ALEXANDRE HENRIQUE DOS REIS PRADO

INFLUÊNCIA DO USO COMBINADO DO EDTA EM PROCEDIMENTO ENDODÔNTICO REGENERATIVO: *ESTUDO EXPERIMENTAL EM MODELO ANIMAL E REVISÃO SISTEMÁTICA*

Faculdade de Odontologia
Universidade Federal de Minas Gerais
Belo Horizonte
2021

Alexandre Henrique dos Reis Prado

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do grau de Mestre em Odontologia – área de concentração em Endodontia

Orientadora: Profª. Dra. Francine Benetti
Coorientador: Prof. Dr. Antônio Paulino Ribeiro Sobrinho

Belo Horizonte
2021

Ficha Catalográfica

P896i 2021 T

Prado, Alexandre Henrique dos Reis.
Influência do uso combinado do EDTA em procedimento endodôntico regenerativo: estudo experimental em modelo animal e revisão sistemática / Alexandre Henrique dos Reis Prado. -- 2021.

127 f. : il.

Orientadora: Francine Benetti.
Coorientador: Antônio Paulino Ribeiro Sobrinho.

Dissertação (Mestrado) -- Universidade Federal de Minas Gerais, Faculdade de Odontologia.

1. Àcido edético. 2. Endodontia. 3. Fatores de diferenciação de crescimento. 4. Polpa dentária. 5. Regeneração tecidual guiada. I. Benetti, Francine. II. Ribeiro Sobrinho, Antônio Paulino. III. Universidade Federal de Minas Gerais. Faculdade de Odontologia. IV. Título.

BLACK - D047

Elaborada por: Miriam Cândida de Jesus - CRB 6/2727.

UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

FOLHA DE APROVAÇÃO

AVALIAÇÃO DA INFLUÊNCIA DO USO COMBINADO DO EDTA NO PROCEDIMENTO ENDODÔNTICO REGENERATIVO: ESTUDO EXPERIMENTAL EM MODELO ANIMAL E UMA REVISÃO SISTEMÁTICA

ALEXANDRE HENRIQUE DOS REIS PRADO

Dissertação submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Odontologia, como requisito para obtenção do grau de Mestre, área de concentração Endodontia.

Aprovada em 29 de julho de 2021, pela banca constituída pelos membros:

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Defesa Homologada pelo Colegiado de Pós-Graduação em Odontologia em 09 / 08 /2021.

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Dedico este trabalho aos meus pais e a minha avó que tanto torceram por mim e me incentivaram a nunca desistir dos meus sonhos. Compartilho minha conquista com vocês. A todos vocês, o meu amor e a minha eterna gratidão.

AGRADECIMENTOS

À Deus pelo amor, capacitação e graça contínua que me foram concedidos.

Aos meus mestres nesta jornada, meu coorientador Prof. Antônio Paulino Ribeiro Sobrinho pela confiança e disponibilidade para que esse trabalho fosse concretizado e, em especial à minha orientadora Profª. Francine Benetti pelo apoio, confiança e pela condução, lado a lado, dos meus passos na pesquisa com dedicação, paciência e amizade. Todas as oportunidades e os seus ensinamentos conduzidos até aqui foram decisivos na minha construção pessoal e intelectual. Você é minha inspiração, e meu maior exemplo de docente, pesquisadora, além de ter tornado uma grande amiga desde os primeiros dias do meu mestrado. Sinto que lhe devo minha eterna gratidão por acreditar em mim! Recorro aos versos de Eugene P. Bertin para expor essa minha gratidão: “Ensinar é deixar um vestígio de si mesmo no desenvolvimento de outro.”

À CAPES pela concessão da minha bolsa de mestrado, apoio financeiro e, dessa forma, possibilitar a minha dedicação exclusiva ao Mestrado.

Aos demais pesquisadores/colaboradores pelo suporte e participação na concretização deste trabalho.

À coordenação e aos professores com quem tive a oportunidade de trabalhar como monitor na disciplina de TCC II durante todo meu mestrado, e aos professores do projeto de extensão “Tratamento Endodôntico em Dentes Permanentes Jovens”. Sou grato pelos ensinamentos e experiências vivenciadas.

Ao nosso grupo de pesquisa, alunos de iniciação científica (Lara, Mariana, Débora, Pedro) e de pós-graduação. Obrigado pela oportunidade de poder trabalhar e aprender com todos vocês, principalmente às alunas Sabrina e Rogéria que foram essenciais para o desenvolvimento deste trabalho. A caminhada continua…

Aos meus amigos de Diamantina, Belo Horizonte e do intercâmbio pelo companheirismo e, em especial, ao Rider pela torcida diária, suporte e incansável incentivo durante esse período tão importante, sendo peça fundamental para a concretização desse sonho.

A todos os amigos que conheci em Araçatuba e, principalmente, ao meu conterrâneo Gabriel pela recepção, risadas, e por ter tornado meus dias de ansiedade e de trabalho mais leves.

A todos que mencionei e àqueles que não nomeei...

O caminhar não se faz sozinho, vocês trouxeram mais esperança nos meus passos.

*"*Para se ter sucesso, é necessário amar de verdade o que se faz. Caso contrário, levando em conta apenas o racional, você simplesmente desiste. É o que acontece com a maioria das pessoas."

Steve Jobs

RESUMO

Os objetivos deste estudo foram: 1. Verificar a influência do uso do ácido etilenodiaminotetracético (EDTA) no processo de reparo após revascularização pulpar em molares imaturos de ratos, avaliando a formação de tecido mineralizado, presença de infiltrado inflamatório, e presença/maturação de fibras colágenas; 2. Verificar a influência do uso do EDTA na presença de fatores de crescimento após revascularização pulpar em molares imaturos de ratos, avaliando a imunomarcação do fator de transformação do crescimento (TGF)-β1 e fator de crescimento de fibroblastos (FGF)-2; 3. Verificar a influência do uso do EDTA em fatores relacionados ao procedimento endodôntico regenerativo, por meio de revisão sistemática. Para as análises experimentais, molares inferiores direito ou esquerdo de 12 ratos Wistar (4 semanas) tiveram as polpas removidas, e foram divididos aleatoriamente nos grupos (n = 6): NaOCl - irrigação com hipoclorito de sódio (NaOCl) 2,5%; e NaOCl-EDTA – NaOCl 2,5%, seguido de EDTA 17%. Após, foi induzido o sangramento intracanal por sobreinstrumentação com lima K #15 e os dentes foram selados. Molares inferiores que não receberam intervenção destes animais, foram aleatoriamente selecionados como controle (controle-15d; n = 3). Outros molares inferiores direito ou esquerdo de três animais que não receberam qualquer intervenção, foram utilizados como controle-imediato (n = 3). Após 15 dias, os animais dos grupos experimentais e controle-15d foram eutanasiados; animais do grupo controle-imediato foram eutanasiados imediatamente no dia da experimentação. As peças foram preparadas para análise histológica (hematoxilina-eosina), análise do colágeno (picrosírius red e Tricrômio de Masson), e imunohistoquímica (TGF-β1 e FGF-2). Os resultados foram submetidos a testes estatísticos específicos (p<0,05). Para a revisão sistemática, dois autores independentes conduziram busca em bases de dados eletrônicas (até fevereiro-2021), extração dos dados e avaliação do risco de viés. Análise dos fatores de crescimento foi considerada desfecho primário. Em relação à formação de tecido mineralizado, o grupo NaOCl-EDTA teve mais espécimes com aumento concomitante da espessura e comprimento da raiz, apesar de todos espécimes dos grupos experimentais mostrarem presença de cemento neoformado; no entanto, houve fechamento parcial do forame apical em NaOCl e completo em NaOCl-EDTA, em alguns espécimes. A maioria dos espécimes dos grupos experimentais apresentaram infiltrado inflamatório até o terço médio do canal radicular. Foi observada maior neoformação tecidual nos espécimes do NaOCl-EDTA (p<0,05). Em relação ao colágeno, apesar do grupo NaOCl-EDTA apresentar maior quantidade de colágeno na extremidade radicular, não houve diferença significativa com o grupo EDTA, e ambos apresentaram maior quantidade de fibras imaturas nessa região; no centro do terço apical do canal radicular, houve equivalência de fibras maduras e imaturas nos grupos, sem diferença significativa. Houve significativa imunomarcação de TGF-β1 no grupo NaOCl-EDTA comparado ao NaOCl (p<0,05), mas não houve diferença na imunomarcação de FGF-2. Conclui-se que: 1. EDTA influenciou positivamente a presença de tecido neoformado nos canais radiculares após revascularização pulpar, mas não influenciou na maturação colágena; 2. EDTA influencia a imunomarcação de TGF-β1, mas não de FGF-2; 3. A revisão sistemática revelou que o EDTA pode aumentar a liberação de TGF-β da dentina, além da migração, adesão e diferenciação celular.

Palavras-chave: Ácido edético. Endodontia regenerativa. Fatores de crescimento-diferenciação. Polpa dentária. Regeneração tecidual guiada. Revisão sistemática.

ABSTRACT

Influence of combined use of EDTA in Regenerative Endodontic Procedure: Animal study and systematic review

The objectives of this study were: 1. To evaluate the influence of the use of ethylenediaminetetraacetic acid (EDTA) in the repair process after pulp revascularization in immature rat molars, by evaluating mineralized tissue formation, presence of inflammatory infiltrate, and the presence/maturation of collagen fibers; 2. To evaluate the influence of EDTA in the presence of growth factors after pulp revascularization in immature molars of rats, by analyzing the immunolabelling of transforming growth factor (TGF)-β1 and fibroblast growth factor (FGF)-2; 3. To evaluate the influence of the use of EDTA on the factors associated with the regenerative endodontic procedure, through a systematic review. For experimental analyses, lower right or left molars of 12 Wistar rats (4-week-old) had pulp tissue removed, and they were randomly separated in the groups (n = 6): NaOCl - irrigation with 2.5% NaOCl; and NaOCl-EDTA - 2.5% NaOCl followed by 17% EDTA. Afterwards, blood clot was induced by inserting a 15 K-file beyond the apex, and teeth were sealed. Lower untreated molars from these animals were randomly selected as control (control-15d; n = 3). Other lower right or left molars of other three animals that did not receive any intervention, they were used as control-immediate (n = 3). After 15 days, the rats from the experimental and the control-15d groups were killed; animals from control-immediate group were killed immediately at the experimental period. The jaws were prepared for histological (haematoxylin-eosin); collagen analysis (picrosirius red and Masson's trichrome); and immunohistochemical analyses (TGF-β1 and FGF-2). Data were submitted to statistical tests (p<0.05). For systematic review, two authors independently performed a search in electronic databases (up to February-2021), data extraction and risk of bias evaluation. The release of growth factors was the primary outcome. Regarding mineralized tissue formation, NaOCl-EDTA group had more specimens with a concomitant increase in root thickness and length, and all specimens from the experimental groups showed neoformed cementum-like tissue; however, some specimens of NaOCl showed partial apical closure, while NaOCl-EDTA had complete apical closure. Most specimens from the experimental groups had inflammatory infiltrate extending to the middle third of root canals. A significant tissue neoformation was observed in the NaOCl-EDTA specimens (p<0.05). For collagen, the NaOCl-EDTA group showed more collagen fibers in the root tip, without significant difference compared to NaOCl group, where both groups showed greater amount of immature fibers in this region; in the center of the apical third, there was similar amount of mature and immature fibers from both groups, without significant differences. There was significant TGF-β1 immunolabeling in the NaOCl-EDTA group compared to NaOCl group (p<0,05), but there was no significant difference in FGF-2 immunolabeling. In conclusion: 1. EDTA positively influenced the presence of newly formed tissue in root canals after pulp revascularization, but it did not influence collagen maturation; 2. EDTA influenced TGF-β1 immunolabeling, but it did not influence FGF-2; 3. The systematic review showed that the use of EDTA can enhance TGF-β release from dentin, in addition to increase cell migration, adherence and differentiation.

Keywords: EDTA. Regenerative endodontics. Growth differentiation factors. Dental pulp. Guided tissue regeneration. Systematic review.

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

AAE	*American Association of Endodontists*
µm	Micrômetro
CEBIO	Centro de Bioterismo
CEUA	Comissão de Ética no Uso de Animais
CT	Comprimento de Tabalho
CTMs	Células-tronco Mesenquimais
DPSCs	Células-tronco da Polpa Dentária (do inglês *Dental Pulp Stem Cells*)
EDTA	Ácido Etilenodiaminotetracético
FGF-2	Fator de Crescimento Fibroblástico–2 (do inglês *Fibroblast Growth Factor-2*)
HDPCs	Células da Polpa Dentária Humana (do inglês *Human Dental Pulp Cells)*
HE	Hematoxilina-eosina
CTMs	Células-tronco Mesenquimais
NaOCl	Hipoclorito de Sódio
PBS	Tampão Fosfato Salino (do inglês *Phosphate-bufferid Saline*)
PBS CIMMO HP	Cimento Biológico Reparador
PDLSCs	Células-tronco do Ligamento Periodontal (do inglês *Periodontal Ligament Stem Cells*)
PSR	Picrosírius Red
SBCA	Sociedade Brasileira de Ciência em Animais de Laboratório
SCAPs	Células-tronco da Papila Apical (do inglês *Stem Cells from the Apical Papila*)
SCR	Sistema de Canais Radiculares
SHEDs	Células-tronco de Dentes Decíduos Esfoliados (do inglês *Stem Cells from Human Exfoliated Deciduous*)
TGF-β	Fator de Crescimento Transformante-β (do inglês *Transforming Growth Factor-β*)
TM	Tricrômio de Masson

SUMÁRIO

1 CONSIDERAÇÕES INICIAIS

Ao longo dos anos, a Endodontia tem buscado preservar a manutenção da dentição natural, de maneira fisiologicamente funcional, contribuindo para a saúde bucal e sistêmica do paciente. Dentes permanentes imaturos podem ter a rizogênese interrompida na presença de uma necrose pulpar oriunda de lesões cariosas profundas, lesões traumáticas ou anomalias dentárias (ANDREASEN; KAHLER, 2015; LAW, 2013; PALMA *et al.*, 2017; SCARPARO *et al*., 2011). Nesses casos, o tratamento endodôntico convencional é desafiador, visto que possíveis fraturas radiculares e/ou extravasamento do material obturador podem ocorrer, devido às finas paredes dentinárias e à ausência de constrição apical (DING *et al*., 2009; SHAH *et al*., 2008; THIBODEAU *et al*., 2007). Diante disso, novos materiais e abordagens terapêuticas vêm sendo propostos para o tratamento dos dentes imaturos. Ainda, a presença de uma reduzida espessura e comprimento das paredes dentinárias, devido a paralização da formação radicular, pode aumentar a susceptibilidade a fraturas do dente ao longo da vida do paciente (BRACKS *et al.*, 2019).

Procedimentos endodônticos regenerativos, como a revascularização pulpar, têm ganhado atenção entre os clínicos, e envolvem procedimentos de base biológica buscando substituir fisiologicamente o complexo dentino-pulpar danificado (BRACKS *et al.*, 2019; CHAE *et al.*, 2018; HARGREAVES *et al.*, 2013; KIM *et al*., 2018; SHAMSZADEH *et al.*, 2019; VERMA *et al.*, 2016). Esses procedimentos podem resultar no término da formação radicular, sendo considerado uma das melhores opções de tratamento para dentes permanentes imaturos com necessidade de tratamento endodôntico (TAWEEWATTANAPAISAN *et al.*, 2019; ULUSOY *et al*., 2019).

Estudos prévios de coorte prospectivo e retrospectivo demonstram resultados bastante promissores quanto à revascularização pulpar, que alcança uma taxa de sucesso de 95% a 100% (ALOBAID *et al*., 2014; ARSLAN *et al.,* 2019; JEERUPAHN *et al*., 2012), promovendo um aumento no crescimento e na espessura radicular, além de fechamento do forame apical (SHAH *et al*., 2008). A revascularização pulpar se baseia em pilares da engenharia tecidual, como: a migração e diferenciação de células-tronco mensenquimais (CTMs); a presença de um *scaffold* tridimensional

que atue como uma matriz de suporte para a organização tecidual, fatores de crescimento que auxiliem na proliferação e diferenciação celular; e um ambiente livre de microrganismos (ALBUQUERQUE *et al*., 2014; BRACKS *et al.*, 2019; CONDE *et al*., 2016; GOMES-FILHO *et al*., 2013; HARGREAVES *et al*., 2013; ZHANG *et al.*, 2014).

Quanto aos sinais de sucesso do tratamento endodôntico regenerativo, esses foram definidos como: ausência de sintomatologia, cicatrização óssea periapical, maturação radicular (ganhos em espessura e comprimento) e resposta positiva aos testes de sensibilidade pulpar (GALLER, 2016). Ainda, alguns fatores que influenciam positivamente os resultados da técnica foram revelados, como: diâmetro do ápice aberto (sendo favorável quando ≥ 0,5 mm), idade do paciente (melhor prognóstico quando de 9 a 18 anos), selamento coronário adequado, e antissepsia do sistema de canais radiculares (SCR) (ESTEFAN *et al*., 2016; JUNG *et al*., 2019; LIN; KAHLER, 2017).

O primeiro relato de caso clínico realizado com sucesso em um dente submetido à técnica foi em 2001, onde foi observada resposta positiva aos testes de sensibilidade e maturação radicular cerca de 30 meses após o procedimento (IWAYA *et al.,* 2001). Ademais, foi descrito que a técnica de revascularização pulpar permite a migração de CTMs da região da papila apical para o interior do canal radicular (DING *et al.*, 2009; GARCIA-GODOY; MURRAY, 2012), promovendo a continuidade do desenvolvimento radicular (LAW, 2013; LOVELACE *et al*., 2011; SHAH *et al*., 2008) e o restabelecimento da imunocompetência e nocicepção (LAW, 2013).

Inicialmente, o conceito de revascularização pulpar foi promulgado por estudos realizados por Ostby em dentes humanos e caninos (HØRSTED; NYGAARD-OSTBY, 1978; NYGAARD-OSTBY, 1961), após indução do sangramento intracanal nesses dentes. Posteriormente, o procedimento endodôntico regenerativo - termo adotado em 2007 pela *American Association of Endodontics* (AAE) (MURRAY *et al*., 2007) vem sendo estudado, de forma a melhorar o prognóstico do dente imaturo comprometido, na tentativa de permitir o restabelecimento de um tecido pulpar (ou tecido semelhante a este) e dos tecidos periapicais, e consequentemente, o desenvolvimento completo das raízes dentárias.

A indução de sangramento no canal radicular é realizada através de estímulo nos tecidos periapicais com limas endodônticas manuais (GALLER, 2016). Lovelace

et al. (2011) relataram um aumento de 400 a 600 vezes da expressão de marcadores de CTMs no sangramento intracanal induzido pelos tecido periapicais, comparado às amostras de sangue sistêmicas. Ainda, o coágulo sanguíneo proveniente dos tecidos periapicais favorece a expressão de vários fatores de crescimento, como o fator de crescimento transformador (TGF)-β e o fator de crescimento fibroblástico (FGF)-2, promovendo angiogênese, migração celular e regulando a diferenciação odontoblástica (JUNG *et al*., 2019).

As CTMs são capazes de se diferenciarem em diversos tecidos (PETERS, 2014), e quando de origem dentária, foram classificadas da seguinte maneira: células-tronco da polpa dentária (DPSCs), células-tronco de dentes decíduos esfoliados (SHEDs), células-tronco da papila apical (SCAPs) e células-tronco do ligamento periodontal (PDLSCs) (SHARMA *et al*., 2010). Em geral, as CTMs retêm a capacidade de adquirir fenótipo condrogênico, odontogênico, osteogênico, e neurogênico, através de sua exposição a diferentes concentrações de fatores de crescimento e outras proteínas (HARGREAVES *et al*., 2008), que se ligam a receptores celulares, induzindo a proliferação e/ou diferenciação destas células (MURRAY *et al*., 2007). Os protocolos clínicos têm evoluído no intuito de induzir o potencial das CTMs e permitir a liberação de fatores de crescimentos aprisionados nas paredes dentinárias (VERMA *et al*., 2016).

Apesar da crescente investigação no campo da endodontia regenerativa, uma análise bibliométrica e cientométrica atual sobre o tema (SHAMSZADEH *et al*., 2019), demonstra que o nível de evidência científica no campo da endodontia regenerativa é baixo. Ademais, deixa claro que os estudos se concentram principalmente na análise da microbiota endodôntica, e na comparação das diferentes medicações intracanais. Os autores salientam a importância de se avaliar os efeitos inflamatórios envolvidos no processo de cicatrização e regeneração tecidual, englobando as diferentes soluções irrigadoras comumente utilizadas no procedimento de revascularização pulpar, como o hipoclorito de sódio (NaOCl) e o ácido etilenodiaminotetracético (EDTA).

Segundo estudos anteriores, a solução irrigante pode apresentar um efeito potencializador na liberação de fatores de crescimento da matriz dentinária (ALGHILAN *et al*., 2017; DENIZ SUNGUR *et al.*, 2019). Diferentes concentrações de NaOCl (0,5% - 6%), e um agente quelante, como o EDTA à 17%, tem sido propostos nesse procedimento (GALLER *et al*., 2011; GALLER, 2016). Os protocolos recentes

da *European Society of Endodontology* (GALLER *et al*., 2016) e da AAE (2018) tem sugerido o uso do EDTA 17% após o uso de concentrações reduzidas de NaOCl, a fim de minimizar a citotoxicidade deste e ampliar a liberação de moléculas bioativas da dentina (CHAE *et al.*, 2018).

Embora o uso do EDTA possa influenciar negativamente na formação do coágulo intracanal (TAWEEWATTANAPAISAN *et al.*, 2019), os resultados são controversos quanto aos seus efeitos no comportamento celular. Enquanto alguns estudos encontraram uma redução na viabilidade e migração células após uso do EDTA (AKSEL *et al*., 2020; DENIZ SUNGUR *et al.*, 2019), outros demonstraram um impacto positivo comparado a outros agentes quelantes no comportamento celular (ATESCI *et al*., 2020; CHAE *et al.*, 2018; GALLER *et al.*, 2011; GALLER *et al.*, 2016; GONÇALVES *et al.*, 2016; IVICA *et al.*, 2019; KUCUKKAYA EREN *et al.*, 2021).

A matriz dentinária é considerada um reservatório para diferentes fatores de crescimento, que apresentam papel importante no recrutamento e diferenciação de CTMs para a área afetada (AKSEL *et al.*, 2020; DENIZ SUNGUR *et al.*, 2019; GONÇALVES *et al*., 2016; SADAGHIANI *et al.*, 2016). O uso do EDTA pode aumentar a liberação desses fatores de crescimento pela remoção da camada de *smear layer* (BRACKS *et al*., 2019; CHAE *et al.*, 2018; GALLER *et al.*, 2011; GONÇALVES *et al*., 2016; GRAHAM *et al*., 2006; PANG *et al*., 2014). Entre esses fatores, o TGF-β e o FGF-2 parecem ser considerados como fatores chaves na regeneração do complexo dentino-pulpar (MATHIEU *et al*., 2013).

O TGF-β atua como um fator quimiotático e estimulante para a ativação e migração de CTMs (GONÇALVES *et al.*, 2016), regulando positivamente a diferenciação odontoblástica (CHAE *et al.*, 2018; KUCUKKAYA EREN *et al*., 2021). Também contribui para a dentinogênese reparadora (CHAE *et al.*, 2018; KUCUKKAYA EREN *et al.*, 2021), além de apresentar efeitos imunossupressores substanciais com a capacidade de inibir a produção de citocinas pró-inflamatórias (MACIEL *et al.*, 2012). Semelhantemente, o FGF-2 mostrou capacidade de induzir a proliferação e diferenciação de células da polpa dentária humana (HDPCs) em linhagens de odontoblastos *in vitro* (MORITO *et al.*, 2009). Esse fator também pode estimular o processo de angiogênese (ABOUT *et al.*, 2000; MATHIEU *et al.*, 2013), induzir à produção de cálcio (MATHIEU *et al.*, 2013), e potencializar a ação do TGF-β (KIM *et al*., 2012). Além disso, TGF-β e FGF-2 são capazes de evocar respostas celulares mesmo em baixas concentrações (GALLER *et al.*, 2011), assumindo um

papel importante na neoformação tecidual no interior dos canais radiculares (BRACKS *et al*., 2019).

Por outro lado, a influência na liberação de fatores de crescimento após o condicionamento da dentina com EDTA permanecem controversos. Enquanto algumas avaliações *in vitro* sugerem não haver influência do EDTA na liberação de TGF-β (AKSEL *et al*., 2020; ATESCI *et al*., 2020), outros estudos demonstraram um aumento significativo na expressão desta proteína (BRACKS *et al*., 2019; CAMERON *et al*., 2019; CHAE *et al*., 2018; GALLER *et al*., 2015; GONÇALVES *et al*., 2016; ZENG *et al*., 2016).

A maioria das avaliações sobre o efeito do EDTA na liberação de fatores de crescimento e de outras moléculas bioativas, consiste em estudos laboratoriais, utilizando matriz de dentina condicionada (DUNCAN *et al*., 2017) ou discos de dentina (ATESCI *et al*., 2020; DENIZ SUNGUR *et al*., 2019), o que não simula um cenário clínico de revascularização pulpar. Apenas um estudo anterior em animais foi conduzido para avaliar a influência da irrigação com EDTA na liberação de TGF-β, enquanto nenhuma avaliação *in vivo* da liberação de FGF-2 foi encontrada. Assim, torna-se necessário conhecer se a liberação de TGF-β e FGF-2 da matriz dentinária pode também ocorrer dentro do espaço do canal radicular apos irrigação, seguindo os protocolos regenerativos recomendados pela *European Society of Endodontology* (GALLER *et al*., 2016) e pela AAE (2018).

Conhecer os efeitos do uso do EDTA na revascularização pulpar pode ser promissor para reduzir falhas na execução desse procedimento e, em última análise, para o seu sucesso final. Contudo, poucos estudos têm avaliado histologicamente os efeitos da irrigação com EDTA na terapia regenerativa (BRACKS *et al*., 2019; BUCCHI *et al*., 2017). Além disso, uma avaliação do conteúdo de colágeno *in vivo* como um arcabouço para o reparo e mineralização de tecidos em dentes imaturos ainda não foi investigada, e nenhuma revisão sistemática prévia foi conduzida para avaliar a influência do uso do EDTA na liberação de moléculas bioativas da dentina, no comportamento celular e no processo de cicatrização e/ou regeneração tecidual.

Dessa forma, o objetivo desse estudo foi avaliar o reparo tecidual a nível histológico, após o uso do EDTA 17% na revascularização pulpar, juntamente com seus efeitos na liberação de TGF-β1 e FGF-2, utilizando um modelo experimental de revascularização em molares imaturos de ratos. Além disso, o presente trabalho realizou uma revisão sistemática para avaliar a influência do uso do EDTA na

liberação de fatores de crescimento da dentina, no comportamento celular e na regeneração tecidual.

2 OBJETIVOS

2.1 Objetivo geral

Este estudo avaliou a influência do uso combinado do EDTA no processo de reparo após procedimento de revascularização pulpar, através de análises histológica e imunohistoquímica em molares imaturos de ratos, e por meio de uma revisão sistemática de estudos *in vivo* e *in vitro*.

2.2 Objetivos específicos

Investigar a influência do uso do EDTA após procedimento de revascularização pulpar em ratos na:

a) Formação de tecido mineralizado nas paredes dos canais radiculares, presença de cemento neoformado, fechamento do forame apical, extensão do infiltrado inflamatório e presença de tecido neoformado no interior dos canais radiculares, por meio da técnica de hematoxilina-eosina (HE);
b) Área de cemento e tecido mole neoformados no interior dos canais radiculares obtidos em μm^2 , por meio de análise histomorfométrica;
c) Identificação e maturação das fibras colágenas dos tecidos neoformados, por meio das técnicas de Tricrômico de Masson e Picrosirius Red, respectivamente;
d) Liberação dos fatores de crescimento TGF-β1 e FGF-2, por meio de análise imunohistoquímica;
e) Liberação de fatores biológicos da dentina ou polpa e tecidos periapicais, por meio de uma revisão sistemática da literatura.

3 METODOLOGIA EXPANDIDA

3.1 Estudo experimental em modelo animal

3.1.1 Animais

Foram utilizados 15 ratos machos (*Rattus albinus*, *Wistar*), pesando aproximadamente 80 g, com idade média de 4 semanas e provenientes do Centro de Bioterismo (CEBIO) da UFMG (Belo Horizonte, MG, Brasil). O cálculo amostral foi baseado em estudos anteriores (BRACKS *et al*., 2019; SCARPARO *et al*., 2014).

Os animais foram mantidos em ambiente com temperatura entre 22ºC e 24ºC e com ciclo de luz controlado (12 horas claro e 12 horas escuro) e em gaiolas coletivas (quatro ratos por gaiola). Cada comedouro e bebedouro foi cheio com ração e água filtrada *ad libitum*, respectivamente. Higienizações periódicas das gaiolas foram realizadas, com troca e limpeza dos comedouros e bebedouros.

Os procedimentos experimentais respeitaram os princípios éticos adotado pela Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL), e o projeto de pesquisa foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da UFMG – protocolo CEUA n° 81/2020 (ANEXO A).

3.1.2 Drogas empregadas

Para anestesia, os animais receberam via injeção intramuscular, um anestésico à base de cloridrato de Cetamina 10% (80 mg/Kg; Dopalen, Vetbrands – Divisão de Saúde Animal, Jacareí, SP, Brasil) e um sedativo à base de cloridrato de Xilazina 2% (20 mg/Kg; Anasedan, Agribands do Brasil Ltda., Paulínia, SP, Brasil). Para eutanásia, foi utilizado um anestésico via intraperitoneal à base de tiopental sódico (150 mg/Kg; Thipentax, Cristália – Produtos Químicos Farmacêuticos Ltda – Itapira, Brasil).

3.1.3 Divisão dos grupos

Os primeiros molares imaturos inferiores dos lados esquerdo ou direto de 12 ratos foram divididos, aleatoriamente, nos grupos NaOCl e NaOCl-EDTA, de acordo com os procedimentos realizados, que podem ser visualizados na Tabela 1. Molares inferiores que não receberam intervenção destes animais, foram aleatoriamente selecionados como controle (controle-15d; n = 3). Outros molares inferiores direito ou esquerdo dos outros três animais que não receberam qualquer intervenção, foram utilizados como controle-imediato (n = 3). Os grupos controles também podem ser observados na Tabela 1.

Tabela 1 - Distribuição dos grupos experimentais, de acordo com o tratamento realizado

Grupos	**Pulpectomia**	**Limpeza dos canais radiculares**	**Revascularização pulpar**	**Eutanásia**
NaOCl (n = 6)	Remoção do tecido pulpar da raiz mesial	NaOCl 2,5% por 5 min, seguido de soro fisiológico	Indução de sangramento periapical e selamento da cavidade	15 dias
NaOCl-EDTA (n = 6)	Remoção do tecido pulpar da raiz mesial	NaOCl 2,5% seguido de irrigação com EDTA 17% por 5 min cada, seguido de soro fisiológico	Indução de sangramento periapical e selamento da cavidade	15 dias
Controle-imediato (n = 3)	------	------	------	Imediato
Controle-15d (n = 3)	------	------	------	15 dias

NaOCl: hipoclorito de sódio; EDTA: ácido etilenodiaminotetraacético. Fonte: Do autor, 2021.

3.1.4 Procedimentos operatórios

Todos os procedimentos experimentais foram realizados em bancada de laboratório com materiais esterilizados e auxílio de um microscópio operatório (Alliance, São Paulo, SP, Brasil), com aumento de 40× (BRACKS *et al*., 2019).

Após a anestesia dos animais, os primeiros molares inferiores imaturos foram isolados com o auxílio de grampos especiais e barreira gengival (Top dam, FGM, Joinville, SC, Brasil), como mostrado na Figura 1 (A-C). O campo operatório foi descontaminado com NaOCl 2,5%, para realização dos tratamentos de acordo com cada grupo.

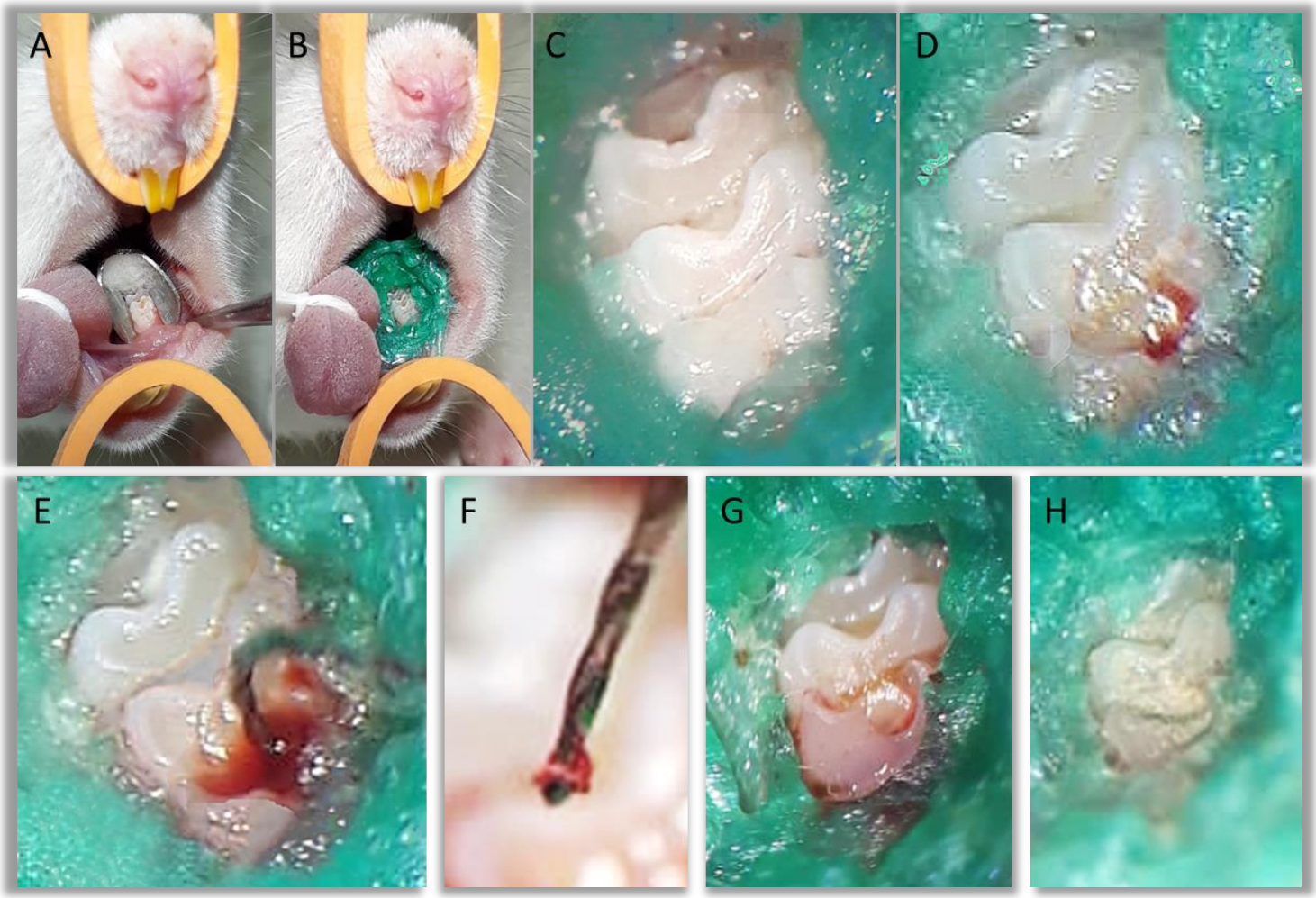

Figura 1 – Procedimento operatório de revascularização pulpar em molares imaturos de ratos. (A, B) Isolamento dos molares inferiores com posicionamento do grampo mais barreira gengival; (C) molares inferiores isolados; (D) exposição da câmara pulpar da raiz mesial do primeiro molar inferior; (E) pulpectomia com lima tipo K #10; (F) tecido pulpar removido do canal mesial; (G) aspecto final do dente após aplicação dos protocolos de irrigação e secagem do canal; (H) selamento coronário com material reparador e cimento de ionômero de vidro. Fonte: Do autor, 2021.

Os princípios clínicos de antissepsia do canal radicular foram baseados nos protocolos sugeridos pela AAE (2018) e Shimizu *et al*. (2013). Primeiramente, após da definição do comprimento de trabalho (CT) por meio de exame radiográfico do canal mesial dos molares inferiores imaturos de ratos com mesma idade e peso em estudo piloto (CT = 4 mm), foi realizada a abertura coronária dos molares (Figura 1, D) com brocas esféricas LN estéreis (Dentisply Maillefer, Tulsa, OK, USA) em baixa rotação acopladas em um motor elétrico com velocidade controlada (Beltec, Araraquara, SP, Brasil), e sob irrigação constante com soro fisiológico. Após a exposição do canal mesial, foi realizada a pulpectomia com lima endodôntica tipo K #10 (Dentsply Sirona, Ballaigues, Switzerland), no CT. Inicialmente, os canais foram irrigados com solução de soro fisiológico para remoção de restos de remanescentes pulpares e secos com pontas de papel absorvente.

Após, foi feita a aplicação dos protocolos de irrigação de acordo com os grupos experimentais (NaoCl e NaOCl-EDTA). Para tanto, foi utilizando seringa descartável de 3,0 mL (BD Plastipak, Curitiba, PR, Brasil) e agulha fina (0,40 x 25 mm, Endo-Eze™ Irrigator tip) (Ultradent, Indaiatuba, SP, Brasil), posicionada a 1 mm aquém do CT. Os grupos receberam os seguintes tratamentos: grupo NaOCl - antissepsia com NaOCl à 2,5% por 5 min (GOMES-FILHO *et al*., 2013; SHIMIZU *et al*., 2013; YAMAUCHI *et al*., 2011), seguido de irrigação final com soro fisiológico 0,9%; grupo NaOCl-EDTA antissepsia do canal mesial, como descrito no grupo NaOCl, seguido irrigação com EDTA 17%, por 5 min, e irrigação final com soro fisiológico. Durante o tempo de aplicação de cada solução usada, foi realizada agitação com movimentos de cateterismo realizados com lima endodôntica tipo K #10 inserida no canal mesial.

Ao final, os canais radiculares mesiais foram secados com cones de papel absorvente, seguido da indução do sangramento intrarradicular. Esse procedimento foi baseado nos protocolos descritos pela AAE (2018) e por Bracks *et al*. (2019): conseguinte à secagem dos canais mesiais, uma lima endodôntica do tipo K #15 foi pressionada 0,5 mm além do forame apical, de forma estimular a formação do coágulo sanguíneo nos grupos NaOCl e NaOCl-EDTA. Posteriormente, com o auxílio de uma bolinha de algodão estéril presa a uma pinça clínica, foi realizada a remoção do sangue presente na câmara pulpar, e uma camada coronária de aproximadamente 1 mm do cimento reparador PBS CIMMO HP (CIMMO, Pouso

Alegre, MG, Brasil) foi acondicionada com o auxílio de condensadores endodônticos, na embocadura do canal mesial (NAMOUR; THEYS, 2014; THIBODEAU *et al*., 2007). Por fim, o a cavidade foi selada com cimento de ionômero de vidro fotopolimerizável (GC America Inc., Alsip, IL, USA) (AAE, 2018; GALLER *et al*., 2016), e acompanhado por 15 dias (Figura 1, G, H). Dipirona sódica (150 mg/Kg) foi aplicada nos animais, via intramuscular, para a analgesia pós-cirúrgica.

3.1.5 Eutanásia e processamento laboratorial

No período imediato (grupo controle-imediato), ou decorrido o período 15 dias (grupos experimentais e grupo controle-15 dias), os animais foram eutanasiados com sobredose de Tiopental, como descrito anteriormente. Após, foi removida toda a pele correspondente à face do animal e com dois cortes realizados com tesoura no ângulo da boca, seguido da separação da mandíbula e maxila. Empregando uma lâmina intercambiável nº 15, montada em cabo de bisturi, foi realizada uma incisão em profundidade ao nível do plano sagital mediano, separando a mandíbula direita da esquerda. Empregando-se novamente uma tesoura, foi feito outro corte de modo a tangenciar a borda da mandíbula, removendo os tecidos em excesso, possibilitando a obtenção das mandíbulas com os primeiros molares, objeto do estudo.

As mandíbulas do lado esquerdo e direito foram imediatamente colocadas em frascos individuais devidamente identificados, contendo solução de formaldeído à 4%, tamponada com pH neutro, durante as primeiras 24 horas e depois lavadas em água corrente por um período de 12 horas, para a remoção de toda solução de fixação. Após a fixação, as peças foram desmineralizadas em solução de EDTA neutro a 10% por um período aproximado de 6 semanas. Posteriormente, essas foram lavadas em água corrente, desidratadas em álcool, diafanizadas em xilol e incluídas em parafina. A orientação no momento da inclusão permitiu a realização de cortes teciduais dos molares inferiores e estruturas de suporte no plano mesiosagital. As peças foram cortadas com cortes semi-seriados, com 5 µm de espessura, realizados em micrótomo (Leica - RM 2045, Buffalo Grove, IL, USA). Os cortes histológicos foram selecionados a partir do momento onde a raiz mesial do primeiro molar inferior fosse visualizada em seu sentido longitudinal pleno (BENETTI *et al*., 2018a).

3.1.6 Forma de análise dos resultados

3.1.6.1 Avaliação histológica

Os cortes histológicos advindo dos molares imaturos inferiores foram submetidos às técnicas de coloração Hematoxilina-eosina (HE), Tricrômio de Masson (TM), Picrosírius red (PSR) e imunohistoquímica (CINTRA *et al*., 2017). Para cada espécime a primeira lâmina histológica obtida foi selecionada e corada com HE, e as duas seguintes preparadas para as análises TM e PSR. Outras duas lâminas por marcador foram selecionadas para técnica de imunohistoquímica (BENETTI *et al*., 2018a; CINTRA *et al*., 2017).

3.1.6.2 Análise histológica (técnica de HE)

Para a análise histológica, três cortes seriados de cada raiz foram selecionados e examinados em microscópio óptico (DM 4000 B, Leica®, Germany), por um observador calibrado e cego quantos aos grupos experimentais.

As secções coradas em HE foram usadas para a análise histopatológica, onde os tecidos intracanal e periapical de cada grupo foram analisados em escores de acordo com parâmetros envolvendo a formação de tecido mineralizado nas paredes dos canais radiculares (PALMA *et al.*, 2017; ZHANG *et al*., 2014), a presença de cemento neoformado (GOMES-FILHO *et al.*, 2013), fechamento do forame apical (PALMA *et al.*, 2017), extensão do infiltrado inflamatório (GOMES-FILHO *et al.*, 2013) e a presença de tecido neoformado no interior dos canais radiculares. Estes parâmetros avaliados foram descritos na Tabela 2.

Tabela 2 - Escores aplicados para cada parâmetro avaliado na análise histopatológica

Parâmetros avaliados na análise histopatológica
Formação de tecido mineralizado nas paredes dos canais radiculares
0 = Ausente
1 = Presente, com aumento do comprimento radicular
2 = Presente, com aumento da espessura radicular
3 = Presente, com aumento da espessura e do comprimento radicular
Cemento neoformado
0 = Ausente
1 = Presente
Fechamento do forame apical
0 = Ausente
1 = Parcial
2 = Completo
Extensão do infiltrado inflamatório
0 = Ausente
1 = Presente, no terço apical
2 = Presente, estendendo para o terço médio
3 = Presente, estendendo para o terço cervical
Presença de tecido neoformado no interior dos canais radiculares
0 = Ausente
1 = Presente, no terço apical
2 = Presente, estendendo para o terço médio
3 = Presente, estendendo para o terço cervical

Fonte: Do autor, 2021.

As secções coradas em HE foram usadas também para análise histomorfométrica, onde a área de cemento neoformado foi obtida em μm^2 (BENETTI *et al*., 2018b; LONDERO *et al*., 2015; PALMA *et al*., 2017).

3.1.6.3 Análise das fibras colágenas (técnicas de PSR e TM)

A identificação e os níveis de maturação das fibras de colágeno foram avaliados pelas técnicas de TM e PSR, respectivamente. As secções coradas pelo PSR foram analisadas sob microscopia de luz polarizada. O programa LAS foi utilizado para avaliar o colágeno presente na extremidade radicular e também no centro do terço apical do canal radicular (400×, Leica LAS 4.12, Leica Microsystems), permitindo a seleção de cores correspondentes para cada tipo de fibra de colágena. Após a seleção da cor, o programa calculou automaticamente a área marcada de cada tipo de fibra colágena. As fibras amarelo-esverdeadas foram consideradas imaturas e finas, enquanto que as fibras laranja-avermelhadas foram classificadas como maduras e grossas (BENETTI *et al.*, 2020; CINTRA *et al*., 2017; JUNQUEIRA *et al*., 1982; TERAYAMA *et al*., 2020).

3.1.6.4 Técnica de imunohistoquímica

Foi avaliada a imunomarcação dos fatores de crescimento TGF-β1 e FGF-2 pela técnica da imunoperoxidase indireta (BENETTI *et al.*, 2019). Os cortes histológicos foram desparafinizados em xilol e hidratados em série decrescente de etanol (100° - 100° - 100° - 90° - 70° GL). A recuperação antigênica foi realizada através da imersão das lâminas histológicas em tampão Diva Decloaker® (Biocare Medical, CA, USA), em câmara pressurizada Decloaking Chamber® (Biocare Medical, CA, USA), a 95°C, por 10 minutos. Após lavagens em tampão fosfato salino (PBS) 0,1M, pH 7,4, as lâminas histológicas foram imersas em peróxido de hidrogênio à 3%, por 1 hora, para o bloqueio da peroxidase endógena. Em prosseguimento, depois de lavagens em PBS, os cortes histológicos foram tratados com 3% de soro albumina bovino por 12 horas, para bloqueio dos sítios inespecíficos.

As lâminas histológicas foram submetidas à incubação com os seguintes anticorpos primários: anti-TGF-β1 (rabbit primary antibodies; Sigma-Aldrich Co. LLC, St. Louis, MO, USA) e anti-FGF-2 (rabbit primary antibodies; Sigma-Aldrich Co.). Os anticorpos primários foram diluídos em PBS acrescido de 0,1% Triton X-100 (PBS-TX), durante 24 horas, em câmara úmida. Nas etapas subsequentes foi empregado o Universal Dako Labeled (HRP) Streptavidin-Biotin Kit® (Dako Laboratories, CA,

USA). Após lavagens, as secções histológicas foram incubadas no anticorpo secundário biotinilado, durante 2 horas, lavadas, e tratadas com estreptavidina conjugada com a peroxidase da raiz forte (HRP), por 1 hora.

Depois de três lavagens em PBS-TX, procedeu-se para a revelação utilizando-se como cromógeno o 3,3'- tetracloridrato de diaminobenzidina (DAB chromogen Kit®, Dako Laboratories, CA, USA). Ao término de uma série de lavagens em PBS, os cortes histológicos foram contra corados com hematoxilina de Harris. Como controle negativo, os espécimes foram submetidos aos procedimentos descritos anteriormente suprimindo-se a utilização dos anticorpos primários.

A imunomarcação foi avaliada em duas regiões diferentes da região apical do dente (aumento de 400×): na ponta da raiz e no centro do tecido pulpar no terço apical. Para isso, a imunomarcação foi definida como a coloração acastanhada presente nas células. Uma análise semi-quantitativa foi realizada para levantar informações referentes a quantidade de células marcadas (BENETTI *et al*., 2017, 2019). Os escores utilizados foram (BENETTI *et al*., 2018a, 2019): 0 – ausência de imunomarcação (completa ausência de células imunorreativas); 1 – baixo padrão de imunomarcação (aproximadamente um quarto das células imunorreativas); 2 – moderado padrão de imunomarcação (aproximadamente metade das células imunorreativas); 3 – elevado padrão de imunomarcação (aproximadamente três quartos das células imunorreativas); 4 – extremamente elevado padrão de imunomarcação (praticamente todas as células imunorreativas).

3.1.7 Análise estatística

A análise estatística foi conduzida utilizando-se o programa *Statistical Package for the Social Sciences* (SPSS for Mac OS, version 19.0, IBM Inc., Armonk, NY, USA), para comparação dos dados referentes aos grupos experimentais. Os grupos controles não foram avaliados estatisticamente. Após a aplicação do teste de normalidade Shapiro-Wilk, os dados paramétricos foram submetidos ao teste-T de Student, e os dados não-paramétricos, ao teste de Mann-Whitney. Para todos os testes foi considerado valor de $p < 0,05$.

3.2 Revisão Sistemática

3.2.1 Protocolo e registro

A presente revisão foi realizada de acordo com as diretrizes do *Preferred Report Items for Systematic Reviews and Meta-analyses* (PRISMA) (DOS REIS-PRADO *et al.*, 2021; PAGE *et al.*, 2021). O protocolo de revisão sistemática foi registrado na base de dados PROSPERO sob o número: CRD42020205417 (https://www.crd.york.ac.uk/PROSPERO/).

3.2.2 População e Critérios de elegibilidade

Foram incluídos somente 1) estudos *in vivo* que avaliaram os efeitos do EDTA sobre os fatores biológicos da dentina ou polpa/tecidos periapicais em procedimentos endodônticos regenerativos, e 2) estudos *in vitro* que avaliaram os efeitos do EDTA na viabilidade celular, migração, fixação ou morfologia celular, e nos fatores biológicos da dentina. Os critérios de exclusão foram 1) estudos que avaliaram os efeitos do EDTA na dentina, células ou polpa/tecidos periapicais, sem grupo controle ou outras soluções irrigantes, sem EDTA; e 2) estudos em que não foi possível obter acesso ao texto completo. Não houve restrições com base no idioma ou data de publicação.

Uma abordagem de população, intervenção, comparação, resultado (PICO) foi usada para abordar a seguinte questão: “O EDTA influencia os fatores biológicos da dentina, células ou tecidos em procedimentos endodônticos regenerativos?” A população do estudo foi composta de dentina, células ou tecidos pulpar/periapicais de humanos ou animais que foram submetidos à irrigação/condicionamento com EDTA. A intervenção consistia na irrigação ou condicionamento com EDTA; e a comparação na irrigação ou condicionamento com outras soluções. O desfecho primário considerado foi o efeito do EDTA na liberação de fatores de crescimento da matriz dentinária. Já os desfechos secundários foram: viabilidade, migração, adesão, morfologia e diferenciação celular, imunomarcação/expressão de outras proteínas, caracterização do coágulo sanguíneo, inflamação e crescimento tecidual, aumento do comprimento e/ou espessura da raiz, diminuição do diâmetro apical, mineralização e reabsorção óssea ou radicular.

3.2.3 Fontes de informação e estratégia de busca

Pesquisas eletrônicas foram conduzidas nas bases de dados PubMed/MEDLINE, Scopus, Cochrane Library, Web of Science e Embase, até fevereiro de 2021. A literatura cinzenta também foi consultada através do OpenGrey, juntamente com a realização de buscas manuais nas listas de referências dos artigos incluídos. A estratégia de busca usou uma combinação de palavras-chaves e termos MeSH (Medical Subject Headings), que foram associados com operadores Booleanos “AND” e “OR”, como mostra a Tabela 3.

Tabela 3 - Estratégia de busca utilizada para cada base de dados

Base de dados	Estratégia de busca
Medline através do PubMed (http://www.ncbi.nlm.nih.gov/pubmed), **OpenGrey** (http://www. opengrey.eu/)	((EDTA [MeSH Terms] OR “ethylenediaminetetraacetic acid” [MeSH Terms] AND “bioactive molecules" [Title/Abstract] OR "tissue engineering" [MeSH Terms] OR "growth factors" [MeSH Terms] OR "stem cells" [MeSH Terms] OR “mesenchymal stem cells” [MeSH Terms] OR "dental pulp stem cells" [Title/Abstract] OR cells [Title/Abstract] OR tissues [MeSH Terms] OR tissue [Title/Abstract] AND "incomplete root formation" [Title/Abstract] OR "immature permanent teeth" [Title/Abstract] OR "immature teeth" [Title/Abstract] OR "immature permanent tooth" [Title/Abstract] OR "immature tooth" [Title/Abstract] OR "incomplete rhyzogenesis" [Title/Abstract] OR "regenerative endodontics" [MeSH Terms] OR "regenerative endodontic therapy" [Title/Abstract] OR "regenerative endodontic procedure" [Title/Abstract] OR "pulp revitalization" [Title/Abstract] OR "pulp revascularization" [Title/Abstract] OR "pulp regeneration" [Title/Abstract] OR "pulp-dentin complex regeneration” [Title/Abstract]))
Scopus (http://www.scopus.com/)	#1 TITLE-ABS-KEY (EDTA) OR TITLE-ABS-KEY (ethylenediaminetetraacetic acid) #2 TITLE-ABS-KEY (bioactive molecules) OR TITLE-ABS-KEY (tissue engineering) OR TITLE-ABS-KEY (growth factors) OR TITLE-ABS-KEY (stem cells) OR TITLE-ABS-KEY (mesenchymal stem cells) OR TITLE-ABS-KEY (dental pulp stem cells) OR TITLE-ABS-KEY (cells) TITLE-ABS-KEY (tissues) TITLE-ABS-KEY (tissue) #3 TITLE-ABS-KEY (incomplete root formation) OR TITLE-ABS-KEY (immature permanent teeth) OR TITLE-ABS-KEY (immature teeth) OR TITLE-ABS-KEY (immature permanent tooth) OR TITLE-ABS-KEY (immature tooth) OR TITLE-ABS-KEY (incomplete rhyzogenesis) OR TITLE-ABS-KEY (regenerative endodontics) OR TITLE-ABS-KEY (regenerative endodontic therapy) OR TITLE-ABS-KEY (regenerative endodontic procedure) OR TITLE-ABS-KEY (pulp revitalization) OR TITLE-ABS-KEY (pulp revascularization) OR TITLE-ABS-KEY (pulp regeneration) OR TITLE-ABS-KEY (pulp-dentin complex regeneration) #1 AND #2 AND #3
Cochrane (https://www.cochranelibrary.com/)	(EDTA [MeSH Terms] OR “ethylenediaminetetraacetic acid” [MeSH Terms]) AND (“bioactive molecules" OR "tissue engineering" [MeSH] OR "growth factors" [MeSH] OR "stem cells" [MeSH] OR “mesenchymal stem cells” [MeSH] OR "dental pulp stem cells" OR cells OR tissues [MeSH] OR tissue) AND ("incomplete root formation" OR "immature permanent teeth" OR "immature teeth" OR "immature permanent tooth" OR "immature tooth" OR "incomplete rhyzogenesis" OR "regenerative endodontics" [MeSH] OR "regenerative endodontic therapy" OR "regenerative endodontic procedure" OR "pulp revitalization" OR "pulp revascularization" OR "pulp regeneration" OR "pulp-dentin complex regeneration”)
Web of Science (https://clarivate.com/webofsciencegroup/solutions/web-of-science-core-collection/)	TOPIC: (EDTA OR "ethylenediaminetetraacetic acid") AND TOPIC: ("bioactive molecules” OR "tissue engineering” OR "growth factors" OR "stem cells" OR "mesenchymal stem cells" OR "dental pulp stem cells" OR cells OR tissues OR tissue) AND TOPIC: ("incomplete root formation" OR "immature permanent teeth" OR "immature teeth" OR "immature permanent tooth" OR "immature tooth" OR "incomplete rhyzogenesis" OR "regenerative endodontics" OR "regenerative endodontic therapy" OR "regenerative endodontic procedure" OR "pulp revitalization" OR "pulp revascularization" OR "pulp regeneration" OR "pulp-dentin complex regeneration")
Embase (https://www.embase.com)	(edta:ti,ab,kw OR 'ethylenediaminetetraacetic acid':ti,ab,kw) AND ('bioactive molecules':ti,ab,kw OR 'tissue engineering':ti,ab,kw OR 'growth factors':ti,ab,kw OR 'stem cells':ti,ab,kw OR 'mesenchymal stem cells':ti,ab,kw OR 'dental pulp stem cells':ti,ab,kw OR cells:ti,ab,kw OR tissues:ti,ab,kw OR tissue:ti,ab,kw) AND ('incomplete root formation':ti,ab,kw OR 'immature permanent teeth':ti,ab,kw OR 'immature teeth':ti,ab,kw OR 'immature permanent tooth':ti,ab,kw OR 'immature tooth':ti,ab,kw OR 'incomplete rhyzogenesis':ti,ab,kw OR 'regenerative endodontics':ti,ab,kw OR 'regenerative endodontic therapy':ti,ab,kw OR 'regenerative endodontic procedure':ti,ab,kw OR 'pulp revitalization':ti,ab,kw OR 'pulp revascularization':ti,ab,kw OR 'pulp regeneration':ti,ab,kw OR 'pulp-dentin complex regeneration':ti,ab,kw)

MeSH – Medical Subject Heading. Fonte: Do autor, 2021.

3.2.4 Seleção dos estudos

A seleção do estudo foi realizada por dois autores (A.H.R.P. e R.R.F.) de maneira independente em um processo de duas etapas. Primeiramente, foi realizado um exercício de calibração entre os revisores, onde foi discutido os critérios de elegibilidade. Após obter um nível de concordância inter-examinador adequado

(Kappa: 0,843 - 1,000), prosseguiu-se para a primeira etapa. Na Etapa 1, ambos os autores avaliaram os títulos e resumos dos estudos recuperados na busca eletrônica. Estudos com títulos e resumos que atenderam aos critérios de elegibilidade foram incluídos. Para os estudos com títulos e resumos que não fornecerem informações suficientes para a tomada de decisão, os textos completos foram baixados e analisados. Os artigos foram organizados em ordem alfabética por titulo e os estudos duplicados puderam ser identificados e removidos manualmente. Na etapa 2, os autores avaliaram os textos completos. Somente estudos que atenderam completamente os critérios de inclusão foram incluídos. As divergências entre os dois autores foram inicialmente resolvidas por meio de discussão e, quando necessário, um terceiro autor (F.B.) foi consultado.

3.2.5 Extração dos dados

Dois revisores (A.H.R.P. e R.R.F.) coletaram de maneira independente e em duplicata, os dados de todos os estudos incluídos, utilizando um formulário de extração de dados piloto em uma planilha do Excel. As planilhas incluíam as seguintes informações: identificação do artigo (sobrenome do primeiro autor e ano de publicação), desenho do estudo, modelo experimental, tamanho da amostra, grupos, protocolo experimental e análises realizadas. Posteriormente, terceiro investigador (F.B.) revisou todos os dados extraídos.

3.2.6 Avaliação do risco de viés

Dois investigadores (A.H.R.P. e S.C.O.) avaliaram de forma independente os risco de viés dos estudos selecionados, com base nos níveis de evidência, conforme proposto pela versão modificada da ferramenta *Joanna Briggs Institute's Critical Evaluation Checklist for Experimental Studies* (DOS REIS-PRADO *et al*., 2021; YAYLALI; KECECI; UREYEN, 2015). Os itens do *checklist* incluíram: objetivo claramente descrito; justificativa do tamanho amostral; randomização dos espécimes; análise cega; equivalência e semelhança entre controle e grupos experimentais; protocolo de tratamento claramente descrito; avaliação padronizada dos grupos; avaliação confiável dos desfechos; e análise estatística apropriada. Cada item foi registrado utilizando uma escala de 2 escores: 0, conteúdo não

reportado ou reportado inadequadamente; e 1, conteúdo reportado e de forma suficiente. Ademais, a ferramenta RoB para estudos de intervenção animal (SYRCLE's RoB tool) (HOOIJMANS *et al*., 2014) com modificação (para melhor caracterizar a descrição dos estudos em animais incluídos, os investigadores também avaliaram se o tamanho amostral foi justificado) foi aplicada nos estudos *in vivo*. Os estudos foram categorizados como tendo risco baixo ("*yes*"), alto ("*no*") ou incerto ("*unclear*") de viés baseado na qualidade da informação fornecida. Dúvidas e discrepâncias entre os pesquisadores eram discutidas para se chegar a um consenso e, caso não resolvidas, um terceiro avaliador (L.G.A.) foi consultado.

4 ARTIGO 1

O artigo 1 “***Histologic Evaluation of the influence of EDTA irrigation on Regenerative Endodontic Procedure in immature rat molar model***” será submetido ao periódico *International Endodontic Journal* (Qualis A1, Fator de Impacto = 3,801).

Abstract

Aim To analyse the influence of the use of ethylenediaminetetraacetic acid (EDTA) on the repair process of immature molars of rats after a regenerative endodontic procedure (REP).

Methodology The lower first molars of 12 4-weeks-old Wistar rats underwent pulpectomy in the mesial root and were divided into the following groups: sodium hypochlorite (NaOCl; n = 6) – the mesial canals were irrigated with 2.5% NaOCl for 5 min; and NaOCl-EDTA (n = 6) – the canals were irrigated with 2.5% NaOCl, followed by 17% EDTA for 5 min each. After evoking bleeding using size 10 K-file, the cavities were sealed. Three molars on the untreated side were randomly used as control (control-15d; n = 3) and three molars from the other three rats untreated were used as immediate control (n = 3). At 15 days (NaOCl, NaOCl-EDTA and control-15d groups) or immediately (control-immediate), the animals were killed and the teeth were subjected to histologic evaluation of tissue regeneration and of collagen fibres. Mann-Whitney test and t-test were used ($P < 0.05$).

Results The experimental groups had newly formed cementum-like tissue on root length and thickness. Half of the specimens in NaOCl-EDTA group showed apical foramen closure, while NaOCl group had partial apical closure. The experimental groups showed inflammatory infiltrate extending mainly to the medium third of the root canal. These parameters were similar between experimental groups ($P > 0.05$). Newly formed connective tissue in the pulp space was significantly higher in the NaOCl-EDTA group than in NaOCl group ($P < 0.05$). Regarding the collagen fibres, the NaOCl-EDTA group had more collagen fibres in the root tip, but there was no significant difference compared to NaOCl group, and both groups showed greater amount of immature fibres in this area; in the centre of the apical third of root canal, there was equivalence between mature and immature fibres from both groups ($P > 0.05$).

Conclusions EDTA irrigation improved newly formed intracanal connective tissue after REP in immature molars of rats; however, EDTA did not influence cementum-like tissue formation, apical closure, inflammatory infiltrate, and maturation of collagen fibres.

Keywords Dental pulp, EDTA, Guided tissue regeneration, Histologic outcomes, Regenerative endodontics, Root canal irrigants.

Introduction

Caries, traumatic dental injuries and dental anomalies may damage the pulp-dentin complex of immature permanent teeth, resulting in pulp necrosis and incomplete root development (Scarparo *et al*. 2011, Palma *et al.* 2017). In these cases, conventional endodontic treatment often presents challenges in these teeth, since there is a major risk of root fractures and overflow of filling materials, due to the presence of thin dentinal walls and open apices (Shah *et al.* 2008, Thibodeau *et al*. 2007). In addition, a presence of thin remaining dentinal walls thickness and length due to disrupted root maturation leaves the tooth even more susceptible to fracture (Bracks *et al*. 2019). Therefore, new materials and approaches have been proposed for the treatment of immature teeth with pulp necrosis.

Regenerative endodontic procedures (REPs) are biologically based treatments that have emerged and it may help to restore the physiologically functional dentition by regenerating tissue in the canal space of immature necrotic teeth (Galler *et al.* 2016, Kim et al. 2018, Shamszadeh *et al.* 2019). These procedures are based on pillars of tissue engineering that contributes to its long-term success, including stem cells, 3-dimensional scaffolds, signalling molecules and a bacteria-free environment (Gomes-Filho *et al*. 2013, Zhang *et al.* 2014, Conde et al. 2016, Bracks *et al.* 2019).

Clinical studies using REP have demonstrated promising results with high survival and success rates ranged between 95%-100% (Alobaid *et al*. 2014, Jeerupahn *et al*. 2012, Arslan *et al.* 2019), and showing increased root length and thickness, and apical closure (Shah *et al*. 2008, Torabinejad & Faras 2012). Despite those results, there is no standard irrigation protocol available in REP (Aksel & Serper 2014, Shamszadeh *et al.* 2019, Aksel *et al.* 2020). Whilst generally, current clinical protocols of the European Society of Endodontology (Galler *et al*. 2016) and the American Association of Endodontics (2018) have proposed the use of 17% ethylenediaminetetraacetic acid (EDTA) after low concentrations of sodium hypochlorite (NaOCl).

The use of EDTA is important to minimise the cytotoxicity of NaOCl and to enhance the release of bioactive molecules from the dentine (Chae *et al.* 2018, Kim et al. 2018). In addition, conditioning dentine with EDTA removes the smear layer, which may expose growth factors entrapped in the dentin matrix (Graham *et al*. 2006, Pang *et al*. 2014, Conde *et al*. 2016, Gonçalves *et al*. 2016, Bracks *et al*. 2019).

The expression of those molecules from conditioned dentine might modulate cellular activity in periapical tissues (Gonçalves *et al*. 2016, Taweewattanapaisan *et al*. 2019), playing a crucial role in the intracanal tissues neoformation (Bracks *et al*. 2019). Thus, the additional irrigation with EDTA has been fully recommended in order to optimize the conditions of cell differentiation, tissue formation and regeneration (Pang *et al.* 2014, Conde *et al*. 2016). Nevertheless, EDTA may be capable of promoting some negative impact on cell viability (Aksel *et al*. 2020), cell migration (Deniz Sungur *et al.* 2019), and blood clot formation (Taweewattanapaisan *et al*. 2019) compared with other solutions, which might affect tissue regeneration.

There have been a few histological analyses evaluating the tissue reaction involved in the repair process and regeneration after the use of NaOCl followed by EDTA irrigation as clinical recommendations for REP. Additionally, an *in vivo* collagenous content evaluation as a relevant component for the tissue repair and mineralisation in immature teeth has not yet been investigated.

The present study aimed to histologically evaluate the influence of the use of EDTA on the repair process of immature molars of rats that underwent a REP. The null hypothesis tested was that there are no differences in the histological findings after REP with or without EDTA.

Materials and Methods

Animals

A total of fifteen 4-week-old male Wistar albino rats (weighing approximately 80 g) were used. The sample size was based on a previous study (Scarparo *et al*. 2014). The animals were housed in a temperature-controlled environment (22°C–24°C) with a 12-h light-dark cycle and *ad libitum* access to water and feed. The study was approved by the local Animal Research Ethics Committee (CEUA 81/2020) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA).

Pulp revascularization procedure

The animals were anaesthetised via intramuscular (IM) injection using ketamine 10% (80 mg/Kg; Ketamina Agener 10%, União Química Farmacêutica Nacional S/A, Embu-Guaçu, São Paulo, Brazil) and xylazine 2% (15 mg/Kg; Xilazin, Syntec do Brasil LTDA, Cotia, São Paulo, Brazil). The lower left or right first immature molars

of twelve animals were randomly separated in the experimental groups NaOCl and NaOCl-EDTA based on the operative procedures. Lower untreated molars from these animals were randomly selected as control (Control-15d, n = 3). Additionally, lower left or right immature molars of other three rats did not receive any intervention, and they were used as Control-immediate (n = 3). These controls were included for histologic reference of natural embryogenesis.

The teeth were isolated with gingival barrier (Top dam, FGM, Joinville, SC, Brazil) and special dental clamps. Then, endodontic access in mesial root canal was performed with a sterile LN round bur (Dentisply Maillefer, Tulsa, OK, USA) in low speed coupled to an electric motor (Beltec, Araraquara, SP, Brazil) and under constant irrigation with saline. Operative procedures were performed using an operating microscope (Alliance, São Paulo, SP, Brazil) with 24× magnification. After exposing the dental pulps, concomitantly pulpectomy was performed using size 10 K-files (Dentsply Sirona, Ballaigues, Switzerland). The canals were irrigated with sterile saline solution and dried with sterile paper points. To define the working length of the mesial root canals, a radiograph was taken in a pilot study with size 10 K-file introduced in mesial canal.

After, the irrigation protocols were performed according to the experimental groups (NaOCl and NaOCl-EDTA). For the NaOCl group, the mesial canals were irrigated with 2.5% NaOCl for 5 minutes; and in the NaOCl+EDTA group, the mesial canals were irrigated with 2.5% NaOCl followed by 17% EDTA for 5 min each. Subsequently, the canals were irrigated with saline solution and dried with sterile absorbent paper points. Intracanal bleeding was induced by introducing a 15 K-file at 0.5 mm beyond the apical foramen, using a counter clockwise push-and-pull motion until the root canal was filled with blood from the periapical tissues (Bracks *et al*. 2019). PBS CIMMO HP (CIMMO, Pouso Alegre, MG, Brazil) was placed for coronal barrier, and the cavities were sealed with resin-modified glass ionomer cement light-cured (GC America Inc., Alsip, IL, USA).

Sample preparation and histological analysis

At the 4-week period (Control-immediate group), or fifteen days after the pulp revascularization procedure (experimental groups and Control-15d), the rats were killed with an overdose of anaesthetic solution (Thiopentax; Cristália—Produtos Químicos Farmacêuticos LTDA, Itapira, São Paulo, Brazil). The right and left jaws

were separated, dissected, fixed in a solution of 4% buffered formaldehyde for 24 h, and then decalcified in 10% EDTA solution for 45 days. The specimens were processed and embedded in paraffin. Serial histological sections (5 μm) were cut in the mesiosagittal plane and were selected from the point where the mesial root of the first molar was seen in all its longitudinal extension. Then, the histological sections were stained with haematoxylin-eosin (HE), Masson's trichrome (MT), and picrosirius red (PSR) (Cintra *et al*. 2017). The first slide obtained with histological sections was selected for HE staining, and each the next two for MT or PSR analysis.

The analyses were performed under light microscopy (400× magnification; DM4000 B; Leica Microsystems, Wetzlar, Germany) by a single calibrated and blinded operator to the experimental groups. Sections stained with HE were used for histopathological analysis. The intracanal and periapical tissues present in each group were analysed and scored according to the histopathological parameters involving mineralized tissues formed on root canal (Zhang *et al*. 2014, Palma *et al.* 2017), newly formed cementum-like tissue (Gomes-Filho *et al.* 2013), apical closed histologic (Palma *et al.* 2017), extension of the inflammatory infiltrate (Gomes-Filho *et al.* 2013), and presence of newly connective tissue. These rated parameters were described in Table 1.

The collagen fibres maturation was analysed in the sections stained by PSR under polarized light microscopy. The images were obtained of the apical tip of the root, and the centre of the apical third of the root canal (400× magnification; Leica QWin V3, Leica Microsystems), allowing the selection of corresponding colours for each type of collagen fibre. After colour selection, the program automatically calculated the marked area of each collagen fibre type inside the pulp chamber. Greenish-yellow fibres were classified as immature and thin, while yellowish-red fibres were considered mature and thick (Cintra *et al*. 2017, Benetti *et al.* 2020, Terayama *et al*. 2020).

Statistical analysis

Nonparametric data were analysed using the Mann-Whitney test, whereas for parametric data, the t-test was used after normality of the data was tested for. Statistical significance was set at $P < 0.05$ for all analyses.

Results

Histologic Findings

The effects of EDTA on immature rats molars after REP were determined by comparing the same parameters between experimental groups. Representative images of the evaluated histopathological parameters are presented in Figure 1, and the results are summarized in Table 1.

After 15 days of REP, most specimens of NaOCl-EDTA group showed a concomitant increase of root length and thickness indicated by a deposition of cellular mineralized tissue compared to NaOCl group, which an increase in the root canal wall thickness was mostly observed. All teeth in the NaOCl and NaOCl-EDTA groups displayed cementum tissue formation, where many cementocyte-like cells were present in the newly layer of mineralized tissue formed on the root canal wall. Histologic evaluation showed progressive apical closure in NaOCl-EDTA group, where half of the specimens exhibited complete closure of the root apex while NaOCl without EDTA showed partial apical closure in one tooth.

Regarding the extension of inflammatory infiltrate, both experimental groups showed a variable inflammatory response to treatment with the presence of polymorphonuclear cells, extending mainly to the medium third of the root canal (Figure 1). These four evaluated parameters did not differ histologically between experimental groups ($P > 0.05$).

Regarding the newly formed connective tissue into the pulp canal space, a partial ingrowth of connective tissue occurred significantly in most specimens of NaOCl-EDTA group, extending mainly from the apical to the medium third of teeth ($P < 0.05$). The tissue was characterized by a continuous layer of connective tissue of the periodontal ligament, with fibroblasts and blood vessels. Additionally, the presence of some islands of cellular cementum in the lumen of the root canal space were also noted in the NaOCl-EDTA group. On the other hand, newly formed connective tissue areas in the root canal space were predominantly absent in the NaOCl group.

Table 1 Histologic evaluation for each parameter in experimental groups after 15 days of REP

Scores for each parameter	Experimental groups (n = 6)		*P*-value
	NaOCl	NaOCl-EDTA	
Mineralized tissue formed on the root canal wall			
0 = absent	0/6	0/6	
1 = present with increased the length of the root	0/6	0/6	= 0.241
2 = present with increased the thickness of the root	5/6	3/6	
3 = present with increased the thickness and length of the root	1/6	3/6	
Newly formed cementum-like tissue			
0 = absent	0/6	0/6	= 1.000
1 = presence	6/6	6/6	
Apical closed histologic			
0 = absent	5/6	3/6	= 0.149
1 = partial	1/6	0/6	
2 = complete	0/6	3/6	
Extension inflammatory infiltrate			
0 = absent	0/6	0/6	
1 = within the apical third	0/6	1/6	= 0.598
2 = extending to the medium third	5/6	4/6	
3 = extending to the cervical third	1/6	1/6	
Presence of newly connective tissue on the root canal			
0 = absent	5/6	1/6	
1 = within the apical third	1/6	2/6	= 0.019*
2 = extending to the medium third	0/6	3/6	
3 = extending to the cervical third	0/6	0/6	

The symbol * indicates presence of significant difference between the groups ($P < 0.05$). NaOCl: sodium hypochlorite; EDTA: ethylenediaminetetraacetic acid.

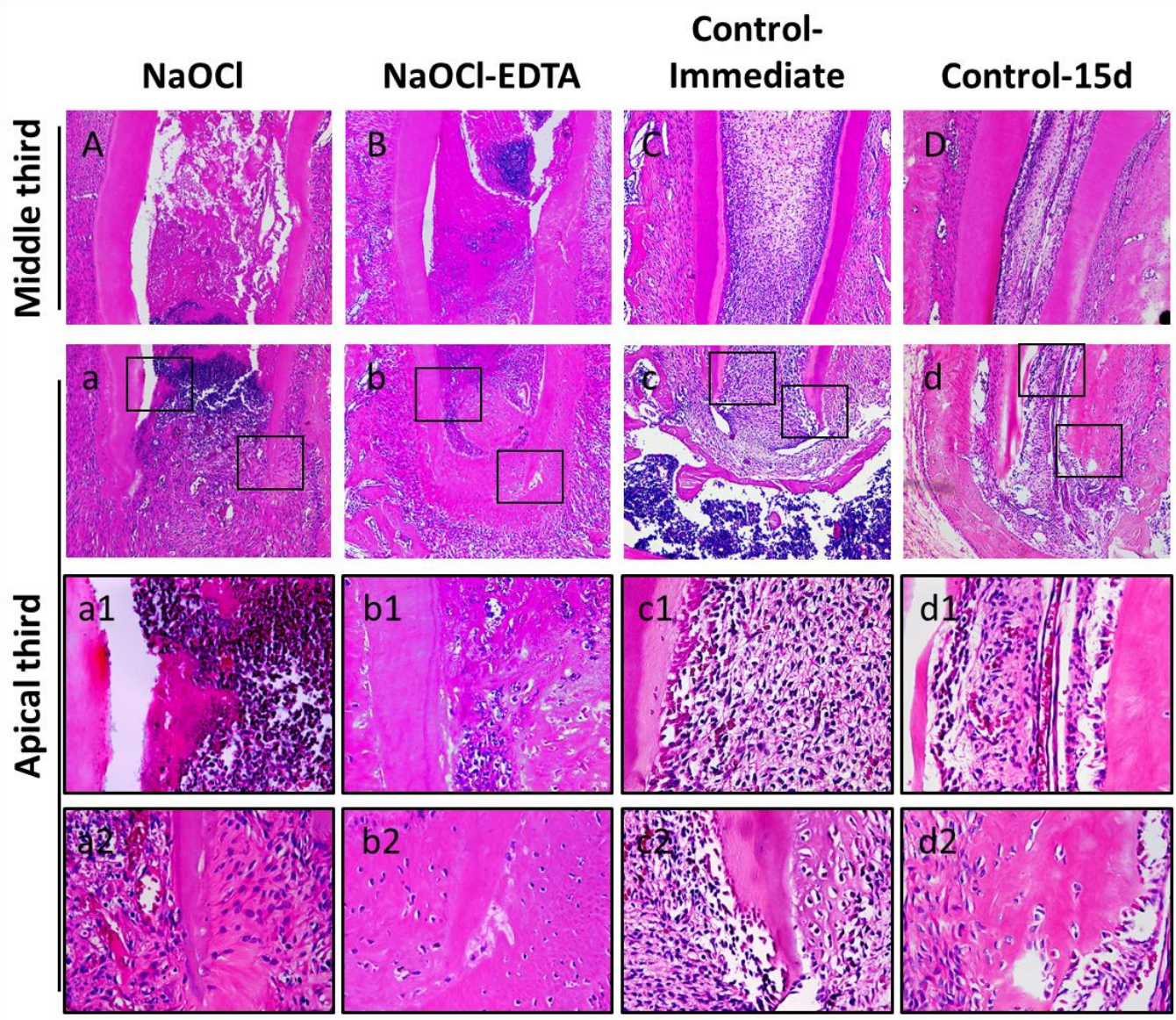

Figure 1 Representative images of histological analysis of the root canal space and periapical region. NaOCl: (A) panoramic microscopic aspect of the middle third with the presence blood clot, (a) panoramic microscopic aspect of the apical third with inflammatory infiltrate, (a1) superior apical area with inflammatory infiltrate and blood clot, and (a2) root tip area with blood vessels, inflammatory cells, cementum-like tissue and ingrowth of fibroblast-like cells; NaOCl-EDTA: (B) panoramic microscopic aspect of the middle third showing the presence of blood clot and inflammatory infiltrate, (b) panoramic microscopic aspect of the apical third with apical closure, and newly formed cementum-like tissue, (b1) presence of blood clot, inflammatory cells, cementum-like tissue, and (b2) root tip area with cementum-like tissue with cells similar to cementoblasts and cementocytes. Control-immediate and Control-15d: (C, D) panoramic microscopic aspect of the middle third showing pulp tissue, (c, d) panoramic microscopic aspect of the apical third showing (c) open apex and (d) advanced root development, (c1, d1) blood vessels and organised pulp tissue, and (c2, d2) root tip area with o areas of cementum. [100×, 400×: haematoxylin–eosin]

Presence and maturation of collagen fibres

Representative images of the TM and PSR staining are displayed in Figure 1, and the results are shown in Figure 2. Whereas NaOCl-EDTA group exhibited more collagen fibres in the root tip region, there was no significant difference compared with the NaOCl group, where both groups showed greater amount of immature fibres in this area. Regarding the centre area of the apical third, equivalent values between mature and immature fibres from both evaluated groups were found, without significant differences.

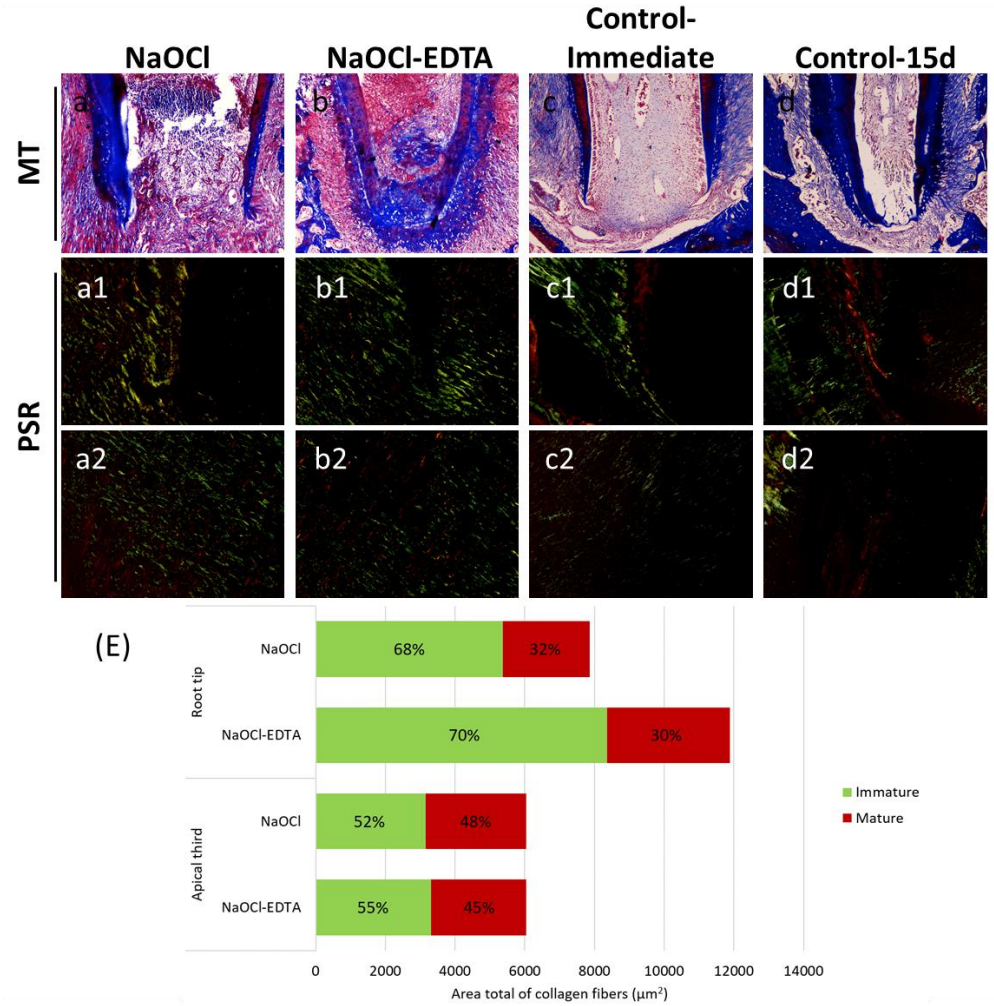

Figure 2. Representative images of collagen analysis. (a-d) Panoramic microscopic aspect of the apical third of the root canal space and periapical region; (a1-d1) collagen fibres maturation viewed under polarized light microscopy in the root tip area with higher amount of immature collagen fibres in all groups; and (a2-d2) collagen fibres maturation viewed under polarized light microscopy in the centre area of pulp apical third with similar amount between immature and mature collagen fibres in the experimental groups. (E) Evaluation of the area and maturation of collagen fibres using PRS staining. There was no significant difference between NaOCl and NaOCl-EDTA groups in the total area of collagen fibres or on collagen maturation in the root tip and at the centre of the pulp apical third ($P > 0.05$). [100×: Masson's trichrome, 400×: Picrosirius red]

Discussion

In the current study, a comparative histologic evaluation of the effect of EDTA on the tissue regenerative potential of immature molars of rats that underwent a REP with or without the use of EDTA was performed. It was found that the use of NaOCl-EDTA group promoted mineralized tissue deposition along the root canal walls, cementum tissue formation, apical closure, and inflammatory response in most specimens similarly to NaOCl group. On the other hand, the former demonstrated a higher newly formed connective tissue within the pulp canal space than NaOCl without EDTA, as confirmed by the HE analysis. A greater number of immature fibres were found in the root tip region, while similar values of mature and immature fibres were observed in the center area of the apical third in the experimental groups. There were no significant differences between the groups at the evaluated areas. Thus, the null hypothesis of the study was partially accepted.

An investigation of the steps of regenerative therapy, such as scaffold formation and irrigation, is necessary to establish promisor clinical protocols for REP (Peters 2014, Bracks *et al*. 2019). Besides showing high repair potential, bleeding induction in immature teeth is necessary to produce a support in-growth of new tissue, including the delivery of stem cells and growth factors during regeneration process (Gomes-Filho *et al*. 2013, Jung *et al*. 2019). Additionally, adequate disinfection before inducing bleeding in the root canal space is important for the success of REP (Verma *et al*. 2017).

EDTA is widely used as the final rinse in the protocols for REP, due to its ability to remove intracanal dressing and smear layer generated by instrumentation (Chae *et al*. 2018), in addition to expose and release the dentin matrix (Wang *et al*. 2010; Scarparo *et al*. 2011). Therefore, irrigation with 17% EDTA has also been incorporated after NaOCl in REP (Galler *et al*. 2016), probably due to its high expression of proteins during calcium quenching associated with the stem cell differentiation, which might positively influence tissue neoformation (Widbiller *et al*. 2017, Bracks *et al*. 2019).

Similarly to the present study, the formation of connective and cementum-like tissues was previously described in the regenerated tissues using other irrigating protocols in animal models submitted to REP (Thibodeau *et al*. 2007, Yamauchi *et al*. 2011, Gomes-Filho *et al*. 2013). These histological findings may be associated with the ingrowth of periodontal ligament stem cells into the canal space. Nevertheless, no

pulp-like tissue with odontoblast-like cell layer was observed in these investigations, similarly to our study. Conversely, Ishizaka *et al*. (2012) also performed an *in vivo* study, which demonstrated the presence of pulp-like tissue regeneration when pulp, adipose and bone marrow derived CD31(-) cells were deposited in the root canal by using a collagen scaffold as a stem cell homing with stromal cell-derived factor 1. Nevertheless, the study of Ishizaka *et al*. (2012) did not perform dentine conditioning, which do not simulate a clinical environment.

There are still uncertainties about the histological improvements of irrigation with EDTA that need to be explored in REP. By using a rat model with immature teeth, we histologically assessed this point in the present report. Groups that did not receive any treatment were used as the immediate and 15-day period controls. The histological sections of the experimental groups were associated with continued root development, mineralized tissue formation, apical closure, inflammatory reaction, newly formed connective tissue, and areas of collagen deposition in immature rat teeth after 15 days of REP. HE evaluation revealed a continued root development, including increased root length and thickness, in most specimens irrigated with EDTA. Furthermore, a layer of cementum-like tissue deposited on the root canal walls of all samples and complete or partial apical closure was noticed. Interestingly, the NaOCl-EDTA showed complete closure in half of the samples, one of the main histologic findings of this study. These results might be related with the presence of calcium ions in the blood clot that play a crucial role in its osteoinductive properties (Yamauchi *et al*. 2011, Taweewattanapaisan *et al*. 2019). In addition, the ability of EDTA to expose signalling molecules following dentin demineralization, such as transforming growth factor (TGF)-β, that might regulate cellular activity (Gonçalves *et al*. 2016, Chae *et al*. 2018, Bracks *et al*. 2019, Kucukkaya Eren *et al*. 2021) might have enhanced biological apical closure in this group.

A considerable inflammatory infiltration composed by polymorphonuclear cells, extending mainly to the medium third of the canal, was observed in the NaOCl and NaOCl-EDTA groups. In accordance with these findings, a previous histologic evaluation of immature dog teeth irrigated with 1.25% NaOCl during REP showed a significant number of inflammatory cells adjacent (and not in it) to the newly formed intracanal tissue (Wang *et al*. 2010). The liberation of bioactive molecules following dentine conditioning is involved in the recruitment of various immune-inflammatory cells (Galler *et al*. 2011). Similarly to Wang *et al*. (2010), the presence of

inflammatory cells was not capable of interfering the cementum-like tissue neoformation in the current study, and it may even accelerate hard tissue deposition by providing factors to stimulate stem cell differentiation into cementoblasts (Wang *et al*. 2010). Thus, the presence of inflammatory cells in the treated area may be related to tissue repair. Moreover, Bracks *et al*. (2019) reported a higher interleukin (IL)-1 mRNA expression in the mice teeth submitted to irrigation to EDTA followed by blood clot formation at 7 and 14 days of analysis. IL-1 is a pro-inflammatory cytokine that activates endothelial cells to participate in the immune-inflammatory response, and also shows proangiogenic effects (Bracks *et al*. 2019).

Histologic reports using human and animal teeth have shown the presence of fibrous tissue, and intracanal islands of cementum-like or bone-like tissues (Wang *et al*. 2010, Yamauchi *et al*. 2011, Gomes-Filho *et al*. 2013, Shimizu *et al*. 2013). Regenerated tissues may have characteristics of the tissue from which stem cells originate (Jung *et al*. 2019). This investigation found ectopic areas of cellular cementum-like tissue in the newly formed tissue in the lumen of the canal space in the NaOCl-EDTA, probably originated from stem cells derived from the periodontal ligament and alveolar bone. Regarding the newly generated tissue onto the pulp canal space, a significant partial ingrowth of connective tissue with fibroblasts and blood vessels that reached the medium third of the teeth irrigated with NaOCl followed by EDTA was identified in comparison with those without EDTA irrigation. These results may be associated to the ability of EDTA to release several growth factors entrapped in dentin, thereby promoting cell migration and differentiation. However, further long-term evaluations are necessary to assess the histologic characteristics of the new tissue formed into the root canal, in addition to the presence of growth factors and other bioactive molecules in immature teeth irrigated with EDTA in comparison to non EDTA irrigation.

The extracellular matrix of dental the pulp is composed with approximately 34% of collagen fibres, which represents one of the most important classes of extracellular macromolecules of the dental pulp (Cintra *et al*. 2017). Whereas type I collagen is the most predominant component in the mineralized tissues, a great distribution of collagen type I and III, or mature and immature fibres has been reported in dental pulp, respectively (Garcia *et al*. 2003, Cintra *et al*. 2017). These proteins participate in the process of tissue organization and mineralization of the tooth. In the present study, a visualization of the collagen was executed by using MT

and PSR techniques. Areas of collagen were found especially in the apical area of the treated groups. Although there were no statistical differences between the experimental groups for total area of collagen fibres, there was histological evidence of more collagen fibres in the tip region of NaOCl-EDTA group, probably indicating faster repair.

Concomitant, a specific analysis for immature and mature collagen fibres detection was performed using polarized light microscopy. A greenish-yellow colour suggests that collagen is poorly packed, whereas a yellowish-red colour suggests better maturity and thick fibre organization (Cintra *et al*. 2017, Benetti *et al*. 2020). Immature fibres were predominant in the root tip region, may indicate a loose connective immature tissue-like, compatible with the animal's age. The increased number of collagen fibres in the tip region of NaOCl-EDTA may have been associated with the presence of ectopic mineralization areas onto the pulp space in this group. On the other hand, similar amount of mature and immature collagen fibres was observed in the center region of the apical third from both experimental groups. These results disagree with the study by Gomes-Filho *et al*. (2013), who found dense collagen fibres in the newly formed tissue inside the root canals. However, the analysis period of this previous study was 3 months after the REP, in addition to using the dog model. To our knowledge, there are no other studies that evaluated the maturation of collagen fibres in the newly formed tissue after pulp revascularization. These data are interesting, as greater collagen maturation can be related to older pulp tissue.

Residual bacteria demonstrate a negative effect on the outcomes of REP, and it is associated with persistent periapical radiolucency, in addition to showing a reduction in newly formed mineralized tissue (Verma *et al*. 2017). Nevertheless, the negative effects of residual contamination on newly formed tissues were excluded from this study, so that any variable could be discarded, as well as performed in other investigations (Nosrat *et al*. 2018, Alexander *et al*. 2019). However, it is important now to assess the influence of EDTA on the revascularization of a tooth with necrotic pulp. As a possible limitation, a human model would be favourable over animal models, to get even closer to the clinical reality, despite the great physiological, biological and anatomical similarity of rat molars with human teeth (Dammaschke 2010, Cintra *et al*. 2016).

This study is important because it shows that despite the existing contradiction about the effects of EDTA on pulp regeneration (Deniz Sungur *et al.* 2019, Taweewattanapaisan *et al*. 2019, Aksel *et al*. 2020), it favoured the neoformation of connective and mineralized tissue, strengthening the root structure, even if they are not original tissues of the pulp-dentin complex. Still, this study was carried out only within the period of 15 days. Perhaps over a longer period, both groups had complete apical closure. However, the present results might indicate acceleration in the repair process by the use of EDTA.

Conclusion

The use of EDTA in REPs improved newly formed intracanal connective tissue in immature molars of rats; however, EDTA did not influence cementum-like tissue formation, apical closure, inflammatory infiltrate, and maturation of collagen fibres.

Conflict of Interest

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

Acknowledgement

This research was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – 88887.489995/2020-00.

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5 ARTIGO 2

O artigo 2 “***The effect of EDTA on TGF-β1 and FGF-2 release from immature teeth submitted to regenerative endodontic therapy: an animal study***” será submetido ao periódico *Journal of Endodontics* (Qualis A1, Fator de Impacto = 2,886).

Abstract

Introduction: This study investigated the influence of ethylenediaminetetraacetic acid (EDTA) irrigation in the cementum-like tissue formation, and in the transforming growth factor (TGF)-β1 and fibroblast growth factor (FGF)-2 release during the regenerative endodontic procedure (REP) in immature rats molars. **Methods:** The lower first molars of 4-weeks-old 12 rats were devitalized and randomly assigned into experimental groups (n = 6): sodium hypochlorite (NaOCl) – irrigation of the mesial canals with 5 min 2.5% NaOCl; and NaOCl-EDTA – irrigation of the mesial canals with 2.5% NaOCl, followed by 17% EDTA for 5 min each. After evoking bleeding using size 10 K-file, the cavities were sealed. Three untreated molars were used as Control-15d (n = 3), and untreated molars from the other three rats were used as immediate control (n = 3). At fifteen days, the animals were killed and the teeth were harvested for histomorphometric analysis, immunohistochemistry, and statistical analysis ($P < 0.05$). **Results:** The histomorphometric analysis showed higher cementum-like tissue formation in the NaOCl-EDTA group than NaOCl group ($P < 0.05$). For the growth factors release, NaOCl-EDTA group enhanced TGF-β1 release in the root tip area and in the center region of apical third of pulp tissue as compared to the NaOCl group ($P < 0.05$); however, there was no significant difference of FGF-2 release between both irrigating protocols in these evaluated areas ($P > 0.05$). **Conclusion:** EDTA in REP improved the area of neoformed cementum-like tissue and the immunolabeling of TGF-β1 in the apical area, while it did not influence on FGF-2.

Keywords EDTA; FGF-2; guided tissue regeneration; regenerative endodontics; root canal irrigants; TGF-beta.

Statement of Clinical Relevance

EDTA irrigation has been suggested in regenerative endodontics procedures (REPs) to create a favorable environment for tissue repair. This *in vivo* study using immature rat molars demonstrated that irrigation with 17% EDTA after 2.5% NaOCl could be more effective for continued root development, due to improved new mineralized tissue formation, and for TGF-β1 release in REPs.

Introduction

Regenerative endodontic procedure (REP) involves biologically based procedures designed to replace damaged structures of the dentine–pulp complex (1, 2, 3), promoting further root development and apical closure (4). REP applies principles of tissue engineering, such as scaffold, stem cells, and the growth factors following dentin demineralization (1, 2, 5, 6).

The dentin matrix is known as a reservoir for several growth factors, which play an important role on the recruitment of undifferentiated pulp cells to the injury area, in addition to stem cell proliferation and differentiation (4, 7, 8). Among these factors, the transforming growth factor (TGF)-β1 and fibroblast growth factor (FGF)-2 play a crucial role in pulp-dentin complex regeneration (9).

TGF-β1 appears to be a substantial chemoattractant and stimulating factor for the activation and migration of stem cells (7), upregulating odontoblastic differentiation (4, 10). This molecule also contributes for reparative dentinogenesis (4, 10), in addition to show potent immunosuppressive effects against pro-inflammatory citokynes (3). Furthermore, FGF-2 may show the ability to differentiate human dental pulp cells (HDPCs) into odontoblast lineages *in vitro* (11). This factor also stimulates angiogenesis, acting as a mitogen for pulp progenitor cells (9), and leading to the mineralized tissue production (9). These growth factors are capable of evoking cellular responses, even at low concentrations (12).

The chosen irrigating solution may show a significant effect on growth factor release (4, 13). The American Association of Endodontists (14) have suggested the use of 17% ethylenediaminetetraacetic acid (EDTA) after NaOCl irrigation in their clinical considerations for REP, to enhance the release of growth factors from dentine (2, 15). However, the findings of the effect of EDTA conditioning in growth factor liberation remain controversial. Whilst, some *in vitro* investigations have found no influence of EDTA on TGF-β1 release (8, 16), other studies have shown an increased amount of this molecule released after EDTA irrigation (2, 3, 7, 17-19). Thus, it is necessary to know whether TGF-β1 and FGF-2 liberation from the dentin matrix could also occur inside the root canal space after irrigation with current regenerative protocol.

To our knowledge, only one previous animal study (3) was conducted to assess the influence of EDTA irrigation on TGF-β1 release, whereas no *in vivo* evaluation of FGF-2 released from dentine was found. Additionally, most investigations are *in vitro* studies using conditioned dentin powder or dentin discs (4, 16, 20), which do not simulate a clinical scenario of REP. Therefore, this study aimed to investigate the influence of EDTA irrigation in the tissue repair, and in the release of TGF-β1 and FGF-2 into the root canal space of

immature rats molars following REP. The null hypothesis was that there is no difference in the amount of growth factors released inside the root canals amongst the evaluated irrigation protocols.

Materials and Methods

Experimental design

Fifteen 4-week-old male Wistar rats (approximately 80 g) were used. The sample size was established on the basis of the findings of a previous study (21). The animals were housed in a temperature-controlled environment (22°C ± 1°C, 70% humidity, 12-h light–dark cycle) with *ad libitum* access to water and feed. This study was approved by the local Ethics Committee (CEUA 81/2020).

Surgical procedure

The animals were anesthezied via intramuscular injection using 10% ketamine (80 mg/Kg; Ketamina Agener 10%, União Química Farmacêutica Nacional S/A, Embu-Guaçu, São Paulo, Brazil) and 2% xylazine (15 mg/Kg; Xilazin, Syntec do Brasil LTDA, Cotia, São Paulo, Brazil). Twelve animals were selected as controls, while other 12 were randomly separated for operative procedures in the experimental groups. After the anesthesia, the molars were isolated with gingival barrier (Top dam, FGM, Joinville, SC, Brazil) and special dental clamps. Thus, coronal access in mesial root canal was performed with a sterile LN round bur (Dentisply Maillefer, Tulsa, OK, USA) in low speed coupled to an electric motor. For these procedures, an operating microscope (Alliance, São Paulo, SP, Brazil) with 40× magnification was used (3). Subsequently, a pulpectomy was performed using size 10 K-files (Dentsply Sirona, Ballaigues, Switzerland). The canals were irrigated with sterile saline solution and dried with sterile paper points. The working length of the mesial root canals was determined during a pilot study, which a radiograph was taken with size 10 K-file into the mesial canal.

After the removal of pulp from mesial canal, the rats were randomly distributed into 2 groups (n = 6): NaOCl, where the mesial canals were irrigated with 2.5% NaOCl for 5 minutes; and NaOCl-EDTA, where the mesial canals were irrigated with 2.5% NaOCl followed by 17% EDTA for 5 minutes. Afterwards, the canals were irrigated with saline solution and dried with sterile absorbent paper points. A sterile 15 K-file was inserted 0.5 mm beyond the apex to induce bleeding and blood clot formation inside the mesial canal. Teeth

were sealed with PBS CIMMO HP (CIMMO, Pouso Alegre, MG, Brazil) followed by resin-modified glass ionomer cement light-cured (GC America Inc., Alsip, IL, USA).

The untreated lower left or right first molars of rats from the experimental groups (NaOCl and NaOCl-EDTA) were randomly selected as control (Control-15d, n = 3). Additionally, lower left or right immature molars of other three animals did not receive any intervention, and they were used as Control-immediate (n = 3). These controls were included for histologic reference, and observation of physiologic root development.

Sample preparation and histological analysis

At the 4-week period (Control-immediate group), or after 15 days, the rats were killed with an overdose of the anesthetic solution. The hemi-mandibles were separated, dissected, and fixed (4% buffered formaldehyde; 24 hours). The specimens were decalcified (10% ethylenediaminetetraacetic acid; 45 days) and dehydrated, clarified, and embedded in paraffin. Serial histological sections (5 µm) were selected from the point where the mesial root of the first molar was at its full longitudinal extension. The two first slides obtained with histological sections were selected for hematoxylin-eosin (H.E.) staining, and each the next two for immunohistochemical analysis.

The analyses were performed under light microscopy (400× magnification; DM4000 B; Leica Microsystems, Wetzlar, Germany) by a single calibrated and blinded operator to the experimental groups. Sections stained with H.E. were used for histomorphometrically analysis, where area of new cementum-like tissue was obtained in μm^2 (22-24).

Immunohistochemical analyses

The histological sections were obtained for immunohistochemical assessments with an indirect immunoperoxidase technique (25, 26) for TGF-β1 and FGF-2. Histological sections were deparaffinized and hydrated. Antigen retrieval was performed by immersing the histological slides in buffer citrate solution (Antigen Retrieval Buffer; Spring Bioscience, Pleasanton, CA, USA) in a pressurized chamber (Decloaking Chamber; Biocare Medical, Concord, CA, USA) at 95°C (10 min). The slides were rinsed with phosphate-buffered saline at the end of each stage of the immunohistochemical reaction. The histological sections were immersed in 3% hydrogen peroxide solution (1h 20 min) and in 1% bovine serum albumin (12 h) to block the endogenous peroxidase activity and nonspecific sites, respectively. The histological slides were divided and incubated with one of the following primary antibodies: anti-TGF-β1 and anti-FGF-2 (rabbit primary antibodies; Sigma-Aldrich Co. LLC, St. Louis,

MO, USA), that were diluted (Antibody Diluent with Background Reducing Components; Dako Laboratories, Carpinteria, CA, USA), and placed in a moist chamber (24 h). Then, the histological sections were incubated with a biotinylated secondary antibody (1,5 h) and were treated with streptavidin–horseradish peroxidase conjugate (1,5 h) (Universal Dako Labelled Streptavidin-Biotin kit; Dako Laboratories). After, the reaction was developed using the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB Chromogen kit; Dako Laboratories) and counterstained with hematoxylin. The negative controls were submitted to the procedures but without the primary antibodies.

For analyses, the immunolabelling was evaluated in two region of teeth apical area (400× magnification): in root tip and in the center of pulp apical third. For that, the immunolabelling was defined as the predominant presence of a brownish color in the cells. A semi-quantitative analysis was performed, which provides information on the numbers of immunolabeled cells (25, 27). The scores were assigned as follows (24, 25): 0, immunolabelling missing (complete absence of immunoreactive cells); 1, low pattern of immunolabelling (approximately one quarter of the immunoreactive cells); 2, moderate pattern of immunolabelling (approximately one half of the immunoreactive cells); 3, severe pattern of immunolabelling (approximately three quarters of the immunoreactive cells); and 4, very severe pattern of immunolabelling (approximately all immunoreactive cells).

Statistical analysis

Nonparametric data were analyzed using the Mann-Whitney test, whereas for parametric data, the t-test was performed after normality test for statistical comparisons between the experimental groups at the significance level of 5% ($P < 0.05$).

Results

Cementum-like tissue analysis

The data from the histomorphometric analysis can be seen in Figure 1, whereas Figure 2 shows representative images of the histological analysis. At 15 days, a significant increase in the area of cementum-like tissue deposited in the apical area was observed most specimens of NaOCl-EDTA in comparison to the NaOCl group ($P < 0.05$). In both groups, cells similar to cementoblasts and cementocytes were noticed in the new mineralized tissue. On the other hand, samples from Control-immediate group had organized pulp tissue and open apices.

Conversely, all teeth from Control-15d showed continued root development with progressive apical closure, and a normal dentin-pulp complex.

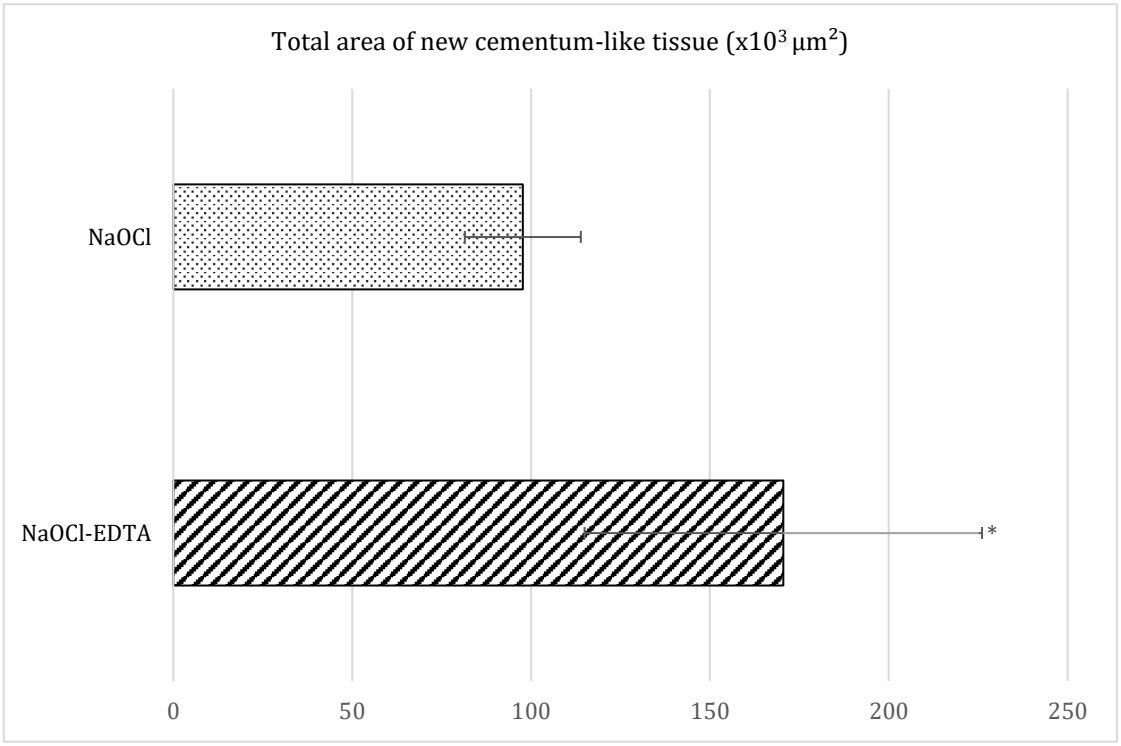

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Figure 1. Data of histomorphometric analysis of new cementum-like tissue in the apical area, at 15 days. The symbol * indicates significant difference between the experimental groups ($P < 0.05$).

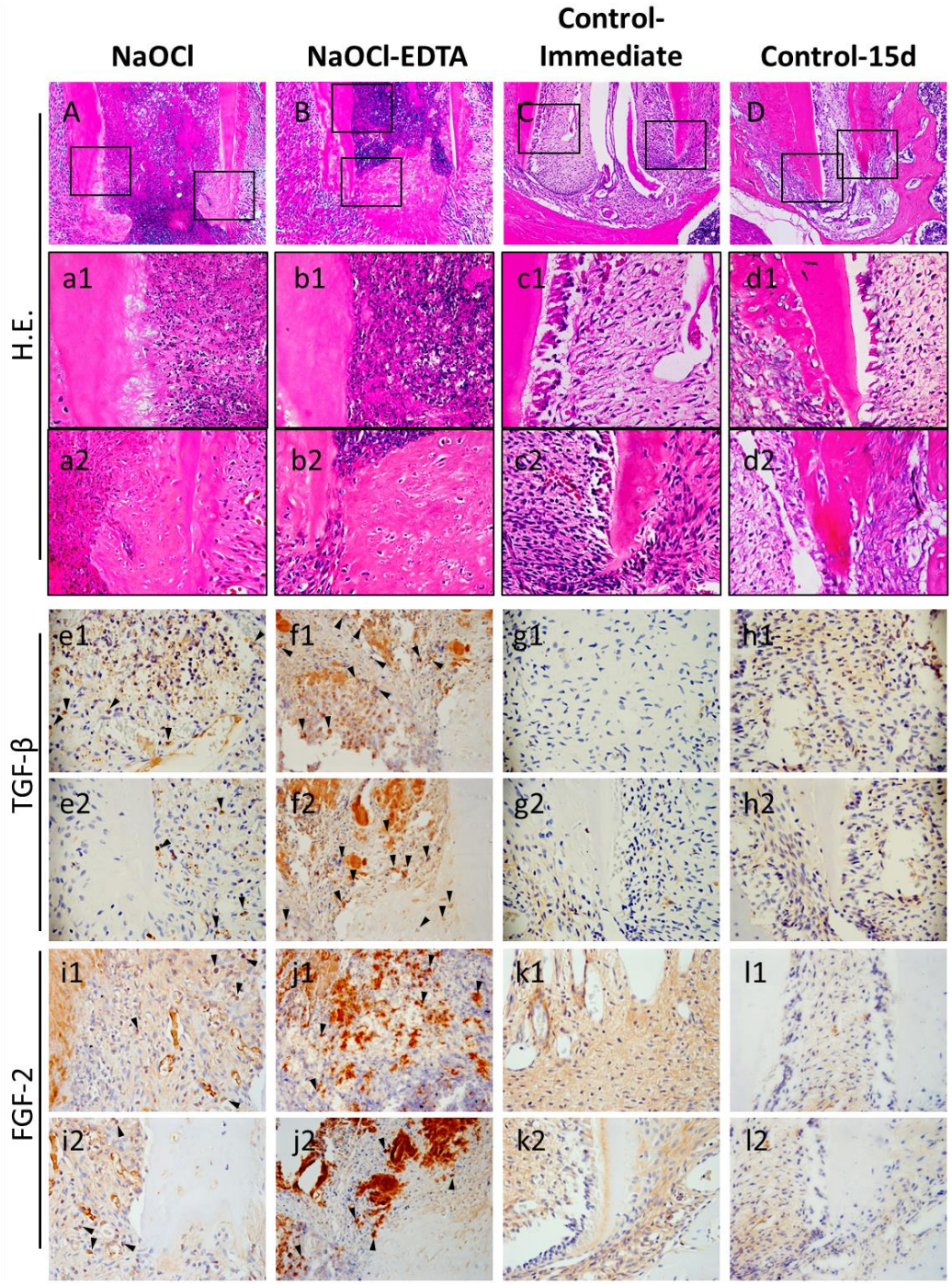

Figure 2. Representative images of histological analysis and of TGF-β1 and FGF-2 immunolabelling. (A-a2, B-b2) NaOCl and NaOCl-EDTA: (A, B) panoramic microscopic aspect of the apical third with new cememtum-like tissue, (a1, b1) apical third with inflammatory cells and blood clot, (a2, b2) root tip area neformed cementum-like tissue, and ingrowth of fibroblast-like cells. (C-c2, D-d2) Control-immediate and Control-15d: (C, D) panoramic microscopic aspect of the apical third showing normal pulp tissue, (C) open apex and (D) continued root development, (c1-c2, d1-d2) presence of organized tissue, and normal dentin-pulp complex. (e1-h1, e2-h2) TGF-β1: (e1, e2) NaOCl showing low-to-moderate immunolabelling, and (f1, f2) NaOCl-EDTA showing moderate-to-very severe immunolabelling. (i1-l1, i2-l2) FGF-2: (e1, e2) NaOCl and (j1, j2) NaOCl-EDTA showing low-to-moderate immunolabelling. Black arrowheads indicate immunolabelled cells. [100×, 400×: Hematoxylin–eosin; 400×: Immunolabeling for TGF-β1 and FGF-2]

Analysis of the release of growth factors

Images representative of the TGF-β1 and FGF-2 analysis can be seen in Figure 2, while the scores for each marker are shown in Table 2. At 15 days, the NaOCl-EDTA groups had mostly very severe TGF-β1 immunolabelling in the root tip area and moderate immunolabeling into the apical third of pulp tissue. Conversely, NaOCl group significantly exhibited low-to-moderate immunolabelling in both locations, compared to NaOCl-EDTA ($P < 0.05$).

Regarding FGF-2 analysis, both experimental groups had low-to-moderate immunolabeling in the root tip location and mostly moderate immunolabeling in the apical third ($P > 0.05$).

Table 1 Scores for immunohistochemical labeling of TGF-β1 and FGF-2 in each location of groups

Analysis		Scores	TGF-β1		FGF-2	
			NaOCl	NaOCl-EDTA	NaOCl	NaOCl-EDTA
Location	**Root tip**	0	0/6	0/6	0/6	0/6
		1	3/6	0/6	2/6	2/6
		2	3/6	2/6	3/6	2/6
		3	0/6	1/6	1/6	2/6
		4	0/6	3/6	0/6	0/6
		Median*	1,5[a]	3,5[b]	2[a]	2[a]
		P value	= 0.015		= 0.818	
	Apical third of pulp	0	0/6	0/6	0/6	0/6
		1	3/6	0/6	2/6	1/6
		2	2/6	1/6	2/6	2/6
		3	1/6	4/6	1/6	2/6
		4	0/6	1/6	1/6	1/6
		Median*	1,5[a]	3[b]	2[b]	2,5[a]
		P value	= 0.026		= 0.589	

*Different letters in the same line indicate statistically significant differences between groups for each immunolabeling in each location ($P < 0.05$).

Discussion

EDTA has been recommended as a final irrigant in REP, since it may provide a favorable condition for tissue repair by the liberation of bioactive molecules following dentin demineralization (15, 17, 28). The present study investigated the effects of EDTA irrigation in

the tissue repair by assessing the hard tissue formation, and in the ability to extract growth factors (TGF-β1 and FGF-2) release in REP using an *in vivo* evaluation. The histological findings demonstrated that final irrigation with 17% EDTA after 2.5% NaOCl significantly increased the area of neoformed cementum-like tissue, and improved TGF-β1 release into the root canal space of immature rat molars. Conversely, there was no impact of the use of EDTA on FGF-2 release. Thus, the null hypothesis of the study was partially rejected.

In REP, immature tooth is chemically treated to enable the removal of microorganisms and necrotic tissue remnants. Furthermore, this therapy induces the release of signaling molecules from dentin, promoting continued root development by new hard tissue formation, and new soft tissue formation by stem cells migration and/or proliferation (7). In the current study, the choice of NaOCl and 17% EDTA was based on the considerations of an international protocol for REP (14), and in the antibacterial and solvent potential of NaOCl, besides the chelating potential of EDTA.

Similarly to other histologic studies in animals (29, 30), hard tissue deposition was observed after REP, especially in the root canal walls. This newly generated mineralized tissue area was improved in the NaOCl-EDTA group compared to irrigation without EDTA. This new tissue had characteristics of cementum tissue, and probably originated from stem cells from the periodontal ligament or the apical papilla. This improved new hard tissue deposition may be attributed with the optimal environment created by EDTA, promoting the liberation of dentin matrix-derived growth factors (17, 28) associated with tissue mineralization, such as TGF-β1.

A previous report stated that higher growth factor release is directly associated with an increased exposure time of EDTA (17), and its effectiveness in removing smear layer. There are various *in vitro* investigations that evaluated the effects of EDTA in growth factors release mostly using either dentin discs or dentin extracts (4, 8, 10, 6, 17). Some of these evaluations investigate the amount of growth factors released during dentin conditioning rather than after irrigation, what does not simulate the clinical environment, where an induction of intracanal canal bleeding is performed after irrigation. Besides this, there are a very limited number of *in vivo* studies focused on the influence of EDTA on growth factors release during REP.

After EDTA irrigation, growth factors exposed from conditioned dentin may diffuse into the blood clot, promoting cell migration, in addition to induce cell differentiation due to contact between stem cells with dentin structure (6). TGF-β is one of the most important growth factors in REP (16), performing a crucial role in odontoblastic differentiation and dentinogenesis (2, 10, 28). Increased immunolabeling of TGF-β1 in the apical region of the

samples irrigated with 17% EDTA was found. These results support *in vitro* studies, in addition to a single existing *in vivo* data using molars of mice (3) that irrigation with EDTA alone (2, 3, 28) or combined with NaOCl (18, 19) was able to effective liberation of TGF-β1 from dentin by using different methods of analysis. This result may be attributed to solubilization of the dentin matrix favored by EDTA (15).

Despite this, no influence of the EDTA was observed in the FGF-2 release. The association of FGF-2 with TGF-β1 is responsible of differentiation of HDPCs into odontoblast-like cells (16). In accordance with this finding, 17% EDTA only showed significant liberation of FGF-2 in human root fragments, when combined with adipose-derived mesenchymal stem cells, presenting the importance of these cells in the secretion of FGF-2. On the other hand, the amount of bioactive molecules released and its distribution in root canal may vary over time, which a significant liberation of these factors may be found over extended periods.

In order to establish applicable protocols of REP for clinical scenario, an investigation of the histological effects of EDTA in REP using human models is also important, in addition to the existing *in vivo* investigations using animals. Moreover, the influence of EDTA on infected root canals has also to be considered in *in vivo* studies. For instance, one previous *in vitro* study using human root segments reported no influence of 17% EDTA on TGF-β1 release under the presence of residual biofilm, even after previous irrigation with 1.5% NaOCl (19), which demonstrates the deleterious impact of residual bacterial on tissue regeneration. Additionally, the data from our study is restricted to a period of 15 days of evaluation. Therefore, differences in the results regarding growth factors' distribution and FGF-2 release may stem from the long-term longitudinal assessment.

Within the limitations of this study, it demonstrated that despite the controversial findings about the effects of EDTA on growth factors release of the previous *in vitro* evaluations, it is capable of promoting continued root development due to increased neoformed cementum-like tissue deposition, and consequently, which may reinforce root walls of immature weakened teeth. Furthermore, EDTA enhanced TGF-β1 release in the apical region, which may be responsible to improve hard tissue deposition.

Conclusion

EDTA irrigation in REP improved the area of neoformed cementum-like tissue and the immunolabeling of TGF-β1 in the apical area, whereas it did not influence on FGF-2.

Conflicts of interest

The authors explicitly state that there are no conflicts of interest in connection with this article.

Acknowledgement

This research was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil - CAPES (n. 88887.489995/2020-00).

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6 ARTIGO 3

O artigo 3 “***Influence of EDTA on regenerative endodontics: A systematic review***” foi submetido ao periódico *Journal of Dental Research* (Qualis A1, Fator de Impacto = 4,914).

Influence of EDTA on regenerative endodontics: A systematic review

Abstract

The findings on the effects of ethylenediaminetetraacetic acid (EDTA) irrigation on biological principles associated with tissue regeneration during regenerative endodontic procedures (REPs) are controversial. This systematic review (PROSPERO register: CRD42020205417) aimed to investigate the influence of EDTA on the biological factors of dentin, cells/tissue during REP. *In vivo* and *in vitro* studies were eligible. Two authors independently conducted a systematic search (PubMed/Medline, Scopus, Cochrane Library, Web of Science, Embase, and OpenGrey as well as in the reference lists of the identified records) up to February-2021. The release of growth factors from the dentin was the primary outcome. Risk of bias was performed according to a modified Joanna Briggs Institute's Critical Evaluation Checklist for Experimental Studies and SYRCLE's RoB tool. The search resulted in 1.848 articles: 36 studies were selected. Among these studies, 32 were *in vitro*, three animal studies, and one that used both models. Meta-analysis was unfeasible due to heterogeneity in concentrations of the EDTA, irrigating protocols, and methods of assessment. Various times of exposure and concentrations of *EDTA* ranging from 3%-15% were observed. Among the selected studies, 17 evaluated growth factors release. Fifteen studies found a significant liberation of transforming growth factor (TGF)-β in the EDTA-treated dentin and four found no influence of EDTA on vascular endothelial growth factor (VEGF) release. Regarding cell behavior (26 studies), eight studies showed no significant influence of EDTA on cell viability; whereas five, nine, and six studies showed higher cell migration, adhesion, and differentiation, respectively when EDTA groups were compared with controls. Elongated and fibroblastic-like cells were observed after EDTA irrigation. Overall, EDTA did not influence blood clot formation and newformed tissue. This systematic review suggests that EDTA irrigation in REP positively influences TGF-β release, cell migration, attachment, and differentiation; however, further research to compare its effectiveness on tissue regeneration is necessary.

Keywords Guided Tissue Regeneration, Growth factor(s), Tissue Engineering, Stem cell(s), Systematic reviews and evidence-based medicine.

Introduction

Incomplete root formation and open apex (Law 2013) make treatment of immature necrotic teeth challenging (Albuquerque et al. 2014). Regenerative endodontic procedure (REP) has gained attention (Verma et al. 2016), as a promising biological approach (Albuquerque et al. 2014) with success rates on the long-term between 95% and 100% (Alobaid et al. 2014). REP may be helpful in restoring the physiologically functional dentition through the repair of damaged dental structures (Law 2013). In addition, sensory and defense mechanisms of the pulp-dentin complex through the pillars of tissue engineering may also be repaired (Hargreaves et al. 2013).

The basis of REP relies on the implantation of scaffolds, stem cells activity and growth factors release (Hashioto et al. 2017). Root canal disinfection and an appropriate conditioned dentin surface for stem cell adhesion are crucial for achieving promising results (Verma et al. 2016). Although there is no standard irrigation protocol for REP (Kim et al. 2018; Shamszadeh et al. 2019), sodium hypochlorite (NaOCl) remains the most common irrigant used (Bucchi et al. 2017; Bracks et al. 2019) due to its well-established properties, such as antimicrobial effect and solvent potential of the tissues (Galler et al. 2011). Complementary irrigation with ethylenediaminetetraacetic acid (EDTA) is also important due to the inability of NaOCl to remove the smear layer from dentin and expose bioactive molecules entrapped in the dentin matrix (Gonçalves et al. 2016; Bucchi et al. 2017; Bracks et al. 2019). Furthermore, EDTA may reduce the amount of endotoxins involved in immune-inflammatory responses (Herrera et al. 2016).

Despite these benefits, EDTA may also provoke some negative effects on stem cell survival and migration (Deniz Sungur et al. 2019; Aksel et al. 2020), which might negatively influence tissue regeneration. Thus, this systematic review evaluated the influence of EDTA on factors associated with REP, through the assessment *in vivo* and *in vitro* studies. The release of growth factors from the dentin matrix was the primary outcome evaluated. The effects of EDTA on cell behavior and different parameters of tissue regeneration were also assessed.

Materials and Methods

Protocol

Reporting of this systematic review was carried out according to the checklist of the Preferred Report Items for Systematic Reviews and Meta-analyses (PRISMA) (Page et al. 2021). A

research protocol was registered at the International Prospective Register of Systematic Review (PROSPERO) database (CRD42020205417).

Eligibility Criteria

The inclusion criteria were: 1) *in vivo* studies that evaluated the effects of EDTA on the biological factors of dentin or pulp/periapical tissues in REP, and 2) *in vitro* studies that determined the effects of EDTA on cell viability, migration, attachment, morphology, and on biological factors from dentin. Exclusion criteria were: 1) studies that examined the effects of EDTA on dentin, cells or pulp/periapical tissues without a control group or another irrigant without the EDTA group, and 2) studies for which the full text was unavailable. There were no restrictions on the language and date of publication.

The population, intervention, comparison, and outcome (PICO) approach was used to address the following question: "Does EDTA influence biological factors of dentin, cells, or tissues in REP?" The study population is composed of dentin, cells or pulp/periapical tissues of humans or animals who/that had been submitted to irrigation/conditioning with EDTA. The intervention was irrigation/conditioning with EDTA; the comparison was irrigation/conditioning with other solutions. The primary outcome assessed was the effect of EDTA on the release of growth factors from the dentin matrix. The secondary outcomes were cell viability, migration, attachment, morphology, protein immunolabeling/expression, blood clot characterization, tissue inflammation, tissue in-growth, root length/root thickness, apical diameter, mineralization, and root/bone resorption.

Search Strategy and Information Sources

Electronic searches were conducted in the PubMed/MEDLINE, Scopus, Cochrane Library, Web of Science, and Embase databases up to February-2021. Grey literature was consulted through OpenGrey, and manual searches were carried out in the reference list of the selected articles. The search strategy used a combination of keywords and Medical Subject Heading (MeSH) terms associated with the Boolean operators "AND" and "OR" as shown in Appendix Table 1.

Appendix Table 1. Search strategy used for the electronic databases

DATABASE	SEARCH STRATEGY
Medline through PubMed (http://www.ncbi.nlm.nih.gov/pubmed), **OpenGrey** (http://www.opengrey.eu/)	((EDTA [MeSH Terms] OR "ethylenediaminetetraacetic acid" [MeSH Terms] AND "bioactive molecules" [Title/Abstract] OR "tissue engineering" [MeSH Terms] OR "growth factors" [MeSH Terms] OR "stem cells" [MeSH Terms] OR "mesenchymal stem cells" [MeSH Terms] OR "dental pulp stem cells" [Title/Abstract] OR cells [Title/Abstract] OR tissues [MeSH Terms] OR tissue [Title/Abstract] AND "incomplete root formation" [Title/Abstract] OR "immature permanent teeth" [Title/Abstract] OR "immature teeth" [Title/Abstract] OR "immature permanent tooth" [Title/Abstract] OR "immature tooth" [Title/Abstract] OR "incomplete rhyzogenesis" [Title/Abstract] OR "regenerative endodontics" [MeSH Terms] OR "regenerative endodontic therapy" [Title/Abstract] OR "regenerative endodontic procedure" [Title/Abstract] OR "pulp revitalization" [Title/Abstract] OR "pulp revascularization" [Title/Abstract] OR "pulp regeneration" [Title/Abstract] OR "pulp-dentin complex regeneration" [Title/Abstract]))
Scopus (http://www.scopus.com/)	#1 TITLE-ABS-KEY (EDTA) OR TITLE-ABS-KEY (ethylenediaminetetraacetic acid) #2 TITLE-ABS-KEY (bioactive molecules) OR TITLE-ABS-KEY (tissue engineering) OR TITLE-ABS-KEY (growth factors) OR TITLE-ABS-KEY (stem cells) OR TITLE-ABS-KEY (mesenchymal stem cells) OR TITLE-ABS-KEY (dental pulp stem cells) OR TITLE-ABS-KEY (cells) TITLE-ABS-KEY (tissues) TITLE-ABS-KEY (tissue) #3 TITLE-ABS-KEY (incomplete root formation) OR TITLE-ABS-KEY (immature permanent teeth) OR TITLE-ABS-KEY (immature teeth) OR TITLE-ABS-KEY (immature permanent tooth) OR TITLE-ABS-KEY (immature tooth) OR TITLE-ABS-KEY (incomplete rhyzogenesis) OR TITLE-ABS-KEY (regenerative endodontics) OR TITLE-ABS-KEY (regenerative endodontic therapy) OR TITLE-ABS-KEY (regenerative endodontic procedure) OR TITLE-ABS-KEY (pulp revitalization) OR TITLE-ABS-KEY (pulp revascularization) OR TITLE-ABS-KEY (pulp regeneration) OR TITLE-ABS-KEY (pulp-dentin complex regeneration) #1 AND #2 AND #3
Cochrane (https://www.cochranelibrary.com/)	(EDTA [MeSH Terms] OR "ethylenediaminetetraacetic acid" [MeSH Terms]) AND ("bioactive molecules" OR "tissue engineering" [MeSH] OR "growth factors" [MeSH] OR "stem cells" [MeSH] OR "mesenchymal stem cells" [MeSH] OR "dental pulp stem cells" OR cells OR tissues [MeSH] OR tissue) AND ("incomplete root formation" OR "immature permanent teeth" OR "immature teeth" OR "immature permanent tooth" OR "immature tooth" OR "incomplete rhyzogenesis" OR "regenerative endodontics" [MeSH] OR "regenerative endodontic therapy" OR "regenerative endodontic procedure" OR "pulp revitalization" OR "pulp revascularization" OR "pulp regeneration" OR "pulp-dentin complex regeneration")
Web of Science (https://clarivate.com/webofsciencegroup/solutions/web-of-science-core-collection/)	TOPIC: (EDTA OR "ethylenediaminetetraacetic acid") AND TOPIC: ("bioactive molecules" OR "tissue engineering" OR "growth factors" OR "stem cells" OR "mesenchymal stem cells" OR "dental pulp stem cells" OR cells OR tissues OR tissue) AND TOPIC: ("incomplete root formation" OR "immature permanent teeth" OR "immature teeth" OR "immature permanent tooth" OR "immature tooth" OR "incomplete rhyzogenesis" OR "regenerative endodontics" OR "regenerative endodontic therapy" OR "regenerative endodontic procedure" OR "pulp revitalization" OR "pulp revascularization" OR "pulp regeneration" OR "pulp-dentin complex regeneration")
Embase (https://www.embase.com)	(edta:ti,ab,kw OR 'ethylenediaminetetraacetic acid':ti,ab,kw) AND ('bioactive molecules':ti,ab,kw OR 'tissue engineering':ti,ab,kw OR 'growth factors':ti,ab,kw OR 'stem cells':ti,ab,kw OR 'mesenchymal stem cells':ti,ab,kw OR 'dental pulp stem cells':ti,ab,kw OR cells:ti,ab,kw OR tissues:ti,ab,kw OR tissue:ti,ab,kw) AND ('incomplete root formation':ti,ab,kw OR 'immature permanent teeth':ti,ab,kw OR 'immature teeth':ti,ab,kw OR 'immature permanent tooth':ti,ab,kw OR 'immature tooth':ti,ab,kw OR 'incomplete rhyzogenesis':ti,ab,kw OR 'regenerative endodontics':ti,ab,kw OR 'regenerative endodontic therapy':ti,ab,kw OR 'regenerative endodontic procedure':ti,ab,kw OR 'pulp revitalization':ti,ab,kw OR 'pulp revascularization':ti,ab,kw OR 'pulp regeneration':ti,ab,kw OR 'pulp-dentin complex regeneration':ti,ab,kw)

MeSH – Medical Subject Heading.

Study selection

Study selection was carried out independently by two authors (A.H.R.P. and R.R.F.) in a two-step process. The records were organized alphabetically by title and duplicates could be

identified and removed manually. In Step 1, the authors appraised titles and abstracts of the studies retrieved from the search. In Step 2, full text of the remaining records was obtained for further evaluation by the authors. Only studies that fulfilled the eligibility criteria were included. Disagreements were solved through discussion, and when necessary, a third reviewer (F.B.) was consulted. Cohen's kappa coefficient for inter-investigator agreement during studies' selection was assessed (Landis and Koch 1977).

Data collection and analyses

Two authors (A.H.R.P. and R.R.F.) undertook data collection independently and in duplicate for all studies using a piloted data extraction form in an excel spreadsheet. The following data were retrieved: first authors' last name, year of publication, study design, experimental model, sample size, groups, experimental protocol, and analyses. A third author (F.B.) revised the data.

Risk of Bias Assessment

Two investigators (A.H.R.P. and S.C.O.) independently assessed the selected studies' methodological quality according to their levels of evidence as proposed by a modified version of the Joanna Briggs Institute's Critical Evaluation Checklist for Experimental Studies (Yaylali et al. 2015; Dos Reis-Prado et al. 2021). Each item was assessed on a 2-point scale: 0, *not reported or reported inappropriately*, and 1, *reported and appropriate*. A 10-criteria tool "Systematic Review Centre for Laboratory animal Experimentation" (SYRCLE's RoB tool) was used for risk of bias assessment of the animal studies (Hooijmans et al. 2014). A judgment of "no" indicated a high risk of bias, "yes" represented a low risk of bias, and "unclear" indicated either lack of information or uncertainty. Discrepancies were resolved with a third examiner (L.G.A.).

Results

Study selection

Figure 1 displays a flowchart of the selection process of the studies. A total of 1,848 articles were identified after searching the databases. After the first screening (Step 1), 55 articles were selected, submitted to a full-text review (Step 2) and 19 studies were excluded, by reasons showed in Figure 1. A total of 36 studies were included in the qualitative analysis (Ring et al. 2008; Huang et al. 2011; Yamauchi et al. 2011; Pang et al. 2013; Martin et al. 2014; Galler et al. 2015a, 2015b; Kim et al. 2015; Park et al. 2015; Ashry et al. 2016;

Gonçalves et al. 2016; Kawamura et al. 2016; Mollashahi et al. 2016; Sadaghiani et al. 2016; Shrestha et al. 2016; Zeng et al. 2016; Alghilan et al. 2017; Duncan et al. 2017; Widbiller et al. 2017; Chae et al. 2018; Hashimoto et al. 2018; Prompreecha et al. 2018; Ranc et al. 2018; Scott et al. 2018; Bracks et al. 2019; Cameron et al. 2019; Ivica et al. 2019; Liu et al. 2019; Widbiller et al. 2019; Deniz Sungur et al. 2019; Taweewattanapaisan et al. 2019; Tunç et al. 2019; Aksel et al. 2020; Atesci et al. 2020; Kucukkaya Eren et al. 2020; Li et al. 2020).

The assessed Cohen's kappa coefficient for inter-investigator agreement during the studies' selection was 0.843 for PubMed, 0.970 for Scopus, 1.000 for the Cochrane Library and OpenGrey, 0.925 for the Web of Science, and 0.876 for the Embase. These values indicated an almost perfect agreement between/among reviewers according to the benchmark scale of Landis and Koch (1977). No additional records were found through manual search in the references lists.

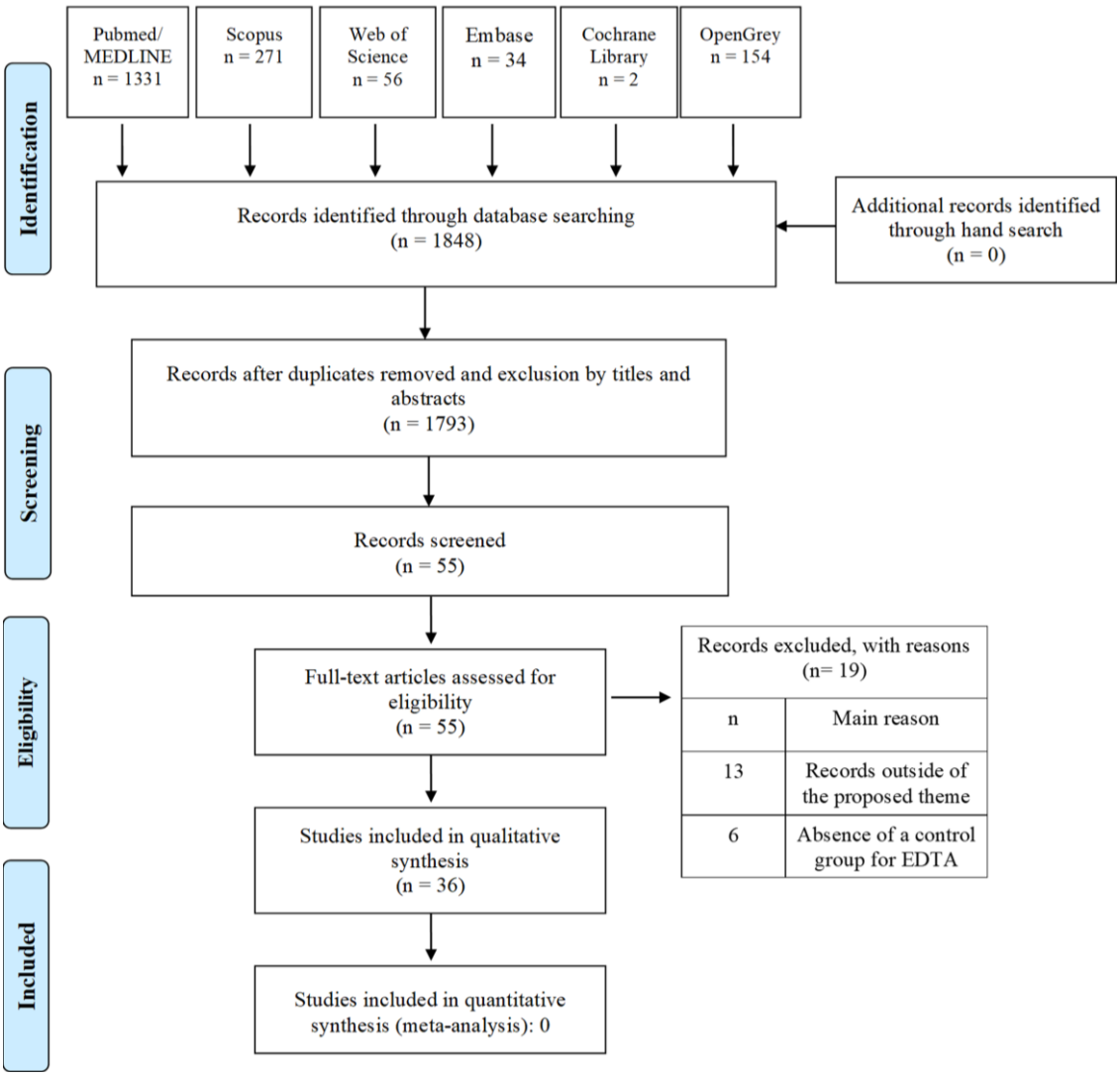

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart.

Characteristics of the included studies

Table 2 summarizes the studies that evaluated growth factors release/expression. Table 3 summarizes the information of the *in vitro* studies that evaluated the cell behavior, and Table 4 shows studies that evaluated the tissue regeneration in immature teeth models. According to the study design, 32 studies were only *in vitro*, three studies were *in vivo*, and one study used both models.

Of the 33 *in vitro* studies, three studies used only dentin discs alone, while most studies used dentin discs and different stem cells. The roots of permanent human teeth with stem cells or alone were also used as an experimental model. Few studies had porcine roots, plastic tooth models, and dentin powder for their experimental model.

The EDTA concentrations ranged from 3-15% at different exposure times. The second most frequent irrigating agent used after EDTA was 0.5-6% NaOCl. The other irrigating that were also evaluated consisted of chlorhexidine (CHX), sterile phosphate-buffered saline (PBS), distilled water, citric acid (CA), phytic acid, phosphoric acid (PHA), Qmix, a mixture tetracycline citric acid and detergent (MTAD), trichostatin, valproic acid, suberoylanilide, etidronic acid, AquatineEC, guanidine hydrochloride (GdnHCl), and hydrochloric acid (HCl).

For the *in vivo* category, 4 animal studies were evaluated. Two studies only used 17% EDTA as the irrigating solution (Yamauchi et al. 2011; Bracks et al. 2019), while other studies also used GdnHCl (Kawamura et al. 2016) and MTAD (El Ashry et al. 2016). Regarding the regenerative protocols, three studies performed pulpectomy associated or not with an induction of periapical lesion in canine and mice posterior teeth, while one used a subcutaneous tooth transplant into immunodeficient mice.

Growth factors release and expression

Among the 17 studies that evaluated the growth factors release/expression (Table 2), transforming growth factor (TGF)-β was assessed in 16 studies. EDTA effectively released TGF-β1 at 7 days (Bracks et al. 2019) or at all periods in 12 studies using concentrations of 10% (Galler et al. 2015a; Gonçalves et al. 2016; Sadaghiani et al. 2016; Duncan et al. 2017; Widbiller et al. 2017), 12% (Liu et al. 2019), and for the most part of studies 17% (Chae et al. 2018; Ranc et al. 2018; Bracks et al. 2019; Cameron et al. 2019; Ivica et al. 2019; Atesci et al. 2020). One available study reported an increase in TGF-β1 release, mainly after ultrasonic activation (Widbiller et al. 2017). Additionally, two other studies only found a significant TGF-β1 release in dentin conditioned with 17% EDTA under sterile conditions without biofilm (Cameron et al. 2019), or when a combination with adipose-derived mesenchymal

stem cells (adMSCs) was applied, also increasing vascular endothelial growth factor (VEGF), bone morphogenetic protein 2 (BMP-2), and fibroblast growth factor (FGF)-2 (Atesci et al. 2020). Conversely, seven studies found no significant differences in TGF-β release using EDTA (Zeng et al. 2016; Sadaghiani et al. 2016; Deniz Sungur et al. 2019; Cameron et al. 2019; Atesci et al. 2020; Aksel et al. 2020; Kucukkaya Eren et al. 2020).

When the growth factors release was evaluated with ELISA and PCR assays, 10% and 17% EDTA without any cell association or technique combination did not influence VEGF release in four studies (Sadaghiani et al. 2016; Bracks et al. 2019; Li et al. 2020; Atesci et al. 2020), FGF release in two studies (Zeng et al. 2016; Atesci et al. 2020), and BMP-2 in one study (Sadaghiani et al. 2016). On the contrary, one study that used immunogold localization visualized by scanning electron microscopy (SEM) showed that conditioning with 10% EDTA (5 and 10 min) enhanced the number of BMP-2 and VEGF particles released (Sadaghiani et al. 2016), in addition to a PCR analysis that found a great expression of nerve growth factor (NGF) at 14 days, and insulin-like growth factor (IGF) at all evaluated periods after irrigation with 17% EDTA (Bracks et al. 2019).

The results of a proteomic essay showed that 10% EDTA extracted a significantly greater quantity of angiogenic-associated growth factors (PDGF-AA, VEGF-A), BMP-7, brain-derived neurotrophic factor (BDNF), placenta growth factor (PIGF), hepatocyte growth factor (HGF), and some integrin growth factor-related family (IGFBP) (Ducan et al. 2017). On the other side, 10% EDTA did not influence fibroblast growth factor members (FGF), glial cell-line-derived neurotrophic factor (GDNF), IGFBP-2, mast/stem cell growth factor receptor (SCF-R), and insulin, in addition to decreasing the expressions of FGF-4, NGF receptor, epidermal growth factor receptor (EGFR-1), and growth/differentiation factor (GFD-15).

Cell behavior using in vitro studies

Among the 19 studies that evaluated cell viability (Table 3), most studies (n = 10) found no influence from the use of 17% EDTA (Ring et al. 2008, Park et al. 2015, Kim et al. 2015, Galler et al. 2015b, Alghilan et al. 2017, Chae et al. 2018, Deniz Sungur et al. 2019, Liu et al. 2019, Widbiller et at 2019, Li et al. 2020). On the other hand, six studies showed a reduction in cell viability, whereas the other six reported higher cell viability after using EDTA; or after performing an irrigation activation protocol.

Out of the 11 studies that evaluated cell morphology, nine studies found the presence of elongated to flattened-shaped cells with fibroblastic-like appearances in EDTA-treated

dentin. Regarding cell migration (n = 7), conditioning with 10-17% EDTA or extracts for different exposure times significantly enhanced cell migration in five studies. Similarly, among 13 studies that evaluated cell attachment, eight found a higher adherence to conditioned dentin with EDTA. One study showed a reduction in cell adhesion after EDTA conditioning (10 and 15 min), while the others reported no influence from EDTA.

For cell differentiation or mineralization protein assay, all seven articles that performed this evaluation using real-time polymerase chain reaction (RT-PCR) found an increased odontoblastic differentiation, and expression of mineralization markers, such as dentin sialophosphoprotein (DSPP) and dentin matrix acidic phosphoprotein (DMP)-1, after EDTA conditioning.

In matrix dentin extracts and dentin discs, 10% EDTA significantly increased alkaline phosphatase (ALP) and osteopontin (OPN) expression at 21 and 14 days, respectively. One study observed that 5-10 minutes of 10% EDTA did not influence runt-related transcription factor 2 (RUNX2).

Tissue regeneration of immature teeth models

Data of tissue regeneration using immature teeth models are presented in Table 4. One animal study using 17% EDTA in REPs protocols found that this solution did not display influence on the presence of inflammatory cells (El Ashry et al. 2016). Regarding tissues in-growth in the root canal space, one article found no statistical difference, while another reported a decrease in the regenerative area in the EDTA group (Kawamura et al. 2016; El Ashry et al. 2016). Two studies using dogs' teeth (Yamauchi et al. 2011; El Ashry et al. 2016), observed no significant improvement of 17% EDTA in the root length/thickening and periapical radiolucencies. No influence on bone resorption was observed. One study showed higher apical diameter closure, while other reported no influence of EDTA.

One record stated that 17% EDTA improves mineralized tissue formation (Yamauchi et al. 2011); another study found no influence of EDTA (El Ashry et al. 2016). Regarding cell differentiation, one study found less odontoblastic/endothelial differentiation of DPSCs in the EDTA group; however, higher cell differentiation was observed with EDTA in the group that used medium with cells (Kawamura et al. 2016).

Table 2. Effects of EDTA on growth factors release/expression

Author	Experi-mental model (n)	Groups	Experimental protocol	Growth factor release	Outcomes
Aksel et al. 2020	Human dentine discs (n = 3)	Second protocols of the study. G1-G5: groups used for analyses not considered in this table; G6: optimized EDTA, G7: EDTA with NBs, G8: EDTA + 5 min USA, G9: EDTA with NBs + USA, G10: PBS	Discs preparation, conditioning protocols, discs placed in 12-well plates with PBS for 24 h, analysis	TGF-β, ELISA (pg/mL). G6: ≅ 780 = G7: ≅ 780 = G8: 872 = G9: 799 = G10: ≅ 780	EDTA did not increase TGF-β release
Atesci et al. 2020	Human roots/dentin discs and adMSCs (n = 4)	NC: DW, G1: 17% EDTA, G2: 10% CA, G3: 1% IP6, G4: 37% PHA, PC: DW + adMSCs, G5: 17% EDTA + adMSCs, G6: 10% CA + adMSCs, G7: 1% IP6 + adMSCs, G8: 37% PHA + adMSCs	Root fragments disinfection, irrigation protocols for 5 min (G1, G2, G3, G5, G6, G7) or 30 s (G4, G8), final irrigation with PBS, cell seeding in half of the group, incubation for 3 d, analysis	ELISA (pg/mg). TGF-β1. NC: ≅ 100 § = G1: > 200 § # G2: ≅ 500 § # G3: ≅ 300 § = G4: ≅ 370; PC: ≅ 370 # G5: ≅ 700 = G6: > 600 = G7: ≅ 580 # G8: ≅ 980; VEGF. NC: ≅ 0 § = G1: ≅ 1 § = G2: ≅ 1 § = G3: ≅ 1 § = G4: ≅ 1; PC: ≅ 8 # G5: ≅ 24 = G6: ≅ 33 = G7: ≅ 20 # G8: ≅. 57; BMP-2. NC: ≅ 60 § # G1: ≅ 240 § = G2: ≅. 250 § = G3: ≅ 250 § = G4: ≅ 230; PC: ≅ 150 # G5: ≅ 400 = G6: ≅ 400 = G7: ≅ 450 = G8: ≅ 520; FGF-2. NC: ≅ 1 § = G1: < 10 § = G2: ≅ 5 § = G3: ≅ 10 § = G4: < 10; PC: < 10 # G5: > 80 # G6: ≅ 40 = G7: > 20 # G8: ≅ 99	There was no significant difference in EDTA without adMSC regarding TGF-β1, VEGF and FGF-2, but EDTA increase BMP-2 release. EDTA with adMSC increased release of all growth factors
Kucukkaya Eren et al. 2020	Human dentin discs and DPSCs (n = 9)	PC: cells, NC: no treatment, G1: 1 min EDTA +cells, G2: 1 min EDTA, G3: 5 min EDTA + cells, G4: 5 min EDTA, G5: 10 min EDTA + cells, G6: 10 min EDTA, G7: 1 min EDTA + BAC + cells, G8: 1 min EDTA + BAC, G9: 5 min EDTA + BAC + cells, G10: 5 min EDTA + BAC, G11: 10 min EDTA+BAC+cells, G12: 10 min EDTA+BAC, G13: cells without disk	Dentin discs preparation, conditioning protocols with or without 17% EDTA or 0.008% BAC, DPSCs seeded on dentin discs, analyses at 24 and 72 h	TGF-β1, ELISA (pg/mL). PC: > 600 = NC: 600 = G1: > 700 = G2: ≅ 600 = G3: > 700 = G4: 600 = G5: ≅ 700 = G6: > 600 = G7: > 700 = G8: > 600 = G9: ≅ 700 = G10: ≅ 600 = G11: > 600 = G12: > 500 # G13: ≅ 1300	EDTA solutions had no significant effect on the TGF-β1 release
Li et al. 2020	Human mandibu-lar single-root premolars and SCAPs (n = 6)	G1: 10 min 1.5% NaOCl (20 mL), G2: NaOCl + 5 min 17% EDTA (10 mL), G3: NaOCl + PDT, G4: NaOCl + EDTA + PDT	Preparation of root segment, irrigating protocols (PDT groups - irradiation for 120 s), final rinse with sterile saline, SCAP with hydroxyapatite-based scaffolds seeded into root canals, incubation for 7 d, analyses	PDGF, qRT-PCR. G1: 1 =, G2: < 1 =, G3: > 3 #, G4: ≅ 2.5 #; VEGF, qRT-PCR. G1: ≅ 1 =, G2: > 2 =, G3: ≅ 7 #, G4 ≅ 7 #	EDTA did not influence PDGF and VEGF expression, with or without PDT

Bracks et al. 2019	Maxillary first molars from Balb/c mice (n = 18)	NC: empty, G1: BC, G2: 17% EDTA + BC	Endodontic access, pulpectomy, groups allocation (G2 - irrigation with 1 min 17% EDTA), dried with sterile paper points, intracanal bleeding with #15 K-file 0.5 mm beyond the apical foramen, coronal seal, euthanasia at 7, 14 or 21 d	TGF-β, RT-PCR. (7 d. NC: ≅ 0 =, G1: ≅ 50 =, G2: < 300 #; 14 d. NC: ≅ 0 =, G1: > 0 =, G2: > 0 =; 21 d. NC: ≅ 0 #, G1: > 100 =, G2: > 100 =). VEGF, RT-PCR. (7 d. NC: ≅ 9 =, G1: ≅ 6 =, G2: ≅ 2 =; 14 d. NC: ≅ 2 =, G1: ≅2 =, G2: ≅ 13 =; 21 d. NC: ≅ 2 =, G1: > 2 =, G2: ≅4 =). IGF, RT-PCR. (7 d. NC: ≅0 =, G1: ≅ 0 =, G2: 40 #; 14 d. NC: > 0 =, G1: > 0 =, G2: > 10 #; 21 d. NC: > 0 =, G1: ≅ 0 =, G2: < 10 #). NGF, RT-PCR. (7 d. NC: ≅ 0 =, G1: ≅ 0 =, G2: > 0 =; 14 d. NC: ≅ 0 =, G1: ≅ 0 =, G2: > 500 #; 21 d. NC: ≅ 0 =, G1: ≅ 0 =, G2: > 0 =)	EDTA increased TGF- β at 7 d, IGF at all periods, and NGF at 14 d. EDTA no influenced in the VEGF expression
Cameron et al. 2019	Human teeth (n = 6)	Sterile versus infected root canals. Control: untreated, G1: 1,5% NaOCl, G2: 17% EDTA, G3: 1.5% NaOCl + 17% EDTA	Sterile (control) and infected root canals with polymicrobial biofilm, disinfection protocols (10 mL of each solution for 10 min), root segments placed in 1 mL HBSS for 24 h, TGF-β1 quantification	TGF-β1, ELISA (ng/gm). Sterile root canal, control: ≅ 2.5 # G1: 0 # G2: ≅ 8.5 = G3: ≅ 7.5; Infected root canals, control: < 2.5 # G1: 0 # G2: ≅ 1 = G3: ≅ 3.5	EDTA increased TGF-β1 release from dentin under sterile conditions. EDTA released more TGF-β1 compared to NaOCl. EDTA did not influence TGF-β1 release in the presence of biofilm compared to control
Ivica et al. 2019	Human dentin discs and human bone marrow–MSCs (n = 3)	G1: 10% CA, G2: 17% EDTA; G3: PBS	Preparation of dentin discs, 300 µL of conditioning agents (CA, EDTA and PBS) for 10 min, analysis	TGF-β1, Slot Blot Protein Immunoassay (ng). G1: 382 ± 30 # G2: 66 ± 3 # G3: no staining	EDTA increased TGF-β1 release compared to PBS.
Liu et al. 2019	Pulp tissue from human premolars or third molars and DPCs (n = 5)	For TGF-β1 release, NC: no EDTA stimulation, G1: 5 min 12% EDTA+ fresh medium for 6 h, G2: 5 min 12% EDTA + fresh medium for 12h, G3: 12% EDTA + fresh medium for 24 h.	Cell culture, cells treated with EDTA in different time points and concentrations, cells maintained in fresh medium for 6, 12 and 24 h, analyses	TGF-β1, ELISA (pg/mL). NC: ≅ 400 =, G1: ≅ 500 #, G2: < 500 #, G3: < 500 #	EDTA increased TGF-β1 release.
Deniz Sungur et al. 2019	Human dentin discs (n = 3)	G1: 17% EDTA, G2: 1% IP6, G3: 9% HEDP, control: DW	Preparation of dentin discs, immersion in the solutions for 5 min, immersion in PBS, incubation at 37 °C up to 28 d, analysis	TGF-β, ELISA (nm). 4h. G1: > 25 = G2: ≅ 25 = G3: < 25 = control: > 25; 1 d. G1: ≅75 = G2: > 50 = G3: < 75 = control < 75; 3 d. G1: < 100 = G2: 75 = G3: 100 = control: ≅ 100; 5 d. G1: 125 = G2: 100 = G3: > 125 = control: ≅ 125; 7 d. G1: < 150 = G2: >	EDTA did not influence TGF- β release compared to other groups

				125 = G3: > 150 = control: 150; 14 d. G1: ≅175 = G2: > 150 = G3: 200 = control: 175; 28 d. G1: < 200 = G2: 175 = G3: 225 = control: > 200	
Chae et al. 2018	Root of permanent human teeth (n = 3)	G1: 5 min saline, G2: 17% EDTA, G3: 10% CA, G4: 10% PHA, G5: 37% PHA, NC: internal surface coated with nail varnish	Preparation of root segments, irrigation with 1.5% NaOCl, randomization, irrigation protocols, dried with paper points, samples placed into 1 mL a- MEM for 24 h, analysis	TGF- β1, ELISA (pg/mL^{-1}). NC: 43 = G1: 53 # G2: 231 # G3: 516 # G4: 240 # G5: 53	EDTA increased TGF- β1 compared to saline solution
Ranc et al. 2018	Immature single-rooted human premolars (n = 5)	NC: no irrigation, PC: 0.9% saline, G1: 5 min 17% EDTA, G2: 20 min 17% EDTA	Samples preparation, randomization, irrigating protocols, rinse with 0.9% saline (PC, G1, and G2), tagging of antibodies and preparation of samples, wash in water, analyses	TGF-β1, Raman intensity. NC: decrease of 70%, G1 increase of 10% compared to the G2, G2 increased 80% compared PC bFGF and BMP-2, Raman intensity. NC: decrease of 50% for bFGF and 82% for BMP-2, G1: NM, G2 increased > 300% compared to PC	EDTA increased TGF-β1 expression, and 20 min EDTA increased bFGF and BMP-2
Duncan et al. 2017	Human dentine discs (n = 3)	G1: 10% EDTA, G2: TSA, G3: VPA, G4: SAHA, G5: PBS	HDACis preparation, isolation of DMCs, DMCs extraction from 5 g of powdered dentine using 25 mL of each solution, constant agitation for 14 d, daily refreshing of extractant, analyses	Proteomic assay (pg/mL^{-1}), TGF- β1. G1: > 100000 # G2: < 20000 = G3: > 10000 = G4: < 30000 = G5: ≅ 30000; GDF-15. G1: > 25 = G2: < 50 = G3: > 50 # G4: > 200 # G5: 150; BMP-7. G1: > 1000 # G2: 0 = G3: 0 = G4: 0 = G5: 0; FGF-2. G1: > 55 = G2: > 45 # G3: > 15 = G4: > 40 = G5: 50; FGF-4. G1: 0 # G2: 160 # G3: 30 # G4: ≅120 = G5: 80; FGF-7. G1: ≅ 70 = G2: ≅ 30 = G3: < 20 = G4: > 60 = G5: < 90; BDNF. G1: ≅ 21 # G2: ≅ 32 = G3: 30 = G4: > 30 # G5: 0; GDNF. G1: 35 # G2: ≅20 = G3: ≅ 18 = G4: ≅ 24 # G5: ≅ 36; PDGF-AA. G1: > 3000 # G2: 0 = G3: 0 = G4: 0 = G5: 0; VEGF-A. G1: 250 #, G2: > 200 =, G3: ≅ 150 #, G4: > 200 =, G5: ≅ 175 #; SCF-R. G1: > 500 # G2: > 1000 = G3: > 1000 # G4: ≅ 2.000 # G5: > 500; IGFBP-1. G1: > 15000 # G2: ≅ 2500 # G3: < 2500 = G4: < 2500 # G5: > 5000; IGFBP-2. G1: > 1000 # G2: < 250 # G3: > 1000 # G4: > 2500 # G5: > 250; IGFBP-3. G1: > 60000 # G2: > 0 = G3: > 0 = G4: > 5000 = G5: 0; IGF-1. G1: 3500 = G2: > 3500 = G3: 4000 = G4: ≅ 3250 # G5: 0; Insulin. G1: > 1300 = G2: > 1400 = G3: 1400 = G4: > 1400 = G5: > 1600; EGFR-1. G1: > 500 # G2: ≅ 1000 # G3: > 1500 # G4: > 2000 # G5: > 1500; NGFR. G1. 0 # G2: > 200	EDTA effectively released TGF- β1, BMP-7, BDNF, PDGF-AA, VEGF-A, IGFBP-1 and -3, IGF-1, HGF and PIGF. EDTA not influenced FGF-2 and -7, GDNF, SCF-R, IGFBP-2, insulin. EDTA decreased GDF-15, FGF-4, EGFR-1, NGFR.

				# G3: > 125 = G4: > 125 # G5: > 300; HGF. G1: 65 # G2: ≅ 20 # G3: 5 # G4: ≅ 30 = G5: ≅ 35; PIGF. G1: 50 # G2: 15 = G3: 10 = G4: > 15 = G5: > 15	
Widbiller et al. 2017	Human dentin discs, and straight roots from first/second molars (n = 9 – dentin discs, n = 12 – root canal)	Dentin discs. One step, G1: 10 min PBS, G2: 10 min PBS+US, G3: 1 min 10% EDTA, G4: 1 min EDTA + US, G5: 3 min EDTA, G6: 3 min EDTA + US, G7: 10 min EDTA; Two step, G1: 10 min PBS + 1 min PBS + US, G2: 1 min EDTA + 1 min PBS + US, G3: 10 min EDTA + 3 min PBS, G4: 10 min EDTA + 3 min PBS + US, G5: 10 min EDTA + 5 min PBS, G6: 10 min EDTA + 5 min PBS+US. Root canal model. G1: 10 min PBS + US, G2: 10 min EDTA + 5 min PBS + US, G3: 3 min EDTA + US	Dentin discs preparation, irrigation protocols, analysis. Root canal model preparation, irrigation protocols, ultrasonic activation, frozen immediately, analysis	TGF-β1, ELISA (pg/mL). Dentin discs. One step. G1: < 50 = G2: < 50 # G3: 197 # G4: 313 = G5: 535 = G6: ≅ 700 = G7: 908; Two step. G1: < 50 # G2: 286 = G3: > 300 # G4: 735 # G5: > 300 # G6: 1334. Root canal model. G1: ≅ 500 # G2: 1023 # G3: 3445	EDTA increased TGF-β1 release, mainly after US activation
Gonçalves et al. 2016	Human dentin samples (n = 10)	G1: PBS with PD, G2: PBS without PD, G3: 1 min 10% EDTA with PD, G4: 1 min 10% EDTA without PD, G5: 5 d 2.5% NaOCl with PD, G6: 5 d 2.5% NaOCl without PD	Tooth slice preparation with or without PD, slices conditioned with irrigating protocols, transferred to 24-well plates incubation at for 3 d, analysis	TGF-β1, ELISA (pg/mL). G1: MC =, G2: MC =, G3: 1.262.175 #, G4: 1.197.095 #; G5: MC =, G6: MC =	EDTA significantly released TGF-β1 from dentin matrix
Sadaghiani et al. 2016	Dentin powder, human dentin slices and DPSCs (n = 6 or 15)	G1: 10% EDTA, G2: 37 % PHA, G3: 10% CA, G4: 25% PA, NC: PBS, PC: CH, *control: no DME or denatured EDTA-extracted DMEs for DME stimulation	Extraction of DME or dentine immersion in the conditioning solutions for 5 or 10 min, frozen, growth factor quantification, washed with DW, immunogold localization, DPSC seeded onto conditioned dentine, cell morphology examined after 1 and 8 d, and analysis	SEM, Immunogold-labeled particles (number of particles). (TGF-β1, 5 min. NC: ≅ 10 =, PC: ≅ 55 #, G1: > 10 =, G2: > 15 #, G3: > 10 =; 10 min. NC: > 10 =, PC: > 40 #, G1: ≅ 15 =, G2: 20 #, G3: 20 #; VEGF, 5 min. NC: MC =, PC: 70 #, G1: ≅ 70 #, G2: > 30 #, G3: > 60 #; 10 min. NC: 0 =, PC: > 80, G1: ≅ 25 #, G2: ≅ 20 =, G3: > 10 =; BMP2, 5 min. NC: 0 =, PC: > 60 #, G1: > 75 #, G2: 20 =, G3: 65 #; 10 min. NC: MC =, PC: ≅ 70 #, G1: ≅ 30 #, G2: > 10 =, G3: ≅ 20 =); ELISA, Concentrations in the conditioned dentine (pg/mL). (TGF-β1, 5 min. NC: 0 =, PC: 0 =, G1: > 650 #, G2: 0 =, G3: 0 =; 10 min. NC: 0 =, PC: 0 =, G1: > 600 #, G2: 0 =, G3: 0 =; VEGF. G3 significantly released VEGF after 5 min among the groups; BMP2, 5 min. NC: 0 =, PC: < 200 =, G1: 0 =, G2: < 200 =, G3: ≅ 600 #; 10 min. NC: 0 =, PC: 0 =, G1: 0 =, G2: < 200 =, G3: 800 #)	EDTA did not influence TGF-b1, and increased BMP-2 and VEGF in SEM analysis, compared to control; but increased TGF-b1, and did not influence BMP-2 and VEGF in ELISA analysis.

Zeng et al. 2016	Human permanent teeth (n = 12)	G1: 1.5% NaOCl + 17% EDTA for 5 min, G2: 2.5% NaOCl + 17% EDTA for 5 min, G3: 5 min 17% EDTA, G4: 5 min DW	Preparation of root segments, growth factor array, irrigating protocols, segments placed into 1 mL a-MEM for 4 h, 1 or 3 d, analyses	ELISA (ng/mL). TGF-β1. 4 h. G1: ≅ 16 =, G2: ≅ 30 =, G3: 4 =, G4: 0.78 =; 1 d. G1: 69.04 #, G2: 59.26 #, G3: 6.92 =, G4: 0.78 =; 3 d. G1: 15.16 =, G2: 13.04 =, G3: 16.25 =, G4: 0.78 =; bFGF 4 h. G1: 0 =, G2: 0.43 =, G3: 0, G4: 0; 1 d. G1: 0 = G2: MC=, G3: 0=, G4: 0 =; 3 d. G1: 0=, G2: MC =, G3: 0=, G4: 0=	EDTA alone did not influence released of TGF-β1 or bFGF
Galler et al. 2015a	Human dentin discs (n = 3)	TGF-β-1 release, G1: 10% EDTA (0.268 mol/L, pH 6), G2: 10% EDTA (0.268 mol/L, pH 7), G3: 17% EDTA (0.456 mol/L, pH 7), G4: 10% CA (0.476 mol/L, pH 2), G5: CB (CA 0.476 mol/L + TCD 1.55 mol/L) pH 5, G6: CAPB (CA 0.476 mol/L + TCD 0.68 mol/L + TP 1.09 mol/L)	Dentin discs preparation, treated with solutions at different concentrations or pH for 5, 10 and 20 min, sample collection, analysis	TGF-β1, ELISA (pg/mL). 5 min. G1: > 200 = G2: > 300 # G3: < 200 # G4: MC = G5: MC = G6: MC; 10 min. G1: > 300 = G2: > 400 = G3: 400 # G4: 57 # G5: MC = G6: MC; 20 min. G1: 449 # G2: 923 = G3: 827 # G4: 57 # G5: MC = G6: MC	EDTA increased the released of TGF-b1

The symbol * indicates additional group per analysis; indicates no significant differences between/among groups; # indicates significant differences between/among groups; ≅ indicates "approximately"; > indicates "greater than"; < indicates "less than".

n.a.: not applicable, G: group, n: number of specimens, d: days, w: week, min: minutes, h: hour, mm: millimeter, mL: milliliter, nm: nanometre, mg: milligram, pg: picogram, ng: nanogram, µL: microliter, °C: degree Celsius, MC: minimal concentration or zero, NM: not mentioned, DMC: dentin matrix component, semi-qRT-PCR: semi-quantitative reverse transcriptase-PCR, DMPs: dentine matrix protein extracts, BC: blood clot, DPCs: dental pulp cells, DPSCs: dental pulp stem cells, EDTA: ethylenediaminetetraacetic acid, NB: nanobubble water, NaOCl: sodium hypochlorite, PBS: sterile phosphate-buffered saline, FBS: fetal bovine sérum, PC: positive control, NC: negative control, adMSCs: adipose-derived mesenchymal stem cells, DW: distilled water, CA: citric acid, IP6: phytic acid; PHA: phosphoric acid, BMP-2: bone morphogenetic protein-2, FGF-: fibroblast growth factor, TAP: triple antibiotic paste (ciprofloxacin, metronidazole and minocycline), CHX: chlorhexidine, CH: calcium hydroxide, BAC: benzalkonium chloride, USA: ultrasonic activation, PDT: photodynamic therapy, SCAPs: stem cells of the apical papilla, a-MEM:alpha- minimum essential medium, RT-PCR: real- time polymerase chain reaction, PDGF: platelet-derived growth factor, IGF: Insulin-like growth factor, NGF: nerve growth factor, IL-: interleukin, HBSS: Hank´s balanced salt solution, MSCs: mesenchymal stem cells, HEDP: etidronic acid, ELISA: enzyme linked immunosorbent assay, HDACis: histone deacetylase inhibitors, TSA: trichostatin A, VPA: valproic acid, SAHA: suberoylanilide, PBS: sterile phosphate-buffered saline, g: grass, BMP-7: bone morphogenetic protein-7, FGF-: fibroblast growth factor-, BDNF: brian-derived neurotrophic factor, GDF-15: growth/differentiation factor 15, GDNF: glial cell-line-derived neurotrophic factor, TGF-: transforming growth factor, nAb TGF-β1: TGF-β1 neutralizing antibody, VEGF-: vascular endothelial growth factor, SCF-R: mast/stem cell growth factor receptor kit, IGFBP: Insulin-like growth factor-binding protein, EGFR: epidermal growth factor receptor, NGFR: tumour necrosis factor receptor, HGF: hepatocyte growth factor, PIGF: placenta growth factor, US: ultrassonic ativation, PD: predentin layer, GdnHCl: guanidine hydrochloride, HCl: hydrochloric acid, MDPSCs: mobilized dental pulp stem cells, , CM: conditioned medium, PA: polyacrylic acid, DME: dentin matrix extracts, TCD: trisodium citrat, CB: citrate buffer, CAPB: citric acid phosphate buffer, TP: trisodium phosphate, M: molar, WMTA: white mineral trioxide aggregate, GMTA: grey mineral trioxide aggregate, NCPs: non-collagenous proteins, GAGs: glycosaminoglycans, ADM: adrenomedullin, BCA: benzalkonium chloride, NCP: non-collagenous protein, MDPC-23: mouse odontoblast-like cells, OD-21: undifferentiated pulp cells, 3T3: mouse fibroblast cells

Table 3. Effects of EDTA on stem cells behavior (*in vitro analyses*)

Author	Experi-mental model (n)	Groups	Experimental protocol	Cell Viability	Cell Migration/ attachment	Cell morphology	Cell differentiation/ mineralization	Outcomes
Aksel et al. 2020	Human dentine discs and DPSC (n = 3)	First protocol, G1: 5 min NaOCl + 3 min PBS, G2: G1 + 5 min EDTA, G3: G2 + 3 min PBS, G4: NaOCl + 5 min EDTA, G5: G4 + 3 min PBS. Second protocol using G3, G6: optimized EDTA protocol, G7: EDTA with NBs, G8: EDTA + 5 min USA, G9: EDTA with NBs + USA, G10: PBS, control: DPSC, PC: DPSC in 10% FBS, NC: DPSCs	Discs preparation, first conditioning protocols, DPSCs seeded on dentin discs, incubation for 3 and 7 d, cell viability/morph ology assays, second conditioning protocols, incubation for 7 d, final analyses	WST-1 analysis. First protocol (3 d: G1: ≅ 0.04 # G2: ≅ 0.01 # G3: ≅ 0.11 # G4: > 0.00 # G5: ≅ 0.07 # control: ≅ 0.15 #; 7 d: G1: ≅ 0.14 # G2: ≅ 0.03 # G3: ≅ 0.24 # G4: ≅ 0.03 # G5: ≅ 0.015 # control: ≅ 0.33); Second protocol (7 d: G6: ≅ 0.23 = G7: ≅ 0.21 # G8: ≅ 0.27 # G9: ≅ 0.24 # G10: 0.18 # control: 0.27)	Transwell migration assay. 7 d: G6: ≅ 0.14 # G7: ≅ 0.17 # G8: ≅ 0.18 = G9: ≅ 0.18 # G10: ≅ 0.13 # NC: ≅ 0.11 # PC: ≅ 0.17	CLSM. Fisrt protocol: G3 and G5 had elongated, fibroblastic-like with flattened cell morphology on the dentin surface; Second protocol: PBS group showed round cell morphology compared to EDTA groups	n.a.	Final rinse with EDTA decreased cell viability compared to others. The activation EDTA protocols increased cell viability/ migration, and influenced cell morphology
Atesci et al. 2020	Human dentin discs and adMSCs (n = 4)	Control: DW, G1: 17% EDTA, G2: 10% CA, G3: 1% IP6, G4: 37% PHA	Root fragments disinfection, irrigation protocols for 5 min (G1, G2, G3) or 30 s (G4), final irrigation with PBS, cell seeding, cultured in GM for 3 d, analysis	n.a.	Attachment, CLSM. 3 d: control: reduced cell attachement, G1: abundance of cells, G4: well attached	CLSM. 3 d: control: round-shaped cells; G1: oblong, flattened, and round-shaped cells; G2: flattened cells with extending cytoplasmic processes, G3: flattened cells and round shape; G4: round and oblong-like cells and flattened and well-attached cells with extending cytoplasmic processes	n.a.	EDTA group had abundance of cells, mostly round-shaped compared to the other groups

Kucukkaya Eren et al. 2020	Human dentin discs and DPSCs (n = 9)	Control: no treatment, G1: 1 min EDTA, G2: 5 min EDTA, G3: 10 min EDTA, G4: 1 min EDTA + BAC, G5: 5 min EDTA + BAC, G6: 10 min EDTA + BAC	Dentin discs preparation, conditioning protocols with or without 17% EDTA or 0.008% BAC, DPSCs seeded on dentin discs, analyses at 24 and 72 h	WST-1 assay (%). 72 h: G1: ≅ 31% = G2: ≅ 29% = G3: ≅ 29% = G4: ≅ 31% = G5: ≅ 30% = G6: ≅ 31% # control: ≅ 7%	Attachment, LDH assay (%). 24 h: G1: ≅ 43% = G2: ≅ 38% = G3: ≅ 34% = G4: ≅ 45% = G5: ≅ 35% = G6: ≅ 38% # control: ≅ 24%	SEM. 72 h: control: mostly spherical and smaller cells; EDTA groups: mostly spindle-shaped cells with elongated cytoplasmic processes	n.a.	EDTA groups increased cell proliferation and attachment, influencing cell morphology
Li et al. 2020	Human mandibular single-root premolars and SCAPs (n = 6)	G1: 10 min 1.5% NaOCl (20 mL), G2: NaOCl + 5 min 17% EDTA (10 mL), G3: NaOCl + PDT, G4: NaOCl + EDTA + PDT	Preparation of root segment, irrigating protocols (PDT groups: irradiation for 120 s), final rinse with sterile saline, SCAP with hydroxyapatite-based scaffolds seeded into root canals, incubation at 37°C for 7 d, analyses	Luminescence analysis (n° of cells). 7 d: G1: ≅ 37 = G2: ≅ 50 # G3: ≅ 62 = G4: ≅ 70	n.a.	SEM. 7 d: G1/G2: cells with small cytoplasmic processes, G3/G4: spindle shaped cells with elongated cytoplasmic processes	n.a.	EDTA did not influenced in the viability or morphology of the cells
Ivica et al. 2019	Human dentin discs/ MSCs (n = 4: viability / migration, n = 3: attach-ment)	G1: 10% CA, G2: 17% EDTA; G3: PBS	Preparation of dentin discs, 300 µL of conditioning agents for 10 min, cells seeded on conditioned dentin discs, analyses after 24 and 48 h	Automatic Cell Counter (cells /dentin area). 48 h: G1: ≅ 6000 # G2: ≅ 2000 = G3: ≅ 2000	Transwell migration assay (nº cells). 24 h: G1: ≅ 5000 # G2: ≅ 2000 # G3: ≅ 100. Attachment, automatic cell counter (cells/ dentin area). 24 h: G1: ≅ $1x10^5$ # G2: ≅ $5x10^4$ # G3: > $5x10^4$. 48 h: G1: ≅	n.a	n.a.	EDTA had higher cell viability and migration than PBS, and higher cell attachment at 24 h

					$6x10^5$ # G2: ≅ $2x10^5$ = G3: > $2x10^5$			
Liu et al. 2019	Pulp tissue from human premolars or third molars and DPCs (n = 5)	Cell viability. According to exposure time: G1: 1 min, G2: 3 min, or G3: 5 min 17% EDTA, control: α-tubulin; and concentration: G1: 3%, G2: 6%, G3: 12%, or G4: 17% EDTA, control: α-tubulin. Cell migration. G1: SDF-1α, G2: EDTA+SDF-1α, G3: EDTA+SDF-1α+siCXCR4, NC: medium without SDF-1α	Cell culture, cells treated with EDTA in different time points and concentrations, cells maintained in fresh medium for 24, 48 and 72 h, analyses	CCK-8 assay (%). Acordding to time of exposure. 24 h: Control: 100 = G1: ≅ 100 = G2: > 100 # G3: ≅ 90; 48 h: Control: 100 = G1: < 100 = G2: < 100 # G3: < 90; 72 h: Control: 100 = G1: ≅ 100 = G2: < 100 # G3: ≅ 90); According to concentration. 24 h: Control: 100 = G1: > 100 = G2: > 100 = G3: > 100 # G4: ≅ 90; 48 h: Control: 100 = G1: ≅ 100 = G2: > 100 = G3: < 100 # G4: < 90; 72 h: Control: 100 = G1: < 100 =G2: >100= G3: ≅100 #G4: ≅ 90)	Transwell migration assay (cells per field): NC: ≅ 80 # G1: ≅ 125 # G2: ≅ 160 # G3: ≅ 90	n.a.	n.a.	17% EDTA for 5 min decreased DPCs viability compared to other EDTA concentration groups and control; no difference in the other exposure times and the control. EDTA significantly enhanced cell migration.
Deniz Sungur et al. 2019	Dentin discs from human third molars and DPSCs (n = 9)	G1: 17% EDTA, G2: 1% IP6, G3: 9% HEDP, G4: DW, PC: 20% FBS, NC: 1% FBS	Dentin discs preparation, *proliferation/ morphology*: disinfection, conditioning (5 min), DPSCs seeded on discs, incubation in GM for 1, 3, 5 d, analysis;	MTT assay. 1 d: G1: ≅ 0.4 =, G2: < 0.4 =, G3: ≅ 0.3 #, G4: < 0.4 =; 3 d: G1: < 0.6 # G2: ≅ 0.4 # G3: < 0.4 # G4: > 0.6; 5 d: G1: ≅ 0.8 =, G2 < 0.6 #, G3: 0.4 #, G4: ≅ 0.8 =	Transwell migration assay. 1 d: G1: ≅ 0.7 =, G2 > 0.6 #, G3: > 0.8 ##, G4: < 0.6 =, NC: ≅ 0.6 =, PC: > 0.6 =; 3 d: G1: < 0.6 =, G2: < 0.6 #, G3: ≅ 0.6 #, G4: < 0.6 =, NC: 0.4 =, PC: < 0.6 =.	SEM. 1 d: G1: contracted cells with a spherical morphology, G2/G3: polygonal morphology and more stretched out onto the dentin, G4: flattened cells compared to G1; 5 d: major cell number in the groups.	n.a.	EDTA decreased cell viability than DW at 3 d, while not influenced at 1 and 5 d. EDTA did not influence cell migration, but influenced morphology cell (contracted cells).

			migration: chambers with culture media, discs in lower chamber, cells incubated for 1 and 3 d, analysis					
Tunç et al. 2019	SHEDs (n = n.a.)	G1: 5% EDTA, G2: 8.5% EDTA, G3: 17% EDTA, G4: 1% NaOCl, G5: 2.5% NaOCl, G6: 5% NaOCl, G7: 5 µg/mL OW, G8: 10 µg/mL OW, G9: 20 µg/mL OW, G10: 0.5 j/cm^2 GaAlAs, G11: 1 j/cm^2 GaAlAs, G12: 1.5 j/cm^2 GaAlAs, Control: no exposure	SHEDs seeded, irrigants and laser application, incubation for 5, 10 and 15 min, analyses	MTT assay (%). 5 min: (G1 ≅ 60, G2: ≅ 60, G3: ≅ 55, G4: >60, G5: >60, G6: 55.3) # (G7: ≅ 100, G8: ≅ 100, G9: >100, G10: ≅ 100, G11: ≅ 100, G12: >100, cont.: 100); 10 min: (G1: >60, G2: >60, G3: 57.2, G4: >60, G5: ≅ 60, G6: ≅ 60) # (G7: >100, G8: >100, G9: 103.7, G10: >100, G11: >100, G12: >100, cont.: 100); 15 min: (G1: ≅ 25, G2: ≅ 25, G3: 21.7, G4: ≅ 30, G5: ≅ 30, G6: ≅ 30) # (G7: >100, G8: 100.7, G9: 100, G10: >100, G11: >100, G12: 99.9, cont.: 100)	n.a.	n.a.	n.a.	EDTA decreased cell viability compared with control

Widbiller et al. 2019	Human dentin discs and SCAPs (n = n.a.)	Control: saline, G1: 2% CHX, G2: 17% EDTA, G3: L-α-lecithin, G4: CHX + L-α-lecithin, G5: CHX + EDTA, G6: CHX + L-α-lecithin + EDTA	Dentin discs preparation, rehydration, conditioning 5 min (mixed or not mixed solutions), SCAPs cultured for 5 d, analysis	Luminescence (%). No mixed solutions: Control: > 10^4 # G1: < 10^2 # G2: > 10^4 = G3 > 10^4; Mixed solutions: control: ≅ 10^4 # G1: < 10^2 # G4: ≅ 10^4 # G5: > 10^3 # G6: < 10^4 =	n.a.	n.a.	n.a.	There was no difference between EDTA and control in cytotoxicity
Chae et al. 2018	Root human teeth and SCAPs (n = 3)	G1: saline, G2: 17% EDTA, G3: 10% CA, G4: 10% PHA, G5: 37% PHA, PC: non-treated dentin	Discs irrigated 1.5% NaOCl, irrigating, surfaces dried, transwell system, SCAPs loaded for 2 in the lower chamber, discs in the upper chamber, cells cultured for 24 h, analysis	MTS assay (nm). PC: ≅ 2.4 =, G1: ≅ 2.3 =, G2: ≅ 2.7 = #, G3: ≅ 2.5 = #, G4: ≅ 2.4 =, G5: ≅ 2.0 #	n.a.	n.a.	n.a.	There was no difference among saline, PC and EDTA in viability
Hashimoto et al. 2018	Dentin discs from bovine teeth and MDP cells (n = 9)	G1: PBS, G2: PBS +10 min 3% EDTA, G3: PBS +10 min 17% EDTA, G4: 1.5% NaOCl, G5: 1.5% NaOCl +1 min 3% EDTA, G6: 1.5% NaOCl +5 min 3% EDTA, G7: 1.5% NaOCl +10 min 3% EDTA, G8: 1.5% NaOCl +20 min 3% EDTA, G9: 1.5% NaOCl +60 min 3% EDTA, G10: 1.5% NaOCl +1 min 17% EDTA, G11: 1.5% NaOCl +5 min 17%	Preparation of dentin discs, irrigating protocols, washes with PBS, MDP cells seeded on dentin discs for 24 and 48 h, analyses	CCK-8 assay (cells/field). G4: ≅ 0.003 = G5: ≅ 0.002 = G6: 0.003 # G7: ≅ 0.010 = G8: ≅ 0.007 = G9: ≅ 0.010 # G10: ≅ 0.002 = G11: ≅ 0.004 # G12: ≅ 0.008 = G13: ≅ 0.007 = G14: ≅ 0.008	Attachment, cell density (cell/field). G1: 158 =, G2: ≅ 260 #, G3: ≅ 350 ##, G4: 24.5 *, G7: ≅ 60 **, G12: ≅ 62 **, G15: 1.5 $, G16: 1.5 $, = G17: 1.5 $. SEM. G1: cytoplasmic process to the smear layer, G3 and G12: cytoplasmic process to the dentin matrix, G4:	Fluorescence photomicrograph. G1/G2/G3: spindle and fibroblastic appearances, G4: round cells, G5 and G6: absence of viable cells, G7: most fibroblastic appearance, G12: most round cells, G15/G16/G17: reduced cells with fibroblastic appearances	RT-PCR. ALP. Control: ≅ $1x10^{-3}$ = G1: ≅ $2x10^{-3}$ # G2: ≅ $4x10^{-3}$ # G3: > $4x10^{-3}$; Control: ≅ $6x10^{-4}$ = G4: ≅ $6x10^{-4}$ # G6: > $6x10^{-4}$ # G12: ≅ $7x10^{-4}$; DMP. Control: 0 = G1: < $2x10^{-3}$ # G2: ≅ $2x10^{-3}$ # G3: ≅ $4x10^{-3}$; Control: ≅ $2.5x10^{-6}$ = G4: < $5x10^{-6}$ # G6: > $1x10^{-5}$ # G12: ≅ $1.5x10^{-5}$; DSPP.	10 min EDTA after 1.5% NaOCl increased cell viability, attachment, and differentiation with cells ≅ odontoblast-like

		EDTA, G12: 1.5% NaOCl +10 min 17% EDTA, G13: 1.5% NaOCl +20 min 17% EDTA, G14: 1.5% NaOCl +60 min 17% EDTA, G15: 6% NaOCl, G16: 6% NaOCl +10 min 3% EDTA, G17: 6% NaOCl +10 min 17% EDTA			few cells; TEM analysis. G7 and G12: cytoplasmic process to the dentin matrix, presence of collagen fiber		control: 0 = G1: < $2x10^{-3}$ # G2: > $2x10^{-3}$ # G3: ≅ $4x10^{-3}$; control: < $1x10^{-7}$ = G4: ≅ $5x10^{-7}$ = G6: < $5x10^{-7}$ = G12: > $1x10^{-6}$	
Prompreecha et al. 2018	Plastic tooth models, human dentin discs, and APCs (n = 9)	NI (G1: 16 min NSS, G2: 15 min EDTA +1 min NSS, G3: 5 min CHX +10 min EDTA +1 min NSS), NI+EA (G4: 16 min NSS, G5: 15 min EDTA +1 min NSS, G6: 5 min CHX +10 min EDTA +1 min NSS), NI+PUI (G7: 16 min NSS, G8: 15 min EDTA +1 min NSS, G9: 5 min CHX +10 min EDTA +1 min NSS), control (non-dinamic irrigation, G10: 16 min NSS, G11: 15 min EDTA+1 min NSS, G12: 5min CHX +10 min EDTA+1 min NSS)	Preparation of immature root canal models (plastic tooth + dentin discs), smear layer removal, samples with CH paste for 1w, samples inserted into the models, irrigating protocols for 16 min, analyses	n.a.	Attachment, immuno-fluorescent assay (positive cells/field) G1: ≅ 230 # G2: ≅ 180 = G3: ≅ 160. G4: ≅ 240 # G5: ≅ 170 = G6: ≅ 150. G7: ≅ 230 = G8: ≅ 160 = G9: ≅ 175. G10: ≅ 80 = G11: ≅ 85 # G12: ≅ 25	n.a.	n.a.	EDTA groups had less cell attachment compared to NSS in the dynamic irrigation; however, no difference was observed between EDTA and NSS in non-dinamic irrigation
Scott et al. 2018	SCAPs, PDL fibroblasts and UMR-106 cells	G1: DW, G2: 10% Endocyn, G3: 6% NaOCl, G4: 17% EDTA, G5: 2% CHX	Cell lines isolated and cultured for 24 h to 48-well plates, exposure to the irrigants,	Autofluorescence (% cell). PDL survival (10 min. G1: ≅ 105 =, G2: ≅ 110 =, G3: ≅18 #, G4: ≅ 10 #, G5: ≅7 #; 1 h. G1:	n.a.	n.a.	n.a.	EDTA and the other irrigants showed more cytotoxicity to PDL, UMR-106, and SCAP cells than DW and Endocyn

	(n = 8)		treatment with calcein AM for 1 h, rinse with PBS, analyses	≅ 100 =, G2: ≅ 90 #, G3: ≅ 7#, G4: ≅ 16#, G5: 20#; 24 h. G1: 100 =, G2: 80#, G3: ≅ 5*, G4: ≅ 5*, G5: ≅ 5*); UMR survival (10 min. G1: ≅ 85 =, G2: ≅ 100 =, G3: 0#, G4: ≅ 0#, G5: ≅ 0#; 1 h. G1: 100=, G2: ≅ 90=, G3: ≅ 5 #, G4: ≅ 5#, G5: ≅ 5 #; 24h. G1: ≅ 90 =, G2: ≅ 100=, G3: 0#, G4 < 0#, G5: ≅ 3#); SCAP survival (10min. G1: > 100=, G2: ≅ 80 =, G3: 20 #, G4: ≅ 10 #, G5: ≅ 10 #; 1h. G1: > 100 =, G2: 20 #, G3: ≅ 5 #, G4: ≅ 18 #, G5: 20 #; 24h. G1: 100 =, G2: 0 #, G3: > 0 #, G4: ≅ 5 #, G5: ≅ 5 #)				
Alghilan et al. 2017	Human dentin samples and DPSCs (n = 10)	G1: TAP, G2: DTAP, G3: DAP, G4: CH, PC: 10 min 17% EDTA, NC: untreated	Dentin samples preparation, intracanal dressing, control groups treated with 200 µL of water, 7d incubation, EDTA and PBS irrigation, DPSC on	WST-1 assay (%). NC: ≅ 116 =, PC: ≅ 95 =, G1: ≅ 10 #, G2: ≅ 90 =, G3: ≅ 20 #, G4: ≅ 36 *	Attachment, LDH assay (%). PC: ≅ 22 =, G1: 40 =, G4: ≅ 28, G3: 15 #, G4: 10 *, NC: 10 *	n.a.	n.a.	EDTA and DTAP had more DPSC proliferation compared to the others, without difference with untreated control. Moreover, EDTA showed higher cell attachment than CH and untreated control

			dentin, incubation for 24h and 3 d, analyses					
Gonçalves et al. 2016	Human tooth slices and SHEDs (n = 10)	G1: PBS with PD, G2: PBS without PD, G3: 1 min 10% EDTA with PD, G4: 1 min 10% EDTA without PD, G5: 5 d 2.5% NaOCl with PD, G6: 5 d 2.5% NaOCl without PD, Control 1: alpha-MEM with 20% FBS, Control 2: alpha-MEM with 10% FBS	Tooth slice with or not PD, slices conditioned with irrigating, transferred to 24-well plates, incubation at for 3 d, cells seeded onto tooth slice CM, migration assay	n.a.	Fluorescent analysis (Arbitrary units). G1: ≅ 4000 # G2: 3000 # G3: ≅ 4000 # G4: 3000 # G5: 4000 # G6: > 3000 # Control 1: > 4000 # Control 2: ≅ 3000	n.a.	n.a.	Dentin slices with PD increased cell migration compared to conditioned slices without PD; however, EDTA does not impact SHED migration
Kawamura et al. 2016	C2C12 and HUVEC (n = 4, cell attachment; n = 3, cell differentiation)	For *in vitro* analyses, G5: nonextracted, G6: EDTA extracts, G7: CM, G8: CM + EDTA extracts, *PC: 1 µg/mL VEGF, FGF-β, and IGF for endothelial differentiation	For proliferation: cells cultured in the extracts for 2, 12, 24, 36, and 48 h; for adhesion: cells cultured on dentin and CM for 48 h; for differentiation: cells cultured in the extracts for 28 d and 14 d, analyses	Proliferation, CCK-8 (absorbance). 2h. G5 < 0.1 = G6: < 0.1 = G7: < 0.1 = G8: < 0.1; 12 h. G5: > 0.1 = G6: > 0.1 # G7: ≅ 0.4 = G8: > 0.4; 24 h. G5: ≅ 0.2 = G6: > 0.2 # G7: ≅ 0.6 # G8: > 0.6; 36 h. G5: > 0.3 = G6: > 0.3 # G7: ≅ 0.7 # G8: 0,8; 48 h. G5: ≅ 0.4 = G6: ≅ 0.4 # G7: ≅ 0.8 # G8: ≅ 1.0	Chemotaxis assay (nº cell). 3 h. G5: > 10 = G6: > 12.5 # G7: ≅ 22.5 # G8: > 25; 6 h. G5: > 12.5 # G6: ≅ 17.5 # G7: > 27.5 # G8: ≅ 32.5; 9 h. G5: ≅ 15 = G6: > 17.5 # G7: ≅ 30 # G8: ≅ 34; 12 h. G5: ≅ 15 = G6: > 17.5 # G7: ≅ 30 # G8: ≅ 35; 15 h. G5: > 15 = G6: > 17.5 # G7: > 30 # G8: ≅ 35; 18 h. G5: > 15 = G6: > 17.5 # G7: > 30 # G8: >35; 21 h. G5: > 15 = G6: > 17.5 # G7: > 30 # G8: > 35; 24 h. G5: ≅ 15 = G6: ≅	n.a.	C2C12 odontoblastic differentiation, DSPP, enamelysin and β-Actin, RT-PCR. G5 and G7: no effect, G6 and G8: induced differentiation; HUVEC endothelial differentiation, Immunocytochemistry of VE-cadherin (fluorescence) G5 and PC: not mentioned, G6: in increase, G7 and G8: induced differentiation	EDTA extracts with CM promoted cell proliferation, migration, and odontoblastic differentiation; however, EDTA extracts showed no increase cell adhesion and in endothelial differentiation

				17 # G7: ≅ 30 # G8: ≅ 35. Adhesion, Giemsa stain (mm^2). G5: ≅ 35 = G6: ≅ 35 # G7: ≅ 56 # G8: ≅ 60 # PC: ≅ 80				
Mollashahi et al. 2016	Stem cells from immature third molars (n = n.a.)	G1: 2% CHX, G2: 17% EDTA, G3: Qmix, G4: 5.25% NaOCl, G5: BioPure MTAD Cleanser, G6: sterile saline, Control: untreated	SCAPs cultured for 1 w, exposure to the solutions for 1.5 and 15 min, MTT assay	MTT assay (%). 1 min. G1: 60 # G2: ≅ 50 = G3: ≅ 57 = G4: ≅ 57 # G5: ≅ 48 # G6: ≅ 100 = Control: 100; 5 min. G1: 60 = G2: 50 = G3: ≅ 53 = G4: ≅ 53 # G5: 40 # G6: ≅ 100 = Control: 100; 15 min. G1: 60 # G2: ≅ 41 = G3: ≅ 43 = G4: ≅ 43 # G5: ≅ 30 # G6: ≅ 100 = Control: 100	n.a.	n.a.	n.a.	EDTA and the other groups had higher cytotoxicity than sterile saline and untreated control
Sadaghiani et al. 2016	Dentin powder, human dentin slices and DPSCs (n = 3)	G1: 10% EDTA, G2: 37 % PHA, G3: 10% CA, G4: 25% PA, NC: PBS, PC: CH	Extraction of dentine matrix, conditioned for 5 or 10 min, frozen, rinse with DW, immunogold localization, DPSC seeded onto conditioned dentine, analysis at 1 and 8 d	n.a.	n.a.	SEM. 2 d. Many cells on conditioned slices. Cell coverage against the open dentinal tubules with G1, G2, G3 and G4 compared with PC; 8 d. G1, G3 and PC: uniform/thick coverage by cells ≅ odontoblast-like, G2: reduced cells; NC: few cells visible	RT-PCR (%). (RUNX2, 3 d. control: > 1.4 =, G1: > 1.2 =, G2: > 1.2 =, G3: 1.2; 21 d. control: 1.2 =, G1: > 1.2 =, G2: > 1.2 =, G3: > 0.8 =; ALP, 3 d. control: MC =, G1: MC =, G2: MC =, G3: MC =; 21 d. control: ≅ 0.25 =, G1: ≅ 0.4 #, G2: > 0.4 #, G3: > 0.3 =; OPN, 3 d. control: MC =, G1: MC =,	EDTA-treated dentin showed a thick coverage by cells with odontoblast-like appearances. In DMEs, EDTA increased ALP and OPN at 21d. In dentin slice, EDTA increased ALP and OPN at 14d.

							G2: MC =, G3: MC =; 21 d. control: MC =, G1: > 0.045 #, G2: > 0.025 #, G3: 0.04 #). Conditioned dentin slices. (ALP, 5 d. NC: MC =, PC: MC =, G1: MC =, G2: MC =, G3: MC =; 14 d. NC: < 0.2 =, PC: < 0.2 =, G1: 0.6 #, G2: > 0.1 =, G3: 0.55 #; OPN, 5 d. NC: < 0.01 =, PC: > 0.01 #, G1: 0.01 =, G2: < 0.01 =, G3: < 0.01 =; 14 d. NC: ≅ 0.03 =, PC: > 0.02 =, G1: 0.05 #, G2: ≅ 0.04 =, G3: ≅ 0.035 =)	
Shrestha et al. 2016	Human dentin discs and SCAPs (n = 6)	G1: control (no treatment), G2: 10 min 5.25% NaOCl, G3: 2 min 17% EDTA, G4: 10 min NaOCl + 2 min EDTA, G5: 10 min NaOCl + 1 min EDTA + 1 min NaOCl	Dentin discs preparation, irrigating protocols, rinse with DW, nanoparticle conditioning, SCAPs cultured on discs for 24 h, staining with calcein-AM, analyses	n.a.	Attachment, fluorescence. G1: unidirectional cells, G2 and G5: less cells, G3: similar cell nº of G1, G4: increase in nº of SCAP adherence when nanoparticle conditioning with CSnp or Dex-CSnp	Fluorescence microscope. G1: fibroblast-like morphology, G2 and G5: rounded cells without cytoplasmic extensions and less ctytoplasmic F-actin compared with the other groups, G3: similar morphology of G1 but more flattened, G4: few cells with cytoplasmic extensions	n.a.	EDTA alone promoted similar cell adherence and morphology to control
Galler et al. 2015b	Human dentin discs and DPSCs	Cell viability. G1: Polystyrene, G2: DW, G3: EDTA, G4: 5.25% NaOCl; Cell migration. PC: αMEM	Cell migration: cell seeding onto dentin discs, chambers with αMEM,	MTT assay (%). 24 h. G1: ≅ 140 # G2: 100 = G3: ≅ 95 # G4: ≅ 10. 48 h. G1:	Crystal violet assay (%). 24 h. PC: 100 # G1: 70 # G2: ≅ 30 = G3: ≅	n.a.	RT-PCR. G1: 1 for mineralization markers, G2: > expression compared to G1,	EDTA did not influence viability, but increased migration and differentiation of DPSCs

	(n = 9, cell viability / migration; n = 3, cell differentiation)	+ 20% FBS, NC: αMEM + 1% FBS, G1: 10 min 10% EDTA, G2: 10 min 5.25% NaOCl, G3: DW; Cell differentiation. G1: DW, G2: EDTA + saline, G3: polystyrene	discs in lower chambers, solutions, cells removed from the membrane after 24 and 48 h, analysis. Cell viability: discs conditioned, cells seeded, assays at 24 and 48 h. Cell differentiation: cells seeded onto discs, immersion in the solutions for 7 d, analysis	≅ 120 = G2: 100 = G3: ≅ 95 # G4: ≅ 15	25 = NC: ≅ 25; 48 h. PC: 10 # G1: ≅ 75 # G2: ≅ 20 # G3: ≅ 30 = NC: ≅30		G3: < expression compared to G1, except for COL1A1 and RUNX2	
Kim et al. 2015	Human dentin discs and DPSCs (n = 4)	G1: 500 mg DAP (metronidazole and ciprofloxacin), G2: 500 mg DAP + 10 min 17% EDTA, G3: 1 mg DAP, G4: 1 mg DAP + 10 min 17% EDTA, G5: 10 min 17% EDTA, Control: no treatment	Dentin discs preparation, DAP medicated, 1w incubation, DAP-samples rinsed with DW, EDTA, DPSCs seeded on discs, LDH assay at 24 h, assay at 3 d	WST-1 assay (%). G1: 0 = G2: 20 # G3: ≅ 65 = G4: ≅ 77 = G5: ≅ 100 = control: 85	Attachment, LDH assay (%). G1: ≅ 7 # G2: ≅ 32 # G3: ≅ 10 # G4: 35 = G5: ≅ 25 = control: ≅ 35	n.a.	n.a.	10 min EDTA may have positive effects cell attachment, but not influenced the proliferation
Park et al. 2015	Human dentin discs and DPSCs (n = 250)	G1: 30 min 5.25% NaOCl, G2: 30 min 5.25% NaOCl +7 d 1 mg/mL CH + PBS, G3: 30 min 5.25% NaOCl +7 d 1 mg/mL CH +3 min 17% EDTA, G4: 30 min 5.25% NaOCl +7 d 1 mg/mL CH +3 min 17% EDTA +24 h	Flow cytometric analysis, dentin slices preparation, cells seeded onto dentin, samples cultured for 7 d, viability and morphology	MTT assay (%). Control: 100 # G1: ≅ 2 = G2: ≅ 17 = G3: ≅ 22 = G4: 20 = G5: ≅ 22	Attachment, RT-PCR. FN-1. G2: 1 #, G3: ≅ 1.7 =, G4: ≅ 2 =, G5: ≅ 2 =; SPP-1. G2: 1 #, G3: ≅ 1.4 =, G4: approx. 1.3 =, G5: ≅ 1.8	SEM analysis. 7 d. G1: DPSCs not attached to the dentin, G2/G3/G4/G5: elongated cells with longer cytoplasmic processes, G5: dentin overlapped by proliferated cell layers	RT-PCR. DMP-1. Control: 1 # G2: ≅ 1.2 # G3: ≅ 1.5 # G4: ≅ 2.3 = G5: ≅ 1.8; DSPP-1. Control: 1 = G2: ≅ 1.2 # G3: ≅ 1.4 # G4: ≅1.7 =G5: ≅2	EDTA did not influence cell viability, but additional treatment with EDTA after NaOCl and CH enhanced cell attachment and differentiation

		culture media, G5: 30 min 5.25% NaOCl +7 d 1 mg/mL CH +instrumentation +3 min 17% EDTA, control: cell culture	assays, cells cultured for 4 w for cell differentiation assay					
Martin et al. 2014	Human root canals and SCAPs (n = 9 – 12)	G1: 10 min 0,5% NaOCl + 5 min saline, G2: 10 min 0,5% NaOCl + 5 min EDTA, G3: 10 min 1.5% NaOCl + 5 min saline, G4: 10 min 1.5% NaOCl + 5 min EDTA, G5: 10 min 3% NaOCl + 5 min saline, G6: 10 min 3% NaOCl + 5 min EDTA, G7: 10 min 6% NaOCl + 10 mL 5% ST+ 5 min saline, G8: 10 min 6% NaOCl + 5 min EDTA, PC: EDTA, NC: saline	Preparation of root segments, scaffold preparation, irrigation protocols, SCAPs with hyaluronic acid–based scaffold seeded into the canals, samples cultured for 7 d, analyses	Luminescence ($x10^3$). NC: 38 =, PC: ≅ 52 #, G1: ≅ 23 =, G2: ≅ 38 #, G3: ≅ 22 =, G4: ≅ 42 #, G5: ≅ 23 =, G6: ≅ 39 #, G7: ≅ 5.5 =, G8: ≅ 30 #	n.a.	n.a.	RT-PCR (DSPP expression). NC: 1.0 =, PC: ≅ 2.2 #, G3: ≅ 1.2 =, G4: ≅ 1.8 #, G5: ≅ 0.5 =, G6: > 1.2 #, G7: 0 =, G8: ≅ 0.4 #	EDTA increased SCAPs survival and DSPP expression
Pang et al. 2013	Human dentin discs and DPSCs (n = 20)	Cell attachment, G1: 17% EDTA, control: no treatment; Cell differentiation/ mineralization, NC: proliferation medium, ND: untreated dentin +proliferation medium, ED: proliferation medium +EDTA-treated dentin, UED: proliferation medium + upper chamber EDTA-treated dentin, PC: differentiation	Attachment: dentin discs preparation, cells seeded on dentin slices, cultured for 3 d, analyses; Differentiation /mineralization: cells placed on dentin for 21 d, dentin slices replenished every 3d, analyses	n.a.	Attachment, cell density. G1: ≅ 2.4 # control: ≅ 1; RT-PCR (FN-1 expression). G1: ≅ 3.1 # control: ≅ 1; SEM analysis. G1: longer cytoplasmic processes with many granules compared to control	n.a.	RT-PCR. DMP-1. NC: 1 = ND: ≅ 1.2 = UED: ≅ 1 # ED: ≅ 3.1 # PC: ≅ 5.2; DSPP. PC: 1 = ND: 1 = UED: ≅ 1 # ED: 3 # PC: ≅ 4.3; ALP. NC: 1 = ND: 1 = UED: ≅ 1.3 # ED: ≅ 1.8 # PC: ≅ 3.2; OCN. NC: 1 = ND: 1 = UED: ≅ 1 = ED: ≅ 1.1 # PC: ≅ 2.7	EDTA-treated dentin promoted cell attachment and odontoblastic/osteo-blastic differentiation

		medium						
Huang et al. 2011	Human single-rooted premolars and HDPSCs (n = 5)	Control: 1 min 5 mL DW, G1: 1 min 5.25% NaOCl, G2: 1 min 17% EDTA, G3: 1 min MTAD	Root slices preparation, irrigating protocols (5 mL), DW rinse, cells onto the root canal, incubation for 72 h, samples dyed, analysis	n.a.	Attachment, fluorescence microscope (cell/field). Control: ≅ 1.6 = G1: ≅ 1.6 # G2: ≅ 6 # G3: 11.4	Fluorescence microscope. Control and G1: round shape cells, G2 and G3: spindle-shaped cells	n.a.	EDTA and MTAD significantly increased HDPC attachment with spindle-shaped cells
Ring et al. 2008	Human root canals, SHEDs and L929 (n = 30, experimental groups; n = 8, control)	G1: 6% NaOCl, G2: 6% NaOCl +15 s EDTA +6% NaOCl, G3: 6% NaOCl +5 min MTAD +15 s MTAD, G4: 2% CHX +15 s EDTA +2% CHX, G5: AquatineEC +15 s EDTA +AquatineEC, G6: MCJ +15 s EDTA +MCJ, G7: saline +DPSCs, G9: saline +15 s EDTA +saline +DPSCs, G10: saline +15 s EDTA +saline +L929 cells, control: saline without DPSCs	Teeth preparation, Protaper and ProFile instrumentation, irrigation, cells seed into root canals, samples cultured for 7 d, analyses at 2, 4 and 7 d	LDH assay (560 nm). G1: ≅ 0.19 = G2: 0.15 = G3: ≅ 0.08 = G4: ≅ 0.08 # G5: ≅ 0.025 = G6: > 0.025 = G7: 0.025 = G8: < 0.025 = G9: < 0.025	Attachment, SEM analysis (cell count). G1: ≅ 2.7 # G2: ≅ 3.2 # G3: 1.5 #, G4: ≅ 2.6 # G5: ≅ 5.7 # G6: ≅ 4.2 # G7: ≅ 5.4 = G8: ≅ 5 = G9: ≅ 5.5	SEM analysis. G1/G2/G3 and G4: round to oblong-shaped cells, G5/G6/G7/G8 and G9: oblong to flattened cells	n.a.	EDTA did not influence cytotoxicity and cell adherence

The simbol * indicates additional group per analysis; = indicates no significant differences between/among groups; # indicates significant differences between/among groups; ≅ indicates "approximately"; > indicates "greater than"; < indicates "less than".

n.a.: not applicable, n: number of specimens, G: group, s: seconds, min: minutes, h: hour, d: days, $mm^{2;}$ square millimeter, °C: degree Celsius, w: week, mg: milligram, nm: nanometre, mL: milliliter, µL: microliter, µg: micrograms, j/cm2: joules per square centimetre, NaOCl: sodium hypochlorite, EDTA: ethylenediaminetetraacetic acid, DPSCs: dental pulp stem cells, NBs: nanobubble water, PBS: sterile phosphate-buffered saline, WST-1: water soluble tetrazolium salts, CLSM: confocal laser scanning microscope, NC: negative control, PC: positive control, adMSCs: adipose-derived mesenchymal stem cells, DW: distilled water, CA: citric acid, IP6: phytic acid, PHA: phosphoric acid, SEM: scanning electron microscope, TEM: transmission electron microscopy, BAC: benzalkonium chloride, LDH: lactate dehydrogenase activity, PDT: photodynamic

therapy, SCAPs: stem cells of the apical papilla, a-MEM: alpha- minimum essential medium, MSCs: mesenchymal stem cells, DPCs: dental pulp cells, α-tubulin: alpha tubulin, SDF-1α: stromal cell–derived factor 1 α, siCXCR4: silencing CXCR4, CCK-8: Cell Counting Kit-8, HEDP: etidronic acid, FBS: fetal bovine serum, GaAlAs: Gallium-Aluminum-Arsenid, OW: ozonated water, SHEDs: stem cells from human exfoliated deciduous teeth, HDPSCs: human dental pulp stem cells, HUVEC: human umbilical vein endothelial cells, CHX: chlorhexidine, MDP: mouse dental papilla, RT-PCR: real-time polymerase chain reaction, APCs: apical papilla cells, NI: needle irrigation, NSS: normal saline solution, EA: EndoActivator, PUI: passive ultrassonic irrigation, USA: ultrasonic activation, CH: calcium hidroxide paste, PDL: human periodontal ligament, GM: growth culture medium, UMR-106: rat osteosarcoma cells, TAP: triple antibiotic paste (ciprofloxacin, metronidazole and minocycline), DTAP: diluted triple antibiotic paste, DAP: double antibiotic paste (ciprofloxacin and metronidazole), PD: predentin layer, C2C12: mouse embryonic muscle myoblast cells, CM: conditioned medium, ST: sodium thiosulfate, MTAD: mixture tetracycline citric acid and detergent, PA: polyacrylic acid, COL1A1: collagen type I, DSPP: dentin sialophosphoprotein, RUNX2: runt-related transcription factor 2, DMP: dentin matrix protein extracts, DMP-1: dentin matrix acidic phosphoprotein-1, ALP: alkaline phosphatase, OCN: osteocalcin, FN-1: fibronectin-1, HDPC: human dental pulp cell, MCJ: morinda citrifolia juice, AquatineEC: aquatine endodontic cleanser, SPP-1: secreted phosphoprotein 1, VEGF: vascular endothelial growth factor, FGF-b: fibroblast growth factors beta, MC: minimal concentration or zero, OPN: Osteopontin, DME: dentin matrix extract, IGF: insulin-like growth factor.

Table 4. The effects of EDTA on blood clot, tissue healing and regeneration of immature teeth models

Author	Experimental model (n)	Groups	Experimental protocol	Tissue inflammation	Tissues ingrowth	Increase in the root length / root thickness	Decrease in apical diameter	Mineralization/ differentiation	Bone or root resorption	Blood clot characterization	Outcomes
Taweewattanapaisan et al. 2019	Human mandibular premolars (n = 7), *in vitro*	G1: 5 min SS, G2: 1 min 17% EDTA + SS (E1N), G3: 5 min 17% EDTA + SS (E5N), G4: 1 min 17% EDTA (E1), G5: 5 min 17% EDTA (E5)	Roots preparation, irrigations, specimens split vertically in half, BC sample collection spread on specimens, analyses	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	SEM. G1 and G2: dense meshwork of fibrins with abundant biconcave erythrocytes, G3: meshwork of fibrins with biconcave and shrinkage erythrocytes, G4 and G5: < and shorter fibrins than G1 with inactivated platelets. Fibrin density. SEM (nº/10 µm). Coronal. G1: 11.60 =, G2: 8.99 =, G3: 8.34 =, G4: 0.22 #, G5: 0.17 #; Middle. G1: 12.38 =, G2: 9.51 =, G3: 9.13 =, G4: 0.22 #, G5: 0.23 #; Apical. G1: 13.33 =, G2: 7.69 =, G3: 11.14 =, G4: 0.23 #, G5: 0.30 #	1 and 5 min EDTA alone decreased BC formation; EDTA with SS final flushing had results similar to the group that used only SS.
El Ashry et al. 2016	Premolars of mongrel dogs (n = 48), *In vivo*	G1: BC, G2: BC + collagen, G3: BC + 2 min 17% EDTA, G4: BC + collagen + 17% EDTA, G5: BC + MTAD, G6: BC + collagen + MTAD, PC: exposed	PA lesion induction, disinfection with 2.6% NaOCl, intracanal dressing with TAP, coronal seal for 3 w, TAP removed by NaOCl, treatment protocols, evaluations	HE (scores). 2 w. G1: 2.1=, G2: 2.4=, G3: 2.3=, G4: 2.6=, G5: 1.4#, G6: 1.6#, PC: 2.7=, NC: 0#; 6 w. G1: 1.3=, G2: 1.6=, G3: 1.4=, G4: 1.7=, G5: 0.9=, G6: 1=, PC: 2.9#, NC: 0#; 12 w. G1: 0.4=, G2: 0.7=, G3:	HE (scores). 2 w. G1: 0.6 =, G2: 0.7 =, G3: 0.9 =, G4: 1 =, G5: 0.4 =, G6: 0.6 =, PC: 0.3 =, NC: NM; 6 w. G1: 1.3 =, G2: 1.4 =, G3: 1.9 #, G4: 2 #, G5: 1 =, G6: 1.1 =, PC: 0.9 =,	RGe (mm). Increase in root length (2 w. G1: 4.9 =, G2: 5.4 =, G3: 5.1 =, G4: 5.7 =, G5: 4.8 =, G6: 5.3 =, PC: 0 #, NC: 6.2 =; 6 w. G1: 13.9 =, G2: 14.6 =, G3: 14 =, G4: 14.8 =, G5: 13.7 =, G6: 14.5 =, PC: 0 #, NC: 15.5 =; 12 w.	RGe (mm). 2 w. G1: 2.9 =, G2: 3.6 =, G3: 3.1 =, G4: 3.8 =, G5: 2.7 =, G6: 3.5 =, PC: 0 #, NC: 6.2 #; 6 w. G1: 16.4 =, G2: 17.3 =, G3: 16.5 =, G4: 17.6 =, G5: 16.2 =, G6: 16.9 =, PC: 0 #,	HE (scores). 2 w. G1: 0.4 =, G2: 0.6 =, G3: 0.6 =, G4: 0.7 =, G5: 0.3 =, G6: 0.4 =, PC: 0 =, NC: NM; 6 w. G1: 0.7 =, G2: 0.9 =, G3: 0.9 =, G4: 1 =, G5: 0.6 =, G6: 0.7 =, PC: 0 =, NC: NM; 12 w. G1: 1.3 =, G2: 1.4 =, G3: 1.4 =, G4: 1.6 =, G5: 1.1	HE (%). 2 w. G1: 75 =, G2: 83.3 #, G3: 75 =, G4: 83.3 #, G5: 75 =, G6: 83.3 #, PC: 83.3 #, NC: 0 #; 6 w. G1: 41.6 =, G2: 58.3 #, G3: 41.6 =, G4: 58.3 #, G5: 41.6 =, G6: 58.3 #, PC: 100 #, NC:	n.a.	The use of EDTA no influenced the inflammation, the root length or thickness, apical diameter, mineralization and bone resorption; EDTA allowed significant tissue ingrowth in the pulp space at 6

		teeth, NC: untouched teeth	at 2, 6 and 12 w	0.6=, G4: 0.9=, G5: 0.5=, G6: 0.6=, PC: 3#, NC: 0#; (cell count) 2 w. G1: 25=, G2: 25.8=, G3: 25.5=, G4: 26=, G5: 20#, G6: 20.9#, PC: 28.4=, NC: 2.5#; 6 w. G1: 16.4=, G2: 16.9=, G3: 16.6=, G4: 17.1=, G5: 14.9=, G6: 15.6=, PC: 36.7#, NC: 2.3#; 12 w. G1: 10.2=, G2: 10.7=, G3: 10.4=, G4: 10.9=, G5: 9.2=, G6: 9.7=, PC: 38.9#, NC: 2.1#	NC: NM; 12 w. G1: 1.9 =, G2: 2 =, G3: 2.6 #, G4: 2.7 #, G5: 1.6 =, G6: 1.7 #, PC: 0.9 #, NC: NM	G1: 16.3 =, G2: 17.1 =, G3: 16.5 =, G4: 17.7 =, G5: 16.1 =, G6: 16.9 =, PC: 0 ± 0 #, NC: 19.7 =); Increase in root thickness (2 w. G1: 4 =, G2: 4.8 =, G3: 4.7 =, G4: 5.3 =, G5: 3.9 =, G6: 4.5 =, PC: 0 #, NC: 6.1 =; 6 w. G1: 11.4 =, G2: 12.1 =, G3: 11.6 =, G4: 12.3 =, G5: 11.2 =, G6: 11.7 =, PC: 0 #, NC: 12.4 =; 12 w. G1: 13.4 =, G2: 14.2 =, G3: 13.6 =, G4: 14.4 =, G5: 12.7 =, G6: 13.8 =, PC: 0 #, NC: 15.8 =)	NC: 20.5 #; 12 w. G1: 30.2 =, G2: 31.9 =, G3: 30.8 =, G4: 32.2 =, G5: 29.8 =, G6: 31.5 =, PC: 0 #, NC: 47 #; HE (%). 2 w. G1: 0 =, G2: 0 =, G3: 0 =, G4: 0 =, G5: 0 =, G6: 0 =, PC: 0 =, NC: 0 =; 6 w. G1: 33.3 =, G2: 41.7 =, G3: 33.3 =, G4: 41.7 =, G5: 33.3 =, G6: 41.7 =, PC: 0 =, NC: 50 =; 12 w. G1: 50 =, G2: 58.3 =, G3: 50 =, G4: 58.3 =, G5: 50 =, G6: 58.3 =, PC: 0 =, NC: 58.3 =	=, G6: 1.3 =, PC: 0 =, NC: NM	0 #; 12 w. G1: 16.6 =, G2: 25 #, G3: 16.6 =, G4: 25 #, G5: 16.6 =, G6: 25 #, PC: 100 #, NC: 0 #		and 12 w.
Kawamura et al. 2016	Porcine roots, immu-nodefi-ciency	P1. Regenera-tive / angiogenes is area, cell	P1 and P2. Cell culture, preparation of CM		P1. Regenerati ve area, (%). G1: ≅80 # G2:			P1. Hoechst 33342+ cells (FLC/mm^2). G1: ≅ 3.000 #, G2: ≅ 2.500		P1 (Cell with collagen). EDTA had less regenerative/

	mice and MDPSC (n = 4), *In vivo*	differentia-tion. G1: nonextrac-ted tooth, G2: HCl-extracted tooth, G3: GdnHCl-extracted tooth, G4: EDTA-extracted tooth. P2. ENM+ cells, PLAP-1, and angiogenic potential. G5: nonextrac-ted, G6: EDTA extracts, G7: CM, G8: CM + EDTA extracts, G9: GdnHCl, G10: CM + GdnHCl, NC: autoclaved teeth	(P2), roots preparation, teeth demineralized with the solutions for 7 d, washing with PBS, MDPSCs were injected into the teeth with collagen (P1) and with CM or not (P2), teeth sealed, subcuta-neous implanta-tion, analyses at 28 d		70 = G3: ≅ 63 # G4: ≅ 7; RECA1 IMS (%) G1: ≅ 16 # G2: 10 = G3: 10 # G4: ≅ 3; PLAP-1 (%) G1: 10 = G2: 13 # G3: ≅ 17 # G4: ≅ 58; P2. PLAP-1 (%) G5: ≅ 12 # autocl.: MC = G6: MC # G7: > 5 = G8: >6 # G9: MC # G10: > 5 =; IHC, PLAP-1 (%) G5: ≅ 15#autocl.: ≅ 1 = G6: ≅ 1 # G7: ≅ 25 # G8: ≅ 16			=, G3: ≅ 2.000 #, G4: > 1.750 #; ODD (ENM+ cells). G1: > 120 =, G2: ≅ 120 =, G3: ≅ 120 =, G4: ≅ 20 #; P2. ODD (ENM+ cells). G5: ≅ 140 = G8: < 140 # G7: > 100 # G6: MC = autocl.: MC.			angiogenesis area and cells; fewer ODD and endothelial differentiation. However, it had more periodontal ligament cells. P2 (cells with CM or not). EDTA did not influence periodontal ligament cells. EDTA allowed higher ODD and endothelial differentiation when associated with CM.
Yamauchi et al. 2011	Double-rooted canine premo-lars (n =	G1: BC, G2: BC + CCS, G3: BC + 2 min 17% EDTA,	PA lesion induction, 2.5% NaOCl irrigation, TAP, IRM	n.a.	n.a.	RGe (%). G1: 64.2 =, G2: 87.5 #, G3: 54.2 =, G4: 83.3 #	HE. G2 and G4: most specimens had apical closure,	HMM (%). DAMT. G1: ≅ 5=, G2: > 10=, G3: > 2.5 =, G4: > 12.5 =; bony islands.	Periapi-cal radiolu-cencies impro-vement. RGe (%).	n.a.	EDTA did not influence the root thickening and periapical radiolucencies

12), *In vivo*	G4: BC + CCS + EDTA, PC: infected only, NC: untreated	for 2 w; 2.5% NaOCl and saline irrigations, protocols, BC inducted, sealing, 3,5 months, analysis		some showed no complete apical closure and forming bony islands	G1: > 5 =, G2: ≅ 14=, G3: 5 =, G4: > 17=; total. G1: > 10 =, G2: ≅ 25#, G3: >7.5=, G4: ≅ 32 #	G1: 56.52 =, G2: 79.2 #, G3: 58.3 =, G4: 87.5#	; EDTA allowed higher apical closure and mineralization .

The symbol # indicates significant differences between/among groups; ≅ indicates “approximately”; > indicates “greater than”; < indicates “less than”.
n.a.: not applicable, G: group, PC: positive control, NC: negative control, n: number of specimens, P1: part one, P2: part two, w: week, d: days, min: minutes, mm: millimeter, µm: micrometer, °C: degree Celsius, EDTA: ethylenediaminetetraacetic acid, NaOCl: sodium hypochlorite, NSS: normal saline solution, RGe: radiographic evaluation, FLC: fluorescence, ODD: odontoblastic differentiation, PA: periapical, BC: blood clot, SEM: scanning electron microscope, HMM: histomorphometry, IHC: immunohistochemistry, HE: hematoxylin and eosin, TAP: triple antibiotic paste (ciprofloxacin, metronidazole and minocycline), MTAD: mixture tetracycline citric acid and detergent, CCS: cross-linked type I collagen scaffold, IRM: intermediate restorative material, DAMT: dentin-associated mineralized tissue, IMS: immunostaining, MC: minimal concentration or zero, NM: not mentioned, MDPSCs: mobilized dental pulp stem cells, ENM: enamelysin, PLAP-1: periodontal ligament-associated protein 1, GdnHCl: guanidine hydrochloride, HCl: hydrochloric acid, PBS: sterile phosphate-buffered saline, CM: conditioned medium.

Risk-of-bias assessment within in vitro and animal studies

Risk-of-bias analyses are presented in Appendix Table 5 and Figure 2. Regarding the critical appraisal of *in vitro* studies (Appendix Table 5, Figure 2A), a high risk of bias was observed only for the absence of sample randomization and justification of the sample size. All studies presented a possibility of comparison between the control and treatment groups at entry, and a reliable outcome assessment tool. A low risk of bias was found in clearly stated aim, baseline equivalence among the groups, clear conditioning protocols, measurement standardization, and appropriate statistical approach. Figure 2B summarizes the risk of bias of animal studies by using the SYRCLE tool. Information to judge most domains was frequently missing. A low risk of bias was observed for incomplete outcome data, selective outcome reporting, and the presence of other sources of bias. Overall, the eligible animal studies showed a high risk of bias.

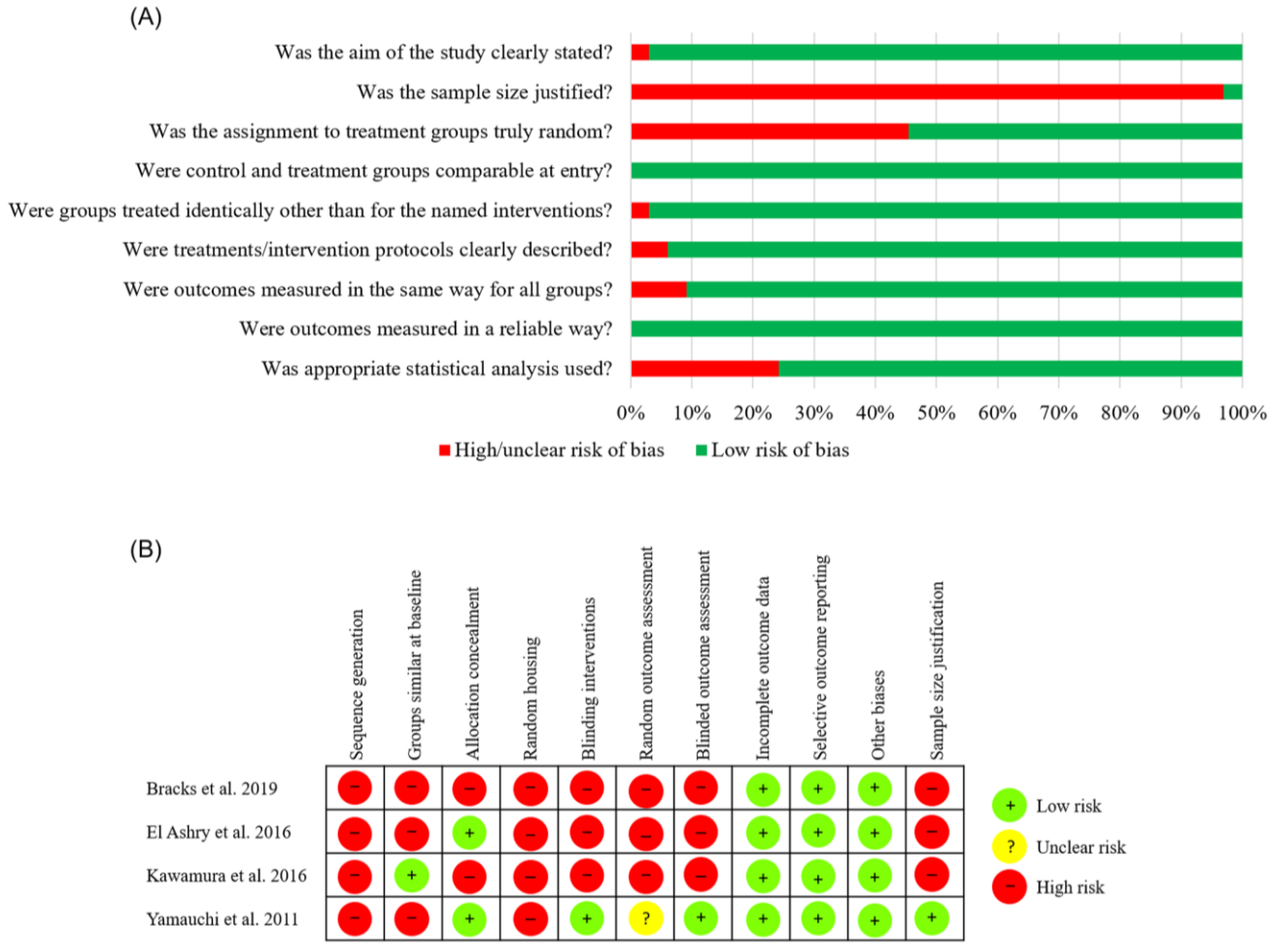

Figure 2. Risk of bias assessment in the included *in vitro* studies according to the percentage of the scores attributed to each evaluated study and risk of bias of the selected animal studies (SYRCLE's RoB tool for assessing risk of bias).

Appendix Table 5 Quality assessment of included in vitro studies

Quality criteria	Was the aim of the study clearly stated?	Was the sample size justified?	Was the assignment to treatment groups truly random?	Were control and treatment groups comparable at entry?	Were groups treated identically other than for the named interventions?	Were treatments/intervention protocols clearly described?	Were outcomes measured in the same way for all groups?	Were outcomes measured in a reliable way?	Was appropriate statistical analysis used?	Total score
Aksel et al. 2020	1	0	1	1	1	1	1	1	1	8
Atesci et al. 2020	1	0	1	1	1	1	1	1	0	7
Kucukkaya Eren et al. 2020	1	0	1	1	1	1	1	1	1	8
Li et al. 2020	1	0	1	1	1	1	1	1	1	8
Cameron et al. 2019	1	0	1	1	1	1	1	1	1	8
Ivica et al. 2019	1	0	1	1	1	1	1	1	0	7
Liu et al. 2019	1	0	0	1	1	1	1	1	1	7
Deniz Sungur et al. 2019	1	0	0	1	1	1	1	1	1	7
Taweewattanapaisan et al. 2019	1	0	1	1	1	1	1	1	1	8
Tunç et al. 2019	1	0	0	1	1	1	1	1	1	7
Widbiller et al. 2019	1	0	0	1	1	1	1	1	1	7
Chae et al. 2018	1	1	1	1	1	1	1	1	1	9
Hashimoto et al. 2018	1	0	0	1	1	1	1	1	1	7
Prompreecha et al. 2018	1	0	1	1	1	1	1	1	1	8
Ranc et al. 2018	1	0	1	1	1	1	1	1	0	7
Scott et al. 2018	1	0	0	1	1	1	0	1	1	6
Alghilan et al. 2017	1	0	1	1	1	1	1	1	1	8
Duncan et al. 2017	1	0	0	1	1	1	1	1	1	7
Widbiller et al. 2017	1	0	0	1	0	1	0	1	0	4
Gonçalves et al. 2016	1	0	0	1	1	1	1	1	1	7
Kawamura et al. 2016	1	0	0	1	1	0	0	1	0	4
Mollashahi et al. 2016	0	0	1	1	1	0	1	1	1	6
Sadaghiani et al. 2016	1	0	0	1	1	1	1	1	1	7
Shrestha et al. 2016	1	0	0	1	1	1	1	1	1	7
Zeng et al. 2016	1	0	1	1	1	1	1	1	1	8
Galler et al. 2015a	1	0	0	1	1	1	1	1	0	6
Galler et al. 2015b	1	0	0	1	1	1	1	1	0	6
Kim et al. 2015	1	0	1	1	1	1	1	1	1	8
Park et al. 2015	1	0	1	1	1	1	1	1	0	7
Martin et al. 2014	1	0	0	1	1	1	1	1	1	7
Pang et al. 2013	1	0	1	1	1	1	1	1	1	8
Huang et al. 2011	1	0	1	1	1	1	1	1	1	8
Ring et al. 2008	1	0	1	1	1	1	1	1	1	8

0: not reported or reported but inadequate, 1: reported and adequate

Discussion

This systematic review primarily evaluated the effects of irrigation with EDTA on the release of growth factors in REP; also analyzed the influence of EDTA on stem cell behavior, and in tissue regeneration with data from 36 included *in vitro* and *in vivo* studies. Only studies evaluating the effects of EDTA on biological factors of the dentin or pulp/periapical tissues, and on cell behavior compared to at least one control group, were eligible. We found that EDTA-treated dentin, with different concentrations, effectively released TGF-β, in addition to improving cell morphology, migration, adherence, and differentiation. Meta-analysis was not performed due to wide variations in methods for assessment, irrigation protocols, and different concentrations and time of exposure of EDTA.

REP is currently considered one of the most favorable treatment for immature permanent teeth with pulp necrosis (Ulusoy et al. 2019; Taweewattanapaisan et al. 2019), promoting root development and apical closure (Deniz Sungur et al. 2019; Ulusoy et al. 2019). The European Society of Endodontology (Galler et al. 2016) and the American Association of Endodontists' clinical guidelines (2018) have recommended the use of 17% EDTA after NaOCl for REP to optimize cell viability and differentiation and to enhance the release of growth factors from the dentin matrix (Chae et al. 2018; Kim et al. 2018).

NaOCl was noted to be the most common irrigation solution after EDTA in this systematic review, probably due to its organic solvent potential and antimicrobial effectiveness (Galler et al. 2011; Gonçalves et al. 2016). The presence of a sterile environment plays a crucial role in the success of REP, since there is an impact on the chemotaxis of mesenchymal stem cells, and consequently, on the mineralized tissue neoformation (Verma et al. 2016). Nevertheless, NaOCl is known for showing negative effects on stem cells' survival (Trevino et al. 2011; Martin et al. 2014), in addition to being a potential irritant for periapical tissues, especially at high concentrations (Gonçalves et al. 2016). Therefore, additional conditioning with EDTA may neutralize the cytotoxicity provoked by NaOCl, enhancing cellular spreading and the liberation of bioactive molecules from the conditioned dentin (Galler et al. 2011; Chae et al. 2018; Aksel et al. 2020).

The expression of signaling molecules following dentin demineralization might modulate cellular activity from the periapical area (Gonçalves et al. 2016; Taweewattanapaisan et al. 2019), playing a crucial role in the intracanal tissues neoformation (Bracks et al. 2019). A total of 16 evaluated the release of endogenous growth factors from dentin using different irrigating protocols with dentin discs. The most assessed protein was TGF-β. This growth factor performs well as a substantial chemoattractant/stimulant of the

activation of stem cells (Gonçalves et al. 2016). In addition, TGF-β shows the ability to induce odontoblastic differentiation and to contribute to dentinogenesis (Chae et al. 2018; Kucukkaya Eren et al. 2021).

Most articles demonstrated an effective release of TGF-β1 after irrigation with 10-17% EDTA. Widbiller et al. (2017) found an increase in TGF-β1 liberation using only ultrasonic activation, which might be associated with an improved superficial erosion in the dentin, dissolution of the smear layer, and other debris removal, thus exposing growth factors entrapped on the dentine surface. A significant release of TGF-β1 in EDTA-treated dentin was also reported in two studies (Cameron et al. 2019; Atesci et al. 2020) under sterile condition or when associated with adMSCs. These findings support the importance of other pillars of tissue engineering for clinical success of REP, such as the presence of a sterile environment and stem cells in the root canal.

Angiogenesis occurs especially during the early stages of wound healing (Liao et al. 2011). Among the studies that investigated VEGF release, a molecule that supports the angiogenic activity (Wibdiller et al. 2017), most reports found no influence on the irrigation with EDTA (Bracks et al. 2019; Atesci et al. 2020; Li et al. 2020). Moreover, the experiment of Sadaghiani et al. (2016) using ELISA assay found no significant effects of EDTA on this protein. The reason for these findings might be explained by the longer period of analyses, which included the very short half-life of VEGF and its basal levels in dentin (Atesci et al. 2020). On the other hand, when adMSCs were added, EDTA effectively released this protein; the presence of these cells, which were not receiving sufficient oxygen, might have also induced VEGF production (Bracks et al. 2019).

Whereas TGF-β may have immunosuppressive effects against the production of pro-inflammatory cytokines (Maciel et al. 2012), EDTA did not influence tissue inflammation (El Ashry et al. 2016). In other analysis, one study found a high expression of IL-1 in the EDTA group using real-time PCR (Bracks et al. 2019). These controversial results may have taken place due to differences in the methods of analysis, since the presence of pro-inflammatory cytokine does not exactly depend on the presence of inflammatory cells (Benetti et al. 2018). Moreover, inflamed areas are associated with an increased vascularity (Liao et al. 2011), especially during the initial phases of healing. Furthermore, the interaction between stem cells in inflamed tissues and their potential to control the inflammatory reaction that promotes tissue healing is less understood (Liao et al. 2011). Taweewattanapaisan et al. (2019) reinforced the importance of a minimal inflammatory reaction for an ideal scaffold for stem cell homing.

In this systematic review, significant differences between the means values of EDTA and the control group were considered, but the comparison between these two groups was not always available (Sadaghiani et al. 2016). This may, however, have reduced the chance of achieving significant differences between groups. Moreover, additional research to produce stronger evidence on the influence of EDTA in the liberation of other bioactive peptides is either desirable and encouraged.

The EDTA did not show superiority in the analysis of cell survival. Differences regarding stem cells' lineages, periods of analysis, solutions, and methods of assessment among the studies may have impacted the results. Overall, most studies showed a positive impact of the use of EDTA on cell migration, attachment, and differentiation. For the cell morphology, irrigation with EDTA was associated with the presence of an oblong and fibroblastic-like appearance with flattened morphology. EDTA treatment is capable of exposing organic components in the superficial dentin layer, such as collagen and glycosaminoglycans, which play a crucial role in cell attachment (Oyarzun et al. 2002; Hashimoto et al. 2018). Moreover, this systematic review found an increased amount of TGF-β released from EDTA-treated dentin, being TGF-β a potent chemoattractant that promotes cell migration into dentin and cell differentiation when in contact with dentinal (Galler et al. 2015; 2016; Gonçalves *et al.* 2016; Chae et al. 2018; Hashimoto et al. 2018).

The few *in vivo* studies included have evaluated blood clot and tissue regeneration using, on their most part, 17% EDTA, as proposed by current clinical protocols (Galler et al. 2016; AAE 2018). This assortment of protocols made it difficult to systematically discuss these data. In most studies, the use of EDTA influenced the majority of the included parameters related to tissue healing or regeneration. Additionally, the animal studies showed a high risk of bias, mostly involving randomization and blinding performances. These limitations need to be considered when interpreting such an evidence. Thus, additional well-designed histological analyses and clear descriptions using immature teeth models and optimal conditions of EDTA treatment for REP are warranted.

Although most *in vitro* studies in the current systematic review had high-quality evidence, some issues, such as the absence of adequate randomization and no justification of sample size, were reported. In addition, *in vitro* and animal studies have the limitation of transferability of findings to the clinical environment. Conversely, these findings provide evidence that EDTA irrigation induces positive clinical effects during REP. Future well-designed histologic analyses and randomized clinical trials comparing effectiveness

between/among EDTA and other irrigation agents for immature permanent teeth with pulp necrosis are needed to address these limitations and to provide a strong level of evidence.

In summary, this systematic review found a significant liberation of TGF-β1 from dentin, and the presence of flattened fibroblastic-like cells after irrigation with different concentrations of EDTA at periods ranging from 1-10 min of exposure; in addition, EDTA also enhanced cell migration, attachment and differentiation.

Author contributions

AH. Reis Prado, contributed to methodology, study selection, data collection, quality assessment, writing (original draft); RR. Fagundes, contributed to study selection, data collection, writing (original draft); SC. Oliveira, contributed to methodology, quality assessment, writing (original draft); LG. Abreu, contributed to methodology, quality assessment, supervision, validation, visualization; MC. Bottino, contributed to conceptualization, project administration, validation, writing (review/editing); A. Ribeiro-Sobrinho, contributed to conceptualization, project administration, resources, supervision, validation, writing (review/editing); F. Benetti, contributed to conceptualization, methodology, study selection, data collection, project administration, resources, supervision, writing (review/editing). All authors gave their final approval and agree to be accountable for all aspects of the work.

Acknowledgments

This research was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – 88887.489995/2020-00. The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Supplementary Material

A supplemental appendix to this article is available online.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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7 CONSIDERAÇÕES FINAIS

O sucesso dos procedimentos endodônticos regenerativos está atrelado aos princípios da engenheira tecidual, como a migração e diferenciação de CTMs; o uso de um *scaffold* que atue como uma matriz de suporte para a organização tecidual; fatores de crescimento que auxiliem na migração, proliferação e diferenciação celular; e um ambiente estéril livre de focos de infecção.

A solução irrigante de escolha pode apresentar um efeito potencializador na liberação de fatores importantes para a regeneração do complexo dentino-pulpar e, consequentemente, para o sucesso final do tratamento. A adição do EDTA 17% após a irrigação com o NaOCl, conforme proposto pelos protocolos e considerações clínicas recentes, pode ser uma alternativa para minimizar os efeitos citotóxicos do NaOCl nas células-troncos, e aumentar a liberação de moléculas bioativas da dentina, devido sua ação quelante.

Os resultados do estudo *in vivo* presente neste trabalho nos permitem concluir que: a) o uso do EDTA 17% resultou em uma maior neoformação tecidual no interior dos canais radiculares de dentes imaturos de ratos submetidos à revascularização pulpar; ainda, este procedimento, independentemente do uso do EDTA, promoveu um aumento no crescimento e espessura das paredes dos canais radiculares, observados pela neoformação cementária presente, indução ao fechamento apical e presença de um infiltrado inflamatório localizado principalmente até o terço médio dos canais mesiais; EDTA não influenciou na maturação colágena presente na região periapical e apical do canal radicular; b) irrigação adicional com EDTA promoveu uma maior imunomarcação de TGF-β1 na região apical; contudo o EDTA não influenciou na imunomarcação de FGF-2. Já na revisão sistemática foi observado que a irrigação com diferentes concentrações de EDTA, em períodos de exposição variando de um a 10 minutos, é capaz de promover uma liberação significativa de TGF-β1 da dentina, com a presença de células semelhantes a fibroblastos achatados; além de aumentar a migração, fixação e diferenciação celular.

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ANEXOS

ANEXO A – Parecer da Comissão de Ética no Uso de Animais

UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO
Certificamos que o projeto intitulado "Análise da influência de dois diferentes protocolos de antissepsia na revascularização pulpar de molares de ratos", protocolo do CEUA: 81/2020 sob a responsabilidade de Francine Benetti que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899 de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE MINAS GERAIS, em reunião de 22/06/2020.

Vigência da Autorização	22/06/2020 a 21/06/2025
Finalidade	Pesquisa
***Espécie/linhagem**	Rato heterogênico / Rattus albinus, Wistar
Nº de animais	6
Peso/Idade	80g / 4(semanas)
Sexo	masculino
Origem	Centro de Bioterismo (CEBIO) da UFMG
***Espécie/linhagem**	Rato heterogênico / Rattus albinus, Wistar
Nº de animais	6
Peso/Idade	80g / 4(semanas)
Sexo	masculino
Origem	Centro de Bioterismo (CEBIO) da UFMG
***Espécie/linhagem**	Rato heterogênico / Rattus albinus, Wistar
Nº de animais	6
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***Espécie/linhagem**	Rato heterogênico / Rattus albinus, Wistar
Nº de animais	6
Peso/Idade	80g / 4(semanas)
Sexo	masculino
Origem	Centro de Bioterismo (CEBIO) da UFMG

Considerações posteriores:

22/06/2020	Aprovado na reunião “on line” do dia 22/06/2020. Validade: 22/06/2020 à 21/06/2025.

Belo Horizonte, 24/04/2021.

Atenciosamente,

Sistema Solicite CEUA UFMG
https://aplicativos.ufmg.br/solicite_ceua/

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ANEXO B – Protocolo da revisão aprovado no PROSPERO (CRD42020205417)

NIHR | National Institute for Health Research

PROSPERO
International prospective register of systematic reviews

Citation

Alexandre Henrique dos Reis Prado, Lucas Guimarães Abreu, Rogéria Fagundes, Sabrina de Castro Oliveira, Antônio Paulino Ribeiro-Sobrinho, Francine Benetti. Influence of EDTA on factors that may interfere with regenerative endodontic procedures: a systematic review. PROSPERO 2020 CRD42020205417 Available from: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020205417

Review question

Does EDTA influence biological factors of dentin, cells or tissues in regenerative endodontic procedures?

Searches

Eletronic searches will be conducted within the following databases from their date of inception until August 2020:
• PubMed/MEDLINE
• Scopus

• Cochrane Library

• Web of Science Core Collection (Web of Science)

• Embase

The review team will hand-search the reference list of the articles included in the systematic review and in literature reviews identified during the searches. Also, grey literature will be searched (OpenGrey). No restrictions based on the language and date of the publication will be considered.

Types of study to be included

Inclusion criteria:

1. Studies in vivo on animal or human that evaluated the effects of EDTA on the biological factors of the dentin or pulp/periapical tissues in regenerative endodontic procedures;

2. Studies in vitro that evaluated the effects of EDTA on cell viability, migration, attachment or morphology;

3. Studies in vitro that evaluated the effects of EDTA on biological factors from dentin.

Exclusion criteria:

1. Studies that evaluated only the effects of EDTA on dentin, cells or pulp/periapical tissues without control group;

2. Studies that evaluated only other irrigating solutions without EDTA group;

3. Studies for which the full text was unavailable.

Condition or domain being studied

Regenerative endodontics is based on principles of tissue engineering, including scaffolds, stem cells and / or growth factors. The complete disinfection of the root canal system, and an adequate dentin surface for cell proliferation and attachment is crucial for promising results (Verma et al. 2016, Hashioto et al. 2017). Although there is no standardized protocol available, sodium hypochlorite (NaOCl) is the main irrigating solution used in the regenerative endodontic treatment. Ethylenediaminetetraacetic acid (EDTA) is another

option due to its chelating action, and ability to release growth factors and other bioactive molecules from the dentin matrix. A few studies with unclear results about its influence on growth-factors release and proliferation and migration of dental stem-cells during the pulp revascularization are observed. Furthermore, according to a scientometric and bibliometric analysis, it is important to access the immunoinflammatory and morphological effects involved in the healing and tissue regeneration process in association with the disinfection protocols used in the treatment (Shamszadeh et al. 2019).

Participants/population
Dentin, cells or pulp/periapical tissues of humans or animals that had been submitted to irrigation/conditioning with EDTA.

Intervention(s), exposure(s)
The intervention includes irrigation/conditioning with EDTA.

Comparator(s)/control
The comparator includes irrigation/conditioning with other solutions, without EDTA.

Main outcome(s)
Release of growth-factors from the dentin-matrix.

Measures of effect
For continuous outcomes, mean difference (MD), or standardised mean difference (SMD) will be used. On the other hand, for dichotomous data, odds ratio (OR) or probability/relative risk (RR) will be considered.

Additional outcome(s)
Cell attachment, cell differentiation, cell viability, cell morphology, dental pulp stem cells (DPSCs) expression, protein immunolabeling/expression, mineralization, tissue inflammation, tissues in-growth, increase in the root length/root thickness, decrease in apical diameter.

Measures of effect
For continuous outcomes, mean difference (MD), or standardised mean difference (SMD) will be used. On the other hand, for dichotomous data, odds ratio (OR) or probability/relative risk (RR) will be considered.

Data extraction (selection and coding)
Selection of the studies

The downloaded set of records from each database will be imported to an Excel spreadsheet. Duplicate records will be identified and will be removed. Two independent authors will scan all the records in a two-step process. In Step 1, both authors will scan the titles and abstracts of the studies retrieved from the research. Both the authors will be blinded to each other's decisions. In Step 2, full text of the remaining records will be obtained for further evaluation. Studies in which the full text fulfilled the eligibility criteria were also included. Any disagreement between the authors will be resolved by discussion, and when necessary, a third autor will be consulted.

Data Extraction

Two authors will undertake data collection independently and in duplicate for all studies meeting the inclusion criteria using a piloted data extraction form in an excel spreadsheet. One author will extract the following data from the included studies: first authors' last name, year of publication, study design, experimental model, sample size, groups, experimental protocol and analyzes. Subsequently, a second author will review data collected by the previous author. Any disagreement between the authors will be resolved by discussion with the third author.

Risk of bias (quality) assessment
Some characteristics of the elegible studies will be assessed, such as clear aim, justification of the sample size, sample randomization, blind treatment allocation, possibility of comparison between control and treatment groups, clear treatment protocol, measurement standartization, method treatment, and adequate

statistical analysis.

Two reviewers will independently evaluate the quality of the studies and disagreement will be solved by discussion with third author. Quality assessment of the included studies will be done using standard checklists. The methodological quality of the selected studies will be assessed based on the levels of evidence as proposed by the Joanna Briggs Institute Clinical Appraisal Checklist for Experimental Studies, and for Randomized Controlled Trials. Also, a RoB tool for animal intervention studies (SYRCLE's RoB tool) will be used. A judgement of 'high' will indicate a high risk of bias, 'low' indicate low risk of bias, and 'unclear' indicate either a lack of information or uncertainty over the potential for bias. Studies will be categorised as being at low, high, or unclear risk of bias.

Strategy for data synthesis

A qualitative synthesis of the data will be perfomed based on the primary and secondary outcomes, using tables. We anticipate that quantitative synthesis (meta-analyses) may be possible. For continuous outcomes, mean difference (MD), or standardised mean difference (SMD) will be used to assess the outcomes. On the other hand, for dichotomous data, odds ratio (OR) or probability/relative risk (RR) will be considered. Heterogeneity among the studies will be assessed by examining the characteristics of the studies/study design, similarity among the experimental models, interventions and the analyses. Meta?analysis will be restricted to studies of similar comparisons that reported the same outcomes. The presence of heterogeneity will be statistically confirmed using a $?^2$ test, where a p value <0.05 will indicate significant heterogeneity (Higgins 2011).

Analysis of subgroups or subsets

Subgroup analyses will be performed depending on the type of study: study with humans / study with animals. It is important to look separately due to the possibility to find both types of studies during electronic search. Moreover, biological specificities and limitations of each methodology may impact those results.

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Professor Francine Benetti. Restorative Dentistry, Universidade Federal de Minas Gerais (UFMG), School of Dentistry, Belo Horizonte, MG, Brazil

Type and method of review

Intervention, Systematic review

Anticipated or actual start date

10 August 2020

Anticipated completion date

10 October 2021

Funding sources/sponsors

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

Grant number(s)

State the funder, grant or award number and the date of award

88887.489995/2020-00

Conflicts of interest
Not applicable.
None known

Language
English

Country
Brazil

Stage of review
Review Ongoing

Subject index terms status
Subject indexing assigned by CRD

Subject index terms
MeSH headings have not been applied to this record

Date of registration in PROSPERO
09 October 2020

Date of first submission
08 September 2020

Stage of review at time of this submission

Stage	Started	Completed
Preliminary searches	Yes	No
Piloting of the study selection process	Yes	No
Formal screening of search results against eligibility criteria	Yes	No
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

The record owner confirms that the information they have supplied for this submission is accurate and complete and they understand that deliberate provision of inaccurate information or omission of data may be construed as scientific misconduct.
The record owner confirms that they will update the status of the review when it is completed and will add publication details in due course.

Versions
09 October 2020