Lilian Vieira Oliveira

Terapias pulpares conservadoras e regeneração pulpar-Uma nova era na endodontia. Análise laboratorial e série de casos.

Conservative pulp therapies and pulp regeneration- A new era in endodontics: Laboratory analysis and case series

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 Concentração de Clínica Odontológica Integrada.

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Uberlândia, 2021

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ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Odontologia				
Defesa de:	Tese de Doutorado, número 62, PPGODONTO				
Data:	Cinco de Abril de Dois Mil e Vinte e Um	Hora de início:	10:00	Hora de encerramento:	12:22
Matrícula do Discente:	11813ODO010				
Nome do Discente:	Lilian Vieira Oliveira				
Título do Trabalho:	Terapias pulpares conservadoras e regeneração pulpar- uma nova era na endodontia: Análise laboratorial e série de casos.				
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				

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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.

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Referência: Processo nº 23117.022154/2021-83 SEI nº 2679883

Ficha Catalográfica Online do Sistema de Bibliotecas da UFU
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O48
2021

Oliveira, Lílian Vieira, 1987-
Terapias pulpares conservadoras e regeneração pulpar- Uma nova era na endodontia: Análise laboratorial e série de casos. [recurso eletrônico] / Lílian Vieira Oliveira. - 2021.

Orientador: 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.2021.203
Inclui bibliografia.
Inclui ilustrações.

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

Em todos os instantes, em todos os momentos, de todas as formas, ***Deus*** permaneceu por perto e jamais me abandonou. Nos dias difíceis, foi Ele que me deu a forca que nenhuma pessoa poderia me dar. Sinto uma gratidão imensa por ter sido rodeada de amigos, de família, de amor.

Aos meus pais e irmão, Aloisio, Margarida e Tiago. Vocês são meus exemplos de amor mais puro, de sabedoria divina, e de Deus perante nossas vidas. Vocês me ensinam, cada um com seu jeito particular de ser, a sempre colocar o amor acima de tudo. Vocês me ensinam a confiar em Deus, TODAS as coisas! Vocês são os meus maiores presentes e os meus grandes tesouros. Obrigada por tudo que são em todos os dias da minha vida. Vocês sempre serão a base de todas as minhas conquistas. Amo vocês

A minha orientadora Camilla Christian Gomes Moura. Para que tudo isso fosse possível em primeiro lugar contei com você, como orientadora e amiga. Obrigada por ser exemplo de garra, persistência e por me dar forças nos dias de medo, inseguranças e angústias. Sei que seus conselhos sempre foram para que o melhor acontecesse na minha vida. Me sinto feliz e abençoada por Deus ter te colocado na minha vida pois mais do que uma orientadora, você é uma grande amiga. Te acho uma pessoa incrível e sei que não importa a situação, você está ali, pronto para enfrentar e ajudar todos aqueles que você ama. Muito obrigada pela sua amizade, por estar sempre presente e dar oportunidade para todos que te procuram. Eu peço a Deus que continue te fortalecendo a cada dia e abençoando sua família e todos que sei que você ama.

Á Profa. Dra. Maria Antonieta que com ela foi construída uma amizade alicerçada em Deus. Obrigada por todo seu conselho, sabedoria e paciência nos dias que eu estava triste e que você me ajudou muito. Que Deus abençoe sua vida, sua família e seus sonhos.

AGRADECIMENTOS

A minha querida amiga Monise, que durante toda a minha trajetória mostrou sua amizade em todos os momentos e principalmente neste grande desafio desta defesa. Muito obrigada por sua amizade e que Deus te abençoe muito.

A doutoranda Gabriella Leite que nunca negou a mim nenhum favor ou ajuda em toda a minha trajetória. Gabi, que sua vida profissional seja abençoada pois além de inteligente, é uma mulher muito dedicada em tudo o que faz.

As alunas de IC Thamara e Gabrielle que me ajudaram a todo momento nos trabalhos, meu muito obrigada a vocês.

Á Profa. Dra. Priscilla Barbosa, por quem tenho um grande carinho. Sempre que procurei sua ajuda, nunca hesitou em me ajudar. Agradeço pela oportunidade que me deu e por me ajudar nas minhas dificuldades. Admiro você pela mulher que é, uma profissional brilhante, que tem muita garra e determinação. Seu reconhecimento profissional é a resposta de toda dedicação que você tem com tudo o que faz.

Ao Prof. Dr. Carlos José Soares, uma pessoa que tenho total admiração. Um homem determinado, com uma inteligência extraordinária e principalmente dedicado a tudo o que faz. Você merece todo reconhecimento pelo seu trabalho.

Aos amigos Andomar, Eric Cerda e Marcio Alex, meu muito obrigada pelo carinho e amizade de vocês.

Aos meus familiares e amigos, Aos professores da Universidade Federal de Uberlândia, Aos amigos da 66ª Turma de Odontologia UFU e amigos da pós-graduação, A CAPES pela bolsa de dourado, Universidade Federal de Uberlândia, Faculdade de Odontologia e CPBio, Ao entrar nesse desafio, eu tive no meu caminho pessoas muito queridas que me ofereceram todo auxílio, apoio e carinho. São vocês que fizeram diferença e permitiram que esse sonho fosse realizado. Muito Obrigada!

EPÍGRAFE

“Você pode ganhar ou perder, mas ser você mesmo é tudo o que você pode fazer”

Autor Desconhecido

SUMÁRIO

RESUMO

O objetivo geral foi avaliar *in vitro*, em modelos que mimetizem pulpotomias, manchamento e citoxicidade de biomaterais, e apresentar por meio de relato de caso exiquibilidade e sucesso de procedimento endodôntico regenerativo para tratamento de molares jovens. Objetivo 1: Simular fratura coronária complicada em incisivo tratado por pulpotomia total com Bio-C Temp (BC-Angelus) em comparação com dois materiais (MTA Flow–MTA, UltraCal XS-UC; Ultradent), avaliando descoloração dentária, radiopacidade e citotoxicidade nas células pulpares. Objetivo 2: Simular pulpotomia parcial com Bio-C Temp (BC) comparado com Bio-C Repair (BCR-Angelus) e Biodentine (BD-Septodont), avaliando descoloração dentária, radiopacidade, citotoxicidade. Objetivo 3: Relatar acompanhamento de série de casos de revascularização em molares permanentes jovens, com preparo mecanizado de canais radiculares. Nos objetivos 1 e 2, dentes bovinos foram preparados para simular fratura coronária extensa em dente com ápice aberto. As raízes foram preenchidas com mistura de ágar/sangue, e os materiais colocados sobre esta. O grupo controle foi preenchido apenas com ágar/sangue. Análises de avaliação da cor foram realizadas utilizando espectrofotômetro. A mudança total de cor (ΔEab, ΔE00) e índice de brancura (WI) foram calculadas com base no CIELAB. Radiografias digitais foram adquiridas para análise de radiopacidade. Células da polpa humana foram usadas para análise da viabilidade celular. Foram utilizados testes ANOVA e Tukey para comparar os grupos experimentais, e o de Dunnett para comparações com o controle. Nas pulpotomias totais (objetivo 1) todos os materiais foram associados com valores de descoloração maiores do que o aceitável e BT resultou em alteração da cor menor ou semelhante ao MTA e UC, respectivamente. Observou-se redução da radiopacidade apenas no MTA ($P=0,007$). A viabilidade celular dos materiais foi semelhante à do controle (DMEM) ($P>0,05$), exceto para BT que teve viabilidade significativamente menor ($P<0,001$). Nas pulpotomias parciais (objetivo 2), BCR apresentou maior radiopacidade e menor alteração de cor (ΔEab/ΔE00) que outros materiais ($P<0,005$; $P<0,001$). BD apresentou viabilidade celular semelhante à do controle nas diluições mais baixas e BCR

foi semelhante ao controle, independentemente da diluição (P>0,05). A série de casos descreve técnica de revascularização modificada em molares permanentes jovens. Os pacientes foram submetidos ao preparo manual, seguido de limas rotativas NiTi e pasta de hidróxido de cálcio. Na segunda consulta, a medicação intracanal foi retirada, com irrigação final com EDTA sob agitação ultrassônica e promoção do coágulo. Selamento com MTA e ionômero de vidro e restauração com resina. Após 15 dias, verificou-se ausência de dor, edema e fístula, fechamento do ápice radicular e redução da luz do canal. Basedos nos resultados dos três objetivos concluiu-se que: (1) em pulpotomias totais, BT teve viabilidade celular aceitável, semelhante à do MTA e UC nas diluições mais altas, e BT resultou em menor alteração da cor do que MTA e UC; em pulpotomias parciais BCR apresentou melhores resultados do que BD e BCT, com menor mudança de cor, maior radiopacidade e viabilidade > 80%; os achados da série de casos confirmam que a regeneração endodôntica após preparo mecanizado do canal radicular é uma opção promissora para manutenção de molares permanentes em adolescentes.

PALAVRAS-CHAVE: Citotoxidade; Pulpotomia; Radiopacidade; Descoloração coronária, Regeneração da polpa; Tratamento endodôntico regenerativo.

ABSTRACT

The general objective was to evaluate in vitro, in models that mimic pulpotomies, staining and cytoxicity of biomaterials, and to present, through a case report, the feasibility and success of a regenerative endodontic procedure for the treatment of young molars. Objective 1: Simulate complicated coronary fracture in an incisor treated by total pulpotomy with Bio-C Temp (BC-Angelus) compared to two materials (MTA Flow – MTA, UltraCal XS-UC; Ultradent), evaluating tooth discoloration, radiopacity and cytotoxicity in pulp cells. Objective 2: Simulate partial pulpotomy with Bio-C Temp (BC) compared with Bio-C Repair (BCR-Angelus) and Biodentine (BD-Septodont), evaluating tooth discoloration, radiopacity, cytotoxicity. Objective 3: To report the follow-up of a series of revascularization cases in young permanent molars, with mechanized preparation of root canals. In objectives 1 and 2, bovine teeth were prepared to simulate extensive coronary fracture in a tooth with an open apex. The roots were filled with an agar / blood mixture, and the materials placed on it. The control group was filled with agar / blood only. Color evaluation analyzes were performed using a spectrophotometer. The total color change (∆Eab, ∆E00) and whiteness index (WI) were calculated based on CIELAB. Digital radiographs were acquired for radiopacity analysis. Human pulp cells were used to analyze cell viability. ANOVA and Tukey tests were used to compare the experimental groups, and Dunnett's for comparisons with the control. In total pulpotomies (objective 1) all materials were associated with discoloration values greater than acceptable and BT resulted in a color change that was less or similar to MTA and UC, respectively. A reduction in radiopacity was observed only in the MTA ($P = 0.007$). The cell viability of the materials was similar to that of the control (DMEM) ($P > 0.05$), except for BT which had significantly lower viability ($P < 0.001$). In partial pulpotomies (objective 2), BCR showed greater radiopacity and less color change (ΔEab / ΔE00) than other materials ($P < 0.005$; $P < 0.001$). BD showed cell viability similar to that of the control at the lowest dilutions and BCR was similar to the control, regardless of the dilution ($P > 0.05$). The case series describes a modified revascularization technique in young permanent

molars. The patients were submitted to manual preparation, followed by rotating NiTi files and calcium hydroxide paste. In the second consultation, the intracanal medication was removed, with final irrigation with EDTA under ultrasonic agitation and promotion of the clot. Sealing with MTA and glass ionomer and restoration with resin. After 15 days, there was no pain, edema and fistula, closure of the root apex and reduced canal light. Based on the results of the three objectives, it was concluded that: (1) in total pulpotomies, BT had acceptable cell viability, similar to that of MTA and UC at higher dilutions, and BT resulted in less color change than MTA and UC; in partial pulpotomies BCR showed better results than BD and BCT, with less color change, greater radiopacity and viability> 80%; the findings from the case series confirm that endodontic regeneration after mechanized root canal preparation is a promising option for maintaining permanent molars in adolescents.

KEYWORDS: Cytotoxicity; Pulpotomy; Radiopacity; Tooth discoloration, Pulp regeneration; Regenerative endodontic treatment.

1. INTRODUÇÃO E REFERENCIAL TEÓRICO

Traumatismos dentários são injúrias que acometem frequentemente crianças e jovens, podendo envolver dentes e estruturas de suporte (Qudeimat *et al.*, 2019), sendo que as fraturas coronárias em dentes permanentes representam entre 26% e 76% dos casos (Castro *et al*., 2005). Essas fraturas são comumente complicadas, com comprometimento pulpar em dentes com formação radicular incompleta. A necrose pulpar em dentes permanentes imaturos paralisa o desenvolvimento radicular, resultando em forame apical aberto e canais radiculares amplos (Castro *et al.*, 2005). Adicionalmente, as paredes de dentina permanecem curtas e finas, estando mais propensas à fraturas da mastigação ou por trauma (Chen *et al*., 2019), uma situação clínica que representa um significante desafio endodôntico e restaurador. Com o intuito de permitir a continuidade do desenvolvimento radicular, aumento em espessura da dentina e fechamento apical, duas modalidades terapeuticas tem sido preconizadas: pulpotomias e revascularizaçãoes. A pulpotomia é indicado para dentes que possuem características de vitalidade pulpar (Chen *et al*., 2019), enquanto as revascularizações, também conhecidas como procedimentos endodônticos regenerativos são utilizadas em dentes necrosados.

A pulpotomia é um procedimento que consiste na amputação cirúrgica da polpa coronária infectada seguido da colocação de um material de proteção pulpar para simultaneamente cobrir a polpa exposta e preservar sua vitalidade (Wells, Dulong & McCormack, 2019). A pulpotomia total remove toda a polpa coronária, o que tem sido relacionado à uma maior fragilidade coronária. Atualmente tem se falado muito em pulpotomia parcial, procedimento que remove apenas parcialmente a polpa coronária, ficando restrita à polpa exposta (Kang *et al*., 2021).

Os procedimentos endodônticos regenerativo foram introduzidos como uma opção de tratamento para dentes permanentes imaturos necróticos no início dos anos 2000 (Iawaya *et al*., 2001; Banchs et al., 2004), um procedimento baseado biologicamente, o qual substitui as células originais do

complexo dentino-pulpar por células tronco mesenquimais (Murray *et al*., 2007), promovendo além da eliminação dos sintomas e reparo ósseo, o aumento de espessura das paredes do canal, aumento do comprimento radicular e maturação apical (Kim *et al*., 2018). Resumidamente, a técnica consiste no uso de uma combinação de antimicrobianos para reduzir a infecção, indução de sangramento apical para formar um coágulo de sangue com células tronco indiferenciadas dentro do canal radicular (Lovelace *et al*., 2011), seguido da aplicação de material baseado em silicato de cálcio sobre o coágulo servindo como uma barreira coronal (Miller *et al*., 2018). Embora seja geralmente indicado para dentes anteriores traumatizados, vários relatos de caso tem demonstrado sua aplicabilidade também para molares cuja necrose ocorreu por uma cárie dental (Martin *et al*., 2013; Ajram *et al*. 2019) está no fim em amarelo.

Para o sucesso destes tipos de tratamento, os materiais devem ter certas características essenciais, como radiopacidade (Ochoa Rodrıguez *et al*., 2019), biocompatibilidade (Lee *et al*., 2017; Parirokh *et al*., 2018; Pedano *et al*., 2018; Cosme-Silva *et al*., 2019), atuar como barreira contra microorganismos, estimular o reparo e não contribuir para a descoloração dentária (Mozynska *et al*., 2017). O Hidróxido de cálcio ($Ca(OH)_2$) e o trióxido de agregado mineral (MTA) são os materiais mais comumente materiais usados em pulpotomias (Liu *et al*., 2011; Musale *et al*., 2018; Parirokh *et al*., 2018). O $Ca(OH)_2$ possui pH alcalino, sendo capaz de ativar a enzima fosfatase da linha alcalina e, consequentemente, estimular a produção de dentina terciária. Por décadas a pasta de hidróxido de cálcio foi considerado o material de primeira escolha para pulpotomias, contudo, o uso do hidróxido de cálcio por longo período tem sido associado a um selamento deficiente e alta solubilidade em fluidos orais (Gandolfi *et al*., 2015), o que o levou a ser substituído mais recentemente pelo agregado de trióxido mineral (MTA).

O MTA possui propriedades físicas e químicas aceitáveis e excelente biocompatibilidade (Camilleri, 2015; Lee *et al*., 2017; Parirokh *et al*., 2018; Nagendrababu *et al*., 2019; Chen *et al*., 2019). No entanto, a maioria dos estudos demosntra que o MTA pode causar descoloração da coroa devido ao presença de óxido de bismuto como radiopacificador (Yoldas *et al*., 2016;

Shokouhinejad *et al*., 2016). Além disso, embora o próprio material possa causar descoloração, a presença de sangue pode intensificar esse fenômeno (Guimarães *et al*., 2015). Outra limitação do MTA está relacionada à dificuldade de inserção do material tanto nos casos de pulpotomias como nas revascularizões.

Para minimizar essas limitações foi introduzido no mercado o MTA branco, o qual não mudou esse cenário, e segundo algumas pesquisas também pode promover manchamento (Parirokh *et al*., 2018). Com o objetivo de facilitar sua inserção surgiram novas formulações do MTA, com maior maleabilidade como MTA Repair HP (Angelus, Londrina, PR, Brasil) e mais recentemente MTA Flow (Ultradent, South Jordan, UT, USA). Porém um grande salto em relação aos materiais utilizados para pulpotomias e revasuscularizações foi dado pelo surgimento de novos materiais biocerâmicos (Beatty & Svec, 2015; Marconyak *et al*., 2016; Parirokh *et al.,* 2018; Pedano *et al*., 2018; Cosme-Silva *et al*., 2019).

Na última década, o Biodentine (BD, Septodont, Saint Maur-des-Fosses, France), um substituto dentinário com propriedades clínicas e biológicas comparáveis à do MTA foi introduzido no mercado. Esse material é um cimento reparador baseado em silicato tricálcico que apresenta como vantagens quando comparado ao MTA seu tempo de presa acelerado, facilidade de manipulação e manuseio, e menor descoloração coronária (Kaur *et al*., 2017). Outros materiais baseados em silicato de cálcio também têm sido recentemente desenvolvidos, destacando-se aqueles que se apresentam em uma formulação pronta para uso, como o Bio-C Repair (BCR, Angelus, Brasil). BCR tem sido indicado em pulpotomias e apresenta propriedades ideias como baixa ciotoxicidade, boa biocompatibilidade e habilidade de biomineralização (Benetti *et al*., 2019). No entanto, esses novos materiais são caros, o que muitas vezes torna seu uso em países emergentes inviável. Portanto, novos materiais de menor custo contendo partículas biológicas em sua composição têm sido desenvolvidos como uma possível alternativa para uso em pulpotomias, como o Bio-C Temp (Angelus, Brasil).

Devido ao grande impacto que as pulpotomias e procedimentos regenerativos podem respresentar no prognóstico e longevidade de dentes imaturos, estudos *in vitro* que mimetizem pulpotomias e permitam avaliar o potencial de discoloração, radiopacidade e biocompatibilidade de novos materiais são extremamentes relevantes, assim como relatos de casos que permitam difundir ao clínico tais procedimentos.

2. PROPOSIÇÃO

Objetivo Geral

Avaliar *in vitro,* em modelos que mimetizem pulpotomias, o manchamento e citoxicidade de novos biomaterais, e apresentar por meio de relato de caso clínico a exiquibilidade e sucesso de procedimento endodontico regenerativo para tratamento de molares jovens cariados.

Objetivos específicos

Objetivo específico 1

Capitulo 1 - A laboratory evaluation of cell viability, radiopacity and tooth discoloration induced by regenerative endodontic materials

Avaliar *in vitro,* em modelos que mimetizem pulpotomias, o manchamento e citoxicidade de novos biomaterais, e apresentar por meio de relato de caso clínico a exiquibilidade e sucesso de procedimento endodontico regenerativo para tratamento de molares jovens cariados.

Objetivo específico 2

Capítulo 2 - Biological parameters, discoloration, and radiopacity of calcium silicate-based materials in a simulated model of partial pulpotomy

Simular uma fratura coronária complicada em um incisivo tratado por pulpotomia parcial com Bio-C Temp (Angelus, Londrina, PR, Brasil) em comparação com outros dois materiais biocerâmicos, Bio-C Repair (Angelus, Brasil) e Biodentine (Septodont, Saint Maur-des-Fosses, France), avaliando descoloração dentária, radiopacidade, citotoxicidade e cicatrização nas células pulpares.

Objetivo específico 3

Capítulo 3 - Modified revascularization technique in permanent molars. A case series

Relatar o acompanhamento de uma série de casos clínicos de revascularização em molares permanentes jovens gravemente comprometidos em adolescentes, realizada com preparo mecanizado de canais radiculares.

3. CAPÍTULOS

3.1. Capítulo 1

Artigo submetido e aceito no periódico International Endodontic Journal

A laboratory evaluation of cell viability, radiopacity and tooth discoloration induced by regenerative endodontic materials

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INTERNATIONAL ENDODONTIC JOURNAL

doi:10.1111/iej.13308

A laboratory evaluation of cell viability, radiopacity and tooth discoloration induced by regenerative endodontic materials

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Abstract

Oliveira LV, da Silva GR, Souza GL, Magalhães TEA, Barbosa GLR, Turrioni AP, Moura CCG. A laboratory evaluation of cell viability, radiopacity and tooth discoloration induced by regenerative endodontic materials. *International Endodontic Journal*, 53, 1140–1152, 2020.

Aim To analyse the cytotoxicity, colour change and radiopacity of MTA Flow (MTA), UltraCal XS (UC) and Bio-C Temp (BT).

Methodology Human dental pulp cells (hDPCs) stimulated with lipopolysaccharide (LPS) were placed in contact with several dilutions of culture media previously exposed to the experimental materials and tested for cell viability using MTT. Bovine teeth were prepared to simulate an open apex and to mimic extensive crown fracture. The roots were filled with a mixture of agar and blood, and the materials placed over this mixture. The control group consisted of teeth filled only with agar and blood. Colour assessment analyses were performed before and immediately after material insertion and repeated at 30, 45 and 60 days using a spectrophotometer. The total colour change (ΔE_{ab}, ΔE_{00} and whiteness index (WI)) was calculated based on the CIELAB colour space. Digital radiographs were acquired for radiopacity analysis. Cell viability was analysed by one-way ANOVA, whilst differences in colour parameters (ΔE_{ab}, ΔE_{00} and WI) were assessed by two-way repeated measures ANOVA ($\alpha = 0.05$). Tukey's test was used to compare the experimental groups, and Dunnett's test was used to compare the experimental groups with the control group.

Results MTA, UC and BT had similar cell viability to that of the control group (DMEM) ($P > 0.05$), except for the BT group at the 1 : 1 and 1 : 2 dilutions, which had significantly lower viability ($P < 0.001$). All materials were associated with discoloration values greater than what is considered to be the acceptable threshold, and BT resulted in less or similar tooth colour change than MTA and UC, respectively. Decreasing radiopacity over time was observed only in the MTA group ($P = 0.007$). Lower values of radiopacity were found in the BT group compared with the UC and MTA groups ($P < 0.001$).

Conclusions The new bioceramic material (BT) had acceptable cell viability, similar to that of MTA and UC at the highest dilutions, and BT resulted in less tooth colour change than MTA and UC. Despite its lower radiopacity, BT was identified radiographically.

Keywords: cytotoxicity, pulpotomy, radiopacity, staining potential, tooth discoloration.

Received 9 January 2020; accepted 7 April 2020

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Introduction

Dental trauma often occurs in young people and may involve teeth and supporting structures (Qudeimat *et al.* 2019). Crown fractures of permanent teeth account for between 26% and 76% of all traumatic injuries (Castro *et al.* 2005). These fractures are often complicated with pulp involvement in teeth with incomplete root formation. In this case, pulpotomy is a viable treatment option because it allows continuous root development and apical closure (Alqaderi *et al.* 2016, Chen *et al.* 2019).

Pulpotomy has an excellent prognosis and comprises surgical amputation of the infected coronal pulp (depth of 1.5–2.0 mm) and placement of a protective material to cover the exposed pulp and in an attempt to preserve its vitality (Tuloglu & Bayrak 2016, Wells *et al.* 2019). For the success of this type of treatment, the materials must have certain essential characteristics, such as radiopacity (Ochoa-Rodríguez *et al.* 2019), biocompatibility and nontoxicity (Lee *et al.* 2017, Parirokh *et al.* 2018, Pedano *et al.* 2018, Cosme-Silva *et al.* 2019), as well as act as a barrier against microorganisms, stimulate tissue healing and not contribute to discoloration (Możyńska *et al.* 2017). Calcium hydroxide ($Ca(OH)^2$) and mineral aggregate trioxide (MTA) are the most commonly used materials in pulpotomies (Liu *et al.* 2011, Musale *et al.* 2018, Parirokh *et al.* 2018).

$Ca(OH)_2$ has an alkaline pH and can activate alkaline phosphatase and consequently stimulate the production of tertiary dentine. Nevertheless, the long-term use of $Ca(OH)_2$ is associated with poor sealing and high solubility in oral fluids (Gandolfi *et al.* 2015). Another material used in pulpotomy is MTA, which has acceptable physical and chemical properties and excellent biocompatibility (Camilleri 2015, Lee *et al.* 2017, Parirokh *et al.* 2018, Nagendrababu *et al.* 2019, Chen *et al.* 2019). Nonetheless, most studies have concluded that MTA may cause crown discoloration due to the presence of bismuth oxide as a radiopacifier (Yoldas *et al.* 2016, Shokouhinejad *et al.* 2016). In addition, although the material itself may cause discoloration, the presence of blood might intensify this phenomenon (Guimarães *et al.* 2015).

Therefore, new bioceramic materials have emerged to overcome these problems (Beatty & Svec 2015, Marconyak *et al.* 2016, Parirokh *et al.* 2018, Pedano *et al.* 2018, Cosme-Silva *et al.* 2019), such as Sealer Plus (MK Life) and Endosequence BC Sealer (Brasseler), but only a few studies have analysed conditions that mimic pulpotomy in traumatized immature teeth. However, these new materials are expensive, which often makes their use in emerging countries infeasible. Therefore, new, lower cost materials containing bioceramic particles in their composition have been developed as a possible alternative for use in pulpotomies. In this sense, the present study aims to compare the cell viability of human dental pulp cells (hDPCs), radiopacity and crown discoloration produced by a new bioceramic material (Bio-C Temp®; Angelus, Londrina, PR, Brazil) and two materials (MTA Flow and UltraCal XS; Ultradent, South Jordan, UT, USA) traditionally used in pulpotomies. The null hypothesis was that no significant differences would be found in the cell viability, radiopacity or coronal discoloration associated with the evaluated materials.

Materials and methods

Preparation of materials for cell viability test

The materials used were MTA Flow (MTA) (Ultradent), UltraCal XS (UC) (Ultradent) and Bio-C Temp® (BT) (Angelus, Londrina). The components of the root canal filling pastes tested are described in Table 1. The MTA Flow samples were prepared according to the manufacturer's recommendations. Then, 0.22 mL of MTA Flow, UC and BT were inserted in 24-well plates under aseptic conditions in a laminar flow cabinet. Immediately, all materials were covered with 2.5 mL of Dulbecco modified Eagle medium (DMEM) for cell culture (Vitrocell Embriolife, Campinas, SP, Brazil) and incubated in the dark for 24 h at 37 °C (Bin *et al.* 2012). The original extracts (1 : 1) were prepared following the recommendations of the ISO 10993 (2009). After incubation, these original extracts were serially diluted in cell culture medium before testing until the dilution of 1 : 32.

hDPC culture

Primary human dental pulp cell (hDPCs) cultures were donated from the School of Dentistry of the Federal University of Uberlândia (UFU), after signing the informed consent form by the guardians (Ethics Committee protocol number 09016219.1.0000.5152). Two healthy primary teeth nearing ($n = 2$) were collected, and the pulp was extracted from the pulp chamber using a sterilize sharp excavator. Afterwards,

Table 1 Components of the root canal filling pastes tested

Material	Technical information	Components
UltraCal XS (Ultradent, Indaiatuba, SP, Brasil)	Paste ready for use	Calcium hydroxide, barium sulphate, aqueous matrix of methylcellulose
MTA Flow (Ultradent, Indaiatuba, SP, Brasil)	Powder and gel (two big ends mixed with two drops and inserted with microtips)	Extremely fine inorganic powder of tricalcium and dicalcium silicate with a water-based gel
Bio-C Temp (Angelus, Londrina, PR, Brasil)	Paste ready for use	Calcium silicate, calcium aluminate, calcium oxide, calcium tungstate in a mixture of ester glycol salicylate and polyethylene glycol and other supplementary agents

the pulp tissue was immersed for 1 h in the following solution: 3 mg mL^{-1} collagenase type I (Sigma-Aldrich, San Louis, MI, USA) and 4 mg mL^{-1} dispase (Sigma-Aldrich). The samples were centrifuged at 250 *g* (centrifuge 80-2B, Centribio, Curitiba, PR, Brazil) for 2 min and resuspended in basal medium. The cells obtained were plated in 25-cm^2 flasks and incubated for 4 days at 37 °C with 5% CO_2. The culture medium was first replaced after 3 days of incubation; thereafter, it was changed twice a week. The cells were expanded up to the 4th passage and frozen for later experimental use.

hDPCs with lipopolysaccharide-induced stress and exposure to extracts

Cells were cultured in DMEM (Vitrocell) supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and 1% penicillin–streptomycin (Sigma-Aldrich) in a humid atmosphere of 5% CO_2 and 37 °C until confluence. The hDPCs were plated on 96-well plates (2×10^4 cells/well) and allowed to adhere overnight. Then, the cells were incubated with 200 μL of the extracts at pre-determined dilutions (1 : 1, 1 : 2, 1 : 4, 1 : 8, 1 : 16 and 1 : 32) and simultaneously with lipopolysaccharide (LPS) (LPS, Ultra-pure grade, *Escherichia coli* O111:B4, Invitrogen, San Diego, CA, USA) at the concentration of 10 μg mL^{-1}, for a period of 24 h. After the incubation period, the cells were immediately tested for viability by MTT formazan. The control group was maintained in DMEM (not LPS-stimulated). This study was repeated twice using five samples for each group at every moment. Cell viability was evaluated proportionally to absorbance and expressed as the percentage of viable cells. The mean values obtained for the control group were considered as 100% of cell viability, and the values of each sample of the experimental groups were obtained proportionally to the control.

Analysis of viability by MTT formazan

The cell viability was evaluated 24 h after the treatment with the extracts. MTT solution (Sigma-Aldrich; 5 mg mL^{-1}) was added to each well, and the cells were incubated at 37 °C for 4 h. The supernatants were removed, and then, 100 μL dimethyl sulphoxide (DMSO; LGC Biotecnologia, Cotia, SP, Brazil) was added. Afterwards, the optical density (OD) at 570 nm was measured using a microplate reader (Biochrom, Cambridge, UK).

Selection of teeth and sample preparation for colour measurements and radiopacity tests

The sample size calculation was based on data from Yoldas *et al.* (2016). Eighteen teeth per group were required to have a 90% chance of detection as significant at the 5% level (2-sided test), with a minimum detectable difference in means of 5.97 with an expected standard deviation of 4.61 with regard to primary outcome (colour discoloration – ΔE_{ab}) evaluated by a sphere spectrophotometer. The calculation was performed using the statistical software package SigmaStat version 12.5 (Systat Software Inc., San Jose, CA, USA).

Seventy-two central incisors from young cows were obtained from a local abattoir (Real, Uberlândia, MG, Brasil). Each tooth was cleaned and stored in distilled water at 4 °C. Extrinsic stains and calculi were removed with an ultrasonic scaler, followed by polishing with pumice paste and water. To create standardized specimens and to mimic traumatic dental injuries in immature permanent incisors, the apical part of each root was removed with a high-speed disc (12 mm from the amelocemental region to the apical region), and a part of the crown of each tooth was removed (8 mm from the cement–enamel region to the incisal edge). Next, the apical opening of the root canal was treated with 37% phosphoric acid for 15 s and then rinsed. The bonding agent (3M ESPE, St. Paul, MN,

USA) was applied and light cured for 20 s, and then, a composite resin material (3M ESPE Z250, Sumaré, SP, Brazil) was placed and cured for 40 s (Fig. 1a). The specimens were then randomly assigned ($n = 18$) to three experimental subgroups (BT, MTA and UC) and a control group (agar + blood). Each tooth was included in polystyrene resin, and the preparation was performed with a PM 82 drill (KG Sorensen, Cotia, SP, Brazil) to obtain similar root canals with a large internal diameter. Then, the root canals were rinsed with 2.5% sodium hypochlorite for 20 min followed by 3 mL of 17% EDTA solution and 5 mL of distilled water. To simulate the pulp and inherent difficulties related to the pulpotomy procedure, a mixture of agar (Kasvi, São José dos Pinhais, PR, Brazil) and bovine blood was prepared (Lenherr *et al.* 2012). Agar was weighed and diluted in warm water according to the manufacturer's recommendations. Then, 6 mL of prepared agar was mixed with 100 μL of fresh uncoagulated blood and inserted on the root canal using pipette tips in a volume of approximately 80 μL per tooth.

Colour assessment

A spectrophotometer (Easyshade Compact Advance 4.0; Vita-Zahnfabrik, Bad Sackingen, Germany) was used to assess tooth colour. A silicone index (Precise SX; Dentsply, Petropolis, RJ, Brazil) containing a 6-mm hole for the placement of the spectrophotometer tip was used to standardize the readings and reposition the Easyshade at each time-point (Fig. 1b). Three assessments were performed on each tooth, and the average was recorded. Five sessions of colour measurements were conducted at the following intervals: T0, before application of the root-end filling material (baseline); T1, immediately after application of the root-end filling material; T30, 30 days after; T45, 45 days after; and T60, 60 days after.

The CIE $L^*a^*b^*$ system (L^*: white/black; a^*: red/green; b^*: yellow/blue) values were noted for each specimen. The mean value of three measurements was calculated at each assessment time/material. The total colour differences (ΔE_{ab}) were calculated using the following equation: $\Delta E_{ab} = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]$ ½. In addition, the whitening indexes (WI) were calculated using the following formula: $WI = 0.551^*L - 2.324^*a - 1.1^*b$ (Pérez Mdel *et al.* 2016), and DE_{00} was also calculated using the formula described in a prior study (Sharma *et al.* 2005).

Radiopacity

For radiopacity analysis of the materials (Fig. 2), all teeth in the four groups were radiographed using the VistaScan Mini Plus® photostimulable phosphor (PSP) system (Dürr Dental, Bietigheim-Bissingen, Germany). Each specimen was placed on the centre of a size 2 (3 × 4 cm) PSP plate along with a 10-mm aluminium step wedge. A Timex 70E X-ray unit (Gnatus, Ribeirão Preto, SP, Brazil) was used, operating at 70 kV, 7.0 mA, 0.14-s exposure time and 28 cm focus/film distance. After exposure, the plates were scanned, and the 8-bit images were exported to ImageJ for Windows software (National Institutes of Health, Washington, WA, USA). For each image, one area of the same square format (50 × 50 pixels) was defined as the region of interest (ROI). This ROI was placed in the area of the radiographs that contained the most homogenous part of the restorative material. The mean grey values of the ROIs were determined using the histogram analysis tool of the software. Radiographs and a grey value analysis were performed before, immediately after and thirty days after filling material application.

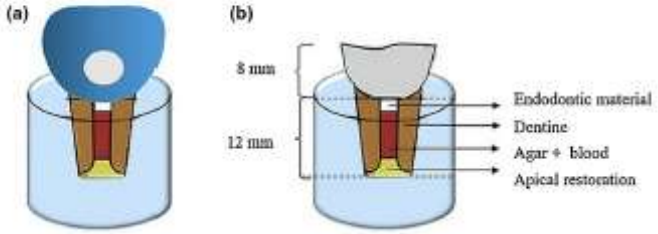

Figure 1 Schematic showing tooth specimen: (a) after coronal and root preparation; (b) after silicone impression material index insertion for colour measurements.

Statistical analysis

Cell viability, colour assessment and radiopacity data were analysed for normality and homoscedasticity using the Shapiro–Wilk and Levene tests. One-way ANOVA followed by Tukey's test was used to compare data of the cell viability intragroup amongst dilutions and amongst the materials at each of the dilutions tested. Two-way repeated measures ANOVA and Tukey's tests were used to compare the radiopacity and colour parameters (*L, *a, *b, ΔE_{ab}, ΔE_{00} and WI), where 'time assessment' was used as a repetition factor, Dunnett's test was used to compare the colour in the experimental groups with the control group. A statistical analysis was performed using SigmaPlot 12.5 statistical software package (Systat Software Inc). The significance level was set at 95% for all data analyses.

Results

Viability by MTT formazan

The cytotoxicity results are presented in Figs 3 and 4. The MTA group was not different amongst the dilutions evaluated ($P = 0.09$). Dunnett's test revealed that the MTA-treated cells had greater viability than the control group cells (DMEM) at 1 : 4 dilution ($P = 0.01$).

Significantly lower percentages of viable cells were obtained after the treatment with UC extracts at 1 : 8 dilution, ($P = 0.0364$). All dilutions tested for UC had similar cell viability compared with the control group (DMEM) ($P > 0.05$).

Significant differences were obtained amongst the dilutions in relation to the BT group ($P < 0.0001$). At 1 : 1 and 1 : 2 dilutions, there was a significantly lower viability compared with other dilutions and with the control group (DMEM) ($P < 0.0001$, Fig. 3). Figure 4 shows the comparison between root-end filling materials at the dilutions tested. BT had significantly lower viability than the other materials at the 1 : 1 and 1 : 2 dilutions ($P < 0.0001$). At the 1 : 4 and 1 : 8 dilutions, UC and BT were similar to each other ($P = 0.16$ and $P = 0.97$, respectively), and MTA led to greater viability ($P = 0.001$) at the 1 : 8 dilution. At 1 : 16 and 1 : 32, the three materials had similar values of cell viability.

Colour assessment

Table 2 presents the mean and standard deviation values of colour alteration (ΔE_{ab} and ΔE_{00}) for all groups immediately after application of the root-end filling material (T1) and over time (T30, T45, T60). Two-way repeated measures ANOVA revealed a significant interaction between material and assessment time (ΔE_{ab}: $P = 0.012$ and ΔE_{00}: $P = 0.023$). UC was associated with less colour alteration at 45 days than MTA (ΔE_{ab}: $P < 0.001$ and ΔE_{00}: $P < 0.001$) and BT (ΔE_{ab}: $P < 0.001$ and ΔE_{00}: $P < 0.001$).

At the 60-day measurement, UC and BT had similar tooth discoloration, whereas MTA had significantly more discoloration ($P < 0.001$). The analysis of MTA and UC over time revealed that the colour tended to stay stable over 30–60 days. BT had a maximum discoloration at 45 days followed by a rebound effect at 60 days. When comparing the colour change of the samples from the initial assessment time (T1) to the other experimental time-points (T30; T45; T60), the control group, UC and MTA all had significant discoloration. Dunnett's test immediately after material placement (T1) revealed a significant discoloration between the control group compared

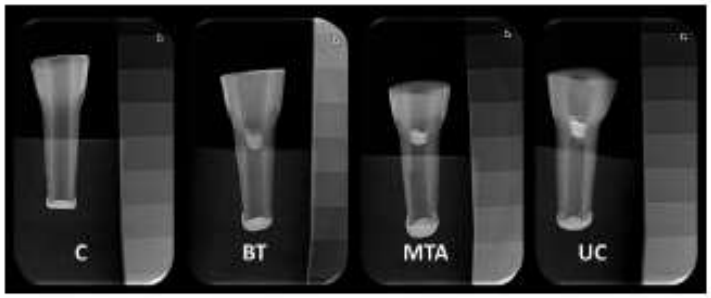

Figure 2 Representative images of radiopacity from each group: C, control (agar + blood); BT, Bio-C Temp; MTA, mineral trioxide aggregate (MTA Flow); UC, ultraCal XS.

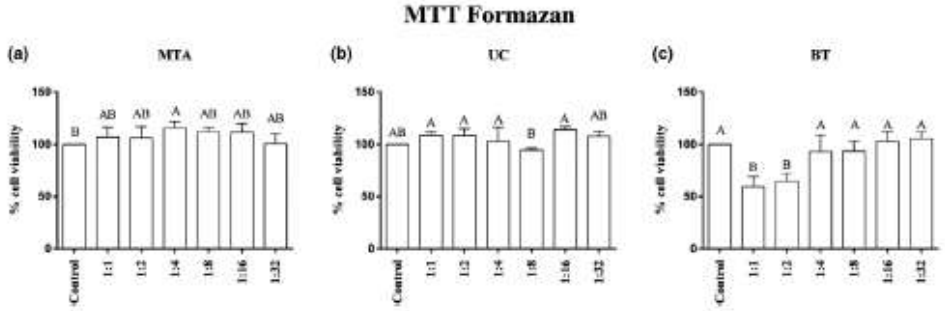

Figure 3 Cell viability percentage of hDPCs after exposure to extracts according to material tested and dilution by the MTT formazan method. (a) MTA, mineral trioxide aggregate (MTA Flow) exposure; (b) UC, UltraCal XS exposure; (c) BT, Bio-C Temp exposure. Capital letters indicate comparison amongst different dilutions of extracts and the control group for each material. One-way ANOVA and Tukey's test ($P < 0.05$).

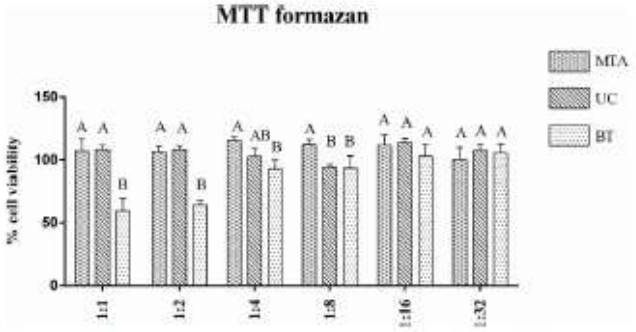

Figure 4 Cell viability percentage of hDPCs after exposure to extracts comparing the materials in the same dilution by the MTT formazan method. MTA, mineral trioxide aggregate (MTA Flow); UC, UltraCal XS; BT, Bio-C Temp. Capital letters indicate comparison amongst different materials at the same dilution. One-way ANOVA and Tukey's test ($P < 0.05$).

Table 2 Mean and standard deviation of discoloration (ΔE_{ab} and ΔE_{00}) in the different groups evaluated after root-end filling material and assessment time

	ΔE_{ab}				ΔE_{00}			
Groups	T1	T30	T45	T60	T1	T30	T45	T60
Control (agar + blood)	1.9 (2.0)c	9.5 (2.6)a	7.6 (2.3)b	7.4 (2.3)b	1.2 (1.2)b	4.85 (1.5)a	4.27 (1.4)a	4.22 (1.4)a
BT	7.4 (2.4)Ab*	8.1 (1.35)Aab	9.6 (2.6)Aa	7.5 (2.4)Bb	4.27 (1.5)Ab*	5.08 (0.8)Ab	5.9 (1.6)Aa*	4.47 (1.4)Bb
UC	2.8 (2.9)Bb	5.6 (2.4)Ba*	6.0 (2.6)Ba	6.6 (3.2)Ba	1.48 (1.3)Bb	3.25 (1.5)Ba*	3.32 (1.6)Ba	3.4 (1.5)Ba
MTA	9.2 (7.8)Ab*	10.7 (5.9)Aa	14.5 (7.9)Aa*	12.9 (5.2)Aa*	5.35 (4.2)Ab*	5.93 (2.9)Aa	8.63 (4.9)Aa*	7.46 (3)Aa*

T1, after application of root-end filling material; T30, 30 days after; T45, 45 days after; and T60, 60 days after.
Different capital letters in columns indicate significant differences between filling materials in the same assessment time, and different lowercase letters in rows indicate significant intragroup differences between the periods analysed (two-way repeated measures ANOVA and Tukey's test - $P < 0.05$).
*Symbol indicates significant differences in columns with the control group (agar + blood) by Dunnett's method, $P < 0.05$.

with BT and MTA ($P < 0.001$). At 30 days, only the UC group differed significantly from the control group, whereas at 60 days, a significant difference from the control group was observed only in the MTA group ($P < 0.001$).

Figure 5 shows the CIELAB parameters. L^* is the parameter that usually represents the major concern from an aesthetic standpoint (darkness to lightness), and it initially presented a similar behaviour between the groups at 30 days. However, at 60 days, the MTA (vs. UC; $P < 0.001$ and vs. BT; $P = 0.031$) and control (vs. UC; $P < 0.001$ and vs. BT; $P = 0.019$) groups had greater darkening than the other groups (low L values). The UC and BT groups had similar behaviour, with a reduction in L values up to 30 days and a tendency of recovery of L values after this period; this was not observed in the control and MTA groups.

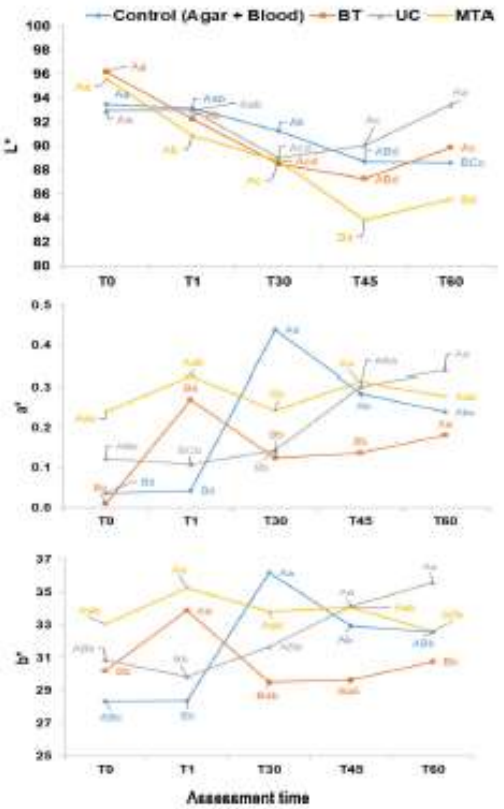

Figure 5 Graphs show the trends in the L^*, a^* and b^* parameters of the materials over time (L^*: white/black; a^*: red/green; b^*: yellow/blue). Different capital letters indicate differences between filling material in the same interval assessment time, and different lowercase letters indicate intragroup differences between the periods analysed.

In relation to the a^* parameter (red-green gradient), there was a fluctuation of mean values in the experimental periods amongst the different materials and the control, with a tendency to equivalence at 60 days. The analysis of the graph (Fig. 5b) reveals that the MTA group had a lower tendency to change in a^* values over time. The BT group had an initial peak after material insertion, a dramatic reduction at 30 days and a subsequent increase at 60 days, whereas the UC group remained stable until 30 days, increasing its a^* values after this period.

The b^* parameter (blue-yellow gradient) was similar amongst groups at 30 and 60 days, except in the BT group. The analysis of the behaviour of each material over time revealed little variation in the mean values of b^* for the BT and MTA groups over the 30- to 60-day period. The UC group had a significant increase in the mean value of b^* at 45 days ($P < 0.001$), and after 60 days, all groups behaved similarly to the control group.

The whiteness index (WI) was significantly influenced by 'material' ($P = 0.044$), 'assessment time' ($P < 0.001$) and the interaction 'material × assessment time' ($P < 0.001$). These data are presented in Fig. 6. Immediately after material insertion, MTA had significantly lower WI values than UC ($P = 0.009$) and BT ($P = 0.032$), which behaved similar to the control group at T1. At T45, UC ($P = 0.018$) and MTA ($P < 0.001$) demonstrated a reduction in WI compared to BT, and all materials retaining this low index at T60. WI was similar at T60 for all materials. In general, the BT group had the most WI changes compared with control group over time. The alterations in colour parameters are illustrated in Fig. 7. To facilitate the visualization of colour changes, the values of L^*, a^* and b^* were converted to an RGB (red, green and blue) system, and coloured rectangles were drawn in RGB using Microsoft® PowerPoint®.

Radiopacity

Table 3 presents the median and standard deviation values for radiopacity expressed as grey values one and thirty days after material insertion. The t-test did not indicate a difference between the initial (104.4 ± 8.1) and final (108.5 ± 8.6) radiopacities of the control teeth, which did not receive the material ($P = 0.159$). BT had a significantly lower initial radiopacity than UC and MTA ($P < 0.001$), which

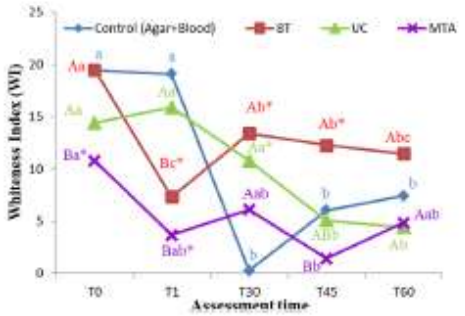

Figure 6 The behaviour of the whiteness index during the entire experiment. T0: baseline, T1: after application of root-end filling material, T30: 30 days after; T45: 45 days after; and T60: 60 days. BT, Bio-C Temp; UC, UltraCal XS; MTA, mineral trioxide aggregate (MTA Flow). Different capital letters indicate significant differences between filling materials in the same assessment time, and different lowercase letters indicate significant intragroup differences between the periods analysed (two-way repeated measures ANOVA, $P < 0.05$); *symbol indicates significant differences with the control group (agar + blood) in the same assessment time by Dunnett's method, $P < 0.05$.

had similar radiopacity ($P = 0.97$), at T1. After 30 days, all materials had different radiopacities, with the highest grey values in the UC group (vs. MTA: $P = 0.045$ and vs. BT: $P < 0.001$) and the lowest in the BT group (vs MTA: $P < 0.002$). There was an interaction factor, 'assessment time × material' ($P = 0.035$), and only MTA had significantly reduced radiopacity after 30 days ($P = 0.007$).

Discussion

The results support the rejection of the null hypothesis tested because significant differences were found between the materials regarding the viability of pulp cells, radiopacity and coronal discoloration in the presence of blood. Previous studies evaluating the cell viability of pulp cells in contact with MTA demonstrated that this material did not affect this parameter in hDPCs (Rodrigues *et al.* 2017, Tomás-Catalá *et al.* 2017, Pedano *et al.* 2018). However, no studies to date have evaluated BT cytotoxicity because this material is new. Biocompatibility is an important property that should be considered when selecting a material for pulpotomies due to its direct contact with vital tissues (Lee *et al.* 2014). Amongst the evaluations that can be performed in this context, the analysis of cytotoxicity and potential adverse effects on cell behaviour is one of the most commonly used. In the present study, the cytotoxicity test selected was MTT formazan. The MTT formazan method is a widely used cytotoxicity test (Pires *et al.* 2016, Collado-González *et al.* 2017) that determines cell viability as a function of their mitochondrial activity through the conversion of tetrazolium salt into formazan crystals by mitochondrial dehydrogenases (Mosmann 1983).

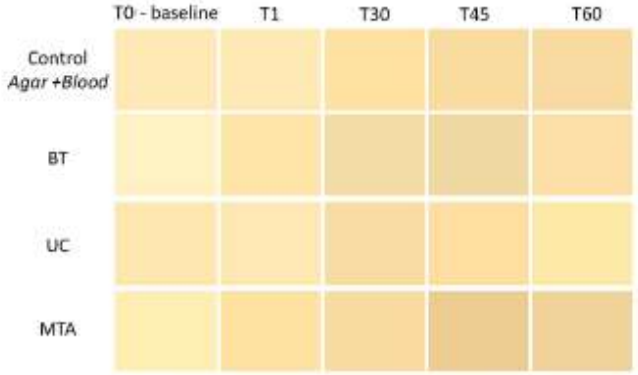

Figure 7 Tooth behaviour illustrated based on data from $L^*a^*b^*$ converted to RGB demonstrating the colour changes of specimens during the experiment. T0: baseline, T1: after application of root-end filling material, T30: 30 days after; T45: 45 days after; and T60: 60 days after. BT, Bio-C Temp; UC, UltraCal XS; MTA, mineral trioxide aggregate (MTA Flow). Illustrative cylinder-shaped composite specimens.

Table 3 Mean and standard deviation of radiopacity (grey value) in the different groups evaluated at day 1 and day 30

	Assessment time	
Groups	T1	T30
BT	187.4 (9.7)Ba	186.2 (9.6)Ca
UC	201.2 (11.5)Aa	202.7 (11)Aa
MTA	200.5 (10.6)Aa	196 (11)Bb

T1: immediately after application of root-end filling material and T30: 30 days after.
Different capital letters in columns indicate significant differences between filling material in the same assessment time, and different lowercase letters in rows indicate significant intragroup differences between the periods analysed (two-way repeated measures ANOVA, $P < 0.05$).

The MTT results revealed that MTA and UC were not cytotoxic for hDPCs at all dilutions. MTA has been reported to induce proliferation of hDPCs by elution components such as calcium ions (Takita *et al.* 2006). The high proliferation of hDPCs at the 1 : 4 dilution of extract corroborates previous studies using the MTT assay (Rodrigues *et al.* 2017, Pedano *et al.* 2018). Few researchers have evaluated the cytotoxicity of $Ca(OH)_2$ paste in the same formulation used in the present study (Althumairy *et al.* 2014). Previously, it was reported that UC extracts caused a significant increase in cell viability (Pires *et al.* 2016), which was not found in the present study. This could be because the referenced study used peripheral blood mononuclear cells, and $Ca(OH)_2$ has the capacity to induce an inflammatory response (Nelson Filho *et al.* 1999). Regarding BT, an increase in cell proliferation was expected because its composition includes calcium silicates, calcium aluminate, calcium oxide and calcium tungstate. However, the lowest BT dilutions had a cytotoxic effect on hDPCs, decreasing the cell viability to approximately 60% compared with the control. A possible hypothesis for this reduction in viability is the presence of TiO_2 in its composition. This component may interfere with a series of cellular events, including those associated with stimulation of the mitogen-activated protein kinase (MAPK) pathway, with a consequent reduction in cell survival (Yu *et al.* 2019). In addition, previous investigations have demonstrated that TiO_2 induces apoptosis in different cell types including murine leukaemic monocyte macrophages (RAW 246.7 cells) (Dhupal *et al.* 2018), lymphocytes (Wang *et al.* 2007), fibroblasts (Jin *et al.* 2008) and mesenchymal stem cells (Yu *et al.* 2019). However, considering that there are no studies evaluating the cytotoxicity of this component in hDPCs, it is not possible to directly relate to the results of these studies. In addition, it is important to note that at higher dilutions, the behaviour of BT-treated cells was similar to the behaviour of cells treated with the other tested materials, and therefore, the use of BT in pulp cells would not be contraindicated.

In pulpotomies, the material is placed directly into tissue containing blood; therefore, aiming to mimic the clinical situation, all materials of the study were applied directly to a mixture of agar containing blood, as agar has a gelatinous consistency similar to that of pulp tissue. The present results revealed that blood was able to increase the discoloration associated with all materials, including colour changes in the negative control group (agar + blood). The discoloration in the negative control group was greater at 30 days, remaining low in the subsequent periods. Additionally, the luminosity and WI of the control group reduced over time, indicating that the presence of blood caused tooth darkening. A possible mechanism explaining the staining caused by blood is related to the accumulation of haemoglobin or other haematin molecules (Marin *et al.* 1997). The haemolysis of these molecules releases haeme groups, which can cause darkening of the tooth structure as they produce black iron sulphide. Therefore, a reduction in lightness values and an increase in redness and yellowness values (Fig. 5) following blood exposure to the specimens could be expected.

Beyond the blood, biomaterials are related to tooth staining (Beatty & Svec 2015). Several studies have reported greater staining for grey MTA associated with blood (Lenherr *et al.* 2012, Guimarães *et al.* 2015). Laboratory studies have indicated that MTA is associated with high staining potential as it comprises heavy metal ions and bismuth oxide as a radiopacifier (Marciano *et al.* 2015). Possible explanations for MTA-related tooth discoloration are related to the dissociation of oxide bismuth into dark crystals (Yoldas *et al.* 2016, Możyńska *et al.* 2017) or overoxidation of this compound due to contact with NaOCl (Camilleri 2014, Marconyak *et al.* 2016), which clinically occurs in pulpotomy. Furthermore, this material in contact with blood exacerbates the discoloration process (Guimarães *et al.* 2015, Shokouhinejad *et al.* 2016). White MTA was developed to overcome this issue. However, even white MTA may cause discoloration, probably due to oxidation and incorporation of the iron content into the calcium aluminoferrite phase of MTA after setting (Marciano *et al.* 2015).

Particularly in the present study, MTA Flow was selected due to its easier insertion using syringes, which clinically results in a smaller amount of material residues on the dentine walls. This is a relatively new material consisting of a grey powder containing dicalcium and tricalcium silicate, bismuth oxide and a liquid vehicle composed of a water-soluble silicone-based gel that can be manipulated in various consistencies. The manufacturer proposes its use in pulp capping, pulpotomies, sealing perforations and resorptions, retrofillings and teeth with an incomplete root apex (Ultradent, 2011, Ultradent, 2017). The other materials used for comparison (BT and UC) are also injectable and come in a ready-to-use form that also makes them easy to insert. UC is based on a calcium hydroxide paste (calcium hydroxide, barium sulphate and aqueous matrix), and it is known to be used in pulpotomies and direct pulp protection but with high solubility (Pereira *et al.* 2019). BT is a paste recommended for intracanal medication and pulp regeneration by the manufacturer (Angelus 2019). BT colour visually differs from UC, having a more yellowish coloration; additionally, its consistency is slightly different, probably related to the vehicle used. Even though the components of BT medication classify it as a bioceramic material, studies using other bioceramics in pulpotomies have shown less discoloration (Camilleri 2015, Shokouhinejad *et al.* 2016, Yoldas *et al.* 2016). Biodentine (Septodont, France) has shown less discoloration than MTA, possibly due to the use of zirconium oxide as a radiopacifier instead of bismuth oxide (Yoldas *et al.* 2016). BT has a titanium oxide radiopacifier, which is not expected to produce dentine staining. However, BT had ΔE_{ab} and ΔE_{00} values similar to those of MTA and higher than those of UC at 45 days. This could indicate a transient interaction between blood and BT compounds because at 60 days, these values were reduced, making them lower than those of MTA and similar to those of the control. The intense white colour of the UC probably blocks the influence of blood on the initial colour measurement, which could be observed at 30 days, when the UC group presented lower ΔE_{ab} and ΔE_{00} values than the control group. However, this difference was attenuated over time, presenting mean values of discoloration similar to those of the control and BT groups at 60 days. It is important to note that all materials tested had values higher than what is considered the acceptable threshold ($\Delta E_{ab} = 2.66$ and $\Delta E_{00} = 1.77$; Paravina *et al.* 2015).

Associated with the global colour change, the whiteness index (WI), a simple linear formulation obtained using the values of the three CIELAB chromatic coordinates, was used (Pérez *et al.* 2016). It represents a significant step for the assessment of colour change because it correlates with the perception of tooth whiteness. The results of this method are more clinically relevant and provide a clearer interpretation: high positive values of the WI index indicate higher whiteness values. Tooth yellowness may not be a perfect antonym of tooth whiteness, but WI could be used to reflect perceptual yellowness (Sullivan *et al.* 2019). Compared with the classic materials used in pulpotomy, BT had WI values similar or lower than those already established in the literature. Moreover, all materials resulted in a slightly greater difference in tooth whiteness (≥5.69) (Pérez Mdel *et al.* 2016) compared with teeth without any root-end filling material.

For this study, the Vita Easyshade spectrophotometer was used to evaluate colour change. This instrument was applied because of the technique's sensitivity to even slight changes in colour and excellent reproducibility. The same equipment was used in previous studies (Guimarães *et al.* 2015, Marconyak *et al.* 2016, Yoldas *et al.* 2016), which used the CIE $L^*a^*b^*$ space system to evaluate colour change. Regarding radiopacity, the ISO standard was not used, which uses pre-established silicone moulds filled with cement. The reason for inserting the material directly into the tooth was for the study design to approximate clinical practice, where this is the only standard of evaluation by the professional. Bovine teeth have been previously used as a substitute for human in studies of tooth discoloration (Beatty & Svec 2015, Yoldas *et al.* 2016). Considering that the coronal dentine of bovine teeth does not differ significantly from that of human teeth in terms of density or diameter of tubules, bovine mandibular incisors may be used in this kind of study (Lenherr *et al.* 2012, Beatty & Svec 2015, Yoldas *et al.* 2016).

Another parameter evaluated was the radiopacity of the materials, which is an important factor to consider when choosing a material for pulpotomy because it enables the visualization of gaps or absence of material through the X-ray and differences in tooth tissues (Guerreiro-Tanomaru *et al.* 2009, Xuereb *et al.* 2016). In the present study, the higher initial radiopacity values (T1) for MTA and UC groups than for BT were probably related to the differences in radiopacifiers present in each material. Nonetheless, it

is possible to observe that only the MTA group had a reduction in a radiopacity after 30 days, which is in agreement with other studies (Camilleri 2008, Cavenago *et al.* 2014, Guimarães *et al.* 2015) and may be caused by the dissociation of bismuth oxide. On the other hand, it is possible that the radiopacity stability provided by the titanium oxide used as a radiopacifier in BT and by the barium sulphate in UC could be related to the smaller colour variations of these materials over time. It is important to observe that despite the lower radiopacity, BT could be a visual differentiated from the tooth tissues (Fig. 2), fulfilling this fundamental requirement for a material used in pulpotomy.

Conclusion

The new bioceramic material BT had acceptable cell viability, similar to that of MTA and UC, at the greatest dilutions, and resulted in less or similar tooth colour change compared with MTA and UC, respectively. Despite its lower radiopacity, BT was identified radiographically.

Acknowledgments

This project was developed at CPBio – Biomechanics, Biomaterials and Cell Biology Research Center of the Federal University of Uberlândia. The research was 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 conflicts of interest in connection with this article.

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3.2 Capítulo 2

Artigo submetido ao periódico International Endodontic Journal

Biological parameters, discolouration, and radiopacity of calcium silicate-based materials in a simulated model of partial pulpotomy**.**

Abstract

Aim: We aimed to analyse the discolouration, radiopacity, pH, and calcium ion release of Biodentine (BD), Bio-C repair (BCR), and Bio-C temp (BCT), as well as their biological effects on human dental pulp cells (hDPCs). **Methodology:** Bovine teeth were prepared to simulate crown fractures with pulp exposition in the open root apex. The roots were filled using a mixture of agar and blood (control), and BD, BCR, or BCT were placed over the mixture. Colour assessment analyses were performed before and immediately after material insertion and repeated at 30 and 90 days, using a spectrophotometer. Total colour change was calculated based on the CIELab colour space. Digital radiographs were acquired for radiopacity analysis. hDPCs were placed in contact with different dilutions of culture media previously exposed to such materials and tested for cell viability using the MTT assay. The pH and calcium ion release of all materials were measured after 24 h; the data were assessed using one-way analysis of variance (ANOVA). Cell viability was analysed by two-way ANOVA. Differences in colour parameters and wound healing data were assessed by two-way repeated-measures ANOVA ($\alpha=0.05$). Tukey's and Dunnett's tests were used to compare the experimental groups with the control group. **Results:** BCR presented higher radiopacity and smaller colour alteration ($\Delta Eab/\Delta E00$) than the other materials tested ($P<0.005$; $p<0.001$). No significant differences in pH were found among the tested materials ($P>0.05$). BCT showed the largest release of calcium ions ($P<0.0001$). BD presented cell viability similar to that of the control at the lowest dilutions, and BCR's was similar to that of the control, regardless of the dilution tested ($P>0.05$). BCT showed a lower percentage of viability than that of the control at all tested dilutions ($P<0.0001$). Cell migration rates in BD and BCR were similar to those in the control group

after 24 h and 48 h (P>0.05), while BCT presented larger open areas than the control in both periods (P<0.0001). **Conclusions:** BCR showed better favourable results than BD and BCT, with lower colour change, higher radiopacity, and hDPC viability of >80%.

Introduction

Complicated crown fractures involving enamel and dentin with pulp exposure should preferably be treated using conservative pulp therapies (CPT), even in adults (Bourguignon *et al*., 2020). CPT includes pulp capping and pulpotomy performed at different levels according to the clinical signs of pulp contamination observed after exposure. In particular, for children with immature permanent teeth, preserving pulp vitality is crucial for complete root development (Bakhtiar *et al*., 2017; Abuelniel *et al*., 2020).

Calcium hydroxide paste has been used for decades as the first choice for pulpotomies but has been replaced in recent years by mineral trioxide aggregate (MTA). Despite all the widely known advantages of MTA of biocompatibility and induction of a calcific barrier, the potential discolouration has been considered a limiting factor for its use in anterior traumatized teeth (Torabinejad *et al*., 2018; Abuelniel *et al*., 2020). In 2009, Biodentine (BD; Septodont, Saint Maur-des-Fosses, France), a tricalcium silicate-based restorative cement, was introduced in the market as a dentine substitute with biological (Kunert & Lukomska-Szymanska, 2020) and clinical properties comparable to those of MTA (Bakhtiar *et al*., 201;, Abuelniel *et al*., 2020). Among those who advocate the use of BD, its accelerated setting time, ease of mixing, handling, and less coronal discolouration are pointed out as advantages (Kaur *et al*., 2017).

Other ready-for-use silicate-based materials recently developed are Bio-C Temp (BCT, Angelus, Londrina, PR, Brazil) and Bio-C Repair (BCR, Angelus). Both have low cytotoxicity (López-García *et al*., 2019; Oliveira *et al*., 2020; Villa *et al*., 2020; Ghilotti *et al*., 2020), good biocompatibility (Benetti *et al*., 2019), and biomineralization ability (Benetti *et al*., 2019; López-García *et al*., 2019). BCT was primarily conceived by the manufacturer as an intracanal dressing

(Villa *et al*., 2020) and is also indicated for full pulpotomy (Oliveira *et al*., 2020). However, there are no data regarding the use of BCT and BCR for partial pulpotomies in immature traumatized permanent incisors. This procedure has been indicated by the International Association of Dental Traumatology (Bourguignon *et al*., 2020) involving the amputation of 2–3 mm from the exposed pulp, followed by placement of the biomaterial over the remaining coronal pulp and composite restoration. Despite its benefits of continuing deposition of cervical dentin and reduction of root canal obliteration (Elmsmari *et al*., 2019), there is a concern about possible staining promoted by new materials in contact with the blood in the crown (Chen *et al.,* 2020). Therefore, the present study aimed to mimic a complicated crown fracture in an incisor treated by partial pulpotomy using BCT or BCR compared to BD, which is currently recommended as the material of choice for CPT for aesthetic purposes. Tooth discolouration, radiopacity, cytotoxicity on pulp cells, and healing were evaluated *in vitro.*

Materials and methods

Three materials used in this study were BCR, BCT, both ready-to-use, and BD, prepared as per the manufacturer's instructions.

Selection and preparation of samples for colour assessment

Based on the primary outcome (crown discolouration), the data from Yoldas *et al*. (2016) were used for sample calculation, which was performed using the statistical software package SigmaStat (version 12.5; Systat Software Inc., San Jose, CA, USA). A sample size of 64 teeth (n=16 per group) was established to have a 90% chance of detection, significant at the 5% level (two-sided test), with a minimum detectable difference of 4.96, with an expected standard deviation of 3.47. Approximately 300 central incisors from zebu cattle aged 30–36 months were initially obtained from a local abattoir (Real, Uberlândia, MG, Brazil), cleaned with scaler, inspected, and selected in four stages according to the method described by Rosatto *et al.* (2020). A dental operating microscope (D. F. Vasconcellos, Valença, RJ, Brazil) at ×3.0 magnification was used to verify cracks or defects and the presence of open

apex, followed by determination of buccolingual and mesiodistal dimensions using a digital calliper (Mitutoyo Sul Americana Ltda., Suzano, SP, Brazil). The selected teeth were stored in distilled water at 4°C.

We performed a simulation of a complicated crown fracture in an immature tooth (Figure 1) (Oliveira *et al.,* 2020). Part of the crown (8 mm above the amelocemental region) and the root (12 mm below the apical region) were removed perpendicular to its long axis under a water-cooled diamond disc. The root canals were enlarged using #1 through # 5 peeso reamers followed by a drill PM 82 (KG, Sorensen) to standardise the samples. Next, each root was flushed with 20 mL of 2.5% sodium hypochlorite followed by 20 mL of distilled water, and the apical region was closed using composite resin (3M ESPE Z250, Brazil). The samples were embedded in a polystyrene resin (Cristal, Piracicaba, SP, Brazil) and polyether impression material (Impregum F, 3M-Espe, Seefeld, Germany) for the periodontal ligament simulation (Soares *et al.* 2005), as well as to serve as support for material insertion and to obtain radiographic images in a standardised manner.

To simulate a partial pulpotomy, 8 mL of agar was prepared (Kasvi, São José dos Pinhais, Brazil) using 800 µL of fresh uncoagulated bovine blood and inserted up to 3 mm above the amelocemental region using tips in a volume of approximately 100 µL per tooth. The specimens were randomly assigned to three experimental groups (BCR, BCT, and BD) and one control group (agar + blood). The materials were placed at approximately 2 mm thickness above the agar + blood. Finally, the crown canals were closed using modified glass ionomer (Riva Light Cure, Australia).

Colour assessment

Colour values of the samples were determined using a spectrophotometer (Easyshade Compact Advance 4.0; Vita-Zahnfabrik, Bad Sackingen, Germany). For reproducible and standardised readings, an individual silicone index (Precise SX; Dentsply, Petropolis, RJ, Brazil) with a 6-mm hole was created for each sample to reposition the Easyshade tip at each time point (Oliveira *et al.* 2020). Three colour measurements were performed

on each tooth, and the average was recorded. Five sessions of colour assessments were conducted at the following intervals: baseline (sound tooth); T0, before application of the root-end filling material; T1, immediately after application of the material; T2, 30 days after application; and T3, 90 days after application. The colour readings were quantified in terms of the L*, a*, and b* coordinate values, established by the Commission Internationale de l'Eclairage (CIELAB system) for each specimen. The colour difference of the same specimen was calculated using two different equations. The first was the CIELAB colour difference (∆Eab) equation, calculated as follows: $\Delta Eab = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$, where ∆L*, ∆a*, and ∆b* refer to lightness, green-red, and blue-yellow differences of T0 and post T1 to T3 colour measurements. The second was the CIEDE2000 colour difference (DE00), which was calculated as follows: $\Delta E00 = [(\Delta L/KLSL)^2 + (\Delta C/KCSC)^2 + (\Delta H/KHSH)^2 + RT\ (\Delta C/KCSC)\ (\Delta H/KHSH)]^{1/2}$, where ∆L, ∆C, and ∆H are the lightness, chroma, and hue differences between colour measurements. KL, KC, and KH are the parametric factors that influence the viewing conditions and illuminating conditions. RT is the function for the hue and chroma interaction differences in the blue region. SL, SC, and SH are the weighting functions for the colour difference adjustment, considering the location variation of the L*, a*, and b* coordinates (Miotti *et al*., 2017). The whiteness index (WI) was calculated using the following formula: WI = 0.551*L -2.324*a -1.1*b (Perez *et al.* 2019).

Radiopacity

For radiopacity analysis, all teeth were radiographed on the same day of insertion of the materials (T1) and 30 days after (T2). A VistaScan Mini Plus® photostimulable phosphor (PSP) system (Dürr Dental, Bietigheim-Bissingen, Germany) was used, and the teeth were radiographed using a size 2 (3 × 4 cm) PSP plate using 0.14 s exposure time and 30 cm focus/film distance (70 kV, 7.0 mA - Timex 70E X-ray unit, Gnatus, Ribeirão Preto, SP). Subsequent to PSP plate scanning, the images were exported to ImageJ software (National Institutes of Health, Washington, USA) for grey value determination based on three points randomly selected in the region corresponding to the restorative

material: one point each mesially, centrally, and distally positioned. The grey value of each point was determined by the software and ranged from 0 to 255 as the radiographs were 8-bit images. The value 0 refers to a black colour and 255 refers to white colour, with 254 different shades of grey between them, representing the variations between the most radiolucent and the most radiopaque tone.

pH and calcium ion release

To determine the pH and calcium ion release, polyethylene tubes that were 10 mm long with 1.6 mm internal diameter (Embramed, Sao Paulo, SP, Brazil) were filled with each material (n = 5) (Zordan-Bronzel *et al*., 2019). For insertion of materials inside the tubes, a #25 K-file (Dentsply Maillefer, Ballaigues, Switzerland) was used followed by condensation using plugger 1-2 (Odous de Deus, Belo Horizonte, MG, Brazil) under ×3.0 magnification (D.F. Vasconcellos) to confirm complete filling. Next, the samples were immersed in flasks containing 10 mL of deionised water (DW) and stored at 37°C. DW was used as control. The pH of the solutions was measured using a previously calibrated digital pH meter (Digimed Analítica Ltda., Sao Paulo, Brazil) after 24 h of immersion. The mean pH values of each group were calculated after each measurement in triplicate. Calcium ion release assessment was performed on the same samples and the experimental period used for pH analysis. The percentage of calcium ions released by the materials was determined from the fluorescence spectra of liquid samples collected at each time point using X-ray fluorescence spectroscopy (model S8 Tiger Series 2, Bruker, Kontich, Belgium). From each sample, 10 mL of liquid was transferred to a cup of 40 mm (model SC-4340, PremierLab Supply, Port St. Lucie, USA) and weighed on an analytical balance for further normalisation of data. Quantitative analysis was performed using Quant-Express. The data acquisition time was 420 s, and the spectra were processed using the Spectra Plus software, which automatically determines the intensities of X-ray peaks for calcium and quantifies its concentration.

Cell culture assays

Preparation of material extracts

The materials were prepared under aseptic conditions in a laminar flow cabinet and inserted into sterile cylindrical polyethylene moulds with a diameter of 5 mm and height of 2 mm, as previously described (López-García *et al.,* 2019). All materials were sterilised using ultraviolet irradiation for 15 min and stored in an incubator at 37°C for 24 h to allow for complete setting. After this period, the materials were stored in Dulbecco's modified Eagle's medium (DMEM) (LGC Biotechnology, Cotia, SP, Brazil) for 24 h at 37°C with 95% humidity and 5% CO_2 to create the extracts. The ratio of material surface area to medium volume was set at approximately 1.5 cm^2/mL as per the guidelines of the International Organization for Standardization 10993-5. The extracts were collected, filtered with sterile filters of 0.22 µm, and serially diluted in cell culture medium before testing for a final dilution of 1:16.

Isolation and culture of human dental pulp cells (hDPCs)

This study was approved by the Ethical Committee of the Federal University of Uberlândia (protocol number 09016219.1.0000.5152). hDPCs were obtained from healthy third molars (n = 3). Fragments of pulp tissues were digested with 3 mg/mL collagenase type I enzymatic solution and 4 mg/mL dispase (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37°C, seeded in DMEM (LGC Biotechnology) supplemented with 10% foetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad, CA, USA) and 100 µg/mL penicillin/streptomycin (Sigma-Aldrich) and then incubated at 37°C in 5% CO_2. The culture medium was replaced every three days. Cells at passages 4-5 were used for the subsequent experiments.

Cell cytotoxicity assay

For cytotoxicity analysis, 2 × 10^4 hDPCs/well were seeded in 96-well/plates (Corning, New York, NY, USA) containing 10% DMEM and maintained at 37°C at 95% humidity and 5% CO_2 for 24 h. After this period, hDPCs were exposed to material extracts (1:1 to 1:16) or not exposed (control)

for 24 h. For each well, 10 µL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent (Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 5 mg/mL was added to 90 µL DMEM and incubated for 4 h. The medium was removed and formazan crystals were dissolved in dimethyl sulfoxide solution (LGC Biotechnology) (100 µL/well) and the absorbance was measured using an automatic microplate reader (Biochrom, Cambridge, UK) at 570 nm. Cell viability was evaluated in proportion to the absorbance and expressed as the percentage of viable cells. The dilution that demonstrated stabilisation of the viability values (1:2) was used for the cell morphology and migration tests.

Cell morphology and migration

Confocal microscopy (Zeiss LM 510 Meta, ZEISS, Oberkochen, Germany) and inverted digital microscopy (EVOS digital microscope, Advanced Microscopy Group, AMG, Life Technologies, Carlsbad, CA, USA) experiments were used to evaluate cell morphology by changes in the actin cytoskeleton and to assess cell migration by wound healing test, respectively. Both analyses were performed in the absence (control group) or the presence of the material eluates. Twenty-four hours after exposure to 1:2 eluates (n=3 per group), hDPCs were fixed in 4% paraformaldehyde for 10 min, washed with phosphate buffer saline (PBS), permeabilised with 0.5% Triton X-100 (Sigma-Aldrich), and incubated using 1:200 Phalloidin– tetramethylrhodamine B isothiocyanate (Sigma-Aldrich) and TO-PRO 3 Iodide (Invitrogen) 1:1000 for 1 h, washed again in PBS and then analysed at ×20 magnification. The results are qualitatively described.

For cell migration, a scratch was performed with a 200-pipette tip, and each well was washed three times with PBS to remove debris. The scratched area was analysed at 24 and 48 h. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to calculate the percentage of healing area in each period relative to the area measured at 0 h in the same well. Migration distances were measured separately during periods 0–24 h and 24–48 h. As variations in scratch width were established, the “relative healing area” (RHA)

was calculated (RHA [%] = healing closure area [pixel] × 100 [%]/x [pixel]), as described by López-García et al. (2019).

Statistical analysis

The pH, calcium release, cell viability, wound healing, colour assessment, and radiopacity data were analysed for normality and homoscedasticity using the Shapiro-Wilk and Levene tests. For cell viability, two-way analysis of variance (ANOVA) and Tukey's test were used to compare the data between the treated groups. For pH and calcium release, one-way ANOVA followed by Tukey's test was used for intragroup comparisons. Two-way repeated-measures ANOVA and Tukey's tests were used to compare wound healing data, where 'time assessment' was used as a repetition factor. One-way ANOVA was used to compare the colour parameters (*L, *a, *b, and WI) at baseline (sound tooth) to standardise the colour samples among the groups. Two-way repeated-measures ANOVA and Tukey's tests were used to compare the colour parameters (ΔEab, ΔE00, and WI), where 'time assessment' was used as a repetition factor. Dunnett's test was used to compare experimental groups with the control group. Statistical analysis was performed using SigmaPlot 12.5, a statistical software package (Systat Software Inc). The significance level was set at 95% for all the data analyses.

Results

Discolouration

One-way ANOVA showed similar colour parameters for samples among groups (*L: P=0.273/*a:P=0.824/*b: P=0.812 /WI:P=0.95) before the experiment. Table 1 presents the results of the overall colour changes (ΔEab and ΔE00) according to the pulpotomy material and assessment time. Two-way repeated measures ANOVA showed statistical significance for "material" (∆Eab: P<0.001/∆E00: P<0.001) and "assessment time" (P< 0.001) for both outcomes. However, there was no statistically significant difference in the interaction (∆Eab: P = 0.215/∆E00: P=0.023).

BCR was associated with a significantly smaller colour alteration followed by BCT, whereas wound healing caused a significant colour change in BD ($P<0.001$). When comparing the colour change of the samples from the initial assessment time before application of the material (T0) to the other experimental time-points (T1, T2, and T3), T1 and T2 presented significantly more discolouration than T3 ($P<0.001$).

In the control group, T2 showed the highest statistically significant colour change compared to T1 and T3 ($P<0.001$). Dunnett's test immediately after material placement (T1) revealed a significant difference in discolouration between the control group and the BCT and BD groups. At T2, only the BCR group differed significantly from the control group, whereas at T3, a significant difference from the control group was observed only in the BD group ($P<0.001$).

The behaviour of the L*, a*, and b* parameters according to the tested materials and assessment times are shown in Figure 2. Some decrease in lightness occurs after the insertion of tricalcium silicate-based materials. The BCR exhibited a more linear behaviour for this parameter. In general, the materials increased redness and yellowness.

For WI data, two-way repeated measures ANOVA showed that assessment time was significant ($P<0.001$); however, there were no differences between "materials" ($P=0.735$) or for the interaction "material x assessment time" ($P=0.135$). The results for WI are presented in Table 2. Similar WI values at baseline were observed between specimens allocated in each group, demonstrating that the randomisation was able to balance the initial colour of specimens among the treatments ($P=0.95$). The use of agar + blood (control group) decreased WI and the immediate application of the pulpotomy material significantly decreased WI (T1-T0) in the experimental groups ($P<0.05$). At 30 days (T2), WI increased slightly ($P<0.05$) and remained stable until 90 days (T3).

Radiopacity

The radiopacity values of the materials are listed in Table 3. BCR presented higher radiopacity than all materials in both periods ($P<0.05$). The

BCT, BD, and control groups had similar grey values in T1 and T2. BD exhibited significantly lower radiopacity than the other selected materials ($P<0.05$).

PH and calcium ions

The pH of distilled water increased in all groups after the immersion of the specimens (Figure 3A). There were no significant differences between the tested materials at 24 h ($P>0.05$). BD and BCR behaved similarly with respect to calcium ion release (Figure 3B, $P>0.05$). BCT showed greater calcium release than the other materials ($P<0.0001$).

Cell cytotoxicity assay

hDPC cell viability by MTT formazan method of BD, BCR, and BCT groups at different extract dilutions is shown in Figure 4. The cell viability of the BD and BCR groups was similar, regardless of the dilution tested ($P>0.05$). BCT showed viability levels similar to those of BCR and BD at 1:8 and 1:16 ($P>0.05$). The 1:1 dilution was the most cytotoxic in the BCT group ($P=0.0010$). Compared to the control group, BD showed similar values in dilutions of 1:1, 1:2, and 1:4 ($P>0.05$), and lower levels at 1:8 and 1:16 ($P<0.0001$). BCR was similar to that of the control in all dilutions ($P>0.05$), except at 1:1, which showed less viability ($P<0.0001$). BCT showed lower cell viability than the control at all tested dilutions ($P<0.0001$).

Cell morphology and migration

Both hDPC cells in the control group (Figure 5A) and treated with BD (Figure 5B), BCR (Figure 5C), and BCT (Figure 5D) extracts showed a typical fibroblast-like appearance with an elongated cytoskeleton. However, the control group had larger cells with a highly organised and stretched fibre assembly compared to the treated groups.

At all time points, cell migration rates in the BD and BCR groups were similar to each other and similar to those in the control group ($P>0.05$) (Figure 6). In the BCT group, no significant differences were observed at 24 h when compared with the other experimental groups ($P>0.05$). In contrast, at 48 h,

the BCT group exhibited significant differences, with larger open areas compared to the BD and BCR groups (P<0.0001). In the control group, both at 24 h and 48 h, BCT showed significant differences (P<0.0001), being unable to heal the wound.

Discussion

In the present study, the null hypothesis was rejected, as significant statistical differences were found between the calcium silicate-based materials in most of the evaluated parameters. Although previous studies have evaluated BD using similar methodologies (Vallés *et al.,* 2015; Marconyak *et al*., 2016; Shokoubinejah *et al*., 2016; Yoldas *et al*., 2016; Farrugia *et al*., 2018; Adl *et al.,* 2019; Oliveira *et al*., 2020; Ochoa-Rodríguez, 2019; Ochoa-Rodríguez, 2020), few studies have used BCT (Oliveira *et al.,* 2019; Villa *et al.,* 2020), and BCR (Benetti *et al.,* 2019; López-García *et al.,* 2019; Ghilotti *et al.,* 2020), new ready-to-use materials. To assess crown discolouration by these materials in the presence of blood components, a model was designed to simulate the clinical situation of partial pulpotomy. The gelatinous consistency of the agar is similar to that of the dental pulp, making the insertion of the pulpotomy material closer to clinical practice (Oliveira *et al.,* 2020). Bovine teeth were used instead of human teeth because they allow the selection of samples with more rigorous standards given the high availability of samples, the greater flat surface for colour analysis, and lack of concern about ethical issues (Yoldas *et al.,* 2016; Shokouhinejad *et al.,* 2019; Oliveira *et al.,* 2020).

Considering that in partial pulpotomy, only part of the coronary pulp is removed, a possible crown staining caused by the biomaterial could become more noticeable, impacting the aesthetics in anterior teeth even more than in a full pulpotomy. However, an increase in WI values found in the agar+blood-control group over time suggests that the discolouration promoted by partial pulpotomies is transient. The same pattern was also observed for WI in the BCT, BCR, and BD groups, corroborating this statement. A previous study using agar+blood in a full pulpotomy simulation (Oliveira *et al.,* 2020) presented a similar ΔEab stain pattern in the agar+blood-control group, with greater

discolouration at 30 days, remaining low in the subsequent periods. Despite the role of blood contamination in tooth discolouration (Lenherr *et al.,* 2012; Felman & Parashos, 2013; Shokoubinejah *et al.,* 2016; Mozynska *et al.,* 2017), the consistency of the Agar+blood mixture resembling the pulp tissue after haemorrhage control may have been responsible for the colour rebound observed in all groups, including the control and lower discolouration when compared to studies in which the materials were placed directly over the blood (Shokoubinejah *et al.,* 2016; Yoldas *et al.,* 2016). To avoid bias caused by contamination of the pulp chamber, the insertion of the agar+blood mixture was conducted using pipette tips in a predetermined volume until reaching the demarcated area on the crown surface.

Red blood cells are related to discolouration mechanisms (Felman & Parashos, 2013; Yoldas *et al.,* 2016). The iron present in haematin and haemoglobin probably causes tooth staining by infiltrating the dentine tubules (Lenherr *et al.,* 2012; Felman & Parashos, 2013) or penetrating the pores and gaps of materials such as BD and MTA (Shokoubinejah *et al.,* 2016; Yoldas *et al.,* 2016). It has been postulated that blood can be absorbed into fresh unset calcium silicate-based materials, causing greater colour changes (Shokouhinejad *et al.,* 2016). The present study does not support this theory as even BCT, which is launched in non-hardening paste (Villa *et al.,* 2020), presents lower discolouration than BD, which offers a reduced setting time of up to 12 min from the time the mixture is prepared. The exact mechanism for the intensified discolouration of BD in the presence of agar+blood compared to that of BCT and BCR is unknown.

Although there are no data in the literature regarding the staining promoted by tricalcium silicate-based materials in this specific procedure, for all groups, the values of $\Delta E00$ were >0.81, which is considered clinically perceptible (Paravina *et al.,* 2015). However, at T3, BCT and BD showed $\Delta E00$ above the acceptability threshold (> 1.77), with BD being significantly higher than BCT (Paravina *et al.,* 2015). The present results differ from those of previous studies (Marconyak *et al.,* 2016; Shokouhinejad *et al.,* 2016; Adl *et al.,* 2019), which did not detect differences between the materials used for CPT in

the presence of blood components. Direct comparison between the refereed studies cannot be performed owing to the differences in the methodologies and materials evaluated; however, the present results suggest that the structure and composition of the studied biomaterials were decisive for its staining potential, particularly the metal constituents such as iron and aluminium. BD and BCR, in particular, contain iron and aluminium in their composition (Benetti *et al.,* 2019; López-García *et al.,* 2019; Ghilotti *et al.,* 2020). Oxidation of the iron content remaining in the set material is considered a possible mechanism for tooth discolouration (Felman & Parashos, 2013; Shokoubinejah *et al.,* 2016).

Regarding the control group, comparisons with the studied biomaterials showed similarity with BCR in the initial (T1) and final (T3) measurements, indicating that the presence of this material does not result in a greater colour change, in contrast to BD. The limitation of the agar+blood-control group should be addressed as the teeth access cavities were restored with a greater thickness of glass-modified ionomer than in the other groups owing to the absence of experimental materials over the agar+blood surface. The complete filling of the chamber by the restorative material may cause loss of translucency (Shokouhinejad *et al.,* 2016). Owing to this, the control group must be considered in the face of the degree of colour change in the absence of calcium silicate-based materials (Shokouhinejad *et al.,* 2016; Oliveira *et al.,* 2020).

High discolouration has been related to the presence of bismuth oxide (Mozysnka *et al.,* 2017; Oliveira *et al.,* 2020; Torabinejad *et al.,* 2018), which is not found in these new tricalcium silicate-based materials. This radiopacifier has been replaced by other materials, such as zirconium oxide, in BD and BCR or titanium oxide in BCT. Despite having the same radiopacifier, BD and BCR exhibited different radiopacity in both experimental time periods, which is probably related to the percentage of zirconium oxide. Previous studies have shown low radiopacity for BD (Farrugia *et al.,* 2018; Ochoa-Rodríguez *et al* 2019; Ochoa-Rodríguez *et al.,* 2020). However, it is not possible to make comparisons between different radiopacity studies because several factors may interfere, such as the X-ray machine, exposure time, tube voltage, source-to-

object distance, conventional radiography, and digital radiography systems (Ochoa-Rodríguez *et al.,* 2019; Ochoa-Rodríguez *et al.,* 2020).

Previous studies have demonstrated the cytotoxicity of these materials in different cell models (Pinheiro *et al.,* 2018; Paula *et al.,* 2019; Benetti *et al*., 2019; Lopéz-Gárcia *et al.,* 2019; Villa *et al.,* 2020). Considering that the present study proposed to simulate the clinical situation of CPT, the evaluation of these materials was carried out using extracts of the materials (1:1–1:16) that mimic the contact of the materials with the pulp tissue, as the concentration decreases with the elimination of leachable components by the extracellular fluid (Mestieri *et al.,* 2015). The analysis of cell viability at 24 h using MTT is a classical assay that is widely used to evaluate the cytotoxic effects of new materials (Mestieri *et al.,* 2015; Lopéz-Gárcia *et al.,* 2019; Ghilotti *et al.,* 2020), and it is considered the gold standard (Pintor *et al.,* 2020).

According to the MTT results, BCT was cytotoxic at a 1:1 dilution, which is as per a previous study on hDPCs, reducing its toxic effects on more diluted extracts (Oliveira *et al*., 2020). Another study in immortalized fibroblasts showed that the viability was less than 30% for BCT at high concentrations (Villa *et al.,* 2020). A possible explanation is related to its calcium release, which in the present study was slightly superior to the other materials, as demonstrated by X-ray fluorescence spectroscopy assays. Additionally, the presence of TiO_2 in its composition may interfere with a series of cellular events, reflecting a reduction in cell survival by induction of apoptosis, as verified in murine macrophages (Dhupal *et al*., 2018) and mesenchymal stem cells (Yu *et al.,* 2019).

The results with BCR are consistent with those from recently published studies (Benetti *et al.,* 2019; Lopéz-Gárcia *et al.,* 2019; Ghilotti *et al.,* 2020), demonstrating excellent cytocompatibility, higher than 80% in all periods and dilutions. The lower cytocompatibility of BD in hDPCs, when compared to control, differs from that of Ghilotti *et al*. (2020), who demonstrated the highest absorbance levels for undiluted BD compared to untreated cells in the control. In the current study, the similarity between the results of viability in BD, which has been considered the new gold standard in pulpotomies, and BCR,

regardless of the time of analysis, makes this material highly suitable for pulpotomies, with the advantage of ready-to-use formulations.

The repair after CPT initially consists of hDPC proliferation and migration (Lin *et al.,* 2011), which are processes that require a coordinated reorganization of the actin cytoskeleton with the formation of stress fibres (Zhu *et al.,* 2014). Thus, cell morphology and migration analysis were used as complementary methods to the cell viability assay, as these parameters may be influenced by the chemical composition of the materials (Akbulut *et al.,* 2016; Ghilotti *et al*., 2020). The scratch wound healing test was used to assess cell migration as it is a reproducible and low-cost assay that does not require any specialised equipment (Liang *et al.,* 2007).

For the analysis of cell migration and morphology, we chose a dilution in which all materials had viability greater than 70%. Cytoskeletal alterations were not observed in the presence of BD and BCR eluates, whereas BCT presented a slightly lower reorganization of the cytoskeleton. Although this is the first study to evaluate changes in the cytoskeleton after contact with BCT eluates, previous studies evaluating other bioceramic materials have demonstrated an optimised stress fibre configuration than the control group (Zhu *et al.,* 2014; Zhang *et al.,* 2015). However, even when they belong to the same class of materials, factors such as composition, particle size, and bioactivity can interfere with cellular architecture, leading to different cell responses (Nakamura *et al.,* 2010; Kingshott *et al.,* 2011). Regarding the BCT group, considering that actin stress filaments are responsible for providing the forces for cell motility (Li *et al.,* 2015), it is possible to speculate that the decreased migratory ability presented by the BCT group in the wound healing assay is linked to less vigorous F-actin stress fibres.

Conclusion

The three calcium-silicate materials tested for partial pulpotomy promoted tooth colour change, with more favourable results for BCR, which presented lower staining, higher radiopacity, and viability than 80% hDPCs.

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Table 1- Mean and standard deviation of discoloration (ΔE_{ab} and ΔE_{00}) in the different groups evaluated after material insertion and assessment times.

Groups	***ΔEab***			***ΔE00***		
	T1	*T2*	*T3*	*T1*	*T2*	*T3*
Control	3.6 ± 2.2^{b}	5.7 ± 1.8^{a}	2.5 ± 1.0^{b}	2.1 ± 1.3^{b}	3.5 ± 1.1^{a}	1.5 ± 0.6^{b}
BCR	3.2 ± 2.0^{Ca}	$3.5\pm1.5^{Ca*}$	1.8 ± 1.1^{Cb}	1.8 ± 1.0^{Ca}	$2.1\pm0.9^{Ca*}$	1.1 ± 0.5^{Cb}
BCT	$6.3\pm4.6^{Ba*}$	4.9 ± 3.4^{Ba}	3.3 ± 2.2^{Bb}	$3.8\pm2.7^{Ba*}$	3.0 ± 2.1^{Ba}	2.0 ± 1.3^{Bb}
BD	$8.8\pm4.9^{Aa*}$	7.2 ± 3.0^{Aa}	$5.3\pm2.1^{Ab*}$	$5.2\pm3.0^{Aa*}$	4.3 ± 1.8^{Aa}	$3.3\pm1.5^{Ab*}$

*T1, immediately after application of the material; T2, 30 days after and T3, 90 days after. Different capital letters in columns indicate significant differences between groups in the same assessment time, and different lowercase letters in rows indicate significant intragroup differences between the periods analyzed (two-way repeated measures ANOVA and Tukey's test - P < 0.05).*Symbol indicates significant differences in columns with the control group (Agar+Blood) by Dunnett's method, P<0.05.*

Table 2- Mean and standard deviation of Whiteness Index (WI) in the different groups evaluated after material insertion and assessment time.

Groups	*Assessment time*			
	T0	*T1*	*T2*	*T3*

Control	26.9±7.9^{Aa}	24.3±8.7^{Ac}	26.0±9.2^{Aab}	25.7±8.3^{Ab}
BCR	28.0±5.6^{Aa}	24.0±7.4^{Ac}	27.6±5.8 ^{Aab}	26.5±5.6 ^{Ab}
BCT	29.3±7.1^{Aa}	21.2±7.8^{Ac}	28.1±7.2 ^{Aab}	26.2±5.3 ^{Ab}
BD	29.0±10.1^{Aa}	16.7±9.6 ^{Ac}	25.3±7.3 ^{Aab}	23.5±6.5 ^{Ab}

T0, before application of the material; T1, immediately after application of the material; T2, 30 days after and T3, 90 days after. Different capital letters in columns indicate significant differences between groups in the same assessment time, and different lowercase letters in rows indicate significant intragroup differences between the periods analyzed (two-way repeated measures ANOVA and Tukey's test - P < 0.05).

Table 3. Mean of radiopacity (gray value) in the different groups evaluated at day 1 (T1) and day 30 (T2).

Groups	*T1*	*T2*
BCR	226^{Aa}	226^{Aa}
BCT	205^{Ba}	205^{Ba}
BD	199^{Ba}	194^{Ca}
Control	203^{Ba}	200^{Bca}

Different capital letters in the columns indicate statistical difference by the Tukey test (P <0.05). Different lowercase letters in the lines indicate statistical difference by the Tukey test (P < 0.05).

Figure

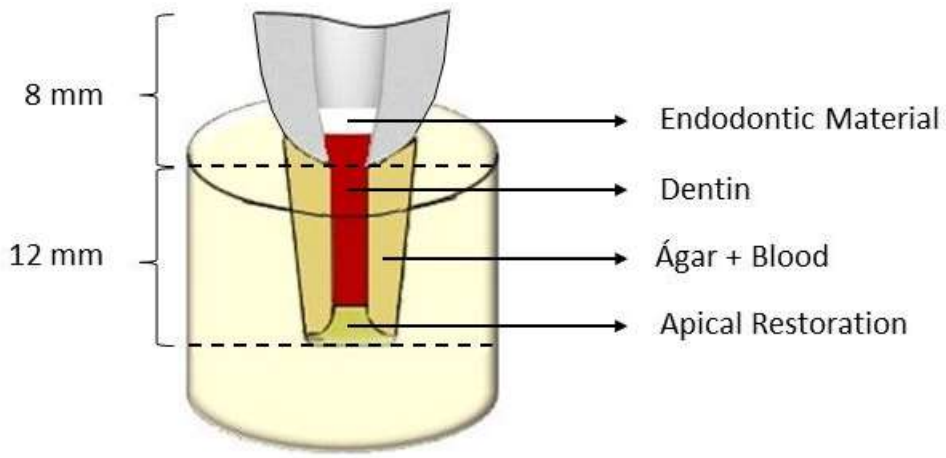

Figure 1. *Schematic showing tooth specimen: a) after coronal and root preparation; b) after silicone impression material index insertion for color measurements*

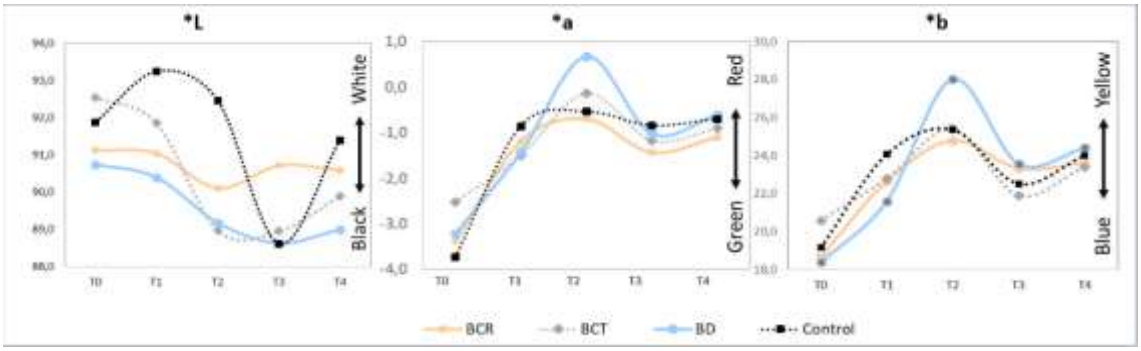

Figure 2. *Graphs show the trends in the *L, *a, *b parameters of groups over time. B, baseline (sound tooth); T0, before application of the material insertion; T1, immediately after application of material; T2, 30 days and T3, 90 days after.*

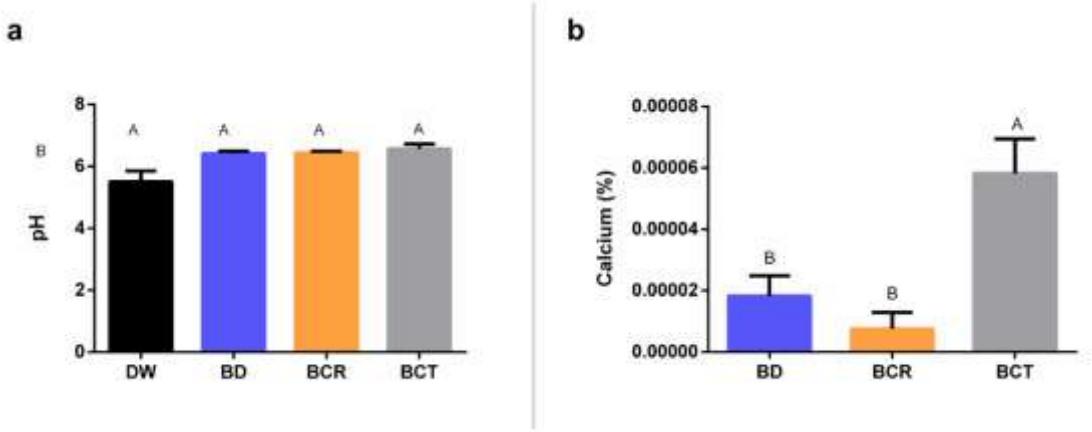

Figure 3. *pH values and percentage of calcium ion released by materials after 24 h of immersion in destiled water. a) pH values; b) percentage of calcium ion. BD: Biodentine exposure; BCR: Bio-C Repair exposure; BCT: Bio-C Temp exposure. Capital letters indicate comparison among materials. One-Way ANOVA and Tukey´s test (P<0.05).*

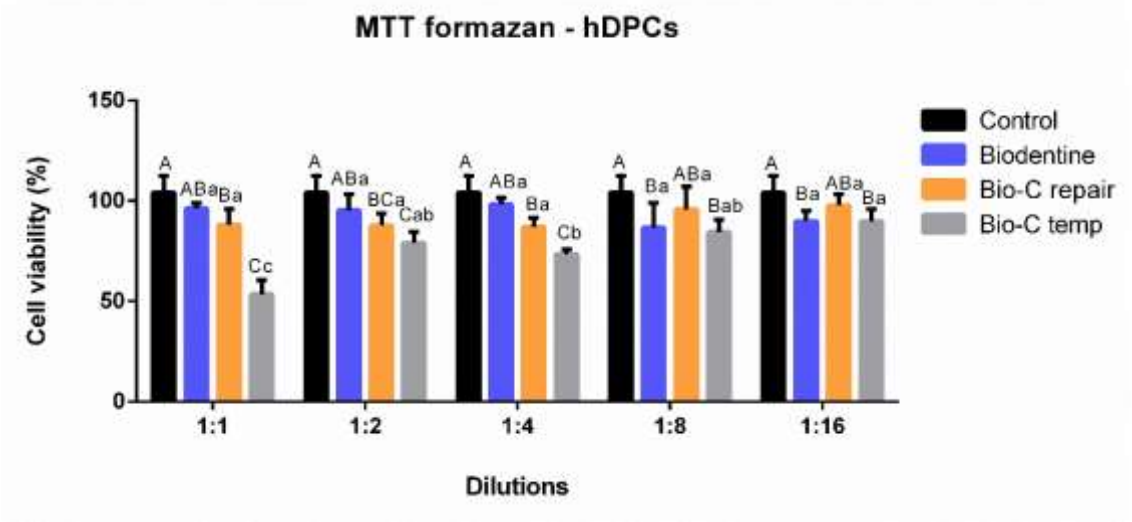

Figure 4. *Cell viability percentage of hDPCs after exposure to extracts according to material tested and dilution by the MTT formazan method. BD: Biodentine exposure; BCR: Bio-C Repair exposure; BCT: Bio-C Temp exposure. Capital letters indicate comparison among different dilutions of extracts and the control group for each material. Lowercase allows comparisons of a material among the different dilutions. Two-way ANOVA and Tukey´s test (P<0.05).*

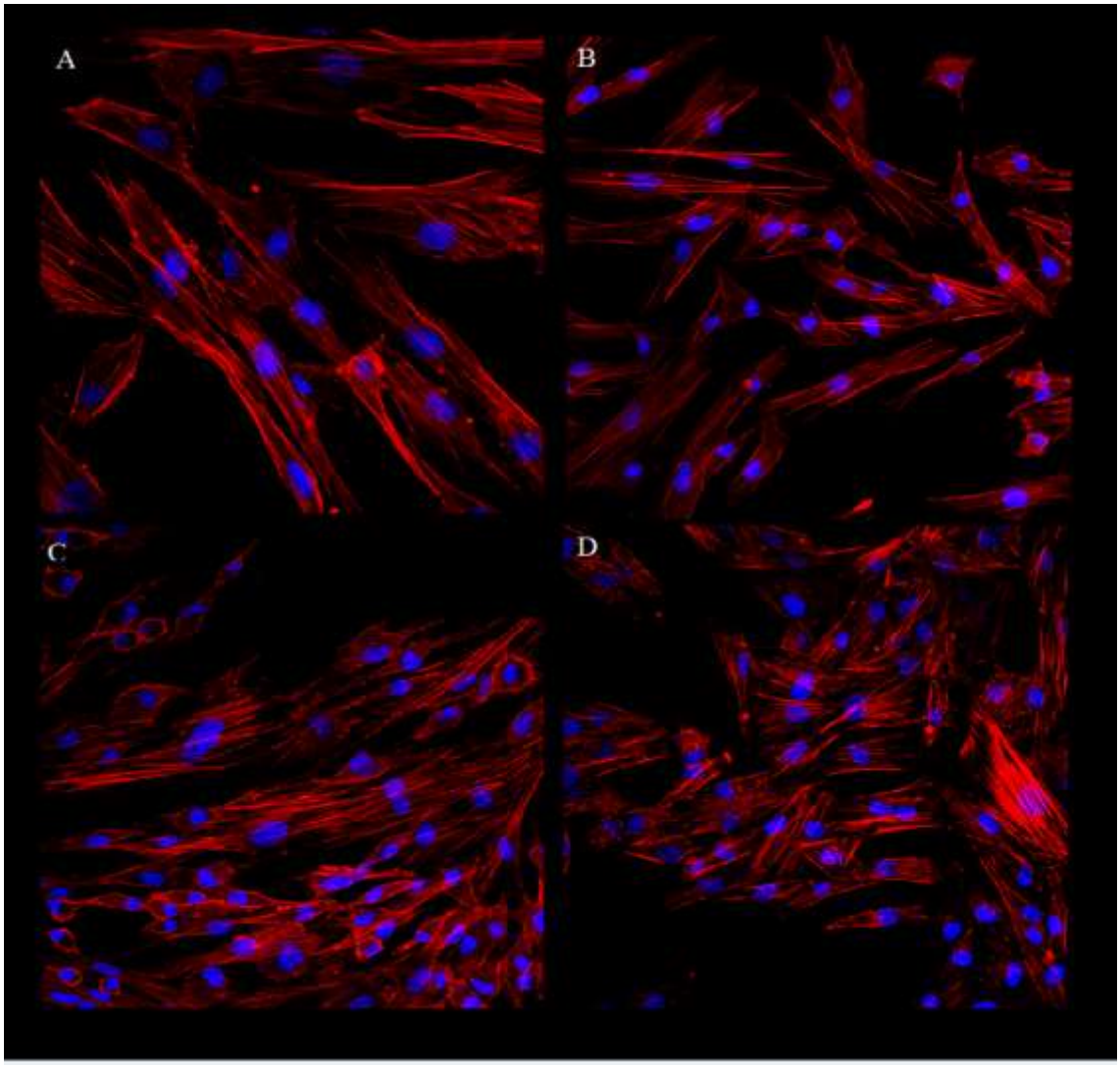

Figure 5. *Immunofluorescence staining to evaluate F-actin cytoskeleton and cell morphology. Staining of actin filaments (phalloidin-FITC) and the nuclei (TO-PRO) of human dental pulp cells (hDPCs) after 24 h of contact with material extracts. a) Control group; b) Biodentine; c) Bio-C repair; d) Bio-C temp.*

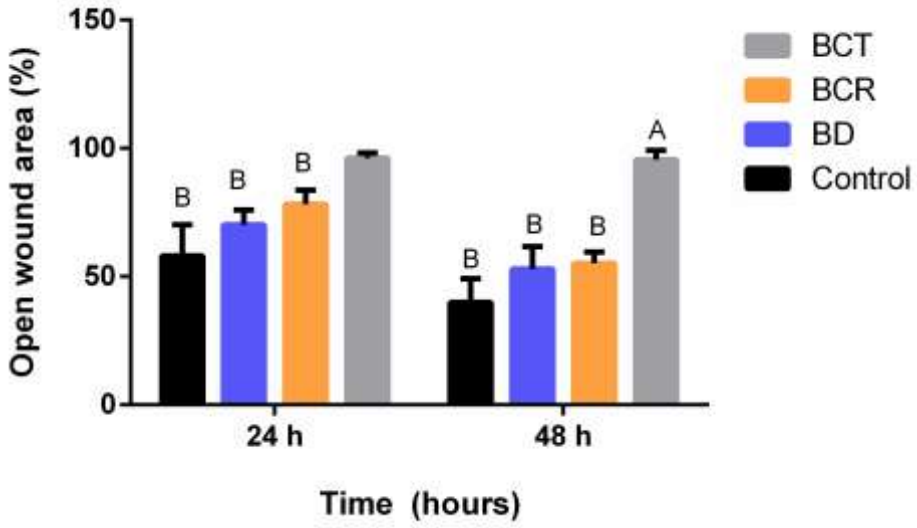

Figure 6. *Migration assay: The closure of the space created in the scratch assay after the contact of the extracts of all three materials with the hDPCs after 24 and 48 hours is represented by a bar graphic after the statistical analysis. Capital letters indicate comparison among materials. Two-way repeated measure ANOVA and Tukey's test (P<0.05).*

3.3 Capítulo 3

Artigo clínico enviado ao periódico Research, Society and Development jornal

Técnica de revascularização modificada em molares permanentes. Uma série de casos

RESUMO

Existem poucos relatos de procedimentos endodônticos regenerativos em molares, e a maioria é usando preparo de canal radicular manual. Esta série de casos descreve uma técnica de revascularização modificada usada em molares permanentes (cinco pacientes entre 9 e 16 anos). Os pacientes foram encaminhados por um serviço de emergência e foi feita o diagnóstico com base no relato do paciente. Na primeira consulta, o preparo coronal foi realizado com limas Hedstroem e brocas Gates Glidden, seguido de preparo completo do canal radicular com limas rotatórias de NiTi. A pasta de hidróxido de cálcio ($Ca(OH)_2$) foi usada como medicação intracanal. Na segunda consulta, a medicação intracanal foi retirada, seguida da irrigação final com EDTA sob agitação ultrassônica e o coágulo foi promovido. As entradas dos canais foram seladas com cimento de agregado trióxido mineral (MTA Fillapex) e restauradas provisoriamente com ionômero de vidro fotopolimerizável. Todos os dentes foram finalmente restaurados com restauração direta de resina composta e foram acompanhados por até 18 meses, verificando dor, edema e fístula durante a avaliação clínica. Exames radiográficos foram realizados para avaliar o reparo apical até 15 meses, onde foi observado fechamento do ápice radicular e redução do canal. Após 6 meses, a evidência de reparo foi observada em todos os casos. Foi possível confirmar que a regeneração endodôntica após o preparo mecanizado do canal radicular com o uso de cimento MTA e restauração em resina composta em molares, é uma opção promissora para a manutenção de molares permanentes em adolescentes.

Palavras-chave: Adolescente; Restauração direta de resina composta; Molar permanente imaturo; Regeneração da polpa; Tratamento endodôntico regenerativo;

Modified revascularization technique in permanent molars. A case series

ABSTRACT

There are few reports of regenerative endodontic procedures in molars, and most using manual root canal preparation. This case series describes a modified revascularization technique used in permanent molars (five patients between 9 and 16 years old). Patients were referred by an emergency service and a diagnostic hypothesis was made based on the patient's reports. At the first appointment, coronal preparation was performed using Hedstroem files and Gates Glidden drills, followed by complete root canal preparation with rotary NiTi files. Calcium hydroxide ($Ca(OH)_2$) paste was used as intracanal medication. At the second appointment, intracanal medication was removed, followed by final irrigation with EDTA under ultrasonic agitation, and the clot was promoted. The entrances of the canals were sealed using a mineral trioxide aggregate sealer (MTA Fillapex) and provisionally restored with a light-cured glass ionomer. All teeth were finally restored using direct composite resin restoration and were followed for up to 18 months, checking pain, edema, and fistula during clinical evaluation. Radiographic examinations were performed to assess apical repair until 15 months, where root apex closure and canal reduction were observed. After 6 months, evidence of healing was observed in all cases. It was possible to confirm that endodontic regeneration after mechanized root canal preparation, use of a MTA sealer, and direct composite resin restoration in molars is a

promising option for maintaining permanent molars in adolescents.

Keywords: Adolescent; Direct composite resin restoration; Immature permanent molar; Pulp regeneration; Regenerative endodontic treatment;

Técnica de revascularización modificada en molares permanentes. Una serie de casos

ABSTRACTO

Hay pocos informes de procedimientos de endodoncia regenerativa en molares y la mayoría utiliza la preparación manual del conducto radicular. Esta serie de casos describe una técnica de revascularización modificada utilizada en molares permanentes (cinco pacientes entre 9 y 16 años). Los pacientes fueron remitidos por un servicio de urgencias y se formuló una hipótesis diagnóstica a partir de los informes de los pacientes. En la primera cita, la preparación coronal se realizó utilizando limas Hedstroem y fresas Gates Glidden, seguida de una preparación completa del conducto radicular con limas rotatorias NiTi. Se utilizó pasta de hidróxido de calcio ($Ca(OH)_2$) como medicación intracanal. En la segunda cita se retiró la medicación intracanal, seguida de la irrigación final con EDTA bajo agitación ultrasónica y se promovió el coágulo. Las entradas de los canales se sellaron con un sellador de agregado de trióxido mineral (MTA Fillapex) y se restauraron provisionalmente con un ionómero de vidrio fotopolimerizable. Todos los dientes fueron finalmente restaurados usando restauración directa de resina compuesta y fueron seguidos durante hasta 18 meses, controlando el dolor, el edema y la fístula durante la evaluación clínica. Se realizaron exámenes radiográficos para evaluar la reparación apical hasta los 15 meses, donde se observó el cierre del ápice radicular y la reducción del conducto. Después de 6 meses, se observó evidencia de curación en todos los casos. Se pudo confirmar que la regeneración endodóntica después de la preparación mecanizada del conducto radicular, el uso de un sellador MTA y la restauración directa de resina

compuesta en los molares es una opción prometedora para el mantenimiento de los molares permanentes en adolescentes.

Palabras llave: Adolescente; Restauración directa de resina compuesta; Molar permanente inmaduro; Regeneración de la pulpa; Tratamiento endodóntico regenerativo;

INTRODUCTION

Revascularization is a modality of regenerative endodontic therapy that enables the root canal filling by a new connective tissue, induces the formation of mineralized tissue, providing apical root closure (apexification), and could also induce root growth (maturogenesis) (Saoud *et al.,* 2014; Antunes *et al.,* 2015). The procedure often begins with chemical disinfection using sodium hypochlorite (NaOCl), root canal preparation, and use of intracanal medication to reduce the number of microorganisms in the root canal system (Galler *et al.,* 2016). At a second appointment, bleeding is induced within the canal, promoting the formation of a blood clot by means of stem cells of the apical papilla can migrate (Diogenes *et al.,* 2017). Studies have demonstrated the success of revascularization in permanent necrotic incisors (Wigler *et al.,* 2013; Saoud *et al.,* 2014; Antunes *et al.,* 2015).

Initially, revascularization was indicated only for teeth with incomplete root development (Saoud *et al.,* 2014; Duggal *et al.,* 2017). However, studies have demonstrated the efficiency of this technique in mature permanent teeth (Paryani *et al.,* 2013; Saoud *et al.,* 2016). There are some reports in the literature regarding the treatment of immature permanent molars with regenerative therapy (Martin *et al.,* 2013; Ajram *et al.,* 2019). Nonetheless, the small adherence of this treatment modality to molars is probably related to the anatomical difficulties of disinfection and filling the canal with an adequate volume of blood. Considering that the early loss of molars in young patients affects occlusion, maxillary bone growth and chewing performance, every effort should be made to maintain these teeth (Rodrigues *et al.,* 2020). Therefore, the aim of this study was to report the follow-up of a series of clinical cases of revascularization in severely compromised young permanent molars in adolescents, performed using mechanized root canal preparation.

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CASE REPORTS

Five young patients (9-16 years old) with molar teeth presenting extensive coronary destruction due to caries were selected for this case series study. The

patients were referred to the Hebiatric Clinic of the Federal University of Uberlândia, Uberlândia, Brazil, which performs endodontic and restorative treatments in molars of patients aged 6 to 18 years. All teeth had been submitted to emergency treatment for pain relief in the emergency service of the university and had already been submitted to coronary opening. The cases were treated after parents or guardians signed an informed consent form (3397587/20).

Anamnesis and clinical examination

The patient's complaints and medical and dental histories were recorded on the anamnesis form. Extraoral and intraoral examinations were conducted, recording the swelling, presence of sinus tract, vertical percussion, and palpation sensitivity. Preoperative digital periapical radiographs were taken (FIT T2 Microimage, Indaiatuba, SP, Brazil). Based on these data and previous history of pain, a pulpal-periapical diagnosis was determined. The data collected from patients was used to generate the diagnostic hypothesis and are summarized in Table 1. Of the five teeth treated, one was mandibular first molar, three were mandibular second molars and one was mandibular first molar; only one tooth had all the root canals with large open apexes. The one maxillary molar treated had complete root development, showing slightly open foramina in the palatal root canal.

Table 1. Clinical and radiographic findings: outcomes.

Patient n°	Sex	Age	Tooth n°	Clinical diagnosis	Time final restoration	Last Control (month)	Periapical status after endodontic regenerative treatment	Root canal
1	M	16	3	Irreversible pulpite	After 2 weeks	15	healed	reduced

2	F	15	31	Pulp necrosis	After month	1	15	healed	reduced
3	M	14	18	Pulp necrosis	After months	6	15	healed	reduced
4	F	13	31	Irreversible pulpite	After months	4	15	healing	normal
5	M	9	30	Irreversible pulpite	After week	1	15	healed	reduced

F, female; M, male

Summary of procedures

1. First Visit

In the first session, the following procedures were conducted: local anesthesia with 2% lidocaine containing 1: 100000 epinephrine (DFL, Rio de Janeiro, RJ, Brazil), rubber dam isolation, and removal of temporary restorative material. After root canal access performed using rounded carbide burs and Endo Z bur (Malleifer, Dentsply, Petrópolis, RJ, Brazil, they were irrigated with approximately 10 ml of 2.5% sodium hypochlorite (Asfer, São Caetano do Sul, SP, Brazil) delivered by a 27-gauge open-ended and explored with #10-15 hand K-files (Dentsply Maillefer, Oklahoma, USA). To avoid the stress caused by rotatory files during the root canal preparations, the coronal third of root canal was prepared with #15-25 H-files (Dentsply Maillefer, Oklahoma, USA) and Gates Glidden drill sizes #2 and #3 (Dentsply Maillefer, Oklahoma, USA). A working length 1 mm short of the radiographic apex was determined using periapical radiographs. Then, root canals were prepared with Protaper Next rotary files (Dentsply) X1-X4 under copious irrigation using NaOCl. The final irrigation was performed with 10 ml of saline solution (Equiplex, Aparecida de Goiania, Brazil). Root canals were dried with sterile paper points and dressed with calcium hydroxide paste (Ultracal, Ultradent, Southern Jordan, UT, USA). The pulp chamber was sealed with a sterile cotton pellet and filled using conventional glass ionomer cement (Maxxion R, FGM, Joinville, SC, Brazil).

2. Second Visit

At least two weeks after the first treatment visit, the asymptomatic teeth were submitted to the revascularization procedure. The procedure was performed with local infiltrative anesthesia of 2% lidocaine (DFL, Brazil) without vasoconstrictor followed by rubber dam isolation and removal of provisional restoration. Calcium hydroxide paste was removed from the root canals using K-files and 20 ml irrigation of 2.5% NaOCl, followed by 10 ml of sterile saline solution and drying with sterile paper points. Then, the root canal was irrigated with 3 ml of 17% EDTA (1 ml/min) under ultrasonic stirring (E1-Irrisonic, Helse, Santa Rosa de Viterbo, Sao Paulo, Brazil) and dried with paper points (Dentsply). Apical bleeding was induced by passing with K-file size #15 (for mandibular mesial or maxillary buccal canals) or #20-25 (for mandibular distal or maxillary palatal canals) approximately 2 mm beyond the apex. The filling of root canals by the clots was accompanied by 3x magnification using a surgical microscope (DFV, Valença, RJ, Brazil). After approximately 10 minutes, when the blood became semi coagulated, MTA Fillapex (Angelus, Londrina, PR, Brazil) was mixed and introduced into the coronal part of each canal using an appropriate device similar to a miniature amalgam carrier (MTA Applicator, Angelus, Londrina, PR, Brazil) and was vertically condensed. The collagen matrix was not used because the space inside the root canal was insufficient. The pulp chamber was filled with resin-modified glass ionomer cement (Riva, SDI, São Paulo, SP, Brazil). A postoperative radiograph was taken using FIT T2.

3. Third Visit

The final coronal restoration was performed by removing partially the resin-modified glass ionomer, and the coronal portion was filled using bulk fill composite resin. Selective enamel etching was performed using 37% phosphoric acid (Condac 37, FGM) for 30 seconds, followed by washing using air/dry spray for 30 seconds and gentle drying using absorbent paper. The self-etching adhesive system (Ambar APS, FGM) was actively applied using a microbrush (Cavibrush, FGM), followed by gently air spray for solvent evaporation. The

adhesive system was light cured for 10 seconds using a light curing unit with 1400 mW/cm^2 (VALO Cordless, Ultradent). The depth of cavity was checked using a periodontal probe (Hu-Friedy, Chicago, IL, USA), and the regular paste composite resin (Opus bulk fill APS, FGM, Joinville, SC, Brazil) was inserted into an increment of 5 mm in thickness and was light cured in each occlusal/mesial and occlusal/distal region for 40 seconds. The second increment was inserted, and the occlusal anatomy was defined using a sculpting spatula (Millenium, Golgran, São Caetano do Sul, SP, Brazil). All procedures were performed under rubber dam isolation. The time between the conclusion of endodontic treatment and definitive restoration with bulk fill composite resin ranged from 7 days to 6 months; however, during this period, glass ionomer cement provisional restoration was maintained and checked periodically.

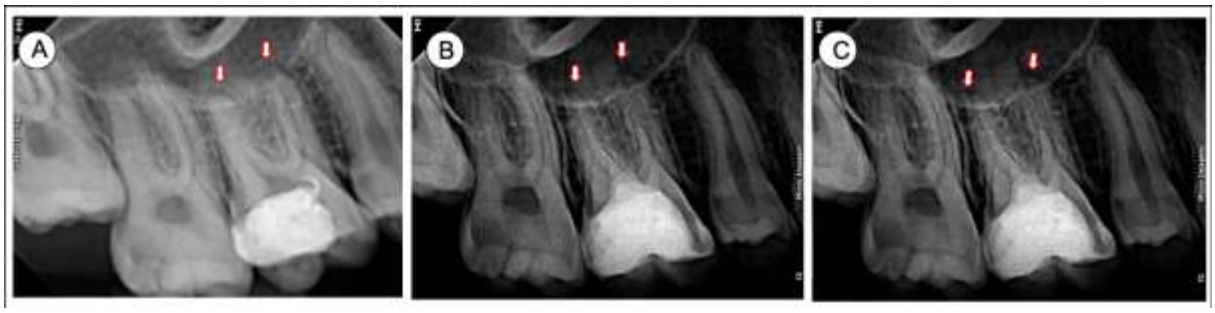
Follow-up examination

In the present study, the follow-up of the treatments was performed based on the parameters described by Orstavik *et al.* (1986) and Saoud *et al.* (2016), which classified the outcome as healed, healing, and disease. Some modifications to the original criteria were added: healed was defined as a tooth without clinical signs and symptoms and periapical radiography with normal appearance; healing was defined as a tooth with reduction of the periapical lesion, without clinical signs and symptoms; and diseased was defined as development or persistence of periapical lesion associated or not with the presence of clinical signs and symptoms. In addition, apical closure or reduction of canal lumen by deposition of mineralized tissue was verified. The clinical and radiographic data obtained from each patient are summarized in Table 1. The follow-up occurred 1 month after the conclusion of the endodontic treatment and afterward every 3 months until 15 months of follow-up.

Clinical case 1 (Figure 1A-1C), of the oldest patients (16 years old), involved treatment performed in the upper molar. At the initial appointment, only the lingual root canal had a slightly open apex, which was confirmed after endodontic file exploration. The other root canals were not large, and periodontal ligament space was slightly thickened, although no radiographic periapical lesion was present.

The tooth was classified as healed at 12 months after endodontic treatment, with a reduction in the space of the periodontal ligament for normal parameters. A slight reduction of the canal lumen was also observed.

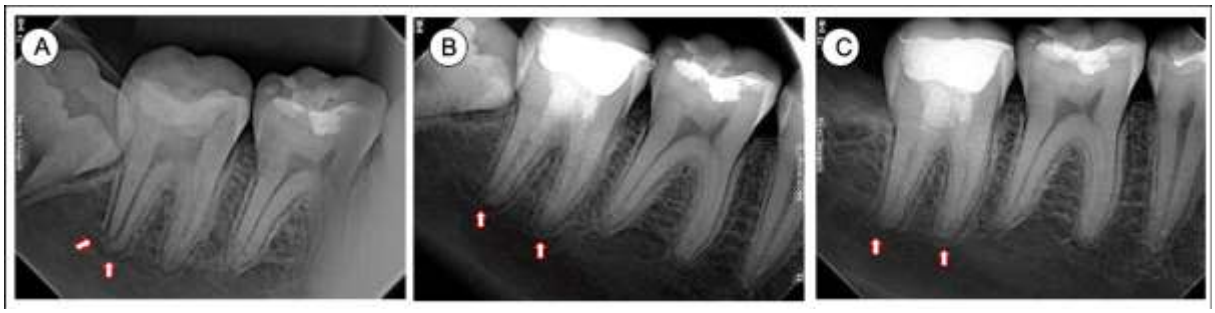
Figure 1



Radiographic follow-ups of the case 1. (A) X-ray image of mandibular first molar showing complete root canal formation and slight thickening of the periodontal ligament (arrows). (B) X-ray image of six-month follow-up showing normal root canal apex appearance (arrow). (C) Final X-ray image after fifteen-month follow-up showing reduction in the canal volume (arrow).

Clinical cases 2 (Figure 2A-2C), 3 (Figure 3A-3C), and 4 (Figure 4A-4C) presented open apex only in the distal root. All teeth received the same treatment protocol. Fifteen months after the regenerative endodontic therapy, all distal root canal apexes were closed and the teeth were classified as healed, reducing the periodontal ligament space to normal parameters. In clinical cases 2 and 3, a reduction in the lumen of the root canals was observed, suggesting possible obliteration (Figure 2A-3C).

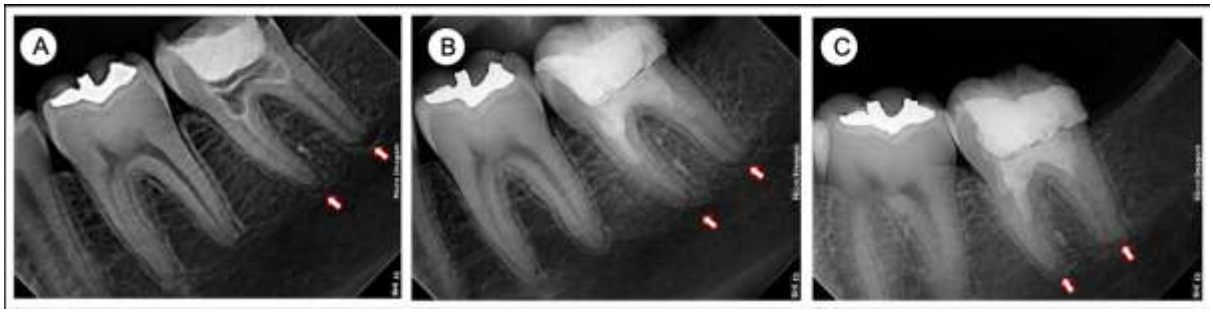
Figure 2



Radiographic follow-ups of the case 2. (A) X-ray image of mandibular first molar

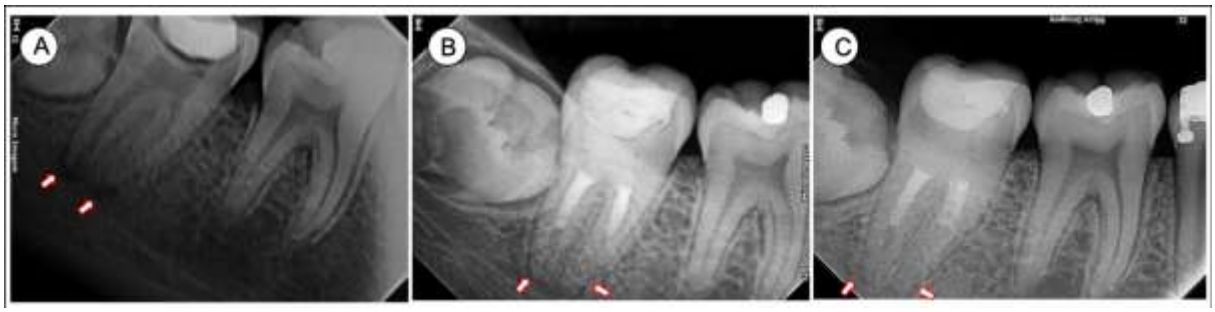
showing larger volume of root canal and open distal canal apex (arrows). (B) X-ray image of six-month follow-up demonstrating normal appearance of both root canal apexes (arrow). (C) Final X-ray image after fifteen-month follow-up showing reduction in the canal volume and the apex closing (arrow).

Figure 3



Radiographic follow-ups of the case 3. (A) X-ray image of mandibular second molar showing open distal canal apex (arrows). (B) X-ray image of six-month follow-up demonstrating normal appearance of both root canal apexes (arrow). (C) Final X-ray image after fifteen-month follow-up showing the apex closing (arrow).

Figure 4

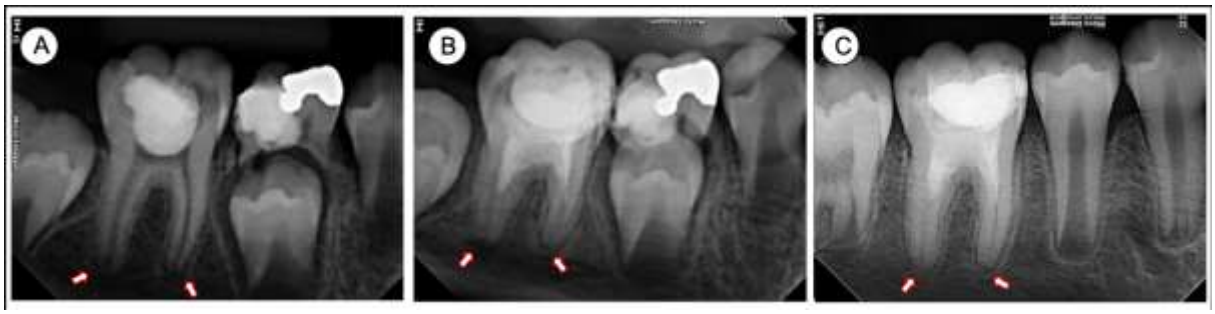


Radiographic follow-ups of the case 4. (A) X-ray image of mandibular first molar showing larger volume of root canal and open distal canal apex (arrows). (B) X-ray image of six-month follow-up demonstrating normal appearance of both root canal apexes (arrow). (C) Final X-ray image after fifteen-month follow-up showing reduction in the canal volume and the apex closing (arrow).

Clinical case 5 (Figure 5A-5C) was the youngest patient (9 years old). At the initial appointment, a wide distal canal with open apex, increased periodontal ligament

space, and initial signs of apical radiolucent development were verified. After treatment, in follow-up visits, a reduction in canal lumen and apical repair were verified, classifying the tooth as healing up to 6 months.

Figure 5



Radiographic follow-ups of the case 5. (A) X-ray image of mandibular first molar showing larger volume of root canal and both open canal apexes (arrows). (B) X-ray image of three-month follow-up demonstrating reduction in canal lumen and normal appearance of both root canal apexes (arrow). (C) Final X-ray image after fifteen-month follow-up showing greater reduction in the canal volume and both apexes closing (arrow).

DISCUSSION

The published case reports of regenerative endodontic treatments enrolling permanent molars (Ajram *et al.,* 2019; Zhujiang *et al.,* 2016) indicated that there is no standardized clinical protocol established for this treatment (Kontakiotis *et al.,* 2015). Some authors report the procedure in a single appointment (Topçuoğlu *et al.,* 2016), whereas others use two or more appointments (Ajram *et al.,* 2019; Zhujiang *et al.,* 2016; Dhaimy *et al.,* 2017). In the present report, the regenerative endodontic procedures were completed in two appointments, because the coronary openings were done previously at the beginning of the treatment in emergency service. The clinical cases were considered healed after 15 months of evaluation. The literature presents several factors responsible for the success of regenerative endodontic treatments, such as root canal disinfection and apical papilla stem cell survival (SCAP) (Diogenes *et al.,* 2017; Duggal *et al.,* 2017).

Previous studies have shown that SCAPs are responsible for tissue repair (Sonoyama *et al.,* 2006; Huang *et al.,* 2010); thus, their survival directly influences the potential for cell differentiation and repair. SCAPs have high proliferative potential and are able to differentiate into several cell subtypes, such as osteogenic, odontogenic, and neurogenic cells (Sonoyama *et al.,* 2006). SCAPs induce tissue repair by depositing a continuous layer of dentin-like tissue (Huang *et al.,* 2010), promoting additional development in thickness and length (Diogenes *et al.,* 2016; Diogenes *et al.,* 2017). Such condition was observed in clinical case 5 when analyzing the apical third of the distal root. Another factor that may also influence the viability of the SCAPs is the concentration of NaOCl used (Martin *et al.,* 2020; Antunes *et al.,* 2015). Although this is the most used irrigating solution in regenerative endodontic treatments, the recommended concentration to be used is variable (Antunes *et al.,* 2015; Kontakiotis *et al.,* 2015). In the present study, 2.5% NaOCl was chosen, which is a potent antimicrobial agent capable of dissolving organic matter (Yang *et al.,* 1995). *In vitro* studies demonstrated that 2.5% NaOCl solutions do not compromise the survival of SCAPs, although higher concentrations are not recommended due to their cytotoxicity (Trevino *et al* 2011; Galler *et al.,* 2011). The use of 17% EDTA as final irrigation in cases of revascularization is also recommended (Galler *et al.,* 2011; Kontakiotis *et al.,* 2015). Its use is supported by chelating capability that promotes the release of growth factors trapped in dentine, favoring the adhesion of SCAPs in dentine walls, without interfering with their survival (Trevino *et al.,* 2011).

The use of intracanal medication in teeth undergoing revascularization is indicated, in most randomized clinical trials and case series reports, the procedure is performed in at least two appointments (Ajram *et al.,* 2019; Dhaimy *et al.,* 2017; Zhujiang *et al.,* 2016). However, there is no consensus about the intracanal medication to be used in regenerative endodontic procedures. While some authors prefer triantibiotic paste (TAP) (Song *et al.,* 2017), others prefer calcium hydroxide ($Ca(OH)_2$) (Ruparel *et al.,* 2012, Althumairy *et al.,* 2014). Based on previous studies that showed that $Ca(OH)_2$ paste is less cytotoxic than TAP to cells in periapical tissues (Ruparel *et al.,* 2012; Althumairy *et al.,* 2014).,

Ultracal®, a ready-to-use $Ca(OH)_2$ paste, was used. In addition, $Ca(OH)_2$ paste is easier to remove than TAP paste (Berkhoff *et al.,* 2014). However, it has been suggested that root canal obliterations are more related to the use of $Ca(OH)_2$ (Song *et al.,* 2017). It was possible to observe that clinical cases 1, 2, 3, and 5 showed a significant reduction in canal volume in the first 12 months, which could be inferred to be related to this statement.

Another important factor for the success of regenerative endodontic treatment is the promotion of the clot, which is induced by the bleeding technique (Duggal *et al.,* 2017). To confirm that there was adequate canal filling by the clot, all cases were treated using a 3x magnification microscope. Molars have canals with a smaller diameter and greater anatomical complexity than incisors, which are usually submitted to regenerative procedures. This fact makes it difficult to induce clotting (Dhaimy *et al.,* 2017), as observed by the operators in this series of cases.

Proper coronal sealing to seal the clot and prevent bacterial contamination is another key factor for successful revascularization (Lin *et al.,* 2014). The MTA was used to seal the canals because it has good sealing properties and excellent biocompatibility (Lin *et al.,* 2014). This was followed by the insertion of resin-modified glass ionomer cement. However, in four teeth, definitive bulk fill composite resin restorations took a long time to perform. This situation is probably related to the fact that RIVA provides proper aesthetics, masticatory efficiency, and sealing capacity, which resulted in the relatives of the adolescents missing consultations. Regarding the biomechanical preparation of root canals, the American Association of Endodontists do not recommend mechanized instrumentation of the root canals (American Association of Endodontics, 2020), although mild instrumentation is generally used (Kontakiotis *et al.,* 2015; Dhaimy *et al.,* 2017). In present report the protocol was modified to in order to meet the specificities observed in young molars.

This is the first series of cases to describe an instrumentation sequence to be used on young molars whose root canals are not as wide and whose apexes are not all opened. The preparation of the cervical third using Hedstroem files and Gates Glidden drills, followed by preparation by Protaper Next files, was used to achieve effective biomechanical preparation, reducing the contamination. To

facilitate the root canal shaping, the mesial canals of the mandibular molars were enlarged, subsequently allowing more effective induction of the clot. This technical modification did not affect the prognosis of the treatments. Even in the adverse conditions found, characterized by teeth largely destroyed by caries and previously exposed to the oral environment, success was obtained, which were considered healed/healing. Moreover, in clinical cases 2, 3, 4, and 5, the apex closure of the distal roots occurred. The lack of response to the pulp sensitivity test during follow-up appointments does not necessarily mean that the tooth has no vitality (Law AS, 2013) because the thickness of sealing material present on the tooth may affect the response to the cold sensitivity test (Torabinejad *et al.,* 2011). All the procedures performed in this series of clinical cases were able to maintain the young permanent molars, contributing to stability of the growing process, masticatory performance, and occlusal stability.

CONCLUSIONS

It is possible to perform regenerative endodontic procedures in young molars severely compromised by caries and obtain success. The instrumentation sequence proposed in this series of clinical cases, which combines hand files, Gates Glidden drills, and a modified Protaper Next technique, did not negatively affect treatment prognosis. Therefore, it is possible to perform this treatment sequence as a method of mechanical disinfection of root canals prior to clot induction in young molars. Another important aspect is that the direct composite resin restoration must be performed as soon as possible after the clot induction.

ACKNOWLEDGEMENTS

The authors are grateful with CAPES finance code 001, FAPEMIG and CNPq for financial support of this clinical service.

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4. CONCLUSÕES

Dentro das limitações metodológicas destes estudos que envolveram dois estudos laboratoriais e um relato de caso clínico pode-se concluir que:

1- O novo material biocerâmico Bioc Temp possui viabilidade, semelhante à do MTA e Ultracal e resultaram em cor de dente semelhante ao MTA e Ultracal, respectivamente. Apesar de sua menor radiopacidade, o BT foi identificado radiograficamente.

2- Os três materiais de silicato de cálcio testados para pulpotomia parcial promoveram mudança na cor do dente, com resultados mais favoráveis para BioC-Repair, que apresentou menor coloração, maior radiopacidade e viabilidade do que 80% hDPCs.

3- É possível realizar procedimentos endodônticos regenerativos em molares jovens gravemente comprometidos por cárie e obter sucesso.

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Anexos

6.1- Normas do Periódico 1 e 2

INTERNATIONAL
ENDODONTIC JOURNAL

Acesse o link:
https://onlinelibrary.wiley.com/page/journal/13652591/homepage/forauthors.html

6.2 - Normas do Periódico 3

Research, Society and Development journal

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