Universidade Federal de Uberlândia

Faculdade de Odontologia

Programa de Pós-Graduação em Odontologia

CAMILA MARIA PERES DE ROSATTO DOMINGOS

Cimentos endodônticos à base de silicato de cálcio: avaliação *in vitro* de diferentes parâmetros de interesse

Calcium silicate-based endodontic sealers: in vitro evaluation by different parameters of interest

Tese apresentada à Faculdade de Odontologia da Universidade Federal de Uberlândia, como requisito parcial para obtenção do título de Doutor em Odontologia na área de Clínica Odontológica Integrada.

Uberlândia, 2021

CAMILA MARIA PERES DE ROSATTO DOMINGOS

Cimentos endodônticos à base de silicato de cálcio: avaliação *in vitro* de diferentes parâmetros de interesse

Calcium silicate-based endodontic sealers: in vitro evaluation by different parameters of interest

Tese apresentada à Faculdade de Odontologia da Universidade Federal de Uberlândia, como requisito parcial para obtenção do título de Doutor em Odontologia na área de Clínica Odontológica Integrada.

Orientadora: Profª. Drª. Camilla Christian Gomes Moura

Banca Examinadora:

Profª. Drª. Camilla Christian Gomes Moura, FOUFU

Prof. Dr. Carlos José Soares, FOUFU

Profª. Drª. Luciana Arantes Porto Carvalho, FOUFU

Prof. Dr. Mário Tanomaru-Filho, FOAr-UNESP

Prof. Dr. Marco Antônio Húngaro Duarte, FOB-USP

Suplentes:

Profª. Drª. Veridiana Resende Novais Simamoto, FOUFU

Profª. Drª. Juliane Maria Guerreiro Tanomaru, FOAr-UNESP

Uberlândia, 2021

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Coordenação do Programa de Pós-Graduação em Odontologia
Av. Pará, 1720, Bloco 4L, Anexo B, Sala 35 - Bairro Umuarama, Uberlândia-MG, CEP 38400-902
Telefone: (34) 3225-8115/8108 - www.ppgoufu.com - copod@umuarama.ufu.br

ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Odontologia				
Defesa de:	Tese Doutorado, número 63, PPGODONTO				
Data:	Trinta e Um de Maio de Dois Mil e Vinte e Um	Hora de início:	14:00	Hora de encerramento:	18:29
Matrícula do Discente:	11713ODO003				
Nome do Discente:	Camila Maria Peres de Rosatto Domingos				
Título do Trabalho:	Cimentos endodônticos à base de silicato de cálcio: avaliação in vitro de diferentes parâmetros de interesse				
Área de concentração:	Clínica Odontológica Integrada				
Linha de pesquisa:	Propriedades Físicas e Biológicas dos materiais Odontológicos e das estruturas dentais				
Projeto de Pesquisa de vinculação:	Propriedades Físicas e Biológicas dos materiais Odontológicos e das estruturas dentais				

Reuniu-se em Web Conferência pela plataforma MConf - RNP, em conformidade com a PORTARIA Nº 36, DE 19 DE MARÇO DE 2020 da COORDENAÇÃO DE APERFEIÇOAMENTO DE PESSOAL DE NÍVEL SUPERIOR - CAPES, pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Odontologia, assim composta: Professores Doutores: Carlos José Soares (UFU); Luciana Arantes Porto Carvalho (UFU); Mário Tanomaru-Filho (UNESP); Marco Antônio Húngaro Duarte (USP); Camilla Christian Gomes Moura (UFU) orientador(a) do(a) candidato(a).

Iniciando os trabalhos o(a) presidente da mesa, Dr(a). Camilla Christian Gomes Moura , apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadores(as), que passaram a arguir o(a) candidato(a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

Aprovado(a).

Esta defesa faz parte dos requisitos necessários à obtenção do título de Doutor.

O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU.

Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

Documento assinado eletronicamente por **Camilla Christian Gomes Moura**, **Professor(a) do Magistério Superior**, em 31/05/2021, às 19:09, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Luciana Arantes Porto Carvalho**, **Professor(a) do Magistério Superior**, em 31/05/2021, às 19:15, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Carlos José Soares**, **Professor(a) do Magistério Superior**, em 31/05/2021, às 19:21, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Marco Antonio Hungaro Duarte**, **Usuário Externo**, em 01/06/2021, às 10:27, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Mario Tanomaru Filho**, **Usuário Externo**, em 01/06/2021, às 12:08, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

A autenticidade deste documento pode ser conferida no site https://www.sei.ufu.br/sei/controlador_externo.php?acao=documento_conferir&id_orgao_acesso_externo=0, informando o código verificador **2806872** e o código CRC **5ED4EDE4**.

Referência: Processo nº 23117.029727/2021-08 SEI nº 2806872

Ficha Catalográfica Online do Sistema de Bibliotecas da UFU
com dados informados pelo(a) próprio(a) autor(a).

D671 2021

Domingos, Camila Maria Peres de Rosatto, 1987-
Cimentos endodônticos à base de silicato de cálcio: avaliação in vitro de diferentes parâmetros de interesse [recurso eletrônico] / Camila Maria Peres de Rosatto Domingos. - 2021.

Orientadora: Camilla Christian Gomes Moura.
Tese (Doutorado) - Universidade Federal de Uberlândia, Pós-graduação em Odontologia.
Modo de acesso: Internet.
Disponível em: http://doi.org/10.14393/ufu.te.2019.2491
Inclui bibliografia.

1. Odontologia. I. Moura, Camilla Christian Gomes , 1979-, (Orient.). II. Universidade Federal de Uberlândia. Pós-graduação em Odontologia. III. Título.

CDU: 616.314

Bibliotecários responsáveis pela estrutura de acordo com o AACR2:

Gizele Cristine Nunes do Couto - CRB6/2091

DEDICATÓRIA

Aos meus pais, Dulce e Cleonir,

Vocês são o meu apoio mais sólido durante todos esses anos dedicados a pós-graduação. Acreditaram junto comigo que o caminho percorrido valeria a pena e me incentivaram constantemente a buscar a realização dos meus sonhos. Foram aqueles que nunca me soltaram a mão, quando eu mesma nem pensei em pedir qualquer tipo de ajuda. Nesse tempo que se passou eu cresci, não só profissionalmente, e vocês nem imaginam o quanto. Eu só tenho a agradecer, por tudo... Tentarei retribuir para sempre... Eu amo vocês!!

Ao meu marido João Paulo,

Você acompanhou toda a minha trajetória até aqui... Começamos a namorar quando eu estava no cursinho pré-vestibular, 15 anos atrás. E desde então, você sempre esteve ao meu lado... Me incentivou a estudar e a realizar o meu sonho de fazer Odontologia na UFU. E sempre foi incentivando todos os outros sonhos que iam aparecendo pelo caminho e até algumas loucuras no percurso: quando eu viajava a semana toda de ônibus pra dar aula em diferentes cidades. No início do nosso casamento, quando eu estava com a carreira estabilizada e nós estávamos na zona de conforto, resolvi largar tudo pra me arriscar no doutorado e você disse: “Estou com você”. Sempre acreditou que eu chegaria em algum lugar... Sempre me disse que eu era capaz. Algumas vezes acreditou até mais do que eu mesma e, por isso, chegamos juntos até aqui. Só nós dois sabemos do caminho percorrido, sempre de mãos dadas. Obrigada por cuidar de mim e por tanto! Meu amor e carinho por você são eternos.

À minha irmã Fernanda,

Você também faz parte desta vitória. Apesar da distância física, sei que você sempre torceu muito por mim e sente orgulho de quem me tornei. Obrigada por vibrar com cada conquista e por ser um incentivo para que eu seja uma pessoa melhor a cada dia. Eu só desejo que você seja uma pessoa muito realizada e feliz.

À Deus,

A minha fé é profunda e eu só tenho a agradecer por ter me dado força, resiliência, sabedoria e discernimento nas minhas escolhas. Eu sou abençoada por amar a minha profissão e ter pessoas boas sempre cruzando os meus caminhos. Obrigada por iluminar e guiar os meus passos, me livrando de todo o mal.

AGRADECIMENTO ESPECIAL

À Prof.ª Drª. Camilla Christian Gomes Moura,

Querida Camilla, sorte daqueles que têm o caminho da vida com algum encontro marcado com o seu... Como eu fui abençoada quando você foi escolhida para me orientar! Eu sempre acreditei que nada é por acaso e nesses 4 anos de convivência, isso só foi confirmado. Serei eternamente grata por cada aprendizado e você nem imagina o quanto aprendi com você... Sobre Endodontia e sobre relações humanas. Estar ao seu lado foi fazer uma nova especialização: sempre me influenciando a buscar conhecimento pra ser uma profissional melhor. Além disso, conheci na vida poucas pessoas com a sua generosidade. Uma orientadora que guia, aconselha e apoia. Uma líder nata. Nesse tempo, também tive certeza que não é atoa que você está onde está. Pessoa extremamente inteligente, curiosa e do raciocínio rápido. Como eu te admiro! E eu desejo que o seu sucesso na pesquisa seja cada vez maior.

Também não posso deixar de dizer que a sua alegria é contagiante... E como eu adoro sua sinceridade extrema, pois é o seu diferencial. Quantos "perrengues" vencidos juntas... Obrigada por confiar em mim e acreditar que eu não te desapontaria. Obrigada por todo apoio, sempre que eu precisei. Eu nunca vou me esquecer de você e da sua família, de como fui acolhida na sua casa. E saiba que pode contar comigo a qualquer momento. Desejo que você seja sempre muito feliz!!

Aos Professores Dr. Carlos José Soares e Drª. Veridiana Resende Novais,

Vocês foram os primeiros a me abrirem as portas na pesquisa, a guiarem os meus passos na pós-graduação e serem um exemplo pra mim. Sempre trabalharam com responsabilidade e dedicação... Vocês exalam amor em tudo o que fazem e com vocês aprendi a amar ainda mais a minha profissão. Com certeza, uma grande parte do que aprendi eu devo a vocês. Obrigada por cada mão estendida, por sempre confiarem em mim e por terem sido luz em toda minha trajetória. Meu carinho e gratidão serão eternos... Vocês foram e são a minha inspiração!

Professores Camilla, Carlos e Veridiana, *algumas pessoas marcam a nossa vida, mudam o nosso destino... Vocês tem responsabilidade nisso! E o carinho que sinto por vocês é realmente especial. Quantos ensinamentos!!! Quantas memórias... Tenho boas histórias pra contar para sempre... Nunca vou me esquecer de vocês, meus mestres e amigos.*

Em especial aos colegas do meu grupo de pesquisa (Gabi, Lilian, Thais Christina e Felipe), sem vocês eu não teria chegado até aqui. A caminhada foi mais feliz tendo companhia, a troca foi maior, o aprendizado foi mais profundo e as memórias ficarão... Obrigada pelos ensinamentos.

À todos do grupo Biaor,

Obrigada pela parceria, seja no laboratório de pesquisa, nas disciplinas, na clínica de extensão de traumatismo dentoalveolar e também pelos momentos alegres de confraternização.

À todos os alunos de Iniciação Científica (em especial Anne, Beathriz Cristinne, Rafael, Anahi e Danilo), a oportunidade de estar próxima de vocês foi um gostoso desafio, de ensinar e aprender. Acho que eu mais aprendi do que ensinei. Tive a certeza que a FOUFU prepara muito bem seus alunos... Obrigada pela confiança, amizade e carinho que sempre tiveram comigo. Obrigada

pela parceria! Com toda a certeza, eu não teria chegado até aqui sem vocês ao meu lado.

À amiga Renata Borges, a minha primeira parceira na pesquisa. Aquela que é amiga pra todas as horas, desde 2009. Foi ela quem me incentivou a fazer mestrado e, com certeza, também foi responsável para que eu chegasse onde estou hoje. Aquela amiga sempre presente, pronta pra escutar e dar conselhos valiosos. Juntas realizamos muitos sonhos que só nós sabemos... Juntas vivemos muitas histórias das quais nos orgulhamos e poderemos contar aos nossos netos. Torço muito por você! Desejo que a nossa amizade só se fortaleça ainda mais.

AGRADECIMENTOS

À toda minha família, eu sei que vocês sempre estão torcendo por mim! Muito obrigada de coração.

Aos meus sogros, eu agradeço todo carinho e incentivo para que eu estudasse e me aprimorasse profissionalmente. Vocês são pra mim um exemplo de dedicação ao trabalho. Obrigada por tudo e por tornarem a minha caminhada mais leve, sendo a minha segunda família em Uberlândia.

À Universidade Federal de Uberlândia, Faculdade de Odontologia e Programa de Pós-Graduação em Odontologia da UFU, por ter sido minha segunda casa desde o início da minha faculdade em 2007. Tenho muito orgulho de ter toda a minha formação na casa, que respira uma Odontologia de excelência, com profissionais tão empenhados e capacitados. Minha gratidão!! Que sorte a minha de poder dizer para sempre: sou FOUFU!

Aos Professores da Área de Endodontia e Materiais Odontológicos (Luciana, Camilla, Maria Antonieta e Cássio), eu só tenho a agradecer pela receptividade nas disciplinas pré-clínicas e clínicas, me dando a oportunidade de aprender e evoluir. Mais uma vez obrigada por terem confiado em mim e acreditarem que eu seria capaz. Nunca me esquecerei da primeira aula que eu dei para os alunos da FOUFU. Foi emocionante, lembrando que a pouco tempo atrás era eu sentada ali. Foi mais emocionante ainda ter tido vocês, meus Professores, na platéia e depois o retorno de cada um. Mais um momento pra nunca mais esquecer... Obrigada a vocês por todo incentivo.

Aos demais Professores da FOUFU e PPGO, obrigada pelos conhecimentos compartilhados e por nos inspirar a ser cada vez melhores para o bem dos nossos pacientes.

Aos Professores que colaboraram com os objetivos deste trabalho (em especial Priscilla Soares, Marcelo Barbosa e Mário Tanomaru-Filho) e técnicos, muito obrigada pelos ensinamentos, disposição em ajudar e parceria.

À todos os técnicos da FOUFU e PPGO, muito obrigada pelo carinho. O trabalho de vocês é muito valioso e a Odontologia da UFU não seria a mesma sem vocês.

Ao conselho da FOUFU, fazer parte das reuniões como representante discente da pós-graduação por quase 2 anos junto aos Professores e gestores da FOUFU, me fez ter a clareza do amor depositado em cada ação pensada e executada com tanta maestria para a melhoria da nossa faculdade. Obrigada por me acolherem e pela troca. Participar da CONFOUFU foi um privilégio e um grande aprendizado.

Aos pacientes atendidos, nas clínicas da graduação e extensão eu pude evoluir como endodontista e me apaixonar ainda mais pela área que escolhi. Eu agradeço imensamente a confiança e o respeito.

Aos Professores, funcionários e alunos das faculdades que fui docente (FAMP, FPM e Unipac), eu aprendi algo a cada semestre, em cada faculdade que passei. Vários momentos e pessoas me marcaram, me impulsionando a buscar os meus objetivos. A ser a cada dia, uma pessoa melhor. Ser professora foi um grande desafio e, na maioria das vezes, não foi nada fácil. Cada obstáculo foi um aprendizado. Mas além das lições, eu também guardo as boas recordações... Quando me perguntam o que eu acho sobre ser professor, eu sempre digo: “Não é fácil, mas é gratificante”. Tenho muitas histórias pra contar dos meus 2 anos de docência... E por causa deles, aprendi a valorizar ainda mais todos os meus Professores e o decorrer do curso de Doutorado.

À todos os Cirurgiões-Dentistas que eu trabalhei, fui muito abençoada por ter tido só pessoas boas no meu caminho. Excelentes profissionais, que contribuíram para que eu amasse a profissão e sempre me ajudaram de alguma forma. Mais uma vez, obrigada por tudo.

Às minhas amigas de infância – Irmãs Marista (Rê, Camilinha, Ana Lú, Natty, Fê, Carol, Babi, Bá, Bru, Mari's, Lú, Paty e Renata), juntas desde os 11 anos de idade eu aprendi a partilhar com vocês. Nossa sintonia é única... Cada uma com seu jeitinho tem um lugar especial no meu coração. E com vocês eu sempre fico mais leve e feliz. Nossa frase: "É sempre bom demais quando estamos juntas", representa o que se passa no nosso coração após cada encontro. Obrigada pela amizade, carinho e incentivo pra conquistar os meus sonhos. Vocês me inspiram a ser cada dia melhor!!

Aos amigos Lele, Thalie, Diego e JN, quantas histórias, algumas viagens e muitas risadas... Os amigos que o João Paulo me deu. Minha vida é mais colorida com vocês ao lado. Obrigada por sempre serem ombro amigo, pra escutar, aconselhar e desejar o melhor. Que a nossa amizade seja eterna...

Às amigas Mari e Tatá, saber que eu tenho vocês ao meu lado me deixa muito feliz! Amigas que eu sei que posso contar, apesar de não nos encontrarmos tanto. Sei que vocês torcem muito por mim e sentem muito orgulho das minhas conquistas. Obrigada pelo carinho e amizade verdadeira.

À amiga Aline Bicalho (Alinoca), você foi um presente que o mestrado me deu e desde então temos boas recordações. Obrigada pelos conselhos maravilhosos e, como te digo: "iluminar os meus pensamentos". Eu só posso te agradecer por sempre estar pronta a me ouvir, trazendo boas energias pra minha vida. Obrigada também por sempre abrir as portas da sua casa. Sua alma é leve e a nossa amizade é valiosa. Nossa sintonia é boa demais! Que tenhamos muitos momentos ainda pra celebrar.

À amiga Marina Roscoe (Marininha), que mesmo estando distante sempre me inspirou para que eu chegasse até aqui. Foi uma grande incentivadora no meu início na pós-graduação, quando comecei a fazer Iniciação Científica. Você faz parte de tudo e eu nunca vou te esquecer! Obrigada pelo carinho... Por ser luz, alegria e leveza.

À Suely Cunha, que sempre me ajudou sem pedir nada em troca... Nunca vou me esquecer do que já fez por mim. Muito obrigada!!

Ao Prof. Márcio Teixeira, que me recebeu muito bem, sendo muito gentil a cada conversa. Me sinto muito honrada por estar próxima daqueles que sempre me inspiraram, meus eternos Professores.

Ao setor de pacientes especiais (Sepae), que me permitiu conhecer um novo mundo... Abriu o meu coração e a minha mente a uma nova realidade. Que delicioso desafio! Agradeço a todas as dentistas do setor, que me acolheram com muito carinho e respeito. Tenho aprendido demais ao lado de vocês, que são profissionais admiráveis!

À Liga de Endodontia da FOUFU (LiEndo), que surgiu a pouco tempo, mas me acolheu com todo o respeito e consideração. Que honra fazer parte dessa equipe super especial, competente e que ama a Endodontia. Tenho certeza que serei muito feliz ao lado de vocês. Agradeço o reconhecimento do meu trabalho e desejo novos desafios e novas conquistas pra nossa liga do coração...

Ao Centro de Pesquisa Odontológico de Biomecânica, Biomateriais e Biologia Celular (CPBio) e seus técnicos, obrigada por toda ajuda no laboratório. Sempre me senti muito acolhida neste lugar. Adorava passar o dia ali e descobrir como funcionava cada equipamento. Fui muito feliz no CPBio!!

À CAPES, FAPEMIG e ao CNPq, pelo apoio financeiro por meio de concessão da bolsa de Doutorado, que recebi nestes 4 anos. Com certeza, esse auxílio fez toda a diferença para que eu vivesse tudo isso e chegasse até aqui.

Muito obrigada!!!

EPÍGRAFE

“Pedras no caminho? Guardo todas, um dia vou construir um castelo....”

Fernando Pessoa

SUMÁRIO

RESUMO

Desde o surgimento do primeiro cimento à base de silicato de cálcio, vários outros têm sido lançados no mercado, alguns na forma de pó e líquido e outros prontos pra uso. Não se sabe ainda como é a atuação desse material em diferentes tipos celulares encontrados na região periapical e as dificuldades que podem ser encontradas durante a limpeza das paredes dentinárias na desobturação para cimentação de pino de fibra. Ou mesmo se podem ocorrer alterações na composição dos cimentos e suas propriedades, devido ao uso de soluções irrigadoras para remoção de resíduos remanescentes destes cimentos. Portanto, este trabalho envolveu cinco objetivos específicos *in vitro*. **Objetivo 1**: avaliar a citotoxicidade e liberação de citocinas após a exposição de monócitos e polimorfonucleares de cultura primária humana frente a diferentes cimentos endodônticos frescos (Sealer 26, AH Plus e EndoSequence BC Sealer). Foi avaliada apoptose e necrose por citometria de fluxo e dosagem de citocinas (IL-4, IL-6, IL-8, IL-10, IL-12) por ELISA. Foi demonstrado que o efeito dos agentes tóxicos liberados variou dependendo do tipo celular e que a composição dos cimentos parece alterar a forma de auto-regulação na produção dessas citocinas pelas células. **Objetivo 2:** avaliar as propriedades físico-químicas e biológicas de cimentos à base de silicato de cálcio prontos pra uso (EndoSequence BC Sealer [END], Bio-C Sealer [BC] e Sealer Plus BC [SPBC]), utilizando cultura primária de osteoclastos. A citotoxicidade foi avaliada por meio do teste de MTT e a inibição da osteoclastogênese por meio do TRAP. A solubilidade dos cimentos foi testada pelo cálculo da perda de massa em meio de cultura e água deionizada. O pH das soluções foi mensurado, assim como a presença de íons cálcio e silício por meio de espectroscopia de fluorescência de raios-x (XRF). END mostrou maior viabilidade celular em relação aos demais cimentos e BC mostrou maior inibição de osteoclastos. Todos os cimentos revelaram alta solubilidade, maior

do que a recomendada pelos padrões da Organização Internacional de Normalização (ISO). A presença de íons silício foi extremamente baixa para todos os cimentos. **Objetivo 3:** avaliar a citotoxicidade, adesão celular e as características químicas dos cimentos à base de silicato de cálcio (BioRoot RCS [BR], Bio-C Sealer [BC] e Sealer Plus BC [SPBC]), utilizando células RAW264.7. A citotoxicidade foi avaliada por meio do teste de MTT e discos dos cimentos foram usados para avaliar a adesão celular, além da caracterização química com XRF, microscopia eletrônica de varredura (MEV) e espectroscopia por energia dispersiva de raios-x (EDX). A composição dos cimentos frescos e após a presa também foi avaliada utilizando a espectroscopia infravermelha transformada de Fourier (FTIR). SPBC revelou a menor viabilidade celular em relação aos demais cimentos. Comparando os grupos experimentais com o grupo controle, a diluição de 1:1 mostrou uma significativa redução na viabilidade para todos os materiais. BC e SPBC apresentaram espectros parecidos em FTIR. EDX demonstrou maior presença de cálcio para BR e SPBC, com presença de alumínio nos cimentos prontos pra uso. XRF também mostrou quantidade de cálcio significativamente maior para BR em relação aos outros cimentos. Precipitados foram encontrados na superfície de todos os materiais. BR apresentou maior grau de pureza e quantidade de cálcio, enquanto BC e SPBC revelaram composição similar. Todos os cimentos mantiveram a viabilidade acima de 70%. **Objetivo 4:** avaliar se soluções (NaOCl e NaOCl+EDTA) e suas ativações (manual, irrigação ultrassônica passiva e com ponta Easy Clean) são capazes de promover a limpeza das paredes dentinárias após desobturação do canal, onde foi utilizado cimento MTA Fillapex, por meio de microtomografia computadorizada (micro-CT); e se estes protocolos de limpeza são capazes de alterar a microdureza da dentina, avaliada por indentação Knoop. Este objetivo mostrou que a ponta Easy Clean removeu mais debris no interior do canal após a desobturação e que o terço cervical foi o que apresentou maior limpeza. Além disso, a solução NaOCl+EDTA teve maior eficácia de limpeza, independente do método de ativação. Nenhum protocolo de limpeza foi capaz de alterar a microdureza dentinária. **Objetivo 5:** avaliar se protocolos de limpeza usando soluções

(NaOCl e NaOCl+EDTA) e a irrigação ultrassônica passiva, alteram a composição de cimentos à base de silicato de cálcio (EndoSequence BC Sealer, BioRoot RCS e Bio-C Sealer) frescos e após a presa em um canal radicular desobturado, por meio de um dispositivo que foi criado para a simulação. A composição dos cimentos após os protocolos de limpeza foi avaliada usando espectroscopia Raman e EDX. Foi observado que independente dos protocolos, a limpeza dos remanescentes em canal desobturado para instalação de pino de fibra de vidro deve ser preconizada somente após a presa do cimento biocerâmico. Pode-se concluir a partir dos objetivos apresentados, que os novos cimentos endodônticos à base de silicato de cálcio apresentam propriedades físico-químicas adequadas, com exceção da solubilidade. Em relação às propriedades biológicas, comportam-se de formas variadas frente a diferentes tipos celulares presentes na região periapical. Além disso, diante da necessidade de reabilitação com pino de fibra de vidro posteriormente a obturação com essa classe de material, os protocolos de limpeza empregados mostraram eficácia, sem causar alterações na composição dos cimentos à base de silicato de cálcio e que a ponta Easy Clean promove melhor limpeza dos remanescentes aderidos às paredes dentinárias.

Palavras-chaves: cimentos endodônticos; citotoxicidade; silicato de cálcio

ABSTRACT

Since the appearance of the first calcium silicate-based sealer, several others sealers have been launched on the market, some in powder and liquid form and others ready-for-use. It is not yet known how is the performance of this material in different cell types found in the periapical region and the difficulties that may be discovered during the cleaning of the dentinal walls in post space for fiber post cementation. Or even if may occur changes in the composition of the sealers and their properties, due to the use of irrigating solutions to remove the remaining residues of these sealers. Therefore, the present study involved five specific *in vitro* objectives. **Objective 1:** to evaluate the cytotoxicity and cytokine release after exposure of monocytes and polymorphonuclears cells obtained from the peripheral blood of humans to different fresh root canal sealers (Sealer 26, AH Plus and EndoSequence BC Sealer). Apoptosis and necrosis was assessed by flow cytometry and cytokine measurement (IL-4, IL-6, IL-8, IL-10, IL-12) by ELISA. It was demonstrated that the effect of toxic agents released varied depending on the cell type and that the composition of the sealers appeared to alter the form of self-regulation in the production of these cytokines by cells. **Objective 2:** to evaluate the physicochemical and biological properties of ready-to-use calcium silicate-based sealers (EndoSequence BC Sealer [END], Bio-C Sealer [BC] and Sealer Plus BC [SPBC]), using osteoclast primary culture. The cytotoxicity was assessed using the MTT test and the inhibition of osteoclastogenesis by TRAP. The solubility of the sealers was tested by calculating the mass loss in culture medium and deionized water. The pH of the solutions was measured, as well as the presence of calcium and silicon ions by X-ray fluorescence spectroscopy (XRF). END showed greater cell viability compared to other sealers and BC showed greater inhibition of osteoclasts. All sealers showed high solubility, greater than that recommended by the standards of the International Organization for Standardization (ISO). The

presence of silicon ions was extremely low for all sealers. **Objective 3:** to evaluate the cytotoxicity, cell adhesion and chemical characteristics of calcium silicate-based sealers (BioRoot RCS [BR], Bio-C Sealer [BC] and Sealer Plus BC [SPBC]), using RAW264.7 cells. The cytotoxicity was assessed by MTT test and sealer discs were used to evaluate cell adhesion, beyond to chemical characterization with XRF, scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX). The composition of unset sealers and after setting was also evaluated by Fourier-transform infrared spectroscopy (FTIR). SPBC revealed the lowest cell viability compared to the other sealers. Comparing the experimental groups with the control group, the 1: 1 dilution showed a reduction significant in viability for all materials. BC and SPBC presented similar spectra in FTIR. EDX demonstrated a greater presence of calcium for BR and SPBC, with the presence of aluminum in ready-to-use sealers. XRF also showed an amount of calcium significantly higher for BR compared to other sealers. Precipitates were found on the surface of all materials. BR showed the greatest degree of purity and the highest calcium content, while BC and SPBC presented similar compositions. All the sealers maintained viability above 70%. **Objective 4:** to evaluate whether solutions (NaOCl and NaOCl+EDTA) and their activations (manual, passive ultrasonic irrigation and Easy Clean tip) are able to promote the cleaning of the dentinal walls after post space preparation, where MTA Fillapex sealer was used, by microcomputed tomography (micro-CT); and whether these cleaning protocols are capable to change the dentin microhardness, assessed by Knoop indentation. This objective showed that the Easy Clean tip removed more debris inside the root canal after post space and that the cervical third presented the greatest cleanliness. In addition, the NaOCl+EDTA solution was more effective in cleaning, regardless of the activation method. No cleaning protocol was able to modify the dentin microhardness. **Objective 5:** to evaluate whether cleaning protocols using solutions (NaOCl and NaOCl+EDTA) and passive ultrasonic irrigation, change the composition of calcium silicate-based sealers (EndoSequence BC Sealer, BioRoot RCS and Bio-C Sealer) fresh and after setting in a post space root canal, using a device that was created for the

simulation. The composition of the sealers after the cleaning protocols was evaluated using Raman and EDX spectroscopy. It was observed that regardless of the protocols, the cleaning of the remnants in a post space root canal for installing a fiberglass post should be recommended only after setting of the bioceramic sealer. It can be concluded from the presented objectives, the new calcium silicate-based endodontic sealers showed adequate physico-chemical properties, except for solubility. Regarding the biological properties, behave in different ways in face of different cell types present in the periapical region. In addition, in the face of need for rehabilitation with a fiberglass post posteriorly the filling with this type of material, the cleaning protocols used showed effectiveness, without causing changes in the composition of calcium silicate-based sealers and that the Easy Clean tip promotes better cleaning of the remnants adhered to the dentinal walls.

Keywords: calcium silicate; cytotoxicity; endodontic sealers

INTRODUÇÃO E REFERENCIAL TEÓRICO

1. INTRODUÇÃO E REFERENCIAL TEÓRICO

Os cimentos endodônticos à base de silicato de cálcio fazem parte de uma nova família de biomateriais da Endodontia Contemporânea formada por cimentos contendo mineral trióxido agregado (MTA) e por cimentos biocerâmicos (Prati & Gandolfi 2015; Jafari & Jafari 2017; Silva Almeida *et al.*, 2017; Donnermeyer *et al.,* 2019). Esta nova classe de cimentos surgiu baseada nos componentes do cimento de Portland (Prati & Gandolfi 2015; Torabinejad *et al.,* 2018; Donnermeyer *et al.,* 2019), apresentando como característica comum a bioatividade, ou seja, a capacidade de induzir a formação de tecido duro tanto no ligamento periodontal quanto no osso (Bryan *et al.,* 2010; Chang *et al.,* 2014; Prati & Gandolfi 2015; Kebudi Benezra *et al.,* 2018; Torabinejad *et al.,* 2018; Zordan-Bronzel *et al.,* 2019; Oh *et al.,* 2020).

Um dos primeiros cimentos introduzido no mercado contendo MTA foi o MTA Fillapex (Angelus, Londrina, PR, Brasil), o qual já está disponível há mais de 5 anos, apresentando inúmeras publicações (Vitti *et al.,* 2013; Kebudi Benezra *et al.,* 2018). No entanto, este cimento apresenta resina em sua composição e o MTA corresponde à apenas uma pequena fração do produto, atuando como um preenchedor. O primeiro cimento biocerâmico lançado foi o iRoot SP (Innovative Bioceramics, Vancouver, CB, Canadá) e, posteriormente, surgiu o cimento EndoSequence BC (Brasseler USA, Savannah, GA, Estados Unidos) com composição similar (Silva Almeida *et al.,* 2017; Donnermeyer *et al.,* 2019). Ambos tornaram-se os materiais com o maior número de estudos publicados (Candeiro *et al.,* 2012; Silva Almeida *et al.,* 2017; Donnermeyer *et al.,* 2019). Dentre esses novos cimentos, existem os que necessitam ser manipulados conforme a indicação do fabricante, apresentados no sistema de pó e líquido (Viapiana *et al.,* 2016; Siboni *et al.,* 2017; Kharouf *et al.,* 2020), e também os prontos pra uso (Alves Silva *et al.,* 2020), disponíveis dentro de seringas para serem injetados no interior do canal radicular otimizando a prática clínica.

Entre os cimentos à base de silicato de cálcio prontos para uso, ganharam destaque no mercado nacional os cimentos Bio-C Sealer (Angelus, Londrina, PR, Brazil) (López-García *et al.,* 2019; Zordan-Bronzel *et al.,* 2019; Alves Silva *et al.,* 2020; Okamura *et al.,* 2020) e Sealer Plus BC (MK Life, Porto Alegre, RS, Brazil) (Mendes *et al.,* 2018; Benetti *et al.,* 2019; Alves Silva *et al.,* 2020; Silva *et al.,* 2021). Embora a literatura já apresente várias pesquisas utilizando estes cimentos, até o momento não foi avaliada a interação do Bio-C Sealer e Sealer Plus BC com células presentes na região periapical envolvidas nos processos inflamatórios e de reabsorção óssea periapical. Estudos *in vitro* têm avaliado o comportamento desses cimentos na presença principalmente de células como os fibroblastos e osteoblastos (Camps *et al.,* 2015; Candeiro *et al.,* 2016; Jung *et al.,* 2018). Porém, é importante avaliar outros modelos celulares envolvidos no reparo periapical como os macrófagos e neutrófilos (Braga *et al.,* 2014; Souza *et al.,* 2019), além do papel dessas células na inibição dos osteoclastos (Zhang *et al.,* 2015; Cheng *et al.,* 2017).

Além da biocompatibilidade, aspectos relacionados à composição química e propriedades físicas dos cimentos endodônticos são foco de constante interesse quando surgem novos materiais no mercado. Particularmente, em relação aos cimentos biocerâmicos, chama à atenção a necessidade de umidade proveniente dos fluidos dentinários para concluir a reação de presa (Prati & Gandolfi 2015; Silva Almeida *et al.,* 2017), caracterizando-os como cimentos hidrofílicos ou hidráulicos (Prati & Gandolfi 2015; Kebudi Benezra *et al.,* 2018). A partir dessa reação de hidratação do cimento, ele forma como sub-produto o hidróxido de cálcio (Camilleri 2011; Bolhari *et al.,* 2014; Ashofteh Yazdi *et al.,* 2019; Grazziotin-Soares *et al.,* 2019) e também ocorre a liberação de diversos íons, principalmente cálcio (Silva Almeida *et al.,* 2017; Mendes *et al.,* 2018; Kharouf *et al.,* 2020) e silício (Prati & Gandolfi 2015; Kebudi Benezra *et al.,* 2018; López-García *et al.,* 2019). Dessa forma, acontece atividade antimicrobiana pelo aumento do pH (Khalil *et al.,* 2016; Silva Almeida *et al.,* 2017; Mendes *et al.,* 2018; Zordan-Bronzel *et al.,* 2019), o que acelera o processo de reparo. Entretanto, diversos estudos demonstraram alta solubilidade desses novos cimentos (Silva Almeida *et al.,* 2017; Tanomaru-

Filho *et al.,* 2017; Mendes *et al.,* 2018; Donnermeyer *et al.,* 2019; Silva *et al.,* 2021), extrapolando as normas definidas pelos padrões da ISO (6876/2012). Portanto, a associação de metodologias para melhor compreender o comportamento dos cimentos biocerâmicos torna-se importante, assim como também é relevante avaliar as propriedades dos materiais em diferentes meios, já preconizado em alguns trabalhos (do Carmo *et al.,* 2018; Kebudi Benezra *et al.,* 2018; Urban *et al.,* 2018; López-García *et al.,* 2019; Torres *et al.,* 2020). Dependendo do meio e do tempo de contato, ocorre a formação de precipitados de fosfato de cálcio e apatita que são responsáveis pela atividade osteogênica destes novos cimentos (Gandolfi *et al.,* 2010; Gandolfi *et al.,* 2011; Taddei *et al.,* 2011; Prati & Gandolfi 2015).

Outro ponto de interesse em relação aos cimentos à base de silicato de cálcio, diz respeito à reabilitação oral em dentes obturados com esses cimentos. Na maioria dos casos, dentes que passaram por tratamento endodôntico possuem pouca estrutura dental e precisam ser reabilitados com retentor intrarradicular (Soares *et al.,* 2012; Rodrigues *et al.,* 2017). Há algum tempo a escolha tem sido pelos pinos de fibra de vidro, devido à distribuição homogênea de tensão dentro da dentina, evitando fratura radicular (Santos-Filho *et al.,* 2008; Veríssimo *et al.,* 2014). Dessa forma, torna-se importante avaliar a remoção dos debris remanescentes da desobturação do canal (Oliveira *et al.,* 2018) para que haja sucesso na cimentação adesiva desses pinos. Atualmente, tem sido preconizada a obturação com cone único utilizando os cimentos à base de silicato de cálcio (Moinzadeh *et al.,* 2015; Chybowski *et al.,* 2018; Guivarc'h *et al.,* 2020). Contudo, esta técnica apresenta maior porcentagem de cimento endodôntico ao redor do cone de guta-percha (Garrib & Camilleri 2020) e torna a desobturação do canal uma etapa muito delicada (Rosatto *et al.,* 2020). Alguns estudos já mostram que os remanescentes biocerâmicos são mais difíceis de serem removidos das paredes dentinárias (de Siqueira Zuolo *et al.,* 2016; Oltra *et al.,* 2017; Donnermeyer *et al.,* 2019; Garrib & Camilleri 2020). Portanto, ainda não está claro na literatura se a limpeza das paredes utilizando diversos protocolos de irrigação e ativação das soluções (Akyuz Ekim & Erdemir 2015; Kato *et al.,* 2016; Lo Giudice *et al.,*

2016; Poletto *et al.,* 2017; Simezo *et al.,* 2017; Pedullà *et al.,* 2019) é eficiente em canais que foram obturados com cimentos biocerâmicos e passaram por alívio. Também não é evidente se esses protocolos podem ocasionar algum prejuízo na microdureza da dentina (Garrib & Camilleri 2020).

Como a desobturação do canal geralmente é realizada imediatamente após a sessão de obturação (Dos Reis-Prado *et al.,* 2021) e o cimento biocerâmico presente no terço apical ainda não tomou presa, a limpeza das paredes da dentina para instalação do pino de fibra usando soluções e/ou ativação torna-se uma etapa preocupante. Os radiopacificadores presentes nestes materiais permitem que eles sejam visualizados em tomadas radiográficas e também sofreram evolução em sua composição (Camilleri & Gandolfi 2010; Kebudi Benezra *et al.,* 2018). O óxido de bismuto tem sido substituído pelo óxido de zircônio (Khalil *et al.,* 2016; Atmeh & AlShwaimi 2017; Zordan-Bronzel *et al.,* 2019), óxido de tântalo (Khalil *et al.,* 2016) e tungstênio de cálcio (Kebudi Benezra *et al.,* 2018), evitando a descoloração dos dentes (Camilleri *et al.,* 2014; Torabinejad *et al.,* 2018) e tornando os cimentos menos tóxicos ao organismo humano (Silva *et al.,* 2014; Li *et al.,* 2017). A maioria dos estudos já realizados mostrou que apesar dessa mudança, os cimentos biocerâmicos exibiram níveis de radiopacidade mais baixos que os cimentos convencionais (Khalil *et al.,* 2016; Jafari & Jafari 2017; Siboni *et al.,* 2017). Portanto, as alterações que podem ocorrer na composição desses cimentos impactam de forma direta no resultado e proservação do tratamento endodôntico em longo prazo. Sendo assim, existe também a possibilidade de realizar a desobturação em um segundo momento, após a presa do cimento, evitando o deslocamento do cone único (Rosatto *et al.,* 2020) e permitindo maior segurança na realização do procedimento. Contudo, no estudo realizado previamente pelo nosso grupo de pesquisa, concluiu-se que quando a desobturação foi realizada tardiamente com termocompactador houve maior presença de resíduos remanescentes da obturação (Rosatto *et al.,* 2020). Dessa forma, torna-se importante analisar melhor os dois momentos (Nagas *et al.,* 2016; Long *et al.,* 2019) assim como as soluções e ativações utilizadas para limpeza dentinária (Akyuz Ekim & Erdemir 2015; Kato *et al.,* 2016; Lo Giudice *et al.,* 2016; Poletto

et al., 2017; Simezo *et al.,* 2017; Pedullà *et al.,* 2019) prévia à instalação de pinos.

Diante do exposto, o objetivo deste trabalho foi comparar as propriedades de diferentes cimentos à base de silicato de cálcio entre si, compreendendo suas características biológicas juntamente com as físico-químicas. Nos capítulos a seguir, iremos apresentar por meio de estudos *in vitro* as características específicas desses novos cimentos, assim como os protocolos de limpeza dos canais após desobturação para cimentação de pinos, que podem ser adotados e não estão bem estabelecidos na literatura. Dessa forma, será possível esclarecer melhor o comportamento desta nova classe de material obturador comparando a dados já existentes na literatura.

2. OBJETIVOS

Objetivo geral

Avaliar por meio de estudos *in vitro* as propriedades biológicas e físico-químicas de cimentos à base de silicato de cálcio. Nesse contexto, avaliar também qual o melhor protocolo de irrigação e ativação para a limpeza das paredes dentinárias em canal radicular que foi obturado com este tipo de cimento e passou por alívio para cimentação adesiva de pino de fibra, e também avaliar o melhor momento para essa limpeza.

Objetivos específicos

Objetivo específico 1

Capítulo 1 – Evaluation of apoptosis/necrosis and cytokine release provoked by three root canal sealers in human polymorphonuclears and monocytes

Avaliar a citotoxicidade e liberação de citocinas após a exposição de monócitos e polimorfonucleares de cultura primária humana frente a diferentes cimentos endodônticos frescos (Sealer 26, AH Plus e EndoSequence BC Sealer).

Objetivo específico 2

Capítulo 2 - Evaluation of premixed calcium silicate-based sealers and their role on osteoclastogenesis

Avaliar as propriedades físico-químicas e biológicas de cimentos à base de silicato de cálcio prontos pra uso (EndoSequence BC Sealer [END], Bio-C Sealer [BC] e Sealer Plus BC [SPBC]), utilizando cultura primária de osteoclastos.

Objetivo específico 3

Capítulo 3 - Evaluation of cytotoxicity, cell attachment and chemical characterization of three calcium silicate-based sealers

Avaliar a citotoxicidade, adesão celular e as características químicas dos cimentos à base de silicato de cálcio (BioRoot RCS [BR], Bio-C Sealer [BC] e Sealer Plus BC [SPBC]), utilizando células RAW264.7.

Objetivo específico 4

Capítulo 4 - Effect of irrigation protocols on root canal wall after post preparation: micro-CT and microhardness study

Avaliar se soluções (NaOCl e NaOCl+EDTA) e suas ativações (manual, irrigação ultrassônica passiva e com ponta Easy Clean) são capazes de promover a limpeza das paredes dentinárias após desobturação do canal, onde foi utilizado cimento MTA Fillapex.

Objetivo específico 5

Capítulo 5 – Effect of solutions and passive ultrasonic irrigation in the composition of calcium silicate-based sealers after post space preparation

Avaliar se protocolos de limpeza usando soluções (NaOCl e NaOCl+EDTA) e a irrigação ultrassônica passiva, alteram a composição de cimentos à base de silicato de cálcio (EndoSequence BC Sealer, BioRoot RCS e Bio-C Sealer) frescos e após a presa em um canal radicular desobturado, que foi criado para a simulação.

3. CAPÍTULOS

CAPÍTULO 1

3.1 Capítulo 1

Artigo publicado no periódico International Endodontic Journal

Souza GL, Rosatto CMP, Silva MJB, Silva MV, Rocha Rodrigues DB, Moura CCG. Evaluation of apoptosis/necrosis and cytokine release provoked by three root canal sealers in human polymorphonuclears and monocytes. **Int Endod J.** 2019;52(5):629-638. **doi: 10.1111/iej.13036**

Evaluation of apoptosis/necrosis and cytokine release provoked by three root canal sealers in human polymorphonuclears and monocytes

Gabriela Leite de Souza[1], Camila Maria Peres de Rosatto[1], Marcelo José Barbosa Silva[2], Marcos Vinícius da Silva[3], Denise Betulucci Rocha Rodrigues[3], Camilla Christian Gomes Moura[1]

[1] Department of Endodontics, School of Dentistry, University of Uberlândia, Uberlândia, MG, Brazil.
[2] Department of Immunology, Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, MG, Brazil.
[3] Department of Microbiology, Immunology and Parasitology, Institute of Biological and Natural Sciences, Federal University of Triângulo Mineiro, Uberaba, MG, Brazil.

Correspondence: Camilla Christian Gomes Moura, Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Av Pará 1720, Campus

Umuarama, Block 4L, annex A, Uberlândia, MG, 38405-328, Brazil (Tel.: (+55) 34 3225-8119; e-mail: camillamoura@ufu.br).

INTERNATIONAL
ENDODONTIC JOURNAL

doi:10.1111/iej.13036

Evaluation of apoptosis/necrosis and cytokine release provoked by three root canal sealers in human polymorphonuclears and monocytes

G. L. Souza[1], C. M. P. Rosatto[1], M. J. B. Silva[2], M. V. Silva[3], D. B. Rocha Rodrigues[3] & C. C. G. Moura[1]

[1]Department of Endodontics, School of Dentistry, University of Uberlândia, Uberlândia, MG; [2]Department of Immunology, Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, MG; and [3]Department of Microbiology, Immunology and Parasitology, Institute of Biological and Natural Sciences, Federal University of Triângulo Mineiro, Uberaba, MG, Brazil

Abstract

Souza GL, Rosatto CMP, Silva MJB, Silva MV, Rocha Rodrigues DB, Moura CCG. Evaluation of apoptosis/necrosis and cytokine release provoked by three root canal sealers in human polymorphonuclears and monocytes. *International Endodontic Journal.*

Aim To evaluate the *in vitro* cytotoxicity and cytokine release of three fresh root canal sealers and to determine the type of cell death they induce.

Methodology The sealers tested were Sealer 26 (S26), AH Plus (AHP), and Endosequence BC Sealer (END). Fresh sealers were cultivated in contact with monocytes and polymorphonuclears (PMNs) obtained from the peripheral blood of humans. Cell viability, apoptosis and necrosis were analysed at 4 h (PMNs) or 24 h (monocytes) using Annexin-V and propidium iodide in a cytometer. The supernatants were used to quantify Interleukin (IL)-4, IL-6, IL-10, IL-12 and tumour necrosis factor-α (TNF-α) in monocytes and IL-8 in PMNs by ELISA. One-way ANOVA and the Tukey post-test were used to compare data for cytotoxicity, and the multiple *T*-test was used to determine the differences between sealers in the release of cytokines that were statistically significant.

Results After 4 h of treatment, S26 was associated with greater cell viability than the other sealers ($P < 0.05$) in the PMN culture and had similar values of necrosis as END ($P > 0.05$). After 24 h of treatment, AHP and END had greater monocyte cell viability than S26 ($P < 0.05$), which had more necrosis ($P < 0.05$). END had the lowest levels of IL-12 compared to the other sealers ($P < 0.05$) and higher levels of IL-6 compared to S26 ($P < 0.05$). The tested sealers did not differ in the release of IL-8, IL-10, TNF-α and IL-4 ($P > 0.05$).

Conclusions The effect of toxic agents released varied depending on the cell type studied. The composition of the sealers appeared to alter the form of self-regulation in the production of these cytokines by cells.

Keywords: cell culture, cell viability, cytokines, cytotoxicity, endodontic sealers.

Received 6 August 2018; accepted 31 October 2018

Introduction

Root filling results in an intimate and complex interface between filling materials and periradicular tissues (Brackett *et al.* 2012). Although sealers and root filling materials are intended to be contained within the root canal space, they may be extruded through the apical constriction, influencing the outcome of root canal treatment (Al-Hiyasat *et al.* 2009). In this sense, it is essential that materials used for root fillings, especially the sealers, are not cytotoxic or mutagenic and do not induce or perpetuate an inflammatory reaction in the periapical tissues, thereby delaying the healing process (Baraba *et al.* 2011).

Correspondence: Camilla Christian Gomes Moura, Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Av Pará 1720, Campus Umuarama, Block 4L, annex A, Uberlândia, MG, 38405-328, Brazil (Tel.: (+55) 34 3225-8119; e-mail: camillamoura@ufu.br).

The biological safety of endodontic materials are currently tested *in vitro* (Brackett *et al.* 2008, 2010, 2012) and *in vivo* (Zmener 2004, Onay *et al.* 2007). Despite criticism involving the *in vitro* tests (Browne 1988), they have fewer ethical limitations and are more rapid and less expensive than *in vivo* animal tests (Brackett *et al.* 2010). Contemporary sealing materials have been evaluated extensively by cell culture tests, which often use primary or immortalized fibroblasts (Camargo *et al.* 2014, Candeiro *et al.* 2016, Cintra *et al.* 2017, Szczurko *et al.* 2018) and osteoblasts (Bryan *et al.* 2010) as models. However, extruded sealer compounds also enter into contact with immune-competent cells in the periapical region, which have been neglected by *in vitro* study models.

The recruitment of inflammatory cells and the release of proinflammatory and immune regulatory mediators occurs in the periapical region (de Brito *et al.* 2012). Among these cells are the polymorphonuclears (PMNs) and monocytes. Polymorphonuclears (PMNs) may elicit the progression of the host immune system by their ability to release inflammatory mediators, such as chemokines and cytokines (Velard *et al.* 2009, Moura *et al.* 2013), influencing the subsequent cell response to biomaterials (Kaplan *et al.* 1996). After colonization of the wound site by neutrophils, monocyte migration occurs (Anderson & Miller 1984). Monocytes work in an orchestrated way with PMNs (Soehnlein & Lindbom 2010) and differentiate into macrophages playing an important role in periapical repair (Sjogren *et al.* 1998). However, little is known about the effect of endodontic sealers on PMNs and their response *in vitro* and/or *in vivo.* Although some studies have evaluated the effects of various endodontic materials on monocyte viability and cytokine secretion (Rezende *et al.* 2005, 2007, de Oliveira Mendes *et al.* 2010), until now, the role of fresh endodontic sealer with different compositions on the mechanisms of cell death has not been evaluated.

The biomaterial chemistry increases or decreases the process of cell death process which is regulated by a variety of extracellular signals (Kaplan *et al.* 1996). In the present study, AH Plus (AHP), Sealer 26 (S26) and Endosequence (END) were evaluated. While AHP is an epoxy resin-based sealer (Marciano *et al.* 2011) and S26 is an epoxy resin-based sealer containing calcium hydroxide (Tanomaru-Filho *et al.* 2011), END contains zirconium oxide, calcium silicates, calcium phosphate monobasic and calcium hydroxide (Hess *et al.* 2011). Thus, it is crucial to evaluate the cytotoxicity of root canal sealers on monocyte and PMN cells, mimicking the possible effects of sealer compounds on the release of key mediators involved in inflammation and cell death pathways.

The objectives of this study were to determine the cytotoxicity of three contemporary freshly prepared root canal sealers using human monocytes and PMNs and to determine the type of cell death they induce. Furthermore, the release of inflammatory mediators induced by these materials was compared using two primary cell cultures that occur in periradicular tissues. The null hypothesis was that the different sealers did not affect PMN and monocyte responses.

Materials and methods

Specimen preparation

Three endodontic sealers commonly used in contemporary endodontic treatment were tested: AH Plus (AHP) (Dentsply, Rio de Janeiro, RJ, Brazil), Sealer 26 (S26) (Dentsply) and Endosequence (END) (Brasseler, Savannah, GA, USA). Specimens were prepared following the manufacturers' directions inside laminar flow. The mixed sealers were dispensed into sterile polyethylene tubes (0.6 mm internal diameter and 10 mm long) (Embramed, São Paulo, SP, Brasil) with the aid of a syringe attached to a needle compatible with the diameter of tubes, ensuring complete filling of the tube without overflow. Immediately, each tube with endodontic sealer was conditioned in a well containing culture medium. Empty tubes were used as controls. PMNs and monocytes were incubated at 37 °C and 5% CO_2 for 4 and 24 h, respectively.

Monocytes and PMN cells isolation from human peripheral blood

Venous blood was collected from 3 healthy donors following approval of the Ethics Committee of Federal University of Uberlândia (Protocol no 2.720.405). PMNs were purified according to a previously published method (Moura *et al.* 2013) from whole human blood using a two-step centrifugation protocol with Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden), followed by Percoll (Pharmacia). The resulting PMNs were resuspended in RPMI 1640 with gentamycin (Sigma-Aldrich, St. Louis, MI, USA) at 120 U L^{-1} and supplemented with 5% heat-inactivated foetal bovine serum (Invitrogen, Carlsbad, CA, USA). PMNs were counted and plated in 24-well culture plates in contact with polyethylene tubes

containing the endodontic sealers at a cell density of 5×10^5 cells/well at 37 °C, 5% CO_2 for 4 h. After that, the supernatant was collected and stored at −20 °C for further cytokine quantification.

Monocytes were obtained from whole human blood using Percoll gradient separation medium (Pharmacia) by centrifugation at room temperature for 30 min at 800 ***g*** to separate monocytes and lymphocytes from the remaining blood elements. The mononuclear cells (1×10^6 cell/well) were plated in 24-well culture plates and were incubated for 3 h in a humidified, 5% CO_2 incubator at 37 °C to allow the monocytes/macrophages to settle on the polystyrene surface. Then, the plates were centrifuged at 400 ***g*** for 10 min, and nonadherent cells were removed by aspiration. Fresh medium was added to each well; the plates were then returned to the incubator and cultivated for 24 h. After this period, the tubes containing fresh sealer were placed in contact with the cells.

The viability of PMN and monocyte suspensions was assayed by Annexin-V/Propidium Iodide (AnnV/PI) double stain using the FACSCalibur cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). All treatments were performed in triplicate for each donor.

Assessment of apoptosis/necrosis by AnnV/PI double stain assay

PMNs and monocytes were washed with phosphate buffered saline (PBS) and detached from the plate using 5 mmol L^{-1} EDTA (Sigma-Aldrich) in PBS for 5 min on ice. They were placed in an incubator for approximately 15 min. After washing, isolated cells were resuspended with PBS, followed by incubation with Annexin-V (AnnV) and propidium iodide (PI) to identify apoptotic and necrotic cells according to manufacturer's instructions (BD Biosciences Pharmingen, San Jose, CA, USA). Flow cytometry analyses were performed using a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). Data were collected from a minimum of $1x10^4$ cells and analysed using cell quest software (Becton-Dickinson). PMNs and monocytes gating were done using side scatter and forward scatter properties. Heat-killed cells (70 °C, 30 min) were used as a positive control for PI staining.

Cytokines and chemokines measurements

Human-specific ELISA kits were used to measure Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-12 (IL-12) and tumour necrosis factor-alpha (TNF-α) in monocyte cultures and Interleukin-8 (IL-8) in PMN cultures. All procedures were performed in triplicate, following the manufacturer's instructions (BD Pharmingen).

Statistical analyses

The data on apoptosis/necrosis and release of cytokines were analysed for normality and homoscedasticity using Shapiro-Wilk's and Levene's tests. One-way ANOVA and the Tukey post-test were used to compare data for cytotoxicity between the treated groups. The multiple *T*-test was used to determine if differences observed among the effects of sealers in the release of cytokines were statistically significant. Statistical significance was set at $\alpha = 0.05$. Statistical analysis was performed using Sigma Plot v.12.0 (Systat Software, San Jose, CA, USA) and GraphPad Prism v.6 (GraphPad Software, La Jolla, CA, USA).

Results

Assessment of apoptosis/necrosis by AnnV/PI double stain assay

Figure 1 a–c shows the effect of various sealers on the viability, apoptosis and necrosis of PMN cells. The exposure of PMNs to AHP and END materials resulted in a significant reduction in the percentage of viable cells compared with the control ($P < 0.0001$). Most of the cells treated with AHP and END underwent apoptosis compared with the control cells ($P = 0.0037$; $P = 0.0030$). END also increased the proportion of necrotic cells compared with the control ($P = 0.0171$), and the AHP ($P = 0.0193$) and had a similar percentage as S26 ($P = 0.8571$; Fig. 1c). S26 had a greater percentage of living cells compared with AHP ($P = 0.0053$) and END ($P = 0.0001$) and a similar percentage as the control group ($P = 0.2129$; Fig. 1a).

Figure 2 a–c shows the effect of different sealer materials on the viability, apoptosis and necrosis of monocytes. The exposure of monocytes to S26 and END sealers resulted in a significant reduction in the percentage of viable cells compared with the control ($P < 0.0001$; $P = 0.0080$). The lowest percentage of viable cells was recorded for the S26 sealer, which was significantly lower compared with other sealers ($P < 0.05$). The greatest percentage of viable cells, similar to that in the control group, was observed for AHP ($P = 0.1457$; Fig. 2a). S26 induced a

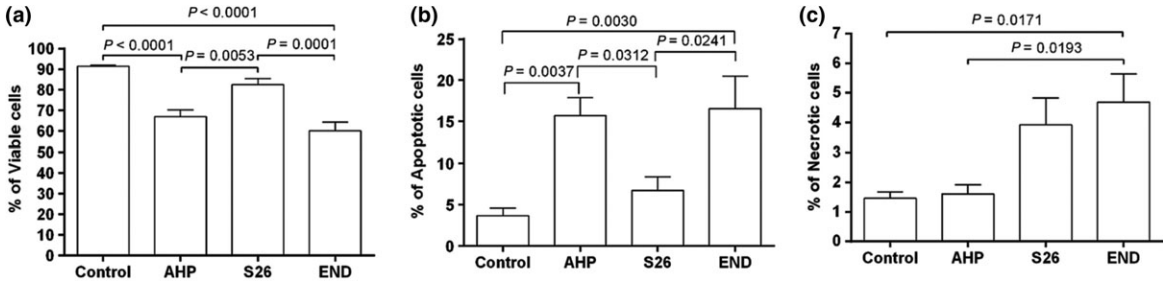


Figure 1 Cell death determination of PMNs after treatment with AH Plus (AHP), Sealer 26 (S26) and Endosequence (END). Effects of sealers on the cell viability (a), apoptosis (b) and necrosis (c) of PMNs.

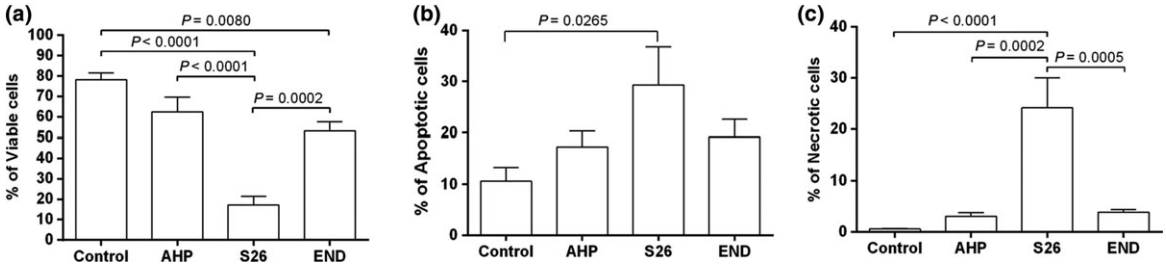


Figure 2 Cell death determination of monocytes after treatment with AH Plus (AHP), Sealer 26 (S26) and Endosequence (END). Effects of sealers on the cell viability (a), apoptosis (b) and necrosis (c) of monocytes.

significantly greater percentage of apoptotic cells compared with the control group ($P = 0.0265$; Fig. 2b) and increased the proportion of necrotic cells in the culture compared with the other materials and control ($P < 0.05$; Fig. 2c).

Cytokine and chemokine measurements

The cytokines and chemokines measured were IL-8 for the PMN cultures and IL-4, IL-6, IL-10, IL-12 e TNF-α for the monocyte cultures.

Figure 3a shows means and standard errors for the release of IL-8 induced by the sealers on PMNs in culture. AHP ($P = 0.0226$) and S26 ($P = 0.0004$) sealers were able to stimulate the PMNs to produce IL-8, with differences in relation to the control group.

Figure 3b–f shows means and standard error for the release of cytokines and chemokines induced by the sealers on monocytes in culture. AHP ($P = 0.0386$) and END ($P = 0.0280$) induced a lower release of IL-10 than the control group (Fig. 3b). END and S26 induced a lower release of IL-12 ($P = 0.0056$) and IL-6 ($P = 0.0263$), respectively, than the control group (Fig. 3c,d). END induced the lowest release of IL-12 in the culture among the sealers evaluated. No significant differences were found among the sealers and the control group regarding the release of IL-4 ($P > 0.05$) and TNF-α ($P > 0.05$; Fig. 3e,f).

Discussion

Studies have shown a relationship between the outcome of root canal treatment with root fillings ending 0–2 mm short of the apex (Schaeffer *et al.* 2005, Ricucci *et al.* 2011). A possible explanation for the lower healing rates of cases associated with overfillings would be related to the cytotoxicity of endodontic sealers, their solubility in tissue fluids, their susceptibility to phagocytosis (Ricucci *et al.* 2016) or induction of a foreign body reaction (Ricucci *et al.* 2009). Although the extraradicular presence of the endodontic filling material may not be directly associated with endodontic post-treatment disease, it has been suggested that the presence of endodontic sealers in periapical tissues may delay the healing process in teeth with apical periodontitis (Fristad *et al.* 2004, Ricucci *et al.* 2016). Therefore, it is important to evaluate cytotoxicity and investigate the activities of immunological biomarkers produced by cells present in the periapical tissues provoked by sealers with different compositions.

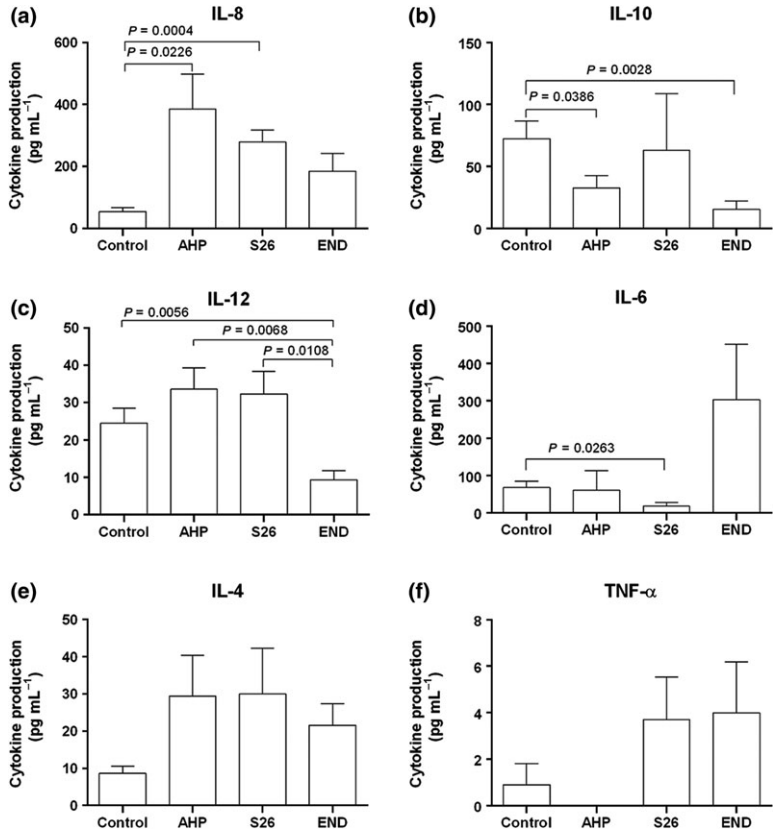


Figure 3 Cytokine production in PMNs and monocytes after incubation with AH Plus (AHP), Sealer 26 (S26) and Endosequence (END). PMN IL-8 production after 4 h of incubation with sealers (a). Monocyte IL-10, IL-12, IL-6, IL-4 and TNF-α production after 24 h of incubation with sealers (b–f).

This is the first study evaluating the effect of fresh endodontic sealers with different compositions, including Endosequence BC Sealer, on monocyte and PMN cell culture models, specifically evaluating the induction of necrosis, apoptosis and secretion of mediators by these cells. In this way, mature nondividing primary cells obtained from peripheral blood were used. Although studies using human monocytic cell lines allow greater control of variables and easy reproducibility, the use of primary cultures are more likely to correspond to PMN and monocyte behaviour *in situ* (Brackett *et al.* 2010). Normal diploid cells have mitotic rates and functions different from those of transformed or tumour cells; thus, their use to record the biomaterials effect on the DNA level, especially on cell death pathways, are preferable (Baraba *et al.* 2011). However, one of the limitations of studies with primary cultures obtained from peripheral blood is the time course, since these cells are not able to multiply and rapidly undergo apoptosis when cultured *in vitro* (Bogdanski *et al.* 2004). Therefore, PMNs were analysed after 4 h of incubation, and monocytes were analysed after 24 h of incubation. Although this model does not allow an analysis of the cytotoxicity of the materials over time, the cells used mimic the classical view of periapical inflammation, which is characterized by an infiltration of leucocytes, primarily PMNs, followed by monocytes (Velard *et al.* 2009, 2010). Furthermore, the cytotoxicity of freshly mixed sealers was evaluated. Previous studies using different experimental designs confirmed that the cytotoxicity of endodontic sealers declines over time (Zhang *et al.* 2010, Loushine *et al.* 2011, Camargo *et al.* 2014). Considering that, clinically, sealer is inserted into root canals in a freshly mixed stage, this strategy was chosen to evaluate the harmful effects considering apoptosis and necrosis.

In the current study, an epoxy resin endodontic sealer (AHP), an epoxy resin containing calcium hydroxide (S26), and a calcium phosphate silicate-based

sealer (END) were tested. These sealers have been extensively evaluated regarding their cytotoxicity in various types of cells (Brackett *et al.* 2008, 2010, Zhang *et al.* 2010, Loushine *et al.* 2011, Camargo *et al.* 2014, Cintra *et al.* 2017), often using filter diffusion models (Zhang *et al.* 2010), extracts (Camargo *et al.* 2014, Cintra *et al.* 2017) or moulds (Brackett *et al.* 2009, 2010, 2012). However, there are no previous studies evaluating AHP, S26 and END, on PMNs and monocytes in human primary cell culture.

The contact of PMNs and monocytes with sealers induced distinct effects regarding apoptosis and necrosis. END and AHP were responsible for apoptosis in PMNs but not in monocytes. Monocytes underwent apoptosis in contact with only S26. Such divergence could be explained by differences in the molecular control of PMN apoptosis compared to monocytes, and the difference regarding Ca^{2+} release over time by the sealers, which result in pH alterations (Tanomaru-Filho *et al.* 2011, Candeiro *et al.* 2012). These results diverge from other cellular analyses, which confirm the biocompatibility of Endosequence BC Sealer (Zhou *et al.* 2015, Alsubait *et al.* 2018). However, the cell types used in these studies were different, and culture models used were based on cellular contact with extracts, not direct contact which could justify the differing results.

The difference in the sensitivity of PMNs and monocytes to sealer components was also confirmed by analysing the percentage of necrotic cells. END was responsible for necrosis in PMNs, but, in monocytes culture, S26 was associated with the greatest percentage of necrotic cells. In these conditions, cell cultures of PMNs and monocytes are more susceptible to necrosis, which could result in intracellular pH alteration that change the behaviour of necrosis molecular regulators such as receptor-interacting protein kinase (RIPK) 1/3, making these cells more susceptible to necrosis (Zhang *et al.* 2017). Calcium hydroxide-based sealers release calcium ions (Ca^{2+}) in the neighbouring environment; however, the concentration of this ion in the medium varies among the studied sealers (Tanomaru-Filho *et al.* 2011, Candeiro *et al.* 2012). According to a previous study, S26 is capable of releasing 0.118 mg mL^{-1} of Ca^{2+} during an experimental period of 3 h and 0.730 mg mL^{-1} of Ca^{2+} during a period of 24 h (Tanomaru-Filho *et al.* 2011), which is probably related to the greater percentage of necrosis after exposure to this sealer in monocyte cells, which was evaluated after 24 h. On the other hand, Endosequence BC sealer releases 0.329 mg mL^{-1} of Ca^{2+} at 3 h and 0.204 mg mL^{-1} of Ca^{2+} at 24 h (Candeiro *et al.* 2012), indicating that such a reduction on Ca^{2+} release could be related to the differences in necrosis observed at 4 and 24 h in the current study.

Additionally, it has been established that an overload of extracellular Ca^{2+} may increase its intracellular concentration (Berridge *et al.* 2003). Intracellular Ca^{2+} has been implicated in most aspects of cell physiology and has been associated with cell death regulation (Zhivotovsky & Orrenius 2011, Smaili *et al.* 2013, Orrenius *et al.* 2015). It has been asserted that necrotic cell death is associated with intracellular Ca^{2+} overload, and multiple functions in the apoptotic process have subsequently been found to be governed by Ca^{2+} signalling (Zhivotovsky & Orrenius 2011, Orrenius *et al.* 2015). Previous studies analysing other biomaterials that are able to release calcium found profound changes in the behaviour of neutrophils cultured in contact with them (Liao *et al.* 2006, Edwards *et al.* 2011). Furthermore, it is important to emphasize that the culture model used was based on direct contact with the sealers, mimicking an extreme condition of the contact of sealers with periapical tissues beyond the apical foramen. Although this study can prove that the endodontic sealers induce apoptosis and/or necrosis, it is not possible to state a relationship of cause/effects for a possible failure of root canal treatment, as the success depends on other factors.

In addition to the possible interference of cell death modalities in the present findings, it is also important to remember that the time of analysis was different between PMNs and monocytes. It is likely that the amount and effect of components released by the sealers at 4 and 24 h were also not the same. Previous studies evaluating other endodontic sealers have shown that the initial cytotoxicity is different from that observed over time (Brackett *et al.* 2008, Pinna *et al.* 2008, Camargo *et al.* 2014). Considering the methodological differences between the present study, regarding the cellular models used and their evaluation methodology, the present findings cannot be directly compared with the literature.

In addition to interfering in the mechanisms of cell death, the characteristics of a biomaterial might activate intracellular motifs that result in the release of mediators that trigger and maintain the inflammatory process or else suppress it (Baraba *et al.* 2011, Brown *et al.* 2012). Until this time, relatively few studies have evaluated the effect of components released by

endodontic materials on the profile of monocytes/macrophages or on the release of cytokines and other mediators related to inflammation (Rezende *et al.* 2005, 2007, Brackett *et al.* 2010, 2012, de Oliveira Mendes *et al.* 2010, Braga *et al.* 2014). However, the referred studies used peritoneal mouse macrophages (Rezende *et al.* 2005, 2007, de Oliveira Mendes *et al.* 2010, Braga *et al.* 2014) or THP-1 macrophages (Brackett *et al.* 2010, 2012) and did not use human primary cultures as a model. Controversy exists regarding the choice of permanent cell lines or primary cell cultures as study models (Honegger 2001, Martinho *et al.* 2018). Despite the greater use of permanent lineage cultures, these have unnatural behaviour, altering the biological responses against materials (Honegger 2001).

In the current study, the proinflammatory mediators IL-6, IL-12, TNF-α and IL-8 (de Brito *et al.* 2012, Márton & Kiss 2014) were evaluated, as well as immunoregulatory/reparative IL-4 and IL-10 (de Brito *et al.* 2012, Márton & Kiss 2014) induced by AHP, S26 and END. These cytokines are key players of a cascade that initiates, maintains and repairs periapical inflammation and bone resorption, including apical periodontitis (Márton & Kiss 2014). Considering that root canal sealer may extrude and establish direct contact with periapical tissues, it is important to know the cytokine profile induced by sealers on monocytes and IL-8, the principal chemokine produced by PMNs. It is important to evaluate these effects because some endodontic materials that are extruded beyond the apical foramen could recruit inflammatory cells that release proinflammatory and immunoregulatory cytokines (Braga *et al.* 2014, Ricucci *et al.* 2016); thus, they were evaluated in the current experimental model.

Previous studies evaluating the cytokines produced by monocytes/macrophages in contact with endodontic materials used macrophage polarization models (Rezende *et al.* 2005, de Oliveira Mendes *et al.* 2010, Braga *et al.* 2014). Macrophages are polarized into two types according to function, receptor relationship, and cytokine production: M1 is characterized by its inflammatory profile, and M2 is recognized by its immunomodulatory/anti-inflammatory activities (Kou & Babensee 2010, de Oliveira Mendes *et al.* 2010, Brown *et al.* 2012). Macrophages can be directed towards M1 phenotypes by Th1-type cytokines, such as IFN-ɣ, or microbial pathogen-associated molecular patterns, such as LPS (Martinez *et al.* 2008). M2 represents a continuum of functionally and phenotypically related cells subdivided into M2a, M2b and M2c (Martinez *et al.* 2008). IL-4 and IL-10 promote the alternative activation of macrophages into M2 cells (M2a and M2c, respectively) and inhibit the classical activation of macrophages into M1 cells (Martinez *et al.* 2008, Kou & Babensee 2010). The absence of significant differences in IL-4 levels among the sealers and the control group could suggest that the sealers do not interfere with the production and secretion of this cytokine. On the other hand, the lower levels of IL-10 detected in AHP and END when compared with the control might indicate a reduction in monocyte/macrophage scavenging activity and prohealing activity. TNF-α has often been evaluated in studies enrolling the cytokine production by macrophage subsets induced by endodontic sealers (Rezende *et al.* 2005, de Oliveira Mendes *et al.* 2010, Braga *et al.* 2014). The lack of difference between the evaluated sealers and control group are in agreement with de Oliveira Mendes *et al.* (2010), which suggests that, in the absence of a residual infection, the sealer does not interfere with TNF-α production. Considering that, in the present study, external agents were not added to induce the polarization of macrophages, it is not possible to make inferences about the role of the sealers studied in the M1 or M2 profile.

IL-12 promotes cell-mediated immune responses, acting at the intersection of the innate and adaptive immune responses (de Oliveira Mendes *et al.* 2010) and increasing in the presence of Gram-positive and Gram-negative bacteria (Rezende *et al.* 2005). In this study, END had the lowest levels of IL-12 compared to the control and other sealers, which differs from previous studies that demonstrated that endodontic materials did not affect the capacity of macrophages to produce IL-12 in the absence of microbial stimuli (Rezende *et al.* 2005, de Oliveira Mendes *et al.* 2010). On the other hand, Braga *et al.* (2014) detected higher levels of IL-12 produced by macrophages in the presence of extracts of endodontic sealers than in control group, in both stimulated and nonstimulated cultures. Despite the differences regarding sealer composition and cellular models used, those results confirm the present study, since they demonstrate that, depending on the composition of the sealer, sealers can stimulate the production of IL-12.

Regarding IL-6, this is the first study to evaluate the interference of sealer composition on the release of this cytokine in monocyte cultures. IL-6 acts as both a proinflammatory and anti-inflammatory cytokine, depending on the induction or inhibition provided by other mediators present in the microenvironment. The

present findings, in which the sealers AHP and END did not differ from control, indicate that these sealers did not modify the pattern of cytokine release by monocytes, which probably indicates that these sealers do not activate a proinflammatory response. The lowest levels of IL-6 were induced by S26, probably because of its cytotoxic effects. A previous study showed similar findings that sealers did not interfere with IL-6 production (Brackett *et al.* 2009). On the other hand, Martinho *et al.* (2018), using pulp cells in contact with extracts of different sealers, among them AHP, found higher levels of IL-6 and an absence of difference for IL-12 when compared to the control group. Those differences could be related to the cell model used, experimental design regarding sealer contact with cells and sealer composition. Further studies using LPS activation are necessary to know the effect of these sealer compounds on activated macrophages.

IL-8 is one of the main PMN inflammatory mediators; it is known to recruit and activate PMNs, as well as other inflammatory cells (Velard *et al.* 2009). In the present study, it was postulated that the products released by the fresh sealers would be able to influence the production of this mediator. In fact, the presence of components of the sealers in the culture medium served as a stimulus for the release of IL-8, which was significantly lower in the control group than in cells cultivated with sealers. Despite the present findings, it is important to take into account that results enrolling cell culture models should not be extrapolated clinically due to this model's lack of a mechanism for the removal of irritating agents (Bryan *et al.* 2010).

The influence of the sealer on viability is dependent on the cell type. AHP was more cytotoxic for PMNs; meanwhile, S26 was more cytotoxicity for monocytes. Both sealers induced apoptosis, justifying the reduction in cellular viability. Sealer composition appeared to alter the form of self-regulation of the production of these cytokines by cells. There was broad variation in the effects of sealers on cytokine production, and it was not possible define a precise cytokine profile. Therefore, more studies must be conducted using other cell models and cocultures in order to understand the steps that may be involved in periradicular reactions to extruded material.

Conclusions

END and AHP increased the percentage of apoptotic and necrotic cells in PMNs and monocytes, respectively, with higher cytotoxicity to these cells. Regarding the release of cytokines, END had the lowest levels of IL-12 compared to the other sealers and higher levels of IL-6 compared with S26. The tested sealers did not differ in the release of IL-8, IL-10, TNF-α and IL-4.

Acknowledgements

This project was developed on CPBio – Biomechanics, Biomaterials and Cell Biology Research Center, and financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, FAPEMIG and CNPq.

Conflict of interest

The authors have stated explicitly that there are no conflict of interests in connection with this article.

References

Al-Hiyasat AS, Tayyar M, Darmani H (2009) Cytotoxicity evaluation of various resin based root canal sealers. *International Endodontic Journal* **43**, 148–53.

Alsubait SA, Al Ajlan R, Mitwalli H *et al.* (2018) Cytotoxicity of different concentrations of three root canal sealers on human mesenchymal stem cells. *Biomolecules* **8**, E68.

Anderson JM, Miller KM (1984) Biomaterial biocompatibility and the macrophage. *Biomaterials* **5**, 5–10.

Baraba A, Zeljezic D, Kopjar N, Mladinic M, Anić I, Miletić I (2011) Evaluation of cytotoxic and genotoxic effects of two resin-based root-canal sealers and their components on human leucocytes *in vitro*. *International Endodontic Journal* **44**, 652–61.

Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signalling: dynamics, homeostasis and remodelling. *Nature Reviews Molecular Cell Biology* **4**, 517–29.

Bogdanski D, Esenwein SA, Prymak O, Epple M, Muhr G, Köller M (2004) Inhibition of PMN apoptosis after adherence to dip-coated calcium phosphate surfaces on a NiTi shape memory alloy. *Biomaterials* **25**, 4627–32.

Brackett MG, Marshall A, Lockwood PE *et al.* (2008) Cytotoxicity of endodontic materials over 6-weeks *ex vivo*. *International Endodontic Journal* **41**, 1072–8.

Brackett MG, Marshall A, Lockwood PE *et al.* (2009) Inflammatory suppression by endodontic sealers after aging 12 weeks *In Vitro*. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* **91**, 839–44.

Brackett MG, Messer RL, Lockwood PE *et al.* (2010) Cytotoxic response of three cell lines exposed *in vitro* to dental endodontic sealers. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* **95**, 380–6.

Brackett MG, Lewis JB, Kious AR *et al.* (2012) Cytotoxicity of endodontic sealers after one year of aging *in vitro*.

Journal of Biomedical Materials Research Part B: Applied Biomaterials **100**, 1729–35.

Braga JM, Oliveira RR, Martins RC, Ribeiro Sobrinho AP (2014) The effects of a mineral trioxide aggregate-based sealer on the production of reactive oxygen species, nitrogen species and cytokines by two macrophage subtypes. *International Endodontic Journal* **47**, 909–19.

de Brito LC, Teles FR, Teles RP, Totola AH, Vieira LQ, Sobrinho AP (2012) T-lymphocyte and cytokine expression in human inflammatory periapical lesions. *Journal of Endodontics* **38**, 481–5.

Brown BN, Ratner BD, Goodman SB, Amar S, Badylak SF (2012) Macrophage polarization: an opportunity for improved outcomes in biomaterials and regenerative medicine. *Biomaterials* **33**, 3792–802.

Browne R (1988) The *in vitro* assessment of the cytotoxicity of dental materials; does it have a role? *International Endodontic Journal* **21**, 50–8.

Bryan TE, Khechen K, Brackett MG *et al.* (2010) *In vitro* osteogenic potential of an experimental calcium silicate-based root canal sealer. *Journal of Endodontics* **36**, 1163–9.

Camargo CH, Oliveira TR, Silva GO, Rabelo SB, Valera MC, Cavalcanti BN (2014) Setting time affects *in vitro* biological properties of root canal sealers. *Journal of Endodontics* **40**, 530–3.

Candeiro GT, Correia FC, Duarte MA, Ribeiro-Siqueira DC, Gavini G (2012) Evaluation of radiopacity, pH, release of calcium ions, and flow of a bioceramic root canal sealer. *Journal of Endodontics* **38**, 842–5.

Candeiro GT, Moura-Netto C, D'Almeida-Couto RS *et al.* (2016) Cytotoxicity, genotoxicity and antibacterial effectiveness of a bioceramic endodontic sealer. *International Endodontic Journal* **49**, 858–64.

Cintra LTA, Benetti F, de Azevedo Queiroz IO *et al.* (2017) Evaluation of the cytotoxicity and biocompatibility of new resin epoxy-based endodontic sealer containing calcium hydroxide. *Journal of Endodontics* **43**, 2088–92.

Edwards FC, Taheri A, Dann SC, Dye JF (2011) Characterization of cytolytic neutrophil activation *in vitro* by amorphous hydrated calcium phosphate as a model of biomaterial inflammation. *Journal of Biomedical Materials Research Part A* **96**, 552–65.

Fristad I, Molven O, Halse A (2004) Nonsurgically retreated root filled teeth–radiographic findings after 20-27 years. *International Endodontic Journal* **37**, 12–8.

Hess D, Solomon E, Spears R, He J (2011) Retreatability of a bioceramic root canal sealing material. *Journal of Endodontics* **37**, 1547–9.

Honegger P (2001) Overview of cell and tissue culture techniques. *Current Protocols In Pharmacology* **4**, 12.1.1–1.12.

Kaplan SS, Basford RE, Jeong MH, Simmons RL (1996) Biomaterial-neutrophil interactions: dysregulation of oxidative functions of fresh neutrophils induced by prior neutrophil-biomaterial interaction. *Journal of Biomedical Materials Research* **30**, 67–75.

Kou PM, Babensee JE (2010) Macrophage and dendritic cell phenotypic diversity in the contexto of biomaterials. *Journal of Biomedical Materials Research Part A* **96**, 239–60.

Liao S, Tamura K, Zhu Y *et al.* (2006) Human neutrophils reaction to the biodegraded nano-hydroxyapatite/collagen and nano-hydroxyapatite/collagen/poly (L-lactic acid) composites. *Journal of Biomedical Materials Research Part A* **76**, 820–5.

Loushine BA, Bryan TE, Looney SW *et al.* (2011) Setting properties and cytotoxicity evaluation of a premixed bioceramic root canal sealer. *Journal of Endodontics* **37**, 673–7.

Marciano MA, Guimarães BM, Ordinola-Zapata R *et al.* (2011) Physical properties and interfacial adaptation of three epoxy resin-based sealers. *Journal of Endodontics* **37**, 1417–21.

Martinez FO, Sica A, Mantovani A, Locati M (2008) Macrophage activation and polarization. *Frontiers in Bioscience* **13**, 453–61.

Martinho FC, Camargo SEA, Fernandes AMM *et al.* (2018) Comparison of cytotoxicity, genotoxicity and immunological inflammatory biomarker activity of several endodontic sealers against immortalized human pulp cells. *International Endodontic Journal* **51**, 41–57.

Márton IJ, Kiss C (2014) Overlapping protective and destructive regulatory pathways in apical periodontitis. *Journal of Endodontics* **40**, 155–63.

Moura CC, Machado JR, Silva MV *et al.* (2013) Evaluation of human polymorphonuclear behavior on textured titanium and calcium-phosphate coated surfaces. *Biomedical Materials* **8**, 035010.

de Oliveira Mendes ST, de Brito LC, Rezende TM *et al.* (2010) A decrease in the innate immune response to infection in the presence of root canal sealers. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **109**, 315–23.

Onay EO, Ungor M, Ozdemir BH (2007) *In vivo* evaluation of the biocompatibility of a new resin-based obturation system. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **104**, 60–6.

Orrenius S, Gogvadze V, Zhivotovsky B (2015) Calcium and mitochondria in the regulation of cell death. *Biochemical and Biophysical Research Communications* **460**, 72–81.

Pinna L, Brackett MG, Lockwood PE *et al.* (2008) *In vitro* cytotoxicity evaluation of a self-adhesive, methacrylate resin-based root canal sealer. *Journal of Endodontics* **34**, 1085–8.

Rezende TM, Vargas DL, Cardoso FP, Sobrinho AP, Vieira LQ (2005) Effect of mineral trioxide aggregate on cytokine production by peritoneal macrophages. *International Endodontic Journal* **38**, 896–903.

Rezende TM, Vieira LQ, Cardoso FP *et al.* (2007) The effect of mineral trioxide aggregate on phagocytic activity and production of reactive oxygen, nitrogen species and arginase activity by M1 and M2 macrophages. *International Endodontic Journal* **40**, 603–11.

Ricucci D, Siqueira Jr JF, Bate AL, Pitt Ford TR (2009) Histologic investigation of root canal treated teeth with apical periodontitis: a retrospective study from twenty-four patients. *Journal of Endodontics* **35**, 493–502.

Ricucci D, Russo J, Rutberg M, Burleson JA, Spangberg LS (2011) A prospective cohort study of endodontic treatments of 1,369 root canals: results after 5 years. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **112**, 825–42.

Ricucci D, Rôças IN, Alves FR, Loghin S, Siqueira Jr JF (2016) Apically extruded sealers: fate and influence on treatment outcome. *Journal of Endodontics* **42**, 243–9.

Schaeffer MA, White RR, Walton RE (2005) Determining the optimal obturation length: a meta-analysis of literature. *Journal of Endodontics* **31**, 271–4.

Sjogren U, Ohlin A, Sundqvist G, Lerner UH (1998) Guttapercha-stimulated mouse macrophages release factors that activate the boné resorptive system of mouse calvarial bone. *European Journal of Oral Sciences* **106**, 872–81.

Smaili SS, Pereira GJ, Costa MM *et al.* (2013) The role of calcium stores in apoptosis and autophagy. *Current Molecular Medicine* **13**, 252–65.

Soehnlein O, Lindbom L (2010) Phagocyte partnership during the onset and resolution of inflammation. *Nature Reviews Immunology* **10**, 427–39.

Szczurko G, Pawińska M, Łuczaj-Cepowicz E, Kierklo A, Marczuk-Kolada G, Hołownia A (2018) Effect of root canal sealers on human periodontal ligament fibroblast viability: *ex vivo* study. *The Journal Odontology* **106**, 245–56.

Tanomaru-Filho M, Saçaki JN, Faleiros FB, Guerreiro-Tanomaru JM (2011) pH and calcium ion release evaluation of pure and calcium hydroxide-containing Epiphany for use in retrograde filling. *Journal of Applied Oral Science* **19**, 1–5.

Velard F, Laurent-Maquin D, Guillaume C *et al.* (2009) Polymorphonuclear neutrophil response to hydroxyapatite particles, implication in acute inflammatory reaction. *Acta Biomaterialia* **5**, 1708–15.

Velard F, Laurent-Maquin D, Braux J *et al.* (2010) The effect of zinc on hydroxyapatite-mediated activation of human polymorphonuclear neutrophils and bone implant-associated acute inflammation. *Biomaterials* **31**, 2001–9.

Zhang W, Li Z, Peng B (2010) *Ex vivo* cytotoxicity of a new calcium silicate-based canal filling material. *International Endodontic Journal* **43**, 769–74.

Zhang ZX, Gan I, Pavlosky A, Huang X, Fuhrmann B, Jevnikar AM (2017) Intracellular pH Regulates TRAIL-induced apoptosis and necroptosis in endothelial cells. *Journal of Immunology Research* **2017**, 1503960.

Zhivotovsky B, Orrenius S (2011) Calcium and cell death mechanisms: a perspective from the cell death community. *Cell Calcium* **50**, 211–21.

Zhou HM, Du TF, Shen Y, Wang ZJ, Zheng YF, Haapasalo M (2015) *In vitro* cytotoxicity of calcium silicate-containing endodontic sealers. *Journal of Endodontics* **41**, 56–61.

Zmener O (2004) Tissue response to a new methacrylate-based root canal sealer: preliminary observations in the subcutaneous connective tissue of rats. *Journal of Endodontics* **30**, 348–51.

CAPÍTULO 2

3.2 Capítulo 2

Artigo submetido no periódico Brazilian Oral Research

Evaluation of premixed calcium silicate-based sealers and their role on osteoclastogenesis

Camila Maria Peres de Rosatto[1], Gabriela Leite de Souza[1], Danilo Cassiano Ferraz[2], Marcelo José Barbosa Silva[3], Mário Tanomaru-Filho[2], Camilla Christian Gomes Moura[1]

[1] Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil.

[2] Department of Restorative Dentistry, School of Dentistry, São Paulo State University, Araraquara, SP, Brazil.

[3] Department of Immunology, Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, MG, Brazil.

Correspondence: Camilla Christian Gomes Moura, Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Av Pará 1720, Block 4L, annex A, class 24, Campus Umuarama, Uberlândia, MG, 38400-902, Brazil (Tel.: (+55) 34 3225-8103; e-mail: camillamoura@ufu.br).

Evaluation of premixed calcium silicate-based sealers and their role on osteoclastogenesis

Abstract: Solubility, pH, ion release, cytotoxicity and osteoclastogenesis inhibition in bone marrow-derived monocyte macrophages (BMMs) were evaluated for: EndoSequence BC Sealer (Brasseler) [END], Bio-C Sealer (Angelus) [BC], and Sealer Plus BC (MK Life) [SPBC]. pH was evaluated after immersion in deionised water (DW) and α-MEM. Solubility was obtained by mass loss. Ion release was measured by using X-ray fluorescence spectroscopy (XRF). Cytotoxicity was evaluated by MTT assay. Inhibition of osteoclastogenesis was evaluated by tartrate-resistant acid phosphatase (TRAP). Statistical analyses were performed using ANOVA, Tukey's and Dunnett's tests or t-tests ($\alpha = 0.05$). END presented highest pH in DW ($p < 0.05$), and BC in α-MEM ($p < 0.05$). Solubility in DW was lower for SPBC ($p < 0.005$). The highest calcium release was observed for BC in DW at 12 h ($p < 0.05$) and in α-MEM at 12 and 24 h ($p < 0.05$). Lowest toxicity was detected for END ($p < 0.05$). BC presented higher inhibitory effect on osteoclast ($p < 0.05$). All calcium silicate-based sealers demonstrated higher solubility than ISO standards. Calcium release was higher for BC. END showed greater cell viability, and BC showed greater inhibition of osteoclasts.

Keywords: Bioceramics; Calcium Silicate; Cytotoxicity; Physicochemical Properties; Root Canal Sealer

Evaluation of premixed calcium silicate-based sealers and their role on osteoclastogenesis

Journal:	*Brazilian Oral Research*
Manuscript ID	Draft
Manuscript Type:	Original Research Report
Specialties:	Dental Materials, Pulp Biology, Endodontic Therapy
Category--Select your categories from the <A HREF='http://www.nlm.nih.gov/mesh/MBrowser.html' target='_new'> MeSH or <A HREF='http://decs.bvs.br/' target='_new'> DeCS lists.:	Bioceramics, Calcium Silicate, Cytotoxicity, Physicochemical Properties, Root Canal Sealer

Evaluation of premixed calcium silicate-based sealers and their role on osteoclastogenesis

Introduction

Root canal sealers may extrude through the foramen, or leachable compounds from the sealers may come into contact with periradicular tissues influencing the healing process.[1] Teeth with periapical periodontitis in which local immunoinflammatory reactions led to alveolar bone resorption adjacent to the root apex and dentin.[1, 2] The reciprocal coordination between osteoblasts and osteoclasts is essential to provide repair of periapical periodontitis.[3] Particularly, the upregulation of osteoclastic activity has been associated with the control of lesion expansion.[4] In recent years, research has evaluated the effect of calcium silicate-based materials on the modulation of osteoclastogenesis.[5-8]

Calcium silicate-based sealers, a new class of endodontic sealers characterised as bioceramics.[9, 10] have been described as bioactive and osteoconductive with the potential to be used as adjuvants in periapical remineralization, favouring regeneration.[11, 12] Bio-C Sealer (Angelus, Londrina, PR, Brazil) and Sealer Plus BC (MK Life, Porto Alegre, RS, Brazil) are premixed and ready-to-use calcium silicate-based endodontic sealers, which were recently evaluated regarding their physiochemical [13, 14] and biological properties.[15-18] However, there is no evidence regarding ions leached in solution by both sealers and their effects on osteoclastic differentiation compared to the gold standard bioceramics sealer, EndoSequence BC Sealer (Brasseler, Savannah, GA, USA).

The aim of this study was to evaluate the effect of premixed calcium silicate-based endodontic sealers EndoSequence BC Sealer, Sealer Plus BC and Bio-C Sealer on bone marrow-derived monocyte-macrophages (BMMs) cytotoxicity, inhibition of osteoclastogenesis, pH, solubility and ion release. The null hypothesis was that there would be no difference between the sealers on all the evaluated parameters.

Methodology

Preparation of sealer discs and extracts

The following endodontic sealers were tested: EndoSequence BC Sealer (Brasseler) – END, Bio-C Sealer (Angelus) - BC, and Sealer Plus BC (MK Life) - SPBC. Acrylic plates, with six perforations each measuring 7.75 mm in diameter x 1.5 mm in thickness,[19] were moulded with silicone. The moulds were filled with the sealers and kept in a humidified chamber at 37 °C for 24 h according to methodology proposed by Tanomaru-Filho *et al.*[20] for analysis of pH, solubility and chemical compounds by X-ray fluorescence spectroscopy.

Sealer extracts were prepared according to standards (ISO 10993–5/2009)[21]. With an insulin syringe, 0.3 mL of each sealer was placed at the bottom of a 24-well plate, and each well was filled with 1.5 mL of Minimum Essential Medium Eagle (α-MEM) (LGC Biotecnologia, Cotia, SP, Brazil), supplemented with 10% heat-inactivated foetal bovine serum (FBS) (LGC Biotecnologia) and 1% penicillin-streptomycin (Sigma-Aldrich, Saint Louis, MO, USA) – 10% α-MEM. The plates were kept for an additional 24 h in a humidified incubator at 37 °C (5% CO_2/air) before extracts preparation.[22] The tested sealers were manipulated according to the manufacturers' directions.

Sealer pH, solubility and ion release

The same samples were used for the three methodologies. The sealer discs were removed from moulds, kept in a desiccator, and weighed on an analytical balance (Shimadzu Corporation, Kyoto, Kansai, Japan) until stabilisation of the initial mass. From each sealer, six discs were placed inside plastic tubes containing 10 mL of deionised water (DW) and the other six on tubes containing 10 mL of α-MEM. These samples were kept in a humidified incubator at 37 °C for a total of 48 h. The pH values were determined after 12, 24 and 48 h of immersion in DW or α-MEM using a previously calibrated digital pH metre (MicroNal B 374, São Paulo, SP, Brazil). The controls were based on both solutions in which no sealers were immersed. The mean pH of each sample in each experimental period for DW and α-MEM was calculated after three

measurements. After each measurement, DW and α-MEM were carefully moved to new tubes for X-ray fluorescence spectroscopy measurement and were replaced by fresh solutions. After 48 h, the discs were removed from the tubes, placed in a desiccator again and then reweighed as described by Torres *et al.*[13] The solubility for samples stored in DW and α-MEM was obtained by calculating the mass loss and is expressed as a percentage.

The percentage of ion released by sealers on DW or α-MEM was obtained by fluorescence spectra of liquid samples collected each time using X-ray fluorescence spectroscopy (XRF; model S8 Tiger Series 2, Bruker, Kontich, Flanders, Belgium). Trace elements can be analysed in concentration of parts per million (ppm). From each sample to be analysed, 10 mL of liquid was transferred to sample cups of 40 mm (model SC-4340, PremierLab Supply, Port St. Lucie, FL, USA) and weighed on an analytical balance for further normalisation of data. Quantitative analysis of constituents was performed by Quant Express for fast and reliable analysis of unknown liquid samples. Data acquisition time for each sample measurement was 420 s, and the spectra were processed using the software Spectra Plus, which automatically determines the intensities of X-ray peaks for the elements and quantifies their concentration.

Bone-marrow derived monocyte-macrophages (BMMs) culture and exposure to the sealer extracts

Animal procedures were performed with approval of the ethics committee in animal experimentation at the Federal University of Uberlândia (protocol number 003/2019). Bone marrow cells (BMCs) were isolated from 6-week-old C57BL/6 mouse femurs as previously described.[23] BMCs were seeded in 6 well/plates containing 10% α-MEM and 30 ng/mL of recombinant murine monocyte colony stimulating factor (M-CSF) (PeproTech, London, United Kingdom). After 3 days, the BMMs were plated on 96-well plates (2×10^4 cells/well) in 10% α-MEM, allowed to adhere overnight, and the cultures were exposed to the sealer extracts at all dilutions (1:20, 1:100, 1:500, and 1:2500) with the presence of M-CSF at 30 ng/mL during 12 h, 24 h and 48 h. After the incubation periods, the cells were immediately tested for cytotoxicity by the MTT assay. The control group was maintained in α-MEM with M-CSF.

MTT assay

Cytotoxicity of sealer extracts on BMMs was evaluated at 12 h, 24 h and 48 h. MTT solution (Sigma-Aldrich) (5 mg/mL) was added to each well, and the cells were incubated at 37 ºC for 4 h. Supernatants were removed, and then 100 µL of dimethyl sulfoxide (DMSO) (LGC Biotecnologia) was added. The optical density at 570 nm was measured using a microplate reader (Biochrom, Cambridge, EN, United Kingdom).

Differentiation of BMMs into osteoclasts (osteoclastogenesis), exposure to the sealer extracts and tartrate-resistant acid phosphatase (TRAP) stain

BMMs were simultaneously exposed to the sealer extracts, 50 ng/mL of recombinant murines RANK Ligand (RANKL) (PeproTech) and 30 ng/mL of M-CSF (PeproTech) during a period of 5 days. The extracts and reagents were replaced on day 3. The positive control group contained cells kept in 10% α-MEM stimulated with M-CSF and RANKL, and the negative control group contained cells maintained only in 10% α-MEM and M-CSF (not induced to differentiation). At day 5, the cells were stained for TRAP using a commercially available staining kit (Sigma-Aldrich) according to the manufacturer's protocol. TRAP-positive multinucleated (>3 nuclei) cells were counted as osteoclasts and expressed as percentage. Images were obtained with 10x magnification using a Leica DM IRB-inverted microscope coupled with a DFC490 camera (Leica, Wetzlar, HE, Germany).

Statistical analysis

All data were collected and tabulated in Microsoft Excel sheets (Microsoft Corporation, Redmond, WA, USA). The data were analysed for normal distribution using the Shapiro-Wilk test and homoscedasticity using the Levene test. Two-way ANOVA, Tukey's and Dunnett's tests were used for pH and osteoclastic differentiation (TRAP stain). Two-way ANOVA and Tukey's tests were used for ion release (XRF). Two-way and three-way ANOVA were used for viability. One-way ANOVA and Tukey's tests for multiple comparisons were used for solubility between the sealers in each solution, and the unpaired t-test was used for comparisons of the same sealer

in different solutions. Statistical analyses were performed with GraphPad Prism software v8.2.0 (San Diego, CA, USA), and the significance was set at $\alpha = 0.05$.

Results

Solubility, pH and ion release

Solubility values were significantly lower for SPBC than END and BC ($p < 0.0001$) after DW immersion. After α-MEM immersion, BC presented significantly higher values than END and SPBC ($p < 0.0001$). The results are shown in Table 1. Figure 1 shows the mean pH values for the three evaluated sealers and respective controls in DW and α-MEM. DW analysis revealed that BC and SPBC groups were associated with pH reduction in the evaluation times ($p < 0.0001$) (Figure 1A); in contrast, END presented high pH values in 48 h ($p < 0.0001$). Comparisons among sealers in DW showed the lowest pH values for SPBC in the three experimental periods ($p < 0.0001$). Samples stored in α-MEM did not demonstrate differences by evaluation time for END and SPBC groups ($p = 0.1748$), while in BC, the lowest pH values were demonstrated in 48 h ($p < 0.0095$) (Figure 1B). Comparisons among sealers in α-MEM showed the highest pH values for BC in 12 h ($p < 0.0087$) and 24 h ($p = 0.0025$). In contrast, in 48 h, the highest pH values were observed for SPBC ($p = 0.0009$). In general, the values after α-MEM immersion for solubility and pH were significantly lower than after DW immersion ($p < 0.0001$).

The results for ion release are shown in Figure 2. BC leached calcium ions in DW, reaching a peak at 12 h with expressive reduction along with time ($p = 0.0073$) (Figure 2A). On the other hand, END exhibited low leaching of calcium in the early stages of hydration with substantial reduction at 48 h ($p < 0.0001$) in both soak solutions (Figure 2A,B**)**. In general, calcium levels were similar for BC and END, except for α-MEM 12 h ($p < 0.0001$) in which BC presented the highest amount of this element (Figure 2B). Calcium levels were practically undetectable for SPBC at all times evaluated with results significantly reduced in comparison to those for END and BC at 12 h ($p < 0.0001$) and 24 h ($p < 0.0001$), for DW solution. In α-MEM, only BC was significantly different from SPBC in both periods ($p < 0.0001$ and $p = 0.0145$,

respectively). The silicon ion release was extremely low in all sealers for both soak solutions ($p > 0.9999$), which is expressed in ppm (Figure 2C,D).

Viability

The dilution and time-dependent effects of the extracts on cell viability for each sealer are shown in Figure 3. Two-way ANOVA demonstrated that END group at different extract dilutions and at different experimental times showed similar viability, except 1:20-1:2500 at 48 h ($p = 0.035$) (Figure 3A). BC (Figure 3B) and SPBC (Figure 3C) groups presented a significant reduction in the percentage of cell viability at 48 h for all dilutions, however no significant differences were observed in the interaction of dilutions and time ($p = 0.560$ and $p = 0.661$, respectively). Compared to the control, significant reduction in the cell viability was observed for all extract dilutions at different experimental times for END, BC, and SPBC sealers ($p = 0.031$, $p < 0.001$, and $p < 0.001$ respectively) (Figure 3D-F). Three-way ANOVA and Tukey's post-tests comparing the sealers ($p < 0.001$), dilutions ($p = 0.002$) and time of evaluation ($p < 0.001$) presented significant differences. END showed the highest viability ($p < 0.001$). SPBC presented a similar viability percentage compared with that of BC ($p = 0.156$).

Effects of the sealer extracts on osteoclastic differentiation and function

The dose-dependent effects of the sealer extracts on TRAP stain are shown in Figure 4. In negative control, unstimulated cells (the negative control) did not show TRAP stain, whereas cultures treated with M-CSF and RANKL (the positive control) exhibited significantly higher values (Figure 4A,B). END inhibited TRAP stain in a dose-dependent manner ($p < 0.001$), except between dilutions 1:500-1:2500 ($p = 0.078$). Cultures exposed to SPBC presented similar values of TRAP-positive cells at dilutions of 1:20 and 1:100 ($p = 0.464$), with lower inhibition in more diluted extracts ($p < 0.001$). BC presented higher inhibitory effects at extract concentrations of 1:20 ($p < 0.001$) with a tendency to stabilise on more diluted samples.

Comparisons between sealers in each extract dilution did not present significant differences in the inhibitory effects at 1:20 dilution ($p < 0.001$). At 1:100 dilution, the highest

number of TRAP-positive multinucleated cells was found in END ($p < 0.003$). Similar TRAP stain was detected in BC and SPBC ($p = 0.215$). At dilutions of 1:500 and 1:2500, there were no significant differences between SPBC-END ($p = 0.860$ and $p = 0.139$, respectively), and BC presented the greater inhibitory values ($p < 0.001$). The number of TRAP-positive multinucleated cells on the positive control was superior to all sealers and dilutions tested ($p < 0.001$).

Discussion

The null hypothesis was rejected since differences were detected between the premixed calcium silicate-based sealers regarding physicochemical and biological parameters evaluated. Although pH, solubility and release of calcium ions promoted by END, BC and SPBC have been previously evaluated,[13, 14, 24-27] the periods of analysis were different. In the present study, these parameters were evaluated at periods corresponding to the cytotoxicity analysis on BMMs culture. Additionally, no studies have compared BC or SPBC with END, which has a relevant number of publications.[9, 12, 24, 26]

Solubility and pH were evaluated both in DW, as recommended by American National Institute/American Dental Association and the International Organization for Standardization tests (nº 57 ANSI/ADA – 2008; and ISO 6876/2012),[28, 29] and in the cell culture medium, which is the condition used for cell treatment. It has been argued that the immersion of endodontic sealers in liquids that simulate body fluids (SBF) better mimics the clinical conditions.[9, 13, 30-32] In agreement with the present results, previous studies have demonstrated reduced solubility of calcium silicate-based materials when immersed in SBF [18, 31] or phosphate buffered saline (PBS).[13, 32] It has been speculated that in such conditions, calcium ions combined with phosphate promote the formation of superficial hydroxyapatite,[33] which may compensate the solubility.[32] In general, the mean solubility values were slightly divergent from the current literature for the three sealers.[12, 13, 25, 27] Nevertheless, the present results confirm that BC presents higher solubility than SPBC, despite the storage solution.[13] As previously reported, the solubility of calcium silicate-based sealers remained above the minimum level recommended by ISO 6876.[10, 13, 14, 25, 29, 30] This kind of solubility test presents limitations when used to evaluate biomaterials

that present water absorption,[11, 13, 20] reflecting the loss of mass overestimated due to the drying process.[14]

Solubility has been straightforwardly related to ion release and pH values,[9, 12, 30] especially calcium ions, which may favour the establishment of an alkaline pH.[12] SPBC stored in DW showed the lowest pH values in the three experimental periods. According to Mendes *et al.,*[25] the alkalizing effect of SPBC remains stable for 7 days, ranging from 9.09 to 10.05. In the current study, SPBC maintained a constant pH over time only when stored in α-MEM. It is not possible to explain exactly why the culture medium and DW results showed different pH patterns, but it is clear that the type of soaking medium used affects the material properties.[30]

The calcium ion release has been often evaluated when analysing tri-calcium silicate-based materials [9, 12, 30, 33] because of its relationship with biomineralization and repair. In the present study, the XRF methodology allowed simultaneous multi-element analysis although only the levels of calcium and silicon have been subjected to quantitative analysis because of their presence on END, BC, and SPBC.[17, 24, 25] In contrast to Mendes *et al.,*[25] the levels of calcium in DW for SPBC were almost undetectable and were not increasing over time, which is probably related to its low solubility.[13] On the other hand, the highest levels of calcium in BC are probably related to the high solubility of this material as previously described.[13, 14] In general, the leaching of calcium in DW was higher than in α-MEM, which is probably related to material solubility and soaking solution.[30] The silicon levels were close to zero in DW and are presented in ppm. The silicon levels were slightly higher in the culture medium than in DW, but this result is different from that in the literature,[17] which is probably related to the method of analysis.

The components leached by endodontic sealers may influence the other physicochemical parameters evaluated and affect cell behaviour.[22, 34] It was postulated that the components eluted by fresh sealers on extracts could affect the viability of BMMs and the osteoclastic differentiation. In fact, sealer extracts caused a dose-dependent reduction in the cell viability, except for END. The lower toxicity of END confirms the data obtained in two systematic reviews [11, 12] although this is a parameter affected by the type of cell evaluated.[34] BC and SPBC showed similar viabilities, close to 80% within the first 24 h of contact, which did not classify them as initially cytotoxic. The biocompatibility of BC and SPBC [15, 16] and cytotoxicity on

distinct cell models [16-18] were previously evaluated, demonstrating that both were biocompatible and that cytotoxicity is reduced in more-diluted extracts. In BMMs, the increased cytotoxicity in 48 h may probably reflect a cumulative effect of toxic compounds on BC and SPBC.

Regarding osteoclastic differentiation, the inhibitory effect of BC in RANKL-induced BMMs was evident when compared to that of the other sealers. RANKL-stimulated BMMs are often used for investigation of osteoclastogenesis.[7, 23] To avoid bias caused by the differences in the number of viable cells, the same number of cells was counted per field. The suppression of osteoclastic differentiation, obtained by TRAP-stain, seems to be an interesting property of BC compared to the other sealers. Although molecular analyses are often used to confirm osteoclastic differentiation,[5, 6, 22, 34] assessments of TRAP-positive cells are an appropriate method for preliminary studies. The increased pH in the inflamed periapical region [32] may neutralise the acid environment promoted by osteoclasts or reduce its differentiation,[12, 35] contributing to hard tissue deposition in resorbed areas.

Taken together, the present results indicate that the material properties and composition were affected by the soaking medium, which is probably influenced by the testing methodology used. The BC and SPBC showed reduced cytotoxicity in higher dilutions, while in END, the cytotoxicity was reduced and remained constant along with time. Although it is not possible, according to the present methodology, to know the mechanisms of osteoclastic inhibition, BC seems to be superior to the other sealers, and inferior results were observed for SPBC.

Conclusions

The biological and physicochemical properties of calcium silicate-based sealers evaluated in this *in vitro* study were affected by the soak solutions. All sealers demonstrated a higher solubility than ISO standards. The presence of calcium was higher for BC, while silicon ions in all sealers were extremely low. END showed significantly greater cell viability, and BC presented higher inhibitory effects of osteoclastogenesis; both END and BC are considered the more favourable materials for periapical tissue repair.

Acknowledgments

The authors are grateful to FAPEMIG, CNPq, and CAPES for their financial support (Funding Code 001). The authors are also grateful for the technical support of Isadora Akemi Uehara and Rafael Ramos Heilbuth. This study was carried out at the CPBio (Biomechanics, Biomaterials and Cell Biology Research Center of the Federal University of Uberlândia - UFU, Minas Gerais, Brazil), at the Institute of Biomedical Sciences (UFU), and in the Faculty of Chemical Engineering at UFU.

References

1. Holland R, Gomes JEF, Cintra LTA, Queiroz IOA, Estrela C. Factors affecting the periapical healing process of endodontically treated teeth. J Appl Oral Sci. 2017;25(5):465-76.
2. Ricucci D, Rocas IN, Alves FR, Loghin S, Siqueira JF, Jr. Apically Extruded Sealers: Fate and Influence on Treatment Outcome. J Endod. 2016;42(2):243-9.
3. Braz-Silva PH, Bergamini ML, Mardegan AP, De Rosa CS, Hasseus B, Jonasson P. Inflammatory profile of chronic apical periodontitis: a literature review. Acta Odontol Scand. 2019;77(3):173-80.
4. Ricucci D, Siqueira JF, Jr., Loghin S, Lin LM. Repair of extensive apical root resorption associated with apical periodontitis: radiographic and histologic observations after 25 years. J Endod. 2014;40(8):1268-74.
5. Cheng X, Zhu L, Zhang J, Yu J, Liu S, Lv F, et al. Anti-osteoclastogenesis of Mineral Trioxide Aggregate through Inhibition of the Autophagic Pathway. J Endod. 2017;43(5):766-73.
6. Tian J, Qi W, Zhang Y, Glogauer M, Wang Y, Lai Z, et al. Bioaggregate Inhibits Osteoclast Differentiation, Fusion, and Bone Resorption In Vitro. J Endod. 2015;41(9):1500-6.
7. Zhang J, Zhu L, Peng B. Effect of BioAggregate on osteoclast differentiation and inflammatory bone resorption in vivo. Int Endod J. 2015;48(11):1077-85.
8. Zhang J, Zhu L, Yan P, Peng B. Effect of BioAggregate on Receptor Activator of Nuclear Factor-Kappa B Ligand-induced Osteoclastogenesis from Murine Macrophage Cell Line In Vitro. J Endod. 2015;41(8):1265-71.

9. Donnermeyer D, Burklein S, Dammaschke T, Schafer E. Endodontic sealers based on calcium silicates: a systematic review. Odontology. 2019;107(4):421-36.

10. Jafari F, Jafari S. Composition and physicochemical properties of calcium silicate based sealers: A review article. J Clin Exp Dent. 2017;9(10):e1249-e55.

11. Lim M, Jung C, Shin DH, Cho YB, Song M. Calcium silicate-based root canal sealers: a literature review. Restor Dent Endod. 2020;45(3):e35.

12. Silva Almeida LH, Moraes RR, Morgental RD, Pappen FG. Are Premixed Calcium Silicate-based Endodontic Sealers Comparable to Conventional Materials? A Systematic Review of In Vitro Studies. J Endod. 2017;43(4):527-35.

13. Torres FFE, Zordan-Bronzel CL, Guerreiro-Tanomaru JM, Chavez-Andrade GM, Pinto JC, Tanomaru-Filho M. Effect of immersion in distilled water or phosphate-buffered saline on the solubility, volumetric change and presence of voids within new calcium silicate-based root canal sealers. Int Endod J. 2020;53(3):385-91.

14. Zordan-Bronzel CL, Esteves Torres FF, Tanomaru-Filho M, Chavez-Andrade GM, Bosso-Martelo R, Guerreiro-Tanomaru JM. Evaluation of Physicochemical Properties of a New Calcium Silicate-based Sealer, Bio-C Sealer. J Endod. 2019;45(10):1248-52.

15. Alves Silva EC, Tanomaru-Filho M, da Silva GF, Delfino MM, Cerri PS, Guerreiro-Tanomaru JM. Biocompatibility and Bioactive Potential of New Calcium Silicate-based Endodontic Sealers: Bio-C Sealer and Sealer Plus BC. J Endod. 2020;46(10):1470-7.

16. Benetti F, de Azevedo Queiroz IO, Oliveira PHC, Conti LC, Azuma MM, Oliveira SHP, et al. Cytotoxicity and biocompatibility of a new bioceramic endodontic sealer containing calcium hydroxide. Brazilian Oral Research. 2019;33:e042.

17. Lopez-Garcia S, Lozano A, Garcia-Bernal D, Forner L, Llena C, Guerrero-Girones J, et al. Biological Effects of New Hydraulic Materials on Human Periodontal Ligament Stem Cells. J Clin Med. 2019;8(8).

18. Lopez-Garcia S, Pecci-Lloret MR, Guerrero-Girones J, Pecci-Lloret MP, Lozano A, Llena C, et al. Comparative Cytocompatibility and Mineralization Potential of Bio-C Sealer and TotalFill BC Sealer. Materials (Basel). 2019;12(19).

19. Carvalho-Junior JR, Correr-Sobrinho L, Correr AB, Sinhoreti MA, Consani S, Sousa-Neto MD. Solubility and dimensional change after setting of root canal sealers: a proposal for smaller dimensions of test samples. J Endod. 2007;33(9):1110-6.

20. Tanomaru-Filho M, Torres FFE, Chavez-Andrade GM, de Almeida M, Navarro LG, Steier L, et al. Physicochemical Properties and Volumetric Change of Silicone/Bioactive Glass and Calcium Silicate-based Endodontic Sealers. J Endod. 2017;43(12):2097-101.

21. ISO -. Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity. London, UK: British Standards Institution ISO 10993-52009.

22. Rodrigues C, Costa-Rodrigues J, Capelas JA, Fernandes MH. Behaviour of co-cultured human osteoclastic and osteoblastic cells exposed to endodontic sealers' extracts. Clin Oral Investig. 2014;18(2):479-88.

23. Soares MPR, Silva DP, Uehara IA, Ramos ES, Jr., Alabarse PVG, Fukada SY, et al. The use of apocynin inhibits osteoclastogenesis. Cell Biol Int. 2019;43(5):466-75.

24. Candeiro GT, Correia FC, Duarte MA, Ribeiro-Siqueira DC, Gavini G. Evaluation of radiopacity, pH, release of calcium ions, and flow of a bioceramic root canal sealer. J Endod. 2012;38(6):842-5.

25. Mendes AT, Silva PBD, So BB, Hashizume LN, Vivan RR, Rosa RAD, et al. Evaluation of Physicochemical Properties of New Calcium Silicate-Based Sealer. Braz Dent J. 2018;29(6):536-40.

26. Rodriguez-Lozano FJ, Lopez-Garcia S, Garcia-Bernal D, Tomas-Catala CJ, Santos JM, Llena C, et al. Chemical composition and bioactivity potential of the new Endosequence BC Sealer formulation HiFlow. Int Endod J. 2020;53(9):1216-28.

27. Zhou HM, Shen Y, Zheng W, Li L, Zheng YF, Haapasalo M. Physical properties of 5 root canal sealers. J Endod. 2013;39(10):1281-6.

28. Association ANSIAD. Laboratory testing methods: endodontic filling and sealing materials. specification n° 57. 2008. p. 10.

29. ISO. Dentistry — Root Canal Sealing Materials. London, UK: British Standards Institution ISO 68762012.

30. Benezra MK, Wismayer PS, Camilleri J. Author Correction: Influence of environment on testing of hydraulic sealers. Sci Rep. 2018;8(1):7209.

31. do Carmo SS, Nespoli FFP, Bachmann L, Miranda CES, Castro-Raucci LMS, Oliveira IR, et al. Influence of early mineral deposits of silicate- and aluminate-based cements on push-out bond strength to root dentine. Int Endod J. 2018;51(1):92-101.

32. Urban K, Neuhaus J, Donnermeyer D, Schafer E, Dammaschke T. Solubility and pH Value of 3 Different Root Canal Sealers: A Long-term Investigation. J Endod. 2018;44(11):1736-40.

33. Abdalla MM, Lung CYK, Neelakantan P, Matinlinna JP. A novel, doped calcium silicate bioceramic synthesized by sol-gel method: Investigation of setting time and biological properties. J Biomed Mater Res B Appl Biomater. 2020;108(1):56-66.

34. Rodrigues C, Costa-Rodrigues J, Capelas JA, Fernandes MH. Long-term dose- and time-dependent effects of endodontic sealers in human in vitro osteoclastogenesis. J Endod. 2013;39(6):833-8.

35. Lee JK, Kwak SW, Ha JH, Lee W, Kim HC. Physicochemical Properties of Epoxy Resin-Based and Bioceramic-Based Root Canal Sealers. Bioinorg Chem Appl. 2017;2017:2582849.

Figure captions

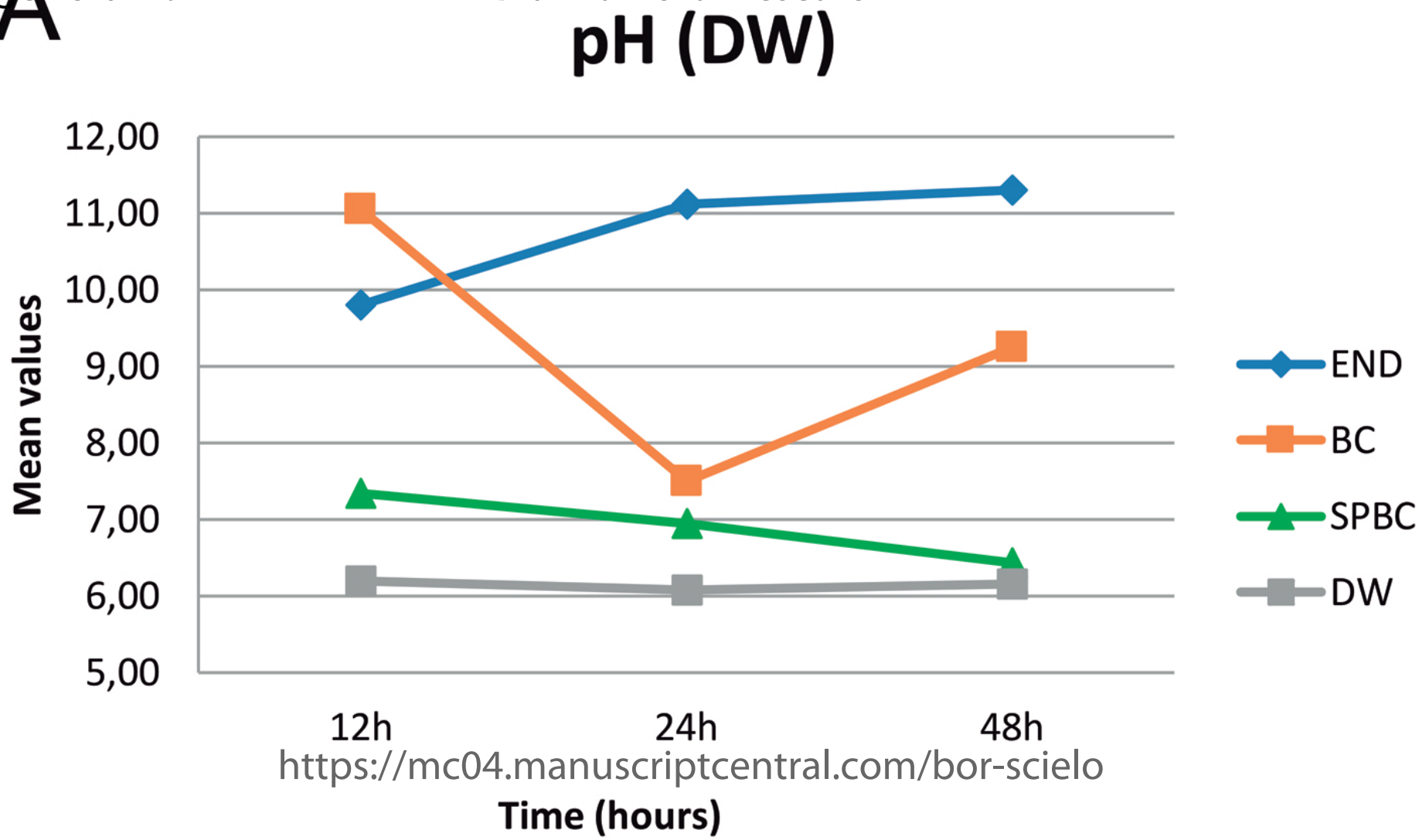
Figure 1. Graphic representation of mean pH curves for all calcium silicate-based sealers comparing with the control soak solutions in the different experimental times. (A) After immersion in DW; and (B) after immersion in α-MEM.

Figure 2. Concentration of ion release between the calcium silicate-based sealers for each soak solution in the evaluation times. (A) Calcium release after immersion in DW; (B) calcium release after immersion in α-MEM; (C) silicon release after immersion in DW; and (D) silicon release after immersion in α-MEM.

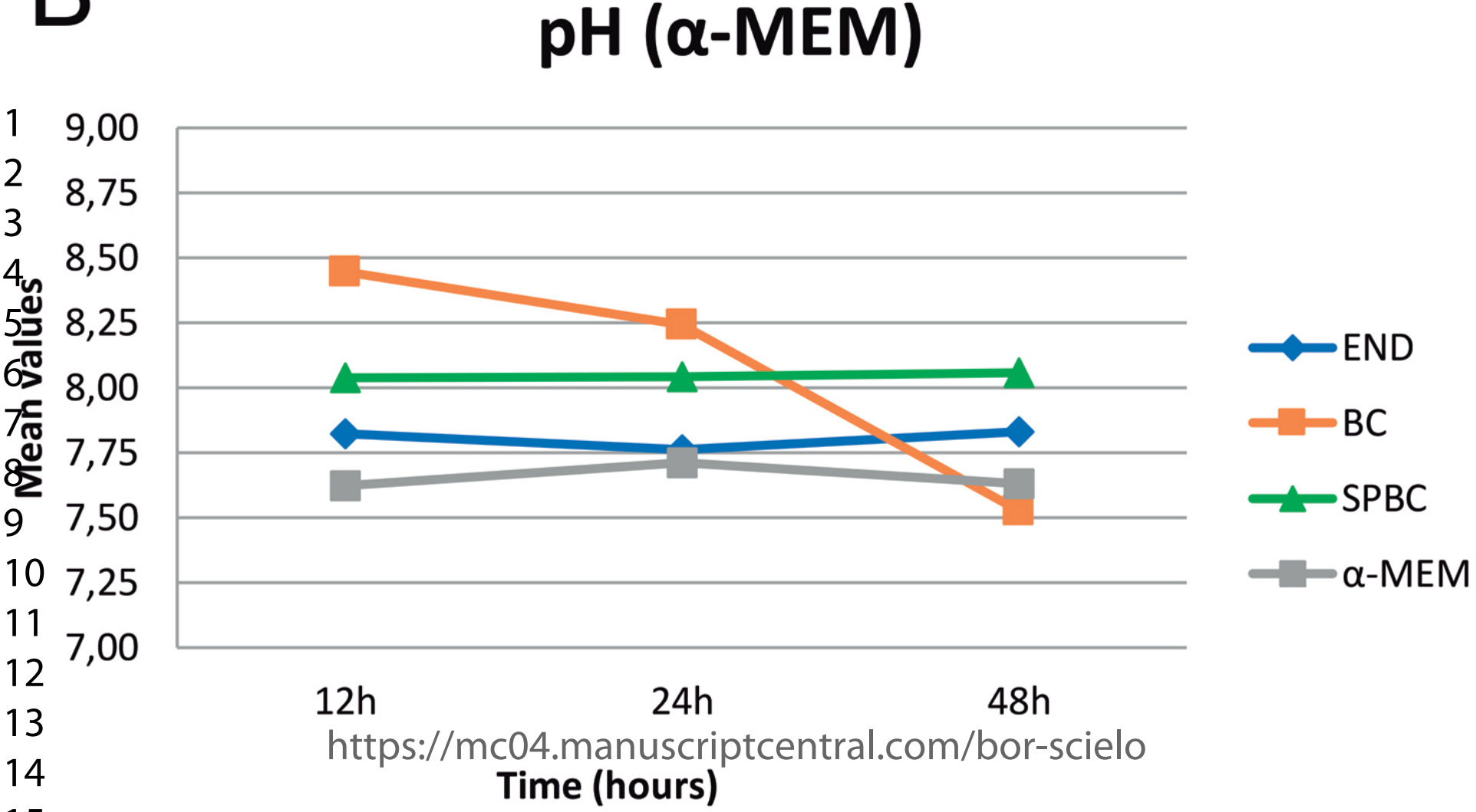
Figure 3. Viable cells (%) by an MTT assay of the sealer extracts in several dilutions at different experimental times. (A) END dilutions; (B) BC dilutions; and (C) SPBC dilutions. Time-dependent effects of (D) END; (E) BC; and (F) SPBC extracts. 100% cell viability was considered for the control group (α-MEM). Asterisk indicated the significant difference ($p < 0.05$).

Figure 4. TRAP stain. (A) Osteoclastic differentiation by TRAP-positive multinucleated cells compared with positive control (RANKL) and negative control group (α-MEM); (B) *$p < 0.05$ compared each sealer at dilutions; **$p < 0.05$ compared between the sealers; ***$p < 0.05$ compared each sealer with the positive control group.

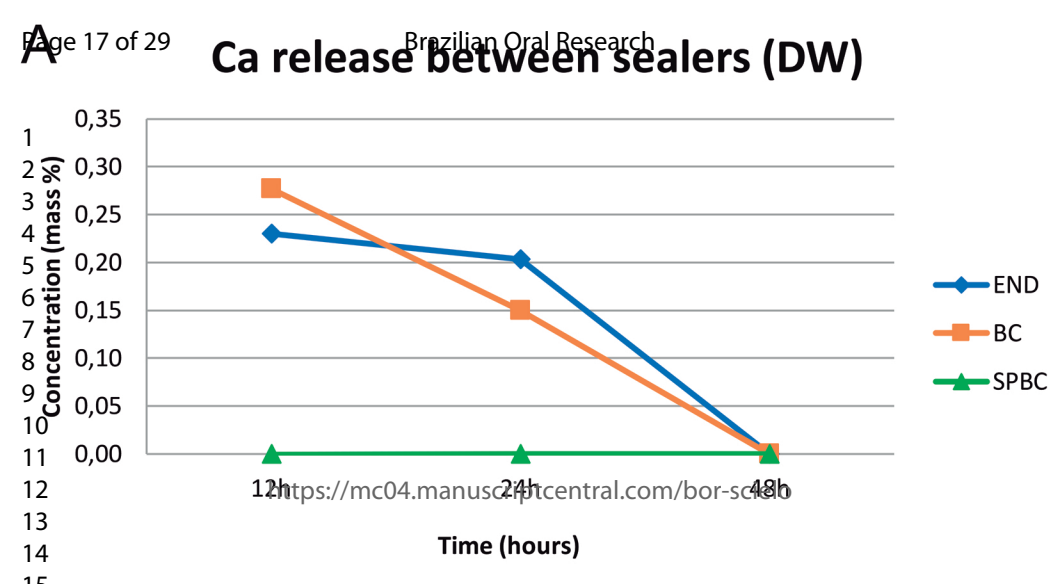
A



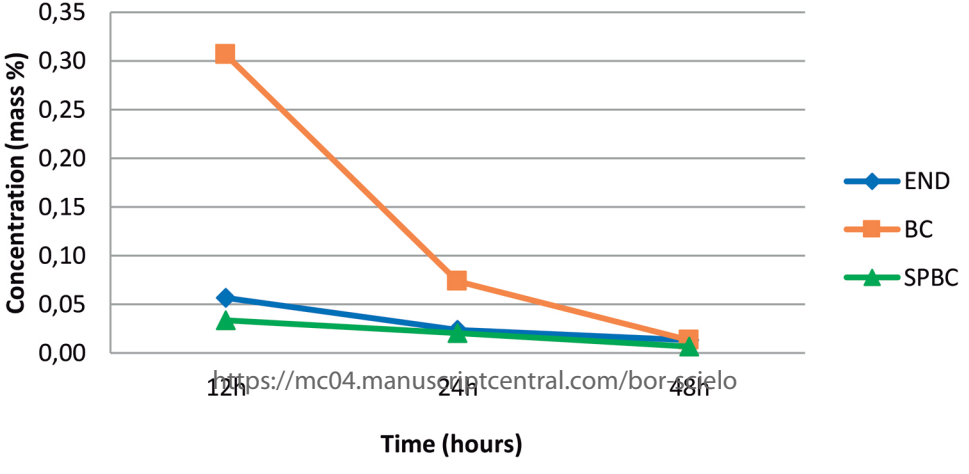
B



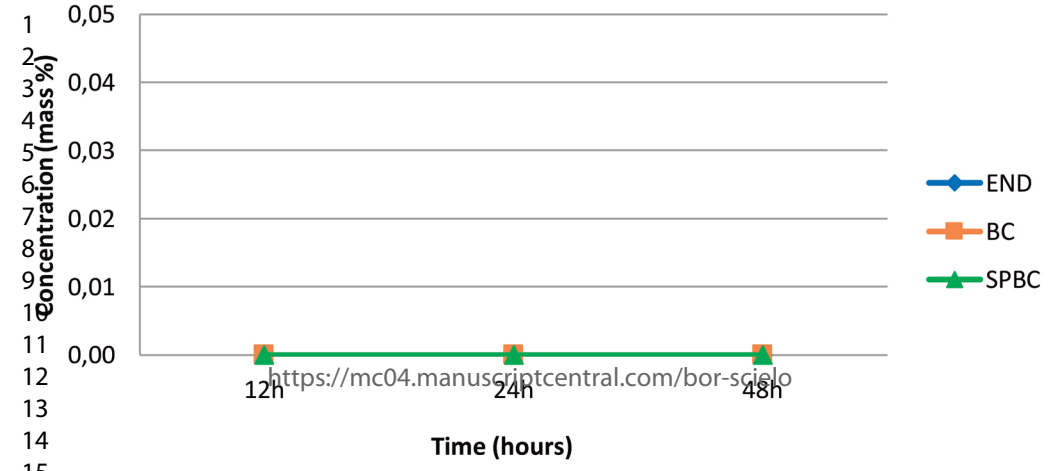
A



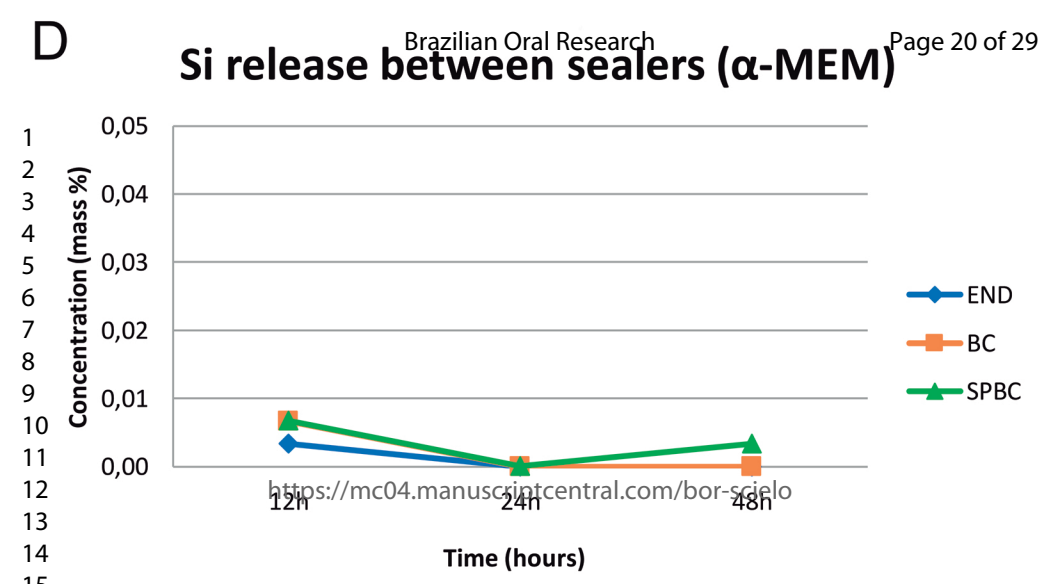
B



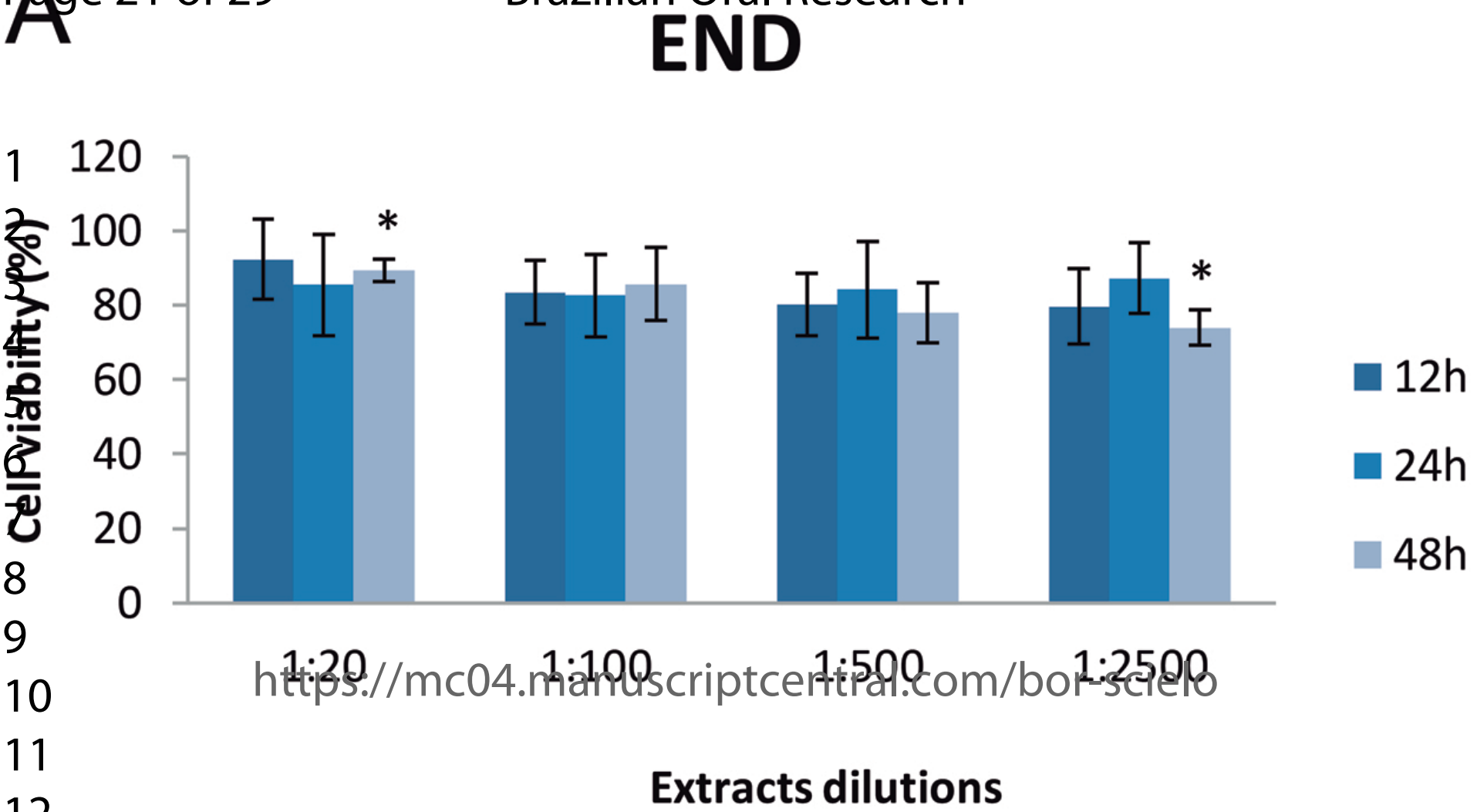
Si release between sealers (DW)



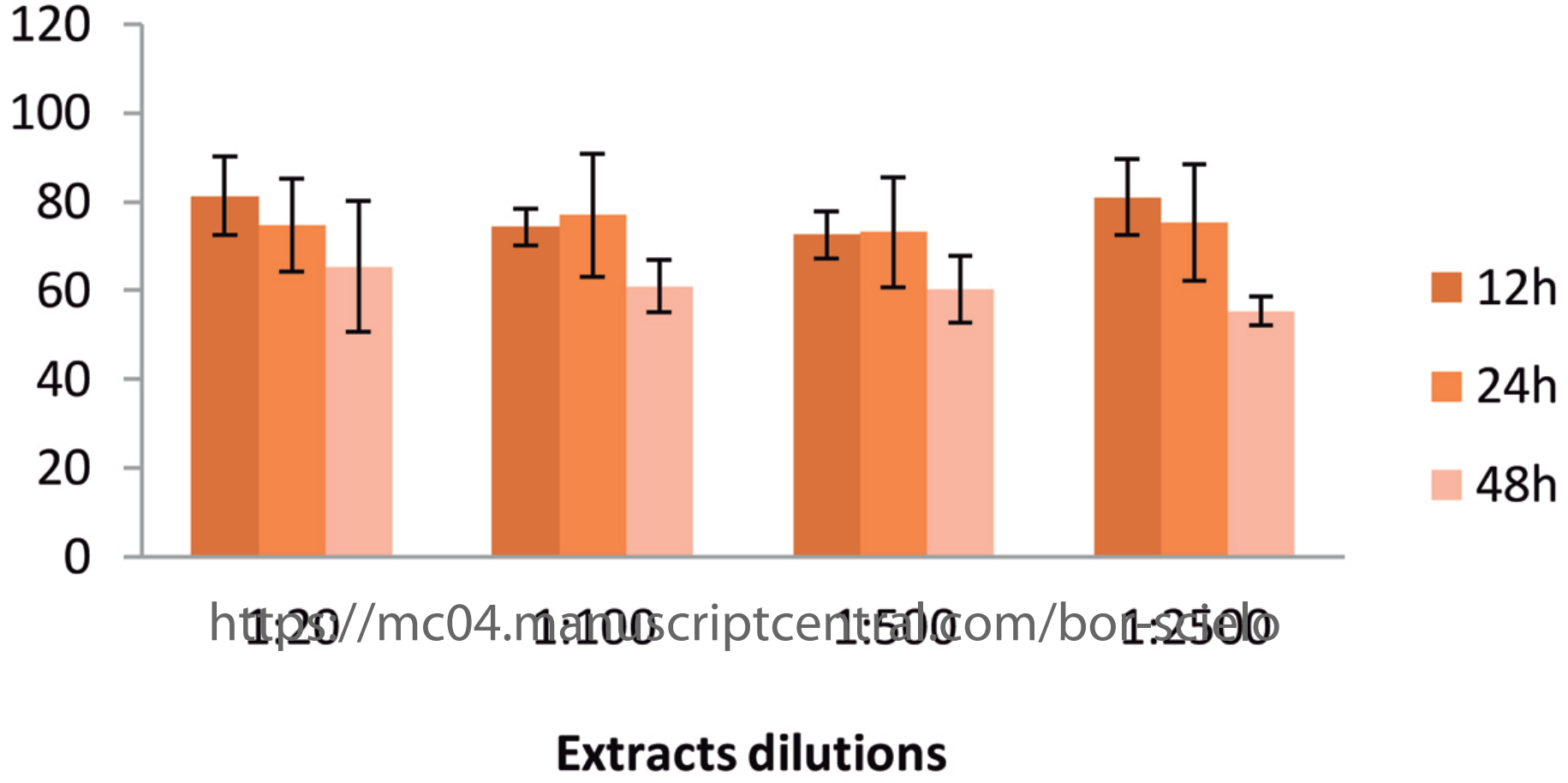
D

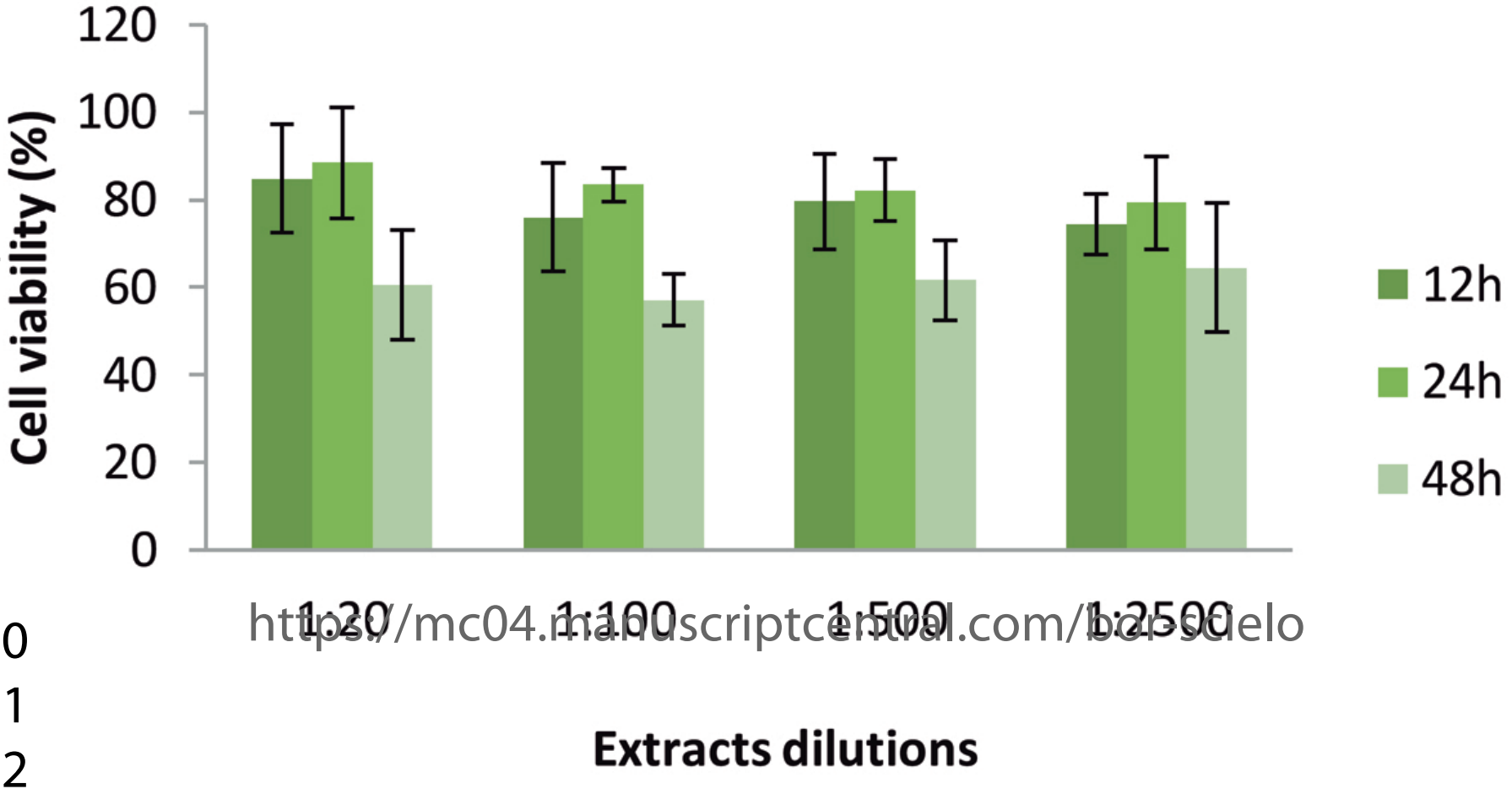


A



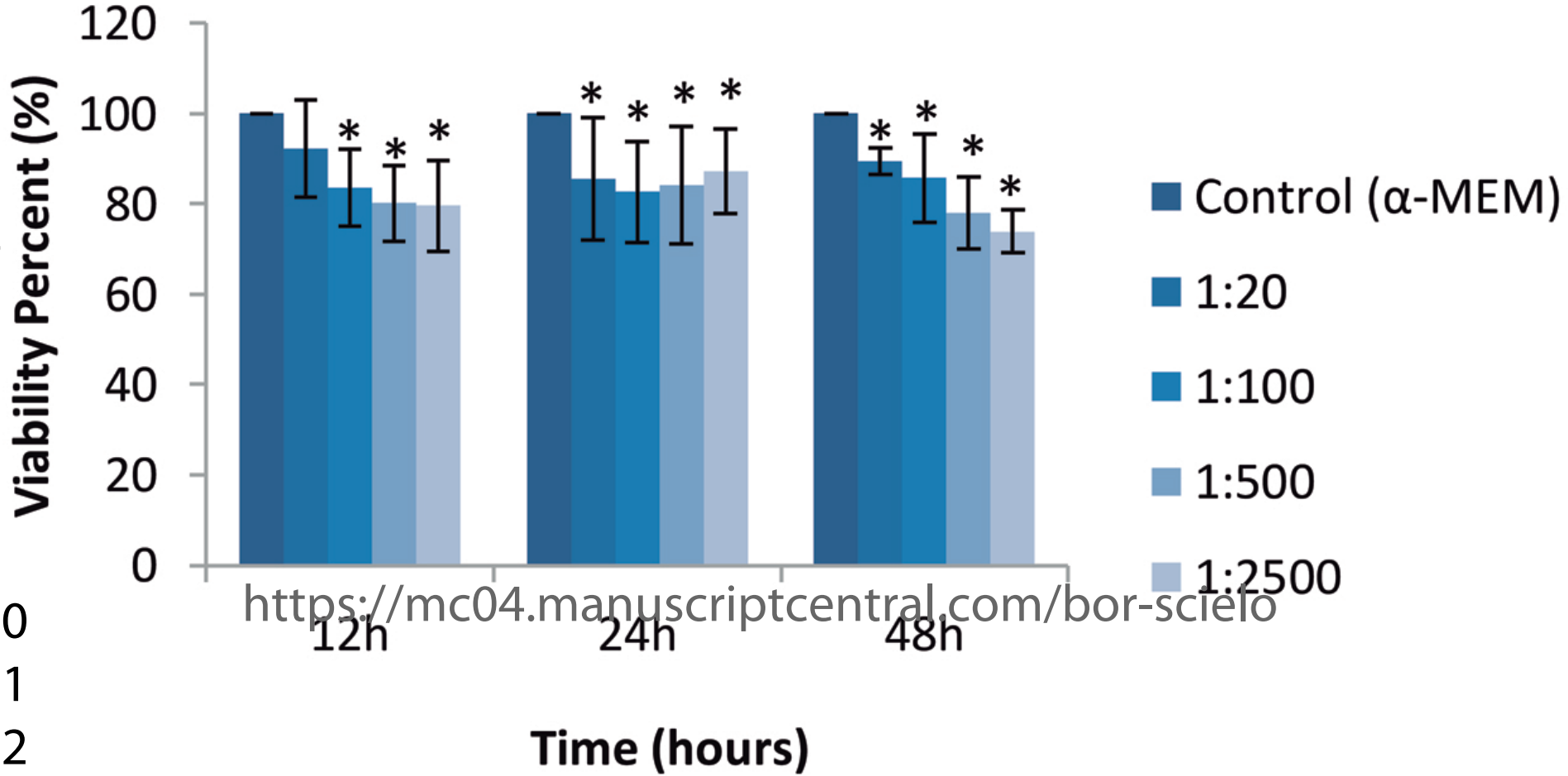
BC

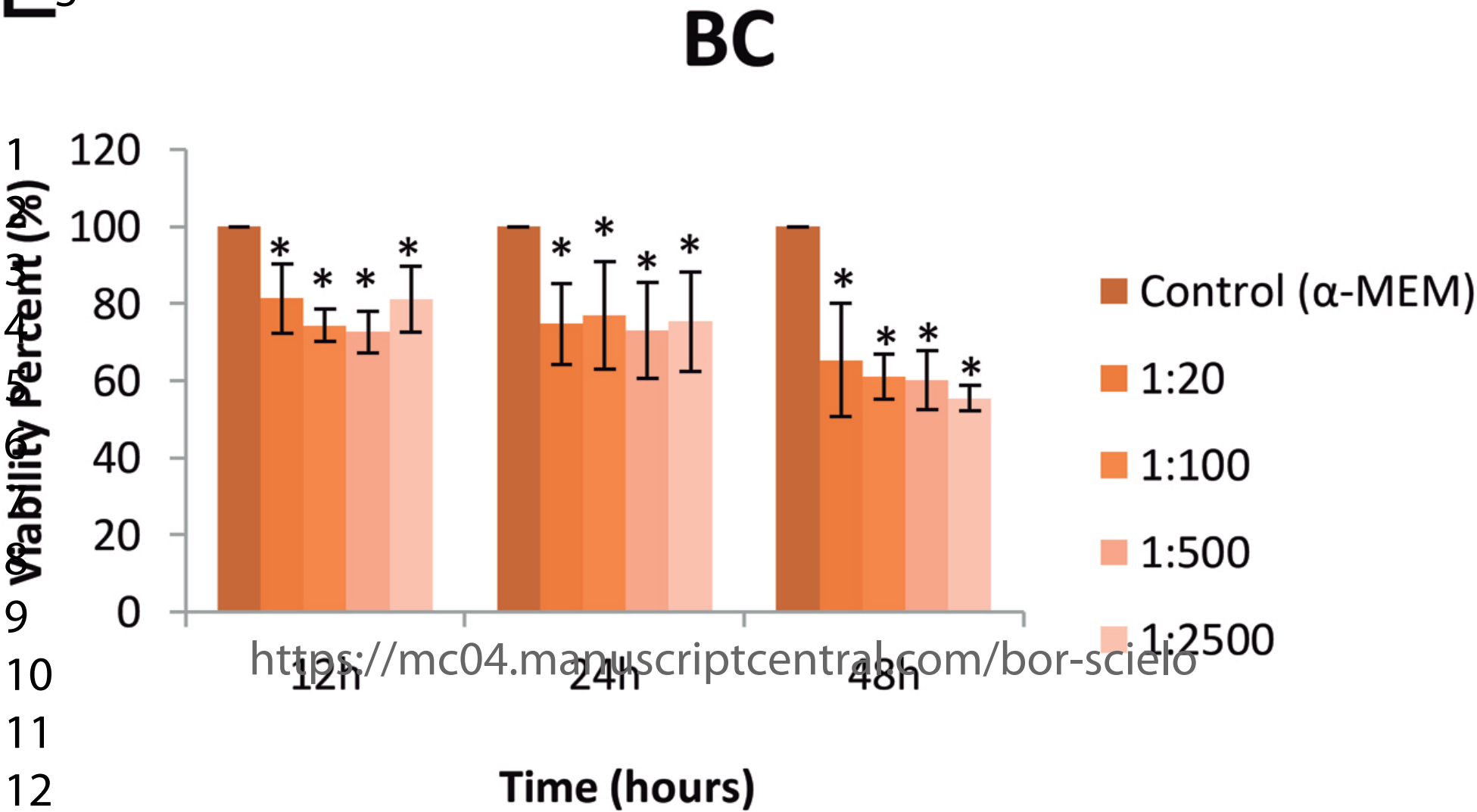


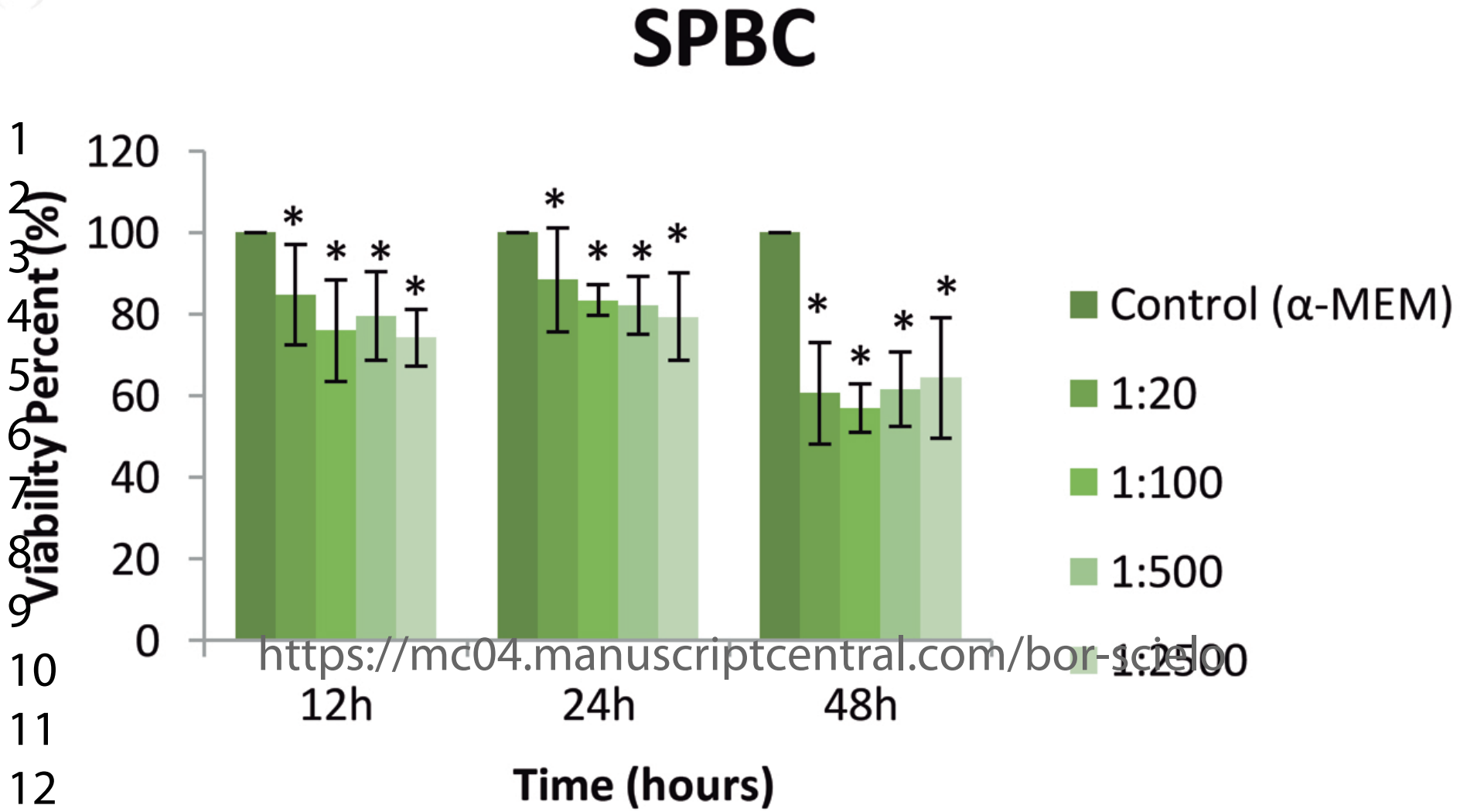


D

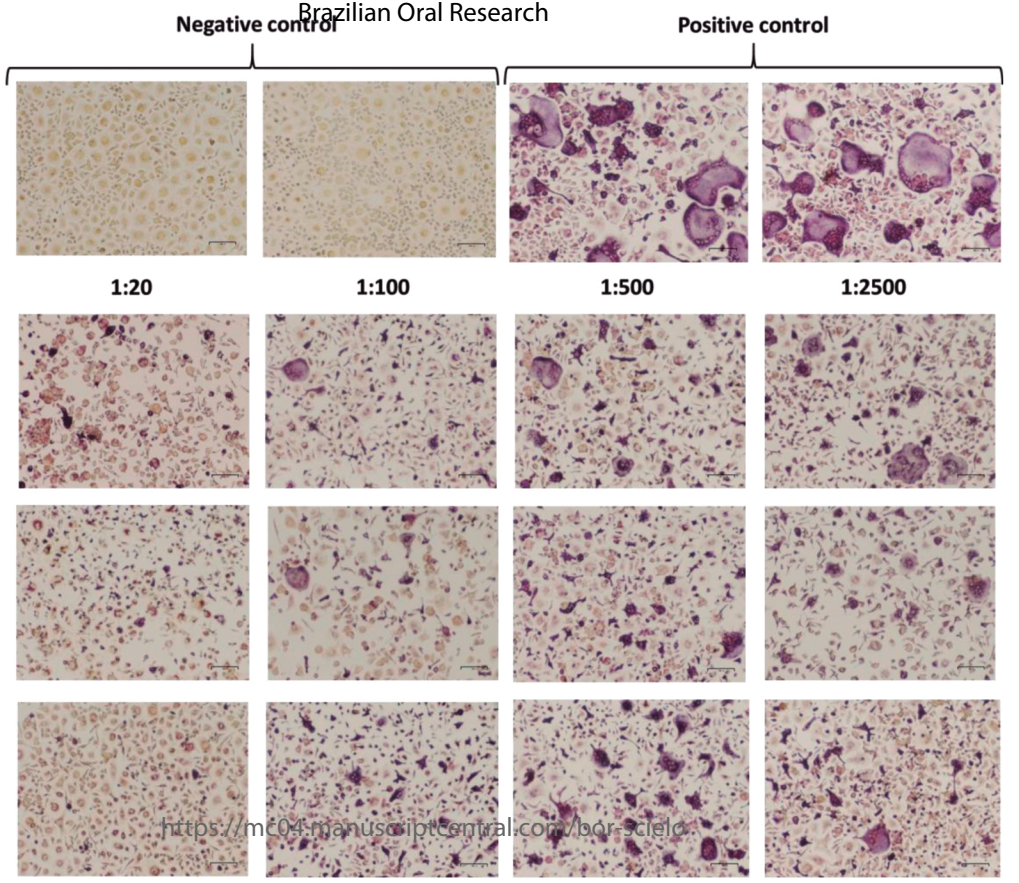
END



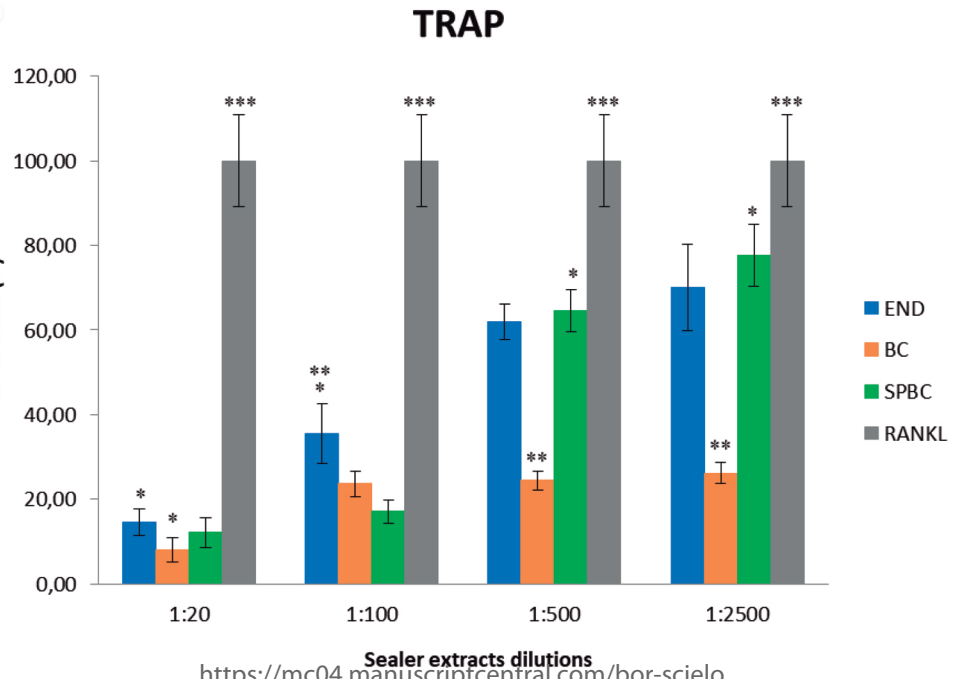




A



B


TRAP
120,00
100,00
80,00
60,00
40,00
20,00
0,00
Osteoclasts (%)
1:20
1:100
1:500
1:2500
Sealer extracts dilutions
END
BC
SPBC
RANKL

TABLES

Table 1. Mean solubility values and (standard deviation) revealed in calcium silicate-based sealers after storage in DW and α-MEM.

Solubility (% mass loss)	Calcium silicate-based sealers		
	END	BC	SPBC
DW	6.82 (0.41) Aa	6.67 (1.21) Aa	4.56 (1.88) Ab
α-MEM	3.84 (1.04) Bb	5.38 (0.87) Ba	3.66 (0.96) Bb

Different uppercase letters in the same column represent significant differences for the same sealer after immersion in different solutions, calculated by using unpaired T test ($p < 0.05$); Different lowercase letters on the same line represent significant differences between different sealers, calculated by using One-Way ANOVA and Tukey's test ($p < 0.05$).

CAPÍTULO 3

3.3 Capítulo 3

Artigo a ser submetido no periódico Restorative Dentistry & Endodontics

Evaluation of cytotoxicity, cell attachment and chemical characterization of three calcium silicate-based sealers

Camila Maria Peres de Rosatto[1], Gabriela Leite de Souza[1], Thamara Eduarda Alves Magalhães[1], Gabrielle Alves Nunes Freitas[1], Marcelo José Barbosa Silva[2], Camilla Christian Gomes Moura[1]

[1] Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil.

[2] Department of Immunology, Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, MG, Brazil.

Correspondence: Camilla Christian Gomes Moura, Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Av Pará 1720, Block 4L, annex A, class 24, Campus Umuarama, Uberlândia, MG, 38400-902, Brazil (Tel.: (+55) 34 3225-8103; e-mail: camillamoura@ufu.br).

Evaluation of cytotoxicity, cell attachment and chemical characterization of three calcium silicate-based sealers

ABSTRACT

Objectives To conduct an *in vitro* investigation to determine the chemical characterization of calcium silicate-based sealers (CSBS), their cytotoxicity, and their attachment to RAW264.7 cells.

Materials and Methods The tested CSBSs were: BioRoot RCS - BR (Septodont), Bio-C Sealer - BC (Angelus), and Sealer Plus BC – SPBC (MKLife). The cytotoxicity of sealer extracts at 1:1, 1:2 and 1:5 dilutions was evaluated using the MTT assay. Sealer discs (n=4) were used to determine chemical characterization by X-Ray Fluorescence Spectroscopy (XRF), Scanning Electron Microscopy (SEM), Energy-Dispersive X-Ray (EDX) and cell adhesion. The composition of the unset and set sealers was detected by Fourier-transform infrared spectroscopy (FTIR). Cell viability and XRF data were analyzed using one-way ANOVA, Tukey's and Dunnett's tests (p<0.05). Qualitative comparisons were performed for other analyses.

Results SPBC at 1:2 dilution presented the lowest cell viability compared with BR (p=0.0456) and BC (p=0.0347). CSBS produced a statistically significant reduction in cellular viability at 1:1 dilution, compared with the control group (p<0.0001). BC and SPBC exhibited similar spectra, in relation to unset and set samples, as detected by FTIR. EDX detected higher calcium content for BR and SPBC, and aluminum only in the premixed sealers. The surface morphology showed irregular precipitates for all sealers. XRF detected the highest calcium release for BR (p<0.05).

Conclusions BR has the greatest degree of purity, and the highest calcium content. BC and SPBC presented similar compositions, with heavy metals, such as aluminum. All the sealers maintained viability above 70%.

Keywords: Bioceramic sealer; Calcium silicate; Cytotoxicity; Physicochemical properties; Root canal filling materials

INTRODUCTION

The concept of foraminal patency [1] and apical enlargement [2] have been recommended in endodontic practice to prevent the packing of debris in the apical third, and to remove the bacteria present in this critical region of the root canal. These procedures can lead to unintentional sealer extrusion through the root apex during obturation [3]. Extrusion of sealer in periapical tissues should not be considered undamaging [3], bearing in mind that there have been reports of postoperative pain occurring in the initial periods [4]. This represents a concern mainly regarding endodontic sealers that have greater fluidity [5] and solubility [6].

Calcium silicate-based sealers (CSBS) in particular have shown a significantly greater amount of extrusion through the root apex than conventional sealers, because of their greater flowability [3,7]. The high solubility of this class of material has already been reported [6,8,9]. However, this aspect is still controversial, and some new endodontic CSBSs have demonstrated higher solubility than that recommended by ISO 6876/2012 [6,10,11].

A possible relationship has been established among the factors of high solubility of CSBS, its increased release of calcium ions, and its high pH values [8,11]. This combination could contribute to greater than 50% cytotoxicity, initially observed in these new sealers [12,13]. However, *in vivo* studies have detected new bone growth and apical healing for CSBS [5,14]. In fact, the biological responses of CSBS obtained in *in vitro* [12,13,15-17] and *in vivo* studies [5,14] may be related to variations of different CSBS compositions and proportions of compounds [8,18,19].

Fibroblasts, pulp cells and osteoblasts are often used *in vitro* evaluations of new endodontic sealers [12,13,15,16]. Macrophages are also an interesting experimental model, because they allow evaluation of the effect of the sealers on cells enrolled in the immunoinflammatory events in the periapical region [17,20]. Bio-C Sealer (BC) (Angelus, Londrina, PR, Brazil) and Sealer Plus BC (SPBC) (MK Life, Porto Alegre, RS, Brazil) are ready-to-use CSBSs recently introduced in the market, and have not yet been tested on macrophage cell culture models. Although BC and SPBC have been characterized by some physicochemical

tests [21,22], few publications have compared the composition of these sealers using complementary tests.

The present research aimed to evaluate the chemical composition and compare the biological properties of three CSBSs (BioRoot RCS, Bio-C Sealer and Sealer Plus BC) in RAW264.7 cells, in relation to cell cytotoxicity, attachment, ion release and surface morphology. The null hypotheses were that there is no difference among the tested sealers in regard to chemical properties and biological effects.

MATERIAL AND METHODS

Material preparation and characterization

BC and SPBC were packed directly into sterile molds. BioRoot RCS (BR) (Septodont, Saint-Maur-des-Fossés, PAR, France) was prepared following the manufacturer's directions, and inserted into syringes immediately before deposition into molds, in order to allow the same conditions for the insertion of all the sealers. The sealers were placed in silicone molds measuring 7.75 mm diameter x 1.5 mm thick, and kept in a humidified chamber at 37°C for 24 h [22] for the cell attachment assay and the chemical composition analysis by X-Ray Fluorescence Spectroscopy (XRF), scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX).

The sealer discs (n = 4) were removed from the molds and placed inside sterile plastic tubes containing 10 mL of Minimum Essential Medium Eagle (α-MEM) (LGC Biotecnologia, Cotia, SP, Brazil) and 1% penicillin-streptomycin (Sigma-Aldrich, Saint Louis, MO, USA) for 24 h. The medium for each sealer sample was carefully moved to a sample cup (model SC-4340, PremierLab Supply, Port St. Lucie, FL, USA) for XRF analysis (model S8 Tiger Series 2, Bruker, Kontich, ANR, Belgium), while the disc was processed for SEM-EDX analysis.

The XRF quantitative analysis of the components released by the sealers on α-MEM at 24 h was performed by Quant-Express, and processed using the Spectra Plus software program, which automatically determines the intensities of X-ray peaks for the elements. The SEM (EVO 10, Carl Zeiss, Oberkochen, BW, Germany) evaluation was performed by washing

hydrated discs (*n* = 4) in 0.05 M Na-cacodylate buffer, fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer, dehydrated in serial ethanol solutions of 50-100% followed by drying using hexamethyldisilazane. After drying, the samples were mounted on stubs and sputter-coated with 30 nm of gold (model Leica EM SCD050 table-top device, Wetzlar, HE, Germany). The samples were analyzed in three distinct regions under 700x, 2340x and 5000x magnifications at 5.0 kV and 20 µm, and under EDX (Oxford model 51-ADD0048, Abingdon, OXON, England), at a working distance of 8.5 mm, and at an acquisition time of 30 s for each point of analysis. The same protocol was used for the cell-plated discs.

Fourier-transform infrared (FTIR) (Vertex 70, Bruker Optik GmbH, Ettlingen, BW, Germany) was used to evaluate unset and set sealers using the OPUS (Bruker) software program. The spectra were obtained over a range of 4000–400 cm^{-1}, with a 4 cm^{-1} resolution, in 32 scans acquired using transmittance spectroscopy.

Cell assays

Cell cytotoxicity

Fresh CSBSs were inserted into 24-well plates under aseptic conditions in a laminar flow cabinet using 0.22 mL. All the materials were immediately covered with 2.5 mL of α-MEM containing 1% penicillin-streptomycin (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and with 10% α-MEM (LGC Biotecnologia), and then incubated in the dark for 24 h at 37°C. The original extracts (1:1) were prepared following ISO 10993-5/2009 recommendations [23]. The medium was collected, filtered at 0.22 µm and diluted at 1:1, 1:2 and 1:5 concentrations [24]. RAW264.7 were seeded in 96-well culture plates (1 x 10^4 cells/mL) in 10% α-MEM. After cell adherence, the effect of the sealer extracts (1:1, 1:2 and 1:5 dilutions) on cell viability in 24 h was assessed using the MTT technique, as previously described [25]. The control group contained cells that did not come into contact with any sealer extract. Each dilution was analyzed in triplicate in three distinct experiments, and the data obtained for each group were expressed as mean ± standard deviation.

Cell attachment

Direct testing was used to determine cell attachment, by seeding 2 x 10^4 RAW264.7 cells (passages 5–7; BCBRJ, Rio de Janeiro, RJ, Brazil) on top of the sealer discs, inside 48-well culture plates in 10% α-MEM for 24 h (n = 4). Afterwards, the samples were processed and analyzed, as described above for SEM. The cells seeded over the glass coverslips were considered as the control.

Statistical analysis

The data were analyzed for normal distribution using the Shapiro-Wilk test, and for homoscedasticity using the Levene test. One-way ANOVA complemented by Tukey's post-hoc test were applied to perform XRF analysis. Cell viability was analyzed initially by comparing different extract dilutions of the sealer. Next, the sealers were compared with one another in the same dilution, and later with the control, using one-way ANOVA, Tukey's and Dunnett's tests. This project was mostly qualitative in nature. The level of significance was set at $p < 0.05$ and all the analyses were performed using the GraphPad Prism software program v8.2.0 (San Diego, CA, USA).

RESULTS

XRF analysis

The results for the leaching of elements by hydrated sealers are shown in Table 1, and are expressed in ppm. BR leached higher amounts of calcium ions into the solution at 24 h and at 3200 ppm, and was significantly different from SPBC (p = 0.0201) and BC (p = 0.0403). On the other hand, all the sealers exhibited a low level of silicon leaching, which was similar (insignificant) among the groups (p = 0.5302). Zirconium was detected only in the BC samples, at 4 ppm (p = 0.4219).

SEM-EDX analysis

Representative SEM images of the hydrated sealer discs are shown in Figure 1. EDX analysis of the components are shown in Figure 2. The surface morphology of all the sealers showed crystallite deposits and crystal precipitates, each belonging to a different type of crystalline. BR exhibited a globular and cubical crystallite appearance, with a mostly grainy microstructure (Figure 1A), and more numerous deposits of crystals than BC and SPBC. BC showed the most regular surface (Figure 1B). In general, the surface particles of SPBC showed varied morphologies, mostly very coarse and elongated (Figure 1C).

The EDX analysis showed carbon, calcium, zirconium, silicon, and oxygen peaks for all CSBSs (Figure 2A, 2B and 2C). Regarding the chemical composition of each sealer, BR and SPBC showed the greatest presence of calcium (Ca) content. BC presented a high percentage of zirconium (Zr), and all the sealers showed low silicon (Si), but especially SPBC. The main difference in the composition was the presence of aluminum (Al) in BC and SPBC, titanium (Ti) in SPBC, and chlorine (Cl) in BR.

FTIR analysis

The chemical composition analyzed using the FTIR spectra of the unset and set CSBSs is displayed in Figure 3. In general, a qualitative comparison of FTIR spectroscopy revealed slight differences for each sealer after the setting reaction, in relation to spectroscopic plots and intensities. Intrinsic comparisons of unset and set sealers demonstrated more relevant differences in BR (Figure 3A and 3B), whereas BC (Figure 3C and 3D) and SPBC (Figure 3E and 3F) exhibited similar spectra. BR had more expressive peaks than the other sealers for the unset sample; in contrast, it had low peak intensities for the set sample.

The unset sealers (Figure 3A, 3C, 3E) showed dicalcium silicate between 494-912 cm^{-1} bands and tricalcium silicate between 573-1112 cm^{-1} bands [26]. Spectra values higher than 1000 cm^{-1} (1101 cm^{-1}, 1246 cm^{-1}, 1348 cm^{-1}, 1456 cm^{-1}, 1647 cm^{-1}, and 2868 cm^{-1}) indicated the dispersing agent component of the sealer as polyethylene glycol [27].

After setting (Figure 3B, 3D, 3F), all the samples indicated absorption intensities of carbonates at 878 and at ~1400 cm^{-1} [28-30]. The formation of calcium silicate hydrate (CSH) was observed in all the sealers as characteristic bands from ~900 to ~1200 cm^{-1} and at 1350 cm^{-1}, indicating a Si-O combination [28,30]. All the specimens showed a small absorption band at ~3640 cm^{-1}, indicating the formation of calcium hydroxide [26,31,32], with a more evident peak for SPBC. BR and SPBC exhibited similar peaks at 560 and 600 cm-1, indicating phosphate ions [30] for unset and set samples.

Cell morphology and adhesion

The qualitative analysis of RAW264.7 morphology, and the adhesion onto the surfaces of the different sealers showed only a few, mainly round cells, and some debris, indicating cell death. In the control groups, the cells adhered to the coverslip surface, and spread over a wider area than the sealer samples (Figure 4).

Cell viability

The effects of CSBS extract dilution on cell viability are shown in Figure 5. Intragroup analysis showed statistical differences between the 1:1 and 1:2 dilutions ($p < 0.0001$ and $p = 0.0001$, respectively), and the 1:1 and 1:5 ($p < 0.0001$) dilutions for the BR and BC groups, with a lower percentage of viable cells at 1:1 dilution. The SPBC was significantly different, as seen by comparing all the extract dilutions ($p < 0.0001$), considering that the higher percentage of viable cells occurred at the 1:5 dilution (Figure 5A). Intergroup analysis found no statistical differences among the sealers at 1:1 and 1:5 dilutions ($p = 0.2886$ and $p = 0.2272$, respectively). At 1:2 dilution, SPBC presented the lowest cell viability compared to BR ($p = 0.0456$) and BC ($p = 0.0347$) (Figure 5B).

The CSBS produced a statistically significant reduction in cellular viability at 1:1 dilution, compared with the control group ($p < 0.0001$). On the other hand, BR and SPBC exhibited higher cell viability at 1:5 dilution, compared with the control condition ($p = 0.0053$ and $p = 0.0068$, respectively) (Figure 5B).

DISCUSSION

The null hypothesis was rejected, since the CSBS showed differences in several of the aspects analyzed. Some *in vitro* studies have been published regarding BR [33-36] and BC [14,19,32]. More recent studies have evaluated the physicochemical [6,11] and biological properties [14,37] of SPBC. However, this was the first *in vitro* study to date that compared the chemical composition of the new ready-to-use CSBS, BC and SPBC, using complementary methodologies such as XRF, FTIR and SEM-EDX.

FTIR and SEM-EDX have already been used to reveal the formation of calcium phosphate and hydroxyapatite deposits on the CSBS after soaking it in different solutions [38-41]. When CSBS comes into contact with humidity, it incorporates the water needed to bring about the setting reaction, and produces CSH [32,42,43] in the form of a gel. Subsequently, the sealer reacts with the CSH gel, thus initiating the setting procedure, and producing calcium hydroxide [42,44-46]. In the present study, small bands (~3640 cm^{-1}) of set sealer indicated the formation of calcium hydroxide, as corroborated by Mollah *et al.* [26]. However, in the present study, the samples were not immersed in water or any other solution for FTIR analysis; they were merely hydrated in a humidified chamber at 37°C for 24 h during the setting reaction. The moisture in this class of endodontic sealer has also been responsible for creating a suitable surface for calcium phosphate and apatite layer formation [47]. Both chemical compounds are precursors of hydroxyapatite [Ca10(PO4)6(OH)2], which may induce hard tissue deposition [12,48]. In previous studies, BR and SPBC showed phosphate ions at 560 and 600 cm-1 [30], in agreement with the current results. Other FTIR analyses for BR [49] and BC [32] revealed similar results.

The difference in the results observed for BR samples, compared with the other sealers, may be attributed to BR being the only two-component sealer evaluated; as such, it must be mixed manually, and any change in its powder-liquid ratio can influence its properties [49,50]. In addition, the premixed sealers used in this study presented organic structures in their compositions, which made them act as thickening agents or fillers, a structure not found in BR [49]. The unset BR sample showed the highest peak intensities, probably due to the calcium

release that was detected in the material by XRF, and that was statistically different from the other sealers.

XRF analysis was carried out to monitor the CSBS ion release. In a clinical situation, tissue fluids in the apical region can penetrate the root canal and degrade the sealer, leading to the leaching of ions by the sealers [51], as demonstrated by BR. Although the biomineralization potential of these sealers in contact with apical tissues was not evaluated in the present study, all sealers were found to release calcium ions. The highest calcium content found in BR, in relation to SPBC and BC, is supported by Kharouf *et al.* [36], who reported that the percentage of calcium was higher for BR than the other premixed sealers tested in their study. XRF revealed the presence of calcium and silicon ions such as EDX, and is distinctly able to detect these components in liquid samples. XRF has the advantage of being able to accurately detect very small amounts of the elements present, in ppm [34]. The three sealers analyzed showed a similar chemical composition in relation to silicon, at low concentrations, as previously described in a literature review [47].

EDX analysis confirmed the presence of carbon, oxygen, silicon, calcium and zirconium [19,35]. This methodology gave different weight percentages of elements for each sealer. The differences among the various CSBSs were not significant for the elements composing the sealers, except for zirconium, which was higher in BC. It is known that zirconium oxide in the new CSBS is a radiopacifying agent, and has acceptable physicochemical properties [52]. Aluminum was detected in both BC and SPBC. The presence of aluminum in the BC composition is in agreement with the findings of a recent study [19]. Chlorine is part of the liquid composition of the BR sealer [36,49], and was not found in the other sealers.

Regarding the CSBS surface morphology, the interaction between the sealer and the fluids needed to trigger the setting reaction may induce the formation of bioactive mineral crystals [53,54]. Although this stage is difficult to explore under SEM [47], BR apparently presented more crystallites on the surface than BC and SPBC. BR previously demonstrated more numerous crystals than another premixed CSBS [36]. In addition, a phosphate band detected for BR using FTIR may be associated with the precipitation of a globular amorphous calcium phosphate over the surface of the set sealer [55,56]. On the other hand, BC showed the

most regular surface, which is probably related to its more homogenous characteristics, seeing that it comes in a package with a ready-to-use premixed vial [32]. This regular surface differs from the irregular crystalline structures noticed in this same sealer in a previous study [19]. However, it must be borne in mind that these studies used different solutions to trigger the setting reaction of the CSBS, and these solutions could have modified the results.

The cell parameters evaluated have already been tested for the same sealers in previous studies, using other cell models [13-15,19,43]. However, it is known that the host response pattern for different biomaterials varies according to the cell type studied [17]. Considering that macrophages are essential for the inflammatory response and repair established in the periapical area, their evaluation in *in vitro* models for new materials is relevant [20]. In the present study, we opted to use a lineage of macrophages, because of the difficulty of working with human primary cultures in today's SARS-COV-2 pandemic scenario. In addition, RAW264.7 cells are a well-known and widely used immortalized cell line for studies in endodontics [57,58]. Regarding the cell viability, the results showed a lower percentage of viable cells for SPBC; however, a comparison with a previously published study cannot be drawn, since fibroblasts were used in that study, and the tested dilutions were much greater [37].

RAW264.7 cells were seeded onto the surfaces of the three sealers to observe the effect of the direct contact of these sealers on cell adhesion and morphology. The parameters were described in previous reports [16,59], and indicate adequate attachment of cells to CSBS. However, in the present study, very few cells with a rounded aspect were found adhered to the surfaces of the materials, unlike the studies referred to. Although we do not know the exact mechanisms that caused the detachment of cells, we can infer that the high number of sprayed cells in the cover slips (control) may indicate that dissolution of the superficial layer of the sealer in the culture medium may be responsible for this detachment.

It is important to address the limitations of this study, which were the use of BR, a hand-mixed sealer, and the RAW264.7 cell lineage, instead of human primary cells, which are more relevant for biocompatibility studies [13]. On the other hand, BR has been on the market for

more than five years, is widely distributed throughout the world, and has been researched in a large number of publications [33-36].

CONCLUSIONS

Among the different CSBSs evaluated in this study, BR features the greatest degree of purity, and the highest calcium content. Both premixed sealers, BC and SPBC, presented a similar composition, and contained heavy metals, such as aluminum. All sealers maintained a viability above 70%.

ACKNOWLEDGMENTS

This project was funded by CAPES – Funding Code 001, FAPEMIG, and CNPq. The study was developed at CPBio - Biomechanics, Biomaterials and Cell Biology Research Center of the Federal University of Uberlândia (UFU; Uberlândia, Minas Gerais, Brazil). The authors are grateful to the School of Chemical Engineering at UFU for letting them use the XRF and SEM-EDX equipment, and for the technical support kindly lent by Rafael Ramos Heilbuth.

Author contributions

Conceptualization: Moura CCG, Silva MJB, Rosatto CMP; *Data curation:* Souza GL, Magalhães TEA, Freitas GAN; *Formal analysis:* Rosatto CMP; *Funding acquisition:* Silva MJB, Moura CCG; *Investigation:* Rosatto CMP, Souza GL, Magalhães TEA, Freitas GAN; *Methodology:* Rosatto CMP, Souza GL, Magalhães TEA, Freitas GAN; *Project administration:* Moura CCG; *Resources:* Silva MJB, Moura CCG; *Software:* Silva MJB, Moura CCG; *Supervision:* Moura CCG; *Validation:* Silva MJB, Moura CCG; *Visualization:* Rosatto CMP, Souza GL, Moura CCG; *Writing - original draft:* Rosatto CMP, Souza GL, Moura CCG; *Writing - review & editing:* Silva MJB, Moura CCG.

Authors' ORCID IDS

Camila Maria Peres de Rosatto (https://orcid.org/0000-0003-0207-8402)

Gabriela Leite de Souza (https://orcid.org/0000-0003-3505-5258)

Thamara Eduarda Alves Magalhães (https://orcid.org/0000-0001-5992-5073)

Gabrielle Alves Nunes Freitas (https://orcid.org/0000-0001-7865-2795)

Marcelo José Barbosa Silva (https://orcid.org/0000-0002-5807-4286)

Camilla Christian Gomes Moura (https://orcid.org/0000-0002-5653-8403)

Conflict of Interest

The authors declare no conflicts of interest, no financial affiliations and no involvement with any commercial organization that may have a direct financial interest in the subject or materials discussed in this manuscript.

REFERENCES

1. Mohammadi Z, Jafarzadeh H, Shalavi S, Kinoshita JI. Establishing Apical Patency: To be or not to be? J Contemp Dent Pract. 2017;18:326-329.

2. Rodrigues RCV, Zandi H, Kristoffersen AK, Enersen M, Mdala I, Ørstavik D, Rôças IN, Siqueira JF Jr. Influence of the Apical Preparation Size and the Irrigant Type on Bacterial Reduction in Root Canal-treated Teeth with Apical Periodontitis. J Endod. 2017;43:1058-1063.

3. Fonseca B, Coelho MS, Bueno CEDS, Fontana CE, Martin AS, Rocha DGP. Assessment of Extrusion and Postoperative Pain of a Bioceramic and Resin-Based Root Canal Sealer. Eur J Dent. 2019;13:343-348.

4. Yu YH, Kushnir L, Kohli M, Karabucak B. Comparing the incidence of postoperative pain after root canal filling with warm vertical obturation with resin-based sealer and sealer-based obturation with calcium silicate-based sealer: a prospective clinical trial. Clin Oral Investig. 2021 Feb 8.

5. Lim ES, Park YB, Kwon YS, Shon WJ, Lee KW, Min KS. Physical properties and biocompatibility of an injectable calcium-silicate-based root canal sealer: in vitro and in vivo study. BMC Oral Health. 2015;15:129.

6. Silva EJNL, Cardoso ML, Rodrigues JP, De-Deus G, Fidalgo TKDS. Solubility of bioceramic- and epoxy resin-based root canal sealers: A systematic review and meta-analysis. Aust Endod J. 2021 Feb 1.

7. Candeiro GT, Correia FC, Duarte MA, Ribeiro-Siqueira DC, Gavini G. Evaluation of radiopacity, pH, release of calcium ions, and flow of a bioceramic root canal sealer. J Endod. 2012;38:842-5.

8. Silva Almeida LH, Moraes RR, Morgental RD, Pappen FG. Are Premixed Calcium Silicate-based Endodontic Sealers Comparable to Conventional Materials? A Systematic Review of In Vitro Studies. J Endod. 2017;43:527-535.

9. Tanomaru-Filho M, Torres FFE, Chávez-Andrade GM, de Almeida M, Navarro LG, Steier L, Guerreiro-Tanomaru JM. Physicochemical Properties and Volumetric Change of Silicone/Bioactive Glass and Calcium Silicate-based Endodontic Sealers. J Endod. 2017;43:2097-2101.

10. ISO-Standards ISO 6876 Dental Root Canal Sealing Materials. 3rd ed. Geneva: International Organization for Standardization; 2012. p1-9.

11. Mendes AT, Silva PBD, Só BB, Hashizume LN, Vivan RR, Rosa RAD, Duarte MAH, Só MVR. Evaluation of Physicochemical Properties of New Calcium Silicate-Based Sealer. Braz Dent J. 2018;29:536-540.

12. Candeiro GTM, Moura-Netto C, D'Almeida-Couto RS, Azambuja-Júnior N, Marques MM, Cai S, Gavini G. Cytotoxicity, genotoxicity and antibacterial effectiveness of a bioceramic endodontic sealer. Int Endod J. 2016;49:858-864.

13. Jung S, Sielker S, Hanisch MR, Libricht V, Schäfer E, Dammaschke T. Cytotoxic effects of four different root canal sealers on human osteoblasts. PLoS One. 2018;13:e0194467.

14. Alves Silva EC, Tanomaru-Filho M, da Silva GF, Delfino MM, Cerri PS, Guerreiro-Tanomaru JM. Biocompatibility and Bioactive Potential of New Calcium Silicate-based Endodontic Sealers: Bio-C Sealer and Sealer Plus BC. J Endod. 2020;46:1470-1477.

15. Camps J, Jeanneau C, El Ayachi I, Laurent P, About I. Bioactivity of a Calcium Silicate-based Endodontic Cement (BioRoot RCS): Interactions with Human Periodontal Ligament Cells In Vitro. J Endod. 2015;41:1469-73.

16. Rodríguez-Lozano FJ, García-Bernal D, Oñate-Sánchez RE, Ortolani-Seltenerich PS, Forner L, Moraleda JM. Evaluation of cytocompatibility of calcium silicate-based endodontic sealers and their effects on the biological responses of mesenchymal dental stem cells. Int Endod J 2017;50:67–76.

17. Souza GL, Rosatto CMP, Silva MJB, Silva MV, Rocha Rodrigues DB, Moura CCG. Evaluation of apoptosis/necrosis and cytokine release provoked by three root canal sealers in human polymorphonuclears and monocytes. Int Endod J. 2019;52:629-638.

18. Donnermeyer D, Bürklein S, Dammaschke T, Schäfer E. Endodontic sealers based on calcium silicates: a systematic review. Odontology. 2019;107:421-436.

19. López-García S, Pecci-Lloret MR, Guerrero-Gironés J, Pecci-Lloret MP, Lozano A, Llena C, Rodríguez-Lozano FJ, Forner L. Comparative Cytocompatibility and Mineralization Potential of Bio-C Sealer and TotalFill BC Sealer. Materials (Basel). 2019;12:3087.

20. Braga JM, Oliveira RR, Martins RC, Ribeiro Sobrinho AP. The effects of a mineral trioxide aggregate-based sealer on the production of reactive oxygen species, nitrogen species and cytokines by two macrophage subtypes. Int Endod J. 2014;47:909-19.

21. Zordan-Bronzel CL, Esteves Torres FF, Tanomaru-Filho M, Chávez-Andrade GM, Bosso-Martelo R, Guerreiro-Tanomaru JM. Evaluation of Physicochemical Properties of a New Calcium Silicate-based Sealer, Bio-C Sealer. J Endod. 2019;45:1248-1252.

22. Torres FFE, Zordan-Bronzel CL, Guerreiro-Tanomaru JM, Chávez-Andrade GM, Pinto JC, Tanomaru-Filho M. Effect of immersion in distilled water or phosphate-buffered saline on the solubility, volumetric change and presence of voids within new calcium silicate-based root canal sealers. Int Endod J. 2020;53:385-391.

23. ISO-Standards ISO 10993-5 Biological Evaluation of Medical Devices-Part 5: Tests for *In Vitro* cytotoxicity. 3rd ed. Geneve: International Organization for Standardization; 2009. p1-34.

24. Zhang J, Zhu L, Yan P, Peng B. Effect of BioAggregate on Receptor Activator of Nuclear Factor-Kappa B Ligand-induced Osteoclastogenesis from Murine Macrophage Cell Line In Vitro. J Endod. 2015;41:1265-71.

25. de Souza GL, Moura CCG, Silva ACA, Marinho JZ, Silva TR, Dantas NO, Bonvicini JFS, Turrioni AP. Effects of zinc oxide and calcium-doped zinc oxide nanocrystals on cytotoxicity and reactive oxygen species production in different cell culture models. Restor Dent Endod. 2020;45:e54.

26. Mollah M, Yu W, Schennach R, Cocke DL. A Fourier transform infrared spectroscopic investigation of the early hydration of Portland cement and the influence of sodium lignosulfonate. Cem. Concr. Res. 2000,30:267–273.

27. Pavia DL, Lampman GM, Kriz GS, Vyvyan JA. Introduction to Spectroscopy. 5th ed. Washington: Cengage Learning; 2014. p784.

28. Ylmén R, Jäglid U, Steenari B, Panas I. Early hydration and setting of Portland cement monitored by IR, SEM and Vicat techniques. Cem. Concr. Res. 2009,39:433–439.

29. Wang P, Li C, Gong H, Jiang X, Wang H, Li K. Effects of synthesis conditions on the morphology of hydroxyapatite nanoparticles produced by wet chemical process. Powder Technol. 2010,203:315–321.

30. Chen B, Haapasalo M, Mobuchon C, Li X, Ma J, Shen Y. Cytotoxicity and the Effect of Temperature on Physical Properties and Chemical Composition of a New Calcium Silicate-based Root Canal Sealer. J Endod. 2020;46:531-538.

31. Yu P, Kirkpatrick RJ, Poe B, McMillan PF, Cong X. Structure of calcium silicate hydrate (C–S–H): Near–, Mid–, and Far–infrared spectroscopy. J. Am. Ceram. Soc. 1999,82:742–748.

32. Okamura T, Chen L, Tsumano N, Ikeda C, Komasa S, Tominaga K, Hashimoto Y. Biocompatibility of a High-Plasticity, Calcium Silicate-Based, Ready-to-Use Material. Materials (Basel). 2020;13:4770.

33. Viapiana R, Moinzadeh AT, Camilleri L, Wesselink PR, Tanomaru Filho M, Camilleri J. Porosity and sealing ability of root fillings with gutta-percha and BioRoot RCS or AH Plus sealers. Evaluation by three ex vivo methods. Int Endod J. 2016;49:774-82.

34. Siboni F, Taddei P, Zamparini F, Prati C, Gandolfi MG. Properties of BioRoot RCS, a tricalcium silicate endodontic sealer modified with povidone and polycarboxylate. Int Endod J. 2017;(Supplement 2):e120-e136.

35. Elyassi Y, Moinzadeh AT, Kleverlaan CJ. Characterization of Leachates from 6 Root Canal Sealers. J Endod. 2019;45:623-627.

36. Kharouf N, Arntz Y, Eid A, Zghal J, Sauro S, Haikel Y, Mancino D. Physicochemical and Antibacterial Properties of Novel, Premixed Calcium Silicate-Based Sealer Compared to Powder-Liquid Bioceramic Sealer. J Clin Med. 2020;9:3096.

37. Benetti F, de Azevedo Queiroz ÍO, Oliveira PHC, Conti LC, Azuma MM, Oliveira SHP, Cintra LTA. Cytotoxicity and biocompatibility of a new bioceramic endodontic sealer containing calcium hydroxide. Braz Oral Res. 2019;33:e042.

38. Gandolfi MG, Taddei P, Tinti A, Prati C. Apatite-forming ability of ProRoot MTA. Int Endod J 2010;43:917–29.

39. Gandolfi MG, Van Landuyt K, Taddei P, Modena E, Van Meerbeek B, Prati C. Environmental scanning electron microscopy connected with energy dispersive x-ray analysis and Raman techniques to study ProRoot mineral trioxide aggregate and calcium silicate cements in wet conditions and in real time. J Endod. 2010;36:851-7.

40. Gandolfi MG, Taddei P, Siboni F, Modena E, Ciapetti G, Prati C. Development of the foremost light-curable calcium–silicate MTA cement as root-end in oral surgery. Chemical–physical properties, bioactivity and biological behaviour. Dent Mater 2011;27:e134–57.

41. Taddei P, Modena E, Tinti A, Siboni F, Prati C, Gandolfi MG. Vibrational investigation on the in vitro bioactivity of commercial and experimental calcium–silicate cements for root-end endodontic therapy. J Mol Struct 2011;993:367–75.

42. Camilleri, J. Characterization and hydration kinetics of tricalcium silicate cement for use as a dental biomaterial. Dent. Mater. 2011;27:836–844.

43. Kebudi Benezra M, Schembri Wismayer P, Camilleri J. Influence of environment on testing of hydraulic sealers. Sci Rep. 2018;8:7209.

44. Bolhari B, Nekoofar MH, Sharifian M, Ghabrai S, Meraji N, Dummer PM. Acid and microhardness of mineral trioxide aggregate and mineral trioxide aggregate-like materials. J Endod. 2014;40:432-5.

45. Ashofteh Yazdi K, Ghabraei S, Bolhari B, Kafili M, Meraji N, Nekoofar MH, Dummer PMH. Microstructure and chemical analysis of four calcium silicate-based cements in different environmental conditions. Clin Oral Investig. 2019;23:43-52.

46. Grazziotin-Soares R, Nekoofar MH, Davies T, Hübler R, Meraji N, Dummer PMH. Crystalline phases involved in the hydration of calcium silicate-based cements: Semi-quantitative Rietveld X-ray diffraction analysis. Aust Endod J. 2019;45:26-32.

47. Prati C, Gandolfi MG. Calcium silicate bioactive cements: Biological perspectives and clinical applications. Dent Mater. 2015;31:351-70.

48. Moon HJ, Lee JH, Kim JH, Knowles JC, Cho YB, Shin DH, Lee HH, Kim HW. Reformulated mineral trioxide aggregate components and the assessments for use as future dental regenerative cements. J Tissue Eng. 2018;9:2041731418807396.

49. Donnermeyer D, Ibing M, Bürklein S, Weber I, Reitze MP, Schäfer E. Physico-Chemical Investigation of Endodontic Sealers Exposed to Simulated Intracanal Heat Application: Hydraulic Calcium Silicate-Based Sealers. Materials (Basel). 2021;14:728.

50. Cavenago BC, Pereira TC, Duarte MA, Ordinola-Zapata R, Marciano MA, Bramante CM, Bernardineli N. Influence of powder-to-water ratio on radiopacity, setting time, pH, calcium ion release and a micro-CT volumetric solubility of white mineral trioxide aggregate. Int Endod J. 2014;47:120-6.

51. Jafari F, Jafari S. Composition and physicochemical properties of calcium silicate based sealers: A review article. J Clin Exp Dent. 2017;9:e1249-e1255.

52. Li X, Yoshihara K, De Munck J, Cokic S, Pongprueksa P, Putzeys E, Pedano M, Chen Z, Van Landuyt K, Van Meerbeek B. Modified tricalcium silicate cement formulations with added zirconium oxide. Clin Oral Investig. 2017;21:895-905.

53. Reyes-Carmona JF, Felippe MS, Felippe WT. A phosphate-buffered saline intracanal dressing improves the biomineralization ability of mineral trioxide aggregate apical plugs. J Endod. 2010;36:1648-52.

54. Abedi-Amin A, Luzi A, Giovarruscio M, Paolone G, Darvizeh A, Agulló VV, Sauro S. Innovative root-end filling materials based on calcium-silicates and calcium-phosphates. J Mater Sci Mater Med. 2017;28:31.

55. Combes C, Rey C. Amorphous calcium phosphates: synthesis, properties and uses in biomaterials. Acta Biomaterialia 2010;6:3362–78.

56. Niu LN, Jiao K, Wang TD, Zhang W, Camilleri J, Bergeron BE, Feng HL, Mao J, Chen JH, Pashley DH, Tay FR. A review of the bioactivity of hydraulic calcium silicate cements. J Dent. 2014;42:517-33.

57. Wang L, Jin H, Ye D, Wang J, Ao X, Dong M, Niu W. Enterococcus faecalis Lipoteichoic Acid-induced NLRP3 Inflammasome via the Activation of the Nuclear Factor Kappa B Pathway. J Endod. 2016;42:1093-100.

58. Kuramoto M, Kawashima N, Tazawa K, Nara K, Fujii M, Noda S, Hashimoto K, Nozaki K, Okiji T. Mineral trioxide aggregate suppresses pro-inflammatory cytokine expression via the

calcineurin/nuclear factor of activated T cells/early growth response 2 pathway in lipopolysaccharide-stimulated macrophages. Int Endod J. 2020;53:1653-1665.

59. Zhou HM, Du TF, Shen Y, Wang ZJ, Zheng YF, Haapasalo M. In vitro cytotoxicity of calcium silicate-containing endodontic sealers. J Endod. 2015;41:56-61.

FIGURE LEGENDS

Figure 1. Scanning electron microscopy (SEM) of hydrated CSBS surface morphology at 700x and 2340x magnification after 24 h in α-MEM: BR (A), BC (B), and SPBC (C).

Figure 2. Energy dispersive X-ray (EDX) results of the CSBS chemical elements: BR (A), BC (B), and SPBC (C).

Figure 3. Graphic illustrations of Fourier-transform infrared spectroscopy (FTIR) for the unset and set CSBS: BR (A, B), BC (C, D), SPBC (E, F).

Figure 4. Cell attachment of RAW264.7 onto the surfaces of the CSBSs (Control: A; BR: B; BC: C; SPBC: D). The arrows indicate areas with cells.

Figure 5. The extract dilution effects of the CSBS on cell viability of RAW264.7 evaluated using the MTT formazan assay. 100% cell viability was considered for the control group: (A) Asterisks allow comparisons among different extract dilution for the same sealer; (B) comparisons among CSBS and with the control group.

*$p < 0.05$ compared intragroup; **$p < 0.05$ compared intergroup at same extract dilution; ***$p < 0.05$ compared with the control.

TABLE

Table 1. Ion release of hydrated CSBS according to XRF analysis (ppms)

	CSBS			
Elements identified (ppm)	BR	BC	SPBC	*p*
Calcium (Ca)	3200	200	800	0.0201
Silicon (Si)	19	20	12	0.5302
Zirconium (Zr)	-	4	-	0.4219

FIGURES

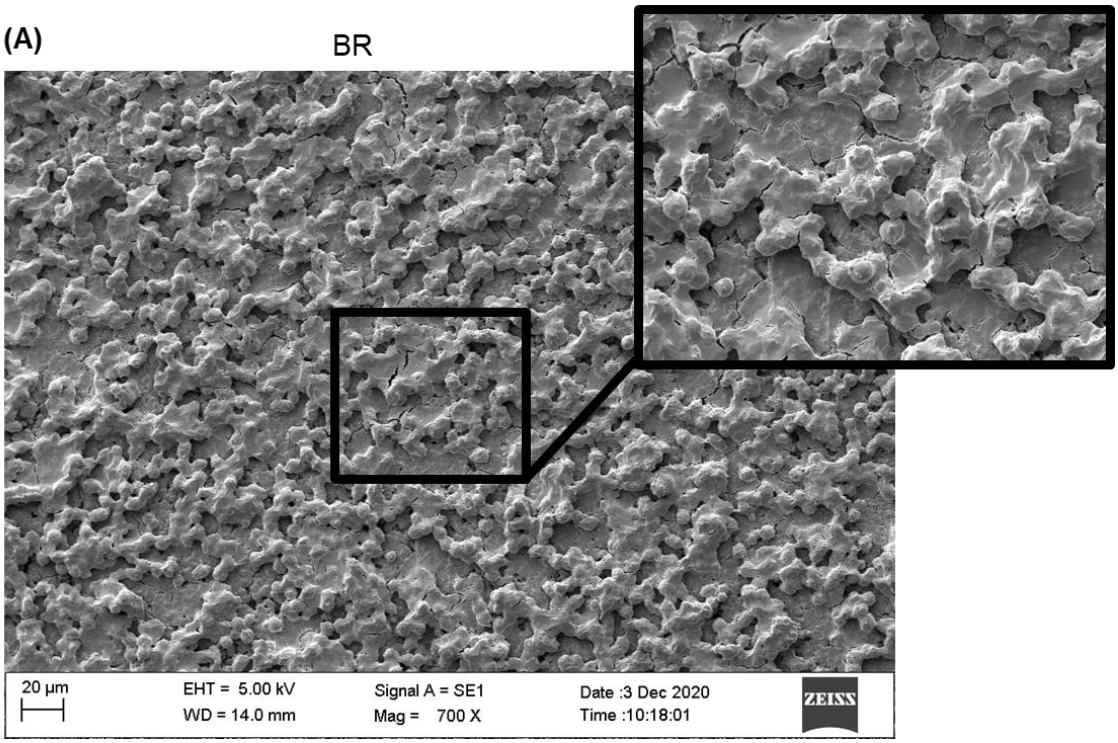


Figure 1. Scanning electron microscopy (SEM) of hydrated CSBS surface morphology at 700x and 2340x magnification after 24 h in α-MEM: BR (A), BC (B), and SPBC (C).

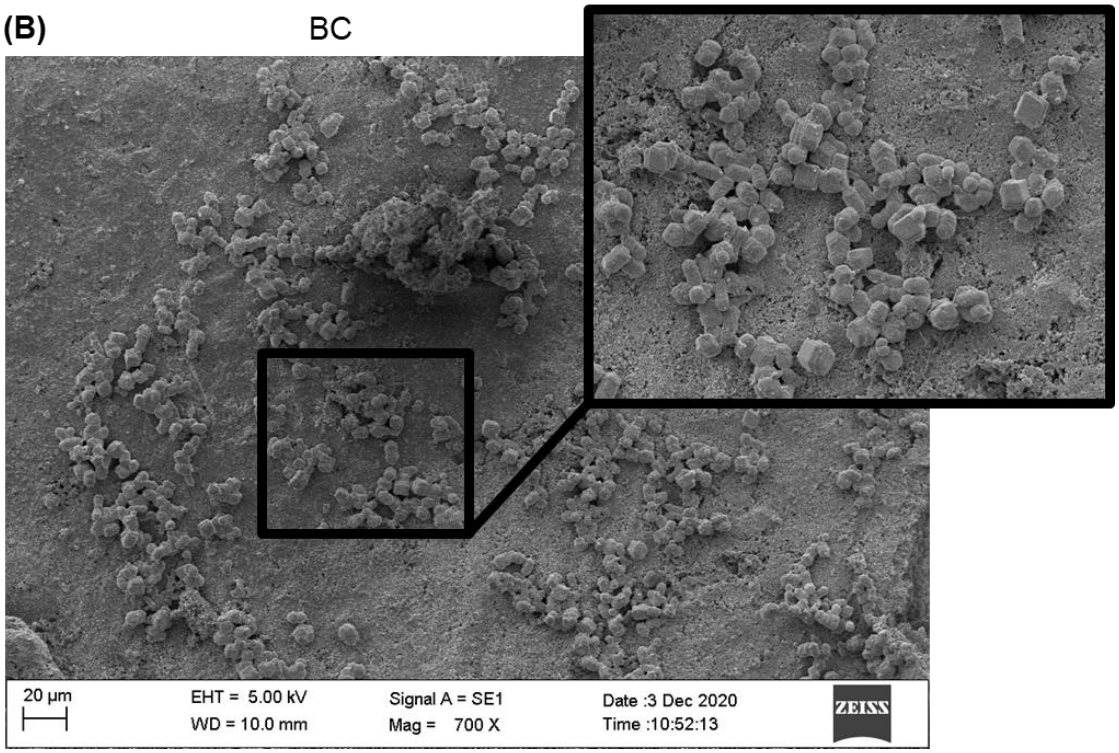


Figure 1. Scanning electron microscopy (SEM) of hydrated CSBS surface morphology at 700x and 2340x magnification after 24 h in α-MEM: BR (A), BC (B), and SPBC (C).

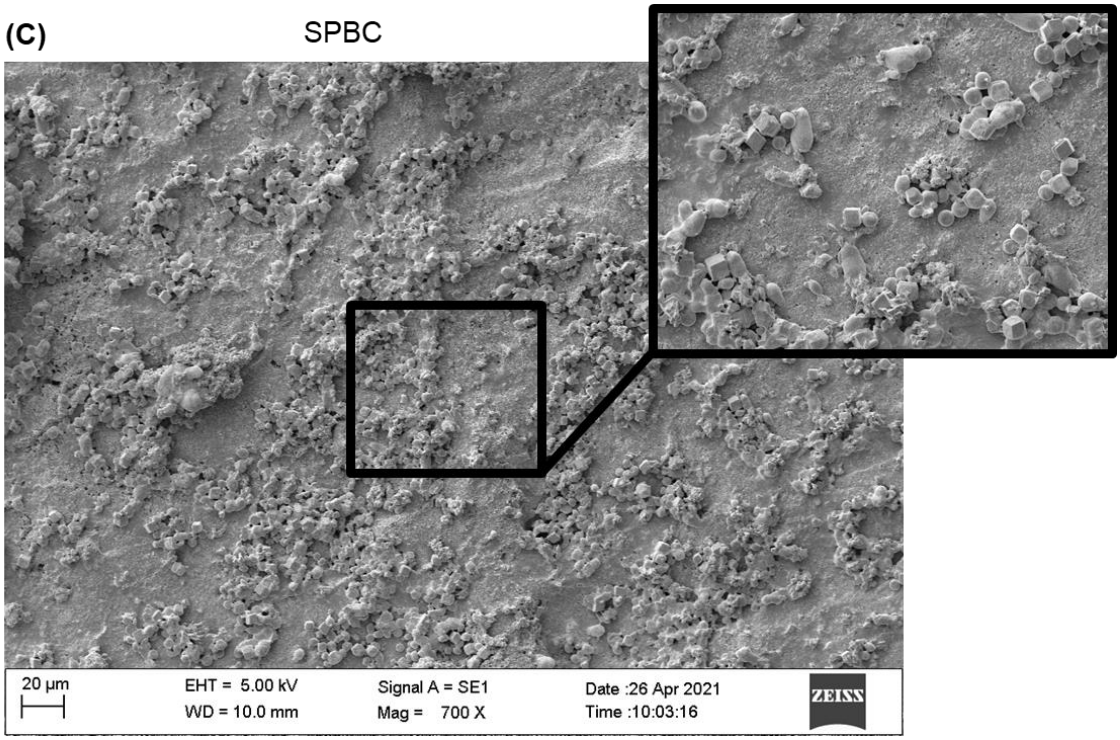


Figure 1. Scanning electron microscopy (SEM) of hydrated CSBS surface morphology at 700x and 2340x magnification after 24 h in α-MEM: BR (A), BC (B), and SPBC (C).

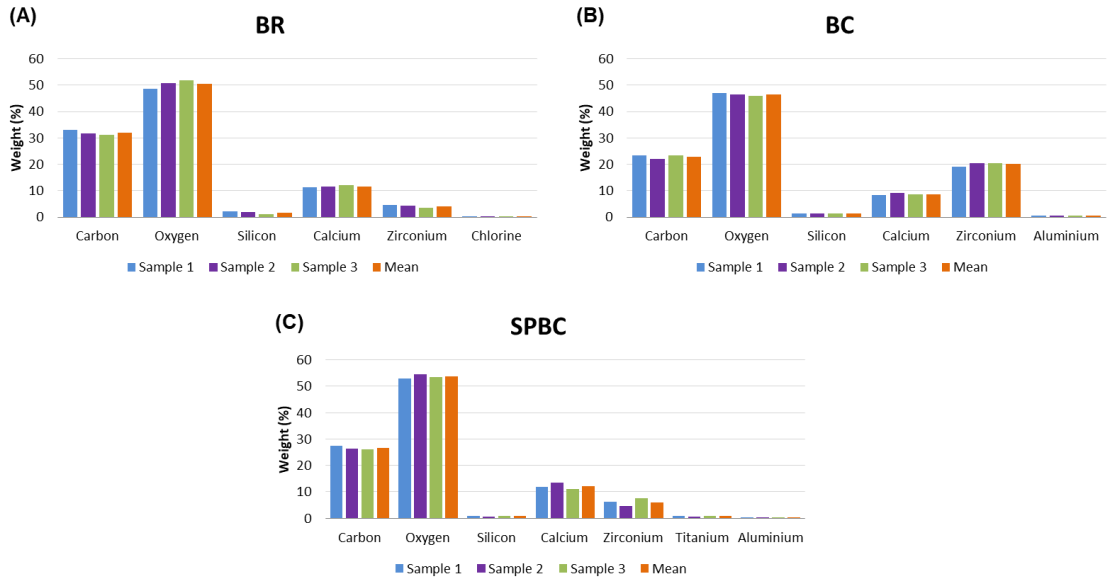


Figure 2. Energy dispersive X-ray (EDX) results of the CSBS chemical elements: BR (A), BC (B), and SPBC (C).

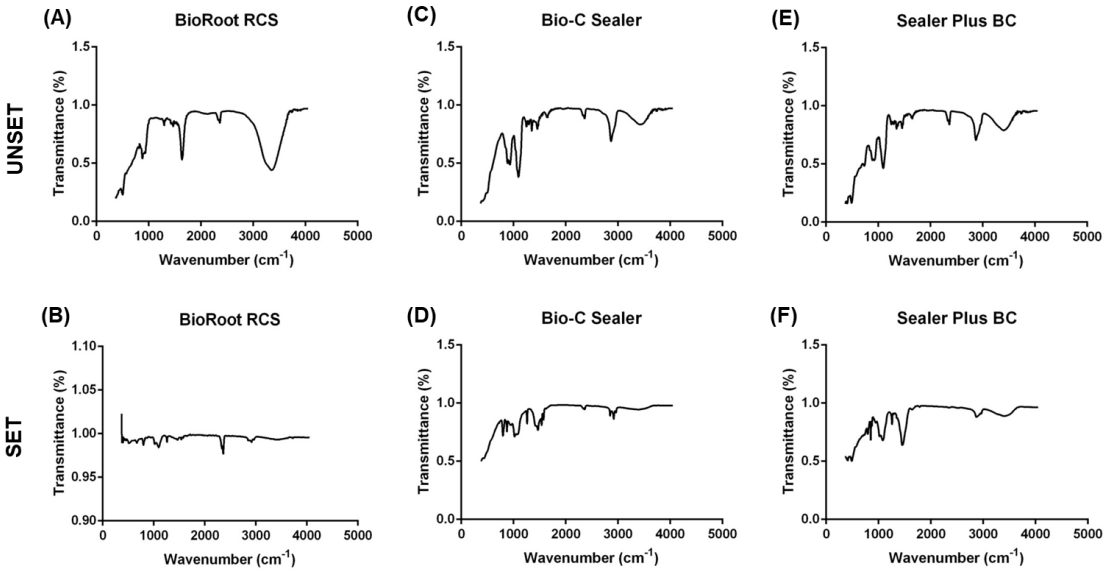


Figure 3. Graphic illustrations of Fourier-transform infrared spectroscopy (FTIR) for the unset and set CSBS: BR (A, B), BC (C, D), SPBC (E, F).

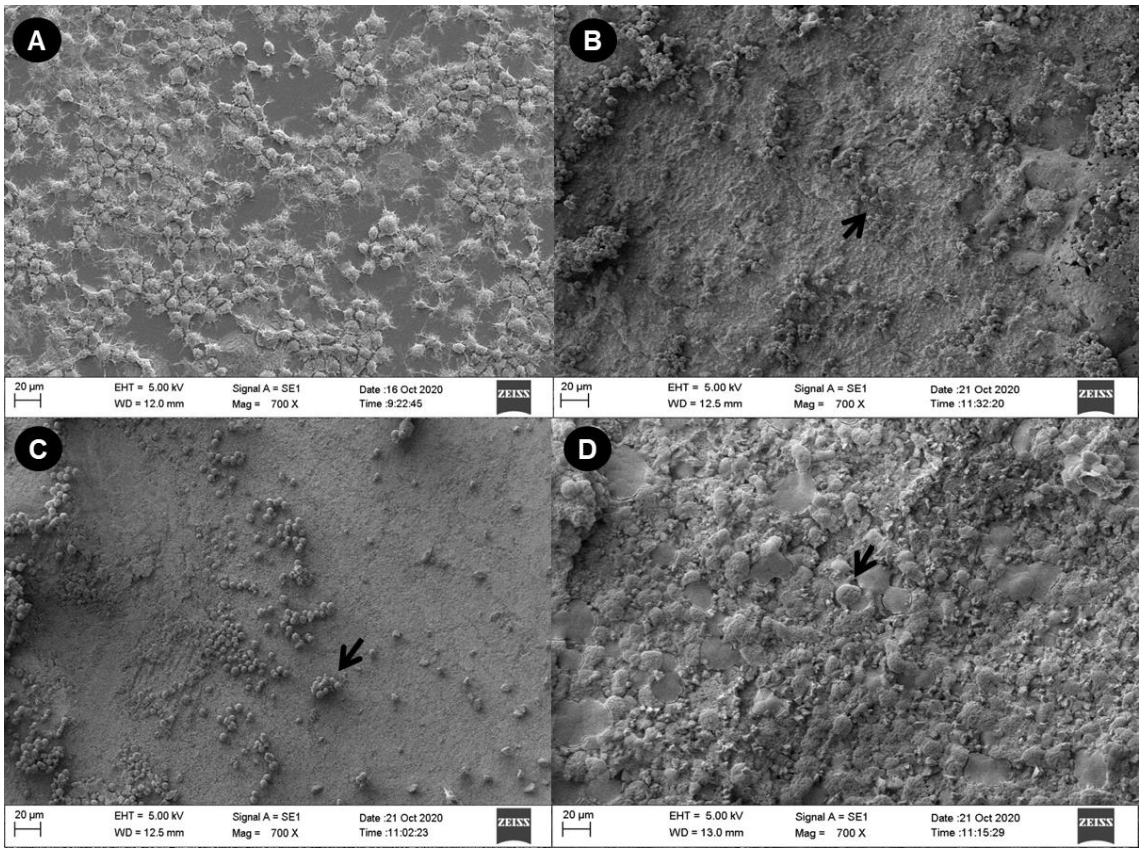


Figure 4. Cell attachment of RAW264.7 onto the surfaces of the CSBSs (Control: A; BR: B; BC: C; SPBC: D). The arrows indicate areas with cells.

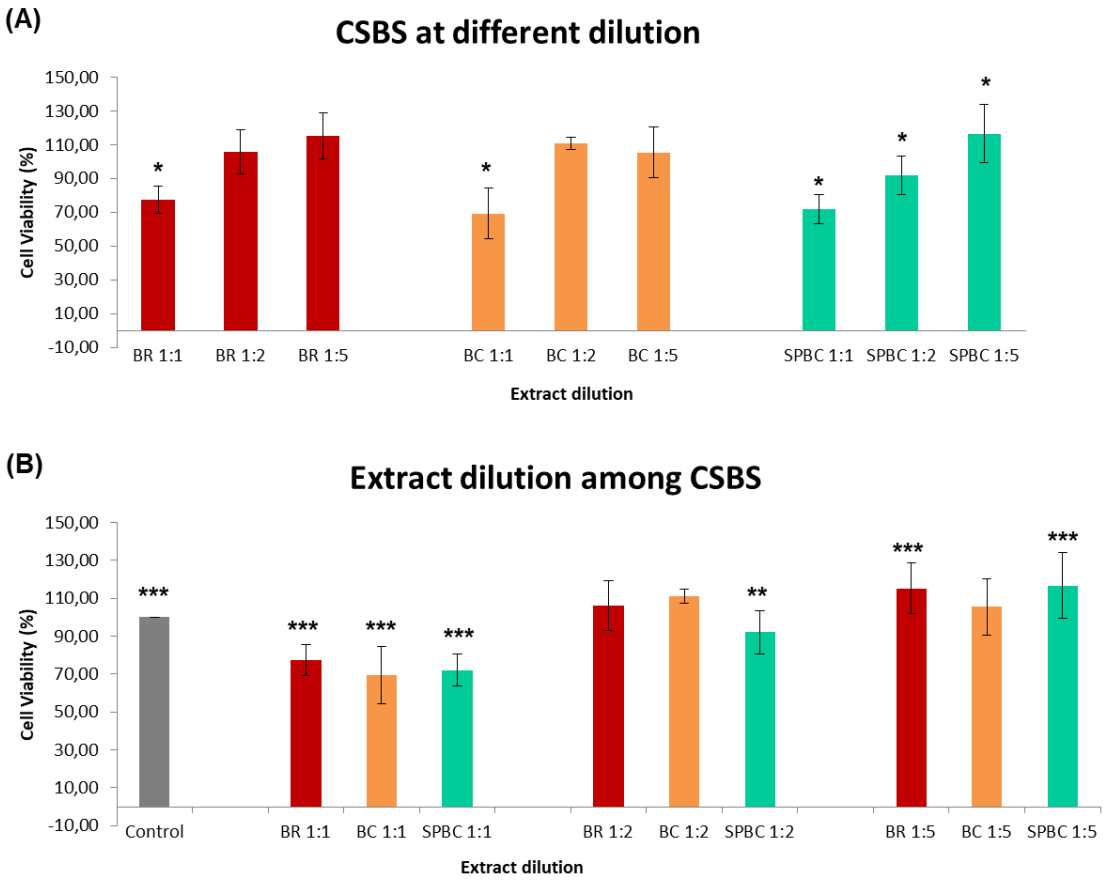


Figure 5. The extract dilution effects of the CSBS on cell viability of RAW264.7 evaluated using the MTT formazan assay. 100% cell viability was considered for the control group: (A) Asterisks allow comparisons among different extract dilution for the same sealer; (B) comparisons among CSBS and with the control group.

*$p < 0.05$ compared intragroup; **$p < 0.05$ compared intergroup at same extract dilution; ***$p < 0.05$ compared with the control.

CAPÍTULO 4

3.4 Capítulo 4

Artigo aceito no periódico Brazilian Oral Research

Effect of irrigation protocols on root canal wall after post preparation: micro-CT and microhardness study

Camila Maria Peres de Rosatto[1], Danilo Cassiano Ferraz[2], Lilian Vieira Oliveira[1], Priscilla Barbosa Ferreira Soares[3], Carlos José Soares[4], Mário Tanomaru-Filho[2], Camilla Christian Gomes Moura[1]

[1] Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil.

[2] Department of Restorative Dentistry, School of Dentistry, São Paulo State University, Araraquara, SP, Brazil.

[3] Department of Periodontology and Implantology, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil.

[4] Department of Operative Dentistry and Dental Materials, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil.

Correspondence: Mário Tanomaru-Filho, Department of Restorative Dentistry, Araraquara Dental School, São Paulo State University - UNESP, Rua Humaitá 1680, Araraquara, SP, CEP 14801-903, Brazil (Tel.: (+55) 16 3301-6390; e-mail: tanomaru@uol.com.br).

BOR.2020-1121 – Original Research – Imaginology

Effect of irrigation protocols on root canal wall after post preparation: a micro-CT and microhardness study

Camila Maria Peres de Rosatto[1] https://orcid.org/0000-0003-0207-8402

Danilo Cassiano Ferraz[2] https://orcid.org/0000-0001-9102-4320

Lilian Vieira Oliveira[1] http://orcid.org/0000-0002-1805-8302

Priscilla Barbosa Ferreira Soares[3] https://orcid.org/0000-0002-4492-8957

Carlos José Soares[4] https://orcid.org/0000-0002-8830-605X

Mario Tanomaru-Filho[2] https://orcid.org/0000-0002-2574-4706

Camilla Christian Gomes Moura[1] https://orcid.org/0000-0002-5653-8403

[1] Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil

[2] Department of Restorative Dentistry, School of Dentistry, São Paulo State University, Araraquara, SP, Brazil

[3] Department of Periodontology and Implantology, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil

[4] Department of Operative Dentistry and Dental Materials, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil

Corresponding author:

Mario Tanomaru-Filho

Email: tanomaru@uol.com.br

ABSTRACT

The aim of this study was to investigate the effects of different post space irrigation protocols for removing residual filling material from dentin walls, by using microcomputed tomography (micro-CT), and the influence of these protocols on dentin microhardness. Bovine incisors (*n* = 35) were filled with the single-cone technique and MTA Fillapex (Angelus, Londrina, PR, Brazil). Post space preparation (PSP) was performed 7 days after filling, using the Odous Touch electrical system (Odous De Deus Ind. e Com., Belo Horizonte, MG, Brazil), followed by post space irrigation using manual irrigation, passive ultrasonic irrigation, or Easy Clean, together with 2.5% sodium hypochlorite (NaOCl), or with 2.5% NaOCl and 17% EDTA (NaOCl/EDTA). Micro-CT scans were performed at three time points. The residual filling material was evaluated at three levels: cervical, middle and apical. The Knoop test was measured with four indentations around the canal lumen at three dentin depths: X (100 µm), Y (200 µm) and Z (400 µm). Statistical analysis was performed using ANOVA ($P < .05$). The effects of the activation method ($P < .001$), and the root level ($P = .013$), as well as the interaction between the irrigant and the activation method ($P = .041$), led to different percentages of residual filling material. Lower amounts of residual filling material were observed at the cervical versus the middle and apical levels ($P < .05$). No significant differences were observed in dentin microhardness ($P > .05$). The best removal of the residual filling material was performed using the Easy Clean tip and NaOCl/EDTA, regardless of the activation methods.

Keywords: Hardness Tests, X-Ray Microtomography, Root canal irrigants

INTRODUCTION

Post space preparation (PSP) leads to the formation of a smear layer that contains the residual filling material used to fill the root canal,[1, 2] and this layer on the root canal wall may influence the retention of fiberglass posts.[3] Considering that the adhesive failure of fiberglass posts is mainly related to debonding at the resin cement/dentin interface,[2, 3, 4, 5] the residual filling material bonded to the canal walls should be removed to improve fiberglass post retention.[3, 6]

Several solutions and protocols have been proposed for post space irrigation,[4, 7, 8] including sodium hypochlorite (NaOCl)[9] and acid ethylenediaminetetraacetic acid (EDTA).[4, 6, 8, 10] NaOCl acts on the organic part of dentin and modifies collagen fibers, whereas EDTA acts on the inorganic part and chelates calcium ions.[11] Both chemical irrigants can change the structural properties of dentin, such as its microhardness.[12, 13]

The effects of irrigating solutions can be enhanced by activation protocols such as passive ultrasonic irrigation (PUI).[1, 10] Easy Clean (EC) (Easy Dental Equipment, Belo Horizonte, MG, Brazil) was developed as a simple alternative to PUI.[14, 15, 16] Neither of these strategies has been studied at length in regard to irrigant activation in the root canal after PSP, but both have been shown to improve debris removal and bond strength of fiber posts.[3, 10, 16] The effect of EC used in continuous rotation was observed for debris removal from the main canal and the isthmus of the mesial root of mandibular molars, and was found to be adequate.[17, 18] However, no studies have investigated the effect of EC under low-speed continuous rotation as a post space irrigation (PSI) strategy.

The aim of this study was to investigate the effects of different post space irrigation protocols for removing the residual filling material on dentin walls, using

microcomputed tomography (micro-CT), and the influence of these protocols on dentin microhardness. The null hypotheses were as follows: 1) the solutions and the activation methods used for post space irrigation would not improve the outcome of residual filling material cleaning; 2) the solutions and activation methods would not affect dentin microhardness.

METHODOLOGY

Tooth selection and root embedment

The sample size was calculated using the BioEstat 5.3 (Mamirauá Institute, Tefé, AM, Brazil) statistical software program.[15, 19] Five teeth per group were required to detect a 90% chance of finding significant differences at the 5% level (2-sided test), with a minimum detectable mean difference of 13.54, and an expected standard deviation of 3.58 with regard to microhardness, the secondary outcome.

Thirty-five bovine incisors with a single root canal, completely formed apices, and similar external morphology were selected. The root dimensions were measured with a digital caliper (Mitutoyo Sul Americana, Suzano, SP, Brazil) in the buccolingual and mesiodistal directions for the 3 thirds of the root. The teeth were also scanned using digital radiography (Sensor Fit T1, Micro Imagem, Indaiatuba, SP, Brazil), so that the mean area of the root canals could be measured by the ImageJ software program (National Institutes of Health, Bethesda, MD, USA). Maximum deviations of 10% from the mean value were included. The teeth were cleaned and then stored in distilled water to prevent the effects of confounding factors on the microhardness tests. The crowns were removed with a diamond disc (KG Sorensen, Barueri, SP,

Brazil), and the mean root length was standardized at 15.0 mm. The working length was determined with a #10 K-file (Dentsply Sirona, York, PA, USA), which was introduced until its tip was visible at the apical foramen, and 1 mm was subtracted from this measurement. The roots were embedded in polystyrene resin (Cristal, Piracicaba, SP, Brazil) to simulate the alveolar bone,[20] and allow the samples to be inserted in the same position during the micro-CT scan (Fig. 1*A*).

Endodontic treatment and post space preparation

All the procedures were performed by different experienced endodontic specialists. The root canals were prepared using a reciprocating nickel-titanium #50.05 file (Reciproc system - VDW, Munich, Germany) with an endodontic motor (Dentsply Sirona, York, PA, USA). Irrigation was performed using 12 mL of 2.5% NaOCl (Asfer Indústria Química, São Caetano do Sul, SP, Brazil) for all the specimens. Next, the root canals were rinsed with 3 cycles of 1 mL of 17% EDTA (Biodinâmica, Ibiporã, PR, Brazil) for 1 minute, followed by 5 mL of 2.5% NaOCl, and a final flush with 5 mL of distilled water. The canals were dried with paper points (VDW), and obturated with the single-cone technique using gutta-percha points R50 (VDW) and MTA Fillapex (batch no. #40006 and #40063; Angelus, Londrina, PR, Brazil).

The specimens were stored at 37°C in a moist environment for 7 days to allow the sealer to set completely. PSP was performed with thermoplasticized gutta-percha for each sample, using M and FM tips from Odous Touch (Odous De Deus Ind. e Com., Belo Horizonte, MG, Brazil); 10 mL of distilled water was used to irrigate a space of 10 mm in length, leaving 4 mm of the apically sealed region untouched.

Post space irrigation protocols

The roots were randomly divided (www.random.org) into two experimental groups (*n* = 15), according to the irrigant: 2.5% NaOCl and 2.5% NaOCl followed by 17% EDTA (NaOCl/EDTA). Each group was further divided into 3 subgroups, according to the following irrigating activation methods (*n* = 5): manual irrigation (MI), PUI, or EC tip, yielding the final groups of NaOCl/MI, NaOCl/PUI, NaOCl/EC, NaOCl/EDTA/MI, NaOCl/EDTA/PUI, and NaOCl/EDTA/EC. As for the controls, the micro-CT evaluations considered manual irrigation as the control for each subgroup (NaOCl/MI and NaOCl/EDTA/MI), and the dentin microhardness assessment considered the root that was obturated and subjected to PSP (*n* = 5) as the control group.

Manual irrigation (*n* = 10) was performed with a 30-g open-ended needle (NaviTip, Ultradent, South Jordan, UT, USA) introduced with an “in-and-out” motion. PUI (*n* = 10) was performed with an Irrisonic E1 (20/01) tip (Helse Dental Technology, Santa Rosa de Viterbo, SP, Brazil) attached to the ultrasonic unit of a Piezon Master 200 (EMS, São Bernardo do Campo, SP, Brazil), on a power level of 2. The EC (*n* = 10) had a 25.04 tip attached to a low-speed contra-angle handpiece (KaVo Kerr Group, Charlotte, USA), running at approximately 20,000 continuous rotations per minute. In all subgroups, the devices were placed 1 mm short of the remaining gutta-percha.

The irrigation sequence for using NaOCl was 1 mL of solution for 20 seconds in 6 cycles.[15, 16] In the NaOCl/EDTA group, 3 mL was used for each solution,[10] with 3

cycles of 1 mL of NaOCl followed by 3 cycles of 1 mL of EDTA, resulting in the same volume and time of irrigation. All irrigants were applied with a 30-g open-ended needle (NaviTip, Ultradent, South Jordan, UT, USA). A final flush with 10 mL of distilled water was performed to remove any chemical solution residue.[13]

Micro-CT analysis

The specimens were scanned in a micro-CT device (SkyScan 1272; Bruker micro-CT, Kontich, Belgium) at three time points to evaluate the residual filling material: after obturation, at PSP and at post space irrigation. Scanning was conducted under the following conditions: 100 KV and 100 μA, 21.7 μm pixel size, 2000 ms exposure time, 180° rotation angle at a rotation step of 0.8, frame averaging of 2, random movement of 20, and Cu filter of 0.11 mm. The images acquired by micro-CT were reconstructed by a NRecon software program (version 1.6.3.3, Bruker micro-CT) with a beam hardening correction of 2%, a smoothing level of 1, and a ring artifact correction level of 7.

A CTAn (version 1.14.4.1, SkyScan, Bruker, Belgium) was used to identify the region of interest (ROI), corresponding to the amount of residual filling material. The ROI selected for each slice was the area around the root canal, used to calculate the volume of residual filling material (mm^3). The presence of residual filling material was processed with the original grayscale in the cervical, middle and apical levels, with 130 slices for each level (Fig. 1*B*). The residual filling material values obtained before applying the irrigating activation method were recorded in mm^3 and converted into a percentage using the following formula: $V_{A\text{-}PSP}$*FP/V_F, where $V_{A\text{-}PSP}$ indicates the object volume after PSP (mm^3), FP indicates the filling percentage (%), and V_F

indicates the filling object volume (mm^3). After the post space irrigation protocols were performed, the $V_{A\text{-}CP}$*$V_{A\text{-}PSP}$/V_{PSP} formula was used, where $V_{A\text{-}CP}$ indicates the object volume after performing the irrigating activation method (mm^3), $V_{A\text{-}PSP}$ indicates the residue after PSP (%), and V_{PSP} indicates the PSP object volume (mm^3).

A CTAn software program was used to generate 3D models for the residual filling material before and after applying the irrigating activation method (Fig. 2). One examiner previously calibrated and blinded to the study groups performed all the measurements.

Dentin microhardness assessment - Knoop test

The roots previously used for micro-CT analysis were prepared according to previously described methods (Fig. 1*C*).[20] The Knoop indentation values were determined with a microhardness tester (FM700; FutureTech, Kawasaki, Japan) at the cervical, middle and apical levels, after a load of 50 g was applied for 15 seconds.[21] Four indentations were made around the canal lumen at three dentin depths (Fig. 1*D*). The average values of dentin microhardness were recorded.

Statistical Analysis

The normality of the data was tested using the Shapiro-Wilk test, and homoscedasticity was tested using the Levene test, after which parametric statistical tests were performed. The micro-CT analysis used three-way ANOVA with Tukey's and Dunnett's tests to evaluate the overall effects of the irrigant activation method and the root level (cervical, medium and apical). The subgroup analysis used post hoc Tukey's test for two-by-two comparisons. Two-way repeated measures ANOVA

was used to compare the irrigants and activation methods at the cervical, medium and apical levels.

The dentin microhardness analysis used two-way ANOVA to compare the irrigants and activation methods for each dentin depth of the canal lumen at the same root level, after which Tukey's and Dunnett's tests were performed. The significance level was set at $\alpha = .05$ for all the tests. Statistical analysis of the data was conducted by using SigmaPlot, version 13.1 (Systat Software, San Jose, CA, USA).

RESULTS

Residual filling material

Three-way ANOVA showed that the activation methods ($P < .001$), the root levels ($P = .013$), and the interaction between the irrigants and the activation methods ($P = .041$) led to significant differences in the percentage of residual filling material. Overall, the samples cleaned with EC showed less residual filling material than those cleaned with PUI or MI ($P < .05$). Significantly more residual filling material was removed at the cervical than middle and apical levels ($P < .05$). Table 1 presents the mean and standard deviation of each post space irrigation subgroup, regardless of the third level.

The repeated measures did not present statistically significant differences among the irrigant solutions at the cervical, middle and apical levels ($P = .991$; $P = .699$; $P = .750$, respectively). The EC group showed lower residue values at the

middle and apical levels than the MI group (control; $P < .027$ and $P = .044$, respectively).

Dentin microhardness

The means and standard deviations of dentin microhardness at the three root levels (cervical, middle and apical) are summarized in Table 2. Two-way ANOVA and Dunnett's test at the cervical, middle and apical root levels, and at X (100 µm), Y (200 µm) and Z (400 µm) distances did not detect any differences in dentin microhardness among the irrigants, the activation methods or the interactions ($P > .05$).

DISCUSSION

Based on the results observed in this study, the null hypotheses were partially rejected, because a statistically significant difference was observed in the cleaning outcome, resulting from the particular activation method used. In contrast, the dentin microhardness values were not affected by the irrigating solutions, or the activation protocols. The effects of PSP on the roots filled using the single-cone technique have been previously evaluated regarding apical displacement and residual filling material.[22, 23] The assessment of residual filling material using micro-CT [9, 22] provides a three-dimensional view of the root canal without damaging the samples.[9] This assessment made it possible to use the same roots in the microhardness test. However, two different control groups were used for the micro-CT [24] and the dentin microhardness analyses. Regarding that of microhardness, a positive control group

was established with no additional irrigation protocols, since the use of chemical solutions can affect dentin microhardness.[12]

The residual filling material was removed with NaOCl or NaOCl/EDTA, both of which have often been recommended as post space cleaning solutions.[6, 8, 9, 25] The volume of irrigants and the number of activation cycles were selected based on preliminary studies that used EC.[14, 15, 16] In the present study, EC was inserted using a low-speed pneumatic contra-angle, enabled by its wide-ranging availability, at no addition cost to dentists. Moreover, this method can be considered more advantageous than that indicated by the manufacturer, which requires reciprocating movement.[14, 16] The lack of a significant difference in the cleaning achieved by EC and PUI, among the subgroup samples irrigated with NaOCl/EDTA, confirms that EC can be used in continuous rotation without affecting the performance of the tips, as previously demonstrated.[17]

The E1 ultrasonic insert has a tip size of 20.01, and promotes cavitation, acoustic microstreaming and shock waves [1] when used properly; it may not have had extensive contact with the canal walls.[16] The contact area could not be covered in the present study, because the Reciproc 50.05 device was used for instrumentation, and its file body has a larger diameter than its ultrasonic tip, thus allowing it to work free of interference. The reduction in the residual filling material adhered to root dentin after application of PUI for post space irrigation corroborates the results of a previous study.[1] However, there are no previous data on EC, regarding its ability to perform adequate cleaning for post installation. Its aircraft wing design and flexibility [14, 15] probably helped clean the gutta-percha and endodontic sealer from the root canals, as suggested by the results of previous studies evaluating its debris removal [14, 16, 17, 26] and irrigant penetration capabilities in lateral canals.[24]

A concern regarding EC is that erosion may occur when it is used with EDTA.[15] Although this issue was not evaluated in the current study, the sequential use of NaOCl and EDTA enhanced by the activation methods may result in the depletion of collagen and apatite in the dentin walls, [11] and a reduction in the calcium/phosphorus ratio,[27] which affects properties such as microhardness.[12, 28] However, the lack of differences observed between Reciproc and EC, regardless of the root level and depth, suggests that the microhardness value eventually became equal to that of the control, when the NaOCl was neutralized by the hydroxyapatite, or when the hydroxyapatite crystals were reprecipitated onto the dentin surface [11] . Nevertheless, microhardness is a complementary parameter, and should not be assessed independently. Although this *in vitro* study had some limitations, such as the use of bovine teeth, non-inclusion of instrument aging, and inability to use the same sample before and after the post space irrigation protocols for dentin microhardness, the results are promising for EC under continuous rotation.

CONCLUSION

In conclusion, irrigation with Easy Clean under continuous rotation showed a lesser amount of residual filling material than PUI or manual irrigation. The cleaning outcomes of the two systems were equivalent when NaOCl/EDTA was used with Easy Clean, PUI and manual irrigation. Dentin microhardness was not affected by the post space irrigation protocols.

ACKNOWLEDGMENTS

The authors are grateful to FAPEMIG, CNPq, and CAPES for their financial support (Funding Code 001). This study was carried out at the CPBio - Biomechanics,

Biomaterials and Cell Biology Research Center of the Federal University of Uberlândia.

Conflict of interest: The authors declare that they have no conflict of interest.

REFERENCES

1. Chen X, Liu H, He Y, Luo T, Zou L. Effects of Endodontic Sealers and Irrigation Systems on Smear Layer Removal after Post Space Preparation. J Endod. 2018;44(8):1293-7.

2. Coniglio I, Magni E, Goracci C, Radovic I, Carvalho CA, Grandini S, et al. Post space cleaning using a new nickel titanium endodontic drill combined with different cleaning regimens. J Endod. 2008;34(1):83-6.

3. Bengoa FP, Arze MCM, Noguera CM, Moreira LFN, Kato AS, Bueno CE. Effect of ultrasonic cleaning on the bond strength of fiber posts in oval canals filled with a premixed bioceramic root canal sealer. Restor Dent Endod. 2020;45(2):e19.

4. Barreto MS, Rosa RA, Seballos VG, Machado E, Valandro LF, Kaizer OB, et al. Effect of Intracanal Irrigants on Bond Strength of Fiber Posts Cemented With a Self-adhesive Resin Cement. Oper Dent. 2016;41(6):e159-e67.

5. Silva NRD, Rodrigues MP, Bicalho AA, Deus RA, Soares PBF, Soares CJ. Effect of Magnification during Post Space Preparation on Root Cleanness and Fiber Post Bond Strength. Braz Dent J. 2019;30(5):491-7.

6. Kul E, Yeter KY, Aladag LI, Ayranci LB. Effect of different post space irrigation procedures on the bond strength of a fiber post attached with a self-adhesive resin cement. J Prosthet Dent. 2016;115(5):601-5.

7. Moura AS, Pereira RD, Rached FJJ, Crozeta BM, Mazzi-Chaves JF, Souza-Flamini LE, et al. Influence of root dentin treatment on the push-out bond strength of fibre-reinforced posts. Braz Oral Res. 2017;31:e29.

8. Oliveira LV, Maia TS, Zancope K, Menezes MS, Soares CJ, Moura CCG. Can intra-radicular cleaning protocols increase the retention of fiberglass posts? A systematic review. Braz Oral Res. 2018;32:e16.

9. Silva E, Belladonna FG, Zuolo AS, Rodrigues E, Ehrhardt IC, Souza EM, et al. Effectiveness of XP-endo Finisher and XP-endo Finisher R in removing root filling remnants: a micro-CT study. Int Endod J. 2018;51(1):86-91.

10. Ekim SNA, Erdemir A. Effect of different irrigant activation protocols on push-out bond strength. Lasers Med Sci. 2015;30(8):2143-9.

11. Ramirez-Bommer C, Gulabivala K, Ng YL, Young A. Estimated depth of apatite and collagen degradation in human dentine by sequential exposure to sodium hypochlorite and EDTA: a quantitative FTIR study. Int Endod J. 2018;51(4):469-78.

12. Aslantas EE, Buzoglu HD, Altundasar E, Serper A. Effect of EDTA, sodium hypochlorite, and chlorhexidine gluconate with or without surface modifiers on dentin microhardness. J Endod. 2014;40(6):876-9.

13. Baldasso FER, Roleto L, Silva VDD, Morgental RD, Kopper PMP. Effect of final irrigation protocols on microhardness reduction and erosion of root canal dentin. Braz Oral Res. 2017;31:e40.

14. Kato AS, Cunha RS, da Silveira Bueno CE, Pelegrine RA, Fontana CE, de Martin AS. Investigation of the Efficacy of Passive Ultrasonic Irrigation Versus Irrigation with Reciprocating Activation: An Environmental Scanning Electron Microscopic Study. J Endod. 2016;42(4):659-63.

15. Simezo AP, da Silveira Bueno CE, Cunha RS, Pelegrine RA, Rocha DG, de Martin AS, et al. Comparative Analysis of Dentinal Erosion after Passive Ultrasonic Irrigation versus Irrigation with Reciprocating Activation: An Environmental Scanning Electron Study. J Endod. 2017;43(1):141-6.

16. Marques AC, Aguiar BA, Frota LM, Guimaraes BM, Vivacqua-Gomes N, Vivan RR, et al. Evaluation of Influence of Widening Apical Preparation of Root Canals on Efficiency of Ethylenediaminetetraacetic Acid Agitation Protocols: Study by Scanning Electron Microscopy. J Contemp Dent Pract. 2018;19(9):1087-94.

17. Duque JA, Duarte MA, Canali LC, Zancan RF, Vivan RR, Bernardes RA, et al. Comparative Effectiveness of New Mechanical Irrigant Agitating Devices for Debris Removal from the Canal and Isthmus of Mesial Roots of Mandibular Molars. J Endod. 2017;43(2):326-31.

18. Rodrigues CT, Duarte MAH, Guimaraes BM, Vivan RR, Bernardineli N. Comparison of two methods of irrigant agitation in the removal of residual filling material in retreatment. Braz Oral Res. 2017;31:e113.

19. Ulusoy OI, Gorgul G. Effects of different irrigation solutions on root dentine microhardness, smear layer removal and erosion. Aust Endod J. 2013;39(2):66-72.

20. Soares CJ, Pizi EC, Fonseca RB, Martins LR. Influence of root embedment material and periodontal ligament simulation on fracture resistance tests. Braz Oral Res. 2005;19(1):11-6.

21. Habelitz S, Marshall SJ, Marshall GW, Jr., Balooch M. Mechanical properties of human dental enamel on the nanometre scale. Arch Oral Biol. 2001;46(2):173-83.

22. Rosatto CMP, Oliveira LV, Ferraz DC, Soares PBF, Soares CJ, Moura CCG. Apical Displacement and Residual Root Canal Filling with Single-Cone After Post Space Preparation: A Micro-CT Analysis. Braz Dent J. 2020;31(1):25-31.

23. Long W, Li J, Liu Y, Jiang H. Effect of obturation technique with immediate and delayed post space preparation on apical voids and bond strength of apical gutta-percha. J Int Med Res. 2019;47(1):470-80.

24. Souza CC, Bueno CE, Kato AS, Limoeiro AG, Fontana CE, Pelegrine RA. Efficacy of passive ultrasonic irrigation, continuous ultrasonic irrigation versus irrigation with reciprocating activation device in penetration into main and simulated lateral canals. J Conserv Dent. 2019;22(2):155-9.

25. Bueno CRE, Cury MTS, Vasques AMV, Sarmiento JL, Trizzi JQ, Jacinto RC, et al. Cleaning effectiveness of a nickel-titanium ultrasonic tip in ultrasonically activated irrigation: a SEM study. Braz Oral Res. 2019;33:e017.

26. Cesario F, Hungaro Duarte MA, Duque JA, Alcalde MP, de Andrade FB, Reis So MV, et al. Comparisons by microcomputed tomography of the efficiency of different irrigation techniques for removing dentinal debris from artificial grooves. J Conserv Dent. 2018;21(4):383-7.

27. Pascon FM, Kantovitz KR, Soares LE, Santo AM, Martin AA, Puppin-Rontani RM. Morphological and chemical changes in dentin after using endodontic agents: fourier transform Raman spectroscopy, energy-dispersive x-ray fluorescence spectrometry, and scanning electron microscopy study. J Biomed Opt. 2012;17(7):075008.

28. De-Deus G, Paciornik S, Mauricio MH. Evaluation of the effect of EDTA, EDTAC and citric acid on the microhardness of root dentine. Int Endod J. 2006;39(5):401-7.

FIGURE LEGENDS

Figure 1 – Schematic figure of the methodologies for micro-CT and Knoop test: (A) Sample placed in a standardized position during the micro-CT scans, using a custom device; (B) the region to be analyzed was divided to represent the cervical, middle and apical levels in the PSP, with 130 slices at each level; (C) two 1-mm-thick root slices were obtained from each PSP level; (D) four indentations were made around the canal lumen at three dentin depths.

Figure 2 – 3D models for residual filling material before and after the cleaning protocols: (A) NaOCl/MI, (B) NaOCl/PUI, (C) NaOCl/EC, (D) NaOCl/EDTA/MI, (E) NaOCl/EDTA/PUI, (F) NaOCl/EDTA/EC.

TABLES

Table 1 - The mean ± standard deviation values of residual filling material (%) for each PSI subgroup, regardless of the third level

	NaOCl	NaOCl/EDTA
MI (control)	8.28 ± 2.08 Cb	3.88 ± 1.64 Aa
PUI	3.23 ± 1.54 Ba	3.66 ± 1.12 Aa
EC	0.41 ± 0.21 Aa	1.02 ± 0.48 Aa

Uppercase letters were used to compare each irrigant among the activation methods (columns); lowercase letters were used to compare the irrigants using the same activation method (rows). Different letters indicate significant differences based on the Tukey test ($P < .05$).

Table 2 - The means and standard deviations of dentin microhardness at the three root levels (cervical, middle and apical)

PSI level	Cervical			Middle			Apical		
Root depth	X	Y	Z	X	Y	Z	X	Y	Z
Control group	37.07± 3.44	39.05± 3.83	41.80± 2.71	35.33± 2.31	37.94± 2.16	41.29± 1.62	35.56± 4.23	37.85± 5.11	40.22± 5.56
NaOCl/ MI	33.50± 0.79	35.77± 0.52	39.13± 0.53	34.42± 4.03	37.83± 4.95	40.61± 4.82	32.45± 3.98	33.79± 4.94	37.38± 5.85
NaOCl/ PUI	33.86± 4.92	37.87± 6.54	41.91± 7.89	31.93± 3.81	36.64± 2.73	41.62± 3.35	31.75± 2.63	35.59± 3.09	40.15± 3.70
NaOCl/ EC	33.98± 2.08	36.82± 2.29	42.43± 2.91	32.48± 4.35	35.15± 4.10	39.21± 4.85	33.90± 2.99	37.02± 4.36	44.03± 2.72
NaOCl/ EDTA/ MI	38.46± 4.35	40.83± 4.61	43.55± 4.96	34.89± 3.13	37.48± 2.20	40.32± 3.62	34.92± 4.74	37.53± 5.26	42.05± 6.19
NaOCl/ EDTA/ PUI	34.85± 4.20	38.43± 3.49	43.48± 2.34	33.28± 2.41	36.13± 2.59	39.64± 3.30	33.11± 1.10	35.61± 1.03	41.31± 2.02
NaOCl/ EDTA/ EC	32.04± 2.51	35.74± 3.74	40.56± 4.74	33.60± 3.91	37.65± 5.84	41.70± 8.57	30.65± 2.22	34.04± 3.12	39.26± 3.84

EDITORIAL CERTIFICATION

TO WHOM IT MAY CONCERN

This document attests that the manuscript below was edited for proper English language usage, grammar, punctuation, spelling and style by the undernamed, a native English-speaking copyeditor, B.A. Hofstra University, NY, and owner of NCristina Martorana Traduções S/C Ltda. [CNPJ: 04.959.659/0001-20]. Neither the research content nor the author's/authors' intentions were altered in any way during the editing process.

Should there be any questions, please contact: ncris.mart@uol.com.br.

Manuscript title:	*Effect of irrigation protocols on root canal wall after post preparation: a micro-CT and microhardness study*
Corresponding author:	Mario Tanomaru-Filho
Co-authors:	Camila Maria Peres de Rosatto, Danilo Cassiano Ferraz, Lilian Vieira Oliveira, Priscilla Barbosa Ferreira Soares, Carlos José Soares, Camilla Christian Gomes Moura
Date:	March 24, 2021
Copyeditor:	Nancy Cristina Martorana

Figures

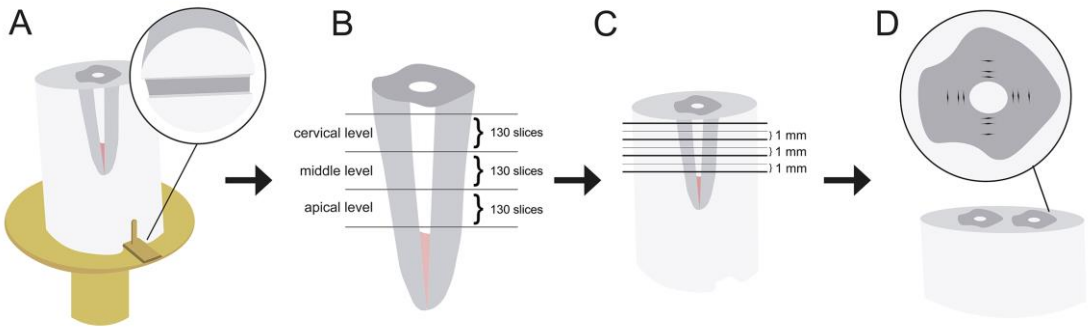


Figure 1 **–** Schematic figure of the methodologies for micro-CT and Knoop test: (A) Sample placed in a standardized position during the micro-CT scans, using a custom device; (B) the region to be analyzed was divided to represent the cervical, middle and apical levels in the PSP, with 130 slices at each level; (C) two 1-mm-thick root slices were obtained from each PSP level; (D) four indentations were made around the canal lumen at three dentin depths.

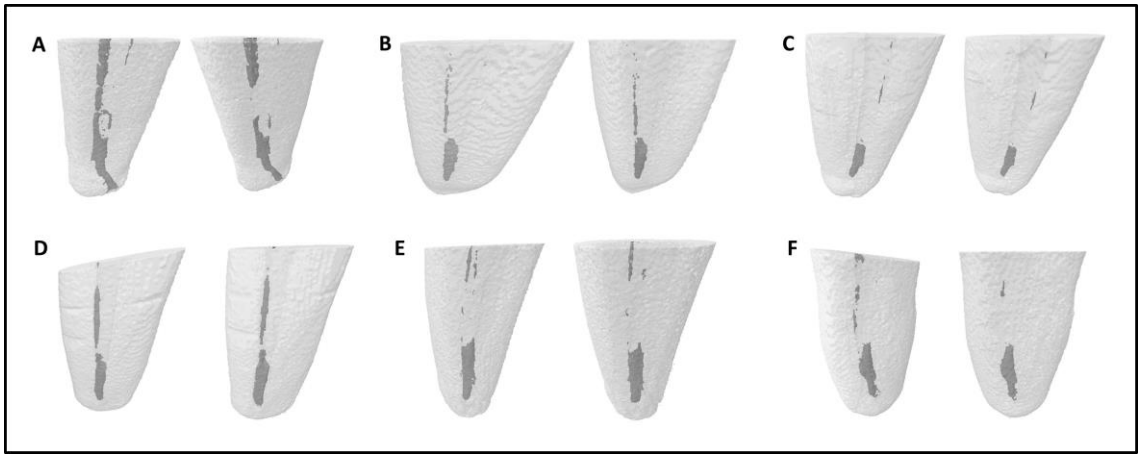


Figure 2 **–** 3D models for residual filling material before and after the cleaning protocols: (A) NaOCl/MI, (B) NaOCl/PUI, (C) NaOCl/EC, (D) NaOCl/EDTA/MI, (E) NaOCl/EDTA/PUI, (F) NaOCl/EDTA/EC.

CAPÍTULO 5

3.5 Capítulo 5

Artigo submetido no periódico Iranian Endodontic Journal

Effect of solutions and passive ultrasonic irrigation in the composition of calcium silicate-based sealers after post space preparation

Camila Maria Peres de Rosatto,[1] Danilo Cassiano Ferraz,[1] Marcelo José Barbosa Silva,[2] Carlos José Soares,[3] Camilla Christian Gomes Moura,[1*]

[1] Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil.

[2] Department of Immunology, Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, MG, Brazil.

[3] Department of Operative Dentistry and Dental Materials, Federal University of Uberlândia, Uberlândia, MG, Brazil.

Correspondence: Camilla Christian Gomes Moura, Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Av Pará 1720, Block 4L, annex A, class 24, Campus Umuarama, Uberlândia, MG, 38400-902, Brazil (Tel.: (+55) 34 3225-8103; e-mail: camillamoura@ufu.br).

Effect of solutions and passive ultrasonic irrigation in the composition of calcium silicate-based sealers after post space preparation

Abstract

Introduction: To evaluate the change in the composition of fresh or set calcium silicate-based sealers after cleaning protocols in a simulated root canal with post space preparation.

Methods and Material: Devices were created with post space preparation. Three calcium silicate-based sealers were tested: EndoSequence BC Sealer, BioRoot RCS, and Bio-C Sealer; moreover, they were tested in two conditions: fresh and set. The devices with sealers (n=5) were irrigated using: 2.5% sodium hypochlorite; 2.5% sodium hypochlorite with passive ultrasonic irrigation; 2.5% sodium hypochlorite followed by 17% ethylenediaminetetraacetic acid (EDTA); and 2.5% sodium hypochlorite followed by 17% EDTA with passive ultrasonic irrigation. Fresh and set calcium silicate-based sealers were used as control. The compositions were analyzed by two common spectra for Raman, zirconium oxide and calcium silicate, and with energy-dispersive x-ray spectroscopy. Raman data were analyzed using ANOVA (α=0.05). Energy-dispersive x-ray spectroscopy data were analyzed qualitatively.

Results: In fresh conditions, sodium hypochlorite followed by EDTA with or without activation resulted in a significant change in both spectra (P<0.05). After setting, only Bio-C Sealer showed a significant change in the calcium silicate spectrum using sodium hypochlorite, followed by EDTA with passive ultrasonic irrigation (P<0.05). Energy-dispersive x-ray spectroscopy showed a reduction in the zirconium, calcium, and silicon levels, mainly when associated with passive ultrasonic irrigation.

Conclusion: Despite the irrigation procotol used, the post space cleaning caused fewer changes in calcium silicate-based sealer composition when performed after setting.

Keywords: Canal Irrigants; Canals Sealer; Raman Spectroscopy; Silicates; X-ray Emission Spectroscopy

Introduction

Endodontically treated structurally compromised teeth may require fiberglass posts (FGPs) to provide adequate retention of crowns [1]. The post space preparation (PSP) may be performed immediately or delayed after obturation [2]. There is no consensus regarding the best moment for PSP [3], especially for teeth obturated with the single-cone technique [4] and calcium silicate-based endodontic sealers (CSBS) [5]. This type of sealer has been used extensively in clinical practice for its bioactivity and biocompatibility [6], antimicrobial activity [7] and hydroxyapatite formation, increasing biomineralization ability [8]. It promotes a hermetical sealing of root canals, due to its good adhesion to dentine walls [9, 10].

Apart from the sealer used in filling, the remnants after PSP become a barrier for adhesive integration to root dentin [11, 12]. Different cleaning protocols and solutions have been proposed to improve canal cleanliness after PSP [13-15]. A recent systematic review suggested the use of sodium hypochlorite (NaOCl) associated with ethylenediaminetetraacetic acid (EDTA) as final irrigants to improve the retention of FGPs [12]. The use of passive ultrasonic irrigation (PUI) has been more effective than syringe needle irrigation in debris removal prior to post installation [14]. However, there is a lack of information on whether irrigation and activation protocols affect CSBS compositions at the apical root canal after PSP. To the best of the authors' knowlegde, no study has tested the effect of PUI with different solutions on fresh or after setting CSBS compositions.

Therefore, this study aimed to analyze the effect of manual irrigation or PUI and solutions used to clean PSP simulated root canal in the composition of fresh or set CSBS.The

null hypothesis was that solutions and the activation protocol do not change the fresh and after setting sealers' compositions.

Material and methods

Device development

The human central incisor was selected for the creation of the device (Ethics Committee of Federal University of Uberlândia #2.570.084). The crown was removed to obtain a remnant of 15.0 mm, which was prepared with ProTaper Next (Dentsply, Sirona, York, Pennsylvania, USA), and obturated with the single-cone technique, using AH Plus (Dentsply, Sirona, York, Pennsylvania, USA). After one day, the PSP was created, using Largo drills #3 and #4 under irrigation with saline solution. Digital radiography was performed to check the permanence of apical sealing at 4 mm.

The root canal was molded with acrylic resin and a metallic core was made. The core was positioned in a glass vial containing a transparent methyl methacrylate (Sigma-Aldrich, Saint Luis, Missouri, USA) solution (MMA) and kept in an incubator at 36 ºC for 48 hours for polymerization. A copy of the PSP was obtained. The device was designed in two parts that fit together (Figure 1A and 1B).

The upper part mimics the PSP, to which the irrigation and activation protocols were applied, and the CSBS was inserted in the acrylic plate at the base. Five devices with two parts each were made, allowing the preparation of samples for each group. However, the acrylic plate with the central hole was made individually for each sample. A central hole on the ending portion of the PSP allowed the fresh or set CSBS to contact the irrigation and activation protocols.

Irrigation and activation protocols

Three CSBS were tested: ENDS, EndoSequence BC Sealer (Brasseler, Savannah, Georgia, USA); BIOR, BioRoot RCS (Septodont, Saint-Maur-des Fossés, Paris, France); and BIOC, Bio-C Sealer (Angelus, Londrina, Paraná, Brazil). A sample size was calculated using G*Power v3.1.9.2 (program written, concept and design by Franz, Universitat Kiel, Germany; freely available Windows application software) based on 0.8 power, α=0.05, and a significant difference of 50% of ZO or CS levels compared to baseline [16].

ENDS and BIOC are ready-to-use and BIOR was prepared according to the manufacturer's recommendations. Each sealer was tested freshly at 1 hour and after setting, waiting for 24 hours, maintaining the sample at 37 ºC in 100% relative humidity [10]. The irrigants were used with or without PUI with a non-cutting E1-Irrisonic tip with .01 taper/20 ISO size (Helse Dental Technology, Santa Rosa de Viterbo, São Paulo, Brazil), mounted on an ultrasonic unit of Piezon Master 200 (EMS, São Bernardo do Campo, São Paulo, Brazil) set to power 2. The activation tip was also placed 1 mm away from the sealer. The fresh and set control groups for each sealer (n=5) did not pass the irrigation and activation protocols, considering that the sealers were only deposited on the acrylic plate for later analyses.

One experienced operator performed the protocols using two solutions: 6 mL of 2.5% NaOCl, (Asfer Indústria Química, São Caetano do Sul, São Paulo, Brazil) in six cycles, 1 mL per cycle [13] for 20 seconds [17, 18]; and NaOCl/EDTA, 2.5% NaOCl followed by 17% EDTA (Maquira, Maringá, Paraná, Brazil) using 3 cycles of 1 mL of NaOCl followed by 3 cycles of 1 mL of EDTA, resulting in the same 6 mL of irrigants during the same 20 seconds. The irrigation was performed with a 30-g open-ended needle (NaviTip, Ultradent, South Jordan, Utah, USA) inserted in the device canal 1 mm short of the ending portion of the PSP. A final flush with 10 mL of distilled water was performed without activation. The experimental group were compared with the same control sealer group after different protocols.

Raman spectroscopy

The sealers were evaluated using a conventional Raman scattering spectroscope (LabRAM HR Evolution, Horiba Scientific, Kyoto, Kansai, Japan), equipped with a 633-nm helium-neon laser beam at room temperature. A x10 numerical aperture objective lens was used with a 4.25 mW power laser beam and a 600 grooves/mm diffraction grating, with 6 acquisitions per point of 15 seconds each. The spectra were analyzed using OriginPro v9.6.5.169 software (OriginLab Corporation, Northampton, Massachusetts, USA).

Analyses of the principal components in the CSBS were selected (n=5): zirconium oxide (ZO) and calcium silicate (CS) spectra. The spectra were standardized by the baseline and normalized to the 1 cm^{-1} Raman peak's height (Figure 2). Preprocessing is used to minimize the variations induced by the experimental set-up. Eleven peaks for the ZO spectrum and four peaks for the CS spectrum were collected using the Peak Analyzer tool (OriginLab Corporation, Northampton, Massachusetts, USA). Data from the same experimental group were averaged and plotted with average spectra of the respective control sealer for quantitative comparison. The mean component peaks were analyzed quantitatively. All analyses were blinded for the CSBS and protocols used.

Energy-dispersive X-ray spectroscopy (EDS)

The elemental compositions of the sealers' principal components were obtained with EDS analysis. The specimens were mounted on aluminum stubs using cyanoacrylate-based adhesive (Super Bonder, Loctite, Itapeví, São Paulo, Brazil) and gold-sputtered with a Leica EM SCD050 table-top device (Wetzlar, Hessen, Germany). At a magnification of 300x and voltage of 20 kV, the EDS detector (Oxford model 51-ADD0048, Abingdon, Oxfordshire, England) coupled to a scanning electron microscopy (model EVO 10, Carl Zeiss, Oberkochen, Baden-Württemberg, Germany) was used to chemically map the CSBS composition at three points for the sample. The principal elements of the CSBS were chosen: zirconium (Zr),

calcium (Ca), and silicon (Si). The average of the three points in each CSBS sample was obtained. The mean proportion of the elements was analyzed qualitatively.

Statistical analysis

The data were analyzed for normal distribution, using the Shapiro-Wilk test, and homoscedasticity, using the Levene test. Two-way ANOVA and Tukey's test were used to analyze control groups for ZO and CS, comparing sealer factor (3 levels) and conditions (2 levels). The ZO and CS data of fresh and set control groups were compared with all experimental groups for each condition, using one-way ANOVA and Dunnett test. To compare the cleaning protocols for each sealer, excluding the control group, two-way ANOVA and Sidak's multiple comparisons tests were used. Statistical significance was set at α=0.05 and all analyses were carried out using the GraphPad Prism software v8.2.0 (San Diego, California, USA).

Results

Raman spectroscopy

The mean Raman spectra obtained after irrigation and activation protocols, compared to the respective fresh or set CSBS control groups, are shown in Figure 3. Raman exhibited vibrations at 140-700 cm^{-1} for the ZO band, and at 710-960 cm^{-1} for the CS band [16]. Comparisons for ZO and CS spectra between the fresh and set control groups are presented in Table 1.

Fresh and set sealer after irrigation and activation protocols, compared with the control groups, are shown in Table 2 and Table 3, respectively. Fresh BIOC and BIOR were not affected by cleaning protocols for the ZO spectrum (P=0.609 and P=0.520). The CS spectrum of fresh BIOC in irrigated and activated groups changed when compared with their respective control groups (P=0.001). Peak intensities of 836 and 891 cm^{-1} decreased for fresh BIOC protocols (Figure 3A). For fresh BIOR, the control group only showed siginificant CS spectrum

alteration for non-activated NaOCl/EDTA (P=0.021), and the 813-882 cm^{-1} peaks showed a decrease using the protocols (Figure 3B). Fresh ENDS (Figure 3C) had significant ZO spectrum change when using NaOCl/EDTA (P=0.049) and NaOCl/EDTA/PUI (P=0.026). An increased 472 peak was observed when NaOCl/EDTA protocol was used and decreased 254-379 peaks when NaOCl/EDTA/PUI was used. The CS spectrum of fresh ENDS was significantly lower in all protocols, compared with their fresh control (P<0.001), presenting a greater reduction in the 809-893 peaks, especially for the NaOCl/EDTA/PUI (Table 2).

Table 3 shows the set CSBS comparisons after cleaning protocols and the control group. For BIOC (Figure 3D), the ZO spectrum was similar for all conditions (P=0.198). However, the CS spectrum changed for BIOC when NaOCl/EDTA/PUI (P=0.048) was used, with reduced intensities of 808 and 907 peaks. For BIOR (Figure 3E) and ENDS (Figure 3F), ZO and CS spectra showed no change produced by cleaning protocols, compared with their controls (P>0.05). The interaction between protocols in the same CSBS had no significance for the ZO and CS bands (P>0.05, Tables 2 and 3).

EDS spectroscopy

The mean proportion (%) of the principal elements in CSBS is presented in Table 4. All control groups detected the presence of Zr, Ca, and Si. Fresh CSBS showed less Zr after the cleaning protocols for all tested sealers, except for ENDS without activation. The Ca and Si levels decreased for activated protocols. In set CSBS, Zr levels were reduced in all protocols for BIOC and ENDS. The BIOR and ENDS groups had predominantly Ca and Si reduction only when NaOCl/PUI was used. When the element was not detected by the EDS, the condition was represented by “ND”.

Discussion

The solutions and activation protocol tested caused significant composition changes of the fresh and set CSBS; therefore, the null hypothesis was rejected. The quantitative elemental maps of the chemical composition of materials obtained by EDS [19] should be interpreted with caution, because they are more predisposed to errors, relative to the wavelength-dispersive spectrometry technique [20]. Therefore, only the qualitative analyses of the chemical elements were determined. The ENDS [21], BIOR [22] and BIOC [6] contain ZO and CS bands and mineral portions composed of Zr, Ca, and Si. The EDS analysis confirmed the presence of these elements in the control groups. Additionaly, the ZO and CS spectra were detected by the Raman analysis.

Raman is used to obtain the chemical profile of the materials [23]. This methodology has been efficiently used to quantify the composition of CSBS influenced by different environmental conditions [16, 24]. The presence of the ZO spectrum or Zr element in the CSBS composition is important to provide adequate radiopacity ISO 6876/2001 [6, 25], replacing bismuth oxide as a radiopacifier. It allows higher and longer release of Ca^{2+}, improving physical properties and biological response [26, 27]. However, their radiopacity levels are lower than those of the other types of endodontic sealers [24, 25, 28]. Significant changes in Zr after PSP and cleaning protocols may change the visualization of the obturation, affecting long-term evaluations.

The presence of the CS or Ca and Si elements is also extremely relevant and the most important component in CSBS [24, 29, 30, 7]. The Ca^{2+} is responsible for antibacterial activity, due to alkaline pH [7]. Associated with Si element, Ca^{2+} results in the bioactive potential for hard tissue formation [31, 32]. Therefore, changes in these components, affected by irrigation and activation protocols, can modify the CSBS biomineralization action. A previous study [33] demonstrated that irrigation with NaOCl and EDTA interferes in calcium release by tricalcium silicate, and negatively influences the bioactivity. Physicochemical properties, such as sealing

ability [31], microhardness [34], and solubility [24, 35], may also suffer interference from the cleaning protocols.

CSBS have high solubility in water [6, 36, 37], which may be related to hydrophilic nanoparticles that increase the surface area, allowing the liquid molecules to come into contact with the sealer [31]. Although the present study did not assess solubility, it is possible to speculate that, for fresh CSBS groups, chemical changes promoted by irrigation and activation protocols may result in increased solubility. The solubilization of CSBS results in the release of OH^- and Ca^{2+} [35], and explains the CS reduction. However, moisture is essential for CSBS setting reaction, given that water connection affects the process of hydroxyapatite layer formation [7, 31, 35]. The presence of voids within the filling [38, 39], caused by ultrasonic activation, may have facilitated the infiltration of irrigating solutions in the remaining CSBS and modified its composition [11].

To simulate this condition, individual devices were created, mimicking a human root prepared to receive a FGP focusing only on sealers, without interference from dentin or gutta-percha. These devices provided more reproducibility, allowing the analysis at the apical third, where the effect of irrigants is more impactful [13, 14]. However, the dynamics of interactions of CSBS with the dentin cannot be evaluated for this device, which is a limitation of the study.

No modification was observed in the ZO spectrum in both fresh and set groups, except for fresh ENDS when irrigated with NaOCl/EDTA associated or not with activation. It is may be related to the oxidizing effect of NaOCl, associated with the chelating action of EDTA. Changes in the CS spectrum observed for fresh BIOC and ENDS indicated that the cleaning procedures should not be performed immediately after PSP, which was observed by EDS analysis.

Despite the limitations of the present study, fresh CSBS should not be subjected to irrigation and activation protocols after PSP. After setting, only BIOC irrigated with NaOCl/EDTA/PUI altered CS spectra; it is conceivable that the acoustic streaming and

ultrasound cavitation have increased the effect of irrigating solutions [11]. Further studies are needed to address clinical recommendations.

Conclusions

This study concluded that despite the irrigation procotol used, the post space cleaning caused fewer changes in CSBS composition when performed after setting. This finding suggests that professionals should wait for the setting reaction of these CSBS before applying cleaning protocols.

Acknowledgments

The authors are grateful for the financial support provided by FAPEMIG, CNPq, and CAPES – Finance Code 001. They are also grateful to the multipurpose laboratories - New Insulating Materials and Semiconductors (LNMIS) at the Institute of Physics and Scanning Electron Microscopy Laboratory of the Faculty of Chemical Engineering at the Federal University of Uberlândia (Uberlândia, Minas Gerais, Brazil). They are also grateful for the valuable assistance of Guilherme de Lima Fernandes and Rafael Ramos Heilbuth with Raman and EDS spectroscopy, respectively. This study was carried out at the CPBio - Biomechanics, Biomaterials and Cell Biology Research Center of the Federal University of Uberlândia.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Ahmed SN, Donovan TE, Ghuman T. Survey of dentists to determine contemporary use of endodontic posts. J Prosthet Dent. 2017;117(5):642–645. doi:10.1016/j.prosdent.2016.08.015
2. Nagas E, Karaduman E, Sahin C, Uyanik O, Canay S. Effect of timing of post space preparation on the apical seal when using different sealers and obturation techniques. J Dent Sci. 2016;11(1):79–82. doi:10.1016/j.jds.2012.05.017
3. Long W, Li J, Liu Y, Jiang H. Effect of obturation technique with immediate and delayed post space preparation on apical voids and bond strength of apical gutta-percha. J Int Med Res. 2019;47(1):470–480. doi:10.1177/0300060518814604
4. Rosatto CMP, Oliveira LV, Ferraz DC, Soares PBF, Soares CJ, Moura CCG. Apical displacement and residual root canal filling with single-cone after post space preparation: a micro-CT analysis. Braz Dent J. 2020;31(1):25-31. doi:10.1590/0103-6440202002597
5. Bengoa FP, Arze MCM, Noguera CM, Moreira LFN, Kato AS, Bueno CEDS. Effect of ultrasonic cleaning on the bond strength of fiber posts in oval canals filled with a premixed bioceramic root canal sealer. Restor Dent Endod. 2020;45(2):e19. doi:10.5395/rde.2020.45.e19
6. Zordan-Bronzel CL, Esteves Torres FF, Tanomaru-Filho M, Chávez-Andrade GM, Bosso-Martelo R, Guerreiro-Tanomaru JM. Evaluation of physicochemical properties of a new calcium silicate-based sealer, Bio-C sealer. J Endod. 2019;45(10):1248–1252. doi:10.1016/j.joen.2019.07.006
7. Donnermeyer D, Bürklein S, Dammaschke T, Schäfer E. Endodontic sealers based on calcium silicates: a systematic review. Odontology. 2019;107(4):421–436. doi:10.1007/s10266-018-0400-3

8. Benetti F, Queiroz ÍOA, Cosme-Silva L, Conti LC, Oliveira SHP, Cintra LTA. Cytotoxicity, Biocompatibility and Biomineralization of a New Ready-for-Use Bioceramic Repair Material. Braz Dent J. 2019;30(4):325-332. doi:10.1590/0103-6440201902457
9. Shokouhinejad N, Gorjestani H, Nasseh AA, Hoseini A, Mohammadi M, Shamshiri AR. Push-out bond strength of gutta-percha with a new bioceramic sealer in the presence or absence of smear layer. Aust Endod J. 2013;39(3):102-106. doi:10.1111/j.1747-4477.2011.00310.x
10. Silva Almeida LH, Moraes RR, Morgental RD, Pappen FG. Are Premixed Calcium Silicate-based Endodontic Sealers Comparable to Conventional Materials? A Systematic Review of In Vitro Studies. J Endod. 2017;43(4):527-535. doi:10.1016/j.joen.2016.11.019
11. Chen X, Liu H, He Y, Luo T, Zou L. Effects of endodontic sealers and irrigation systems on smear layer removal after post space preparation. J Endod. 2018;44(8):1293–1297. doi:10.1016/j.joen.2018.05.014
12. Oliveira LV, Maia TS, Zancopé K, Menezes MS, Soares CJ, Moura CCG. Can intra-radicular cleaning protocols increase the retention of fiberglass posts? a systematic review. Braz Oral Res. 2018;32:e16. doi:10.1590/1807-3107bor-2018.vol32.0016
13. Akyuz Ekim SN, Erdemir A. Effect of different irrigant activation protocols on push-out bond strength. Lasers Med Sci. 2015;30(8):2143–2149. doi:10.1007/s10103-015-1772-z
14. Lo Giudice G, Lizio A, Giudice RL, Centofanti A, Rizzo G, Runci M, Alibrandi A, Cicciù M. The effect of different cleaning protocols on post space: a SEM study. Int J Dent. 2016;2016:1907124. doi:10.1155/2016/1907124
15. Poletto D, Poletto AC, Cavalaro A, Machado R, Cosme-Silva L, Garbelini CCD, Hoeppner MG. Smear layer removal by different chemical solutions used with or without ultrasonic activation after post preparation. Restor Dent Endod. 2017;42(4):324–331. doi:10.5395/rde.2017.42.4.324

16. Atmeh AR, AlShwaimi E. The effect of heating time and temperature on epoxy resin and calcium silicate-based endodontic sealers. J Endod. 2017;43(12):2112–2118. doi:10.1016/j.joen.2017.08.008

17. Kato AS, Cunha RS, da Silveira Bueno CE, Pelegrine RA, Fontana CE, de Martin AS. Investigation of the efficacy of passive ultrasonic irrigation versus irrigation with reciprocating activation: an environmental scanning electron microscopic study. J Endod. 2016;42(4):659–663. doi:10.1016/j.joen.2016.01.016

18. Simezo AP, da Silveira Bueno CE, Cunha RS, Pelegrine RA, Rocha DGP, Martin AS, Kato AS. Comparative analysis of dentinal erosion after passive ultrasonic irrigation versus irrigation with reciprocating activation: an environmental scanning electron study. J Endod. 2017;43(1):141–146. doi:10.1016/j.joen.2016.09.016

19. Prasad PS, Sam JE, Kumar A, Kannan. The effect of 5% sodium hypochlorite, 17% EDTA and Triphala on two different rotary Ni-Ti instruments: an AFM and EDS analysis. J Conserv Dent. 2014;17(5):462–466. doi:10.4103/0972-0707.139842

20. Newbury DE, Ritchie NW. Is scanning electron microscopy/energy dispersive X-ray spectrometry (SEM/EDS) quantitative? Scanning. 2013;35(3):141–168. doi:10.1002/sca.21041

21. Carvalho CN, Grazziotin-Soares R, de Miranda Candeiro GT, Martinez LG, Souza JP, Oliveira PS, Bauer J, Gavini G. Micro push-out bond strength and bioactivity analysis of a bioceramic root canal sealer. Iran Endod J. 2017;12(3):343–348. doi:10.22037/iej.v12i3.16091

22. Parirokh M, Torabinejad M, Dummer PMH. Mineral trioxide aggregate and other bioactive endodontic cements: an updated overview - part I: vital pulp therapy. Int Endod J. 2018;51(2):177–205. doi:10.1111/iej.12841

23. Wang Y, Yao X. Morphological/chemical imaging of demineralized dentin layer in its natural, wet state. Dent Mater. 2010;26(5):433–442. doi:10.1016/j.dental.2010.01.002

24. Siboni F, Taddei P, Zamparini F, Prati C, Gandolfi MG. Properties of BioRoot RCS, a tricalcium silicate endodontic sealer modified with povidone and polycarboxylate. Int Endod J. 2017;50(suppl 2):e120-e136. doi:10.1111/iej.12856
25. Khalil I, Naaman A, Camilleri J. Properties of tricalcium silicate sealers. J Endod. 2016;42(10):1529–1535. doi:10.1016/j.joen.2016.06.002
26. Silva GF, Bosso R, Ferino RV, Tanomaru-Filho M, Bernardi MIB, Guerreiro-Tanomaru JM, Cerri PS. Microparticulated and nanoparticulated zirconium oxide added to calcium silicate cement: evaluation of physicochemical and biological properties. J Biomed Mater Res A. 2014;102(12):4336–4345. doi:10.1002/jbm.a.35099
27. Li X, Yoshihara K, De Munck J, Cokic S, Pongprueksa P, Putzeys E, Pedano M, Chen Z, Van-Landuyt K, Van-Meerbeek B. Modified tricalcium silicate cement formulations with added zirconium oxide. Clin Oral Investig. 2017;21(3):895–905. doi:10.1007/s00784-016-1843-y
28. Jafari F, Jafari S. Composition and physicochemical properties of calcium silicate based sealers: a review article. J Clin Exp Dent. 2017;9(10):e1249–e1255. doi:10.4317/jced.54103
29. Moinzadeh AT, Aznar Portoles C, Schembri Wismayer P, Camilleri J. Bioactivity potential of EndoSequence BC RRM putty. J Endod. 2016;42(4):615–621. doi:10.1016/j.joen.2015.12.004
30. Al-Haddad AY, Kutty MG, Abu Kasim NH, Che Ab Aziz ZA. The effect of moisture conditions on the constitution of two bioceramic-based root canal sealers. J Dent Sci. 2017;12(4):340–346. doi:10.1016/j.jds.2017.03.008
31. Al-Haddad A, Che Ab Aziz ZA. Bioceramic-based root canal sealers: a review. Int J Biomater. 2016;2016:9753210. doi:10.1155/2016/9753210

32. Giacomino CM, Wealleans JA, Kuhn N, Diogenes A. Comparative biocompatibility and osteogenic potential of two bioceramic sealers. J Endod. 2019;45(1):51–56. doi:10.1016/j.joen.2018.08.007

33. Neelakantan P, Berger T, Primus C, Shemesh H, Wesselink PR. Acidic and alkaline chemicals' influence on a tricalcium silicate-based dental biomaterial. J Biomed Mater Res B Appl Biomater. 2019;107(2):377-387. doi:10.1002/jbm.b.34129

34. Loushine BA, Bryan TE, Looney SW, Gillen BM, Loushine RJ, Weller RN, Pashley DH, Tay FR. Setting properties and cytotoxicity evaluation of a premixed bioceramic root canal sealer. J Endod. 2011;37(5):673–677. doi:10.1016/j.joen.2011.01.003

35. Borges RP, Sousa-Neto MD, Versiani MA, Rached-Júnior FA, De-Deus G, Miranda CES, Pécora JD. Changes in the surface of four calcium silicate-containing endodontic materials and an epoxy resin-based sealer after a solubility test. Int Endod J. 2012;45(5):419–428. doi:10.1111/j.1365-2591.2011.01992.x

36. Tanomaru-Filho M, Torres FFE, Chávez-Andrade GM, Almeida M, Navarro LG, Steier L, Gerreiro-Tanomaru JM. Physicochemical properties and volumetric change of silicone/bioactive glass and calcium silicate-based endodontic sealers. J Endod. 2017;43(12):2097–2101. doi:10.1016/j.joen.2017.07.005

37. Torres FFE, Zordan-Bronzel CL, Guerreiro-Tanomaru JM, Chávez-Andrade GM, Pinto JC, Tanomaru-Filho M. Effect of immersion in distilled water or phosphate-buffered saline on the solubility, volumetric change and presence of voids within new calcium silicate-based root canal sealers. Int Endod J. 2020;53(3):385–391. doi:10.1111/iej.13225

38. Al-Haddad A, Abu Kasim NH, Che Ab Aziz ZA. Interfacial adaptation and thickness of bioceramic-based root canal sealers. Dent Mater J. 2015;34(4):516–521. doi:10.4012/dmj.2015-049

39. Viapiana R, Moinzadeh AT, Camilleri L, Wesselink PR, Tanomaru Filho M, Camilleri J. Porosity and sealing ability of root fillings with gutta-percha and BioRoot RCS or AH Plus

sealers. Evaluation by three ex vivo methods. Int Endod J. 2016;49(8):774–782. doi:10.1111/iej.12513

Figure legends

Figure 1. Device images. (A) Schematic of the device design describing the dimensions in mm. (B) Device created in MMA.

Figure 2. Demonstrative spectra of Raman with the ZO and CS bands. The data for further analysis was taken from the highest point of the peak, represented by the asterisk.

Figure 3. Representative Raman spectra for fresh and set CSBS. (A) BIOC/fresh. (B) BIOR/fresh. (C) ENDS/fresh. (D) BIOC/set. (E) BIOR/set. (F) ENDS/set.

Figures

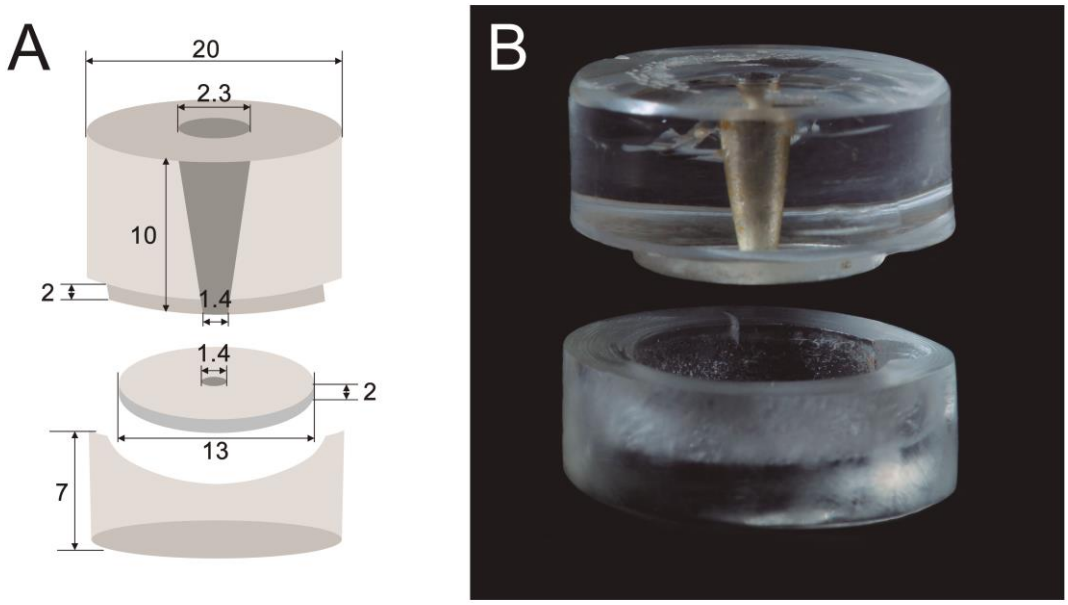


Figure 1

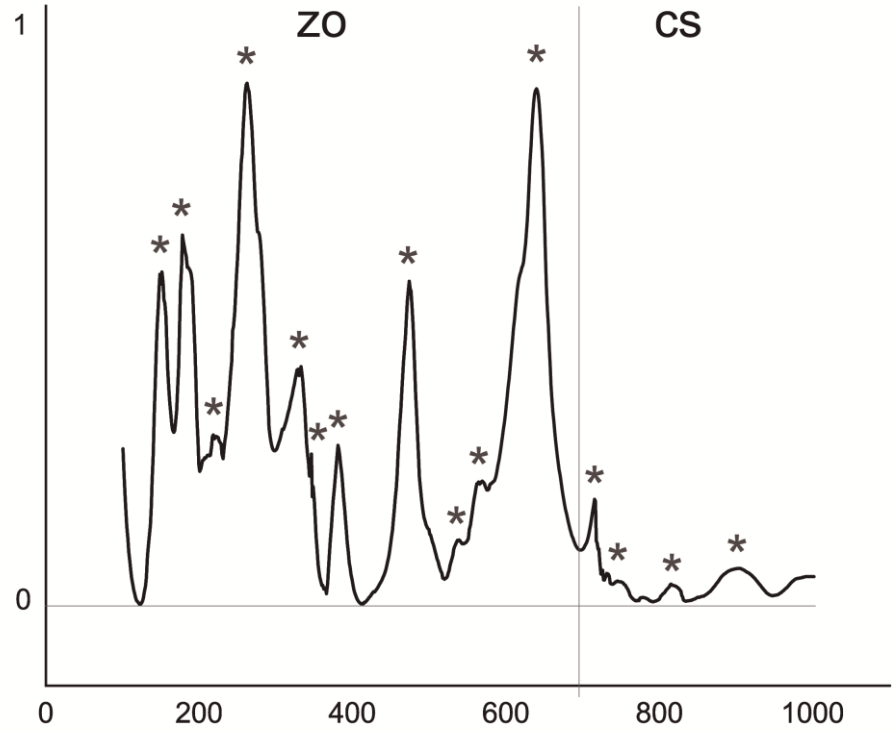


Figure 2

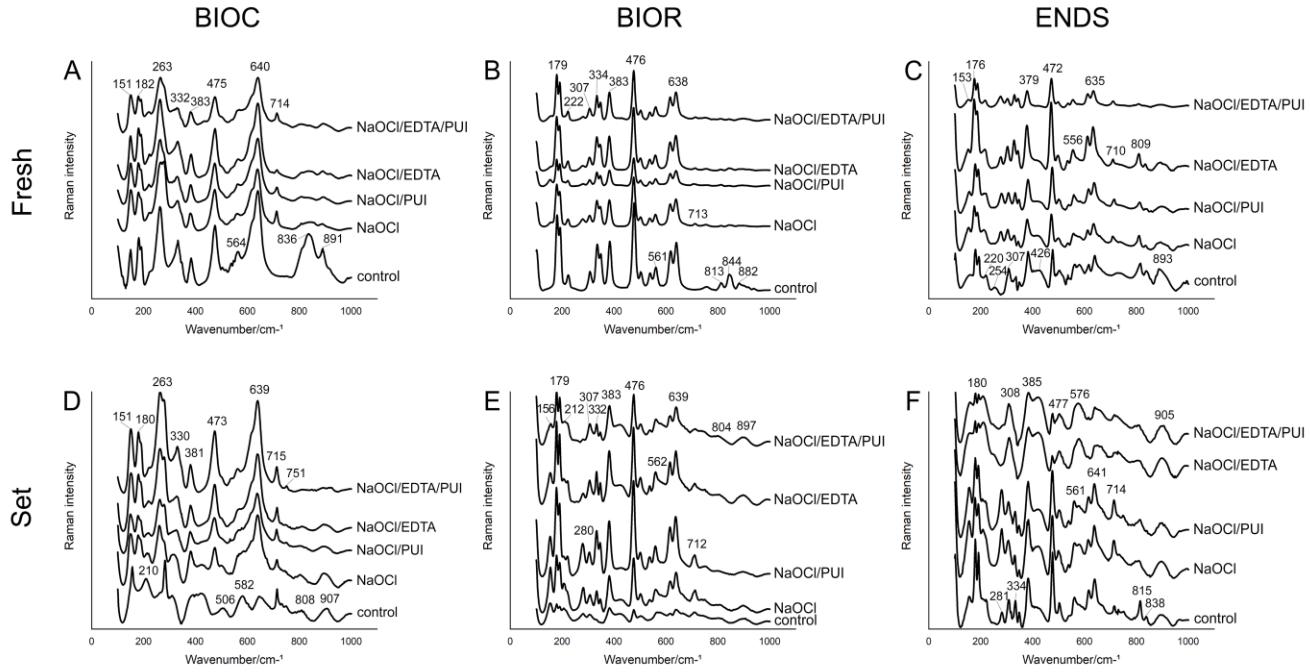

BIOC
BIOR
ENDS
Fresh
Set
A
B
C
D
E
F
Raman intensity
Wavenumber/cm-1
NaOCl/EDTA/PUI
NaOCl/EDTA
NaOCl/PUI
NaOCl
control
263
640
151 182
332
383
475
714
564
836
891
179
476
307
334
383
638
222
713
561
844
813
882
176
153
379
472
635
556
710
809
220
307
254
426
893
639
151
180
330
473
381
715
751
210
582
506
808
907
179
476
307
383
639
156
212
332
804
897
562
280
712
180
308
385
477
576
905
641
561
714
281
334
815
838
0
200
400
600
800
1000

Figure 3

Tables

Table 1. Comparisons of ZO and CS spectra for the fresh and set control groups for all tested CSBS

Control sealers	ZO spectrum		CS spectrum	
	Fresh	Set	Fresh	Set
BIOC	0.51±0.03 Ba	0.46±0.10 Ba	0.31±0.02 Ba	0.31±0.06 Aa
BIOR	0.53±0.01 Bb	0.61±0.10 Aa	0.11±0.02 Cb	0.34±0.07 Aa
ENDS	0.63±0.01 Aa	0.52±0.04 ABb	0.54±0.03 Aa	0.22±0.09 Bb

Different uppercaser letters represent the comparison between different CSBS in each condition; different lowercase letters represent the comparison between conditions for the same control CSBS in the same spectrum, calculated by using Tukey test ($P<0.05$).

Table 2. Comparison of the fresh CSBS control groups with their respective irrigated and activated groups as well as the interaction between protocols in the same CSBS, in ZO and CS spectra

Groups	ZO espectrum			CS spectrum		
	BIOC	BIOR	ENDS	BIOC	BIOR	ENDS
Fresh control	0.51±0.03	0.53±0.01	0.63±0.01	0.31±0.02	0.11±0.02	0.54±0.03
NaOCl	0.49±0.06 a	0.50±0.04 a	0.55±0.06 a	0.14±0.02 a*	0.05±0.01 a	0.28±0.08 a*
NaOCl/PUI	0.53±0.03 a	0.52±0.03 a	0.56±0.03 a	0.19±0.12 a*	0.10±0.07 a	0.19±0.10 a*
NaOCl/EDTA	0.54±0.02 a	0.50±0.02 a	0.51±0.13 a*	0.12±0.03 a*	0.04±0.01 a*	0.21±0.13 a*
NaOCl/EDTA/PUI	0.52±0.07 a	0.51±0.01 a	0.50±0.07 a*	0.13±0.03 a*	0.06±0.02 a	0.19±0.13 a*

* Means significance difference between control group and each experimental group for the same CSBS, calculated by using one-way ANOVA and Dunnett tests (P<0.05); Different lowercase letters represent the interaction between protocols in the same CSBS excluding control group, calculated by using two-way ANOVA and Sidak's multiple comparisons tests (P<0.05).

Table 3. Comparison of the set CSBS control groups with their respective irrigated and activated groups as well as the interaction between protocols in the same CSBS, in ZO and CS spectra

Groups	ZO spectrum			CS spectrum		
	BIOC	BIOR	ENDS	BIOC	BIOR	ENDS
Set control	0.46±0.10	0.61±0.10	0.52±0.04	0.31±0.06	0.34±0.07	0.22±0.09
NaOCl	0.61±0.10 a	0.59±0.06 a	0.54±0.11 a	0.29±0.22 a	0.25±0.11 a	0.28±0.05 a
NaOCl/PUI	0.54±0.12 a	0.55±0.05 a	0.55±0.05 a	0.23±0.13 a	0.16±0.14 a	0.28±0.07 a
NaOCl/EDTA	0.54±0.12 a	0.62±0.10 a	0.62±0.07 a	0.14±0.09 a	0.26±0.21 a	0.35±0.12 a
NaOCl/EDTA/PUI	0.49±0.06 a	0.60±0.07 a	0.62±0.12 a	0.10±0.03 a*	0.28±0.15 a	0.33±0.09 a

* Means significance difference between control group and each experimental group for the same CSBS, calculated by using one-way ANOVA and Dunnett tests (P<0.05); Different lowercase letters represent the interaction between protocols in the same CSBS excluding control group, calculated by using two-way ANOVA and Sidak's multiple comparisons tests (P<0.05).

Table 4. The mean proportion (%) of the principal elements in CSBS

	ZO spectrum			CS spectrum		
	Zr (%)			Ca and Si (%)		
Groups	BIOC	BIOR	ENDS	BIOC	BIOR	ENDS
Fresh control (F)	29.9	7.5	15.8	11.8	7.2	11.5
(F) NaOCl	27.7	ND	29.0	5.6	12.5	4.5
(F) NaOCl/PUI	25.9	6.1	ND	7.5	6.8	4.6
(F) NaOCl/EDTA	25.4	ND	22.9	7.1	17.9	14.0
(F) NaOCl/EDTA/PUI	25.3	ND	ND	2.6	7.1	0.3
Set control (S)	36.2	17.9	56.0	8.8	21.7	7.3
(S) NaOCl	17.9	ND	39.1	6.3	19.1	4.5
(S) NaOCl/PUI	11.0	ND	14.7	4.8	3.2	2.4
(S) NaOCl/EDTA	ND	16.4	36.8	1.3	25.4	13.1
(S) NaOCl/EDTA/PUI	9.6	18.2	32.6	2.4	15.9	7.2

4. CONLUSÕES

Frente às limitações dos cinco estudos *in vitro* apresentados, pode-se concluir que:

1- Cada tipo celular liberou um agente tóxico diferente e a composição dos cimentos testados parece alterar a forma de auto-regulação na produção de citocinas pelas células.

2- Foi encontrada maior viabilidade celular para o cimento EndoSequence BC Sealer. O cimento Bio-C Sealer mostrou maior inibição de osteoclastos. Todos os cimentos revelaram alta solubilidade, maior do que a recomendada pelos padrões ISO.

3- O cimento BioRoot RCS apresentou o maior conteúdo de cálcio e grau de pureza. Os cimentos Bio-C Sealer e Sealer Plus BC mostraram ter composição semelhante e presença de alumínio. Na superfície de todos os cimentos testados foram encontrados precipitados, e todos mantiveram a viabilidade acima de 70%.

4- A ponta Easy Clean removeu mais debris após a desobturação e o terço cervical foi o que apresentou maior limpeza. A solução NaOCl+EDTA teve maior eficácia de limpeza, independente do método de ativação. Nenhum protocolo foi capaz de alterar a microdureza dentinária.

5- A limpeza dos remanescentes em canal desobturado para cimentação de pino de fibra de vidro deve ser preconizada somente após a presa do cimento à base de silicato de cálcio, independente do protocolo utilizado.

5. REFERÊNCIAS

Akyuz Ekim SN, Erdemir A. Effect of different irrigant activation protocols on push-out bond strength. **Lasers Med Sci.** 2015;30(8):2143–2149. https://doi.org/10.1007/s10103-015-1772-z

Alves Silva EC, Tanomaru-Filho M, da Silva GF, Delfino MM, Cerri PS, Guerreiro-Tanomaru JM. Biocompatibility and Bioactive Potential of New Calcium Silicate-based Endodontic Sealers: Bio-C Sealer and Sealer Plus BC. **J Endod.** 2020;46(10):1470-1477. https://doi.org/10.1016/j.joen.2020.07.011

Ashofteh Yazdi K, Ghabraei S, Bolhari B, Kafili M, Meraji N, Nekoofar MH, et al. Microstructure and chemical analysis of four calcium silicate-based cements in different environmental conditions. **Clin Oral Investig.** 2019;23(1):43-52. https://doi.org/10.1007/s00784-018-2394-1

Atmeh AR, AlShwaimi E. The Effect of Heating Time and Temperature on Epoxy Resin and Calcium Silicate-based Endodontic Sealers. **J Endod.** 2017;43(12):2112-2118. https://doi.org/10.1016/j.joen.2017.08.008

*De acordo com a Norma da FOUFU, baseado nas Normas de Vancouver. Abreviatura dos periódicos com conformidade com Medline (Pubmed).

Benetti F, de Azevedo Queiroz ÍO, Oliveira PHC, Conti LC, Azuma MM, Oliveira SHP, et al. Cytotoxicity and biocompatibility of a new bioceramic endodontic sealer containing calcium hydroxide. **Braz Oral Res.** 2019;33:e042. https://doi.org/10.1590/1807-3107bor-2019.vol33.0042

Benetti F, Queiroz ÍOA, Cosme-Silva L, Conti LC, Oliveira SHP, Cintra LTA. Cytotoxicity, Biocompatibility and Biomineralization of a New Ready-for-Use Bioceramic Repair Material. **Braz Dent J.** 2019;30(4):325-332. https://doi.org/10.1590/0103-6440201902457

Bolhari B, Nekoofar MH, Sharifian M, Ghabrai S, Meraji N, Dummer PM. Acid and microhardness of mineral trioxide aggregate and mineral trioxide aggregate-like materials. **J Endod.** 2014;40(3):432-5. https://doi.org/10.1016/j.joen.2013.10.014

Braga JM, Oliveira RR, Martins RC, Ribeiro Sobrinho AP. The effects of a mineral trioxide aggregate-based sealer on the production of reactive oxygen species, nitrogen species and cytokines by two macrophage subtypes. **Int Endod J.** 2014;47(10):909-19. https://doi.org/10.1111/iej.12234

Bryan TE, Khechen K, Brackett MG, Messer RL, El-Awady A, Primus CM, et al. In vitro osteogenic potential of an experimental calcium silicate-based root canal sealer. **J Endod.** 2010;36(7):1163-9. https://doi.org/10.1016/j.joen.2010.03.034

Camilleri J, Gandolfi MG. Evaluation of the radiopacity of calcium silicate cements containing different radiopacifiers. **Int Endod J.** 2010;43(1):21-30. https://doi.org/10.1111/j.1365-2591.2009.01621.x

Camilleri J. Characterization and hydration kinetics of tricalcium silicate cement for use as a dental biomaterial. **Dent Mater.** 2011;27(8):836-44.

https://doi.org/10.1016/j.dental.2011.04.010

Camilleri J. Color stability of white mineral trioxide aggregate in contact with hypochlorite solution. **J Endod.** 2014;40(3):436-40.

https://doi.org/10.1016/j.joen.2013.09.040

Camps J, Jeanneau C, El Ayachi I, Laurent P, About I. Bioactivity of a Calcium Silicate-based Endodontic Cement (BioRoot RCS): Interactions with Human Periodontal Ligament Cells In Vitro. **J Endod.** 2015;41(9):1469-73.
https://doi.org/10.1016/j.joen.2015.04.011

Candeiro GT, Correia FC, Duarte MA, Ribeiro-Siqueira DC, Gavini G. Evaluation of radiopacity, pH, release of calcium ions, and flow of a bioceramic root canal sealer. **J Endod.** 2012;38(6):842-5.
https://doi.org/10.1016/j.joen.2012.02.029

Candeiro GTM, Moura-Netto C, D'Almeida-Couto RS, Azambuja-Júnior N, Marques MM, Cai S, et al. Cytotoxicity, genotoxicity and antibacterial effectiveness of a bioceramic endodontic sealer. **Int Endod J.** 2016;49(9):858-864.
https://doi.org/10.1111/iej.12523

Chang SW, Lee SY, Kang SK, Kum KY, Kim EC. In vitro biocompatibility, inflammatory response, and osteogenic potential of 4 root canal sealers: Sealapex, Sankin apatite root sealer, MTA Fillapex, and iRoot SP root canal sealer. **J Endod.** 2014;40(10):1642-8.

https://doi.org/10.1016/j.joen.2014.04.006

Cheng X, Zhu L, Zhang J, Yu J, Liu S, Lv F, et al. Anti-osteoclastogenesis of Mineral Trioxide Aggregate through Inhibition of the Autophagic Pathway. **J Endod.** 2017;43(5):766-73.

https://doi.org/10.1016/j.joen.2016.12.013

Chybowski EA, Glickman GN, Patel Y, Fleury A, Solomon E, He J. Clinical Outcome of Non-Surgical Root Canal Treatment Using a Single-cone Technique with Endosequence Bioceramic Sealer: A Retrospective Analysis. **J Endod.** 2018;44(7):1199.

https://doi.org/10.1016/j.joen.2018.02.019

de Siqueira Zuolo A, Zuolo ML, da Silveira Bueno CE, Chu R, Cunha RS. Evaluation of the Efficacy of TRUShape and Reciproc File Systems in the Removal of Root Filling Material: An Ex Vivo Micro-Computed Tomographic Study. **J Endod.** 2016;42(2):315-9.

https://doi.org/10.1016/j.joen.2015.11.005

do Carmo SS, Nespoli FFP, Bachmann L, Miranda CES, Castro-Raucci LMS, Oliveira IR, et al. Influence of early mineral deposits of silicate- and aluminate-based cements on push-out bond strength to root dentine. **Int Endod J.** 2018;51(1):92-101.
https://doi.org/10.1111/iej.12791

Donnermeyer D, Bürklein S, Dammaschke T, Schäfer E. Endodontic sealers based on calcium silicates: a systematic review. **Odontology.** 2019;107(4):421-436.

https://doi.org/10.1007/s10266-018-0400-3

Dos Reis-Prado AH, Abreu LG, Tavares WLF, Peixoto IFDC, Viana ACD, de Oliveira EMC, et al. Comparison between immediate and delayed post space preparations: a systematic review and meta-analysis. **Clin Oral Investig.** 2021;25(2):417-440.
https://doi.org/10.1007/s00784-020-03690-x

Gandolfi MG, Taddei P, Siboni F, Modena E, Ciapetti G, Prati C. Development of the foremost light-curable calcium-silicate MTA cement as root-end in oral surgery. Chemical-physical properties, bioactivity and biological behavior. **Dent Mater.** 2011;27(7):e134-57.
https://doi.org/10.1016/j.dental.2011.03.011

Gandolfi MG, Taddei P, Tinti A, Prati C. Apatite-forming ability (bioactivity) of ProRoot MTA. **Int Endod J.** 2010;43(10):917-29.
https://doi.org/10.1111/j.1365-2591.2010.01768.x

Gandolfi MG, Van Landuyt K, Taddei P, Modena E, Van Meerbeek B, Prati C. Environmental scanning electron microscopy connected with energy dispersive x-ray analysis and Raman techniques to study ProRoot mineral trioxide aggregate and calcium silicate cements in wet conditions and in real time. **J Endod.** 2010;36(5):851-7.

https://doi.org/10.1016/j.joen.2009.12.007

Garrib M, Camilleri J. Retreatment efficacy of hydraulic calcium silicate sealers used in single cone obturation. **J Dent.** 2020;98:103370.

https://doi.org/10.1016/j.jdent.2020.103370

Grazziotin-Soares R, Nekoofar MH, Davies T, Hübler R, Meraji N, Dummer PMH. Crystalline phases involved in the hydration of calcium silicate-based

cements: Semi-quantitative Rietveld X-ray diffraction analysis. **Aust Endod J.** 2019;45(1):26-32.

https://doi.org/10.1111/aej.12226

Guivarc'h M, Jeanneau C, Giraud T, Pommel L, About I, Azim AA, et al. An international survey on the use of calcium silicate-based sealers in non-surgical endodontic treatment. **Clin Oral Investig.** 2020;24(1):417-424.

https://doi.org/10.1007/s00784-019-02920-1

International Organization for Standardization. ISO 6876: Dental Root Canal Sealing Materials. Geneva, Switzerland: International Organization for Standardization; 2012.

Jafari F, Jafari S. Composition and physicochemical properties of calcium silicate based sealers: a review article. **J Clin Exp Dent.** 2017;9(10):e1249–e1255.
https://doi.org/10.4317/jced.54103

Jung S, Sielker S, Hanisch MR, Libricht V, Schäfer E, Dammaschke T. Cytotoxic effects of four different root canal sealers on human osteoblasts. **PLoS One.** 2018;13(3):e0194467.
https://doi.org/10.1371/journal.pone.0194467

Kato AS, Cunha RS, da Silveira Bueno CE, Pelegrine RA, Fontana CE, de Martin AS. Investigation of the efficacy of passive ultrasonic irrigation versus irrigation with reciprocating activation: an environmental scanning electron microscopic study. **J Endod.** 2016;42(4):659–663.
https://doi.org/10.1016/j.joen.2016.01.016

Kebudi Benezra M, Schembri Wismayer P, Camilleri J. Influence of environment on testing of hydraulic sealers. **Sci Rep.** 2018;8(1):7209.

https://doi.org/10.1038/s41598-018-24847-5

Kebudi Benezra M, Schembri Wismayer P, Camilleri J. Interfacial Characteristics and Cytocompatibility of Hydraulic Sealer Cements. **J Endod.** 2018;44(6):1007-1017.

https://doi.org/10.1016/j.joen.2017.11.011

Khalil I, Naaman A, Camilleri J. Properties of Tricalcium Silicate Sealers. **J Endod.** 2016;42(10):1529-35.

https://doi.org/10.1016/j.joen.2016.06.002

Kharouf N, Arntz Y, Eid A, Zghal J, Sauro S, Haikel Y, et al. Physicochemical and Antibacterial Properties of Novel, Premixed Calcium Silicate-Based Sealer Compared to Powder-Liquid Bioceramic Sealer. **J Clin Med.** 2020;9(10):3096.
https://doi.org/10.3390/jcm9103096

Li X, Yoshihara K, De Munck J, Cokic S, Pongprueksa P, Putzeys E, et al. Modified tricalcium silicate cement formulations with added zirconium oxide. **Clin Oral Investig.** 2017;21(3):895-905.

https://doi.org/10.1007/s00784-016-1843-y

Lo Giudice G, Lizio A, Giudice RL, Centofanti A, Rizzo G, Runci M, et al. The effect of different cleaning protocols on post space: a SEM study. **Int J Dent.** 2016;2016:1907124.

https://doi.org/10.1155/2016/1907124

Long W, Li J, Liu Y, Jiang H. Effect of obturation technique with immediate and delayed post space preparation on apical voids and bond strength of apical gutta-percha. **J Int Med Res.** 2019;47(1):470–480. https://doi.org/10.1177/0300060518814604

López-García S, Lozano A, García-Bernal D, Forner L, Llena C, Guerrero-Gironés J, et al. Biological Effects of New Hydraulic Materials on Human Periodontal Ligament Stem Cells. **J Clin Med.** 2019;8(8):1216. https://doi.org/10.3390/jcm8081216

López-García S, Pecci-Lloret MR, Guerrero-Gironés J, Pecci-Lloret MP, Lozano A, Llena C, et al. Comparative Cytocompatibility and Mineralization Potential of Bio-C Sealer and TotalFill BC Sealer. **Materials (Basel).** 2019;12(19):3087. https://doi.org/10.3390/ma12193087

Mendes AT, Silva PBD, Só BB, Hashizume LN, Vivan RR, Rosa RAD, et al. Evaluation of Physicochemical Properties of New Calcium Silicate-Based Sealer. **Braz Dent J.** 2018;29(6):536-540.

https://doi.org/10.1590/0103-6440201802088

Moinzadeh AT, Zerbst W, Boutsioukis C, Shemesh H, Zaslansky P. Porosity distribution in root canals filled with gutta percha and calcium silicate cement. **Dent Mater.** 2015;31(9):1100-8.

https://doi.org/10.1016/j.dental.2015.06.009

Nagas E, Karaduman E, Sahin C, Uyanik O, Canay S. Effect of timing of post space preparation on the apical seal when using different sealers and obturation techniques. **J Dent Sci.** 2016;11(1):79–82. https://doi.org/10.1016/j.jds.2012.05.017

Oh H, Kim E, Lee S, Park S, Chen D, Shin SJ, et al. Comparison of Biocompatibility of Calcium Silicate-Based Sealers and Epoxy Resin-Based Sealer on Human Periodontal Ligament Stem Cells. **Materials (Basel).** 2020;13(22):5242.
https://doi.org/10.3390/ma13225242

Okamura T, Chen L, Tsumano N, Ikeda C, Komasa S, Tominaga K, et al. Biocompatibility of a High-Plasticity, Calcium Silicate-Based, Ready-to-Use Material. **Materials (Basel).** 2020;13(21):4770.
https://doi.org/10.3390/ma13214770

Oliveira LV, Maia TS, Zancopé K, Menezes MS, Soares CJ, Moura CCG. Can intra-radicular cleaning protocols increase the retention of fiberglass posts? A systematic review. **Braz Oral Res.** 2018;32:e16.

https://doi.org/10.1590/1807-3107bor-2018.vol32.0016

Oltra E, Cox TC, LaCourse MR, Johnson JD, Paranjpe A. Retreatability of two endodontic sealers, EndoSequence BC Sealer and AH Plus: a micro-computed tomographic comparison. **Restor Dent Endod.** 2017;42(1):19-26.

https://doi.org/10.5395/rde.2017.42.1.19

Pedullà E, Abiad RS, Conte G, Khan K, Lazaridis K, Rapisarda E, et al. Retreatability of two hydraulic calcium silicate-based root canal sealers using rotary instrumentation with supplementary irrigant agitation protocols: a laboratory-based micro-computed tomographic analysis. **Int Endod J.** 2019;52(9):1377-1387.

https://doi.org/10.1111/iej.13132

Poletto D, Poletto AC, Cavalaro A, Machado R, Cosme-Silva L, Garbelini CCD, et al. Smear layer removal by different chemical solutions used with or without ultrasonic activation after post preparation. **Restor Dent Endod.** 2017;42(4):324–331.

https://doi.org/10.5395/rde.2017.42.4.324

Prati C, Gandolfi MG. Calcium silicate bioactive cements: Biological perspectives and clinical applications. **Dent Mater.** 2015;31(4):351-70.

https://doi.org/10.1016/j.dental.2015.01.004

Rodrigues MP, Soares PBF, Valdivia ADCM, Pessoa RS, Veríssimo C, Versluis A, et al. Patient-specific Finite Element Analysis of Fiber Post and Ferrule Design. **J Endod.** 2017;43(9):1539-1544.

https://doi.org/10.1016/j.joen.2017.04.024

Rosatto CMP, Oliveira LV, Ferraz DC, Soares PBF, Soares CJ, Moura CCG. Apical Displacement and Residual Root Canal Filling with Single-Cone After Post Space Preparation: A Micro-CT Analysis. **Braz Dent J.** 2020;31(1):25-31.

https://doi.org/10.1590/0103-6440202002597

Santos-Filho PC, Castro CG, Silva GR, Campos RE, Soares CJ. Effects of post system and length on the strain and fracture resistance of root filled bovine teeth. **Int Endod J.** 2008;41(6):493-501.

https://doi.org/10.1111/j.1365-2591.2008.01383.x

Siboni F, Taddei P, Zamparini F, Prati C, Gandolfi MG. Properties of BioRoot RCS, a tricalcium silicate endodontic sealer modified with povidone and polycarboxylate. **Int Endod J.** 2017;50 Suppl 2:e120-e136.
https://doi.org/10.1111/iej.12856

Silva Almeida LH, Moraes RR, Morgental RD, Pappen FG. Are Premixed Calcium Silicate-based Endodontic Sealers Comparable to Conventional Materials? A Systematic Review of In Vitro Studies. **J Endod.** 2017;43(4):527-535.

https://doi.org/10.1016/j.joen.2016.11.019

Silva EJNL, Cardoso ML, Rodrigues JP, De-Deus G, Fidalgo TKDS. Solubility of bioceramic- and epoxy resin-based root canal sealers: A systematic review and meta-analysis. **Aust Endod J.** 2021.
https://doi.org/10.1111/aej.12487

Silva GF, Bosso R, Ferino RV, Tanomaru-Filho M, Bernardi MI, Guerreiro-Tanomaru JM, et al. Microparticulated and nanoparticulated zirconium oxide added to calcium silicate cement: Evaluation of physicochemical and biological properties. **J Biomed Mater Res A.** 2014;102(12):4336-45.

https://doi.org/10.1002/jbm.a.35099

Simezo AP, da Silveira Bueno CE, Cunha RS, Pelegrine RA, Rocha DGP, Martin AS, et al. Comparative analysis of dentinal erosion after passive ultrasonic irrigation versus irrigation with reciprocating activation: an environmental scanning electron study. **J Endod.** 2017;43(1):141–146.
https://doi.org/10.1016/j.joen.2016.09.016

Soares CJ, Valdivia AD, da Silva GR, Santana FR, Menezes Mde S. Longitudinal clinical evaluation of post systems: a literature review. **Braz Dent J.** 2012;23(2):135-740.

https://doi.org/10.1590/S0103-64402012000200008

Souza GL, Rosatto CMP, Silva MJB, Silva MV, Rocha Rodrigues DB, Moura CCG. Evaluation of apoptosis/necrosis and cytokine release provoked by three root canal sealers in human polymorphonuclears and monocytes. **Int Endod J.** 2019;52(5):629-638.
https://doi.org/10.1111/iej.13036

Taddei P, Modena E, Tinti A, Siboni F, Prati C, Gandolfi MG. Vibrational investigation on the in vitro bioactivity of commercial and experimental calcium–silicate cements for root-end endodontic therapy. **J Mol Struct.** 2011;993:367–75.
https://doi.org/10.1016/j.molstruc.2010.12.058

Tanomaru-Filho M, Torres FFE, Chávez-Andrade GM, de Almeida M, Navarro LG, Steier L, et al. Physicochemical Properties and Volumetric Change of Silicone/Bioactive Glass and Calcium Silicate-based Endodontic Sealers. **J Endod.** 2017;43(12):2097-2101.
https://doi.org/10.1016/j.joen.2017.07.005

Torabinejad M, Parirokh M, Dummer PMH. Mineral trioxide aggregate and other bioactive endodontic cements: an updated overview - part II: other clinical applications and complications. **Int Endod J.** 2018;51(3):284-317.
https://doi.org/10.1111/iej.12843

Torres FFE, Zordan-Bronzel CL, Guerreiro-Tanomaru JM, Chávez-Andrade GM, Pinto JC, Tanomaru-Filho M. Effect of immersion in distilled water or phosphate-buffered saline on the solubility, volumetric change and presence of

voids within new calcium silicate-based root canal sealers. **Int Endod J.** 2020;53(3):385-391.

https://doi.org/10.1111/iej.13225

Urban K, Neuhaus J, Donnermeyer D, Schafer E, Dammaschke T. Solubility and pH Value of 3 Different Root Canal Sealers: A Long-term Investigation. **J Endod.** 2018;44(11):1736-40.

https://doi.org/10.1016/j.joen.2018.07.026

Veríssimo C, Simamoto Júnior PC, Soares CJ, Noritomi PY, Santos-Filho PC. Effect of the crown, post, and remaining coronal dentin on the biomechanical behavior of endodontically treated maxillary central incisors. **J Prosthet Dent.** 2014;111(3):234-46.

https://doi.org/10.1016/j.prosdent.2013.07.006

Viapiana R, Moinzadeh AT, Camilleri L, Wesselink PR, Tanomaru Filho M, Camilleri J. Porosity and sealing ability of root fillings with gutta-percha and BioRoot RCS or AH Plus sealers. Evaluation by three ex vivo methods. **Int Endod J.** 2016;49(8):774-82.
https://doi.org/10.1111/iej.12513

Vitti RP, Prati C, Silva EJ, Sinhoreti MA, Zanchi CH, de Souza e Silva MG, et al. Physical properties of MTA Fillapex sealer. **J Endod.** 2013;39(7):915-8.

https://doi.org/10.1016/j.joen.2013.04.015

Zhang J, Zhu L, Yan P, Peng B. Effect of BioAggregate on Receptor Activator of Nuclear Factor-Kappa B Ligand-induced Osteoclastogenesis from Murine Macrophage Cell Line In Vitro. **J Endod.** 2015;41(8):1265-71.
https://doi.org/10.1016/j.joen.2015.03.021

Zordan-Bronzel CL, Esteves Torres FF, Tanomaru-Filho M, Chávez-Andrade GM, Bosso-Martelo R, Guerreiro-Tanomaru JM. Evaluation of physicochemical properties of a new calcium silicate-based sealer, Bio-C sealer. **J Endod.** 2019;45(10):1248–1252.
https://doi.org/10.1016/j.joen.2019.07.006

Zordan-Bronzel CL, Tanomaru-Filho M, Rodrigues EM, Chávez-Andrade GM, Faria G, Guerreiro-Tanomaru JM. Cytocompatibility, bioactive potential and antimicrobial activity of an experimental calcium silicate-based endodontic sealer. **Int Endod J.** 2019;52(7):979-986.

https://doi.org/10.1111/iej.13086

6. ANEXOS

Anexo 6.1 – Parecer do Comitê de Ética em Pesquisa referente ao capítulo 2

Universidade Federal de Uberlândia
– Comissão de Ética na Utilização de Animais –

Comissão de Ética na Utilização de Animais CEUA

CERTIFICADO

Certificamos que o projeto intitulado "Avaliação in vitro da citotoxicidade e atividade de fosfatase alcalina promovida por cimentos endodônticos biocerâmicos em cultura de osteoclastos.", protocolo nº 003/19, sob a responsabilidade de **Camilla Christian Gomes Moura** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, 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 **APROVADA** pela COMISSÃO DE ÉTICA NA UTILIZAÇÃO DE ANIMAIS (CEUA) da UNIVERSIDADE FEDERAL DE UBERLÂNDIA, em reunião **10 de maio de 2019.**

(We certify that the project entitled "Avaliação in vitro da citotoxicidade e atividade de fosfatase alcalina promovida por cimentos endodônticos biocerâmicos em cultura de osteoclastos.", protocol 003/19, under the responsibility of Camilla Christian Gomes Moura - involving the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata, for purposes of scientific research - is in accordance with the provisions of Law nº 11.794, of October 8th, 2008, of Decree nº 6.899 of July 15th, 2009, and the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and it was approved for ETHICS COMMISSION ON ANIMAL USE (CEUA) from FEDERAL UNIVERSITY OF UBERLÂNDIA, in meeting of May 10th, 2019).

Vigência do Projeto	Início: 01/06/2019 Término: 01/06/2020
Espécie / Linhagem / Grupos Taxonômicos	Camundongo isogênico C57BL/6
Número de animais	36
Peso / Idade	25g/ 6 a 8 semanas
Sexo	Macho
Origem / Local	Biotério Central da Rede de Biotérios de Roedores da UFU (REBIR-UFU)
Local onde serão mantidos os animais:	Biotério Central da REBIR

Uberlândia, 16 de Maio de 2019.

Prof. Dr. Lúcio Vilela Carneiro Girão
Coordenador da CEUA
SEI Nº 1201, DE 12 DE DEZEMBRO DE 2018

Anexo 6.2 – Comprovante de submissão do artigo referente ao capítulo 2

Brazilian Oral Research

Home

Author

Submission Confirmation

Print

Thank you for your submission

Submitted to
Brazilian Oral Research

Manuscript ID
BOR-2021-0424

Title
Evaluation of premixed calcium silicate-based sealers and their role on osteoclastogenesis

Authors
de Rosatto, Camila Maria
de Souza, Gabriela
Ferraz, Danilo
Silva, Marcelo José
Tanomaru-Filho, Mário
Moura, Camilla

Date Submitted
19-Apr-2021

Author Dashboard

Anexo 6.3 - Comprovante de aceite do artigo referente ao capítulo 4

Fwd: ENC: Brazilian Oral Research - Decision on Manuscript ID BOR-2020-1121.R1

Camilla Moura <camillamoura@ufu.br>
Seg, 01/03/2021 16:04
Para: camilamaria_pr@hotmail.com <camilamaria_pr@hotmail.com>

Enviado do meu iPhone

Início da mensagem encaminhada:

De: Mario Tanomaru Filho <tanomaru@uol.com.br>
Data: 1 de março de 2021 14:55:18 BRT
Para: camillamoura@ufu.br, danilocasferraz@hotmail.com
Assunto: ENC: Brazilian Oral Research - Decision on Manuscript ID BOR-2020-1121.R1

Mario Tanomaru Filho
Professor Titular da Disciplina de Endodontia - Faculdade de Odontologia de Araraquara - UNESP
Full Professor, Discipline of Endodontics - Araraquara School of Dentistry - UNESP

De: "Saul Paiva" <onbehalfof@manuscriptcentral.com>
Enviada: 2021/03/01 13:48:27
Para: tanomaru@uol.com.br
Assunto: Brazilian Oral Research - Decision on Manuscript ID BOR-2020-1121.R1

01-Mar-2021

Dear Prof. Tanomaru-Filho:

It is a pleasure to accept your manuscript entitled "Effect of irrigation protocols on root canal wall after post preparation: micro-CT and microhardness study" in its current form for publication in the Brazilian Oral Research. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the Brazilian Oral Research, we look forward to your continued contributions to the Journal.

Sincerely,
Dr. Saul Paiva
Editor-in-Chief, Brazilian Oral Research
smpaiva@uol.com.br

Anexo 6.4 - Parecer do Comitê de Ética em Pesquisa referente ao capítulo 5

UNIVERSIDADE FEDERAL DE UBERLÂNDIA/MG

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Influência de diferentes protocolos de irrigação após alívio na limpeza do canal radicular obturado com cimentos endodônticos biocerâmicos

Pesquisador: Camilla Christian Gomes Moura

Área Temática:

Versão: 1

CAAE: 83210817.8.0000.5152

Instituição Proponente: Universidade Federal de Uberlândia/ UFU/ MG

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.570.084

Apresentação do Projeto:

O protocolo pretende "desvendar qual o protocolo de irrigação após alívio do canal radicular promove melhor limpeza dos resíduos remanescentes de cimento obturador biocerâmico e gutapercha, assim como se existe uma técnica ideal de agitação dessas soluções irrigantes, por meio de ensaio in vitro". E continua a proposta de investigação deste: "esclarecer se os protocolos de irrigação alteram a microdureza dentinária, assim como a composição destes novos cimentos endodônticos e se estes são citotóxicos aos tecidos periapicais". Por fim, conclui a exposição do protocolo:
"para uso clínico, afim de que a posterior reabilitação funcional e estética tenha a adesão preconizada e seja possível conhecer melhor o comportamento dos novos cimentos endodônticos biocerâmicos".

Objetivo da Pesquisa:

O protocolo tem como objetivo geral: "este estudo tem como proposta determinar qual o melhor protocolo de irrigação após alívio do canal radicular e como a agitação das soluções irrigantes influenciam na limpeza dos resíduos remanescente, nas propriedades da dentina radicular, na presa e composição de cimentos endodônticos obturadores biocerâmicos, assim como avaliar sua biocompatibilidade aos tecidos do periápice evitando injúrias".
E como objetivos específicos: "análise da influência do protocolo de irrigação e agitação das

Endereço: Av. João Naves de Ávila 2121- Bloco "1A", sala 224 - Campus Sta. Mônica
Bairro: Santa Mônica **CEP:** 38.408-144
UF: MG **Município:** UBERLANDIA
Telefone: (34)3239-4131 **Fax:** (34)3239-4335 **E-mail:** cep@propp.ufu.br

soluções utilizado após alívio do conduto radicular na remoção de resíduos aderidos as paredes do canal, nas propriedades da dentina e seu aquecimento, em dentes obturados com cimentos endodônticos obturadores biocerâmicos; análise da influência do protocolo de irrigação e agitação das soluções utilizado após alívio do conduto radicular na remoção de resíduos aderidos as paredes do canal, na presa e composição de cimentos endodônticos obturadores biocerâmicos; avaliação da toxicidade e biocompatibilidade destes novos cimentos biocerâmicos em cultura de osteoclastos, afim de avaliar se esses cimento endodônticos obturadores estimulam essas células a realizar reparação óssea e quais mediadores são liberados; confecção de uma revisão sistemática sobre estudos bimecânicos in vitro utilizando o cimento bioativo Endosequence BC Sealer; confecção de caso clínico a ser publicado em periódico nacional abordando a seguinte sequência: instrumentação reciprocante em molar após odontometria realizada em raio x digital, obturação pela técnica do cone único com cimento biocerâmico, alívio imediato, protocolo de irrigação e agitação das soluções irrigantes propostas, cimentação de pino de fibra de vidro conforme o grupo de pesquisa preconiza e confecção de restauração em resina composta, com base realizada em resina bulk-fi".

Avaliação dos Riscos e Benefícios:

Segundo o protocolo, 'não há riscos evidentes que estejam relacionados com este projeto, que comprometa a saúde dos pacientes doadores dos dentes com indicação de extração, cuja finalidade é de contribuir com a pesquisa no Centro de Pesquisa de Biomecânica, Biomateriais e Biologia Celular (CPBio) - FOUFU". Contudo, o protocolo afirma que "os riscos para a coleta de dentes consistem em possível sintomatologia pós-operatória, presença de edema e algum sangramento" E que para isto serão tomadas os devidos cuidados com utilização de materiais estéreis e todas as medidas de biossegurança serão tomadas como protocolo de rotina durante as extrações dentárias nas clínicas de cirurgia do curso de graduação em Odontologia da UFU ou no Pronto-Socorro Odontológico (PSO)".

E em relação aos benefícios, o protocolo afirma que "os doadores de dentes participantes da pesquisa terão benefícios indiretos, pois tendo alcançado o objetivo do presente estudo novo protocolo de tratamento endodôntico será proposto, e isso beneficia não só o paciente participante como também toda a sociedade, pois poderá melhorar o prognóstico do tratamento a ser realizado".

Comentários e Considerações sobre a Pesquisa:

Estrategicamente, o protocolo apresenta a proposta metodológica, expondo o plano de

recrutamento dos doadores de dentes e define com clareza os critérios de inclusão e exclusão dos participantes doadores da investigação, vinculados aos objetivos da amostra. Os participantes doadores serão os atendidos no Hospital Odontológico da UFU.

Considerações sobre os Termos de apresentação obrigatória:

Foram apresentados.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

De acordo com as atribuições definidas na Resolução CNS 466/12, o CEP manifesta-se pela aprovação do protocolo de pesquisa proposto.

O protocolo não apresenta problemas de ética nas condutas de pesquisa com seres humanos, nos limites da redação e da metodologia apresentadas.

Considerações Finais a critério do CEP:

Data para entrega de Relatório Parcial ao CEP/UFU: Janeiro de 2019.

Data para entrega de Relatório Parcial ao CEP/UFU: Janeiro de 2020.

Data para entrega de Relatório Final ao CEP/UFU: Janeiro de 2021.

OBS.: O CEP/UFU LEMBRA QUE QUALQUER MUDANÇA NO PROTOCOLO DEVE SER INFORMADA IMEDIATAMENTE AO CEP PARA FINS DE ANÁLISE E APROVAÇÃO DA MESMA.

O CEP/UFU lembra que:

a- segundo a Resolução 466/12, o pesquisador deverá arquivar por 5 anos o relatório da pesquisa e os Termos de Consentimento Livre e Esclarecido, assinados pelo sujeito de pesquisa.

b- poderá, por escolha aleatória, visitar o pesquisador para conferência do relatório e documentação pertinente ao projeto.

c- a aprovação do protocolo de pesquisa pelo CEP/UFU dá-se em decorrência do atendimento a Resolução CNS 466/12, não implicando na qualidade científica do mesmo.

Orientações ao pesquisador :

• O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 466/12) e deve receber uma via original do Termo de Consentimento Livre e Esclarecido, na

íntegra, por ele assinado.

• O pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS 466/12), aguardando seu parecer, exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade de regime oferecido a um dos grupos da pesquisa que requeiram ação imediata.

• O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS 466/12). É papel de o pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

• Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projetos do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma, junto com o parecer aprobatório do CEP, para serem juntadas ao protocolo inicial (Res.251/97, item III.2.e).

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1012829.pdf	16/02/2018 12:18:33		Aceito
Declaração de Instituição e Infraestrutura	Declaracao_Instituicao_Coparticipante_assinada.jpg	16/02/2018 12:17:55	Camilla Christian Gomes Moura	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_CEP_doutoradoCamilaRosatto.doc	07/02/2018 11:50:19	Camilla Christian Gomes Moura	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_DoutoradoCamila.doc	07/02/2018 11:49:42	Camilla Christian Gomes Moura	Aceito
Outros	Destinacaomaterialbiologico_assinada.jpg	07/02/2018 11:48:55	Camilla Christian Gomes Moura	Aceito
Folha de Rosto	Folhaderosto_assinada.pdf	07/02/2018 11:46:26	Camilla Christian Gomes Moura	Aceito
Declaração de Pesquisadores	LinksCurriculosLattesPesquisadores.doc	11/10/2017 15:51:20	Camilla Christian Gomes Moura	Aceito

Endereço: Av. João Naves de Ávila 2121- Bloco "1A", sala 224 - Campus Sta. Mônica
Bairro: Santa Mônica **CEP:** 38.408-144
UF: MG **Município:** UBERLANDIA
Telefone: (34)3239-4131 **Fax:** (34)3239-4335 **E-mail:** cep@propp.ufu.br

Declaração de Pesquisadores	EquipeExecutoraAssinada.jpg	11/10/2017 15:48:10	Camilla Christian Gomes Moura	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Modelo_de_instrumento_de_coleta_de_dados.doc	11/10/2017 15:47:51	Camilla Christian Gomes Moura	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

UBERLANDIA, 28 de Março de 2018

Assinado por:
Sandra Terezinha de Farias Furtado
(Coordenador)

Endereço: Av. João Naves de Ávila 2121- Bloco "1A", sala 224 - Campus Sta. Mônica
Bairro: Santa Mônica **CEP:** 38.408-144
UF: MG **Município:** UBERLANDIA
Telefone: (34)3239-4131 **Fax:** (34)3239-4335 **E-mail:** cep@propp.ufu.br

Anexo 6.5 – Comprovante de submissão do artigo referente ao capítulo 5

[IEJ] Submission Acknowledgement

Prof. Mohammad Jafar Eghbal via Journals Portal, Shahid Beheshti University of Medical Sciences <journalsserver@sbmu.ac.ir>

Dom, 23/08/2020 13:55

Para: Camilla Christian Gomes Moura <camillamoura@ufu.br>; Camila Maria Peres de Rosatto <camilamaria_pr@hotmail.com>; Danilo Cassiano Ferraz <danilocasferraz@hotmail.com>; Marcelo José Barbosa Silva <majbsilva@gmail.com>; Carlos José Soares <carlosjsoares@ufu.br>

Hello,

Professor Camilla Christian Gomes Moura has submitted the manuscript, "Effect of solutions and passive ultrasonic irrigation in the composition of calcium silicate-based sealers after post space preparation " to Iranian Endodontic Journal.

If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Prof. Mohammad Jafar Eghbal