zircônio, hidróxido de cálcio e propileno glicol. Consiste em uma mistura pronta em pasta que exige a presença de umidade para tomar presa. Ainda não existem estudos na literatura que avaliem algumas propriedades físico-químicas e as propriedades biológicas do cimento Sealer Plus BC justificando o objetivo desse estudo.

OBJETIVOS

O presente estudo tem como **objetivo geral** avaliar as propriedades biológicas e físico-químicas dos cimentos endodônticos à base de resina epóxi, AH Plus e Sealer Plus, e a base de silicato de cálcio, MTA Fillapex e Sealer Plus BC.

Os **objetivos específicos** deste estudo são:

- Avaliar as seguintes propriedades físico-químicas dos cimentos estudados: tempo de presa, pH nos períodos experimentais de 1, 3 e 24 horas e 7, 14, 21 e 28 dias, radiopacidade, escoamento e espessura de película.
- Analisar através de espectroscopia de dispersão de energia por raios-X (EDX) a composição de cada cimento na sua superfície.
- Avaliar a citotoxicidade dos cimentos endodônticos em células obtidas da papila dentária humana (APCs), por meio de MTT em dois tempos diferentes (24 e 72 horas) e SRB.
- Avaliar a bioatividade, em cultura de células obtidas da papila apical humana (APCs) por meio do ensaio da Fosfatase Alcalina e Alizarin Red.
- Avaliar a migração das células da papila apical humana (APCs) expostas aos diferentes cimentos endodônticos para o fechamento da ferida, através do ensaio de “*scratch*”.
- Avaliar a resposta tecidual em relação às características do componente celular inflamatório; condensação fibrosa e presença de abscesso em tecido subcutâneo de ratos nos diferentes períodos experimentais de 7, 30 e 90 dias.

ARTIGO 1

Physicochemical properties of calcium silicate-based and epoxy resin-based root canal sealers: *in vitro* study*.

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ABSTRACT

INTRODUCTION: The aim of this study was to evaluate the physicochemical properties of a calcium silicate-based (MTA Fillapex - MF and Sealer Plus BC - BC) and epoxy resin-based (AH Plus - AHP, Sealer Plus - SP) root canal sealers.
METHODS: For the radiopacity, flow and film thickness, International Organization for Standardization 6876 (2012) specifications were followed. To measure the initial and final setting times, the ASTM C266-08 (2008) specification of the American Society for Testing and Materials was used. pH was evaluated in the time intervals of 1, 3, 24 hours and 7, 14, 21 and 28 days. EDX analysis was used to determine the elemental composition of sealers. ANOVA and Tukey's *post hoc* tests were used for radiopacity, setting time, and pH. Two-way ANOVA and post hoc Tukey's test was perfomed to pH when evaluated the same sealer in the different experimental periods. For flow and film thickness, Kruskal-Wallis and Dunn's *post hoc* tests were used ($p<.05$).
RESULTS: BC presented higher pH values in all periods than other sealers ($p<.05$), except for the 28-day period for MF ($p>.05$). AHP sealer showed the highest radiopacity (8.3 mm Al) ($p<.05$) and all sealers tested presented radiographic densities higher than 3 mm Al (recommended by ISO). MF presented higher film thickness compared to sealers tested ($p<.05$). There was no statistical difference among the analyzed sealers at flow analysis ($p>.05$). AHP sealer showed the higher initial setting time (452.7 min) ($p<.05$) and MF, after seven days, didn't present final setting time. EDX analysis showed peaks of calcium (Ca), for MF and BC. SP did not show Ca.
CONCLUSIONS: Tested properties of epoxy resin-based sealers as well as the bioceramic-based sealers except MTA Fillapex (setting time) were in agreement with ISO 6876/2012 and ASTM C266-08. Sealer Plus BC presented higher pH values. Calcium was found on the surfaces of Sealer Plus BC and MTA Fillapex by EDX analysis.

KEY WORDS: Calcium silicate, resin epoxy, root canal filling material, EDX, physical and chemical properties.

INTRODUCTION

The main objectives of root canal obturation are three-dimensional and hermetic sealing. As a result of the gutta percha does not adhere to the walls of the root canal, the association of an endodontic sealer is necessary to avoid apical microleakage (1). Taken together, biocompatibility, dimensional stability, radiopacity, flow, low solubility are also characteristics that an ideal endodontic sealer should present (2, 3).

Standardized assessment requirements and tests should be needed to determine the physical and chemical properties of an endodontic sealer, as defined by the International Organization for Standardization (ISO) 6876:2012 (4) and the American Society for Testing and Materials ASTM C266-08 (2008) (5). Currently, AH Plus (Dentsply, York, PA, USA), an epoxy resin material filling is considered the gold standard root canal sealer for comparison purposes due to its excellent physicochemical properties (6-11).

A new resin epoxy based sealer, Sealer Plus (MK Life - Medical and Dental Products, Porto Alegre, RS, Brazil) presents calcium hydroxide in the composition and, according to Vertuan et al. (12) showed radiopacity, flow, solubility in accordance with ANSI/ADA No. 57 (13) and ISO 6876:2012 (4).

Aiming combines physicochemical properties of a root canal sealer and the biocompatibility/bioactivity of Mineral Trioxide Aggregate (MTA), calcium silicate-based sealers have been proposed (14). MTA Fillapex (Angelus, Londrina, PR, Brazil) is a sealer that is composed of MTA, salicylate resin, natural resin, calcium tungstate and silica. Studies demonstrated suitable physicochemical properties, such as good radiopacity, flow, working and setting times, solubility, and alkaline pH (14,15).

Sealer Plus BC (MKLife - Medical and Dental Products, Porto Alegre, RS, Brazil) is a bioceramic material that presents zirconium oxide, tri-calcium silicate, di-calcium silicate, calcium hydroxide, and propylene glycol in the composition. This type of material promises, from their expected properties as high pH value and appropriate solubility, a bioactivity potential. So, this sealer is quite new, and only one study in the literature have analysed its properties (16), in which do not include properties tested herein like flow, film thickness and EDX analysis.

Thus, the aim of this study was to evaluate physicochemical properties and EDX analysis of this new calcium silicate-based sealer in comparison with AH Plus, Sealer Plus, MTA Fillapex.

MATERIALS AND METHODS

1. Tested materials

It was included two epoxy resin (AH Plus and Sealer Plus) and two calcium silicate (Sealer Plus BC and MTA Fillapex) based sealers. Chemical composition of the tested materials is shown in Table 1.

Table 1. Chemical composition and manufacter of the endodontic sealers tested.

SEALER/ALLOTMENT	MANUFACTURER	CONTENTS
AH Plus 350680K	Dentsply, York, PA, USA	Paste A: Bisphenol epoxy resin–A, Bisphenol epoxy resin–F, calcium tungstate, zirconium oxide, silica, iron oxide pigments. Paste B: Dibenzyldiamine, aminodiamantana, tricyclodecane–diamine, calcium tungstate, zirconium oxide, silica, silicone oil.
Sealer Plus PS170330010410	MK Life, Porto Alegre, RS, Brazil	Basic Paste: Bisphenol A-co-epichlorohydrin, Bisphenol F epoxy resin (formaldehyde, oligomeric product with 1-chloro-2,3-epoxypropanol and phenol); zirconium oxide, silicone and siloxanes, iron oxide (pigment), calcium hydroxide. Catalyzer Paste: Hexamethylenotetramine, zirconium oxide, silicone and siloxanes, calcium hydroxide, calcium tungstate.
MTA Fillapex 43663	Angelus, Londrina, PR, Brazil	Base Paste: Salicylate resin, natural resin, calcium tungstate, nanoparticulated silica, pigments; Catalyst Paste: diluting resin, Mineral Trioxide Aggregate, nanoparticulated silica, pigments;
Sealer Plus BC WR770100	MK Life, Porto Alegre, RS, Brazil	Zirconium oxide, tri-calcium silicate, di-calcium silicate, calcium hydroxide, propylene glycol.

2. Physicochemical tests

pH analysis

To determine the pH, 10 samples of each sealer were produced (n = 10). Polyethylene tubes measuring 10 mm length and internal diameter of 1.6mm

were used. Sealers were inserted inside the tubes using 1mL syringes until they were completely filled. Each specimen was placed in test glass tubes containing 10mL deionized water and stored at 37°C. Before pH measurement, the specimens were removed, and the test tubes were shaken for 5 seconds. The pH assessment was performed in 1, 3 and 24 hours and 7, 14, 21 and 28 days of immersion with a digital pH meter (Digimed DM-21, São Paulo, São Paulo, Brazil) previously calibrated with solutions of known pH (4, 7 and 14). Samples were kept at 37°C throughout the experimental period. pH evaluations were performed always in the same tubes containing deionized water at each evaluation period.

Radiopacity analysis

The radiopacity test was performed according to ISO 6876:2012 (4). Three samples were produced for each sealer. The sealers were manipulated according to manufacturer's instruction, except Sealer Plus BC (ready to use) and introduced into silicone ring molds with 10mm in diameter and 1mm in height. The filled rings were kept at 37^0C until the sealers were completely set. The samples were placed on an intraoral image plate (Durr Dental, Bietigheim-Bissingen, Germany) along with an aluminum step wedge graduated from 1 to 8 mm Al (in 1 mm increments). Radiographs were taken by using a radiographic unit (Timex 70E, Saevo, Ribeirão Preto, São Paulo, Brazil) operating at 60 kV and 10 mA, with the exposure set at 0.1 seconds and a focus-film distance of 30 cm. Images were digitalized in a VistaScan Mini Easy system (Durr Dental, Germany) and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD). Each image was coded to identify the material used and not manipulated. The average and standard deviation of the greyscale pixel values of the six areas selected were measured using the histogram tool and were recorded. The obtained pixel value of the material was compared to the pixel value obtained in the 3-mm aluminium stepwedge and converted, according to the scale, in mm of Al.

Film thickness analysis

The film thickness was determined as the difference in thickness between two 5-mm-thick glass plates with a size of ± 25mm with and without the sealer

interposed by a micrometer caliper (Mitutoyo, Suzano, SP, Brazil) according to the method described in ISO 6876:2012 (4). The mixed sealers were placed between the glass plates (n=3). After 180 ± 10s from the start of mixing, a load of 150 N was applied vertically on top of the glass plate, ensuring that the material filled the entire area between the top and bottom glass plates. After 10 minutes from the start of mixing, the thickness of the combined glass plates and sealer was measured by using a micrometer caliper. Three measurements were done for each sealer.

Flow analysis

In accordance with ISO 6876:2012 (4), three samples were produced for each material (n=3). Afterward, 0.5mL (± 0,005) sealer was placed on a glass plate with dimensions of 40mm (height) x 40mm (width) x 5mm (thickness) using a 1mL disposable syringe. Another glass plate of the same dimensions was placed centrally on top of the sealer, and a 100g load was centrally applied to the material for 10 minutes. The force load was removed and the longest and the shortest diameter of the sealer disks produced were measured using a digital caliper (Digimess, São Paulo, São Paulo, Brazil). If the disks were not uniformly circular (the maximum and minimum diameters were not within 1 mm), the test was repeated. For each experimental group, the test was conducted three times, and the mean value recorded.

Setting time analysis

The setting time test was performed according to the C266-08 specification of the American Society for Testing and Materials (5). Briefly, silicone ring molds with an inner diameter of 10 mm and a height of 2 mm were used. The molds were placed on the glass plate, and then the materials were mixed for 120 seconds, except Sealer Plus BC, and inserted into the molds (n=3). The whole assembly was then mantained to an incubator (37 ^{0}C, > 95% relative humidity) during the experimental period. A Gilmore needle with a weight of 100 g and an active tip of 2.0 mm diameter was used. The needle was lowered vertically onto the horizontal surface of the sealer, and the setting time was identified as the point when the indenter needle failed to make an indentation.

The materials were tested every 10 minutes or every hour, depending on the setting time stated by the manufacturers. The needle tip was cleaned before each test. The time from the start of mixing until the sealer was set was taken as the initial setting time. The evaluation of the final setting time began immediately after the initial setting time was defined. At this point, a 456g Gilmore needle with a 1.0mm active tip was vertically positioned on the sealer surface. The same interval of time repetitions was used.

Energy-dispersive X-ray spectroscopy Analysis (EDX)

EDX analysis was performed in 3 samples of each sealer (n=3). Samples presenting 10mm in diameter and 2mm thickness were previously prepared according to the manufacturers' instructions. After final setting time, the samples were fixed on a metallic stub and sputter-coated with carbon. A Zeiss-Auriga electronic microscope (Zeiss, Oberkochen, Germany) equipped with a secondary electron detector model X-ACT (EDX; Oxford INCA 350 EDS; Oxford Diffraction, Abingdon, UK) with an acceleration voltage of 15kV and exposed in a high vacuum (10-5 mbar) was used to determine the elemental composition of the sealers. EDX by analytic area (0.01 mm^2) of the surfaces was carried out of each sample.

Statistical analysis

One-way ANOVA and post hoc Tukey’s test was perfomed to radiopacity, setting time and pH. Two-way ANOVA and post hoc Tukey’s test was perfomed to pH when evaluated the same sealer in the different experimental periods. Kruskall-Wallis and post hoc Dunn test was used for flow and film thickness. A significance level was of 5% (GraphPad Software, San Diego, CA, USA). For EDX, the chemical compositon of each sealer was described.

RESULTS

The results obtained are presented in the form of tables and graphics.

Table 2. pH values of the sealers in function of the experimental period.

Sealer	1h	3h	24h	7d	14d	21d	28d
AH plus	6.8 (±0.10)bA	6.82 (±0.11)bcAB	7.27 (±0.11)bC	7.36 (±0.22)bC	6.97 (±0.03)dBD	7.09 (±0.05)bD	7.07 (±0.09)bD
Sealer Plus	6.49 (±0.20)bA	6.55 (±0.18)bA	7.35 (±0.07)bB	7.50 (±0.15)bC	7.21 (±0.10)cD	7.17 (±0.10)bD	7.23 (±0.21)bBD
MTA Fillapex	6.44 (±0.11)bA	6.92 (±0.15)cB	8.21 (±0.28)cC	8.21 (±0.04)cD	8.06 (±0.10)bCD	7.94 (±0.07)cC	7.99 (±0.07)aC
Sealer Plus BC	7.78 (±1.09)aA	9.23 (±0.52)aB	8.41 (±0.33)aB	8.41 (±0.10)aC	8.41 (±0.04)aC	8.17 (±0.05)aAC	7.95 (±0.10)aAC

*Different lowercase letters represent statistical difference among sealers in the same period.
*Different capital letters represent statistical difference in the same material in different periods.

Sealer Plus BC presented higher pH values in all experimental periods compared to epoxy resin sealers and MTA Fillapex ($p<.05$), except for the 28-day period for MTA Fillapex ($p>.05$). When the same sealer was evaluated in the different experimental periods, all of them presented pH elevation between 1h and 24h ($p <.05$) and, between 14 and 28 days, there was no statistical difference in pH values ($p>.05$) (Table 2).

Radiopacity, Film thickness, Flow and Setting time

Table 3. Means and standard deviations of radiopacity (mm Al), film tickness (µm), flow (mm) and setting time (minutes) of each sealer.

SEALER	RADIOPACITY (mm Al)	FILM THICKNESS (micrometers)	FLOW (milimeters)	INICIAL SETTING TIME (minutes)	FINAL SETTING TIME (minutes)
AH PLUS	8.30^{a} (± 0.18)	30^{a} (± 26.67)	22.37^{a} (± 22.99)	452.7^{a} (± 14.87)	864.0^{a} (± 15.26)
SEALER PLUS	5.37^{b} (± 0.09)	30^{a} (± 23.33)	22.74^{a} (± 22.87)	120.7^{b} (± 6.28)	212.3^{b} (± 7.81)
MTA FILLAPEX	3.11^{c} (± 0.05)	40^{b} (± 43.33)	24.55^{a} (± 24.96)	365.7^{c} (± 11.54)	No final setting time
SEALER PLUS BC	3.06^{c} (± 0.06)	20^{a} (± 26.67)	24.97^{a} (± 24.04)	240.0^{d} (± 13.42)	368.0^{c} (± 12.1)
ISO 6876:2012	**>3**	**<50**	**>20**	**-**	**-**

*Different letters represent statistical difference among materials in the same column ($p\leq0.05$).

AH Plus sealer showed the highest radiopacity ($p < .05$) compared to other materials. The MTA Fillapex and Sealer Plus BC presented lower radiopacity,

with no statistical difference between them (p > .05) (Table 3). MTA Fillapex showed higher film thickness compared to all other materials studied (p < .05) (Table 3). There was no statistical difference among the analyzed sealers at flow analysis (p > .05) (Table 3). All materials tested obtained difference among them in inicial and final setting time analysis (p < .05). AH Plus sealer revealed the higher initial setting time (p < .05). MTA Fillapex, after seven days, didn't presented final setting time (Table 3).

Energy-dispersive X-ray sprectroscopy Analysis (EDX)

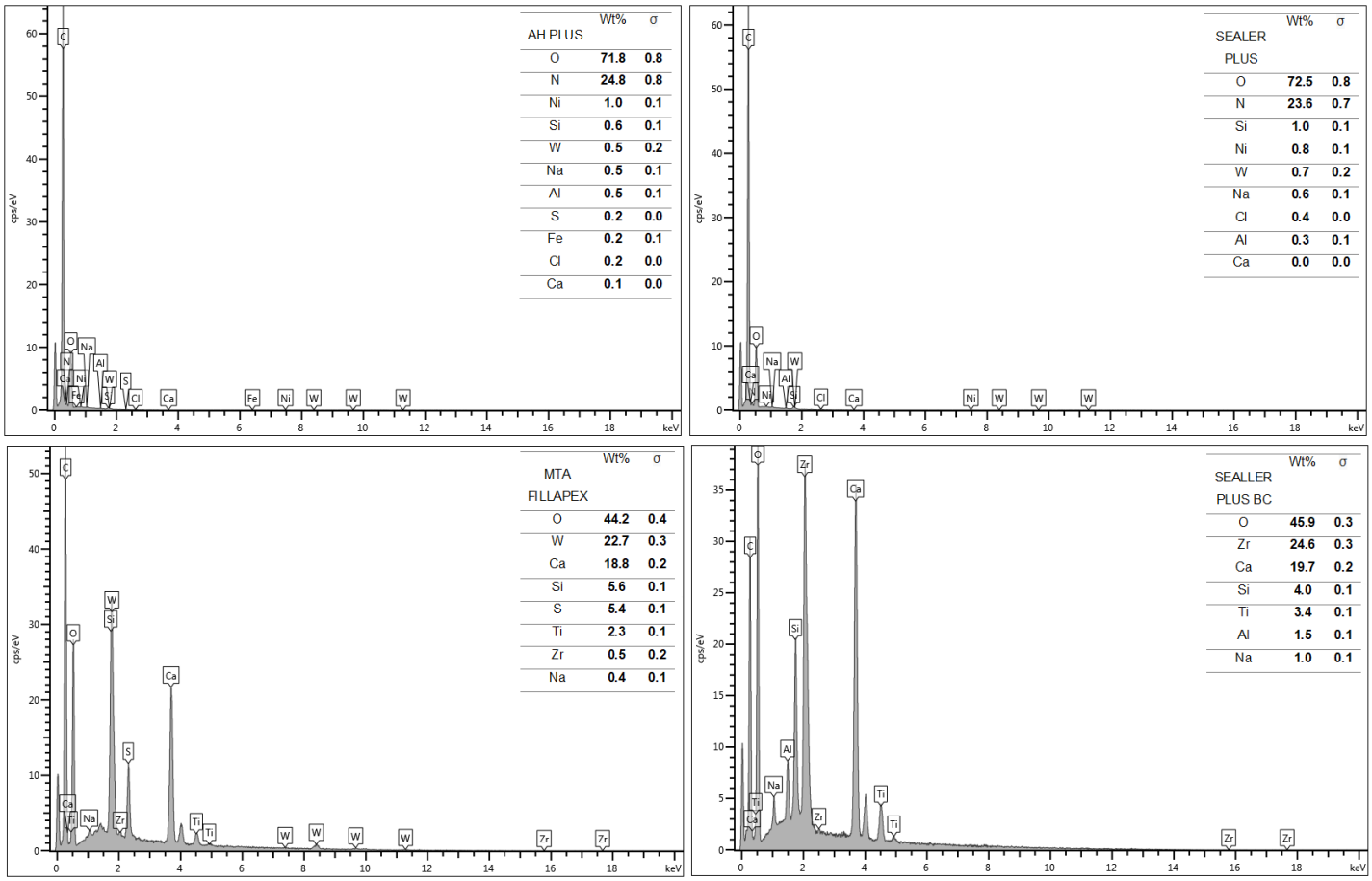

Figure 1: EDX spectra and elemental microanalysis demonstrating the sealers chemical elements' composition and quantification.

EDX analysis revealed peaks of calcium (Ca), 18.08% wt and 19.7% wt, for MTA Fillapex (from calcium tungstate, tri-calcium silicate, di-calcium silicate, calcium hydroxide) and Sealer Plus BC (from tri-calcium silicate, di-calcium silicate, calcium hydroxide), respectively. No traces of Ca (from calcium hydroxide and

calcium tungstate) were found in Sealer Plus surface. Silicon (Si) was detected in all materials. Zr (zirconia) was detected in Sealer Plus BC from the zirconium oxide and W (tungsten) was found in MTA Fillapex, AH Plus and Sealer Plus from calcium tungstate, both are radiopacifiers.

DISCUSSION

Materials that claim the best physicochemical and biological performances are continuously introduced. It is, therefore, necessary to investigate the new materials available. In this study, were tested two epoxy resin-based sealers (AH Plus and Sealer Plus) and two calcium silicate-based sealers (MTA Fillapex and Sealer Plus BC). Few reports are available about the physicochemical properties of Sealer Plus and Sealer Plus BC (12, 16). The procedures were performed as outlined in the ISO 6876/2012 (4) and ASTM C266-03 (5) specifications.

pH increases or alkalinization capacity can be considered a significant chemical property of a sealer, because it may induce periapical repair through mineralization process (17,18,19,20). Sealer Plus BC presented the most alkaline pH value in all periods evaluated, except after 28 days compared to MTA Fillapex. Mendes et al. (16) findings alkaline pH values for Sealer Plus BC compared to AH Plus. Presence of calcium hydroxide in composition may have contributed to this result.

On the other hand, for the release of the hydroxyl ions, the solubilization of the endodontic sealers is necessary, which can compromise dimensional stability (36). Sealer Plus BC has already demonstrated high solubility (Mendes *et al.* 2018) and Duarte et al (33), incorporated calcium hydroxide to AH Plus and observed increased the solubility and In this way, Eldeniz et al. (37) have stated that the problematic dissolution of the root canal sealers is limited because the surface area of the root canal sealer that is exposed to the tissue fluid is limited.

AH Plus and Sealer Plus showed an initial acid pH (1h and 3h) and a weak alkaline pH after 7 days, with no statistical difference between them. Notwithstanding, these two sealers presenting similar base composition, Sealer Plus presents a differential in its formulation: calcium hydroxide. However, this did not influence the pH result as expected. In spite of this chemical element

being present in Sealer Plus formula compounds', negative result of calcium presence was shown in the EDX.

Recently, Vertuan et al. (12), founded acid pH value (5.51 and 5.65) in 3 hours to AH Plus and Sealer Plus, respectively, this acidity was maintained over time for 7 days, unlike our study which showed a slight increase of pH in the same period. MTA fillapex behaved similarly to the resin-based materials studied in the initial periods (1h and 3h), though after 28 days period, it obtained a pH statistically similar to Sealer Plus BC. Poggio et al. (22), reported an alkaline pH (8.02) to MTA Fillapex in 24 hours period and this result corroborates our findings. However, Silva et al. (14), found that the initial pH of MTA Fillapex was alkaline (9.3) and gradually declined over time to 7.76 after one week.

The flow and film thickness of sealers determines the capacity of fills the accessory canals, dentinal tubules, constitute a thin layer among guta-percha points, in order to fill the empty spaces, irregularities and prevent fluid percolation through the root canal system (3, 32). A high-flow can lead to apical leakage of the material and it can cause injury to periapical tissue due to cytotoxicity (33). ISO 6876/2012 (4) determines a minimum of 20 mm as a desirable flow and film thickness under 50 µm. In our study, all sealers tested were in compliance with established standards. In flow analysis, there was not statistical difference among sealers. MTA Fillapex had the thickest film thickness (40 µm) compared to all other materials studied ($p<0.05$) and it was similar to that reported by the manufacturer (39.6 µm).

An important factor from the clinical point of view is setting time property. Setting time of an ideal root canal sealer should permit adequate working time, allowing the clinician to fill, evaluate and make necessary corrections during obturation process (27). No standard for the setting time of endodontic sealers exists according to ISO 6876 (4). However, it can not be too slow because a long setting time may cause irritation with periapical tissues affecting biocompatibility (3, 15, 28). Material composition, particle size, temperature and relative humidity are important variables that interfere with the sealers setting time (29).

For this property, the difference among all sealers tested was significant ($p \leq .05$) and it was not in agreement with the values stated by the manufacturers, excepted Sealer Plus BC. Final setting time values in increasing order were

Sealer Plus < Sealer Plus BC < AH Plus < MTA Fillapex. MTA Fillapex was not set after seven days, and this result was similar to Lee et al. (20). Nevertheless, several reports have shown set to this sealer (19, 30, 31). This discrepancy could be explained by differents methodos some studies used only the 100g needle to determine setting time.

Sufficient radiopacity enables clinicians to make a distinction between adjacent anatomical structures and sealers and to evaluate the quality of root fillings (23). According to ISO 6876/2012 (4), radiopacity of root canal sealers should be at least 3 mm aluminum thickness and all sealers tested presented radiographic densities higher than the recommended. In the present study, AH Plus showed statistically higher radiopacity value (8.3 mm Al) other study found values greater than 10 mm Al for this sealer (20). Nevertheless, a too much high radiopacity of a sealer may hide obturation failures and this is not desired. The MTA Fillapex (3.11 mm Al) and Sealer Plus BC (3.06 mm Al) presented lower radiopacity, studies corroborate our findings (12,16, 20, 24).

As Vitti et al. (15) suggested, the differences between the radiopacity of endodontic sealers were probably caused by different radiopacifying agents. Radiopacity of AH Plus and Sealer Plus is provided by zirconium oxide and calcium tungstate, while MTA Fillapex presents calcium tungstate and Sealer Plus BC zirconium oxide only. Vertuan et al. 2018 (12) concluded that the proportion of radiopacifying agents in AH Plus and Sealer Plus was certainly lower in the composition of Sealer Plus. Previous studies have reported lower radiopacities values for calcium silicate-based sealers compared to AH Plus showing a similar result to this study (25, 26). In EDX analysis, not all chemical elements presented in radiopacifiers were detected. Zirconia, from zirconium oxide, was detected in Sealer Plus BC and tungsten, from calcium tungstate, was found in MTA Fillapex, AH Plus and Sealer Plus. However, no zirconia (zirconium oxide) peaks appeared on Sealer Plus and the manufacturer reports the use of this radiopacifier. On the other hand, MTA Fillapex showed zirconia peaks, and this is not a reported radiopacifier.

Energy-dispersive X-ray spectroscopy (EDX) is a technique to reveal which elements are present in a specific sample. EDX analysis consists of detecting the characteristic X-rays produced by each element after bombarding a sample with high-energy electrons in an electron microscope. What makes EDX

useful is that the number of X-rays emitted by each element present in a sample has a direct relationship to its concentration. This is why it is possible to convert X-ray measurements into a final X-ray spectrum and to evaluate the concentrations of the various chemicals present in a sample (34). Borges et al. (35), compared the AH Plus, iRoot SP, MTA Fillapex, Sealapex and MTA-Angelus and obtained as results the presence of high levels of calcium and carbon on the surface of all tested materials except of the AH Plus, this result is similar to our study. Sealer Plus showed no calcium peaks, however, according to manufacturer there is presence of calcium hydroxide in its composition. This might be explained because the elemental mapping EDX shows the distribution of the elements only on the surface of the sample. EDX revealed the presence of zirconia in the MTA Fillapex, however, according to the manufacturer, there is no zirconium oxide in its composition.

Based on the present results, the tested properties of epoxy resin-based sealers as well as the bioceramic-based sealers except the MTA Fillapex (setting time) were in agreement with ISO 6876/2012 and ASTM C266-08. Sealer Plus BC presented higher pH values. Calcium was found on the surfaces of Sealer Plus BC and MTA Fillapex by EDX analysis.

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ARTIGO 2

Biological properties of calcium silicate-based and epoxy resin-based root canal sealers: *in vitro* and *in vivo* study*.

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ABSTRACT

AIM: To evaluate the biological properties through cytocompatibility, bioactivity and would healing assays and, moreover, reaction in the subcutaneous tissue of rats using Sealer Plus BC (BC) compared to AH Plus (AHP), Sealer Plus (SP) and MTA Fillapex (MF).

METHODOLOGY: Apical papilla cells (APCs) were exposed to 10% extract from the tested material in different periods. Cytotoxicity assessment was performed using the 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) and sulforhodamine B (SRB) assays. Bioactivity were evaluated by alkaline phosphatase enzyme activity (ALP), and Alizarin Red coloring (ALZ). A scratch wound healing model was used to determine sealers effects on cell migration. For *in vivo* evaluation, polyethylene tubes containing the sealers were implanted in rats' subcutaneous tissue and histologically evaluated after the periods of 7, 30 and 90 days. Inflammatory content and collagen fibers condensation were scored. Data were submited to statistical tests ($p \leq .05$).

RESULTS: For MTT, in 24h, BC was similar ($p \geq .05$) with all groups. For SRB, BC demonstrated the highest cell viability ($p \leq .05$). At scratch, after 48h, MF, BC and control promoted cell migration and the area's closure. After 72h, AHP and SP not showed scratch healing. At ALP 24h, SP and MF presented lower values compared to control ($p \leq .05$). All sealers were similar to control in 7 and 14-days period, except SP, which was lower ($p \leq .05$). At ALZ, BC had the highest deposition of mineralized nodules ($p \leq .05$). *In vivo*, no material showed significant difference for the inflammatory infiltrate to control in 7 days. MF in 90 days, showed the highest score to inflammatory infiltrate ($p \leq .05$). MF presented eosinophils at 7 days ($p \leq .05$). There was fiber condensation formation at 30 and 90 days ($p \geq .05$). At 30 and 90-day period, MF and BC presented the highest score of macrophages ($p \geq .05$).

CONCLUSIONS: BC, MF and SP showed cell viability while AHP mild cytotoxicity and cell death in a scratch assay. All materials obtained calcium and phosphate deposits and alkaline phosphatase activity, however, BC presented superior results for mineralized nodules production. Despite being citocompatible, calcium-silicate based sealers showed higher macrophages activity over time,

mainly, MF that also presented giant cell and eosinophils, presenting moderate biocompatibility.

Key words: endodontics, canals sealer, calcium silicate, cytotoxicity, bioactivity, biocompatibility, cell migration, subcutaneous connective tissue.

INTRODUCTION

The contact of the endodontic sealers with the periradicular tissues effects on cell metabolism and regeneration. The composition of the sealers influences its biocompatibility and it may influence the outcome of the endodontic treatment. In this way, a cytotoxic material can trigger cell degeneration, causing adverse effects, and delay periapical healing (Pinheiro *et al.* 2018).

It is recommended that the sealers be carried out by initial tests (cytotoxicity, mutagenicity, and systemic toxicity when taken orally), followed by other preliminary tests (subcutaneous, muscular and osseous implants and sensitization and irritation tests) and then preclinical animal usage studies. Only then should tests with humans be carried out. Cell culture studies may elucidate mechanisms that involve the different biological responses of the materials (Collado-Gonzalez *et al*. 2017), in this regard, cytotoxicity and bioactivity assays are the first screening tests to evaluate dental material (Bae *et al*. 2010). Taken together, a widely used method to determine biocompatibility consists in implementation of endodontic materials in connective tissue of rats (Mittal *et al*. 1995, Silveira *et al*. 2011). Thus, the ability to generate inflammatory effect can be determined by histopathological analysis of tissues that involve these implants containing the materials inserted in animals (Zmener *et al*. 1988, Economides *et al.* 1995, Grecca *et al*. 2011).

Calcium silicate-based sealers was introduced as root repair material and root canal sealer and promove important role due to appropriate biological responses (Zhang & Li 2009, Alanezi *et al*. 2010, Damas *et al*. 2011, Loushine *et al.* 2011, Pinheiro *et al.* 2018). According to studies, bioceramic sealers have the potential to promote bone regeneration when involuntarily extruded through the apical foramen, as well as repairing root perforations (Prati & Gandolfi 2015, Rodríguez-Lozano *et al*. 2017).

Different sealers based on calcium silicate have been proposed, such as MTA Fillapex (Angelus, Londrina, PR, Brazil), Endosequence BC Sealer (Brasseler USA, Savannah, GA), TotalFill BC Sealer (FKG Dentaire SA, La Chaux-de-Fonds, Switzerland), and Sealer Plus BC (MKLife, Porto Alegre, RS, Brazil), a premixed sealer, among others. Sealer Plus BC presents zirconium oxide, tri-calcium silicate, di-calcium silicate, calcium hydroxide, and propylene glycol in the composition and showed physicochemical properties as setting time,

pH, calcium release, flow, and radiopacity following the required standards (Mendes *et al.* 2018). However, even though there are many available bioceramic material, no study has analyzed this sealer about its cytotoxicity or effect on cell proliferation, as well as its potential for bioactivity and tissue tolerance, justifying this study.

Thus, the aim of this study was to evaluate the biological properties through cytocompatibility, bioactivity and scratch assays and, moreover, reaction in the subcutaneous tissue of rats using Sealer Plus BC compared to AH Plus, Sealer Plus and MTA Fillapex.

METHODS

This study was approved by the Research Ethics Committee (protocol number 38542614.6.0000.5347) and the Animals Use Ethics Committee (protocol number 34629) Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

Evaluated sealers

It was included two epoxy resin (AH Plus and Sealer Plus) and two calcium silicate (Sealer Plus BC and MTA Fillapex) based sealers. The chemical composition of the tested materials is shown in Table 1.

Sealers were manipulated according to the manufacturer, seemed in a two 12-well plate (Kasvi, Curitiba, PR, Brasil) (n = 3) and, before setting, filled with DMEM supplemented with 10% FBS and 1% P/S (volume = 360mm³ per well). The plates were incubated at 37°C, 100% humidity and 5% de CO_2 for 24 hours and the extracts were collected in sterile Falcon tubes. Sealer's extracts at 10% concentration were obtained using DMEM as medium.

Table 1**.** Chemical composition and manufacter of the endodontic sealers used in this study**.**

SEALER/ALLOTMENT	MANUFACTURER	CONTENTS
AH Plus 350680K	Dentsply, York, PA, USA	Paste A: Bisphenol epoxy resin–A, Bisphenol epoxy resin–F, calcium tungstate, zirconium oxide, silica, iron oxide pigments. Paste B: Dibenzyldiamine, aminodiamantana, tricyclodecane–diamine, calcium tungstate, zirconium oxide, silica, silicone oil.
Sealer Plus PS170330010410	MK Life, Porto Alegre, RS, Brazil	Basic Paste: Bisphenol A-co-epichlorohydrin, Bisphenol F epoxy resin (formaldehyde, oligomeric product with 1-chloro-2,3-epoxypropanol and phenol); zirconium oxide, silicone and siloxanes, iron oxide (pigment), calcium hydroxide. Catalyzer Paste: Hexamethylenotetramine, zirconium oxide, silicone and siloxanes, calcium hydroxide, calcium tungstate.
MTA Fillapex 43663	Angelus, Londrina, PR, Brazil	Base Paste: Salicylate resin, natural resin, calcium tungstate, nanoparticulated silica, pigments; Catalyst Paste: diluting resin, Mineral Trioxide Aggregate, nanoparticulated silica, pigments;
Sealer Plus BC WR770100	MK Life, Porto Alegre, RS, Brazil	Zirconium oxide, tri-calcium silicate, di-calcium silicate, calcium hydroxide, propylene glycol.

Cell culture

Apical papilla cells (APCs) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin and 10% streptomycin (PenStrep, Gibco, Invitrogen, Life Technologies, Canyon City, Oregon, USA) in an incubator at 37°C, 100% humidity and 5% CO_2. Successive passages were prepared for cellular expansion, and the experiments were performed at the fourth cell passage. At 80% confluence, cells were treated with 0.25% Trypsin-EDTA solution (Sigma-Aldrich) to plate.

The experiments hereafter were performed in triplicate, n = 10 samples per group for the cytotoxicity and bioactivity assays, and n = 3 samples per group for the scratch assay. DMEM medium was used as a control for all experiments.

Cytotoxicity assays

Cytotoxicity was assessed by 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich), and sulforhodamine B (SRB, Sigma-Aldrich).

The MTT assay was analyzed in two experimental periods, 24h and 72h, after cells exposure to the extracts at 10% concentration. For this assay, SCAPs were seeded in 96-well plates (Kasvi, Curitiba, PR, Brasil) at a concentration of $5x10^3$ cells/mL, and 10 µL of a 5 mg/mL MTT solution were added in each well, followed by incubation for 3h at 37°C, 100% humidity and 5% de CO_2. After this period, the wells contents were removed and the colorimetric product solubilized in 100 µL of acidified isopropanol (HCl 0.4N, Sigma-Aldrich). The optical densities of the solutions were measured in a spectrophotometer (Thermo Fischer Scientific Inc., Waltham, MA, USA) at 570 nm wavelength.

For SRB evaluation, APCs were seeded in 96-well plates (Kasvi, Curitiba, PR, Brasil) at a concentration of $5x10^3$ cells/mL, exposed to the 10% eluate for 72h, and the, 25 µL of a 50% trichloroacetic acid solution (Sigma-Aldrich) were added in each well, followed by incubation for 1h at 4°C. After this period, the wells contents were removed and 100 µL of SRB dye (0.4%) were added in each well for 30 minutes. The plate was washed with 1% acetic acid solution (Sigma-

Aldrich) and 100 µL of Trizma Base (10mM) (Sigma-Aldrich) were added to solubilize the colorimetric product. The optical densities of the solutions were measured in a spectrophotometer (Thermo Fischer Scientific Inc., Waltham, MA, USA) at 570 nm wavelength.

The MTT and SRB absorbance readouts were normalized with the absorbance of the control group (DMEM) and represented the activity of the viable cells.

Would-Healing (Scratch assay)

APCs were seeded in two 12-well plates (Kasvi, Curitiba, PR, Brasil) at a concentration of $3x10^5$ cells/mL. After 48h incubation at 37ºC, 5% CO^2 and 95% humidity, a scratch (cross risk) was realized in the cell monolayer with a pipette tip P10 (TPP Techno Plastic Products, Trasadigen, Switzerland). Culture medium was replaced by the extract of the 10% sealers. From this moment, the plates were taken to the Axio Observer Z1 inverted microscope (Zeiss, Göttingen, Germany) with a coupled photographic camera (Axiocam mrn, Zeiss, Göttingen, Germany) using a 10x objective (Ecplan-Neofluar 10x / 0.3 aperture, Zeiss, Göttingen, Germany) and images were captured at first period and every 6 hours during 72 hours. Images of cell migration were analyzed using ImageJ Software (National Institutes of Health, Bethesda, MD).

Bioactivity assays

Bioactivity were evaluated by alkaline phosphatase (ALP) enzyme activity, and Alizarin Red (ALZ) coloring. ALP was assessed at the experimental periods of 1, 7, 14 days and ALZ at 14 days. For both analyses, APCs were seeded at a concentration of $1x10^4$ cells/mL and exposed to 10% sealers eluate.

For ALP, at each experimental time point, the culture medium containing material extracts was removed from the wells, and the cells were washed with 500 µL of PBS 1X. Then, 500 µL of sodium dodecyl sulfate solution 1% (SDS, Sigma-Aldrich) was added to each well. Samples were let to rest for 30 minutes at room temperature, and the protocol was followed according to the manufacturer's instructions (Labtest, Lagoa Santa, MG, Brazil). Samples were

transferred to a 96-well plate for the measurement of absorbance using a spectrophotometer at 590 nm wavelength (ELx800; Bio-Tek Instruments). Data were expressed as unit/liter (U/L), according to the formula below:

$$\text{Alkaline phosphatase (U/L)}: \frac{\text{test absorbance}}{\text{standard absorbance}} \times 45$$

For ALZ assay, after experimental time point, extracts were removed from the wells, and the cells were washed with 500 µL of PBS 1X and then fixed with 500 µL of formaldehyde solution 10% (Sigma-Aldrich) at room temperature for 15 minutes. Subsequently, the wells were washed with 500 µL of distilled water, and 500 µL of AZR solution 2%, pH 4.2 (Sigma-Aldrich), was added to each well and stored at room temperature for 20 minutes. The nodules were solubilized with 10% cetylpyridinium chloride (Sigma-Aldrich) for 15 min, and the optical density was measured in a 96-well plate at 562 nm wavelength in a spectrophotometer (Thermo Fischer Scientific Inc.).

Biocompatibility assay (in vivo)

A total of 30 male Wistar rats (Rattus norvegicus) weighing between 220 and 300g at 6 weeks old were used. They were divided into 3 experimental periods (7, 30, and 90 days) (n = 10). The animals were anesthetized with 0.008 mL/100 g ketamine (Francotar® - Virbac do Brasil Indústria e Comércio Ltda., Roseira, SP) and 0.004 mL/100 g 2% xylazine hydrochloride (Virbaxyl® 2% - Virbac do Brasil Indústria e Comércio Ltda., São Paulo, SP). The area was disinfected with alcohol-iodine solution (Quinta Essência Cosméticos e Medicamentos Ltda., Porto Alegre, RS, Brazil) after the dorsal skin was shaved. Five incisions measuring 1.0-cm long were made in the animals' backs. Using blunttipped scissors, lateral tearing of the subcutaneous tissue provided 5 surgical cavities in quadrants equidistant from the center of the animals' backs. Polyethylene tubes approximately 10-mm long and 1.3 mm in diameter (Abbott Laboratories do Brasil, São Paulo, SP, Brazil) were filled with the sealers, which were prepared according to the manufacturers' instructions. The filled tubes were inserted into the surgical cavities parallel to the incision. An empty tube was used

as the control. The incisions were closed using 4-0 silk thread (Johnson & Johnson Produtos Profissionais Ltda, São José dos Campos, SP, Brazil).

Analgesia was performed in the postoperative period for pain control. It was administered anti-inflammatory (Ketofen 1% injectable - 0.2 mL / kg - 1 time daily for 3 days - as indicated) and antimicrobial (Flotril 2.5% injectable - 0.2 mL / kg - once daily for 5 days - according to the indicated dosage) intraperitoneal route, according to responsible veterinarian prescription.

After each experimental period, the animals were euthanized with inhalational anesthetic, Isoflurane (Instituto Biochimico LTDA, Itatiaia, RJ, Brazil). Trichotomy and excision of the implant area were performed, and the resulting specimens were placed on paper discs and fixed in 10% formalin for 48 hours.

After fixation, the polyethylene tubes containing the test materials were removed, and the tissue was embedded in paraffin. Sections 5- to 6-µm thick were taken along the axis of the tube, mounted on slides, and stained with hematoxylin-eosin. Slices were examined under a light microscope by a single-blinded and calibrated examiner (kappa >7 for all evaluated variables).

Cellular and inflammatory events were determined by the presence of neutrophils, giant cells, eosinophils, macrophages, and inflammatory infitrate intensity scored according to the following scale (Figueiredo *et al*, 2001):

1. Absent, no inflammation
2. Mild, cells were present but sparse or in reduced clusters
3. Moderate, cells were present but did not dominate the microscopic field
4. Intense, cells were present in the form of an infiltrate similar to the material used

Fiber condensation was classified according to the following scale:

1. Absence
2. Presence of a thin layer
3. Presence of a thick layer of collagen fibers

Abscess formation was classified as follows:

1. Absence
2. Presence of an abscess in contact with the surgical cavity where the material was inserted
3. Presence of an abscess far from the surgical cavity.

Statistical Analysis

One-way ANOVA and the Tukey's *post hoc* were used to compare data for cytotoxicity, bioactivity, and cell migration. For *in vivo* tissue response, statistical analyses were performed using Kruskal-Wallis test and Dunn's *post hoc* among materials in the same experimental period, and One-way ANOVA and the Tukey's *post hoc* were used to compare the same material in different periods. The significance level was set at .05. Statistical analysis was performed using GraphPad Prism v.6 (GraphPad Software Inc., San Diego, CA, EUA).

RESULTS

Cytotoxicity assays

For MTT, in 24-hour period, Sealer Plus BC presented statistical similarity ($p \geq .05$) with all sealers tested including control group. After 3 days, Sealer Plus and Sealer Plus BC showed viability higher than 100%, statistically higher than the control group, AH Plus and MTA Fillapex ($p \leq .05$). For SRB, Sealer Plus BC demonstrated the highest cell viability ($p \leq .05$). Only AH Plus presented values lower than control group ($p \leq .05$) (Figure 1).

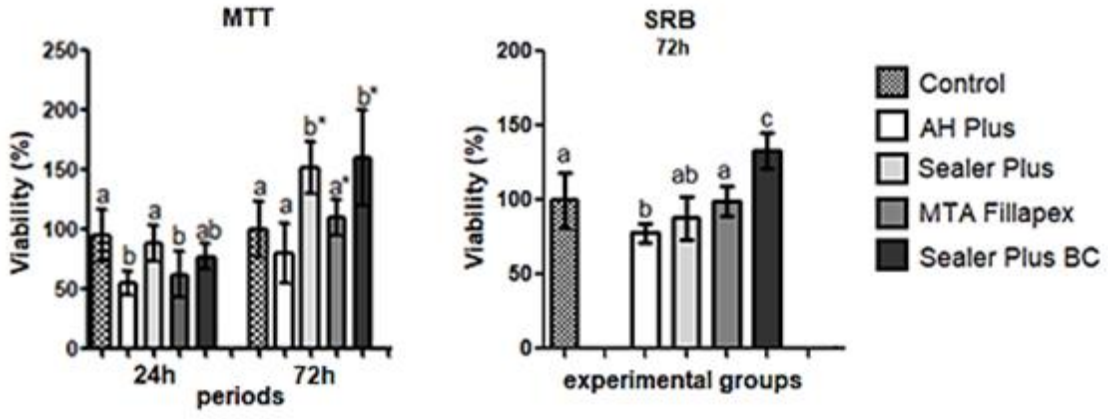

Figure 1. MTT and SRB Sealers' cytotoxicity assays at APCs. Different lowercase letters represent statistical difference among sealers at the same evaluated period ($p \leq .05$). * Indicate significant difference among the same material in different periods (MTT) ($p \leq .05$).

Would-healing

To determine the effects of sealers extracts on would-healing, an *in vitro* scratch healing assay was used. In the period of 48 hours, MTA Fillapex, Sealer Plus BC and control group promoted the cell migration and the closure of the area in 100%. After 72 hours of experimental period, AH Plus and Sealer Plus not showed scratch healing, however AH Plus presented the lowest value of cell covered área ($p \leq .05$) (Figure 2 and 3).

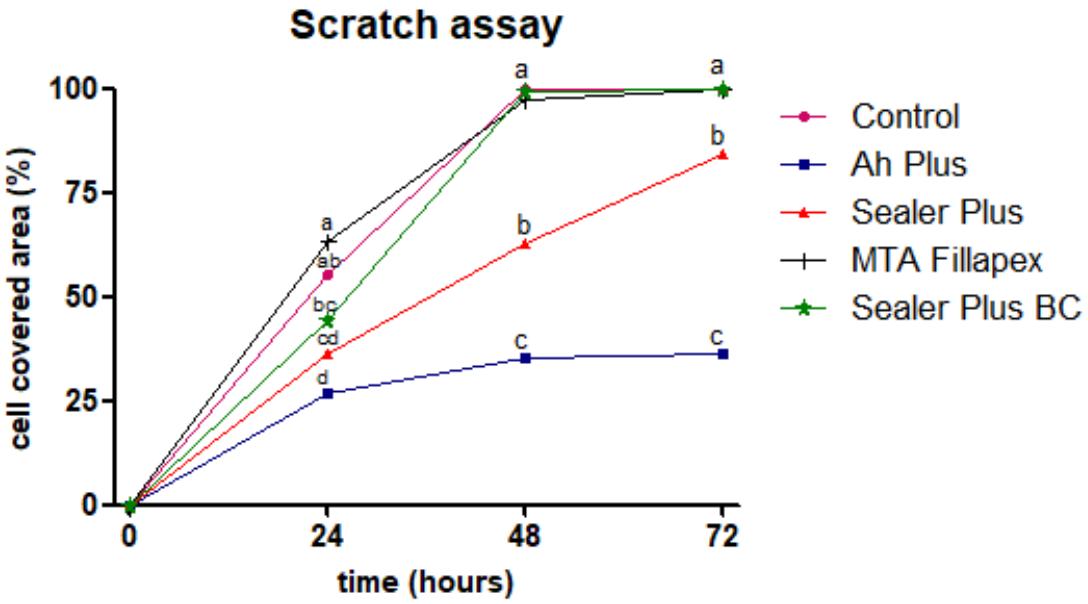

Figure 2. Scratch evaluation of sealer extracts at APCs. Different letters indicate a significant difference among materials in the same experimental period ($P \leq .05$).

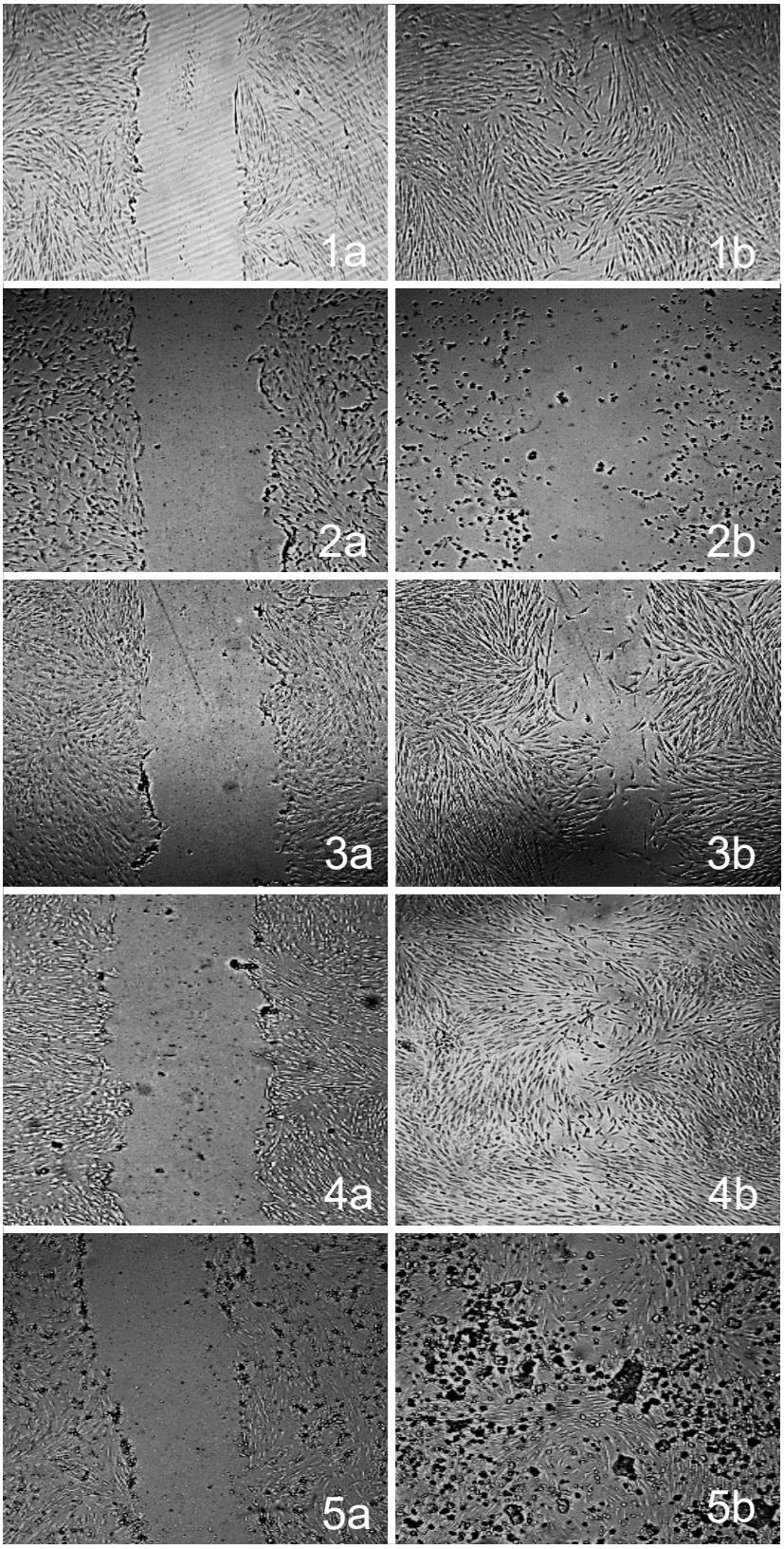

Figure 3. Images corresponding to the scratch assay according to the evaluated sealer. Letter "a" corresponds to the initial period (t = 0) and letter "b" corresponds to the final period of 72 hours. 1 Control. 2 AH Plus. 3 Sealer Plus. 4 MTA Fillapex. 5 Sealer Plus BC. (10x)

Bioactivity assays

At ALP 24h experimental period, Sealer Plus and MTA Fillapex presented lower values compared to control ($p \leq .05$). All sealers were like control in 7 days period, except Sealer Plus ($p \leq .05$). Enzyme activity outcomes in all materials were similar to control at 14 days ($p \geq .05$) (Figure 4).

At ALZ assay, Sealer Plus BC demonstrated the highest deposition of mineralized nodules compared to other sealers ($p \leq .05$). AH Plus and MTA Fillapex were similar ($p \geq .05$). Control did not present deposition of mineralized nodules (Figure 4).

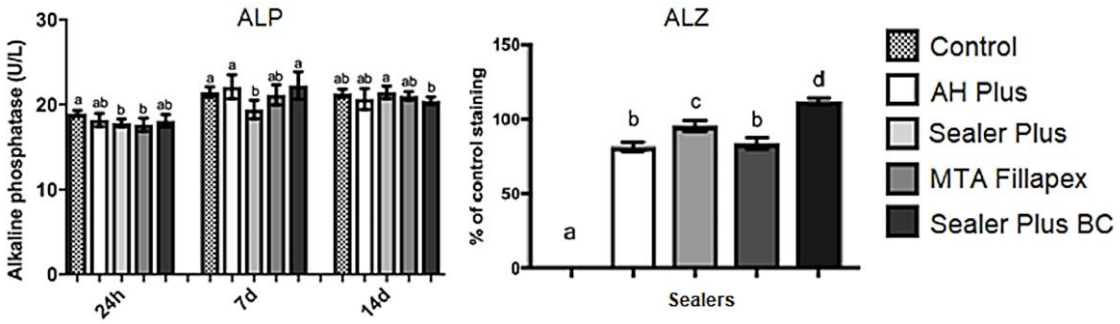

Figure 4. ALP enzyme activity assays of the sealers at APCs. Different lowercase letters represent statistical difference between them at the same evaluated experimental period ($P \leq .05$). ALZ assay of the sealers at APCs. Different lowercase letters represent statistical difference between the sealers ($P \leq .05$).

Biocompatibility assay (in vivo)

There were 20 implants lost: - control group: 5 implants; - AH Plus: 5; - Sealer Plus: 3; - MTA Fillapex: 5; - Sealer Plus BC: 2.

The results are expressed in table 2 and figures 5 and 6.

No abscesses were found in any groups. Only at 7 days, neutrophils were present in control group and Sealer Plus BC, however, without significant difference with other sealers ($p \geq .05$). Giant cell was present at 30 days for MTA Fillapex and Sealer Plus BC, however, without significant difference with other sealers ($p \geq .05$). At 7 days, no fiber condensation formation was observed for sealers. There was fiber condensation formation at 30 and 90 days without statistical difference between them ($p \geq .05$). At 30 and 90-day period, MTA

Fillapex and Sealer Plus BC presented the highest score for the presence of macrophages in relation to the other sealers.

No material showed significant difference for the inflammatory infiltrate compared to control in 7-days period ($p \geq .05$). Nevertheless, after 30 days Sealer Plus BC showed the highest presence of inflammatory infiltrate and, after 90 days, MTA Fillapex showed the highest score ($p \leq .05$) among materials and control. It was observed eosinophils only at 7 days for MTA Fillapex ($p \leq .05$).

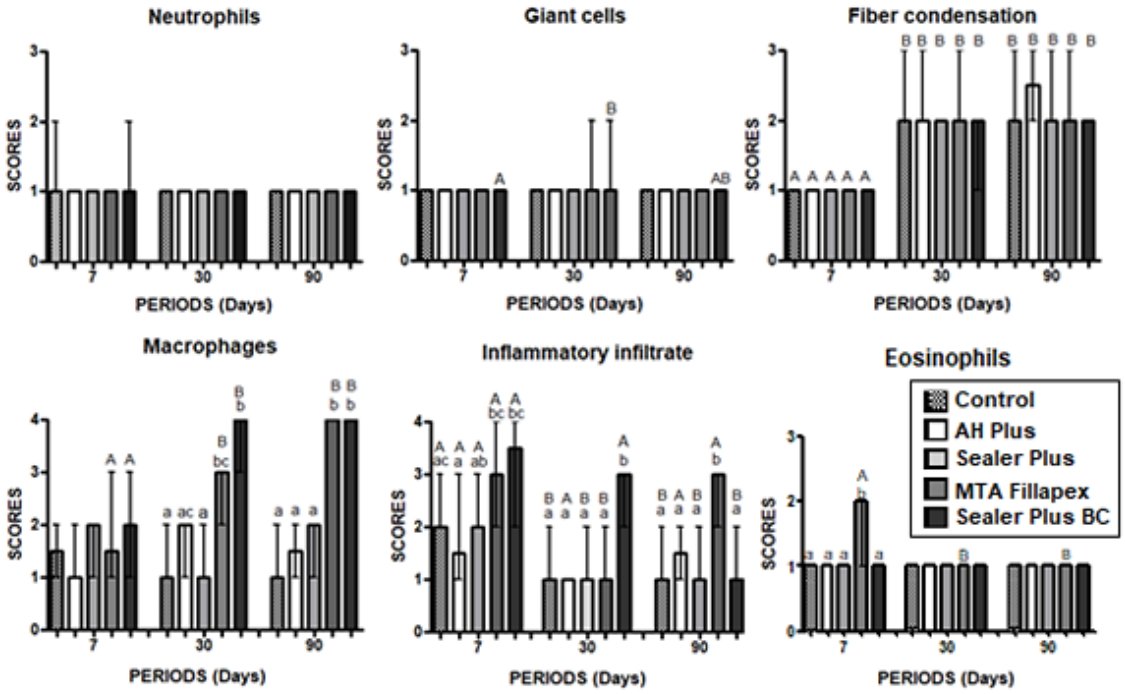

Figure 5. Comparison among materials and experimental periods for each variable including neutrophils, giant cells, fiber condensation, macrophages, eosinophils, and inflammatory infiltrate. No abscesses were found; thus, it was not classified in the figure. Different lowercase letters indicate significant differences among different sealers in the same period ($p \leq .05$). Different uppercase letters indicate a significant difference in the same material in the different experimental periods ($p \leq .05$).

Table 2. Absolute and relative frequencies for observed histologic features according to periods and groups.

SCORES	CONTROL			AH PLUS			SEALER PLUS			MTA FILLAPEX			SEALER PLUS BC		
	7	30	90	7	30	90	7	30	90	7	30	90	7	30	90
INFLAMMATORY INFILTRATE INTENSITY															
1	-	6 (66,6%)	7 (87,5%)	4 (50%)	9 (100%)	4 (50%)	-	5 (55,5%)	6 (75%)	-	8 (88,8%)	-	-	-	5 (55,5%)
2	5 (62,5%)	3 (33,3%)	1 (12,5%)	3 (37,5%)	-	4 (50%)	6 (60%)	4 (44,5%)	2 (25%)	3 (30%)	1 (11,2%)	-	1 (10%)	3 (33,3%)	4 (44,5%)
3	3 (37,5%)	-	-	1 (12,5%)	-	-	4 (40%)	-	-	5 (50%)	-	6 (100%)	4 (40%)	6 (66,6%)	-
4	-	-	-	-	-	-	-	-	-	2 (20%)	-	-	5 (50%)	-	-
NEUTROPHILS															
1	7 (87,5%)	9 (100%)	8 (100%)	8 (100%)	9 (100%)	8 (100%)	10 (100%)	9 (100%)	8 (100%)	10 (100%)	9 (100%)	6 (100%)	8 (80%)	9 (100%)	9 (100%)
2	1 (12,5%)	-	-	-	-	-	-	-	-	-	-	-	2 (20%)	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GIANT CELLS															
1	8 (100%)	9 (100%)	8 (100%)	8 (100%)	9 (100%)	8 (100%)	10 (100%)	9 (100%)	8 (100%)	10 (100%)	7 (77,8%)	6 (100%)	10 (100%)	5 (55,5%)	9 (100%)
2	-	-	-	-	-	-	-	-	-	-	2 (22,2%)	-	-	4 (44,5%)	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EOSINOPHILS															
1	-	-	-	-	-	-	-	-	-	3 (30%)	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	7 (70%)	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MACROPHAGES															
1	4 (50%)	7 (77,8%)	7 (87,8%)	5 (62,5%)	3 (33,3%)	4 (50%)	3 (30%)	6 (66,7%)	3 (37,5%)	5 (50%)	-	-	3 (30%)	-	-
2	4 (50%)	2 (22,2%)	1 (22,2%)	3 (37,5%)	6 (66,7%)	4 (50%)	7 (70%)	3 (33,3%)	5 (62,5%)	4 (40%)	1 (11,2%)	-	5 (50%)	-	-
3	-	-	-	-	-	-	-	-	-	1 (10%)	8 (88,8%)	-	2 (20%)	3 (33,3%)	-
4	-	-	-	-	-	-	-	-	-	-	-	6 (100%)	0 (0%)	6 (66,6%)	9 (100%)
FIBER CONDENSATION															
1	8 (100%)	-	-	8 (100%)	-	-	10 (100%)	-	-	10 (100%)	-	-	10 (100%)	1 (11,2%)	-
2	-	7 (77,8%)	7 (87,5%)	-	6 (66,7%)	4 (50%)	-	9 (100%)	7 (87,5%)	-	8 (88,8%)	5 (83,4%)	-	8 (88,8%)	9 (100%)
3	-	2 (22,2%)	1 (12,5%)	-	3 (33,3%)	4 (50%)	-	-	1 (12,5%)	-	1 (11,2%)	1 (16,6%)	-	-	-

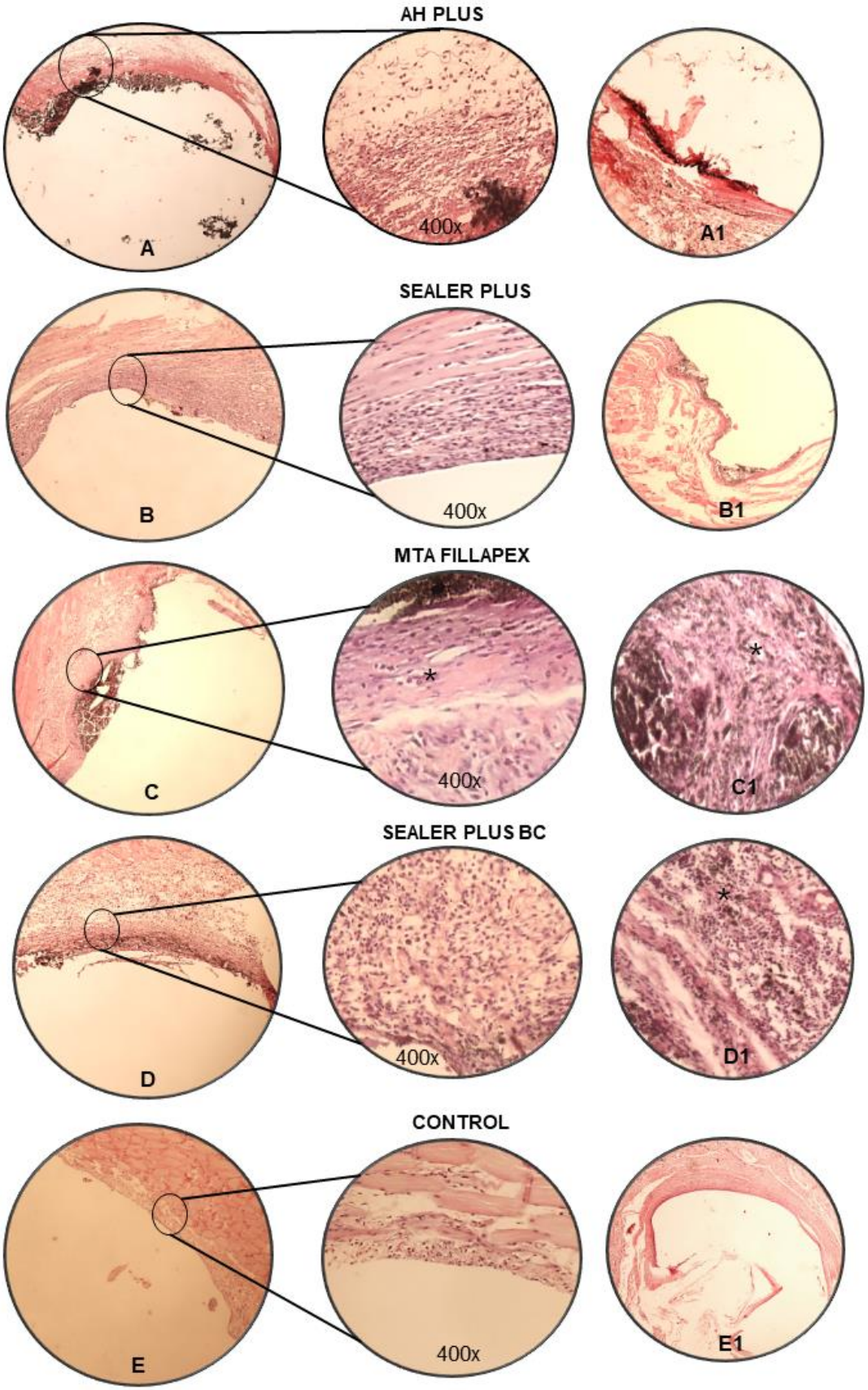

Figure 6. Connective tissue response to the different endodontic sealers. A- AH Plus 7 days (40x): slight inflammatory infiltrate. A1: AH Plus 90 days (40x): presence of thin layer of fibrous condensation. B- Sealer Plus 7 days (40x): Mild inflammatory infiltrate. B1- Sealer Plus 90 days (40x): presence of thin layer of fibrous condensation. C- MTA Fillapex 7 days (40x): Moderate

inflammatory infiltrate, presence of sparse eosinophils (*). C1- MTA Fillapex 90 days (400x): presence of thin layer of fibrous condensation, intense presence of macrophages (*). D-Sealer Plus BC 7 days (40x): Moderate inflammatory infiltrate, slight presence of neutrophil and macrophages. D1- Sealer Plus BC 90 days (100x): presence of thin layer of fibrous condensation, intense presence of macrophages (*). E- Control 7 days (40x): Mild inflammatory infiltrate. E1- Control 90 days (40x): presence of thin layer of fibrous condensation.

DISCUSSION

The intimate contact of the periapical tissues with the endodontic sealers requires material's biocompatibility, no cytotoxicity and not inducing a long-term inflammatory reaction, without producing adverse effects, such as activation of a host response or direct induction of cell death. In order to evaluate sealers' biological response, as well as their possible risks, cell culture methodologies (*in vitro* assay) and histological evaluation (*in vivo* assay) were associated in this study.

Endodontic materials' cytocompatibility is currently tested in different cell lines (Rodríguez-Lozano *et al.* 2015, Poggio *et al*. 2017, Pinheiro *et al*. 2018, Jung *et al*. 2018, Zordan-Bronzel *et al*. 2019). Apical papilla cells (APCs) were used to realize the assays considering that they differentiate into odontoblast-like cells forming dentin (Sonoyama *et al*. 2006) and appear to be the source of primary odontoblasts that are responsible for the formation of root dentin (Huang *et al*., 2008).

Cytotoxicity was assessed by MTT and SRB assays. MTT analyzes mitochondrial succinate dehydrogenase, one of the enzymes responsible for cellular respiration, and SRB determines protein synthesis in cells. According to ISO 10993-5 (2009) a material exhibits slight cytotoxicity above 60% cell viability and is considered non-cytotoxic above 90%. When cell respiration was analyzed, Sealer Plus BC, MTA Fillapex and Sealer Plus could be considered non-cytotoxic in 72h-period, the same occurred when protein synthesis (SRB) was analyzed. AH Plus presented moderate cytotoxicity for both assays. Cintra *et al*. (2017) obtained greater cell viability to Sealer Plus after 24 hours to pure extract while AH Plus showed moderate cytotoxicity in fibroblasts (L929). In human periodontal ligament stem cells, MTT showed greater cell viability for TotalFill BC Sealer and the lowest for AH Plus (Rodríguez-Lozano *et al*. 2015). On the other hand, MTA Fillapex presented severely cytotoxicity on human

gingival fibroblast and primary human osteoblast (Zhou *et al.* 2015, Jung *et al*. 2018), the authors suggested that the resinous components' presence, mainly salicylate resin may induce cell apoptosis. Moreover, the radiopacifier used in previous research with MTA Fillapex may have been the bismuth-containing, present in its past composition rather than calcium tungstate.

Silicate-based sealers are reported as an inducer of cell differentiation and odontogenic proliferation (Peng *et al*. 2011). In the first 24 hours, all materials promoted some level of cell migration. Sealer Plus BC and MTA Fillapex supported cell survival and exhibited excellent cell migration in SCAPs with wound closure at 48h, similar to control. AH Plus and Sealer Plus, resin-based sealers, did not promote scratch closure in 72h, however AH Plus, showed a stagnation and consequent cell death. Using living/death and Richardson staining, the odontoblasts did not survive at the 10% concentration for AH Plus and showed an altered morphology (Jung *et al*. 2018). Rodriguez-Lozano *et al.* (2015), also observed this cell stagnation for AH Plus after 24 hours in human periodontal ligament stem cells, without cell death. However, in this same study, cells treated with MTA Fillapex did not promote wound healing, probably because the different composition. Sealer Plus showed a growing cell migration, not enough to close in 72h, this material has a similar composition to AH Plus, but in its formula, there is calcium hydroxide's presence and that may have influenced this better result. Black granules' formation was observed for Sealer Plus BC, which were present in initial period sparingly, and over time, increased in quantity.

Alkaline phosphatase enzyme activity makes it possible to determine the inductive potential of mineralized tissue formation. The bioceramic sealers did not show higher enzymatic activity to alkaline phosphatase compared to resin-based sealers, in agreement with Benezra *et al.* 2018 and Bronzel *et al*. 2019, which used human osteoblast-like cells (Saos-2) and human gingival fibroblast cells, respectively.

ALZ identifies calcium and phosphate deposits in cell culture, all sealers evaluated induced the production of mineralized nodules. Sealer Plus BC showed a greater and significant deposition of calcium nodules, followed by Sealer Plus. Sealer Plus, although it is a resin-based sealer, presents calcium hydroxide in the composition, MTA Fillapex, who presents calcium hydroxide, and AH Plus

showed lower production of mineralized nodules. In osteoblast precursor cell line, AH Plus did not present mineralized nodules, while EndoSequence BC Sealer presented a high osteogenic potential (Giacomino *et al.* 2019).

The bioactive potential of calcium silicate-based sealers is a consequence of the pH increases, alkalinization capacity, and solubility of these materials even after setting (Donnermeyer *et al.* 2018). Sealer Plus BC has already demonstrated alkaline pH and high solubility (Mendes *et al.* 2018). One possible explanation for the results of Sealer Plus BC is the difference in presentation, because it is a premixed sealer, and only starts its set-in contact with humidity, while the others started soon after their spatulation.

Taken into account, the sealers interact with cells of the periapical region and immunocompetent cells, which are ignored by in vitro study models (Souza *et al*. 2018). *In vitro* cytotoxicity studies results should be interpreted with wariness, so it is necessary to complement *in vivo* models. The human body defenses mechanisms' do not exist in a culture plate and must be considered for interpretations for biocompatibility (Loushine *et al.* 2011).

In this sense, for the *rat's* assay, to prioritize animal welfare and to disregard adverse contaminations and inflammation due to surgery, an antibiotic (Flotril 2.5%) and an anti-inflammatory (Ketofen 1%) have been administered, so the tissue reaction is given purely in response to the implanted material.

Sealer Plus BC presented a moderate initial inflammatory response (7 days), which was like MTA Fillapex. As well the reduction of this inflammation over time, no granuloma, eosinophils, or abscesses were observed, similar to control group. The calcium silicate-based sealers were the only ones that presented giant cells and showed, over time, an increase in the presence of macrophages, showing intense even after 90 days, which demonstrates the body's attempt to eliminate the foreign material via phagocytosis, probably due to higher pH. MTA Fillapex, although presenting good results in cell viability and migration, was the only material that presented a slight presence of eosinophils, which are related to hypersensitivity reactions, marking a tendency toward increased potential for tissue irritation. This sealer showed moderate

inflammatory infiltrate, without reduction, not exhibiting biocompatibility, exactly as Zmener *et al.* (2012) and Bosio *et al.* (2014) studies. MTA Fillapex is a resin based and not the calcium silicate base sealer, and this resin may be irritating to the periapical tissues. In contrast there are studies that show a good tissue reaction to MTA Fillapex (Gomes-filho *et al*. 2011, Saraiva *et al*. 2018). In addition, MTA Fillapex was not set after seven days (Lee *et al*. 2017), and this could be associated to hypersensitivity reactions.

Compared to the bioceramic sealers, AH Plus showed a lower inflammatory infiltrate and despite having mild cytotoxicity for viability assays and cell death in scratch assay, showed an adequate tissue response in the connective tissue of rats, in accordance with Tavares *et al*. 2013 and da Silva *et al*. 2018., that also obtained better results for AH Plus compared to MTA Fillapex. Sealer Plus, presented good biocompatibility results with an initial mild inflammatory infiltrate, corroborating with the findings of Cintra *et al.* 2017.

In the 90-day period, all groups (including the control group) obtained the formation of a thin fibrous tissue capsule, and this indicates that they are being tolerated by the tissues (Yaltirik *et al*. 2004). More over, collagen fibers condensation is substantially associated with the healing process together with inflammatory content (Figueiredo *et al*. 2001).

CONCLUSIONS

The composition of the sealers influences its biocompatibility when in contact with periapical tissue. Sealer Plus BC, MTA Fillapex and Sealer Plus showed cell viability while AH Plus moderate cytotoxicity and cell death in a scratch assay. All materials obtained calcium and phosphate deposits and alkaline phosphatase activity, however, Sealer Plus BC presented superior results to mineralized nodules production. Despite being citocompatible, calcium-silicate based sealers showed higher macrophages activity over time, mainly, MTA Fillapex that also presented giant cell and eosinophils, presenting moderate biocompatibility.

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CONSIDERAÇÕES FINAIS

Adequadas propriedades físico-químicas e biológicas são requeridas quando do lançamento de um cimento obturador em Endodontia. Atualmente, esforços estão sendo despendidos no estudo e comercialização de cimentos a base de silicato de cálcio, por estes apresentarem características de bioatividade, ou seja, são capazes de interagir com as células do tecido promovendo o selamento biológico através da deposição de tecido mineralizado, sem, no entanto, esquecer das características físico-químicas. Esta dissertação objetivou a avaliação das propriedades físico-químicas e biológicas (*in vitro* e *in vivo*) do cimento Sealer Plus BC, um novo cimento obturador a base de silicato de cálcio.

No Artigo 1, as propriedades físico-químicas do Sealer Plus BC foram avaliadas comparando aos cimentos já existentes a base de resina epóxi (AH Plus e Sealer Plus) e a base de silicato de cálcio (MTA Fillapex). No Artigo 2, os mesmos materiais foram avaliados quanto a sua citotoxicidade, migração celular, bioatividade em células-tronco da papila apical (SCAPs) e biocompatibilidade em tecido conjuntivo de ratos.

Para a metodologia do primeiro estudo, foram realizados testes de acordo com as normas pré-estabelecidas pela ISO e pra ASTM, as quais determinam requisitos mínimos para que os materiais sejam aprovados para uso. Além disso, foi realizada análise do pH por 28 dias para avaliação das variações de alcalinidade ao longo do tempo e determinação da composição elemental da superfície dos cimentos através de espectroscopia de energia dispersiva (EDX), com a finalidade de comparação com o fornecido pelo fabricante. O cimento Sealer Plus BC apresentou os maiores valores de pH associado à presença de cálcio no EDX, além disso também cumpriu todos os requisitos mínimos estipulados pela ISO e ASTM. Todos outros materiais estudados estavam de acordo com as normas, exceto o cimento MTA Fillapex, o qual não mostrou tempo de presa final adequado.

Com relação às metodologias realizadas em cultura celular, no segundo artigo, células da papila apical (SCAPs) foram escolhidas por apresentarem

elevado potencial de proliferação e diferenciação, representando uma população de células progenitoras, correlacionadas à formação radicular (HUANG et al., 2008). Ademais, os ensaios realizados são comumente empregados para verificação da citotoxicidade e bioatividade de materiais (REIS et al., 2017). Para os ensaios de viabilidade (MTT e SRB) o único cimento que demonstrou uma leve citotoxicidade foi o AH Plus, enquanto o Sealer Plus BC, MTA Fillapex e Sealer Plus mostraram viabilidade. Em se tratando de migração celular, apenas os cimentos Sealer Plus BC e MTA Fillapex conseguiram fechar a ferida, inclusive sem diferença estatística para o grupo controle. E, no ensaio de Alizarin, todos cimentos estudados formaram depósitos de cálcio e fosfato, entretanto o que mostrou melhores resultados foi o Sealer Plus BC.

Para determinar biocompatibilidade, a implantação dos cimentos endodônticos em tubos no tecido conjuntivo do dorso de ratos foi utilizada (SILVEIRA et al., 2011) devido ao rápido metabolismo deste animal (MORETTON et al., 2000), fácil manuseio e possibilidade de extrapolar os resultados em humanos, dado que nestes há uma resposta de todo organismo envolvido (SPÅNGBERG, 1978). Sealer Plus BC apresentou moderado infiltrado inflamatório no tempo inicial de 7 dias, entretanto esse diminuiu ao longo dos 90 dias. Além disso, não apresentou granulomas, eosinófilos, abscessos em nenhum período experimental. O cimento MTA Fillapex, embora tenha apresentado resultados satisfatórios em cultura celular, apresentou toxicidade tecidual, foi o único material a apresentar eosinófilos e, somado a isso, seu infiltrado inflamatório não diminuiu com o passar do tempo. O AH Plus juntamente com o Sealer Plus mostrou adequada resposta tecidual, com formação de cápsula fibrosa e diminuição do infiltrado inflamatório com o tempo.

Por fim, conclui-se que o cimento Sealer Plus BC, por apresentar propriedades físico-químicas adequadas, citocompatibilidade, potencial para bioatividade e, por ter obtido resultados satisfatórios *in vivo,* como diminuição do infiltrado inflamatório com o passar do tempo e formação de cápsula fibrosa ao redor do tubo, é um cimento biocompatível.

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ANEXOS

Sistema Pesquisa - Pesquisador: Fabiana Soares Grecca Vilella

Caixa de entrada

Retornar

Dados Gerais:

Projeto Nº:	32780	**Título:**	AVALIACAO DAS PROPRIEDADES FISICO-QUIMICAS DO CIMENTO OBTURADOR A BASE DE RESINA EPOXI SEALER PLUS		
Área de conhecimento:	Endodontia	**Início:**	01/06/2017	**Previsão de conclusão:**	31/05/2018
Situação:	Projeto em Andamento				
Origem:	Faculdade de Odontologia Programa de Pós-Graduação em Odontologia	**Projeto da linha de pesquisa:** BIOMATERIAIS E TECNICAS TERAPEUTICAS EM ODONTOLOGIA			
Local de Realização:	não informado				

Não apresenta relação com Patrimônio Genético ou Conhecimento Tradicional Associado.

Objetivo:

que somado ao dela, totalizando 100 gramas) será colocada sobre o cimento. Dez minutos após a espatulação, a carga será removida, e os diâmetros maior e menor do cimento comprimido serão medidos utilizando um paquímetro digital, e o valor médio será registrado. Será utilizado o teste ANOVA, se os resultados obtidos forem paramétricos, se estes forem não paramétricos, será utilizado o teste de Kruskal-Wallis. O nível de significância será de 95%.

Palavras Chave:

CIMENTOS ENDODÔNTICOS, PROPRIEDADES FÍSICO-QUÍMICA

Equipe UFRGS:

Nome: FABIANA SOARES GRECCA VILELLA
Coordenador - Início: 01/06/2017 Previsão de término: 31/05/2018

Nome: PATRICIA MARIA POLI KOPPER MORA
Pesquisador - Início: 01/06/2017 Previsão de término: 31/05/2018

Pessoas registradas mas não confirmadas como membros da equipe UFRGS:

Nome: ALEXANDER POMPERMAYER JARDINE
Outra: Aluno de Doutorado - Início: 01/06/2017 Previsão de término: 31/05/2018
Participação aguardando confirmação do pesquisador

Nome: Cláudia Daniela Schuster
Outra: Aluno de Especialização - Início: 01/06/2017 Previsão de término: 31/05/2018
Participação aguardando confirmação do pesquisador

Nome: Lucas Siqueira Pinheiro
Outra: Aluno de Doutorado - Início: 01/06/2017 Previsão de término: 31/05/2018
Participação aguardando confirmação do pesquisador

Nome: RENATA GRAZZIOTIN SOARES
Pesquisador - Início: 01/06/2017 Previsão de término: 31/05/2018
Participação aguardando confirmação do pesquisador

Avaliações:

Comissão de Pesquisa de Odontologia - Aprovado em 05/05/2017 Clique aqui para visualizar o parecer

Anexos:

Projeto Completo	**Data de Envio:** 06/04/2017
Concordância de Instituição	**Data de Envio:** 07/04/2017
Concordância de Instituição	**Data de Envio:** 07/04/2017

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 34629
Título: AVALIACAO DA BIOCOMPATIBILIDADE E CITOTOXICIDADE DOS CIMENTOS ENDODONTICOS SEALER PLUS BC, SEALER PLUS, MTA FILLAPEX E AH PLUS EM CULTURA DE CELULAS E TECIDO CONJUNTIVO DE RATOS

Vigência: 01/05/2018 à 30/04/2020

Pesquisadores:

Equipe UFRGS:

FABIANA SOARES GRECCA VILELLA - coordenador desde 01/05/2018
ROBERTA KOCHENBORGER SCARPARO - pesquisador desde 01/05/2018
PATRICIA MARIA POLI KOPPER MORA - pesquisador desde 01/05/2018
GABRIELA CARDOSO FERREIRA - Aluno de Mestrado desde 01/05/2018
Lucas Siqueira Pinheiro - Aluno de Doutorado desde 01/05/2018

Comissão De Ética No Uso De Animais aprovou o mesmo em seus aspectos éticos e metodológicos, para a utilização de 30 ratos Wistar machos com 6 semanas de idade, pesando entre 220 e 300 g, provenientes do CREAL-UFRGS; de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Quinta-Feira, 6 de Dezembro de 2018

MARCELO MELLER ALIEVI
Coordenador da comissão de ética