

Gabriela Leite de Souza

**Tratamento endodôntico de dentes permanentes imaturos: avaliações
in vitro e computacionais de diferentes parâmetros de interesse.**

*Endodontic treatment of immature permanent teeth: in vitro and
computational evaluations of different parameters of interest.*

Tese apresentada à Faculdade de
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Doutor em Odontologia na Área de
Clínica Odontológica Integrada.

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Orientadora: Profa. Dra. Camilla Christian Gomes Moura

Banca Examinadora:

Profa. Dra. Camilla Christian Gomes Moura

Profa. Dra. Pricilla Barbosa Ferreira Soares

Prof. Dr. Carlos José Soares

Profa. Dra. Graziela Bianchi Leoni

Profa. Dra. Fabiane Carneiro Lopes Olhê

Uberlândia, 2023.



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



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“Quanto mais aumenta nosso conhecimento, mais evidente fica nossa ignorância”

John F. Kennedy

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RESUMO

Este trabalho teve como objetivo avaliar *in vitro* e por ensaios computacionais diversos procedimentos e materiais utilizados em terapias pulpares e regeneradoras em dentes imaturos. Para isso, no capítulo 1, vidro bioativo $\text{SiO}_2\text{--Na}_2\text{O--CaO--P}_2\text{O}_5$ (SNCP) e SNCP dopado com titânio 10% (SNCP:10Ti) foram sintetizados, caracterizados e avaliados sob a forma de solução na viabilidade, migração e expressão de fosfatase alcalina (ALP) em células da polpa dental humana. No capítulo 2, nanocristais de óxido de zinco dopado com cálcio 1% (ZnO:1.0Ca) foram manipuladas com propilenoglicol (PRG) ou polietilenoglicol com propilenoglicol (PEG+PRG) e as pastas tiveram sua citotoxicidade, radiopacidade, pH e penetração em túbulos dentinários investigadas e comparadas com pastas de hidróxido de cálcio (Ca(OH)_2). No capítulo 3, dentes permanentes imaturos com reabsorção radicular por substituição simulados foram obturados com Biodentine (BD), Bio-C Repair (BCR), e através de apexificação com agregado de trióxido mineral (MTA) e foram testados para resistência à fratura. Adicionalmente, avaliou-se os efeitos desses materiais na osteoclastogênese induzida *in vitro*. No capítulo 4, a distribuição de tensões de dentes revascularizados com diferentes desenvolvimentos radiculares (RET) e de dentes permanentes imaturos tratados com apexificação com MTA e reforçado com resina composta intra-radicular (RC) foi avaliada durante ao carregamento de mordida usando método de análise bidimensional de elementos finitos. Por fim, no capítulo 5, relatou-se o tratamento multidisciplinar de uma menina de 9 anos que sofreu uma fratura complicada de coroa no incisivo central superior esquerdo e uma necrose tardia no incisivo lateral superior esquerdo, que foram respectivamente tratados através de pulpotomia parcial seguido de tratamento endodôntico radical, e procedimento endodôntico regenerativo. Os vidros bioativos SNCP e SNCP:10Ti foram corretamente sintetizados. Os níveis de citotoxicidade foram altos na diluição de 1:1 ($p<0.05$) e similares ao controle em 1:8 e 1:16 ($p>0.05$). Entretanto, SNCP:10Ti foi menos citotóxico na diluição de 1:8 ($p<0.05$). Os grupos apresentaram migração e expressão de ALP similares ($p>0.05$). As pastas de ZnO:1.0Ca mostraram menor viabilidade em 1:1 e 1:2 comparadas às de Ca(OH)_2 ($p<0.0001$). As pastas de Ca(OH)_2 apresentou maiores valores de pH e menor radiopacidade que ZnO:1.0Ca ($p<0.05$). As pastas de ZnO:1.0Ca tiveram profundidade de penetração maior que Ca(OH)_2 no terço apical ($p<0.0001$). Nenhuma diferença significativa na resistência à fratura foi observada entre dentes

obturados com BCR, BD ou apexificação com MTA ($p>0.05$). BD inibiu a osteoclastogênese de maneira similar ao BCR e MTA ($p>0.05$), contudo BCR mostrou uma porcentagem menor de osteoclastos comparado o MTA ($p<0.0001$). O aumento da espessura da dentina radicular após RET não teve benefício significativo na distribuição de tensões de dentes permanentes imaturos. RC apresentou menor acúmulo de tensões intrarradicular na região cervical em comparação aos modelos de RET. Quanto ao relato de caso, o acompanhamento de 4 anos da paciente confirma evidência de sucesso clínico do tratamento proposto, uma vez que a paciente se apresentou assintomática, e todos os dentes envolvidos demonstraram completa formação radicular e aspecto de normalidade periapical.

Palavras-chave: Apexificação, Biovidro, Cimento de silicato, Óxido de zinco, Pulpotomia, Resistência à fratura, Sobrevivência celular, Tratamento endodôntico regenerativo.

ABSTRACT

This study aimed to evaluate in vitro and through computational tests several procedures and materials used in pulpal and regenerative therapies in immature teeth. In chapter 1, bioactive glass $\text{SiO}_2\text{--Na}_2\text{O--CaO--P}_2\text{O}_5$ (SNCP) and SNCP doped with 10% titanium (SNCP:10Ti) were synthesized, characterized and evaluated in solution for viability, migration and expression of alkaline phosphatase (ALP) in human dental pulp cells. In chapter 2, zinc oxide nanocrystals doped with 1% calcium (ZnO:1.0Ca) were manipulated with propylene glycol (PRG) or polyethylene glycol with propylene glycol (PEG+PRG) and were investigated for cytotoxicity, radiopacity, pH and penetration into dentinal tubules compared with calcium hydroxide pastes (Ca(OH)_2). In Chapter 3, immature permanent teeth with simulated replacement root resorption were filled with Biodentine (BD), Bio-C Repair (BCR), and through apexification with mineral trioxide aggregate (MTA) and were tested for fracture toughness. Additionally, the effects of these materials on in vitro induced osteoclastogenesis were evaluated. In Chapter 4, the stress distribution of revascularized teeth with different root development (RET) and of immature permanent teeth treated with apexification with MTA and reinforced with intraradicular composite resin (RC) was evaluated during bite loading using method of analysis two-dimensional finite elements. Finally, in chapter 5, the multidisciplinary treatment of a 9-year-old girl who suffered a complicated fracture of the crown in the upper left central incisor and a late necrosis in the upper left lateral incisor, which were respectively treated through partial pulpotomy followed by endodontic treatment radical and regenerative endodontic procedure, has been reported. The SNCP and SNCP:10Ti bioactive glasses were correctly synthesized. Cytotoxicity levels were high at the 1:1 dilution ($p<0.05$) and similar to the control at 1:8 and 1:16 ($p>0.05$). However, SNCP:10Ti was less cytotoxic at the 1:8 dilution ($p<0.05$). The groups showed similar ALP migration and expression ($p>0.05$). ZnO:1.0Ca pastes showed lower viability in 1:1 and 1:2 compared to Ca(OH)_2 ($p<0.0001$). Ca(OH)_2 pastes showed higher pH values and lower radiopacity than ZnO:1.0Ca ($p<0.05$). ZnO:1.0Ca pastes had greater penetration depth than Ca(OH)_2 in the apical third ($p<0.0001$). No significant difference in fracture toughness was observed between teeth filled with BCR, BD or MTA apexification ($p>0.05$). BD inhibited osteoclastogenesis similarly to BCR and MTA ($p>0.05$), however BCR showed a lower percentage of osteoclasts compared to MTA ($p<0.0001$). Increasing root dentin thickness

after RET had no significant benefit in the stress distribution of immature permanent teeth. RC showed less accumulation of intraradical stresses in the cervical region compared to the RET models. In the presented clinical case, the 4-year follow-up of the patient confirms evidence of clinical success of the proposed treatment, since the patient was asymptomatic, and all teeth involved demonstrated complete root formation and periapical normal appearance.

Keywords: Apexification, Bioglass, Silicate cement, Zinc oxide, Pulpotomy, Fracture resistance, Cell survival, Regenerative endodontic treatment

1 INTRODUÇÃO E REFERENCIAL TEÓRICO

O traumatismo dentário contabiliza aproximadamente 5% de todas as injúrias e acomete principalmente crianças e adolescentes (Andersson 2013), acarretando em diversas complicações, como descoloração do dente, mobilidade, má oclusão, necrose pulpar e eventualmente perda do elemento dental (Matoug-Elwerfelli et al. 2022). A perda de vitalidade pulpar, especialmente em dentes imaturos, pode reduzir significativamente o prognóstico a longo prazo de dentes traumatizados (Nazzal & Duggal, 2017). Portanto, a preservação da vitalidade pulpar por meio de terapias vitais pulpares (TVP) é um componente chave no manejo de dentes traumatizados (Cohenca et al., 2013; da Rosa et al., 2018).

As TVP podem ser definidas como “estratégias destinadas a manter a vitalidade da polpa” (ESE, 2019). Estas incluem capeamento pulpar direto e pulpotomias. Especificamente nos casos de capeamento pulpar direto e pulpotomias, a técnica requer a remoção da irritação microbiana e tecidos inflamados seguido da proteção da polpa vital exposta pela inserção de um biomaterial de selamento (Duncan, 2022). Nos últimos 20 anos, vários avanços revigoraram a área de TVP, incluindo o advento dos cimentos de silicato de cálcio bioativos (CSCs) (Parirokh et al. 2018, Duncan 2022), bem como melhorias técnicas e biológicas na irrigação e manuseio do tecido exposto (Ballal et al., 2021; Bjørndal et al., 2019).

Dentre as soluções utilizadas para lavar a superfície pulpar exposta atingindo a desinfecção e hemostasia, o hipoclorito de sódio e a clorexidina têm sido amplamente indicados (Mente *et al.* 2014, Kundzina *et al.* 2017, Harms *et al.* 2019). Contudo, o desenvolvimento de soluções irrigadoras que possam conter a hemorragia pulpar, possuam propriedades antimicrobianas e sejam capazes de estimular o processo de biomineralização, são o novo desafio das TVP.

O vidro bioativo é um biomaterial biodegradável e biocompatível, composto de diferentes porcentagens de SiO_2 – Na_2O – CaO – P_2O_5 (Hench & Anderson, 1993) que exhibe uma capacidade única de ligação óssea formando uma camada superficial de hidroxiapatita após incubação em fluidos fisiológicos (Martin et al. 2009) e

simultaneamente suportam processos regenerativos biológicos, como angiogênese e osteogênese durante sua dissolução (Kim et al. 2017, Yan et al. 2020). Além disso, composições específicas de BGs podem fornecer atividade antibacteriana (Saha et al. 2020) e induzir uma resposta anti-inflamatória (Björkenheim et al. 2021). Nesse contexto, diante dos resultados promissores do seu uso como material de proteção em TVP (Long et al. 2017, Zhu et al. 2019), a aplicação do vidro bioativo como solução irrigante em TVP pode ser interessante.

No entanto, nem sempre é possível realizar TVP em dentes imaturos uma vez que em determinadas situações estes podem evoluir para necrose pulpar. Nos casos de necrose de dentes permanentes imaturos, uma abordagem de tratamento endodôntico alternativa deve ser usada devido à presença de ápice aberto e amplo, raízes divergentes e paredes dentinárias finas (Torabinejad et al. 2017). Além disso, esses dentes apresentam maior risco de fraturas catastróficas, especialmente relacionadas à região cervical (Cvek, 1992).

Atualmente, temos como principais alternativas de tratamento para estes casos: apicificação por trocas consecutivas de medicação intracanal, tampão apical com biomateriais ou revascularização. A abordagem de apicificação por meio de trocas consecutivas de medicação intracanal é uma conduta terapêutica tradicional que consiste na utilização de hidróxido de cálcio (Ca(OH)_2) à longo prazo para induzir a apicificação através da formação de uma barreira calcificada no ápice radicular após desinfecção dos canais radiculares (Shabahang, 2013). A finalização do tratamento endodôntico é postergada até que o fechamento radicular seja finalizado. Entretanto, dificuldades em obter uma barreira apical bem como falhas na continuidade do desenvolvimento radicular (Shabahang, 2013) com aumentado risco de fratura subsequente durante ou após o tratamento (Cvek, 1992) tornam a manutenção desse dente imprevisível. Além disso, pastas a base deste material precisam ser trocadas no máximo a cada 30 dias, embora o veículo usado possa determinar períodos maiores ou menores de trocas (Ballal et al. 2010), levando a uma falta de cooperação do paciente durante o período de tratamento.

Diante das desvantagens das contínuas trocas de medicação intracanal em dentes traumatizados, uma medicação intracanal em pasta à base de hidróxido de cálcio, óxido de zinco e clorexidina gel 2%, que não requer trocas periódicas tem sido proposta por um grupo de pesquisadores da FOP-UNICAMP (Soares et al. 2013, Soares et al. 2014).

Inspirados na possibilidade de podermos produzir dentro da Faculdade de Odontologia da Universidade Federal de Uberlândia (UFU) uma pasta obturadora temporária que possua baixo custo e propriedades físico, químicas e biológicas desejáveis para a apexificação, desenvolvemos uma pasta a base de nanopartículas de óxido de zinco dopados com cálcio (ZnO:Ca). Este material teve sua citotoxicidade testada *in vitro* na forma nanoparticulada e em pó (Souza et al. 2020), no entanto, até o momento, ainda não havia sido associado a nenhum veículo para uso dentro do canal radicular.

Em casos em que as paredes radiculares encontram-se paralelas e o diâmetro apical menor que 1,1 mm, uma das alternativas é o uso de uma barreira apical artificial com Agregado de Trióxido Mineral (MTA) (Simon et al. 2007, Bonte et al. 2015,). Este procedimento reduz a possibilidade de fraturas a longo prazo quando comparado ao Ca(OH)_2 (Bonte et al. 2015), resulta em menos consultas para completar o tratamento e na formação de barreira apical mais previsível (Shabahang, 2013). Além disso, o MTA é um material bioativo, com alta resistência mecânica (Marciano et al. 2019). Apesar de apresentar características e benefícios que são favoráveis ao seu uso, MTA tem suas limitações, como longo tempo de presa (Bortoluzzi et al. 2009), dificuldade de manuseio (Ber et al. 2007), baixa adesividade às paredes (Inada et al. 2023) e manchamento (Jesus et al. 2023).

Por essas razões, outros cimentos baseados em trióxido mineral tem sido sugeridos como possibilidades promissoras para a terapia de apexificação (Girish et al. 2017, Sarraf et al. 2019). O Biodentine (BD; Septodont, Saint Maur-des-Fosses, France) é um cimento restaurador baseado em silicato tri-cálcio indicado como “substituto dentinário”, comparável ao MTA em termos de biocompatibilidade e indução de barreira calcificada (Kaur et al. 2017), mas com melhorias em relação à manipulação, manuseio, tempo de presa e manchamento dental (Kaur et al. 2017). Outro material biocerâmico que também pode ser citado é o Bio-C repair, o qual foi recentemente lançado, sendo apresentado em uma forma “pronta para uso”, dispensando manipulação. Este produto apresenta baixa citotoxicidade (Ghilotti et al. 2020, Oliveira et al. 2021), boa biocompatibilidade (Benetti et al. 2019), boa habilidade de mineralização (Benetti et al. 2019, Santiago et al. 2021), sendo indicado para tratamentos endodônticos regenerativos e reparativos.

Apesar da variedade de cimentos endodônticos bioativos disponíveis no mercado, a técnica de apexificação por barreira apical, não fortalece a estrutura dental, visto que o canal radicular permanece com paredes dentinárias finas (Hargreaves et al. 2013). Nesse sentido, para melhorar o selamento dos terços cervical e médio de raízes imaturas, a literatura tem sugerido a obturação completa do sistema de canal radicular com os cimentos baseados em silicato de cálcio (Bayram & Bayram 2016, Aksel et al. 2017, Ürkmez & Erdem 2020, Żuk-Grajewska et al. 2020), uma vez que as partes cervical e média da raiz fornecem a maior parte da resistência do dente. A obturação completa com MTA tem sido proposta também em dentes reimplantados, onde comumente surgem complicações como anquilose e reabsorção radicular por substituição (RRS) (Panzarini et al. 2007, Panzarini et al. 2012, Ferreira et al. 2017). Diante da necessidade de inibir ou atrasar os efeitos da reabsorção radicular em dentes traumatizados sem os inconvenientes das contínuas trocas de Ca(OH)_2 , o MTA foi proposto como material obturador em dentes reimplantados (Panzarini et al. 2007, Panzarini et al. 2012, Ferreira et al. 2017) exibindo resultados promissores, com menor ocorrência de anquilose dental quando comparado ao procedimento com Ca(OH)_2 (Zare Jahromi et al. 2019)). Contudo, até o momento nenhum estudo avaliou aspectos biomecânicos de dentes que já apresentem reabsorção radicular por substituição e que foram completamente obturados com materiais bioativos.

Procedimentos endodônticos regenerativos também conhecidos como revascularização, têm sido propostos como uma alternativa de tratamento para dentes imaturos com necrose pulpar (Banchs et al. 2004, Hargreaves et al. 2013). Esse tratamento tem como objetivo regenerar a função do tecido pulpar, resultando em eliminação dos sintomas, reparo ósseo e maturação radicular, caracterizada pelo fechamento do ápice radicular com aumento da espessura das paredes dentinárias e possibilidade de ganho em comprimento radicular (Belli et al. 2018). Em relação à técnica, resumidamente ela consiste no uso de uma combinação de antimicrobianos para reduzir a infecção, indução de sangramento apical para formar um coágulo contendo células tronco indiferenciadas (Lovelace et al. 2011, Kontakiotis et al. 2015, Anthrayose et al. 2021), seguido da aplicação de material baseado em silicato de cálcio sobre o coágulo, servindo como uma barreira coronal (Miller et al. 2018). Hipoteticamente, o desenvolvimento radicular seguido do tratamento de revascularização deveria aumentar a resistência do dente

permanente imaturo e portanto, oferecer uma vantagem biomecânica à apexificação com MTA. Entretanto, o desenvolvimento radicular atingido após a revascularização pode variar de 2 à 40% (Ong et al. 2020) , sendo que o terço cervical radicular permanece enfraquecido devido ao vedamento coronário colocado na junção cimento-esmalte (Demirel et al. 2021). Sendo assim, ainda são necessários estudos que avaliem a performance biomecânica de diferentes resultados possíveis obtidos no tratamento endodôntico regenerativo e a distribuição de tensões do dente permanente imaturo.

Por fim, com o objetivo de difundir ao clínico abordagens atuais utilizadas no tratamento endodôntico de dentes permanentes imaturos traumatizados, será apresentado ao final desta tese um relato de caso que contempla a abordagem endodôntica conservadora através de pulpotomia parcial de um incisivo central superior do lado esquerdo com fratura extensa de coroa e procedimento endodôntico regenerativo do incisivo lateral superior do lado esquerdo, o qual foi diagnosticado com necrose pulpar 9 meses após o trauma. Nas diretrizes recentemente publicadas pela Associação Internacional de Traumatologia Dentária (IADT), houve uma mudança na preferência pelo tratamento conservador da polpa, e as pulpotomias parciais são agora o tratamento recomendado para dentes com desenvolvimento radicular completo e incompleto (Bourguignon et al. 2020). Apesar das lesões dentárias geralmente afetarem apenas o único dente que sofreu o impacto, lesões secundárias como necrose pulpar podem ser observadas após várias semanas ou meses em dentes adjacentes que não foram impactados diretamente, mesmo quando a resposta após o trauma foi positiva (Aas & Skaare 2011; Santiago et al. 2015; Elbay et al. 2015) .

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar *in vitro* e por ensaios computacionais o comportamento biomecânico de dentes permanentes imaturos traumatizados submetidos à diferentes protocolos endodônticos e desenvolver materiais nanoparticulados e bioativos com potencial aplicabilidade nas diversas modalidades de tratamento endodôntico de dentes imaturos.

2.2 OBJETIVOS ESPECÍFICOS

I) Sintetizar e caracterizar um vidro bioativo $\text{SiO}_2\text{--Na}_2\text{O--CaO--P}_2\text{O}_5$ (SNCP) e SNCP dopado com titânio (wt 10%) (SNCP:10Ti) para aplicação como solução irrigante em TVP. Adicionalmente avaliar a citotoxicidade das soluções de biovidro, migração celular e liberação de fosfatase alcalina de células da polpa dental humana (hDPCs).

II) Avaliar as propriedades químicas e física com potenciais implicações clínicas (radiopacidade, pH, penetração tubular) e citotoxicidade de uma pasta obturadora temporária nanoparticulada de óxido de zinco com cálcio manipulada com dois diferentes veículos (propilenoglicol e polietilenoglicol+propilenoglicol) em macrófagos (RAW264.7).

III) Avaliar e comparar o efeito da obturação completa com diferentes materiais (Biodentine, Bio-C repair e plug de MTA), na resistência a fratura de dentes permanentes imaturos simulando reabsorção radicular por substituição.

IV) Avaliar o padrão de distribuição de tensões em dentes permanentes revascularizados com diferentes estágios de maturação radicular (10% de aumento em espessura e comprimento, 10% em comprimento e 20% em espessura, 10% em comprimento e 40% em espessura) comparado com um dente permanente imaturo com o canal preenchido completamente com resina composta através de modelos bidimensionais simulados por elementos finitos.

V) Relatar através de um caso clínico a abordagem multidisciplinar no tratamento de dentes permanentes imaturos traumatizados, envolvendo a realização de pulpotomia parcial e revascularização pulpar.

CAPÍTULO 1

Synthesis and characterisation of two bioactive glass solutions for vital pulp therapy – A preliminary study.

Biotechnology Letters

Synthesis and characterisation of two bioactive glass solutions for vital pulp therapy - A preliminary study --Manuscript Draft--

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Corresponding Author:	Camilla Christian Gomes Moura Federal University of Uberlandia: Universidade Federal de Uberlandia Uberlândia, Minas Gerais/MG BRAZIL	
Corresponding Author Secondary Information:		
Corresponding Author's Institution:	Federal University of Uberlandia: Universidade Federal de Uberlandia	
Corresponding Author's Secondary Institution:		
First Author:	Gabriela Leite de Souza	
First Author Secondary Information:		
Order of Authors:	Gabriela Leite de Souza Júlio César Tsukide Rogerio Valentim Gelamo Anielle Christine Almeida Silva Marcos Vinícius da Silva Rodrigo César Rosa Camilla Christian Gomes Moura	
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Abstract:	Purpose: This study synthesised, characterised, and assessed the effects of bioactive glass SiO₂-Na₂O-CaO-P₂O₅ (SNCP) and SNCP doped with titanium (SNCP:10Ti) on human dental pulp cells (hDPCs) viability, migration, and alkaline phosphatase (ALP) expression. Methods and materials: SNCP and SNCP:10Ti were prepared via a melt-quenching , and atomic force microscopy showed their morphologies. Fourier-transform infrared spectroscopy showed the formulations' structural changes and qualitative characteristics. hDPSCs were cultured in aqueous solutions of SNCP, SNCP:10Ti, and Ca(OH)₂ prepared by mixing 1 g of powder in 10 mL of distilled water, then diluted from 1:1 to 1:16. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide formazan assay, cell migration by scratch assay, and ALP activity was assessed on day 7. Cell viability, pH, and wound healing were analysed using analysis of variance (ANOVA) and Tukey's test. ANOVA was used to compare ALP fluorescence (α=0.05). Results: The materials were 1 µm thick. SNCP:10Ti presents the Ti nanoparticles	

	located on the edge. The samples' chemical integrity was unaffected by incorporation of TiO₂ into SNCP. Cytotoxicity levels were high in a 1:1 dilution ($p < 0.05$); similar at 1:4, 1:8, and 1:16 ($p > 0.05$); and similar to the control at 1:8 and 1:16 ($p > 0.05$). However, SNCP:10Ti was less cytotoxic in the 1:8 dilution ($p < 0.05$). The migration test showed no significant differences between the samples ($p > 0.05$). Fluorescence intensity was similar in all experimental groups ($p > 0.05$) and lower in the control ($p < 0.05$). Conclusion: SNCP and SNCP:Ti have potential for use in vital pulp therapies Doping with Ti reduced the cytotoxicity of SNCP in hDPCs.
Suggested Reviewers:	

Synthesis and characterisation of two bioactive glass solutions for vital pulp therapy - A preliminary study

Gabriela Leite de Souza¹, Júlio César Tsukide², Rogerio Valentim Gelamo³, Anielle Christine Almeida Silva⁴, Marcos Vinícius da Silva⁵, Rodrigo César Rosa⁶, Camilla Christian Gomes Moura⁷

¹PhD Student, Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil. <https://orcid.org/0000-0003-3505-5258>

²MSc Student, Department of Physiological Sciences, Federal University of Triângulo Mineiro, Uberaba, MG, Brazil <https://orcid.org/0000-0003-0972-1907>

³Professor, Institute of Technological and Exact Sciences, Federal University of Triângulo Mineiro, Uberaba, MG, Brazil. <https://orcid.org/0000-0003-2124-3450>

⁴Professor, Functional and New Nanostructured Materials Laboratory, Physics Institute, Federal University of Alagoas, Maceió, AL, Brazil. <https://orcid.org/0000-0003-1959-2870>

⁵Professor, Department of Microbiology, Immunology and Parasitology, Federal University of Triângulo Mineiro, Uberaba, MG, Brazil. <https://orcid.org/0000-0002-2966-7621>

⁶Professor, Department of Structural Biology, Federal University of Triângulo Mineiro, Uberaba, MG, Brazil. <https://orcid.org/0000-0003-3157-0826>

⁷Professor, Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil. <https://orcid.org/0000-0002-5653-8403>

***Correspondence author:** Camilla Christian Gomes Moura, PhD. Department of Endodontics, School of Dentistry, Federal University of Uberlândia. Av Pará 1720, Campus Umuarama, Block 4L-A, Uberlândia, MG, Brazil. Zip-Code: 38405-328. *E-mail address:* camillamoura@ufu.br ; *Phone number:* +55 34 3225-8119

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Author contributions

Conceptualization: Moura CCG, Gelamo RV, Silva MV, Rosa RC ; methodology: Souza GL, Tsukide JC, Silva ACA, Gelamo RC; formal analysis: Souza GL, Moura CCG, Silva MV; resources: Silva ACA, Moura CCG; writing- Souza GL, Rosa RC, Tsukide JC; original draft: Silva MV, Gelamo RV; writing-review and editing: Souza GL; supervision: Moura CCG. All authors read and approved the final manuscript.

ABSTRACT

Purpose: This study synthesised, characterised, and assessed the effects of bioactive glass $\text{SiO}_2\text{--Na}_2\text{O--CaO--P}_2\text{O}_5$ (SNCP) and SNCP doped with titanium (SNCP:10Ti) on human dental pulp cells (hDPCs) viability, migration, and alkaline phosphatase (ALP) expression.

Methods and materials: SNCP and SNCP:10Ti were prepared via a melt-quenching , and atomic force microscopy showed their morphologies. Fourier-transform infrared spectroscopy showed the formulations' structural changes and qualitative characteristics. hDPSCs were cultured in aqueous solutions of SNCP, SNCP:10Ti, and Ca(OH)_2 prepared by mixing 1 g of powder in 10 mL of distilled water, then diluted from 1:1 to 1:16. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide formazan assay, cell migration by scratch assay, and ALP activity was assessed on day 7. Cell viability, pH, and wound healing were analysed using analysis of variance (ANOVA) and Tukey's test. ANOVA was used to compare ALP fluorescence ($\alpha=0.05$).

Results: The materials were 1 μm thick. SNCP:10Ti presents the Ti nanoparticles located on the edge. The samples' chemical integrity was unaffected by incorporation of TiO_2 into SNCP. Cytotoxicity levels were high in a 1:1 dilution ($p<0.05$); similar at 1:4, 1:8, and 1:16 ($p>0.05$); and similar to the control at 1:8 and 1:16 ($p>0.05$). However, SNCP:10Ti was less cytotoxic in the 1:8 dilution ($p<0.05$). The migration test showed no significant differences between the samples ($p>0.05$). Fluorescence intensity was similar in all experimental groups ($p>0.05$) and lower in the control ($p<0.05$).

Conclusion: SNCP and SNCP:Ti have potential for use in vital pulp therapies Doping with Ti reduced the cytotoxicity of SNCP in hDPCs.

Keywords: Bioglass; Endodontics; Pulpotomy.

INTRODUCTION

Current scientific evidence recommends vital pulp therapies (VPT) to preserve vitality after injuries resulting from trauma, caries, or restorative procedures, both in immature and mature teeth (AAE, 2021; Duncan *et al.* 2022). VPT procedures have traditionally included cappings and pulpotomies (AAE, 2020) and require removal of microbial irritation, damage, and inflamed tissues, and protection of the

exposed vital pulp from external stimuli by placing a well-sealed covering biomaterial (Nunez et al. 2021). Specifically, in relation to pulpotomies, the containment of pulpal haemorrhage is considered an important point for decision-making regarding the performance of the procedure, as it is clinically related to the success of the procedure (Duncan et al. 2022, Matoug-Elwerfelli et al. 2022).

Several haemostatic agents have been employed for haemorrhage control, such as sodium hypochlorite, chlorhexidine, and calcium hydroxide solutions (Tüzüner et al. 2012, Accorinte et al. 2005, Duncan et al. 2022, Matoug-Elwerfelli et al. 2022). Calcium hydroxide (Ca(OH)_2) solution is recommended because it exerts biological activity through the dissociation of calcium and hydroxyl ions (Mohammadi & Dummer 2011), and causes fewer metabolic alterations in the pulp tissue compared to sodium hypochlorite (Accorinte et al. 2005). Considering the physical and biological properties of bioactive glasses (BGs), as confirmed by *in vitro* biomineralisation studies (Li et al. 2023, Liu et al. 2023), and their antimicrobial properties (Saha et al. 2020), their use in irrigating solutions could become an alternative in pulpotomies.

BG composites are biodegradable and biocompatible biomaterials with bases containing different percentages of $\text{SiO}_2\text{--Na}_2\text{O--CaO--P}_2\text{O}_5$ (SNCP) (Hench & Anderson, 1993). BGs exhibit a unique bone-bonding ability by forming a hydroxyapatite surface layer after incubation in physiological fluids (Martin et al. 2009) and they simultaneously support biological regenerative processes, such as angiogenesis and osteogenesis, during their dissolution (Kim et al. 2017, Yan et al. 2020). Furthermore, specific BG compositions can provide antibacterial activity (Saha et al. 2020) and induce anti-inflammatory responses (Björkenheim et al. 2021).

The specific use of BG in VPT has been explored in animal models, showing the formation of reparative dentin and only a mild inflammatory response during pulp capping procedures (Long et al. 2017, Zhu et al. 2019). A clinical trial on primary teeth demonstrated that BG promotes the formation of a dentin bridge (Ahmadvand et al. 2016). However, a recent randomised controlled trial demonstrated that tailored amorphous multiparous BG induces persistent inflammation, negating the ability of the pulp to heal and regenerate (Elhamouly et al. 2021).

One strategy used to modify the undesirable properties of materials and improve their functionality is the intentional insertion of doping agents into the material structure (Asif et al. 2014; Zhu et al. 2019). Previous studies have reported that the incorporation of titanium (Ti) into BGs can improve their bioactivity (Asif et al. 2014), biocompatibility (Moss et al. 2010), and induce cell proliferation (Abou Neel et al. 2007).

However, the biological effects of BG on human dental pulp cells (hDPCs) are largely unknown. In this context, the purpose of this preliminary *in vitro* study was to synthesise and characterise a BG SNCP and SNCP doped with Ti (wt 10%) (SNCP:10Ti) for application as an irrigation solution in VPT. In addition, the cytotoxicity of the BG solutions was evaluated and compared to that of the $\text{Ca}(\text{OH})_2$ solution in hDPCs. The null hypothesis was that there would be no significant differences in cell viability, migration, or alkaline phosphatase expression after exposure to the evaluated materials.

MATERIALS AND METHODS

Preparation of SNCP and SNCP:10Ti

The BGs were synthesised at the Laboratory of New Nanostructured and Functional Materials of the Physics Institute of the Federal University of Alagoas, Maceió, AL, Brazil, and is currently in the patent application process, (Brazilian National Institute of Industrial Property Process: 20230000622). Both types of BGs were synthesised using fusion techniques. Briefly, SiO_2 , Na_2O , CaO , and P_2O_5 , pure or with the addition of 10% wt of Ti ions, were mixed and melted at a temperature of 1000 to 2000°C. Then, the mixture was transferred to a cooling plate at a temperature of 0 to 25°C. The BG macromolecules were pulverised using an agate mortar or ball mill to obtain BG microparticles doped (SNCP:10Ti) or not (SNCP) with Ti ions.

Atomic force microscopy

The morphologies of SNCP and SNCP:10Ti were obtained using atomic force microscopy (AFM) in the dynamic mode (Shimadzu microscope model SPM9700) (Barueri, SP, Brazil). Scans in an area of 1 μm were used to obtain the distribution of particle diameters. The results obtained were processed using the software Gwyddion 2.40 (Department of Nanometrology, Czech Metrology Institute, Okružní, Czech Republic), and the particle size distributions were determined using the software UTHSCSA Image Tool (University of Texas Health Science Center, San Antonio, TX, USA) through the random selection of a large number of particles present in the images obtained by AFM; thus, allowing the calculation of the average size of the particles and their standard deviation.

Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) analysis was used to study the structural changes and qualitative characteristics of the different formulations. To record the FTIR spectra, a small amount of sample powder (Ca(OH)_2 , SNCP, and SNCP:10Ti) was deposited in contact with the attenuated total reflectance (ATR) area. The FTIR analysis was performed using an ATR-FTIR Vertex 70/80 (Bruker, Ettlingen, Germany), and the scanning range was set from 370 to 4000 cm^{-1} with a resolution of 4 cm^{-1} and 64 scans. The spectra were then subjected to baseline correction. The FTIR measurements and curve fitting analysis were performed using the OPUS 6.5 software (Bruker).

Preparation of BG solutions

In this study, Ca(OH)_2 powder, SNCP, and SNCP:10Ti were used to prepare aqueous solutions. The SNCP and SNCP:10Ti samples were prepared according to the similar recommendations as for the Ca(OH)_2 solution, and 1 g of the powder was added to 10 mL of distilled water (Accorinte et al. 2005). The solutions prepared were stored in an incubator at 37°C for 24 h. Then, the solution was collected and filtered with sterile filters of 0.22 μm and serially diluted in cell culture medium before testing for a final dilution of 1:16.

pH-measurement

Ca(OH)_2 , SNCP, and SNCP:10Ti solutions were prepared as previously described. The pH was measured using a pH metre (Digimed Analítica Ltda., São Paulo, SP, Brazil), which was previously calibrated, after 24 h and 7 days of immersion ($n = 10$). Distilled water (DW) was used as control.

Culture of hDPCs

Primary cultures of cells from the dental pulp were used to simulate a clinical scenario of pulp exposure in which mesenchymal stem cells were present. Fresh pulp tissue was collected from two healthy third molars with complete root formation immediately after extraction (protocol no. 09016219.1.0000.5152; Federal University of Uberlândia). The teeth were placed in sterile gauze and fractured using a surgical hammer. In a laminar flow hood under aseptic conditions, the pulp tissues were collected and minced into small pieces and digested using 3 mg/mL collagenase type I solution (Sigma-Aldrich, St. Louis, MO, USA) and 4 mg/mL dispase (Sigma-Aldrich) for 3 h at 37°C. hDPCs were subcultured in a complete Dulbecco's modified Eagle's medium (DMEM) (LGC Biotechnology, Cotia, SP, Brazil) supplemented with 10% foetal bovine serum (Gibco, Langley, OK, USA), 1%

penicillin/streptomycin (Sigma-Aldrich), at 37°C and 5% CO₂ in a humidified environment. The culture medium was replaced every 3 days. Cells at passages 4–5 were used for subsequent experiments. All analyses (cell cytotoxicity, cell migration, and immunostaining) were performed in triplicate for each material and in two independent experiments.

Cell cytotoxicity assay

For cytotoxicity analysis, hDPCs were plated in 96-well plates (Costar Corp., Cambridge, MA, USA) (2×10^4 cells/well) and allowed to adhere overnight. After this period, cells were exposed to experimental solutions (Ca(OH)₂, SNCP, and SNCP:10Ti) at different dilutions (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 supernatant was removed and formazan crystals were dissolved in 100 µL of dimethyl sulfoxide solution (LGC Biotechnology). The optical density was measured at 570 nm using a microplate reader (Biochrom, Cambridge, UK). Cell viability was evaluated in proportion to the absorbance and expressed as the percentage of viable cells. The dilution that stabilised the viability values (1:4) was used for the migration test and immunostaining of hDPCs.

Migration test

When hDPCs were 95% confluent, a scratch was made with a 200-µL pipette tip in each well of 24-well plate, and each well was washed thrice with phosphate buffer saline (PBS) to remove debris and the solutions. The medium containing Ca(OH)₂, SNCP, or SNCP:10Ti at 1:4 dilution was added, and digital images of the scratched area were obtained immediately and after 24 h and 48 h (inverted digital microscopy: EVOS digital microscope; Advanced Microscopy Group AMG, Life Technologies, Carlsbad, CA, USA). The control group contained cells maintained in DMEM only. Microscopic images were processed using ImageJ (National Institutes of Health, Bethesda, MD, USA) to calculate the percentage of the healing area in each period relative to the area measured at 0 h in the same well. Migration distances were measured separately during the periods of 0–24 h and 24–48 h, as described by Oliveira et al. 2021.

Cell attachment, cytoskeleton and ALP staining

hDPCs were plated on glass coverslips in 24-well plates in the absence (control group) or presence of BG solutions at a 1:4 dilution (SNCP or SNCP:10Ti) for 7 days. The culture medium was replaced every

2 days. After the exposure period to the materials, hDPCs were fixed in 4% paraformaldehyde for 10 min, washed with PBS, permeabilised with 0.5% Triton X-100 (Sigma-Aldrich) and incubated using 1:100 anti-ALP antibody (Developmental Hybridoma Bank, B4-78), 1:200 Alexa Fluor 488 phalloidin (Sigma-Aldrich), and 1:1000 TO-PRO 3 Iodide (Invitrogen) for 1 h, washed again in PBS and then analysed at $\times 20$ magnification (inverted digital microscopy: EVOS digital microscope). Cell images in five randomly selected areas were acquired and processed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA) to calculate the fluorescence intensity related to ALP expression in each experimental group. The results are qualitatively and quantitatively described.

Statistical analysis

Cell viability, wound healing, and ALP fluorescence data were analysed for normality and homoscedasticity using the Shapiro–Wilk and Levene tests. For cell viability and pH measurements, the two-way analysis of variance (ANOVA) and Tukey’s test were used to compare data between the treated groups. 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 ALP fluorescence. The Dunnett’s test was used to compare experimental and control groups. Statistical analysis was performed using the GraphPad Prism 6 software package (La Jolla, CA, USA). The significance level was set at 95% for all the data analyses.

RESULTS

AFM

Figure 1 presents contrast AFM images and a histogram of the dimensions of the pure BG samples. The tonal homogeneity in the image contrast demonstrated that the composition was equally distributed, corresponding to the SNCP (Figure 1A). Measurements of sample size and thickness (Figure 1B) generated a histogram showing 1 μm samples (Figure 1C).

Figure 2 presents contrasting AFM images and a histogram of the dimensions of SNCP:10Ti. In the contrast analysis of the AFM image of the nanostructured BG, two distinct shades were observed (Figure 2A), confirming that the nanoparticles present in the BG were located at the edges. When performing the measurements of sample size and thickness (Figure 2B), a histogram was generated showing samples of 1

μm nanostructured BG (Figure 2C). Based on the AFM analysis, it was confirmed that the samples have dimensions of 1 μm and that the nanostructured samples have nanoparticles located on the edges of the BG.

FTIR

The functional groups of BG before and after doping with Ti, and Ca(OH)_2 were determined from the FTIR spectra shown in Figure 3. The incorporation of TiO_2 into the SNCP had no remarkable effect on the chemical integrity of these samples, as all of them possessed several functional groups related to the BG base. In particular, a band corresponding to the Si-O-Si bending vibration mode at 498 cm^{-1} and the Si-O-Si symmetric stretching vibration of the bridging oxygen between the tetrahedral, which was observed at 780 cm^{-1} , were detected (Li et al. 2017). These two bands confirm the presence of SiO_2 , the main component of the glass system, as a network former. Moreover, the presence of phosphate groups was indicated by the band corresponding to the P-O stretching mode in the $1043\text{--}1024\text{ cm}^{-1}$ region (Wang et al. 2011). The presence of Ti within the glass network was confirmed by the Ti-O vibration band at 635 cm^{-1} (Mabrouck et al. 2021). According to the spectra of the Ca(OH)_2 , a thin 3642 cm^{-1} band was attributed to the vibration of the free hydroxyl groups. The bands at 1515 , 877 , and 856 cm^{-1} were attributed to the C-O vibration, carbonate bond. The band at 435 cm^{-1} is attributed to the vibration of the stretch Ca-O (Darroudi et al., 2016).

pH-measurement

The pH values of the SNCP, SNCP:10Ti, and Ca(OH)_2 solutions are shown in Figure 4. All tested solutions showed an alkaline pH. Ca(OH)_2 presented the highest pH value (12.16), followed by SNCP (11.7) and SNCP:10Ti (10.4), in the two evaluated periods ($p = .001$). The pH values remained stable over time in all the groups ($p > .05$).

Cell cytotoxicity assay

Cell viability by the MTT formazan method of the SNCP, SNCP:10Ti, and Ca(OH)_2 groups at different extract dilutions for 24 h is shown in Figure 5. All materials showed high cytotoxicity at a 1:1 dilution ($p < .05$). The evaluated materials were similar to each other at 1:4, 1:8, and 1:16 ($p > .05$), and similar to the control at 1:8 and 1:16 ($p > .05$), except for SNCP:10Ti, which presented lower values at the 1:8 dilution ($p < .05$).

Migration test

No significant differences were found between any of the materials and the negative control (basal growth medium) at 24 h and 48 h ($p > .05$) (Figure 6A-B). At all time-points, the cell migration rates in the $\text{Ca}(\text{OH})_2$, SNCP, and SNCP:10Ti groups were similar to each other and to those in the control group ($p > .05$).

Cell attachment, cytoskeleton and ALP staining

hDPCs were well-adhered and oriented on glass coverslips, exhibiting positive phalloidin staining with fibroblast morphology (green). ALP staining in hDPCs was higher in the presence of the test materials than in the basic growth medium (control) on day 7. For all materials, ALP labelling was mainly observed throughout the cytoplasm, whereas extracellular labelling was barely observed (red) (Figure 7A). The staining intensity for ALP was similar in all experimental groups ($p > .05$). The control group presented lower fluorescence intensity than the experimental groups ($p < .05$) (Figure 7B).

DISCUSSION

BGs have been studied in dentistry, particularly as endodontic repair materials (Chitra et al. 2022, Ribeiro et al. 2023). Several methods are currently available for the synthesis of BGs. Although currently the sol-gel method and solid-state reaction have been widely cited for this purpose (Omidian et al. 2022, Ghițulică et al. 2021), the melt quenching and subsequent heat treatment technique can be considered as the most common way of synthesising glass-ceramics (Kaur et al. 2015, Filip et al. 2022). The effect of surface modification on short-range structural changes in the BGs was successfully investigated using FTIR. The spectral changes observed for SNCP in comparison with SNCP:Ti can be explained in terms of the change in the nature of the network modifier cation, which in turn influences the chemical environment of the nonbridging oxygen (Lopes et al. 2017). Additionally, the effect of Ti doping on the glass surface morphology was investigated by AFM, which confirmed that the smoothness of the glass surface was diminished, and the surface became less homogeneous after surface modification. Thus, the characterisation results demonstrate that the traditional melting synthesis methodology produces pure, homogeneous, and uncontaminated BGs.

The pH analysis of the materials after 7 days confirmed that all materials had an alkaline pH, although the pH of the SNCP solutions was lower than that of the $\text{Ca}(\text{OH})_2$ solution, with the lowest values

for SNCP:10Ti. A previous study demonstrated that the pH generated in an aqueous environment by BGs 28S5 and 45S5 was similar to that of calcium hydroxide (Gubler et al. 2008). However, in this study the materials were tested by immersion of pressed samples into simulated body fluid. Another study demonstrated slightly lower pH values for Ti-doped BG than for pure BG (Asif et al. 2014), corroborating the present results.

In this study, the null hypothesis was partially rejected as significant differences were found between the studied materials and the control group only in the cytotoxicity assay. Considering the possible use of aqueous BG solutions in the VPT, hDPCs were selected as the cellular models for evaluation in this preliminary study. The proportion of biomaterials to be incorporated into the DW was determined according to a previous study that evaluated an aqueous solution of calcium hydroxide (Accorinte et al. 2005). To the best of our knowledge, there are no laboratory or in vivo studies that use aqueous solutions of BG as a possible irrigation solution for VPT. However, according to the ISO 10993-5 guideline (ISO/EN10993-5 2009), the evaluation of biomaterials in cell culture must be performed using different dilutions because in vitro models do not guarantee blood flow and refreshment or exchange of the material. Despite these limitations, this is a well-established method in the study of biomaterials (Souza et al. 2020, Oliveira et al. 2021), which considers a threshold of 70% cell viability to be cytotoxic. Both evaluated SNCPs solutions presented higher percentages of viable cells than those recommended from the 1:2 dilution, differing from the Ca(OH)_2 solution, which was initially more cytotoxic. Other studies demonstrated a reduction in cell viability upon contact with Ca(OH)_2 (Souza et al. 2020, Manaspon et al. 2021). However, comparisons between these studies should be performed with caution, as in the aforementioned studies, the Ca(OH)_2 used was nanoparticulated (Souza et al. 2020) and in the form of cement (Manaspon et al. 2021). A possible hypothesis for the cytotoxicity observed in this group is that cells in contact with Ca(OH)_2 experience an overload of extracellular Ca^{2+} which may increase their intracellular concentration (Berridge et al. 2003) interfering with cell physiology and cell death pathways (Smaili et al. 2013, Orrenius et al. 2015, Naumova & Sachl 2020). However, in the present study, the lack of quantitative analysis of the release of calcium ions in the evaluated bioglass solutions can be considered a limitation.

Previous studies evaluating the effect of BG on the cytotoxicity of hDPCs (Spagnuolo et al. 2021) have demonstrated no negative cytotoxic effects of BG on these cells. However, it is important to emphasize that the size and shape of the biomaterials are essential features that may affect their toxicity (Verma & Stellacci 2010, Kladko et al. 2021). However, in the present study, no significant differences were found in

the viability of SNCP and SNCP:10Ti, which is probably related to the high biocompatibility of titanium (Kumarage et al. 2023). Previous studies have demonstrated that hDPCs in contact with TiO₂ nanoparticles for 24 h show more than 70% viability; therefore, they are considered biocompatible (Tabari et al. 2017).

In addition to low toxicity, it is desirable that the materials used for VPT do not negatively affect cell migration, adhesion and differentiation (Rodriguez-Lozano et al. 2021). The *in vitro* scratch assay is still often the method of choice for analysing cell migration in the laboratory because it does not require any specialised equipment and is a straightforward and economical method (Liang et al. 2007). Although previous studies have demonstrated that Ca(OH)₂ (Chen et al. 2016) and BGs (Kermani et al. 2020, Wang et al. 2020) can increase cell migration, this was not observed in the present study, indicating an optimal biological response to these dilutions. In this study, both SNCPs and Ca(OH)₂ were evaluated in the form of diluted extracts, specifically for migration and immunostaining, at a dilution of 1:4. This dilution was chosen because it resulted in a viability greater than 70%. The cell migration results correlated with the cell attachment and spreading observed in the immunostaining results, as cells require adequate attachment and spreading for subsequent migration.

One of the main goals of VPT is to stimulate the pulp to initiate reparative tertiary dentin formation. ALP immunostaining and migration assay were performed in this study as complementary methods to cell viability assay. ALP activity is considered a marker of osteoblast differentiation, and its assessment allows the evaluation of the biological activity of cells towards a mineralising phenotype (Macri-Pellizzeri et al. 2018). As ALP is secreted in the early stages of differentiation, ALP activity is generally assessed at relatively early phases *in vitro* (Arora et al. 2022). In the present study, the expression of ALP was evaluated after 7 days, and it was observed that the evaluated materials were able to induce the expression of this enzyme in a similar way (Gungor et al. 2023, Hyvari et al. 2023)

CONCLUSION

Within the limitations of the present study, we conclude that the melt-quenching method successfully produced both pure and Ti-doped BGs. Both tested BGs have the potential for use in VPT, considering their similar results to Ca(OH)₂ in terms of cell migration and alkaline phosphatase release. However, doping with Ti seems to reduce the cytotoxic potential of SNCP BGs in pulp cells.

DATA AVAILABILITY

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

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COMPETING INTERESTS

The authors have no financial or proprietary interests in any material discussed in this article.

FIGURE LEGENDS

Fig.1 Atomic force microscopy (AFM). A and B: SNCP contrast AFM images. C: Histogram generated from the AFM image in Figure 1B.

Fig. 2 Atomic force microscopy (AFM). A and B: SNCP:10Ti contrast AFM images. C: Histogram generated from the AFM image in Figure 2B.

Fig. 3 SNCP and SNCP:10Ti chemical characterization. The chemical composition was assessed by the Fourier transform infrared spectra, where the Ti-O vibration band recorded at 635 cm^{-1} is assigned to the presence of Ti in the glass structure.

Fig. 4 pH values after 24 h and 7 days of immersion of $\text{Ca}(\text{OH})_2$, SNCP, and SNCP:10Ti in distilled water.

Ca(OH)₂: calcium hydroxide solution; SNCP: SiO₂–Na₂O–CaO–P₂O₅ solution; SNCP:10Ti: wt 10% Ti-doped SNCP; DW: distilled water. Capital letters indicate comparison amongst materials. Lowercase letters indicate comparisons between evaluation times. Two-way analysis of variance and Tukey's test ($p < .05$).

Fig. 5 Cell viability percentage of human dental pulp cells after exposure to experimental solutions (Ca(OH)₂, SNCP, and SNCP:10Ti) at different dilutions (1:1 to 1:16), by the MTT formazan method. Capital letters indicate comparison amongst different dilutions of extracts and the control group for each material. Lowercase letters indicate comparison between different materials at the same dilution. Two-way analysis of variance and Tukey's test ($p < .05$).

Fig. 6 Migration assay. (A) Images of the scratch healing of all groups acquired at 0, 24, and 48 h with an EVOS digital microscope at magnification of 10×. (B) The closure of the space created in the scratch assay after the contact of the solutions at a 1:16 dilution with the human dental pulp cells after 24 and 48 h is represented by a bar graphic after the statistical analysis. Capital letters indicate comparison between time for each material. Lowercase letters indicate comparison between materials in the same period. Two-way repeated measure analysis of variance and Tukey's test ($p < .05$)

Fig. 7 Cell attachment, cytoskeleton and ALP staining. A) Representative images of staining for alkaline phosphatase by human dental pulp cells treated with Ca(OH)₂, SNCP, and SNCP:10Ti. (B) Fluorescence intensity related to ALP expression for SNCP, SNCP:Ti, and Ca(OH)₂. Capital letters indicate comparison amongst materials. One-way analysis of variance and Dunnett's test ($p < .05$).

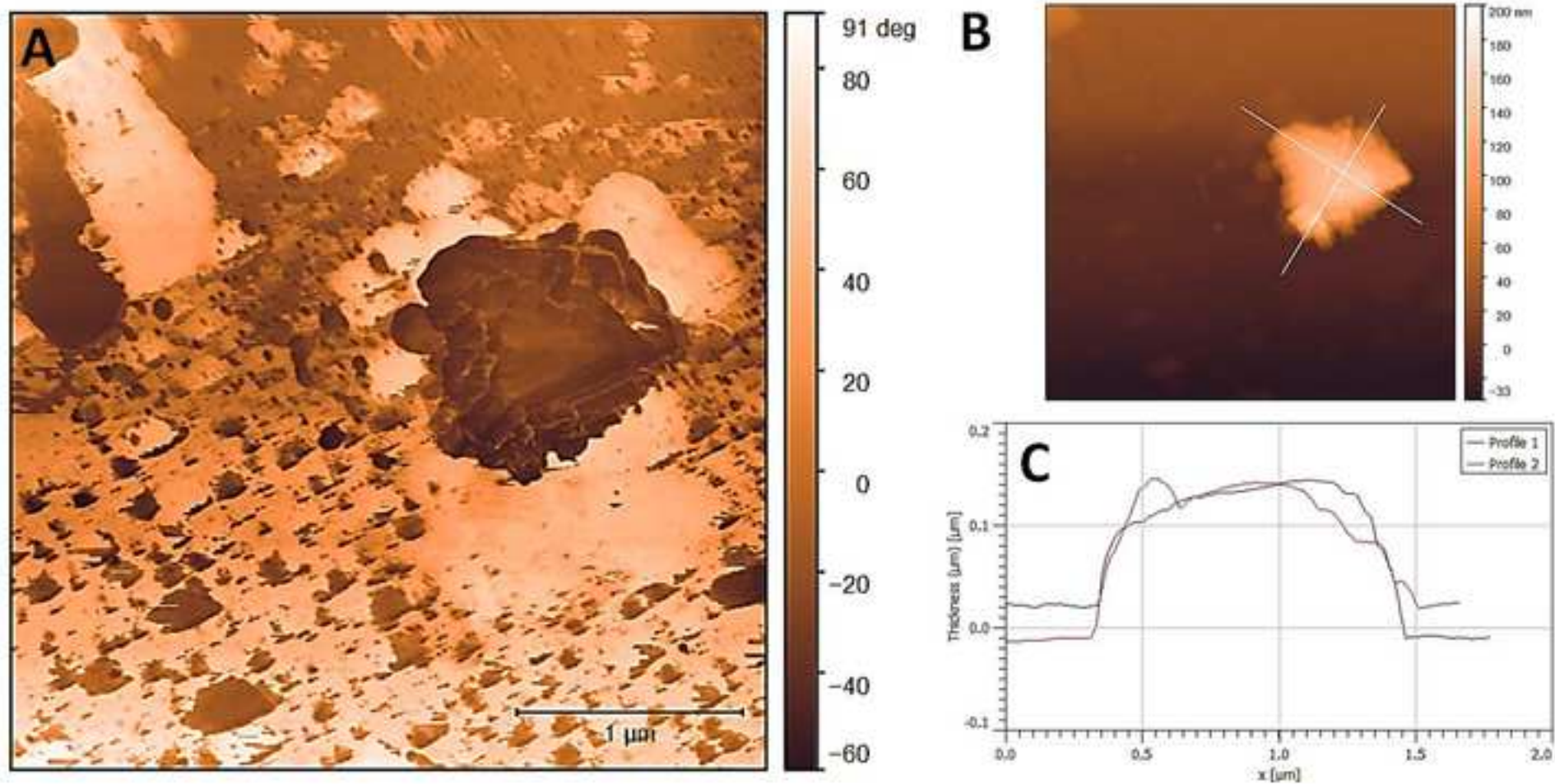
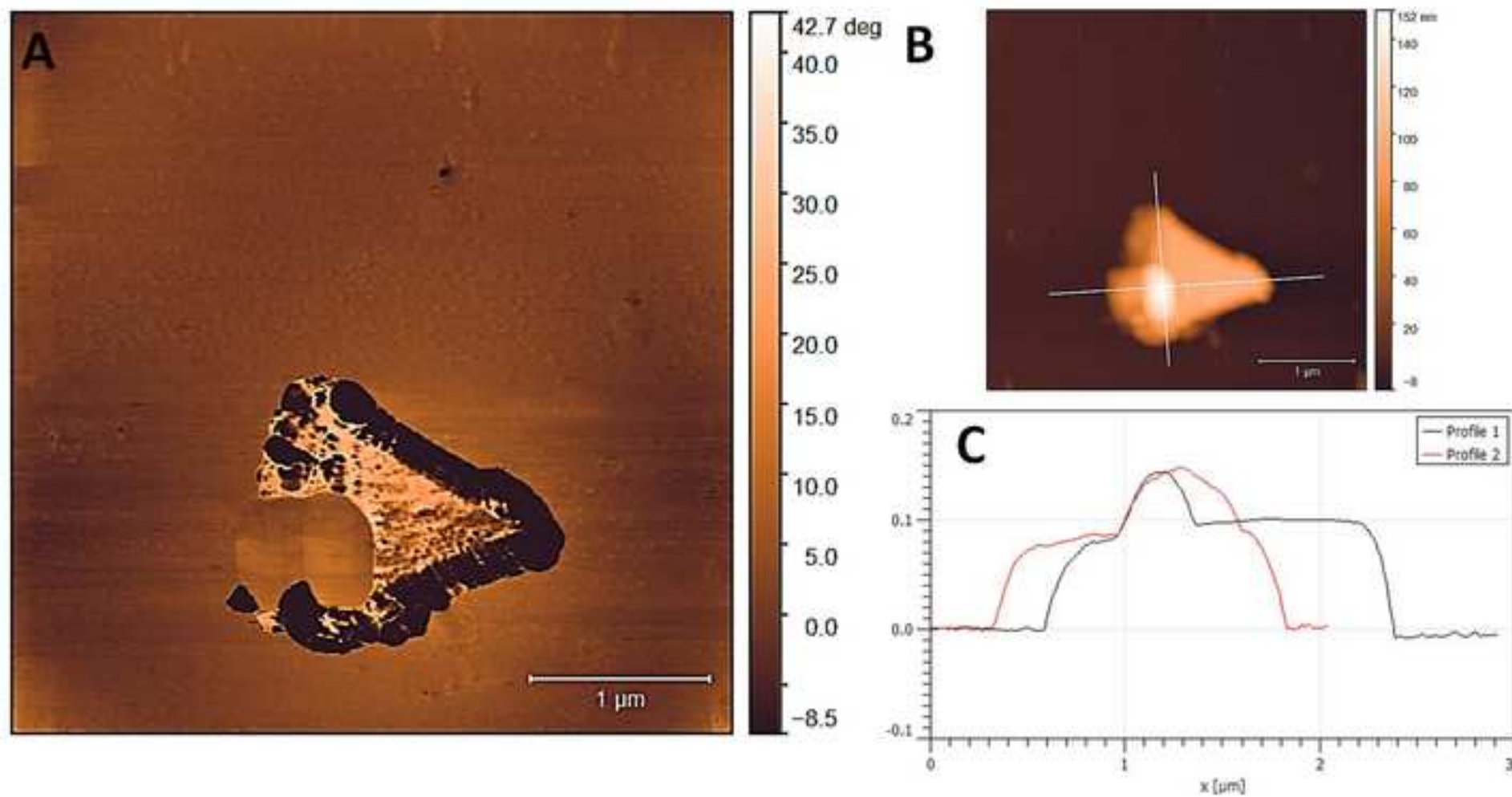
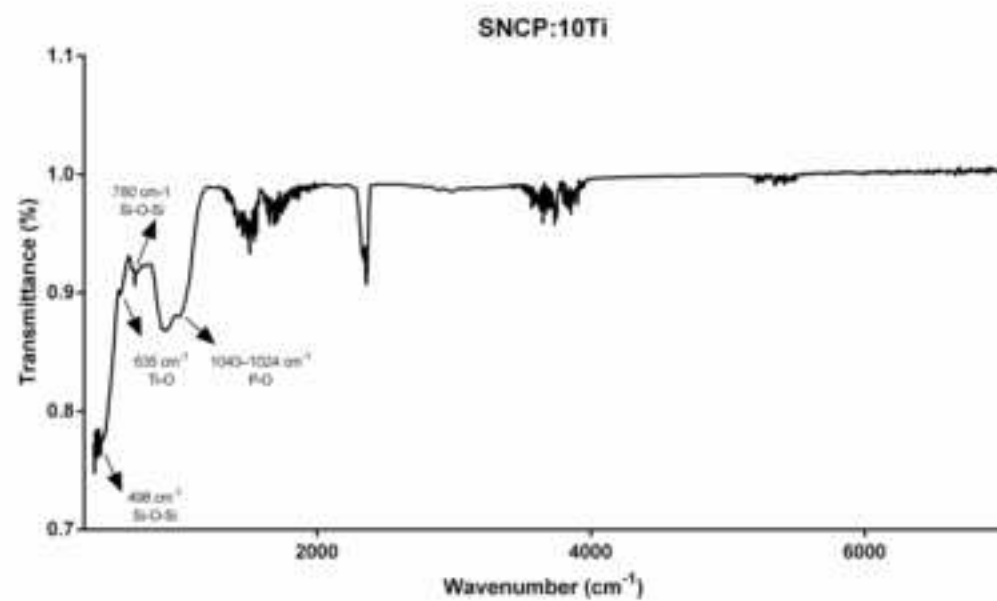
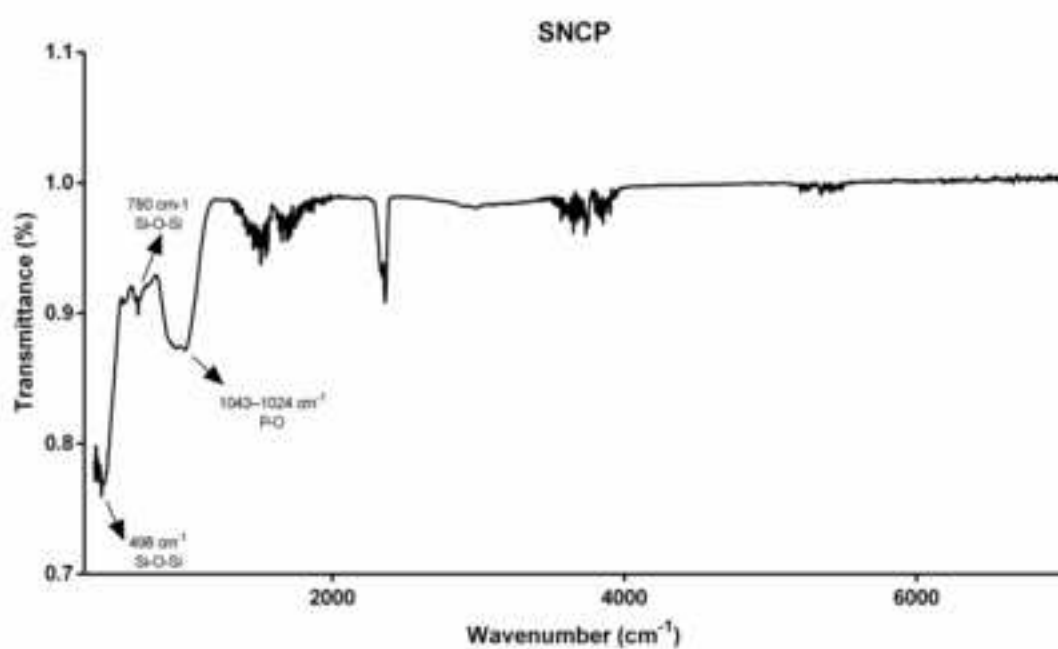
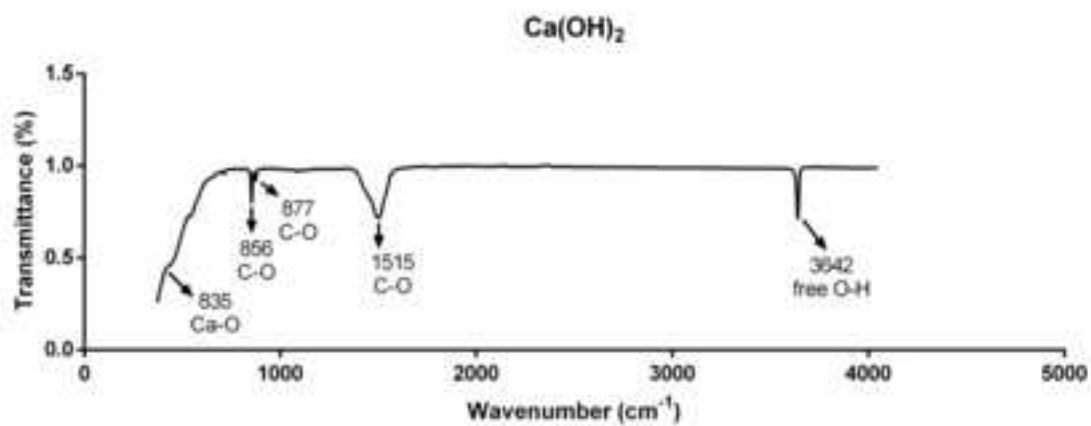


Figure 2

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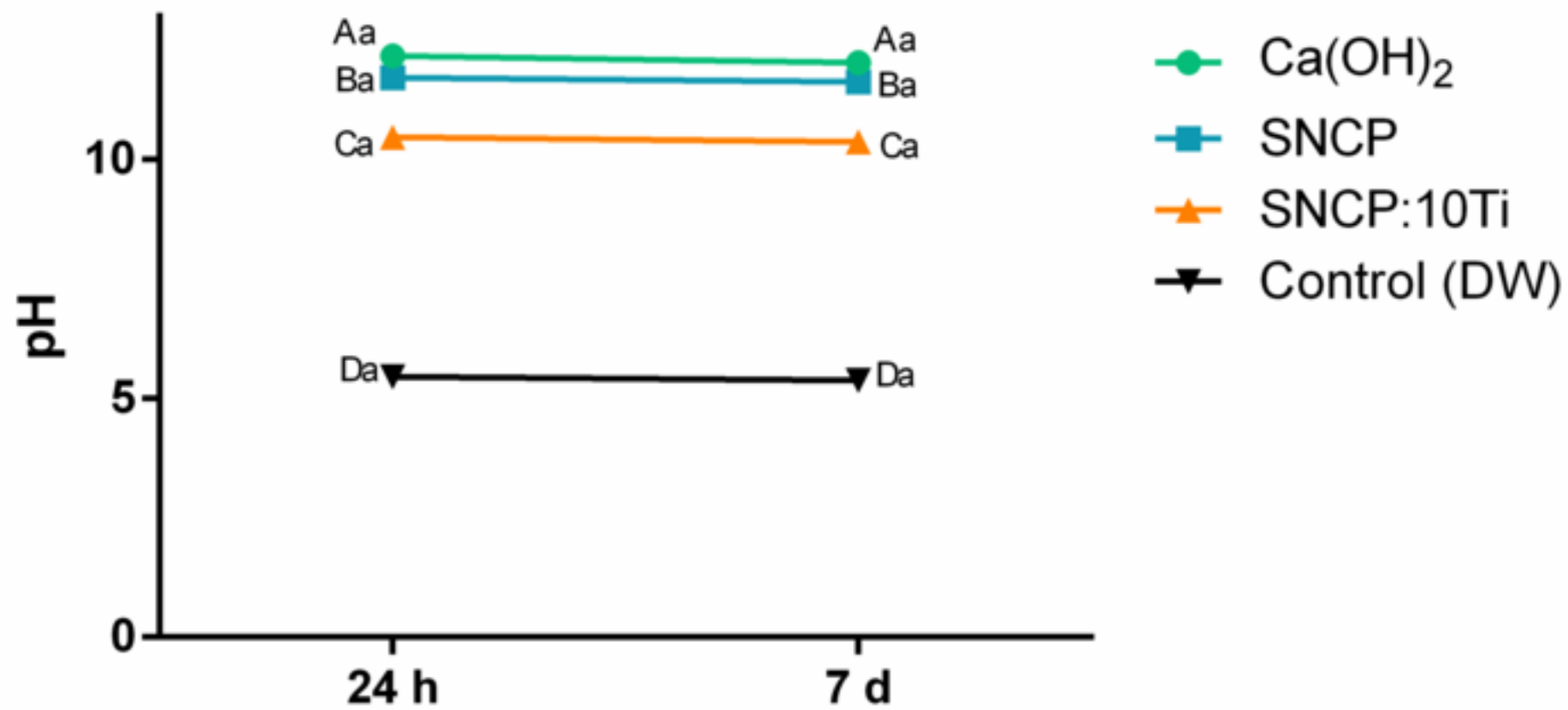
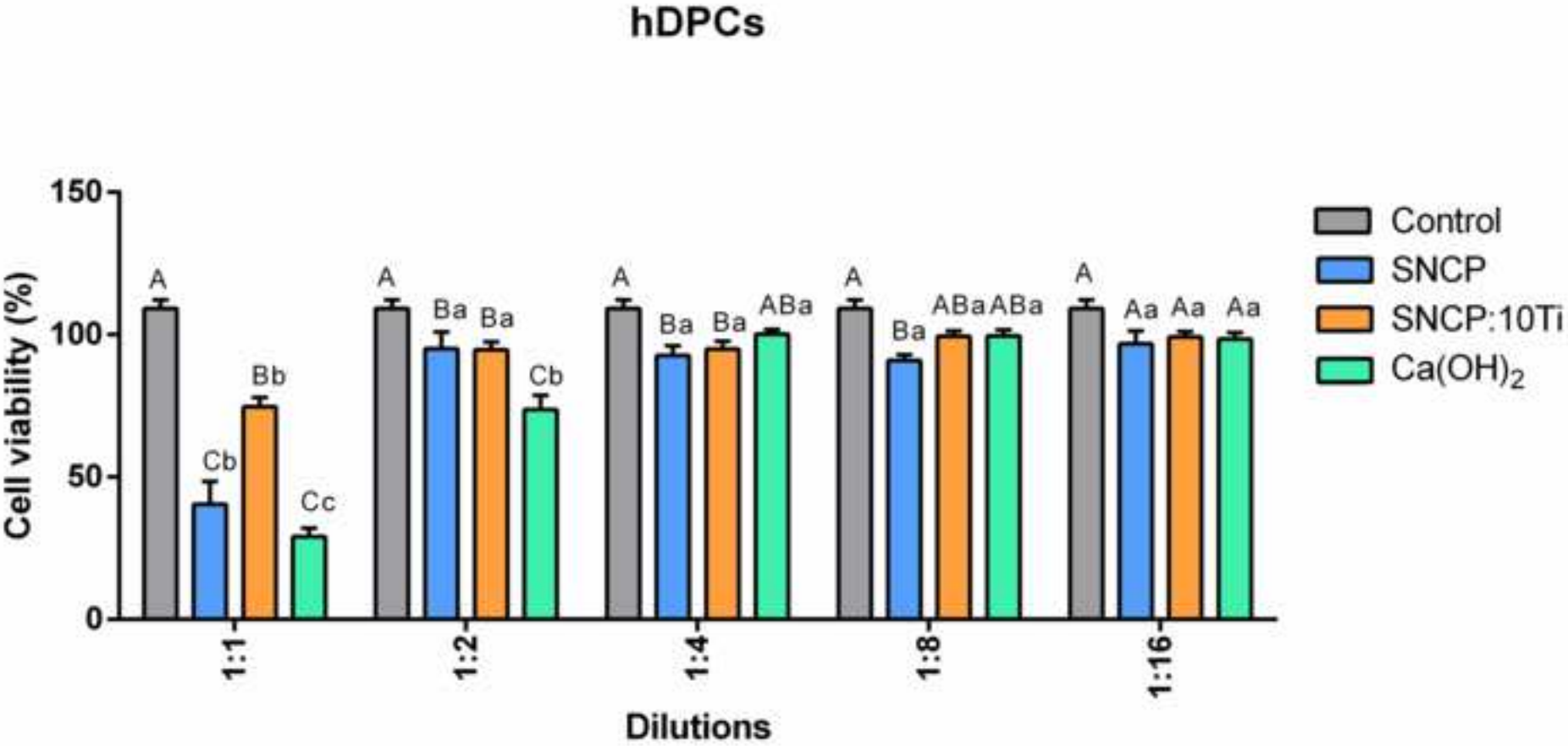
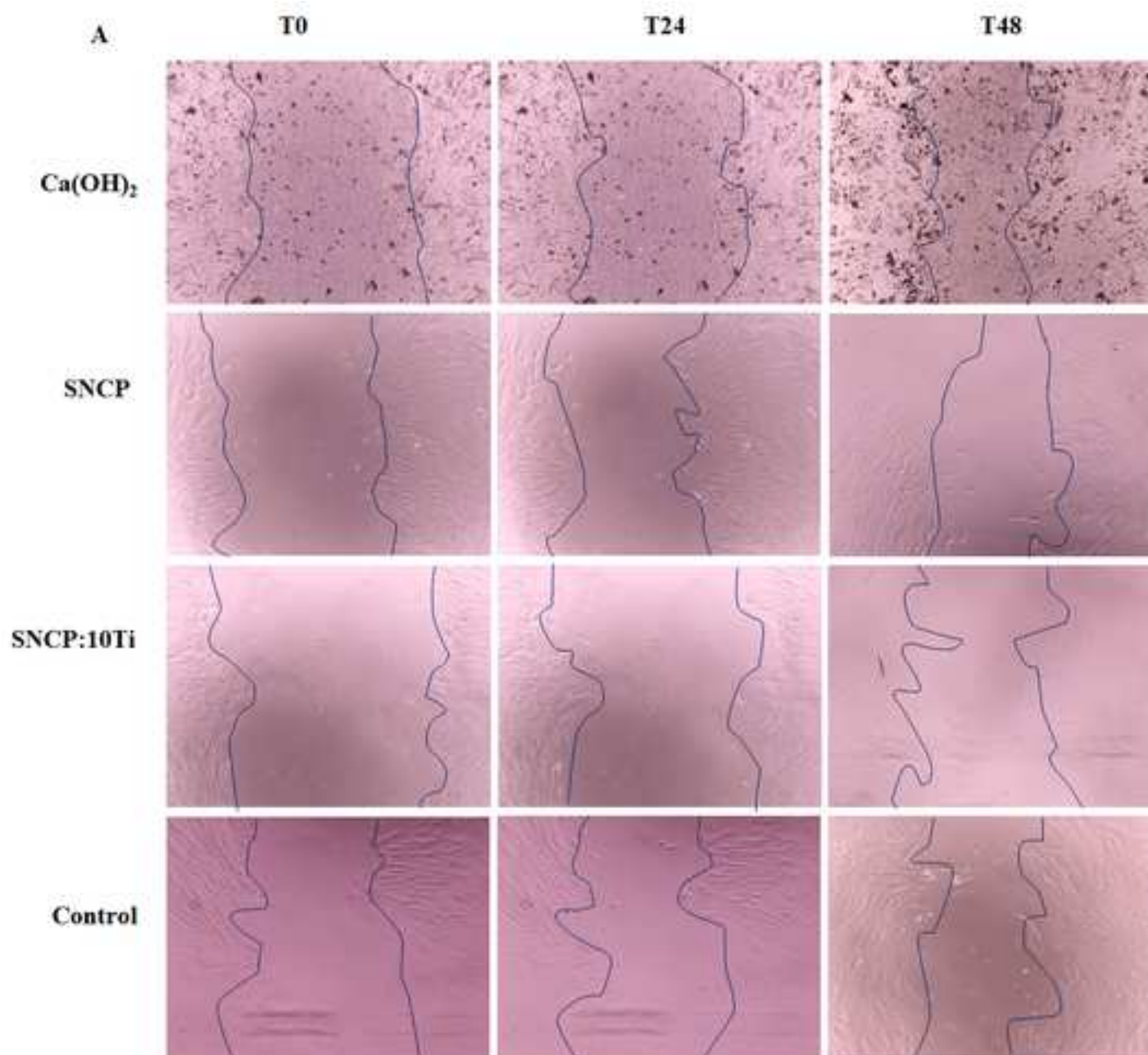


Figure 5





B

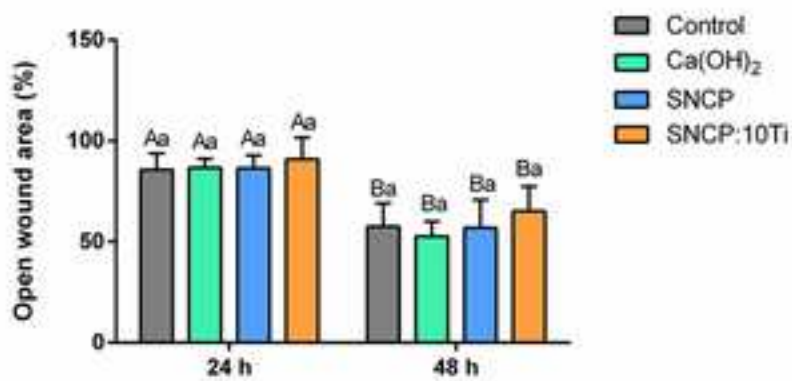
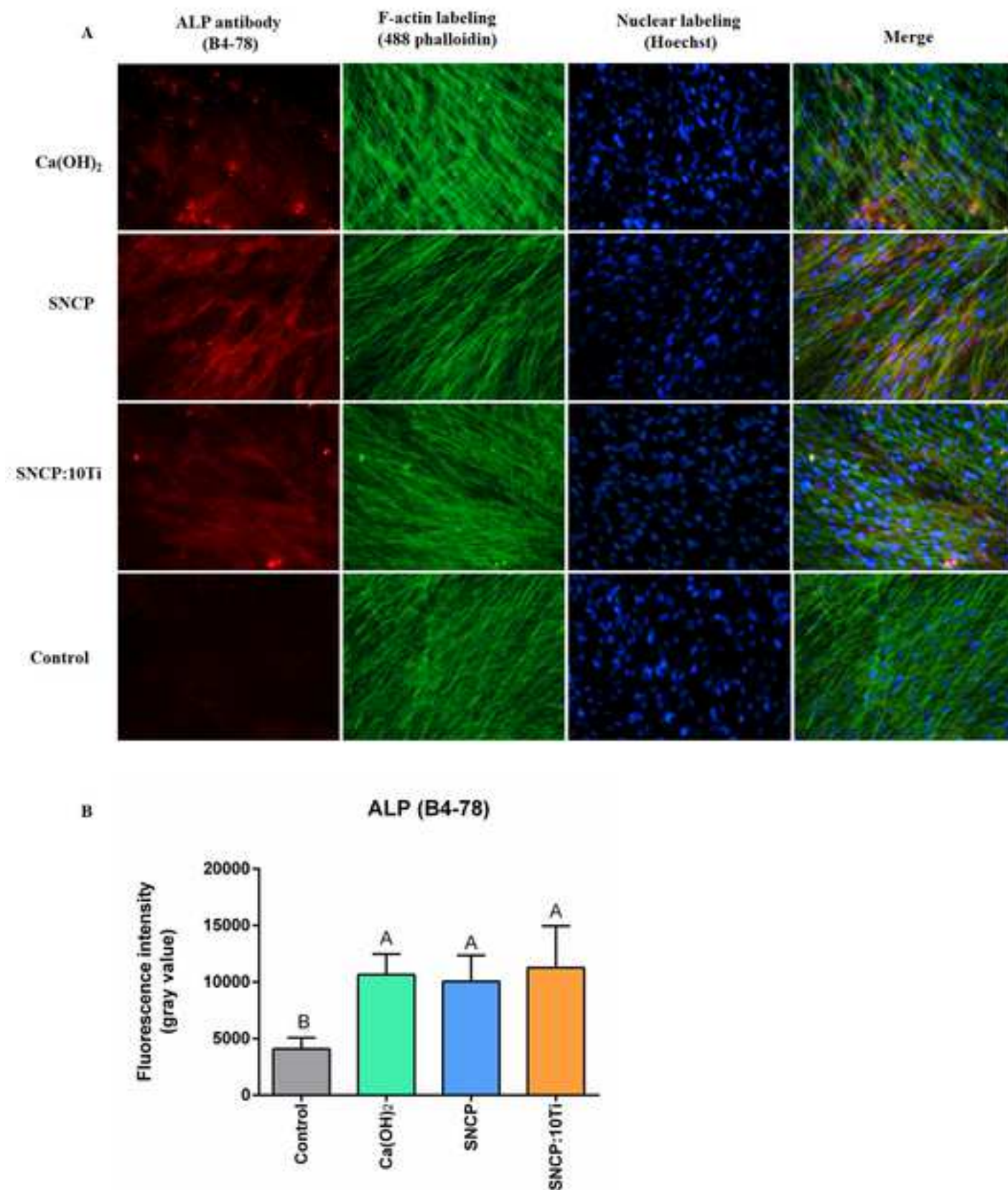


Figure 7

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CAPÍTULO 2

Calcium-doped zinc oxide nanocrystals as an innovative intracanal medicament: a pilot study.

Research Article



Calcium-doped zinc oxide nanocrystals as an innovative intracanal medicament: a pilot study

Gabriela Leite de Souza ,¹ Thamara Eduarda Alves Magalhães ,²
Gabrielle Alves Nunes Freitas ,² Nelly Xiomara Alvarado Lemus ,¹
Gabriella Lopes de Rezende Barbosa ,³ Anielle Christine Almeida Silva ,⁴
Camilla Christian Gomes Moura *

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

²School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil

³Department of Oral Diagnosis, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil

⁴Functional and New Nanostructured Materials Laboratory, Physics Institute, Federal University of Alagoas, Maceió, AL, Brazil



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de Souza GL, Magalhães TEA, Freitas GAN,
Lemus NXA, Barbosa GLR, Silva ACA, Moura
CCG

*Correspondence to

Camilla Christian Gomes Moura, PhD

Professor, Department of Endodontics, School
of Dentistry, Federal University of Uberlândia.
Av Pará 1720, Campus Umuarama, Block 4L-A,
Uberlândia, MG 38405-328, Brazil.
Email: camillahistologia@yahoo.com.br

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Conflict of Interest

No potential conflict of interest relevant to this
article was reported.

ABSTRACT

Objectives: This study investigated the cytotoxicity, radiopacity, pH, and dentinal tubule penetration of a paste of 1.0% calcium-doped zinc oxide nanocrystals (ZnO:1.0Ca) combined with propylene glycol (PRG) or polyethylene glycol and propylene glycol (PEG-PRG).

Materials and Methods: The pastes were prepared by mixing calcium hydroxide [Ca(OH)₂] or ZnO:1.0Ca with PRG or a PEG-PRG mixture. The pH was evaluated after 24 and 96 hours of storage in deionized water. Digital radiographs were acquired for radiopacity analysis and bubble counting of each material. The materials were labeled with 0.1% fluorescein and applied to root canals, and images of their dentinal tubule penetration were obtained using confocal laser scanning microscopy. RAW264.7 macrophages were placed in different dilutions of culture media previously exposed to the materials for 24 and 96 hours and tested for cell viability using the MTT assay. Analysis of variance and the Tukey test ($\alpha = 0.05$) were performed.

Results: ZnO:1.0Ca materials showed lower viability at 1:1 and 1:2 dilutions than Ca(OH)₂ materials ($p < 0.0001$). Ca(OH)₂ had higher pH values than ZnO:1.0Ca at 24 and 96 hours, regardless of the vehicle ($p < 0.05$). ZnO:1.0Ca pastes showed higher radiopacity than Ca(OH)₂ pastes ($p < 0.01$). No between-material differences were found in bubble counting ($p = 0.0902$). The ZnO:1.0Ca pastes had a greater penetration depth than Ca(OH)₂ in the apical third ($p < 0.0001$).

Conclusions: ZnO:1.0Ca medicaments presented higher penetrability, cell viability, and radiopacity than Ca(OH)₂. Higher values of cell viability and pH were present in Ca(OH)₂ than in ZnO:1.0Ca.

Keywords: Cell survival; Calcium hydroxide; Nanoparticles; Physicochemical properties; Zinc oxide

Author Contributions

Conceptualization: Souza GL, Moura CCG, de Rezende Barbosa GL, Silva ACA. Data curation: Souza GL, de Rezende Barbosa GL. Formal analysis: Souza GL, Moura CCG, de Rezende Barbosa GL, Silva ACA. Funding acquisition: Moura CCG, Silva ACA. Investigation: Souza GL, Magalhães TEA, Freitas GAN, Lemus NXA. Methodology: Souza GL, Magalhães TEA, Freitas GAN, Lemus NXA. Project administration: Moura CCG. Resources: Moura CCG, de Rezende Barbosa GL, Silva ACA. Software: Souza GL, Magalhães TEA, Freitas GAN, Lemus NXA, de Rezende Barbosa GL. Supervision: Moura CCG. Validation: Souza GL, Moura CCG, de Rezende Barbosa GL. Visualization: Souza GL, Moura CCG. Writing - original draft: Souza GL, Magalhães TEA, Freitas GAN, Lemus NXA, de Rezende Barbosa GL, Silva ACA, Moura CCG. Writing - review & editing: Souza GL, Moura CCG.

ORCID iDs

Gabriela Leite de Souza 
<https://orcid.org/0000-0003-3505-5258>
Thamara Eduarda Alves Magalhães 
<https://orcid.org/0000-0001-5992-5073>
Gabrielle Alves Nunes Freitas 
<https://orcid.org/0000-0001-7865-2795>
Nelly Xiomara Alvarado Lemus 
<https://orcid.org/0000-0002-9658-2433>
Gabriella Lopes de Rezende Barbosa 
<https://orcid.org/0000-0002-5305-7671>
Anielle Christine Almeida Silva 
<https://orcid.org/0000-0003-1959-2870>
Camilla Christian Gomes Moura 
<https://orcid.org/0000-0002-5653-8403>

INTRODUCTION

The vast majority of dental trauma in permanent teeth has been reported to occur in children and adolescents [1]. The combination of 2 different types of traumatic injuries on the same tooth—luxation and a concomitant crown fracture injury—may significantly increase the risk of pulp necrosis and infection, compromising root development in immature teeth [1,2]. Among the more recent treatment modalities proposed for necrotic immature permanent teeth are apical plugs using mineral trioxide aggregate (MTA) and revascularization [3]. However, these treatment modalities require a substantial learning curve and are not performed by most dental surgeons, who continue to perform conventional endodontic therapies with multiple visits and routine use of pastes containing calcium hydroxide $[\text{Ca}(\text{OH})_2]$ for apexification [4]. Additionally, the European Society of Endodontology recommends that in cases of external infection-related resorption, the duration of $\text{Ca}(\text{OH})_2$ paste application should be between 4 weeks and several months [3]. In such situations, the use of $\text{Ca}(\text{OH})_2$ pastes for inter-visit dressings is justified by their antimicrobial properties and by their ability to stimulate hard tissue formation [4].

Some properties of $\text{Ca}(\text{OH})_2$ pastes have been related to the availability of hydroxyl ions, which in turn is associated with the type of vehicle used. Although aqueous vehicles such as water and saline are commonly used, other nonaqueous preparations, such as polyethylene glycol (PEG) and propylene glycol (PRG), have also been recommended due to their ability to create a reservoir for prolonged release of hydroxyl ions [4,5]. Another advantage of PEG and PRG is that both increase the viscosity of the medication, act as thickeners, and improve the consistency of the product, which makes it easier to insert into the root canals [4,6]. Regardless of the vehicle used, one of the challenges for apexification using $\text{Ca}(\text{OH})_2$ pastes involves the need for periodic changes of the intracanal dressing. To solve this limitation, other long-term medications have been proposed, such as a combination of $\text{Ca}(\text{OH})_2$, zinc oxide (ZnO), and chlorhexidine [7-9]. The formulation of this paste is based on a proportional mixture, in which $\text{Ca}(\text{OH})_2$ is dissolved in a few weeks and ZnO acts as an inert sealing material [7].

Previously, the pH and antimicrobial activity of $\text{Ca}(\text{OH})_2$ paste combined with nanoparticulate ZnO were evaluated [10]. Instead of mixing substances, such as $\text{Ca}(\text{OH})_2$ and ZnO, doping techniques have been used to modify the composition of materials by adding dopants to improve their functionality, resulting in synergistic effects. A previous study synthesized calcium-doped zinc oxide (ZnO:Ca) nanocrystals with different percentages of calcium ion (Ca^{2+}) by weight, evaluating their chemistry, physical properties, and cytotoxicity [11,12]. That investigation indicated the potential applicability in dentistry of 1.0% Ca^{2+} -doped ZnO (ZnO:1.0Ca) nanocrystals. However, to enable the use of ZnO:1.0Ca as a temporary medication for traumatized teeth, it is necessary to combine it with vehicles already established in endodontic practice, to achieve an adequate consistency for root canal insertion.

Therefore, the present study examined selected chemical-physical properties of materials with potential clinical implications (radiopacity, pH, dentinal tubule penetration) and the cytotoxicity of a paste of ZnO:1.0Ca combined with PRG or PEG-PRG on a leukemia macrophage murine cell line (RAW 264.7). The null hypothesis tested was that all pastes would present similarities in the evaluated parameters.

MATERIALS AND METHODS

Materials

The pastes were prepared in a laminar flow chamber with 0.28 g of $\text{Ca}(\text{OH})_2$ nanocrystals or 0.28 g of ZnO:1.0Ca with 240 μL of PRG or 240 μL of the vehicle mixture (120 μL PEG + 120 μL PRG). This proportion was previously tested to obtain a consistency similar to Coltosol (Coltene-Vigodent S/A Indústria e Comércio, Rio de Janeiro, RJ, Brazil), facilitating its clinical use. The pastes were used for experiments using teeth as the experimental unit and for material characterization.

Cell viability assay

RAW 264.7 cells (Cell Bank of Rio de Janeiro, Rio de Janeiro, RJ, Brazil) were plated at a density of 2×10^4 cells/well in 96-well plates (Corning, New York, NY, USA) containing Dulbecco's modified Eagle's medium (DMEM) (Vitrocell Embriolife, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (Vitrocell) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were incubated at 37°C in 5% CO_2 and 95% humidity for 24 hours to allow them to adhere to the plates.

The freshly prepared pastes were inserted into a sterile cylindrical polyethylene tube (diameter: 5 mm; height: 2 mm), kept in an incubator at 37°C for 24 hours, and sterilized by ultraviolet irradiation for 1 hour. Subsequently, the pastes were transferred to a 24-well culture plate and covered with 2.5 mL of DMEM (Vitrocell) for 24 hours at 37°C. The undiluted extraction media were filtered with a 0.22 μm filter (Kasvi, São José dos Pinhais, PR, Brazil) and used to prepare serially diluted extracts until a dilution of 1:16. The culture medium was then removed, and the cells were incubated with 200 μL of the extracts at predetermined dilutions (1:1, 1:2, 1:4, 1:8, and 1:16). The positive control group contained cells maintained in 10% DMEM. Three independent experiments were performed in triplicate.

Cell viability analysis was performed with incubation periods of 24 hours and 96 hours using the MTT method as previously described [12]. After exposure to each conditioned medium, 1 mg/mL MTT solution (Sigma-Aldrich) was added and incubated for an additional 4 hours. To solubilize the formazan crystals, the supernatant was removed, 10% dimethyl sulfoxide solution (LGC Biotecnologia, Cotia, SP, Brazil) was added to each well, and the plates were incubated at 37°C for 10 minutes. The optical densities were measured in a 570-nm wavelength filter (Biochrom, Cambridge, UK). The absorbance readings were normalized in proportion to the absorbance of the positive control, which represented succinate dehydrogenase activity (cell metabolism).

pH

To determine the pH, polyethylene tubes that were 10 mm long with a 1.6 mm internal diameter (Embramed, Sao Paulo, SP, Brazil) were filled with each material ($n = 5$). To insert the materials inside the tubes, a #25 K-file (Dentsply Maillefer, Ballaigues, Switzerland) was used, followed by condensation with a 1-2 plugger (Odous de Deus, Belo Horizonte, MG, Brazil) under $\times 3.0$ magnification (D.F. Vasconcellos, Valença, RJ, Brazil) to confirm complete filling. Next, the samples were immersed in flasks containing 10 mL of deionized water (DW) and stored at 37°C. DW was used as a control. The pH of the solutions was measured using a previously calibrated digital pH meter (Digimed Analítica Ltda., Sao Paulo, Brazil) after 24 hours and 96 hours of immersion. The mean pH values of each group were calculated after each measurement in triplicate.

Radiopacity and bubbles

Using a silicone mold, 4 specimens (ZnO:1.0Ca+PRG ; ZnO:1.0Ca+PEG-PRG ; $\text{Ca(OH)}_2\text{+PRG}$; $\text{Ca(OH)}_2\text{+PEG-PRG}$), 6 mm in thickness and 5.4 mm in diameter, were prepared from each material. All specimens were radiographed once on the same day of preparation. A VistaScan Mini Plus® photostimulable phosphor (PSP) system (Dürr Dental, Bietigheim-Bissingen, Germany) was used, and the samples were imaged by a size 2 (3×4 cm) PSP plate using the settings of 70 kV, 7.0 mA, 0.14 seconds exposure time, and a focus-film distance of 30 cm (Timex 70E X-ray unit, Gnatus, Ribeirão Preto, SP). Subsequently, PSP plate scanning was performed, and the images were exported to ImageJ software (National Institutes of Health, Bethesda, MD, USA) for radiopacity analysis and bubble counting.

To determine the gray values, 3 points were randomly selected on each image in the region corresponding to the restorative material: one positioned to the right, one centrally, and one to the left. The gray value of each point was determined by the software, and it ranged from 0 to 255, as the radiographs were 8-bit images. The value 0 corresponded to black and 255 indicated white, with 254 different shades of gray between them, representing the variations between the most radiolucent to the most radiopaque tone.

The same radiographic images were used to count the number of bubbles on each material. For this analysis, a round region of interest of 250 pixels was selected, encompassing all of the specimen areas. Subsequently, the image was duplicated and made binary. A sequence of imaging tools was applied to remove outliers and perform despeckling. The resultant image was eroded and dilated once to reduce noise. To obtain a more homogeneous image and perform a proper bubble count, a closing tool was applied. The image was inverted, and a software tool to analyze particles was used to determine the number of bubbles.

Dentinal tubule penetration

Twenty bovine central incisors (30-38 months) with similar dimensions and pulp spaces were selected according to Oliveira *et al.* [13] and stored in distilled water at 4°C until use. All teeth were standardized in a cut machine (Isomet 5000; Buehler, Lake Bluff, IL, USA) to remove the dental crown, leaving a remaining height of 12 mm. The root canals were enlarged with Peeso reamers #1 to #6 (1.50 mm). Then, each root was flushed with 20 mL of 2.5% sodium hypochlorite followed by 20 mL of distilled water. The root canals were dried with paper points, and the external surfaces were dried by wipers (Kimberly Clark, Irving, TX, USA). The external surface was covered with a double layer of nail polish (Colorama, L'Oréal Brasil LTDA., Rio de Janeiro, RJ, Brazil), and each root was embedded in a polystyrene resin (Cristal, Piracicaba, SP, Brazil) and polyether impression material (Impregum F; 3M ESPE, Seefeld, Germany) to simulate periapical tissues. The prepared roots were randomly divided into 6 experimental groups ($n = 5$) based on the material used as follows: ZnO:1.0Ca+PRG ; ZnO:1.0Ca+PEG-PRG ; $\text{Ca(OH)}_2\text{+PRG}$; $\text{Ca(OH)}_2\text{+PEG-PRG}$. The pastes were mixed with 0.1% fluorescein (Sigma-Aldrich) as a fluorescence tracer for confocal laser scanning microscopic analysis. The materials were then incrementally placed in an orthograde direction into the root canals until they appeared from the apex and up to the cervical line with a medium-sized stainless steel endodontic plugger (Easy, Belo Horizonte, MG, Brazil). Then, the access cavities were sealed with temporary filling material (Cavit W; 3M ESPE), and the samples were incubated at 37°C for 24 hours before analysis. Three 1-mm-thick slices perpendicular to the long axis of the root canal, corresponding to the cervical, middle, and apical thirds, were obtained from each specimen using a slow-speed, water-cooled 0.3-mm microtome saw (Isomet 5000). All specimens were mounted onto glass slides and scanned under a

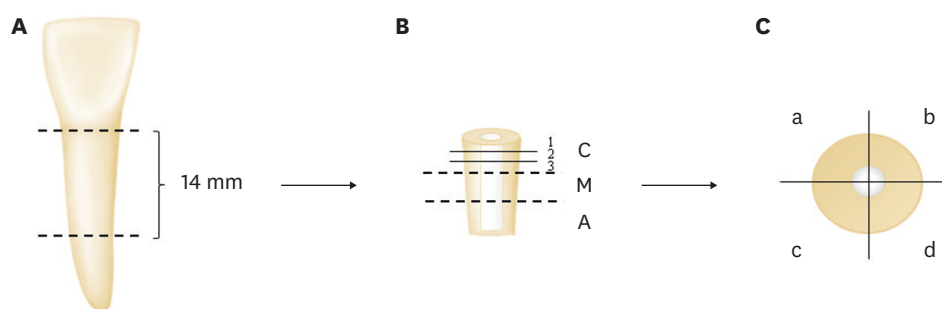


Figure 1. Schematic diagram showing dentin penetration in a tooth sample. (A) Delimitation of the section region; (B) after preparation and insertion of the medications, the roots were sectioned in the cervical, middle, and apical thirds to evaluate dentinal penetration under fluorescence microscopy; (C) penetration measurements were made in quadrants: upper left (a), upper right (b), lower left (c), and lower right (d).

confocal laser scanning microscope (LSM Pascal; Carl Zeiss, Jena, Germany) using the 543-nm wavelength of a helium laser under $\times 10$ magnification (numeric aperture = 0.12). The depth at which the material penetrated the dentin tubules was measured. This measurement was performed in 4 quadrants: upper left (a), upper right (b), lower left (c), and lower right (d) (Figure 1). The measurement started from the wall of the root canal, following the trajectory of the dentinal canaliculus to the maximum point of penetration of the intracanal medicament.

Statistical analysis

The cell viability, pH, radiopacity, bubble count and dentinal tubule penetration data were analyzed for normality and homoscedasticity using the Shapiro–Wilk and Levene tests. For cell viability, pH and dentinal tubule penetration, 2-way analysis of variance (ANOVA) and the Tukey test were used to compare the data between the treated groups. One-way ANOVA and the Tukey test were used to compare the radiopacity of the materials. The Dunnett test was used to compare the experimental groups with the control group. The statistical analysis was performed using GraphPad Prism, a statistical software package (GraphPad LLC, San Diego, CA, USA). The significance level was set at 95% for all analyses.

RESULTS

Cell viability assays

At 24 (Figure 2A) and 96 hours (Figure 2B), it was observed that all materials were more cytotoxic at the 1:1 dilution than at the other dilutions ($p < 0.0001$). At dilutions of 1:4 and higher, the cytotoxic effects stabilized, with similar cell viability values between the dilutions for each material ($p > 0.05$). At 24 hours and 96 hours, the ZnO:1.0Ca+PRG and ZnO:1.0Ca+PEG-PRG groups at 1:1 and 1:2 dilutions presented the lowest percentages of viability ($p < 0.0001$). Within 24 hours, the cells in contact with extracts of ZnO:1.0Ca+PRG and ZnO:1.0Ca+PEG-PRG at 1:4, 1:8, and 1:16 showed the highest levels of viability ($p < 0.0001$), while those in contact with extracts of Ca(OH)₂+PEG-PRG had the lowest values ($p < 0.0001$) (Figure 2A). After 96 hours, all groups showed similar viability values at 1:8 and 1:16 ($p > 0.05$) (Figure 2B). For both periods evaluated, the experimental groups had lower viability than the control group ($p < 0.0001$).

pH

Ca(OH)₂ had higher pH values than ZnO:1.0Ca at 24 and 96 hours, regardless of the vehicle used ($p < 0.05$). The pH of the Ca(OH)₂ mixtures decreased over time ($p < 0.05$). ZnO:1.0Ca

did not show pH variations between the evaluated periods, regardless of the vehicle used ($p > 0.05$) (Figure 3).

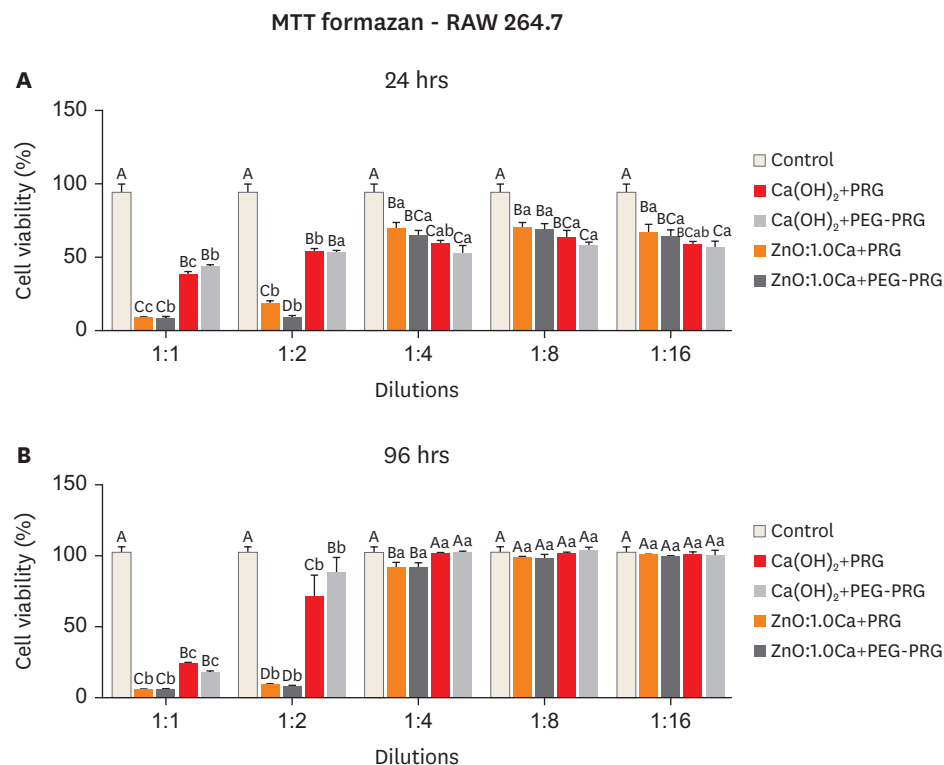


Figure 2. Cell viability percentage of RAW264.7 cells after (A) 24 hours and (B) 96 hours of exposure to extracts according to the material tested and dilution by the MTT formazan method. Capital letters indicate comparisons among different dilutions of extracts and the control group for each material. Lowercase letters indicate comparisons of the same material among the different dilutions. Two-way analysis of variance and the Tukey test ($p < 0.05$). ZnO:1.0Ca, 1.0% Ca²⁺-doped zinc oxide nanocrystals; Ca(OH)₂, calcium hydroxide; PRG, propylene glycol; PEG-PRG, polyethylene glycol and propylene glycol.

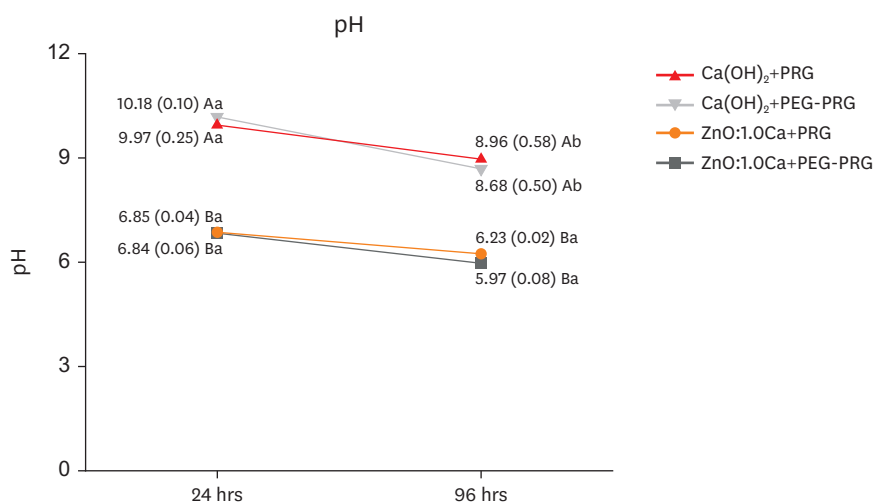


Figure 3. pH values after 24 hours and 96 hours of immersion in distilled water. Capital letters indicate comparisons among materials. Lowercase letters indicate comparisons of the same material among the different times of exposure. Two-way analysis of variance and the Tukey test ($p < 0.05$). ZnO:1.0Ca, 1.0% Ca²⁺-doped zinc oxide nanocrystals; Ca(OH)₂, calcium hydroxide; PRG, propylene glycol; PEG-PRG, polyethylene glycol and propylene glycol.

Table 1. Mean radiopacity (gray values) in the different groups evaluated

Groups	Gray value
Ca(OH) ₂ +PRG	56.83 ± 6.83 B
Ca(OH) ₂ +PEG-PRG	59.92 ± 5.28 B
ZnO:1.0Ca+PRG	168.92 ± 14.46 A
ZnO:1.0Ca+PEG-PRG	177.83 ± 8.13 A

The values are mean ± standard deviation.

ZnO:1.0Ca, 1.0% Ca²⁺-doped zinc oxide nanocrystals; Ca(OH)₂, calcium hydroxide; PRG, propylene glycol; PEG-PRG, polyethylene glycol and propylene glycol.

*Different capital letters represent statistically significant differences between groups by the Tukey test ($p < 0.05$).

Radiopacity

Table 1 presents the mean and standard deviation values for radiopacity, expressed as gray values immediately after material insertion. The ZnO:1.0Ca pastes showed higher radiopacity than the Ca(OH)₂ pastes ($p < 0.01$).

Bubble count

Table 2 presents the mean bubble count and standard deviation. There were no statistically significant differences between the materials evaluated ($p = 0.0902$).

Dentinal tubule penetration

The levels of penetration of the materials into the different radicular thirds were observed (**Figure 4**). The experimental materials ZnO:1.0Ca+PRG and ZnO:1.0Ca+PEG-PRG exhibited similar penetration depths in all radicular thirds ($p > 0.05$). CaOH₂+PRG, and Ca(OH)₂+PEG-PRG had higher penetration in the cervical third than in the apical third ($p = 0.01$). All materials had similar depths of penetration in the dentinal tubules in the cervical third ($p > 0.05$). In the middle third, Ca(OH)₂+PRG, ZnO:1.0Ca+PRG, and ZnO:1.0Ca+PEG-PRG had similar penetration depths ($p > 0.05$), and Ca(OH)₂+PEG-PRG presented the lowest values. ZnO:1.0Ca pastes had a greater penetration depth than Ca(OH)₂ pastes in the apical third ($p < 0.0001$) (**Figure 5**).

DISCUSSION

The null hypothesis was rejected, as the paste formulations affected the cell viability, radiopacity, and penetrability. The cytotoxicity of materials can vary depending on the cell model evaluated, the methodology of analysis, and the type of vehicle used for intracanal dressing [6,12,14]. The RAW 264.7 macrophage lineage was chosen for this study because monocytes and macrophages play a pivotal role in periapical repair, releasing mediators related to the recruitment and differentiation of other cells involved in the formation of the mineralized apical barrier [15]. Previously, the same doped materials (ZnO:xCa) with different percentages of Ca²⁺ by weight ($x = 0.7\%$; 1.0% ; 5.0% ; 9.0%) were tested

Table 2. Mean bubble count in the different groups evaluated

Groups	Number of bubbles
Ca(OH) ₂ +PRG	13.5 ± 3.87 A
Ca(OH) ₂ +PEG-PRG	11 ± 2.45 A
ZnO:1.0Ca+PRG	8.5 ± 3.42 A
ZnO:1.0Ca+PEG-PRG	8.25 ± 1.71 A

The values are mean ± standard deviation.

ZnO:1.0Ca, 1.0% Ca²⁺-doped zinc oxide nanocrystals; Ca(OH)₂, calcium hydroxide; PRG, propylene glycol; PEG-PRG, polyethylene glycol and propylene glycol.

*Different capital letters represent statistically significant differences between groups by the Tukey test ($p < 0.05$).

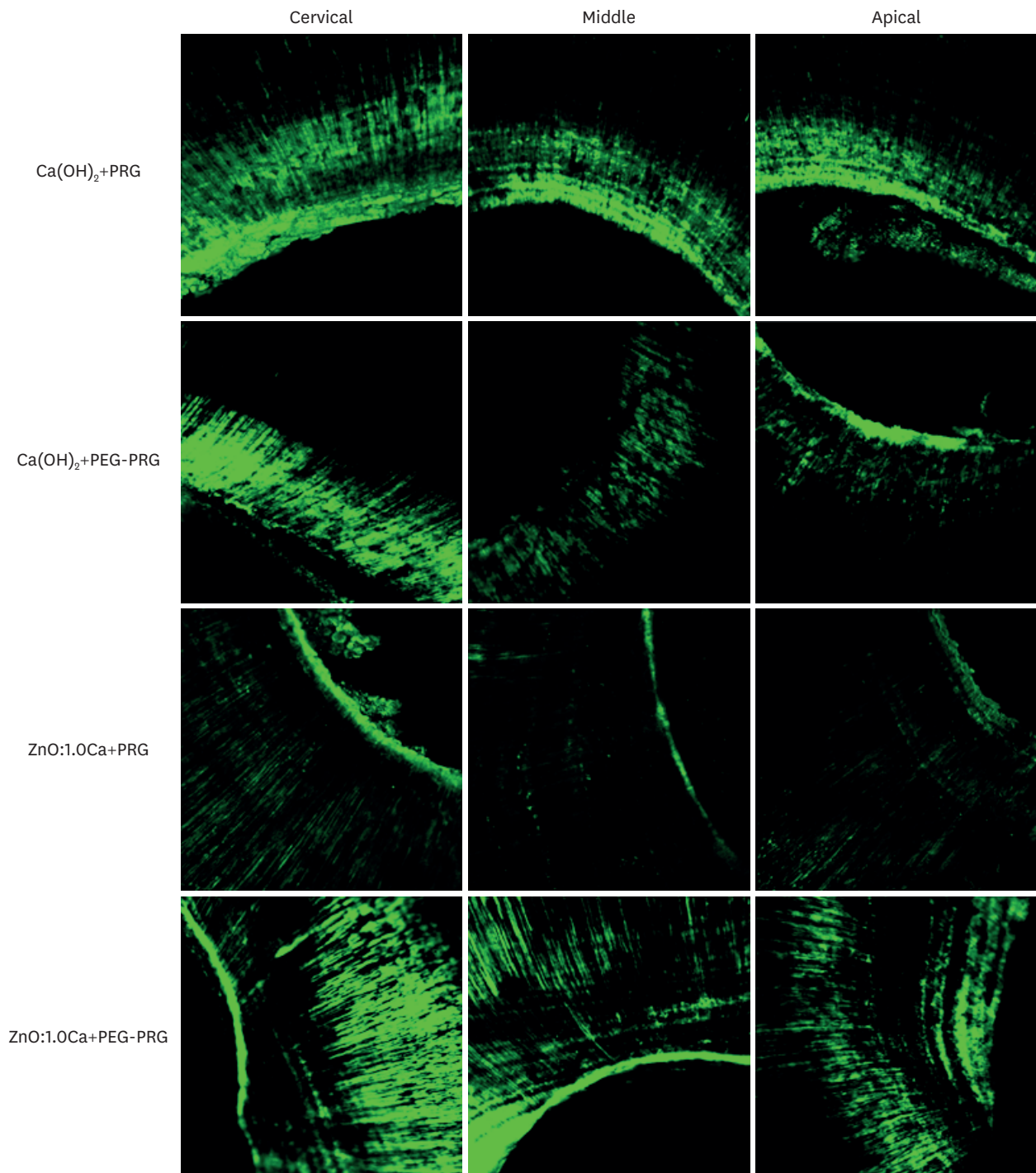


Figure 4. Representative confocal laser scanning microscopy images from each experimental group at $\times 10$ magnification. ZnO:1.0Ca, 1.0% Ca^{2+} -doped zinc oxide nanocrystals; $\text{Ca}(\text{OH})_2$, calcium hydroxide; PRG, propylene glycol; PEG-PRG, polyethylene glycol and propylene glycol.

against RAW 264.7, SAOS-2, and human dental pulp cells, supporting the use of ZnO:0.7Ca nanocrystals as pulp protection material [12]. However, in that study, the assessments were performed with materials in powder form, which does not represent clinical use, as the combination of $\text{Ca}(\text{OH})_2$ [4,6,16] or ZnO with different vehicles is necessary to obtain an adequate consistency of the material [10,17]. Specifically, in this study, the paste consistency

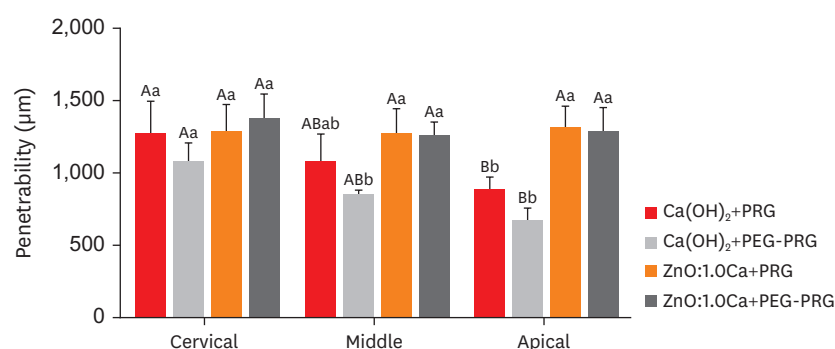


Figure 5. Dentinal tubule penetration depth (µm) of the experimental groups ($n = 15$). Capital letters indicate comparisons of the same material among the different root thirds. Lowercase letters indicate comparisons among the different materials. Two-way analysis of variance and the Tukey test ($p < 0.05$). ZnO:1.0Ca, 1.0% Ca^{2+} -doped zinc oxide nanocrystals; $\text{Ca}(\text{OH})_2$, calcium hydroxide; PRG, propylene glycol; PEG-PRG, polyethylene glycol and propylene glycol.

was chosen to facilitate insertion in immature teeth with large canals, minimize the dispersion of the materials into the tissues, and retain the material in the desired area for a longer time [18]. Thus, an initial test was performed in order to verify the consistency of the material after manipulation with the proposed vehicle, demonstrating that the ZnO:0.7Ca nanocrystals did not have the desired consistency after manipulation. Since ZnO:1.0Ca also exhibited favorable cell viability and low production of reactive oxygen species and nitric oxide, such nanocrystals were selected for conducting the present study [12]. To assess the cytotoxicity of ZnO:1.0Ca and $\text{Ca}(\text{OH})_2$, extracts obtained from freshly prepared materials were used [13,19]. This is because, in the clinical setting, the cells present in periapical tissues come into contact with progressively lower concentrations of leachable compounds that are continually cleared by extracellular fluids [13]. The MTT assay was chosen for cytotoxicity screening of these materials because it is often used for determining the cytotoxicity of endodontic materials without resulting in overestimation or underestimation [14].

The results revealed that the ZnO:1.0Ca and $\text{Ca}(\text{OH})_2$ formulations both presented greater cytotoxic effects at higher concentrations (1:1 and 1:2) regardless of the vehicle or contact time, which is in accordance with other studies using different materials [13,19]. However, the behavior of RAW 264.7 against these same formulations at 24 and 96 hours was the opposite at 1:4 extracts, which is considered an intermediate dilution. At a 1:4 dilution, the lower cytotoxicity for ZnO:1.0Ca formulations compared to $\text{Ca}(\text{OH})_2$ at 24 hours suggested that the nano-doped ZnO:1.0Ca formulations initially promoted less damage to cell metabolism and consequently affected cell viability to a reduced degree. The alteration of this scenario at 96 hours, with $\text{Ca}(\text{OH})_2$ samples showing less cytotoxicity than ZnO:1.0Ca, may indicate that the leachate released by the doped material was altered rapidly, resulting in differences in their chemical composition. A possible explanation for the results at 24 hours may be related to the nanocrystal morphology, which is one of the most important characteristics affecting its cytotoxicity [20]. While the $\text{Ca}(\text{OH})_2$ nanocrystals used in the present study exhibit a hexagonal structure, the ZnO:1.0 Ca nanocrystals have a rod shape [12,21]. Hexagonally shaped particles possess edge surface atoms, which makes it easier for them to enter into cells without disrupting the cells, while rod-shaped nanoparticles bind to the positively charged cell surface and disrupt them, resulting in cell death [20]. The results observed at 96 hours at the same dilution (1:4) may also be related to the zinc ion (Zn^{2+}) release by doped ZnO:1.0Ca [22].

ZnO nanocrystals have the potential to be a potent means for Zn^{2+} delivery into intracellular compartments [22]. A previous study demonstrated that the toxicity of ZnO nanoparticles is caused by the dissolution and subsequent release of toxic Zn^{2+} ions into the culture medium [22,23]. The dissolution of Zn^{2+} ions increases with the size of the particles [23]. The presence of Zn^{2+} may affect several cellular mechanisms, such as the cell cycle, differentiation, and death, via different pathways [22,24]. In models using neural cells, the entrance of Zn^{2+} into the cells was observed after several days, and it is possible to speculate that the stronger cytotoxic effects of ZnO:1.0Ca at 96 hours are related to the same phenomena. However, despite the possible toxic effect of Zn^{2+} ions, ZnO has been used for decades in dentistry, including its use in pediatric dentistry in pulpotomies. In addition, ZnO nanoparticles have been shown to be effective against Gram-negative and Gram-positive bacteria [25,26-28].

The methodology followed in the present study for pH assessment is outlined in ISO 6876, which defines the standard for evaluating the physicochemical properties of root canal sealing materials [29]. Although the materials tested are intracanal medications and are not intended to permanently obturate the canals, we chose to follow the ISO standard due to the “paste-like” consistency of the materials. Previous studies have also utilized this methodology, immersing the plastic tubes containing medications in distilled water [13,30]. Oliveira *et al.* [13] used tubes measuring 10 mm in length and 1.6 mm in internal diameter to evaluate the pH of BioC Temp, BioC Repair, and Biodentine. Villa *et al.* [30] evaluated the pH using BioC Temp and UltraCal in tubes measuring 10 mm in length and 1 mm in diameter.

The low pH values of doped ZnO:1.0Ca formulations indicate that the presence of Ca^{2+} , a high-valence metal cation, was not able to modify the acid pH characteristic of ZnO nanoparticles produced via the coprecipitation technique, despite the vehicle used [12]. Previously, Guerreiro-Tanomaru *et al.* [10] evaluated the pH of nanoparticulate ZnO+PEG and compared it to nanoparticulate $\text{Ca}(\text{OH})_2$ +ZnO+PEG and observed slightly higher pH values than the present study. The method of producing the ZnO nanocrystals, which is a determining factor in the material's characteristics, was different in that study from the method used here [24]. In addition, the doping of materials modifies the composition and properties of nanocrystals through the intentional introduction of dopants [11]. In this regard, the synthetic strategies of doped nanocrystals certainly influence the biological and physicochemical properties of nanocrystalline materials.

Regarding $\text{Ca}(\text{OH})_2$ formulations, pH values between 11 and 12 were expected, which were higher than the findings observed in the present study [4]. The reason for this may be attributed to the time of analysis, the vehicles used, differences in methodology and differences regarding the bulk $\text{Ca}(\text{OH})_2$ and nanocrystals [4,16,26,31-33]. No previous studies have compared the dissociation of $\text{Ca}(\text{OH})_2$ formulations into OH^- and Ca^{2+} between bulk $\text{Ca}(\text{OH})_2$ and nanocrystals or pH values. In this sense, it is not possible to directly compare the extant literature and the present findings. Considering that a pH of approximately 8.6 to 10.3 is required for the biological activity of $\text{Ca}(\text{OH})_2$ to promote the activation of alkaline phosphatase enzymes, the pH values obtained by $\text{Ca}(\text{OH})_2$ +PRG and $\text{Ca}(\text{OH})_2$ +PEG-PRG are within an acceptable range [32].

Interestingly, the vehicle did not affect the evaluated parameters of the ZnO:1.0Ca and $\text{Ca}(\text{OH})_2$ nanocrystal formulations. The reason for this may be that both PEG and PRG are viscous vehicles with a high molecular weight [34,35]. The characteristics of PEG and PRG were previously evaluated in a study of antibiotic pastes in a 1:1 ratio, demonstrating

favorable results regarding cytotoxicity [6]. According to Mohammadi and Dummer [35], viscous vehicles promote the slow and sustained release of Ca^{2+} and OH^- ions from $\text{Ca}(\text{OH})_2$ pastes compared with aqueous vehicles. In addition, the use of viscous vehicles has been suggested to optimize the intratubular penetration of intracanal medications [18].

The antimicrobial and penetrability effects of intracanal medications can be affected by the size and shape of the particles and the type of vehicle used [17,36,37]. A previous study demonstrated that as the particle size increased, the particle shape became more rectangular, but as the size decreased, the particle shape became rounder, which was more desirable for deep penetration [37]. Considering that ZnO:1.0Ca nanocrystals used in the present study have a rod shape and are approximately 20 nm in diameter, while the $\text{Ca}(\text{OH})_2$ nanocrystals have a hexagonal structure and are approximately 80 nm in diameter, the greater ability of the ZnO:1.0Ca paste to penetrate into the middle and apical thirds could be attributed to these properties [12,21]. However, the $\text{Ca}(\text{OH})_2$ paste presented a higher fluorescence intensity than ZnO:1.0Ca . Considering that the fluorescence intensity of sodium fluorescein is related to the presence of Ca^{+2} ions and the opposite effect occurs in the absence of these ions, it is possible to suggest that ZnO:1.0Ca+PRG released a smaller amount of Ca^{+2} ions than the other evaluated pastes [38]. However, additional studies need to be performed to confirm this speculation.

Another parameter evaluated was the radiopacity of the pastes, an important factor that enables the visualization of the material radiographically, as well as the presence of bubbles or an absence of material in the desired region [39], which facilitates clinical follow-up [30]. Among several radiopacifiers used in endodontic materials, ZnO has often been evaluated [40,41]. $\text{Ca}(\text{OH})_2$ pastes, regardless of the vehicle used, do not present adequate radiopacity unless radiopacifying substances are added to the mixture [34]. In this sense, the higher radiopacity of the ZnO:1.0Ca formulations was in agreement with the current literature.

To maximize the benefit of dressing materials, the pastes must be in direct contact with the root canal walls and fill the total extension of the main canal space [42]. In the present study, all groups presented bubbles, without significant differences among them, which may be related to the similar consistency of the materials. However, the method used for bubble count based on binarized radiographic images has limitations, especially regarding the use of molds to obtain the images and a lack of volumetric analysis. Micro-computed tomography (μCT) would be a better alternative since it is the method of choice to calculate the volume of filling materials, providing high-resolution 3-dimensional assessment [43,44]. Despite the advantages of μCT , it does not represent a tool that is available for clinicians to assess the filling of a given material, which is why radiographic images were chosen to assess the presence of bubbles.

One of the concerns regarding the materials tested as long-term medications is the difficulty of root canal removal, as the remnants of dressing material may prevent the adaptation of endodontic sealers to the root canal wall and the penetration of sealers into dentinal tubules [45]. Considering that intracanal medication remnants can be found in roots after irrigation, regardless of the vehicle used for their manipulation, the combination of chemical removal and mechanical activation of the irrigant solutions should be proposed in order to obtain more favorable results [46-48]. Further studies need to be conducted to assess the difficulty of removing the medications proposed in this study.

CONCLUSIONS

In conclusion, ZnO:1.0Ca nanocrystals associated with low-surface-tension vehicles presented higher penetrability, cell viability, and radiopacity than Ca(OH)₂ nanocrystals. In contrast, higher values of cell viability and pH were present in Ca(OH)₂ nanocrystals than in ZnO:1.0Ca nanocrystals. None of the parameters were influenced by the vehicle (PRG or PEG-PRG).

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CAPÍTULO 3

Effects of different calcium-silicate based materials on fracture resistance of immature permanent teeth with replacement root resorption and osteoclastogenesis.

Research Article



Effects of different calcium-silicate based materials on fracture resistance of immature permanent teeth with replacement root resorption and osteoclastogenesis

Gabriela Leite de Souza ,¹ Gabrielle Alves Nunes Freitas ,²
Maria Tereza Hordones Ribeiro ,³ Nelly Xiomara Alvarado Lemus ,¹
Carlos José Soares ,³ Camilla Christian Gomes Moura ^{1*}

¹Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Uberlândia, Brazil

²School of Dentistry, Federal University of Uberlândia, Uberlândia, Brazil

³Department of Operative Dentistry and Dental Materials, School of Dentistry, Federal University of Uberlândia, Uberlândia, Brazil



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Souza GL, Freitas GAN, Ribeiro MTH, Lemus NXA, Soares CJ, Moura CCG

*Correspondence to

Camilla Christian Gomes Moura, PhD

Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Av Pará 1720, Campus Umuarama, Block 4L, annex A, Uberlândia, MG 38405-328, Brazil.
Email: camillamoura@ufu.br

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ABSTRACT

Objectives: This study evaluated the effects of Biodentine (BD), Bio-C Repair (BCR), and mineral trioxide aggregate (MTA) plug on the fracture resistance of simulated immature teeth with replacement root resorption (RRR) and *in vitro*-induced osteoclastogenesis.

Materials and Methods: Sixty bovine incisors simulating immature teeth and RRR were divided into 5 groups: BD and BCR groups, with samples completely filled with the respective materials; MTA group, which utilized a 3-mm apical MTA plug; RRR group, which received no root canal filling; and normal periodontal ligament (PL) group, which had no RRR and no root canal filling. All the teeth underwent cycling loading, and compression strength testing was performed using a universal testing machine. RAW 264.7 macrophages were treated with 1:16 extracts of BD, BCR, and MTA containing receptor activator of nuclear factor-kappa B ligand (RANKL) for 5 days. RANKL-induced osteoclast differentiation was assessed by staining with tartrate-resistant acid phosphatase. The fracture load and osteoclast number were analyzed using 1-way ANOVA and Tukey's test ($\alpha = 0.05$).

Results: No significant difference in fracture resistance was observed among the groups ($p > 0.05$). All materials similarly inhibited osteoclastogenesis ($p > 0.05$), except for BCR, which led to a lower percentage of osteoclasts than did MTA ($p < 0.0001$).

Conclusions: The treatment options for non-vital immature teeth with RRR did not strengthen the teeth and promoted a similar resistance to fractures in all cases. BD, MTA, and BCR showed inhibitory effects on osteoclast differentiation, with BCR yielding improved results compared to the other materials.

Keywords: Fracture strength; Non-vital tooth; Osteoclastogenesis; Root resorption; Silicate cement

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Author Contributions

Conceptualization: Souza GL, Moura CCG, and Soares CJ. Data curation: Souza GL, Moura CCG, and Soares CJ. Formal analysis: Souza GL, Moura CCG, and Soares CJ. Funding acquisition: Moura CCG. Investigation: Souza GL, Ribeiro MTH, Freitas GAN, and Lemus NXA. Methodology: Souza GL, Ribeiro MTH, Freitas GAN, and Lemus NXA. Project administration: Moura CCG. Resources: Moura CCG. Software: Souza GL and Ribeiro MTH. Supervision: Moura CCG. Validation: Souza GL, Moura CCG, and Soares CJ. Visualization: Souza GL, Moura CCG, and Soares CJ. Writing – original draft: Souza GL, Freitas GAN, Ribeiro MTH, Lemus NXA, Soares CJ, and Moura CCG. Writing – review & editing: Souza GL, Freitas GAN, Ribeiro MTH, Lemus NXA, Soares CJ, and Moura CCG.

ORCID iDs

Gabriela Leite de Souza 
<https://orcid.org/0000-0003-3505-5258>
Gabrielle Alves Nunes Freitas 
<https://orcid.org/0000-0001-7865-2795>
Maria Tereza Hordones Ribeiro 
<https://orcid.org/0000-0002-5126-7931>
Nelly Xiomara Alvarado Lemus 
<https://orcid.org/0000-0002-9658-2433>
Carlos José Soares 
<https://orcid.org/0000-0002-8830-605X>
Camilla Christian Gomes Moura 
<https://orcid.org/0000-0002-5653-8403>

INTRODUCTION

Trauma to immature permanent teeth can lead to pulp necrosis, thereby interrupting root development and making root canal treatment challenging. Although pulp necrosis is the most common post-traumatic complication, replacement root resorption (RRR) is the most concerning complication as it may affect the long-term survival of teeth, mainly after lateral luxation and avulsion [1,2].

In patients with RRR, new alveolar bone is formed and deposited in the space previously occupied by the periodontal ligament (PL) fibers that were lost, and the root is progressively resorbed and replaced by bone tissue [3]. Thus, the development of the RRR process invariably leads to the exposure of the filling material to periodontal tissues. Therefore, choosing root canal filling materials with greater biocompatibility is crucial [4]. Additionally, as resorption is mediated by osteoclasts/odontoclasts, filling the root canal with materials that reduce or inhibit osteoclastic activity could delay the progression of root resorption. This enables dentists to plan adequate rehabilitation for hebiatric patients, in whom the early loss of teeth has a greater psychosocial impact.

Mineral trioxide aggregate (MTA) has been suggested for filling reimplanted teeth due to its sealing ability, biocompatibility, low solubility, ability to inhibit osteoclastogenesis, adequate radiopacity, and high pH [4]. However, MTA has several disadvantages, including prolonged setting time, potential for coronal staining, and handling difficulties [5]. Thus, other calcium-silicate based materials (CSM) materials have been suggested as alternatives.

Biodentine (BD; Septodont, Saint Maur-des-Fossés, France) is a tricalcium silicate-based restorative cement indicated as a “dentine replacement” material. This material is comparable to MTA in terms of its biocompatibility and induction of a calcific barrier [6]. In addition, it has advantages in terms of several other properties, including improved mixing, handling, shorter initial setting time, and less coronal discoloration after its use [6]. Recently, a new ready-to-use CSM, Bio-C Repair (BCR; Angelus, Londrina, Brazil), has become available for reparative or regenerative endodontic treatments. This material has low cytotoxicity, appropriate biocompatibility, and biomineralization ability [7-10].

Although previous studies have evaluated the fracture resistance of immature teeth restored with various intra-radicular filling materials, to our knowledge, no study has evaluated the intra-radicular filling materials in immature permanent teeth in conditions that mimic RRR [11,12]. Thus, in this study, we aimed to evaluate the fracture resistance after cyclic loading of simulated immature teeth with RRR filled with BD, BCR, or MTA plugs. Additionally, we evaluated the cytotoxicity and anti-osteoclastic effects of these materials. The null hypothesis tested was: 1) treatment with different intra-radicular filling materials would not affect the fracture resistance of immature permanent teeth with RRR, 2) the tested materials would not affect RAW264.7 macrophage viability, and osteoclast-like maturation.

MATERIALS AND METHODS

This manuscript has been written according to the Preferred Reporting Items for Laboratory Studies in Endodontology (PRILE) 2021 guidelines [13] (**Supplementary Figure 1**).

Table 1. Calcium-silicate based materials, chemical compositions, and manufacturers of the materials employed in this study

Materials	Chemical composition	Manufacturer	Technical information
Biodentine	Powder: Tricalcium silicate, zirconium oxide, calcium oxide, calcium carbonate, yellow pigment, red pigment, and brown iron oxide Liquid: Calcium chloride dihydrate, sand, and purified water	Septodont	Five drops of liquid are placed in the capsule and it is shaken at 4,000 rpm for 30 sec
White MTA	Powder: Silicon dioxide, potassium oxide, aluminum trioxide, sodium oxide, iron trioxide, sulfur oxide, calcium oxide, bismuth oxide, magnesium oxide Liquid: Water and plasticizer	Angelus	One sachet of powder mixed with one drop of distilled water for 30 sec
Bio-C Repair	Calcium silicate, calcium aluminate, calcium oxide, zirconium oxide, iron oxide, silicon dioxide and dispersing agent	Angelus	Product ready for use, mixture not being necessary

MTA, mineral trioxide aggregate.

Materials

The three materials used in this study were: BCR, which was ready-to-use (Angelus); BD, which was prepared by adding 5 drops of liquid to the capsule and shaking it for 30 seconds at 4,000 rpm (Septodont); and MTA, which was prepared by mixing the contents of one sachet with one drop of distilled water (Angelus) for 30 seconds. Based on the manufacturers' instructions, the components of the CSM tested are described in **Table 1**.

Fracture resistance

Sample size calculation

The sample size was based on the data from Tanalp *et al.* [14] and was calculated using the G*Power version 3.1.9.6 software (<http://www.psych.uni-duesseldorf.de/abteilungen/aap/gpower3/>) to allow for analysis with $\alpha = 0.05$, power ($1 - \beta$ prob) = 0.99, and effect size $f = 1.52$. The analysis of covariance (fixed and main effects, and interactions) statistical test was performed on the data. The type of power analysis was set a priori to compute the required sample size given the α , power, and effect size. Therefore, a minimum of 12 teeth were allocated to each experimental group, with a total of 60 teeth.

Selection of samples

Bovine central incisors (30–36 months) were obtained from a local abattoir (Real, Uberlândia, MG, Brazil), cleaned, and stored in distilled water at 4°C. Using a microscope (D. F. Vasconcellos, Valença, RJ, Brazil) at $3.0 \times$ magnification, the teeth were analyzed for the presence of cracks and fractures. Teeth without cracks, fracture lines, or other deformities underwent measurements of the mesiodistal and buccolingual dimensions of the roots using a digital caliper (Mitutoyo Sul Americana Ltda., Suzano, SP, Brazil). Samples with a maximum deviation of 10% from the mean were included in the study. The selected teeth were stored in distilled water at 4°C until further use.

Simulation of immature permanent teeth

To simulate complicated crown fractures in immature teeth, the samples were sectioned 8 mm above and 12 mm below the cemento-enamel junction (CEJ) while under a water-cooled diamond disc [8]. The root canals were instrumented with Peeso reamers (size 1–5, Dentsply Maillefer) followed by a 3017 HL diamond bur (KG Sorensen, Barueri, SP, Brazil) to create a standard open apex with a diameter of approximately 2.5 mm [15]. Next, the root canals were rinsed with 20 mL of 2.5% sodium hypochlorite followed by final irrigation with 3 mL of 17% ethylenediaminetetraacetic acid (EDTA) solution and 5 mL of distilled water [8]. The samples were re-evaluated for the presence of cracks or defects using the transillumination method. All teeth with cracks were excluded from this study.

Root canal filling and simulation of RRR

The RRR simulation was performed on the palatal surface of the prepared samples based on the protocol previously described by Vieira *et al.* [16]. First, perforations were made perpendicular to the palatal surface in the cervical, middle, and apical thirds of the root using cylindrical drills with diameters of 1.2 mm (1093; KG Sorensen), 1.8 mm (2094; KG Sorensen), and 0.6 mm (1090; KG Sorensen), respectively. The distance between each perforation was approximately 4 mm. Subsequently, the root regions containing the perforations were exposed to an acid demineralization process through treatment with a 5% nitric acid solution for 12 hours, 8% sodium hypochlorite solution for 10 minutes, and 5% nitric acid solution for 12 hours for resorption lesions. The teeth were washed and stored in deionized water to arrest the process. Randomization (blocked random scheme) was performed using the website (www.sealedenvelope.com). Randomization and allocation of the samples to one of the following five groups ($n = 12$) were performed by the same researcher (CCGM), who was not involved in the implementation (GANF) and evaluation (GLS) processes.

- *RRR (negative control group)*: RRR simulated; no apical plug or root canal filling was performed.
- *Normal PL (positive control group)*: no RRR, apical plug, or root canal filling was performed.
- *MTA Plug*: RRR simulated; MTA was prepared according to the manufacturer's instructions, placed into the root canals with an MTA carrier, and condensed with hand pluggers to create a 4-mm-thick MTA. After MTA apexification, the root canal was filled with a Bio-C sealer (Angelus) and gutta-percha cones (Dentsply-Malleifer).
- *BCR*: RRR was simulated, and the root canals were filled with BCR. The ready-to-use cement was inserted incrementally and condensed in the root canal with hand pluggers up to the CEJ.
- *BD*: RRR-simulated BD was prepared according to the manufacturer's instructions and inserted into the root canal as described for the previous group, up to the CEJ.

Following the procedures described above, the teeth were radiographed again to confirm the quality of the fillings, and endodontic access was restored using an adhesive system (Adper Single Bond 2; 3M ESPE, St. Paul, MN, USA) and composite resin (Filtek Z350 XT; 3M, Sumaré, SP, Brazil). The samples were stored at 37°C and 100% humidity for 24 hours. After this period, the teeth were inserted into resin cylinders to simulate RRR regions and regions with normal PLs. The external surfaces of the root were then covered with a 0.2–0.3 mm thick wax layer until it reached 2 mm below the CEJ. Subsequently, the wax-covered roots were embedded in polystyrene resin cylinders (Classico, Rio de Janeiro, RJ, Brazil). After resin polymerization, the roots were removed from the cylinder, and all the wax was removed from the root surface, creating a space in the resin cylinder. Next, the polyether impression material Impregum F (3M ESPE) was mixed and distributed over the buccal root surface. Simultaneously, the polystyrene resin was manipulated and distributed over the palatal root surface, filling the regions where the perforations were made and the regions that were perforation-free. According to the procedure by Soares *et al.* [17], the entire roots of the teeth without RRR and with only simulated PLs were soaked in Impregum F (3M). The teeth were then re-inserted into the cylinder, and excess material was removed using a scalpel blade.

Mechanical cycling test and fracture testing

Chewing cycles were simulated to induce mechanical fatigue (Biocycle; Biopdi, São Paulo, SP, Brazil) by immersing the samples in water at 37°C and cycling 1,200,000 times. A load of 0–50 N at 45° and a frequency of 2 Hz was applied to the palatal surface of the teeth using a stainless-steel cylindrical-shaped tip. Each specimen was arranged in a mounting jig such that

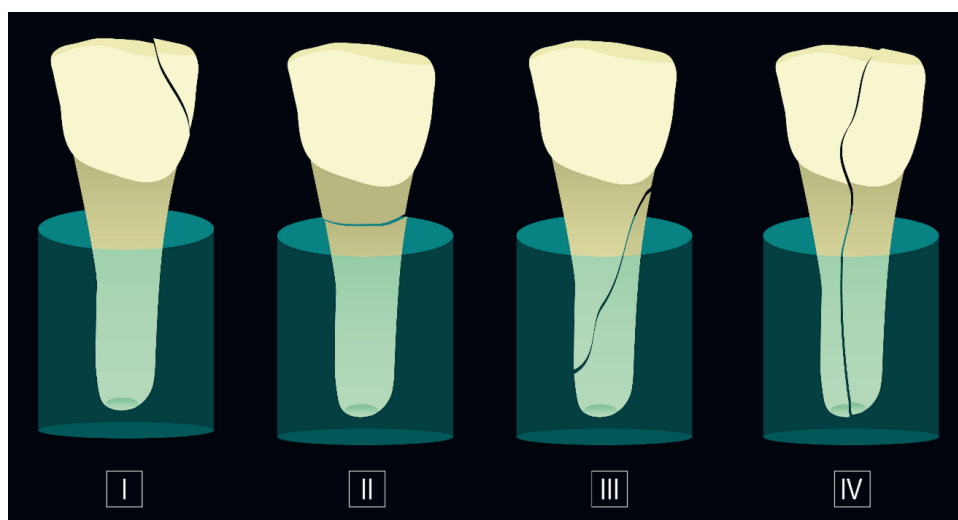


Figure 1. Classification of the fracture modes – (I) enamel fracture; (II) one-third cervical root fracture; (III) one-third middle root fracture; (IV) vertical root fracture.

the load was applied to the palatal surface at a 45° angle with the long axis of the tooth. The fracture was induced in the specimens by applying compressive loads with crosshead speeds of 0.5 mm/min in a universal testing machine (Instron model 3344, Instron; Norwood, MA, USA) fitted with a 5000 N load cell [18]. Both the fracture load (N) and the fracture location were recorded. The fracture modes were analyzed using the scale shown in **Figure 1**.

Cell culture assays

Preparation of material extracts

MTA, BCR, and BD were mixed according to the manufacturers' instructions under sterile conditions, and the extracts were prepared as previously described [8]. The materials were shaped in cylindrical polyethylene molds (diameter, 5 mm; height, 2 mm) and incubated under humidified conditions at 37°C for 24 hours to allow for complete setting. After this period, the specimens were immersed in α -minimal essential medium (α -MEM) (LGC Biotechnology, Cotia, SP, Brazil) for 24 hours at 37°C and 5% CO₂ in a humid atmosphere. Following the ISO 10993-5 guidelines, a material surface area/medium volume ratio of approximately 1.5 cm²/mL was obtained [19]. After incubation, the original extracts were filtered and serially diluted in cell culture medium to a dilution of 1:16 before testing.

Cell cytotoxicity assay

A murine RAW 264.7 macrophage cell lineage was obtained from the Cell Bank of Rio de Janeiro (Rio de Janeiro, RJ, Brazil). The cells were cultured until confluence in α -MEM supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA). After this period, RAW 264.7 cells were seeded in a 96-well plate and exposed or not (control group) to diluted extracts (1:1-1:16) for 24 hours. Next, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Thermo Fisher Scientific, Waltham, MA, USA) was prepared at 5 mg/mL, and 10% MTT diluted in α -MEM was added to each well for 4 hours. After this period, 100 μ L of dimethylsulfoxide (Sigma-Aldrich) was added to each well for approximately 2 minutes. Subsequently, the optical density (OD) at 570 nm of the cells was measured using a microplate reader (Biochrom, Cambridge, UK). Cell viability was expressed as the percentage of OD of the experimental wells relative to that of the control, according to the following formula:

$$\text{Percentage of Viable Cells} = (A/B) \times 100,$$

where A = viable cells in the experimental well and B = viable cells in the control well. The assay was performed in quadruplicate ($n = 4$), and the trial was repeated twice under the same conditions. A dilution that demonstrated a cell viability higher than 80% was used for the osteoclastogenesis assay (1:16).

Osteoclastogenesis induction and exposure to the extracts

RAW 264.7 cells were seeded (2×10^4 cells/well) on a 96-well plate in α -MEM supplemented with 10% FBS and 1% penicillin-streptomycin and allowed to adhere overnight. For osteoclast-like formation, the cells were stimulated with 1:16 diluted extracts of materials and 100 ng/mL receptor activator of nuclear factor-kappa B ligand (RANKL) (PeproTech, Rocky Hill, NJ, USA) for 5 days. On day three, the medium and RANKL were changed [20,21]. The negative control group contained cells maintained in 10% α -MEM (not induced to differentiation), and the positive control group contained cells kept in RANKL without extract treatment. Samples were then prepared for quantification of tartrate-resistant acid phosphatase (TRAP) activity. The assay was performed in quadruplicate ($n = 4$), and the trial was repeated twice under the same conditions.

Tartrate-resistant acid phosphatase staining

The cells were washed with phosphate-buffered saline (PBS; Gibco) and fixed for 30 seconds with a liquid containing citrate, acetone, and formaldehyde. Next, the cells were stained using an acid phosphatase leukocyte TRAP kit (Sigma-Aldrich) in accordance with the manufacturer's recommendations. TRAP-positive cells were evident due to the appearance of cytoplasmic granules marked by dark red to purple deposits. The number of osteoclasts formed per well was represented by TRAP-positive multinucleated cells with 3 or more nuclei [20,21]. Four images of each well were taken at the top left, top right, bottom left, and bottom right, with $10 \times$ magnification using a Leica DM IRB-inverted microscope coupled to a DFC490 camera (Leica, Wetzlar, HE, Germany). The results were expressed as the percentage of TRAP-positive cells identified per field in each well compared to that observed in the positive control group [20].

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). All data were analyzed for normality and homoscedasticity using Shapiro-Wilk's and Levene's tests. Fracture loads and osteoclast numbers were analyzed using 1-way analysis of variance (ANOVA) and Tukey's multiple comparison test to ascertain any significant differences between groups. The fracture modes were also qualitatively evaluated. To determine cell viability, 2-way ANOVA and Tukey's test were used to compare the data between the treated groups. Dunnett's test was used to compare the experimental groups to the control group. Statistical significance was set at $\alpha = 0.05$.

RESULTS

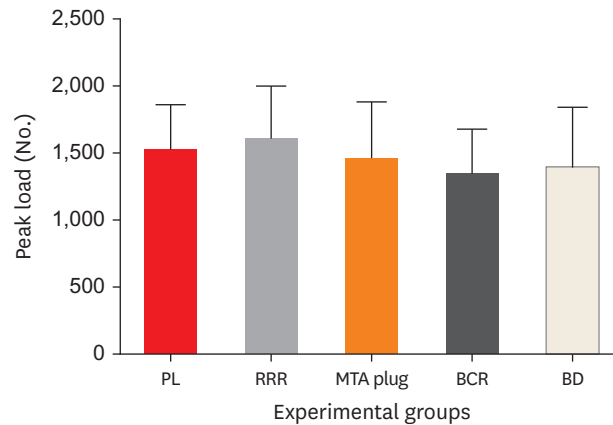
Fracture testing

The results of the fracture testing are presented in **Table 2** and **Figure 2**. There were no statistically significant differences in fracture resistance among the 5 groups evaluated (1-way ANOVA, $F = 0.872$, $p = 0.487$). All teeth filled with MTA plugs, BCR, and BD were fractured

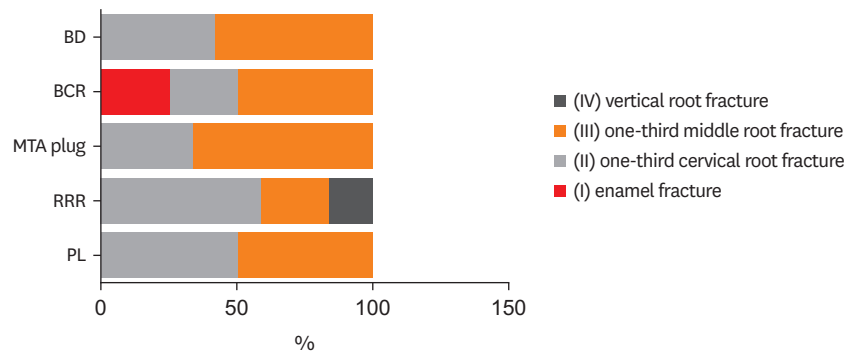
Table 2. Mean $\pm$ standard deviation of fracture resistance in the experimental groups

Experimental groups	Values
PL	1,519 $\pm$ 343.3 ^A
RRR	1,609 $\pm$ 390.5 ^A
MTA Plug	1,453 $\pm$ 375.5 ^A
BCR	1,337 $\pm$ 342.3 ^A
BD	1,390 $\pm$ 354.1 ^A

The mean values $\pm$ standard deviations are provided. One-way ANOVA with Tukey's test, $n = 12$, $p < 0.05$. PL, periodontal ligament; RRR, replacement root resorption; MTA, mineral trioxide aggregate; BCR, Bio-C Repair; BD, Biodentine.


Figure 2. The number of mean peak load and standard deviations required to cause root fractures in each experimental group.

PL: normal periodontal ligament; RRR: replacement root resorption; MTA plug: apical plug with MTA and root canal filling with Bio-C sealer and gutta-percha; BCR: Bio-C repair root canal filling; BD: Biodentine root canal filling.


Figure 3. Fracture modes of the specimens after load tests.

BD: Biodentine; BCR: Bio-C Repair; MTA: mineral trioxide aggregate; RRR: replacement root resorption; PL: periodontal ligament.

in the cervical and middle thirds, except for three samples in the BCR group, which showed enamel fractures. In addition, the teeth in the RRR group tended to fracture mainly in the cervical and middle thirds; however, 2 samples had vertical root fractures (**Figure 3**).

Cell cytotoxicity assay

RAW 264.7 viability results of BCR, BD and MTA groups with different extract dilutions are shown in **Figure 4**. The “dilution” and “material” factors significantly influenced the viability of macrophages ($p < 0.0001$). In addition, the interaction of the factors had a significant effect on the MTT assay result (2-way ANOVA, $F = 77.97$, $p < 0.0001$). There were

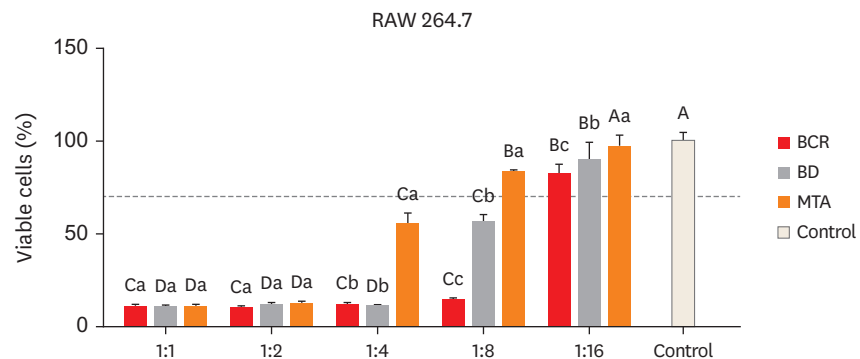


Figure 4. Cell viability percentage (%) of RAW 264.7 cells after exposure to extracts. Capital letters indicate comparisons between different material's dilutions and the control group for each material. Lowercase letters indicate comparisons between materials and dilutions. Groups identified by the same letter are not significantly different ($p > 0.05$). Two-way analysis of variance and Tukey's test ($p < 0.05$). BCR: Bio-C Repair; BD: Biodentine; MTA: mineral trioxide aggregate.

no statistically significant differences in the viability of the cells exposed to the 1:1 and 1:2 dilutions, regardless of the material evaluated ($p > 0.05$). The MTA group showed the highest viability values at dilutions of 1:4, 1:8, and 1:16 ($p < 0.0001$). For all the materials evaluated, the highest percentage of viable cells was found with the 1:16 dilution ($p < 0.0001$). All the groups showed lower viability than the control group ($p < 0.0001$), except for MTA at 1:16 dilution, which showed similar values ($p > 0.05$).

TRAP staining

Compared with the negative control RAW 264.7 cells, the RANKL-induced RAW 264.7 cells differentiated into TRAP-positive multinucleated osteoclasts. However, the number of TRAP-positive osteoclasts decreased after treatment with the extracts ($p < 0.0001$). The percentage of TRAP-positive cells was similar comparing BD and MTA, and BD and BCR ($p > 0.05$). BCR group showed a lower percentage of osteoclasts than the MTA group ($p < 0.0001$) (Figure 5).

DISCUSSION

In this study, the null hypothesis was rejected as significant differences were found between groups treated with different calcium silicate-based materials in the cell culture assays. However, the evaluated materials did not affect the fracture resistance of the immature permanent teeth subjected to RRR. Previous studies have emphasized the importance of strengthening the teeth that are in the early stages of development [12,15]. Dentin deposition on the root canals is not enough to reinforce the teeth, which are consequently predisposed to fracture [22]. Furthermore, it is important that the *in vitro* tests can reproduce the bone capacity to absorb masticatory load and support compressive and tangential forces in a fracture resistance test even under adverse conditions such as RRR. In this study, RRR was simulated through a combined protocol employing cylindrical drill wear and acid demineralization, with the selective inclusion of roots with elastomeric impression materials and polystyrene resin [16]. The biomechanical characteristics of the polystyrene resin differ from those of bone tissue, which represents a limitation of the study. Nonetheless, the simulation was performed in the same manner in all groups, to closely resemble the clinical situation. Furthermore, it is not possible to replicate the irregularities of the resorption cavities and regions with the absence of PL and ankylosis; therefore, the results may vary in clinical situations and should be analyzed with caution.

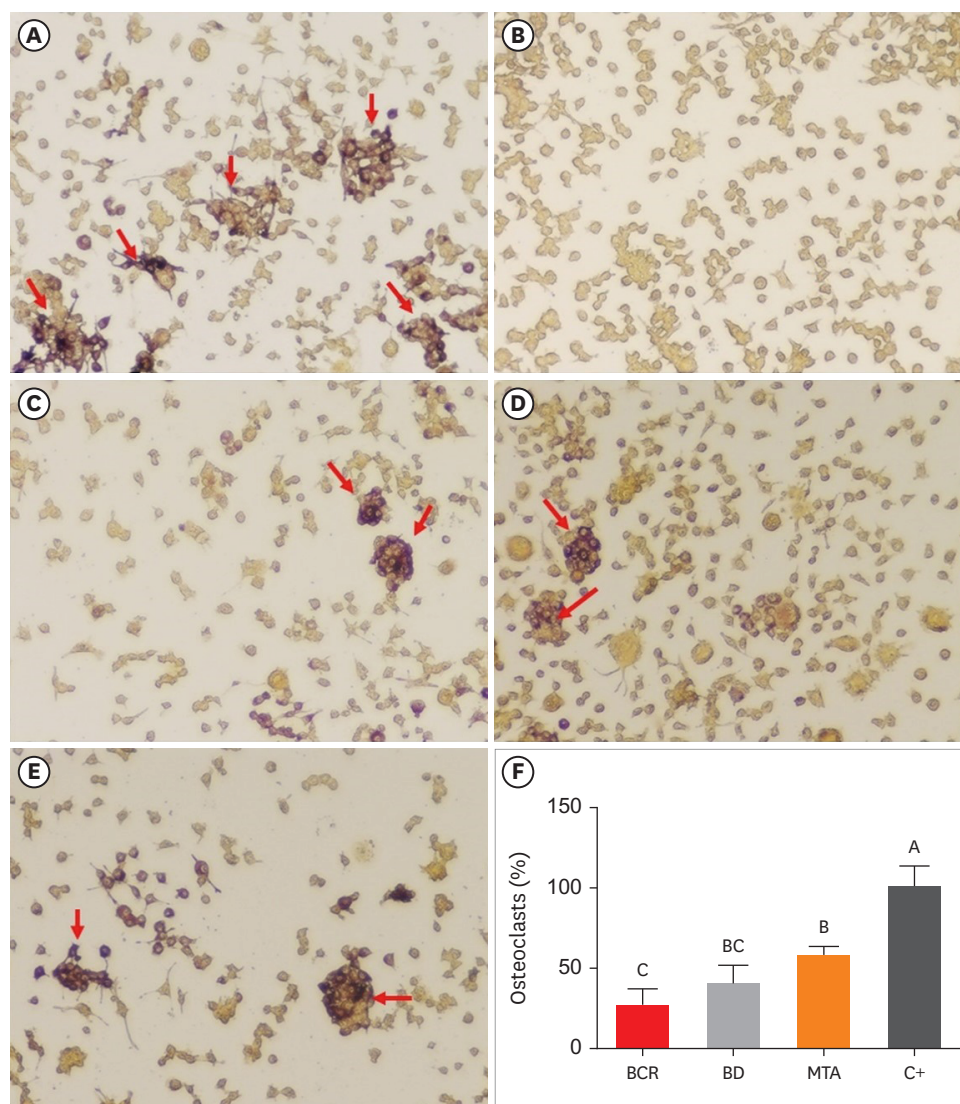


Figure 5. TRAP staining. (A) Positive control (RANKL); (B) Negative control (α -minimal essential medium); (C) Cells treated with Bio-C repair extract; (D) Cells treated with Biodentine extract; (E) Cells treated with MTA extract. TRAP-positive multinucleated cells (arrow); 10 $\times$ magnification. (F) TRAP activity: capital letters indicate comparisons among the materials tested. Groups identified by the same letter are not significantly different ($p > 0.05$). BCR: Bio-C Repair; BD: Biodentine; MTA, mineral trioxide aggregate; TRAP: tartrate-resistant acid phosphatase; RANKL, receptor activator of nuclear factor-kappa B ligand.

RRR occurs progressively, and it irregularly advances to the point of complete replacement of the root by bone [3]. Considering this pattern and the fact that ankylosis occurs significantly more often on the rounded labial and lingual surfaces than on the flat or proximal concave surfaces, this study simulated ankylosis and RRR only on the palatal root surface [23]. In addition, previous studies have demonstrated that immature maxillary incisors exhibit a high concentration of tensile stress on the palatal root surface, which is located within the structure that is more prone to failure [15].

In this study, the group with simulated ankylosis and RRR had a fracture load similar to that of the normal PL group. Notably, a previous study showed that teeth embedded directly in polystyrene resin have lower fracture resistance than those in which PDL simulation is performed

[17]. In these cases, PDL acts as a sustaining pad, thereby reducing alveolar bone stress [24]. When a force is applied, the tooth moves away from the rotation center that is compressing the periodontal fibers and causes bone distortion in the direction of the root movement [25]. Initially, the resistance of the periodontal fibers against the tooth displacement is low; however, as the tooth is forced within its alveolus, the load progressively increases [17]. In this study, the PL was partially simulated, which could explain the similarity between the groups.

In a previous study, CSM enhanced the resistance to horizontal root fractures when used as obturating materials for immature teeth [12]. Nonetheless, our results suggest that these materials cannot increase the fracture resistance of immature teeth with RRR. Moreover, there were no differences between the MTA plug group and the groups filled with the repair materials. These findings are consistent with those of previous studies and may be related to the limited ability of the CSM materials to bond to root dentin [26-28]. In addition, a retrospective clinical study demonstrated that immature teeth treated with MTA apexification and filled with gutta-percha or filled with MTA exhibited a similar rate of root fracture [29]. However, it is important to emphasize that the results of laboratory studies must be considered with caution and cannot be directly compared to those of clinical studies.

The mechanical tests in this study were carried out in bovine teeth because they have similar ultimate tensile strengths, dentine moduli of elasticity, and similar numbers, distributions, and diameters of dentinal tubules to human teeth [30-32]. Furthermore, a prior study established that bovine teeth can be used safely as substitutes for human teeth in fracture strength tests [33].

BCR is a CSM that was launched in the second half of 2019 with a ready-to-use protocol containing calcium silicate, calcium oxide, zirconium oxide, iron oxide, silicon dioxide, and a dispersing agent [9]. The absence of significant differences between the fracture values of the BD and BCR groups in this study could be attributed to the chemical/molecular similarities between these materials. Although there are no studies evaluating the fracture resistance of teeth filled with BCR, to the best of our knowledge, this material has cytotoxicity and mineralization-inducing abilities similar to those of BD [7,10]. However, a recent study showed that the compressive and adhesion strengths of this material are lower than those of BD [34].

The fractures in this study were mainly concentrated in the cervical and middle thirds of the roots. However, in the group simulating a normal PL, the fracture pattern was more homogeneous compared to that in the groups with RRR. This is because the PL transfers the stresses produced by the application of pressure over the coronal tooth structure to all the root surfaces [17]. Therefore, the teeth in the RRR, BCR, BD, and MTA plug groups presented more heterogeneous fracture patterns. Despite the similar fracture patterns between the teeth filled with CSM and gutta-percha, previous studies have shown that complete root canal filling with calcium silicate-based materials can be advantageous for an optimal biomechanical response [35]. This is because filling with CSM creates a monoblock with an interface that extends between the material and the canal walls [36]. In particular, BD is an ideal dentin replacement material, and it has been reported to decrease stress at the cervical root region by keeping the stress in its center while transferring stress to the mid-coronal region [35].

In this study, the murine macrophage lineage RAW 264.7 was used for the induction of osteoclastogenesis as these cells express RANK and can indefinitely grow as osteoclast precursors or differentiate into osteoclasts when co-cultured with RANKL. Although the

choice of permanent cell lines as study models is controversial, the RAW osteoclasts exhibit the essential characteristics of osteoclasts, notably the expression of TRAP and cathepsin K [37,38]. Furthermore, the use of immortalized cell cultures in a preliminary study assessing new materials, such as BCR, allows greater control of variables and easy reproducibility, and has been recommended in the literature [20,21].

Although previous studies have evaluated the cytotoxicity of these materials, this parameter is influenced by the cell type evaluated. Therefore, our viability analysis was conducted using RAW 264.7 to define a concentration at which all experimental groups presented with viability parameters > 70% and would not interfere with future osteoclastogenesis assays.

According to ISO 10993-5:2009, biomaterials that cause a reduction in cell viability by more than 30% are considered cytotoxic [19]. In the present study, the RAW 264.7 lineage showed cell viability rates > 70% only in the 1:16 dilution, suggesting that this cell line can be sensitive to the components present in the materials. These results differ from other studies that employed the same methodology, which reported human dental pulp cells viabilities > 80% even at 1:1 dilutions [8,39]. In another study, the biocompatibility and osteogenesis-inducing potential of BD, BCR, and MTA in cultures of human osteoblastic cells (SAOS-2) were evaluated, and the resulting cells demonstrated similar cell viability rates to those of the controls [10]. Different cell lineages may present divergent biological responses to each other, highlighting the relevance of performing cell viability analyses in different culture models [10]. Previous studies have shown that MTA did not affect RAW 264.7 viability after 24 hours and that BD and MTA were not cytotoxic to macrophages after 48 hours or 72 hours of incubation [21,40]. Despite that, aspects such as setting time, direct or indirect contacts and the preparation of the material extracted can directly influence the biocompatibility and bioactivity of the material, which prevents direct comparisons between studies [10]. The cell viability analysis method using different materials dilutions has wide support in the literature [8,10,40]. However, this method has its own limitations, as it is not possible to accurately quantify the components released by each CSM in the extracellular fluid.

RAW 264.7 osteoclasts express active TRAP enzymes, which are key markers involved in osteoclastic bone resorption. The results of this study showed that TRAP-positive multinucleated osteoclasts were induced at the highest rate in the positive control group, which is consistent with previous results [40]. All calcium-silicate cements evaluated showed a reduction in the number of differentiated osteoclasts. This is also in agreement with recent studies that reported that calcium silicate cements prevent osteoclastogenesis and bone resorption, consequently enhancing bone remodeling [40,41]. In addition, significant differences were not observed in the number of osteoclasts between the BD and MTA groups, as observed in other studies [40,42]. This may be associated with the similarity in the composition of the 2 materials [7]. Nevertheless, structural differences in osteoclasts, such as osteoclast size, the number of nuclei per cell, and the relationship between the contact of osteoclasts with the bone surface, are parameters directly associated with the cells' resorptive activity [43]. Moreover, laboratory studies in rats have demonstrated increased bone losses in regions of furcation perforation sealing when using BD compared to MTA, despite the lack of statistical differences in the number of osteoclasts [42].

In contrast to the other materials, BCR presented the lowest number of osteoclasts, which may be due to the lower concentration of calcium in its structure and the lower amount of calcium ions released in its solution compared to MTA and BD [7,8]. High extracellular

calcium concentrations modulate osteoclast differentiation and function, and osteoclastic bone resorption is directly regulated by calcitonin, and locally by ionized calcium (Ca^{2+}) generated as a result of osteoclastic bone resorption [44]. Furthermore, high concentrations of extracellular calcium cause cellular changes that markedly inhibit the TRAP enzyme and bone resorption [21].

A recent study demonstrated that BD microparticles and soluble leachable components at non-cytotoxic concentrations have anti-inflammatory, immunomodulatory effects and osteoprotective properties [45]. The mechanisms responsible for these included the suppression of several cytokines and the RANKL/Osteoprotegerin (OPG) ratio decrease [45]. In the clastic maturation process, RANKL binds its cognate RANK receptor on the surface of monocytes and macrophages and triggers their differentiation into mature multinucleated osteoclasts with bone resorption activity [46]. The action of RANKL is suppressed by its natural inhibitor OPG. OPG blocks RANKL/RANK interaction, and by doing this, it inhibits osteoclast activation and subsequent bone resorption [46]. In this sense, future studies should evaluate the effects of other materials based on calcium-silicate on the OPG/RANK/RANKL ratio, which could provide important information about the osteoprotective mechanism of these materials.

Traumatized immature permanent teeth have thin walls, are more prone to fracture, are difficult to obturate using conventional methods, and have accelerated resorptive processes. Therefore, it is essential to choose biomaterials that facilitate execution do not affect fracture resistance, and can control the healing process/resorption until the patient completes their craniofacial development.

CONCLUSIONS

In conclusion, the treatment options evaluated for non-vital immature teeth with RRR did not strengthen the teeth and provided similar resistance to fractures. BD, MTA, and BCR showed inhibitory effects on osteoclast differentiation, with BCR yielding more promising results.

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SUPPLEMENTARY MATERIAL

Supplementary Figure 1

Study methods.

[Click here to view](#)

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CAPÍTULO 4

Regenerative endodontic and intra-radicular resin composite filling on stress distribution of an immature maxillary central incisor: a 2D finite element analysis.

REGENERATIVE ENDODONTIC AND INTRA-RADICULAR RESIN COMPOSITE
FILLING ON STRESS DISTRIBUTION OF AN IMMATURE MAXILLARY CENTRAL
INCISOR: A 2D FINITE ELEMENT ANALYSIS.

SHORT TITLE: Biomechanics in Regenerative Endodontics.

Gabriela Leite de Souza,¹ Gabriel Felipe de Bragança,² Andomar Bruno Fernandes Vilela,² Bill Kahler,³ Carlos José Soares,⁴ Camilla Christian Gomes Moura.⁵

1 Gabriela Leite de Souza, Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil. ORCID: 0000-0003-3505-5258; e-mail: gabrielaleiteodon@gmail.com

2 Gabriel Felipe de Bragança, Department of Operative Dentistry and Dental Materials, School of Dentistry, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil. ORCID: 0000-0002-5082-4919; e-mail: gabriel.braganca@hotmail.com

2 Andomar Bruno Fernandes Vilela, Department of Operative Dentistry and Dental Materials, School of Dentistry, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil. ORCID: 0000-0003-2322-7067; e-mail: andomarbruno@live.com.

3 Bill Kahler, School of Dentistry, The University of Queensland, Brisbane, Australia. ORCID: 0000-0002-4181-3871; e-mail: w.kahler@uq.edu.au

4 Carlos José Soares, Department of Operative Dentistry and Dental Materials, School of Dentistry, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil. ORCID: 0000-0002-8830-605X ; e-mail: carlosjsoares@ufu.br

5 Camilla Christian Gomes Moura, Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil. ORCID: 0000-0002-5653-8403; e-mail: camillamoura@ufu.br

CORRESPONDING AUTHOR: Carlos José Soares

Department of Operative Dentistry and Dental Materials, School of Dentistry, Federal
University of Uberlândia.

E-mail address: carlosjsoares@ufu.br

Phone: 55 34 999713472; 55 34 3225811

SUMMARY

This study aimed to investigate the stress distribution during biting load on teeth undergoing regenerative endodontic treatment (RET) with different continued root development outcomes and compared with a tooth treated with MTA apexification reinforced with intra-radicular resin composite bulk fill, using the 2D finite element analysis (FEA) method. Five different 2D FEA models of an immature permanent maxillary incisor were created. These involved immediate post-op model after RET, models after RET varying root length and thickness (RET10%: 10% increase in root length and root thickness, RET20%: 10% increase in root length and 20% increase in root dentine thickness; RET40%: 10% increase in root length and 40% increase in root dentine thickness), and post-op model after MTA apexification reinforced with resin composite inserted into the root canal (RC). A biting load of 100 N was applied, and the stress distributions and residual shrinkage stress were analyzed using modified von Mises stress. Higher stress values in dentine and enamel were observed for models RET10%, RET20% and RET40% compared with RET model. RC presented the lowest dentine stress values. RC model had lower stress concentration at cervical region compared to the other models. The increase in root dentine thickness after RET had no significant benefit on the stress distribution of immature permanent teeth. MTA apexification followed by filling with bulk fill resin composite reduced intraradicular stress in cervical region compared to RET. Thus, MTA apexification followed by tooth filling using bulk fill resin composite is promising for the management of immature non-vital maxillary incisors.

KEYWORDS: apexification, regenerative endodontics, finite element analysis, nonvital tooth.

INTRODUCTION

Traumatic dental injuries represents one of the primary causes of tooth loss and pulp necrosis in adolescents and children. Pulp necrosis in immature teeth arrests further root development, resulting in open apical foramina and large root canals (1). Incompletely formed teeth remain with short and thin dentine walls, which have been shown to experience higher incidences of cervical root fracture by physiological biting load or secondary trauma, leading to reduced prognosis (2). The strengthening of immature necrotic teeth represents a significant endodontic and restorative challenge.

At the beginning of the 2000s, regenerative endodontic treatment (RET) was introduced as a treatment option for necrotic immature permanent teeth (3,4). It is a biology-based procedure which replaces the original cells of the pulp-dentine complex by mesenchymal stem cells. The primary and essential goal of the RET is the elimination of the symptoms and bone healing (5). However the root maturogenesis is an additional benefit that improve the long-term survival of immature teeth from biomechanical perspective (5,6). The continuation of the dentine deposition, promotes by RET, results on thickening of the canal walls, lengthening of the root, and apical maturation (5).

Root development achieved by RET ranging from 2-40% (7), but cervical third of the root remains weakened due to coronal plugs placed in the cementoenamel junction (8). Although the biological repair of the access cavity in the cervical root level with hard tissue bridge is reported among the favorable outcomes of regenerative endodontic procedures (3,4), it is not included in the goals of regenerative endodontic procedures. Several materials have been recommended for filling the cervical area of the root canal and reinforce immature teeth (9). Resin composite, that has similar stiffness with root dentine, inserted into the root canal may increase the mechanical resistance of the roots by bonding interaction with the dentine walls (10,11). Dentine is a hard but elastic tissue, with high deformation capacity (12). This tissue has the physiological function to accommodate chewing forces and resultant stresses, distributing them equally to adjacent structures and minimizing the stress concentration. Thus, a material with elastic modulus similar to dentine, like resin composite, can mimic the root dentine, reducing stress concentrations and producing better biomechanical results in endodontically treated teeth.

There are a lack of studies that evaluate the biomechanical performance of RET in relation to root development. In theory, teeth treated with RET should be stronger compared to

another therapeutic approaches indicated to treat necrotic immature permanent teeth (13). However, this has not to be proved. This study aimed to investigate the stress distribution during biting load on teeth treated with RET with different continued root development outcomes compared with a tooth treated with MTA apexification reinforced with intra-radicular resin composite, using the 2D finite element analysis (FEA) method. The null tested hypothesis was that both MTA apexification reinforced with intra-radicular resin composite and the increase in root length and thickness promoted by RET would not affect the stress distribution on immature permanent traumatized teeth.

MATERIALS AND METHODS

This study was approved by the local ethics committee (CAAE: 05397412.0.0000.5152). Images from a cone-beam computed tomography (CBCT) scan of a child aged 7 years old was obtained from the image bank of the School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil (Figure 1A), and was used to create two-dimensional (2D) models. An image of the sagittal cross-section of the maxilla and mandible was exported to an image processing software program (Image J, Public domain, National Institute of Health, Bethesda, MD, USA). Points were recorded on the outlines of cortical and trabecular bone, enamel, dentine, pulp, periodontal ligament, and surrounding soft tissue using the multi-point tools (Figure 1B). The coordinates obtained were exported to an FEA software package (Marc/Mentat, version 2010.2, MSC software, Santa Ana, CA, USA) and cubic-spline curves were drawn through them, recreating the tissue outlines for the FEA. The element mesh was created using four-node isoparametric arbitrary quadrilateral plane strain elements with reduced integration (one integration point per element, type number 115 in the Marc/Mentat software) (Figure 1C). Five FEA models simulating different treatment scenarios of an immature maxillary central incisor with thin walls, wide root canal and divergent apical opening at stage 3 based on Cvek's classification of root development (1) of immature maxillary central incisor were created as follows:

RET, post-operative scenario immediately using 3 mm coronal MTA plug in the root canal following RET (Figure 1D);

RET10%, post-operative scenario with 10% increase in root length and thickness using a 3 mm coronal MTA plug in the root canal following RET (Figure 1E);

RET20%, post-operative scenario with 10% increase in root length and 20% increase in root thickness using a 3 mm coronal MTA plug in the root canal following RET (Figure 1F);

RET40%, post-operative scenario with 10% increase in root length and 40% increase in root thickness using a 3 mm coronal MTA plug in the root canal following RET (Figure 1G);

RC, immediate post-operative scenario using a 3 mm apical MTA plug following filling of the root canal with a bulk-fill resin composite (Figure 1H).

All interfaces were considered bonded contact preventing relative motion along all model interfaces. Nonbonded contact was prescribed between the antagonist tooth and the maxillary incisor. The models were submitted to a 100 N loading, simulating a bite through the antagonist contact envelope movement between the maxillary and mandibular incisors. The functional contact loading was simulated in 50 increments, with 2N of load for each increment. A nodal displacement constraint (model fixation) was applied on top of the bone structure on the x- (horizontal) and y- (vertical) axes. A nonlinear structural analysis was performed, and all materials were considered linear, isotropic and homogeneous. The mechanical properties required for FEA expressed by elastic modulus (E) and Poisson's ratio (ν), and the compressive strength (CS) and diametral tensile strength (DTS) for all structures necessary for modified Von Mises stress calculation are shown in Table 1 (14-23). The stress distributions and residual shrinkage stress in all models were analyzed using modified von Mises equivalent stress (MVM). The modified von Mises criterion incorporates differences in compressive and tensile strengths in the classic von Mises criterion by including the hydrostatic stress (I_1) (15). The expression for MVM stress becomes: $\sigma_{mvm} = \frac{(SDE-1)I_1 + \sqrt{(SDE-1)^2 I_1^2 + 12SDEJ_2}}{2SDE}$ (15), where I_1 is the first invariant of stress tensor: $I_1 = (\sigma_1 + \sigma_2 + \sigma_3)$ and J_2 is the second invariant of the deviatoric stress tensor: $J_2 = [(\sigma_1 - \sigma_2)^2 + (\sigma_2 - \sigma_3)^2 + (\sigma_3 - \sigma_1)^2]/6$, where σ_1 , σ_2 and σ_3 are the first, second and third principal stress, respectively. The mean values of the 10% highest MVM stresses were determined for the enamel and dentine at shrinkage and at the final loading application. The modified von Mises stresses values were analyzed qualitatively. The stress values are expressed using a linear color scale bar, where yellow and light gray represent the highest stress values and blue and dark gray represent the lowest stress values. The ratio between the highest MVM stress value concentrated in dentine at the final loading application and dentine area for each model was determined. The dentine area was calculated with the aid of an image processing software program (Image J) through the region of interest manager.

RESULTS

Modified von Mises shrinkage stress generated for all models simulating RET and MTA apexification resin composite restored model is shown in Table 2 and Figure 2. All models showed high shrinkage stress concentrations at the increment interfaces (Figure 2).

The mean of the 10% highest modified von Mises stress values (MPa) in enamel and dentine at the peak of final biting loading are shown in Table 3. Higher stress values in dentine and enamel were observed for models with newly formed dentine (RET10%, RET20% and RET40%) compared with the model simulating the immediate preoperative of the RET (RET). The RC model had the lowest dentine stress values.

Modified von Mises equivalent stress distributions for the different models are shown in Figure 3. The models RET10%, RET20% and RET40% showed the highest stress concentrations at the cervical region, especially in the palatal face. For the model simulating the complete filling of the root canal with bulk fill resin composite (RC) lower stress concentration was observed at the cervical region, and more homogeneous stress distribution at root dentine compared to the other models was observed.

The ratio between the highest modified von Mises equivalent stress value in dentine at the final loading application and dentine area to each model are shown in Table 4. Higher values were demonstrated for RET10%, RET20% and RET40% compared to RET. RC model had the highest values.

DISCUSSION

The null hypothesis was accepted since the different outcomes in RETs promoted no significant variation on the stress values in dentin compared to immediate postoperative scenario and MTA apexification showed more homogeneous stress distribution for root dentine. Although it was expected that the root development would reduce the stress values, as previously reported (6,8) the deposition of mineralized tissue led to a slight increase in stress values after RET, regardless of the level of root development.

The rate of root maturogenesis after RETs is variable due individual parameters such as age of the patient, apical diameter and stage of root development that could influence the degree of root development after RET (24). A prospective study demonstrated that the root length can vary from 2.7% to 25.3% and the root dentine thickness from 1.9% to 72.6% (24). Considering

this high variability, the present study fixed the increase in root length at 10%, varying the root dentine wall thickness from 10% to 40%. Furthermore, Alobaid et al. (25) had suggested that an increase of 20% in root dentine thickness could be considered clinically meaningful. The results of the current study do not support that assumption, since our results suggest that in severely immature teeth even a 40% increase in dentin thickness does not seem to significantly improve the distribution of stresses in the dentine.

Greater predisposition to fracture in traumatized teeth according to the stage of root development has been reported (1). In the present study the stresses distribution was similar for all RET models. The similarity between the simulated conditions is in accordance with a recent study that demonstrated a similar fracture resistance and failure pattern for teeth treated with RET in both early and late stages (13). The root tip at stage 3 on Cvek's classification could provide biomechanical behavior similar to that of mature teeth (13). Although the methodologies are not the same, analysis of stress distribution by FEA complements the information of mechanical tests, such as fracture resistance, and offers an explanation of the failure patterns (11).

In this study a 100N functional biting load was used, based on values from previous studies which were found to vary between 59.5 and 155 N (26, 27). In order to mimic the antagonist contact envelop movement that occurs in the anterior bite, the lower incisor touches the upper incisor and slides backwards until it reaches the palatal middle third of the crown. To calculate the stress distribution on experimental models modified Von Mises stress failure criterion was used. Stress distribution pattern analysis in dentine showed that the cervical third of the root developed peak stresses in all the models. This area is, therefore, more prone for development of fracture line on loading and is in agreement with other FEA studies (8,11,28). This may be because of the cervical constriction and also due to the cervical region acting as a fulcrum on masticatory load application, leading to stress concentration (2). At physiological biting, the crown tends to move in the buccal direction while the root apex moves in the palatal direction. Since the palatal cortical bone surfaces surrounding the tooth move away from the rotation center, stress is expected to increase in the palatal cervical area with the force absorbed by periodontal ligament (2,29).

Endodontic access cavities in immature teeth can result in a wide access cavity to gain access to the wide canal. Usually this access cavity is restored at the cervical level of the root with biocompatible/bioactive calcium silicate plugs combined with a composite resin

restoration, which hinder the reinforcement of the immature tooth at the cervical level (8). Curiously, most of the regenerative literature focused on the continuous root development and the dentin wall thickening potential and little attention was given to the cervical area. The biological repair of the cervical region is as important as continuous root development and dentine wall thickening for the long-term prognosis of regenerative endodontic procedures and might account for the true fortification of the necrotic immature teeth (30). In the present study, the similar behavior between RET groups regarding the stress distribution pattern is justified since all groups remain with thin and fragile cervical root dentine.

In vitro studies have suggested a core built-up using resin composite into the root canal aiming to reinforce the immature teeth by bonding interaction to root dentine mediated by adhesive procedures (10,11). The conventional resin composite can have a limited polymerization at the deeper area into the root canal due to light attenuation caused by the distance from the light curing unit tip. Therefore, a bulk-fill composite resin was chosen to restore the MTA treated immature anterior teeth. Bulk-fill resin composites allow application of increments from 4-5 mm thickness, with a high degree of conversion associated with reduced shrinkage stress (31). Shrinkage stress generated during the resin composite bonded to dentine surface may result in crack propagation, marginal loss, secondary caries and postoperative sensitivity in vital teeth (31). Into the root canal, high shrinkage stress can cause debonding at the adhesive/resin composite/dentine interfaces (11). Shrinkage stresses cannot be measured directly; the use of finite element analysis (FEA) as a computational numerical analysis is considered the most comprehensive method to calculate the complex condition of the stress inside materials and dental structures (31,32). The RC group presented the highest shrinkage stress value due to the presence of additional resin composite increment for filling the root canal, supporting the theory that a higher number of increments can increase shrinkage stresses (32). On the other hand, this group showed the lowest values of stresses in the dentine, which may be related to the similar elastic modulus between bulk fill resin composite and dentine. Elastic modulus is defined as the ratio of stress to the corresponding strain in a material under loading (22). The resin composite elastic modulus influence on the residual stress distribution in the remaining tooth structures and at the tooth/restoration interfaces during the loading (11,29). The similar stiffness between the resin composite and root dentine associated with adequate bonding interface can create the unique body that facilitate the dissipation of the stress reducing the stress concentration (22).

The present study demonstrated that the deposition of mineralized tissue after RET increased the stress. Despite that, the increase in root dentine distributes the stresses over a larger area, as shown in the ratio between the highest modified von Mises equivalent stress value and dentine area. This is related to the fact that the neoformed tissue has a higher modulus of elasticity compared to the pulp. Dentine itself is a hard but elastic tissue and has the physiological function to bear chewing forces and distribute them equally to minimize stress (12). In this sense, studies have suggested that the deposition of tubular dentine might also have biomechanical advantages over reparative tissue due to a broader stress distribution along the root (6). Furthermore, according to the stress distribution pattern, the highest stresses were concentrated in the superficial dentine. Dentine becomes weaker closer to the pulp than near the dentine enamel junction, with average values of ultimate tensile strength of 61 MPa for superficial dentine and 33 MPa for deep dentine (33). The maximum stress values found in the present study were lower than values of ultimate tensile strength, assuming that it would not be able to lead to failure.

Few studies in the literature have evaluated the stress distribution in teeth that have undergone RET (6,8), which demonstrated reduced stress by using finite element simulation. However, in these studies, immature teeth with a more advanced root development stage were simulated compared to the present study. The analyses were performed for Von Mises equivalent stresses using a three-dimensional (3D) FEA. While the equivalent Von Mises stresses integrate all stress components into an equivalent stress value, the modified von Mises criterion is interesting because it considers the ratio between compressive and tensile strength, providing greater emphasis on regions that are more likely to fail under tension in fragile structures like dentine. Considering the averages of the 10% highest modified von Mises stresses for each structure eliminates the influence of singular peak values on the results, which would not happen when calculating the maximum principal stress. In addition, the loading scenario evaluated was different, being applied to a traumatic horizontal force load of 400 N (8) or a biting scenario with a load of 240 N in the incisal edge with an angle of 120° to the long axis of the tooth and a traumatic horizontal force load of 300 N (6). Considering that the force direction had an impact on stress distribution, these studies cannot be directly compared to what is being presented in the current study.

We conducted an FEA using a 2D model instead of a 3D model. Although 3D models can better reproduce the clinical characteristics, in some cases, this model can hinder the obtaining of a fine mesh, especially in thin regions, which can produce highly distorted elements

and unreliable numerical results (34). Moreover, there are studies validating the use of 2D models to assess different conditions in anterior teeth in the cross-sectional plane (35,36,37). The models outlined in the present study followed the same principles previously proposed (36,37), however, the referred studies evaluated the distribution of impact stresses during dental trauma and the present work evaluated a physiological loading scenario.

Despite the limitations of this study, since FEA presents idealized and simplified assumptions, in cases where it is not possible to test real situations in vivo or in laboratory conditions, this method has been shown to be an useful tool (2,22). Based on the current results, it is suggested that MTA apexification followed by tooth filling using bulk fill resin composite is promising for the management of immature non-vital maxillary incisors. Since there was not lower stress shown cervically in RET, this treatment modality did not have significant advantages for its recommendation.

A further limitation of this study is that RET protocols is a process of repair rather than true regeneration (38). These authors in an immunofluorescence study of a human tooth reported the neo-mineralized tissue exhibited more collagen and low mineral content that did interlock into the dentinal walls. However, it was suggested that the locking projections may lack strength as the newly formed tissue was shown to easily dissociate from the canal walls. Therefore, it is possible that the results of the current study for RET may be overstated as models assumed dentine regeneration rather than repair.

The results presented should be extrapolated with caution, and clinical decisions on treatment options for immature teeth should also take into consideration factors such as root development stage and patient's preference. Further scientific investigations should address more conservative access cavity design and development of regenerative protocols that can induce continuous root development, dentinal wall thickening, and biological repair of the radicular access opening. Such measures, together with improved restorative methods, could strengthen the tooth, especially in the cervical region.

Within the limitations of this study it can be concluded that the increase in root dentine thickness after RET had no effect on the stress distribution pattern of immature permanent teeth under physiological loading. Also, the treatment of immature teeth with MTA apexification followed by tooth filling using intra-radicular bulk fill resin composite reduced root dentine stress at cervical region compared to RET.

RESUMO

Este estudo teve como objetivo investigar a distribuição de tensões durante a carga mastigatória em dentes submetidos à tratamento endodôntico regenerativo (RET) com diferentes resultados na continuação do desenvolvimento radicular e comparar com um dente tratado com apicificação com MTA reforçado com resina composta bulk fill intra-radicular, usando o método de análise de elementos finitos 2D (FEA). Cinco diferentes modelos 2D FEA de um incisivo maxilar permanente imaturo foram criados. Estes envolveram um modelo de pós-operatório imediato após RET, modelos após RET variando o comprimento e espessura da raiz (RET10%: aumento de 10% no comprimento e espessura da raiz, RET20%: aumento de 10% no comprimento da raiz e aumento de 20% na espessura da dentina radicular; RET40%: aumento de 10% no comprimento da raiz e 40% na espessura da dentina radicular), e modelo pós-operatório após apicificação com MTA reforçado com resina composta inserida no canal radicular (RC). Uma carga de mordida de 100 N foi aplicada, e as distribuições de tensão e tensão de contração residual foram analisadas usando tensão de von Mises modificado. Maiores valores de tensão em dentina e esmalte foram observados para os modelos RET10%, RET20% e RET40% em comparação com o modelo RET. RC apresentou os menores valores de tensão na dentina. O modelo RC apresentou menor concentração de tensões na região cervical em relação aos demais modelos. O aumento da espessura da dentina radicular após o RET não teve benefício significativo na distribuição de tensões de dentes permanentes imaturos. A apicificação com MTA seguida de preenchimento com resina composta bulk fill reduziu o acúmulo de tensões intrarradicular na região cervical em comparação ao RET. Assim, a apicificação com MTA seguida de obturação do dente com resina composta bulk fill é promissora para o manejo de incisivos superiores imaturos não vitais.

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TABLES

Table 1. Mechanical properties for dental structures, MTA and resin composite applied in FEA.

Materials /Structures	Elastic Modulus (MPa)	Poisson's ratio	Diametral Tensile Strength (MPa)	Compressive Strength (Mpa)	References
Enamel	84100	0.30	10.3	384.0	Zarone et al. (14); Versluis and Tantbirojn (15)
Dentin	18600	0.31	98.7	297.0	Ausiello et al. (16); Versluis and Tantbirojn (15)
Pulp	2	0.45	2.94	2.94	Farah et al. (17); Tanaka et al. (18)
Periodontal ligament	50	0.45	-	-	Rees and Jacobsen (19)
Cortical bone	13700	0.33	-	-	Carter and Hayes (20)
Trabecular bone	1400	0.31	-	-	Carter and Hayes (20)
Soft Tissue	1.80	0.30	-	-	Holberg et al. (21)
MTA	11760	0.31	10.5	53.5	Belli et al. (22)
Tetric N-Ceram	12300	0.24	39.5	224.1	Borges et al. (23)

Table 2. Mean and standard deviation of top 10% stresses (MPa) on enamel and dentine at shrinkage of FEA.

Groups	Enamel	Dentine
RET	17.4 (3.8)	4.4 (2.7)
RET10%	17.5 (3.8)	4.4 (1.3)
RET20%	17.5 (3.9)	4.4 (1.3)
RET40%	17.5 (3.9)	4.4 (1.3)
RC	18.1 (4.6)	6.4 (1.5)

Table 3. Mean and standard deviation of top 10% stresses (MPa) on enamel and dentine at a final movement of FEA.

Groups	Enamel	Dentine
RET	19.1 (8.1)	19.0 (3.5)
RET10%	22.0 (8.7)	21.5 (4.1)
RET20%	22.0 (8.7)	21.4 (4.1)
RET40%	22.0 (8.7)	21.1 (4.2)
RC	22.5 (8.7)	15.3 (6.7)

Table 4. Ratio between the highest MVM stress value in dentine (MPa) at a final movement of FEA and dentine area (mm²).

Groups	Area (mm ²)	Highest MVM stress value (MPa)	Highest MVM stress value/area (MPa/mm ²)
RET	35.9	27.3	0.76
RET10%	37.9	31.3	0.83
RET20%	39.0	31.2	0.80
RET40%	39.8	31.2	0.78
RC	35.9	30.4	0.85

FIGURES LEGENDS

Figure 1. Generation of cross-sectional finite element models. A, CT-tomography image of an immature maxillary central incisor; B, Points traced on the CT image in an image processing software; D, RET- immediately after RET; E, RET10%- 10% increase in root length and thickness after RET; F, RET20%- 10% increase in root length and 20% increase in root thickness after RET; G, RET40%- 10% increase in root length and 40% increase in root thickness after RET; H, RC- 3 mm apical MTA plug following filling of the root canal with a bulk-fill resin composite.

Figure 2. Shrinkage stress (MPa) distribution for RET, immediately after RET; RET10%, 10% increase in root length and thickness after RET; RET20%, 10% increase in root length and 20% increase in root thickness after RET; RET40%, 10% increase in root length and 40% increase in root thickness after RET; RC, 3 mm apical MTA plug following filling of the root canal with a bulk-fill resin composite.

Figure 3. Modified Von Mises stress distributions at final movement after applying a 100 N force mimicking bite loading. RET, immediately after RET; RET10%, 10% increase in root length and thickness after RET; RET20%, 10% increase in root length and 20% increase in root thickness after RET; RET40%, 10% increase in root length and 40% increase in root thickness after RET; RC, 3 mm apical MTA plug following filling of the root canal with a bulk-fill resin composite.

FIGURES

FIGURE 1

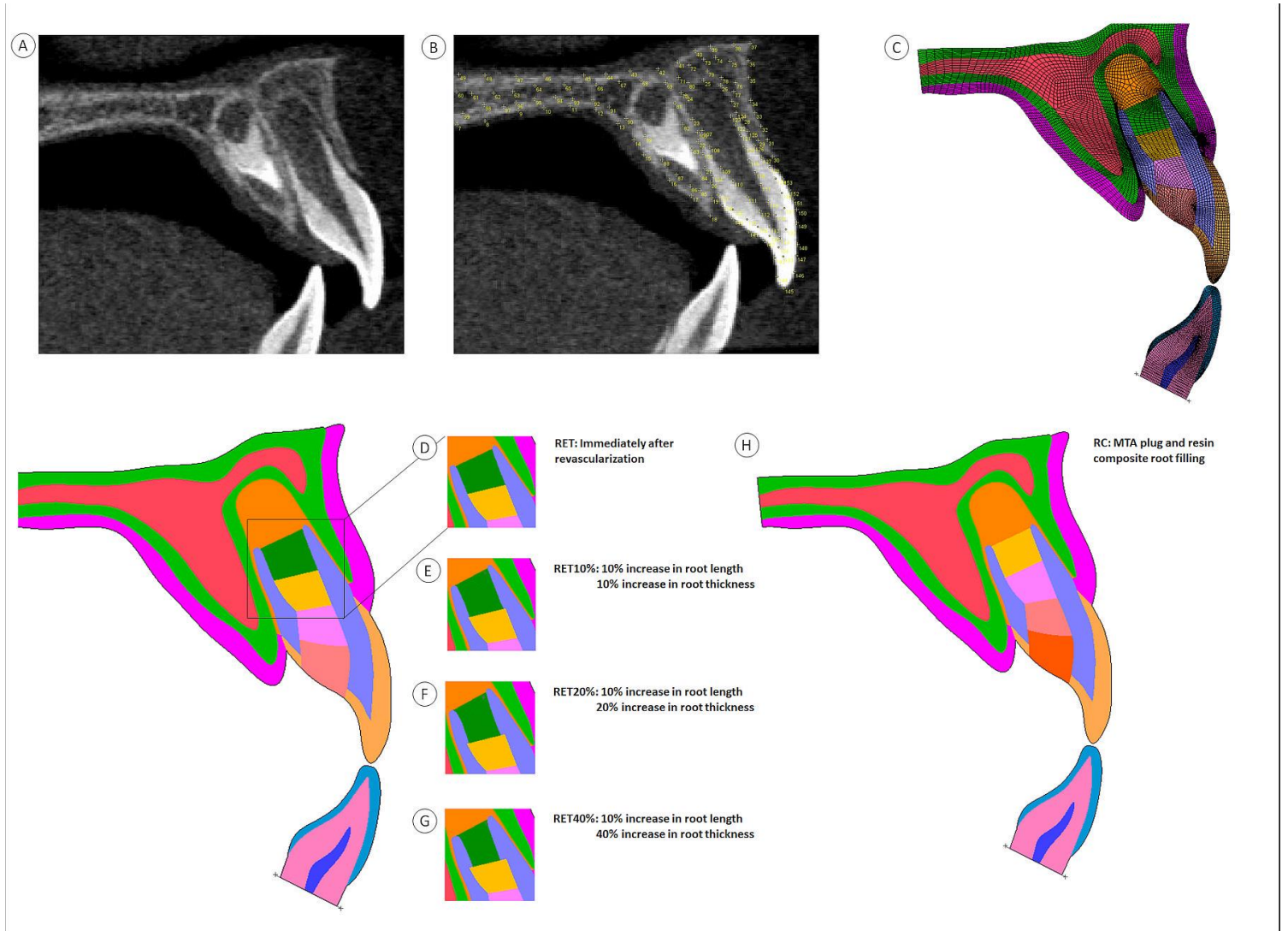


FIGURE 2

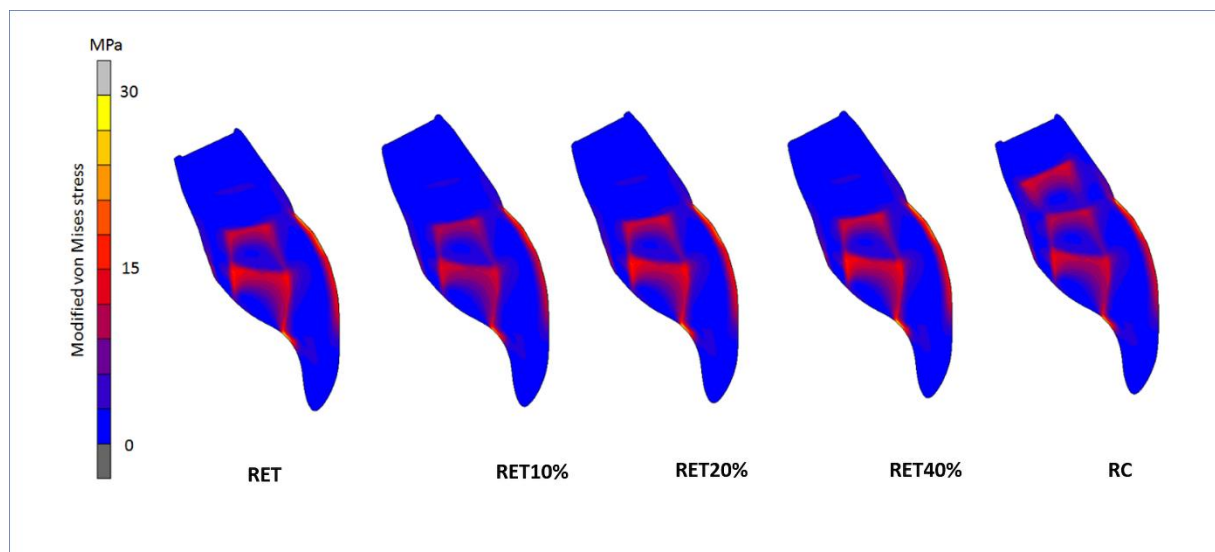
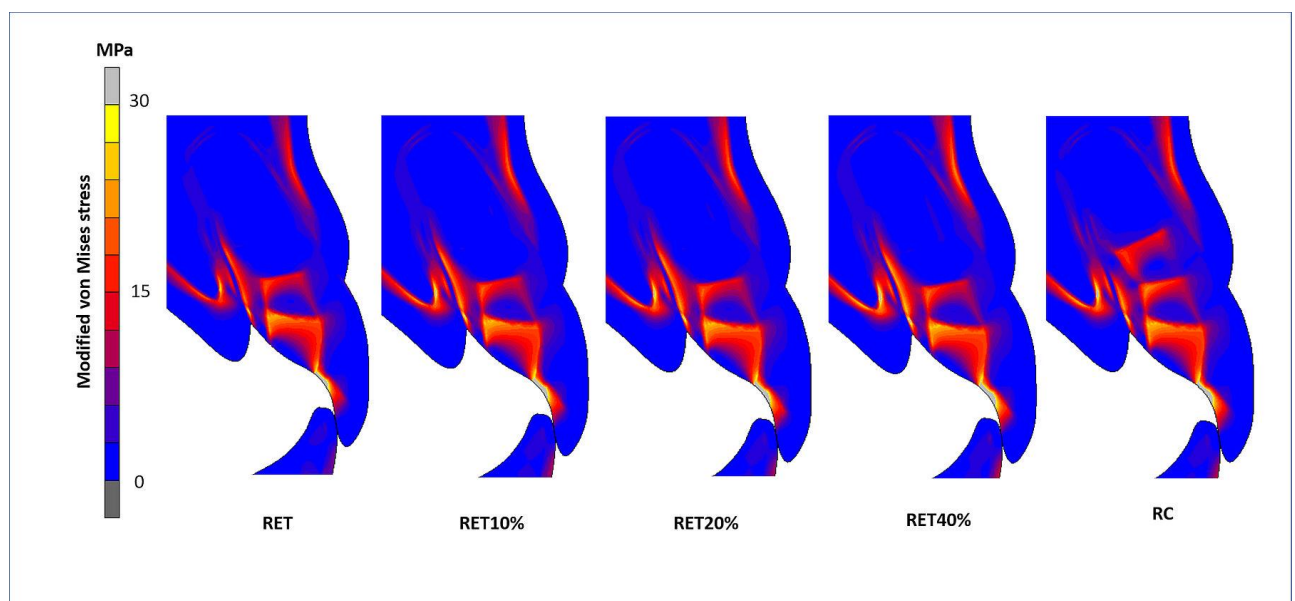


FIGURE 3



CAPÍTULO 5

Terapias pulpares conservadoras: uma abordagem multidisciplinar para dentes traumatizados

TERAPIAS PULPARES CONSERVADORAS: UMA ABORDAGEM MULTIDISCIPLINAR PARA DENTES TRAUMATIZADOS

Gabriela Leite de Souza¹, Camilla Christian Gomes Moura^{2*}, Carlos José Soares³, Sávio Gabriel Silva Rende⁴, Priscilla Barbosa Ferreira Soares⁵.

¹ Programa de pós graduação em odontologia, Faculdade de Odontologia, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brasil. ORCID: 0000-0003-3505-5258; e-mail: gabrielaleiteodon@gmail.com.

² Departamento de Endodontia, Faculdade de Odontologia, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brasil. ORCID: 0000-0002-5653-8403; e-mail: camillamoura@ufu.br

³ Departamento de Dentística Operatória e Materiais Dentários, Faculdade de Odontologia, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brasil. ORCID: 0000-0002-8830-605X ; e-mail: carlosjsoares@ufu.br

⁴ Residente em pacientes com necessidades especiais, Faculdade de Odontologia, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brasil. ORCID: 0000-0001-5907-7852; e-mail: rendesavio@gmail.com

⁵ Departamento de Periodontia, Faculdade de Odontologia, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brasil. ORCID: 0000-0002-4492-8957; e-mail: pbfsoares@gmail.com

***Autor de correspondência:** Camilla Christian Gomes Moura, Professora Doutora, Departamento de Endodontia, Faculdade de Odontologia, Universidade Federal de Uberlândia. Avenida Pará 1720, Campus Umuarama, Bloco 4L, Anexo A, Uberlândia, MG, 38405-328, Brasil. Email: camillamoura@ufu.br

RESUMO

Esse relato de caso discute o tratamento multidisciplinar de uma menina de 9 anos de idade que sofreu uma fratura complicada de coroa no incisivo central superior esquerdo (dente 21) e uma necrose tardia no incisivo lateral superior esquerdo (dente 22), devido à uma queda de própria altura. No tratamento emergencial, foi feita proteção pulpar direta no dente 21 e, após 2 meses, realizou-se o tratamento especializado dos dentes traumatizados. O dente 21 foi tratado com pulpotomia parcial a fim de preservar a vitalidade pulpar e permitir o desenvolvimento radicular, seguido de tracionamento ortodôntico, cirurgia a retalho para restabelecimento de espaço supracrestal e restauração transcirúrgica em resina composta. Nove meses após a realização da pulpotomia (12 meses após o trauma), o dente 21 e o dente 22 foram diagnosticados com necrose pulpar. O dente 21 havia terminado o desenvolvimento radicular, e por isso, foi realizado o tratamento endodôntico convencional. Já o dente 22, apresentava-se com desenvolvimento radicular incompleto, sendo proposto para ele tratamento endodôntico regenerativo, pela técnica tradicional de revascularização, utilizando coágulo como arcabouço. Nos acompanhamentos clínico, radiográfico e tomográfico, a paciente apresentou-se assintomática, e todos os dentes envolvidos demonstraram completa formação radicular e aspecto de normalidade periapical, com preservação de lâmina dura e do espaço do ligamento periodontal. A paciente foi acompanhada por 4 anos, com evidência de sucesso clínico do tratamento proposto.

INTRODUÇÃO

O traumatismo dentário contabiliza aproximadamente 5% de todas as injúrias e acomete principalmente crianças e adolescentes (Andersson 2013), podendo gerar diversas complicações, como descoloração do dente, mobilidade, má oclusão, necrose pulpar e eventualmente perda do elemento dental (Matoug-Elwerfelli et al. 2022). A perda de vitalidade pulpar, especialmente em dentes imaturos, pode reduzir significativamente o prognóstico a longo prazo de dentes traumatizados (Nazzal & Duggal, 2017). Portanto, a preservação da vitalidade pulpar por meio de terapias vitais pulpares (TVP) é um componente chave no manejo de dentes traumatizados (Cohenca et al., 2013; da Rosa et al., 2018).

Dentre as TVP, a pulpotomia envolve a remoção do tecido pulpar inflamado e contaminado, variando o nível de amputação pulpar de acordo com a quantidade de tecido irreversivelmente comprometido após a exposição (Bimstein & Rotstein 2016). Enquanto na pulpotomia total, toda a polpa coronária é removida até o nível dos orifícios do canal (Taha et al., 2018), na pulpotomia parcial (PP) também chamada de pulpotomia Cvek, apenas 2-3 mm de tecido coronário inflamado é removido (Bimstein & Rotstein 2016; Yang et al., 2020). De acordo com as diretrizes recentemente publicadas pela Associação Internacional de Traumatologia Dentária (IADT), a PP é agora o tratamento recomendado para exposições pulpares traumáticas em dentes com desenvolvimento radicular completo e incompleto devido às suas altas taxas de sucesso (86%-100%) (Bourguignon et al. 2020).

Apesar dos esforços para preservar a vitalidade pulpar, a necrose pulpar tem sido considerada a complicação pós-traumática mais comum para todos os tipos de trauma dentário (Anderson 2013, Bourguignon 2020). A terapia endodôntica para dentes permanentes imaturos com necrose pulpar é um desafio, devido às limitações anatômicas tais como paredes dentinárias finas e ápice aberto (Diogenes et al. 2016, Matoug-Elwerfelli et al. 2022). Nesse sentido, a terapia endodôntica regenerativa (TER) passou a representar a primeira opção de tratamento para esses casos (Associação Americana de Endodontia - AAE, 2023), permitindo a formação de um novo tecido reparador vascularizado com algumas respostas imune e fisiológicas (Diogenes et al. 2016), continuação do desenvolvimento radicular, fechamento e cicatrização apical.

No entanto, lesões dentárias em dentes permanentes imaturos podem ser complexas, exigindo uma abordagem multidisciplinar para permitir o desenvolvimento radicular contínuo, restaurando a função e a estética do paciente. O objetivo deste relato de caso foi apresentar uma abordagem de gerenciamento de uma menina de 9 anos de idade que incluiu PP, TER, tratamento de canal radicular convencional, considerações periodontais e restauradoras assistidas por extrusão ortodôntica (Comitê de Ética em Pesquisa da Universidade Federal de Uberlândia – CAAE: 08680919.0.0000.5152). Este caso fornece um exemplo de cooperação entre diferentes disciplinas, lidando com processos de tomada de decisão em situações clínicas complexas em um sistema público de saúde.

RELATO DE CASO

Paciente do sexo feminino, 9 anos de idade, compareceu à clínica de Traumatismo Dentoalveolar da Faculdade de Odontologia da Universidade Federal de Uberlândia (Uberlândia, Minas Gerais, Brasil) com a queixa principal de fratura coronária. Um termo de consentimento válido para futura avaliação foi obtido da sua mãe. Seu histórico médico foi revisado e não contribuiu para o desenvolvimento do caso.

Sua história dental revelou que teve o rosto atingido após uma queda no chão, resultando na injúria dental traumática e laceração do lábio superior. A terapia de emergência foi realizada no pronto atendimento odontológico do Hospital de Clínicas da Universidade Federal de Uberlândia (UFU) onde exame dental e tratamento foram providenciados. O incisivo central superior esquerdo (dente 21) apresentou fratura complicada de coroa em terço cervical com exposição pulpar (o paciente não encontrou o fragmento fraturado) e foi tratado com capeamento pulpar direto utilizando-se cimento de hidróxido de cálcio (Dycal, Dentsply Sirona, São Paulo, SP, Brasil) seguido de restauração provisória com ionômero de vidro quimicamente ativado (Maxxion R, FGM, Joinville, SC, Brasil). Os incisivos laterais superiores (dente 12 e 22) ainda não haviam irrompido. O incisivo central superior direito (dente 11) não foi acometido pelo trauma. Após o tratamento de urgência, a paciente foi referenciada para o atendimento especializado na clínica de Traumatismo Dento-alveolar da Faculdade de Odontologia da UFU para dar continuidade ao tratamento.

Após 2 meses, a paciente compareceu ao atendimento especializado. Nenhum achado extra-oral anormal foi encontrado. Os dentes 12 e 22 estavam em processo de

irrupção, não sendo possível ainda realizar o teste de sensibilidade pulpar. O dente 11 apresentava teste de sensibilidade pulpar ao frio normal e o dente 21 inconclusivo, devido ao pequeno remanescente de estrutura coronária. O exame clínico e radiográfico do dente 21 revelou uma fratura complicada de coroa, envolvendo terço cervical, restauração em ionômero de vidro parcialmente fraturada, com indícios de infiltração (Figura 1A-D). A linha de fratura estava localizada 1 mm supragengivalmente no aspecto vestibular, e subgengivalmente a nível de crista alveolar no aspecto palatino (Figura 1A-B). Nenhuma sensibilidade à percussão ou palpação foi observada em nenhum dos dentes. Após uma discussão multidisciplinar, incluindo uma avaliação ortodôntica e periodontal, o plano de tratamento foi formulado. Indicou-se para os dentes 12, 11 e 22 a preservação, e para o dente 21, devido às características acima relatadas, planejou-se pulpotomia seguido de tracionamento ortodôntico, aumento de coroa clínica e restauração em resina composta.

Sob anestesia local com lidocaína 2% e adrenalina 1:100000 (Alphacaine, DFL, Rio de Janeiro, RJ, Brasil) e isolamento absoluto modificado, foi realizado o acesso cavitário do dente 21 com uma broca multilaminada esférica #2 (KG Sorensen, Barueri, SP, Brasil). Devido às características do tecido pulpar, optou-se pela realização da pulpotomia parcial, amputando aproximadamente 4 mm do tecido pulpar. A hemostasia foi atingida pela irrigação com hipoclorito de sódio (NaOCl) 2,5% (Asfer, São Caetano do Sul, SP, Brasil) e por colocação gentil de algodão estéril sobre a polpa amputada. Em seguida, o tecido pulpar remanescente foi tratado com uma medicação corticosteroide baseada em sulfato de neomicina (5 mg/mL), hidrocortisona (10 mg/mL) e sulfato de polimixina (10.000UI) (Otosporin, FQM, São Paulo, SP, Brasil) por 5 minutos, e protegido com uma camada de 2 mm de espessura de MTA repair (Angelus, Londrina, PR, Brasil) (Figura 2A), o qual foi manipulado de acordo com as instruções do fabricante. Uma restauração provisória de cimento de ionômero de vidro quimicamente ativado (Maxxion R, FGM) foi feita, para que posteriormente o dente fosse restaurado com resina composta (Figura 2B).

Após 4 semanas realizou-se uma restauração em resina composta para instalação do braquete ortodôntico no elemento 21. A restauração foi feita utilizando-se tira de poliéster para confecção da camada palatina. Os protocolos adesivos foram seguidos, e a inserção da resina foi feita utilizando-se técnica incremental, com resina Filtek Z350 cor EA2 para esmalte e DA2 para dentina (3M-ESPE, St. Paul, MN, EUA), seguido de fotopolimerização. O dente apresentava-se assintomático.

Após 2 meses, observou-se indícios radiográficos de continuação do processo de rizogênese com diminuição do diâmetro apical e iniciou-se o tracionamento ortodôntico (Figura 3A). O braquete ortodôntico foi ancorado em uma posição ligeiramente mais cervical em relação ao alinhamento com os dentes adjacentes (Figura 3B). Foi utilizado um arco adaptado 0,018 polegadas de Níquel-Titânio. A cada 2 a 3 semanas o tracionamento foi ativado, tendo alcançado 4 mm de extrusão em 7 semanas. Após esse período, o aparelho ortodôntico foi removido e realizou-se o procedimento cirúrgico a retalho para restabelecimento de espaço supracrestal seguido da restauração em resina composta definitiva.

Após anestesia infiltrativa local empregando solução anestésica a base de lidocaína 2% com adrenalina 1:100.000 (Alphacaine, DFL), realizou-se incisão intrasulcular da mesial do dente 22 à mesial do dente 11 por vestibular e palatina empregando lâmina de bisturi n. 15C (Solidor, Barueri, SP, Brasil). Em seguida fez-se o descolamento do retalho de espessura total e removeu-se todo o tecido de granulação por meio de raspagem e alisamento radicular com auxílio de curetas periodontais. Verificou-se por mensuração com sonda milimetrada a quantidade necessária de osso a ser removida para o reestabelecimento da distância biológica, então foi realizada a osteotomia com cinzéis de Ochsenbien nas faces vestibular e palatina e nas faces mesial e distal confirmando o restabelecimento da distância biológica de 3 mm conseguida entre o término da fratura e a crista óssea.

Após a osteotomia foi realizado o isolamento absoluto do campo operatório com dique de borracha e uso de grampo n. 212 modificado para que houvesse exposição do limite da fratura. A reconstrução coronária foi realizada após a hibridização do substrato dentário por meio de condicionamento com ácido fosfórico a 37% (Condac 37, FGM) e posterior aplicação e fotoativação do sistema adesivo simplificado (Adper SingleBond, 3M-ESPE) seguindo as especificações do fabricante. Em seguida, foi reconstruída de forma incremental a face palatina utilizando a resina Filtek Z350 A2E e Filtek Z350 A2B (3M-ESPE) para a mimetização da dentina coronária. Uma última camada de resina cor A2E foi inserida na face vestibular para promover translucidez semelhante ao esmalte (Figura 4A). Após a remoção do isolamento absoluto, procedeu-se a remoção dos excessos de restauração com pontas diamantadas fina e ultrafina (KG Sorensen) e polimento com pontas de silicone (KG Sorensen) na área subgengival, seguido da sutura do retalho gengival (Figura 4B). Após 15 dias, a paciente

retornou para remoção de sutura, possibilitando verificar normalidade do processo de cicatrização. Nesta seção foi realizado refinamento do acabamento e novo polimento com pontas de silicone e disco de feltro (Diamondflex, FGM).

A paciente foi reavaliada após 30 dias e 90 dias do procedimento cirúrgico. Nesses acompanhamentos, os testes de percussão, palpação e sensibilidade pulpar foram realizados em todos os dentes anteriores. No acompanhamento de 30 dias, observou-se sensibilidade positiva nos elementos 11, 12 e negativa nos elementos 21 e 22, sem dor à percussão vertical, horizontal e nem à palpação. Em 90 dias, o teste de sensibilidade pulpar apresentou os mesmos resultados, contudo no teste de percussão vertical, a paciente relatou dor nos elementos 21 e 22. Desta forma, diagnosticou-se os elementos 21 e 22 com necrose pulpar, procedendo-se então a intervenção endodôntica através de tratamento endodôntico convencional, no elemento 21, e TER no elemento 22, visto que os exames radiográficos periapicais (Figura 5A) e tomografia computadorizada de feixe cônico (CBCT) revelaram formação radicular incompleta, com ápice amplo e paredes dentinárias finas e divergentes (Figura 5B-C).

Imediatamente após o diagnóstico, deu-se início à primeira sessão do procedimento de TER, após anestesia infiltrativa e papilar com lidocaína 2% + 1:100.000 (Alphacaine, DFL) e isolamento absoluto. A abertura coronária foi feita com ponta esférica diamantada 1012 (KG Sorensen) e broca endo Z (Malleifer, Dentsply Sirona), seguida de irrigação abundante com NaOCL 2,5% (Asfer). O comprimento de trabalho foi estabelecido em 1 mm aquém do ápice radiográfico com uma lima K #25 (17 mm) (Malleifer, Dentsply Sirona). As paredes do canal não foram ampliadas; limas de pequeno calibre foram usadas em movimentos circunferenciais suaves para deslocar o tecido pulpar necrótico e a irrigação foi realizada com 30 mL de NaOCL 2,5% (Asfer). Uma lavagem final com solução salina foi realizada e o canal foi seco com pontas de papel absorvente (Malleifer, Dentsply Sirona). Em seguida uma medicação intracanal com hidróxido de cálcio (Ultracal®, Ultradent, Indaiatuba, SP, Brasil) foi aplicada no comprimento de trabalho e selamento provisório com cimento de ionômero de vidro fotopolimerizável (Riva Light Cure®, SDI, São Paulo, SP, Brasil) foram feitos.

A segunda sessão da revascularização foi realizada 15 dias após a primeira. Sob anestesia infiltrativa com lidocaína sem vasoconstritor 2% (Cristália, SP, Itapira, Brasil) e isolamento absoluto, a remoção da medicação foi feita com irrigação abundante de NaOCl 2,5% (Asfer), e ativação com ponta EasyClean® (Easy, Belo Horizonte, MG,

Brasil), seguido de protocolo em 6 ciclos, sendo 3 ciclos de 20s de NaOCl 2,5% (Asfer) e 3 ciclos de 20s EDTA trissódico a 17% (Maquira, Maringá, PR, Brasil). A promoção de coágulo foi executada com lima tipo Kerr #40 (Malleifer, Dentsply Sirona) gentilmente introduzida no canal, em 2 mm além do comprimento de trabalho (19 mm), com suaves movimentos de rotação, para promover o sangramento da região periapical até que este fosse visível na abertura coronal do canal. Após 15 minutos, houve completa geleificação de coágulo à nível cervical, e um tampão cervical de MTA repair (Angelus, Londrina, PR, Brasil) de 3 mm de espessura foi colocado sem pressão sobre o coágulo sanguíneo (Figura 6A). A cavidade de acesso foi restaurada com um ionômero de vidro Ketac™ Molar-Applicap (3M ESPE) e compósito fotopolimerizável Filtek Z350 XT (3M-ESPE).

O tratamento endodôntico do dente 21 começou 1 mês após a finalização da revascularização. Sob anestesia infiltrativa local (lidocaína 2% com adrenalina 1:100.000) e isolamento absoluto, realizou-se abertura coronária e o preparo químico-mecânico foi realizado pela técnica de Goering modificada com a lima memória K#70. Em seguida uma medicação intracanal com hidróxido de cálcio (UltraCal, Ultradent) foi aplicada no comprimento de trabalho e selamento provisório com cimento de ionômero de vidro fotopolimerizável (Riva Light Cure®, SDI, São Paulo, SP, Brasil) foram feitos (Figura 7A).

Devido à pandemia, e à suspensão de atendimentos, a paciente retornou apenas 15 meses após o início do tratamento endodôntico do dente 21. Nesse momento, foi feita a reinstrumentação do canal radicular e nova troca de medicação intracanal. Após 15 dias, realizou-se a obturação do canal radicular pela técnica do cone rolado, com cimento endodôntico Sealer 26 (Dentsply Sirona) (Figura 7B-C). A cavidade de acesso foi restaurada com um ionômero de vidro (Maxxion R, FGM) e compósito fotopolimerizável Filtek Z350 (3M-ESPE). Neste momento também foi feito o acompanhamento clínico e radiográfico do elemento 22, sendo observado desenvolvimento radicular em espessura e comprimento, fechamento apical, espaço do ligamento periodontal e lâmina dura normais, com ausência de rarefação óssea periapical (Figura 6B).

Após 3 meses do tratamento endodôntico do dente 21 finalizado (1 ano e 10 meses após a finalização da revascularização do dente 22), realizou-se novo acompanhamento clínico, radiográfico e tomográfico (Figura 6C). Os elementos 11 e 12 mantiveram a sensibilidade pulpar positiva. O dente 22, que passou pelo tratamento de revascularização, apresentava sensibilidade pulpar negativa. Todos os dentes apresentaram ausência de dor

nos testes de percussão vertical e horizontal, e ausência de mobilidade. Radiograficamente e tomograficamente, os dentes 21 e 22 apresentaram normalidade periapical, com espaço do ligamento periodontal e lâmina dura preservados. Radiograficamente, observou-se a completa obliteração do canal radicular. Após analisar a tomografia, foi possível concluir que houve um estímulo à formação radicular do dente revascularizado, com formação de conduto radicular atrésico. Em 4 anos de acompanhamento, a paciente apresentou-se com ausência de dor nos testes de percussão vertical e horizontal, ausência de mobilidade, e pequena alteração de cor no dente 21 (Figura 8A-B). Radiograficamente, todos os dentes envolvidos no trauma mantiveram o aspecto de normalidade periapical (Figura 8C-D).

DISCUSSÃO

No presente caso o dente 21 foi o principal acometido pelo trauma, apresentando uma fratura complicada de coroa com exposição pulpar. O atendimento de urgência foi realizado após 4 dias no Pronto Atendimento, por meio de capeamento pulpar direto. Neste contexto temos duas tomadas de decisão que podem comprometer a longevidade pulpar, uma vez que a ausência de isolamento absoluto adequado favorece a contaminação do remanescente pulpar por saliva, e a opção por capeamento pulpar direto, segundo a literatura recente, apresenta prognóstico inferior ao das pulpotomias parciais (Wang et al. 2017, Donnelly et al. 2022). Considerando-se a extensão da exposição pulpar, o tempo até a realização do capeamento (Bourguignon et al. 2020; American Academy of Pediatric Dentistry, 2021; European Society of Endodontology 2021) e o fato de que o agente capeador direto ocupa partes do espaço de dentina prejudicando a adesão da futura restauração (Wang et al. 2017), no atendimento especializado, optou-se pela realização da pulpotomia parcial.

Nas pulpotomias parciais, é indicado a remoção de aproximadamente 3 mm de tecido coronário inflamado, sendo a quantidade de tecido a ser removido determinada pelas características pulpareas como cor, consistência ao corte e tempo de sangramento (Donnelly et al. 2022). Clinicamente, caso o tamanho da área do defeito não seja suficiente para colocar uma restauração, 1 a 2 mm adicionais de tecido pulpar precisa ser removido para aumentar a área de união utilizando a câmara pulpar para retenção (Hu et al. 2023). No presente caso, aproximadamente 4 mm de tecido pulpar coronal foi removido até atingir sangramento saudável, estabelecer a hemostasia adequada e permitir uma restauração satisfatória. A literatura indica que idealmente a pulpotomia parcial deva

ser feita de 24 h até 9 dias após a exposição (American Academy of Pediatric Dentistry 2021, Matoug-Elwerfelli et al. 2022). Dessa forma, é importante ressaltar que o atraso de dois meses na procura do atendimento especializado, a possível infiltração devido à fratura da restauração provisória e a dificuldade no isolamento devido ao remanescente coronário insuficiente e frágil, levando a necessidade de uso de isolamento absoluto modificado, são fatores que podem ter impactado na manutenção a longo prazo da vitalidade. Contudo é importante ressaltar que o tempo de exposição pulpar isoladamente não é determinante para o sucesso da pulpotomia parcial, desde que o tecido pulpar superficial inflamado seja amputado ao nível do tecido pulpar saudável, sendo relatado sucesso em pulpotomias feitas até 21 dias após a exposição (Hu et al. 2023).

Como o desenvolvimento radicular do dente em questão estava incompleto, o objetivo primário do tratamento escolhido foi manter a vitalidade pulpar e promover o desenvolvimento fisiológico da raiz, o que foi alcançado, e pode ser observado ao comparar radiograficamente o desenvolvimento radicular dos dentes 21 e 11. Segundo o guia de endodontia clínica da Associação Americana de Endodontia (AAE, 2023), a pulpotomia em dentes permanentes imaturos deve ser considerada como procedimento provisório para permitir o desenvolvimento radicular contínuo (apicigênese) o que de fato ocorreu no presente relato, uma vez que após 11 meses, o dente foi submetido à pulpectomia devido à necrose pulpar. Recentemente, uma análise retrospectiva avaliando o desfecho de pulpotomias em dentes anteriores traumatizados demonstrou que 45,9% dos casos tratados com pulpotomia parcial evoluíram para necrose pulpar em um período médio de 12 meses (Yu et al. 2022). Dessa forma, a necrose pulpar observada no dente 21 após pulpotomia está dentro do esperado na literatura e pode ser considerada um desfecho positivo, visto que o tratamento permitiu a continuação do desenvolvimento radicular, atingindo fechamento apical e aumento de espessura radicular, possibilitando a realização do tratamento endodôntico convencional.

Outro aspecto que deve ser levado em consideração no presente caso refere-se à violação do espaço biológico e as possíveis implicações restauradoras e na saúde periodontal. Sabe-se que a violação do espaço biológico pode levar à falha restauradora, acarretando em inflamação gengival, reabsorção de crista alveolar e recessão (Padbury et al. 2003). Além disso, 2 a 3 mm de espaço biológico deve ser preservado em todas as faces dos dentes a fim de protegê-lo da progressão de infecção do sulco gengival para o periodonto e deve ser reestabelecido antes da recuperação estética e funcional

(Trushkowsky 1998). Para alcançar tal objetivo optou-se por combinar tracionamento ortodôntico e tratamento periodontal cirúrgico.

No presente caso o tracionamento ortodôntico teve como objetivo induzir a extrusão dentária com migração gengival e leve migração óssea alveolar. Esta abordagem é indicada para melhorar o estado periodontal de dentes traumatizados que apresentam perda óssea localizada (Bajaj et al. 2015, Reichardt et al. 2021). Com este protocolo, a migração de tecidos moles e duros são estimuladas pela aplicação de força extrusiva fraca (0,2–0,3 N) a moderada ($>0,3$ N e $<0,6$ N) por meio de fios de níquel-titânio (Reichardt et al. 2021). Após 55 dias de extrusão, 4 mm de raiz foi exposto, corroborando com a literatura (Thakur et al. 2019; Jaiswal et al. 2020). Em pacientes jovens o tempo necessário para extrusão é menor (até seis semanas) comparado com pacientes adultos (até 10 semanas) (Reichardt et al. 2021), uma vez que o metabolismo ósseo é mais dinâmico nas fases de crescimento. Complementar ao tracionamento foi realizado uma cirurgia à retalho e uma restauração transcirúrgica para a correta adaptação e manutenção do espaço supra-crestal. Este é outro fator de grande relevância nos atendimentos a serem realizados nos serviços públicos de saúde, uma vez que a restauração estética da área com coroas de resina composta pode se adaptar às mudanças esperadas dos maxilares durante o período de crescimento craniofacial.

Apesar das lesões dentárias geralmente afetarem apenas o único dente que sofreu o impacto, lesões secundárias como necrose pulpar podem ser observadas após várias semanas ou meses em dentes adjacentes que não foram impactados diretamente, mesmo quando a resposta aos testes de sensibilidade pulpar após o trauma foi positiva (Aas & Skaare 2011; Santiago et al. 2015; Şermet Elbay et al. 2015). No caso apresentado, o dente 22 apresentou necrose 11 meses após o trauma, contudo, o mesmo não se encontrava irrompido no momento do trauma, o que também ascende a suspeita de que tenha ocorrido um trauma secundário não relatado.

Considerando que os métodos de apicificação atuais não promovem crescimento radicular em espessura ou comprimento, mantendo paredes dentinárias frágeis e propensas à fratura em dentes necrosados imaturos (Duggal et al. 2017), optamos por realizar TER pela técnica tradicional preconizada pela Associação Americana de Endodontia (2023). Essa técnica utiliza indução do sangramento apical objetivando o preenchimento do interior do espaço pulpar, fornecimento de células mesenquimais, e criação de um arcabouço biológico formado por coágulo (Diogenes et al. 2016,

Anthrayose et al. 2021). Embora atualmente tenha aumentado os estudos utilizando arcabouços alternativos obtidos à partir de concentrados sanguíneos como plasma rico em plaquetas e fibrina rica em plaquetas (Caviedes-Bucheli et al. 2022), estes se mostram dispensáveis quando se obtém sangramento suficiente durante o processo de indução do coágulo, apresentando resultados similares quanto ao estímulo ao desenvolvimento radicular (Araújo et al. 2022, Vatankhah et al. 2022).

O uso de irrigação copiosa com NaOCl e complementação com ácido etilenodiaminotetracético (EDTA) 17% também seguiu o protocolo recomendado pela AAE (2023) e Sociedade Europeia de Endodontistas (2021). Já em relação à medicação intracanal, optou-se pelo uso de uma pasta pronta para uso à base de hidróxido de cálcio (Ca(OH)_2), o qual apresenta propriedades antibacterianas altamente favoráveis principalmente quando se trata de infecções primárias (Martinho et al. 2023). Embora o Ca(OH)_2 e a pasta tri-antibiótica (TAP) sejam medicações comumente utilizadas como medicação intracanal em procedimentos endodônticos regeneradores, a TAP apresenta alta citotoxicidade, podendo induzir resistência antibiótica e descoloração (Alfadda et al. 2021). Contudo, um dos questionamentos em relação ao uso de Ca(OH)_2 , está relacionado à uma possível associação com a completa calcificação do canal radicular (Almutairi et al. 2022). Apesar do Ca(OH)_2 ser removido antes da indução do sangramento, resíduos podem permanecer no canal radicular e iniciar a calcificação intracanal (Almutairi et al. 2022), o que foi confirmado por meio de análises radiográficas e tomográfica.

Uma das preocupações em utilizar revascularização associada à MTA em dentes anteriores é o manchamento dentário, o qual tem sido verificado mesmo para o MTA branco (Kaur et al. 2017; Tripathi et al. 2020). No presente estudo, foi utilizado MTA repair, o qual apresenta uma formulação com propriedades físicas melhoradas para suprir as desvantagens relacionadas à descoloração, longo tempo de presa e dificuldade de manuseio (Palczewska-Komsa et al. 2021). Notamos no dente 22 leve alteração na coloração da coroa, a qual pode ter sido induzida tanto pelo MTA como pelo coágulo.

CONCLUSÃO

A manutenção destes incisivos após 4 anos de acompanhamento demonstra que a disseminação de prática endodônticas conservadoras e TER associada à outras especialidades se faz necessária para capacitação do dentista do século XXI em todos os

âmbitos, tanto privado como público, visando evitar a perda de dentes em pacientes jovens, o que impõe um desafio ao tratamento devido ao desenvolvimento vertical contínuo dos maxilares durante a fase de crescimento.

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FIGURAS E LEGENDAS



Figura 1: Aspecto clínico inicial . A) Vista frontal; B) Vista palatina; C) Vista lateral; D) Radiografia periapical inicial.



Figura 2: Aspecto clínico (A) e radiográfico (B) imediatamente após a pulpotomia parcial.

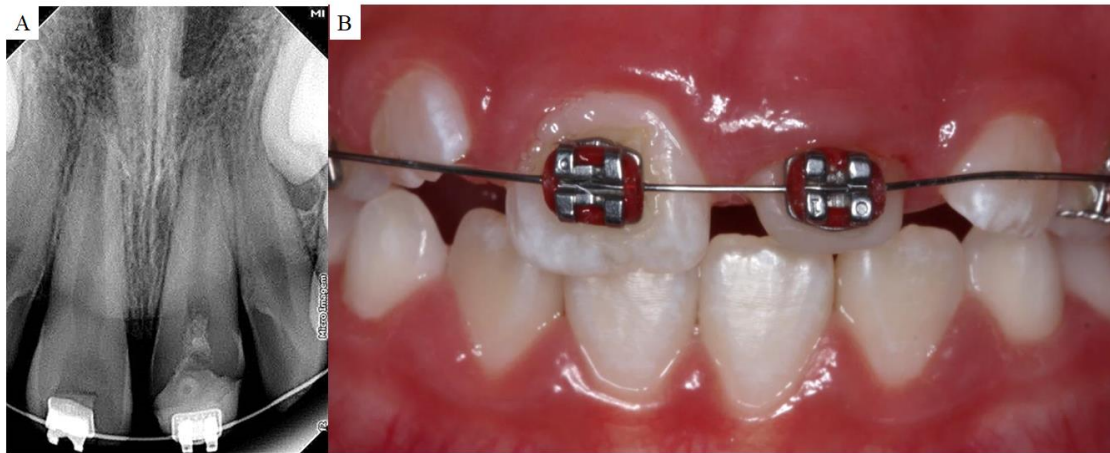


Figura 3: Imagem radiográfica imediatamente após a instalação do aparelho ortodôntico para tracionamento. Observe que o dente 21 apresentou continuação do processo de rizogênese com diminuição do diâmetro apical (A). Aspecto clínico imediatamente após a instalação do aparelho ortodôntico (B)

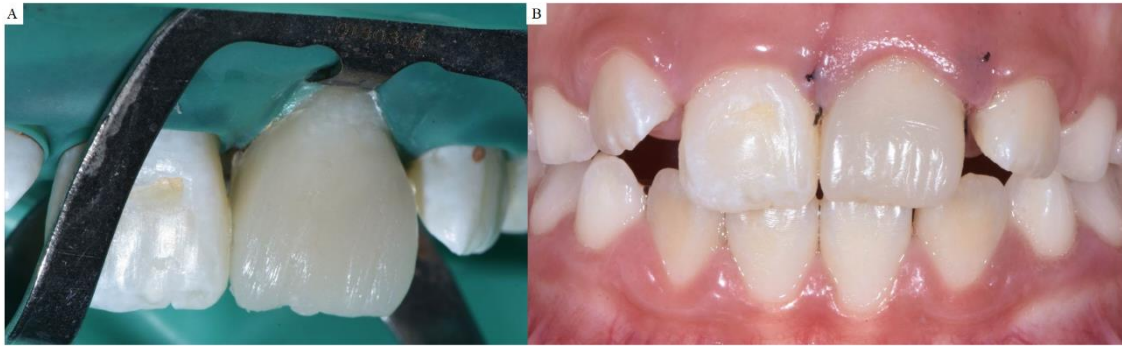


Figura 4: Reconstrução coronária em resina composta imediatamente após a osteotomia, com visualização direta do limite da fratura (A). Aspecto imediato após a remoção do isolamento absoluto e sutura do retalho gengival (B).

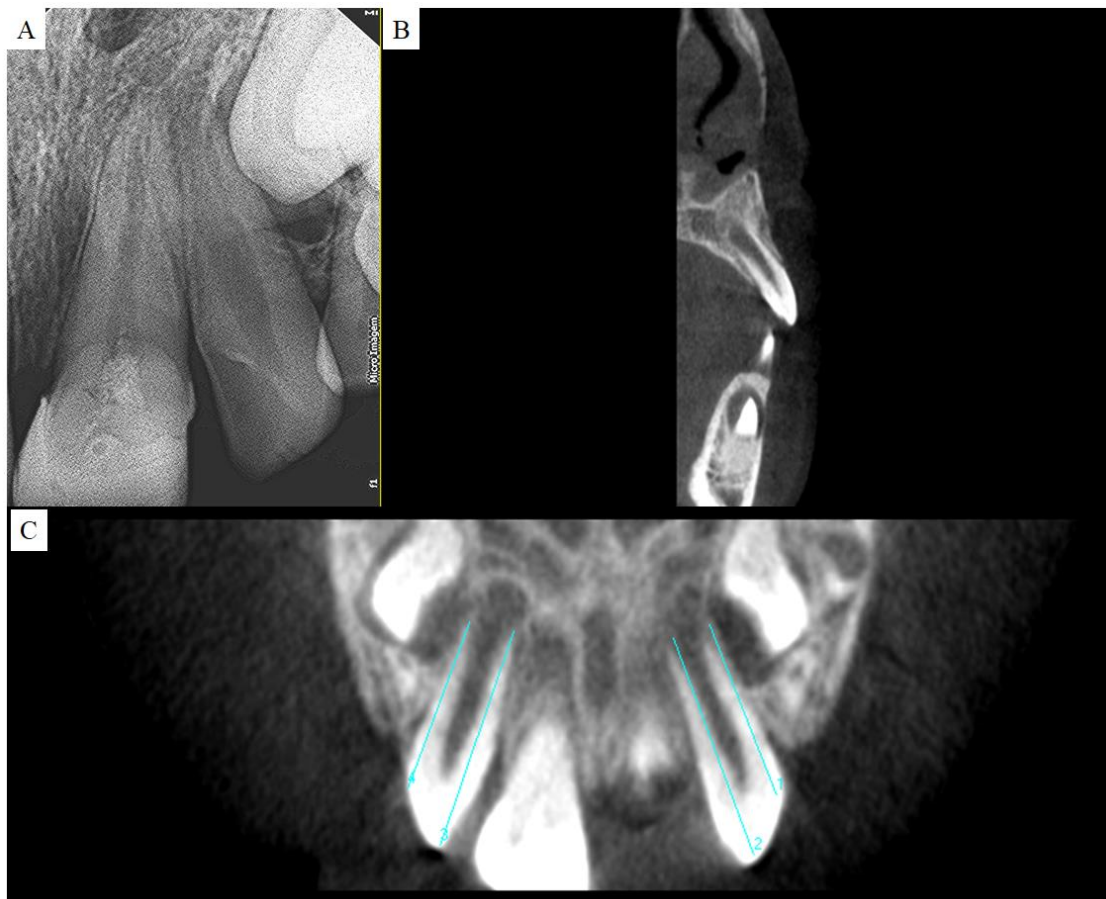


Figura 5: Radiografia inicial para realização da terapia endodôntica regenerativa, evidenciando raízes curtas, com paredes dentinárias finas e canal amplo (A). Tomografia computadorizada para análise de amplitude do diâmetro foraminal e do canal radicular, plano sagital (B) e plano frontal (C)

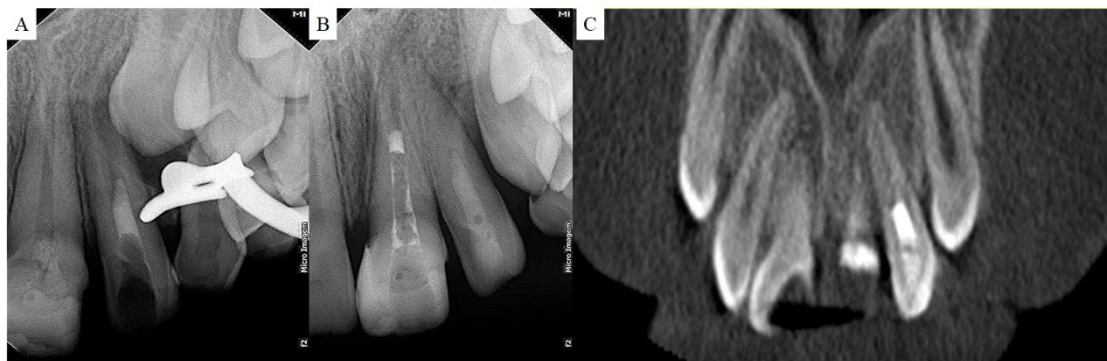


Figura 6: Aspecto radiográfico imediatamente após terapia endodôntica regenerativa (A). Acompanhamento radiográfico após 15 meses de finalização da terapia endodôntica regenerativa (B). Aspecto tomográfico após 18 meses de finalização da terapia endodôntica regenerativa (C).

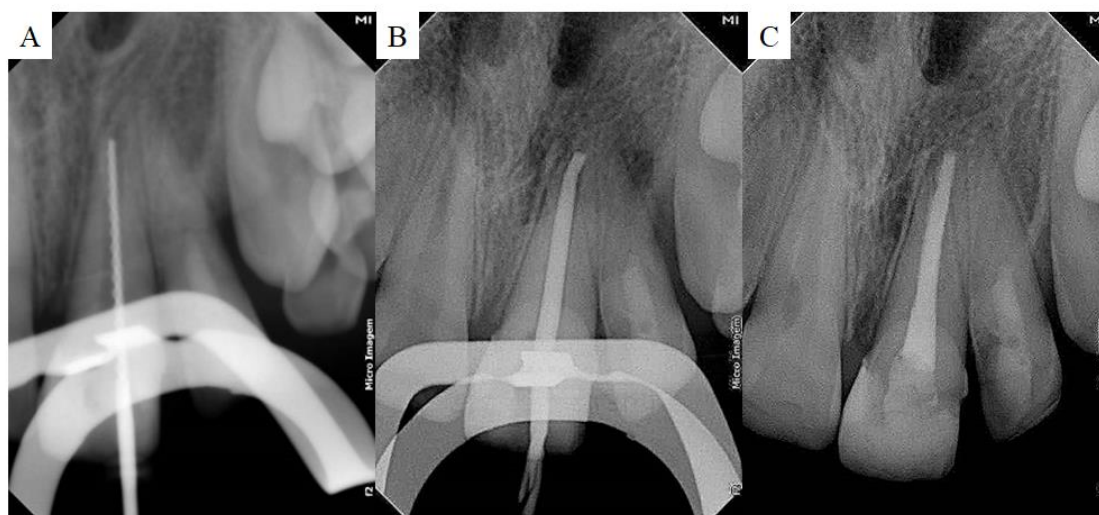


Figura 7: Tratamento endodôntico do dente 21. A) Radiografia de odontometria; B) Radiografia de prova do cone; C) Radiografia final.

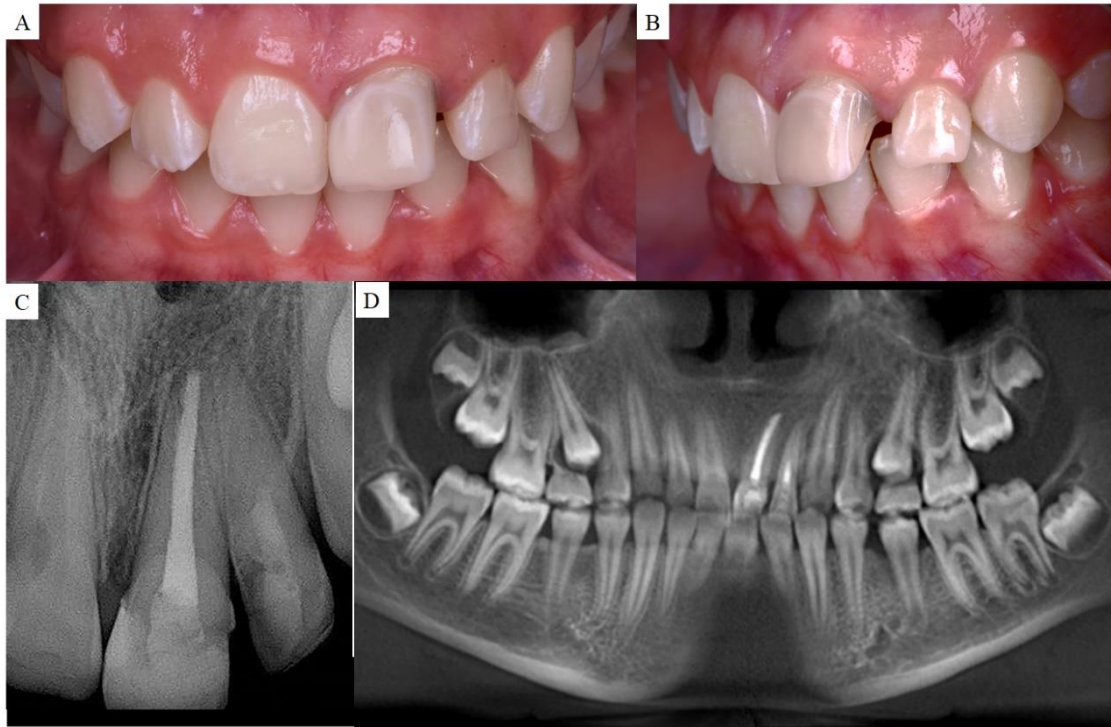


Figura 8: Acompanhamento clínico e radiográfico após 4 anos em tratamento. A) Vista frontal. B) Vista lateral. C) Radiografia periapical dos dentes 21 e 22. D) Radiografia panorâmica dos maxilares em oclusão. É possível observar o completo desenvolvimento radicular dos dentes traumatizados e aspecto de normalidade periapical, com espaço do ligamento periodontal e lâmina dura preservados.

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ANEXO I



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Procedimentos regenerativos em dentes anteriores que sofreram traumatismo dento-alveolar

Pesquisador: Camilla Christian Gomes Moura

Área Temática:

Versão: 3

CAAE: 08680919.0.0000.5152

Instituição Proponente: Universidade Federal de Uberlândia/ UFU/ MG

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.397.587

Situação do Parecer:

Aprovado

Necessita apreciação da CONEP:

Não

UBERLÂNDIA, 17 de Junho de 2019

Assinado por:
Karine Rezende de Oliveira
(Coordenador(a))

ANEXO II



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: EFEITO DE DIFERENTES BIOMATERIAIS PARA TERAPIA VITAL PULPAR EM CULTURA DE CÉLULAS PULPARES DE DENTES DECÍDUOS

Pesquisador: Camilla Christian Gomes Moura

Área Temática:

Versão: 3

CAAE: 09016219.1.0000.5152

Instituição Proponente: Universidade Federal de Uberlândia/ UFU/ MG

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.655.656

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

UBERLÂNDIA, 22 de Outubro de 2019

Assinado por:
Karine Rezende de Oliveira
(Coordenador(a))