DANIELA ALVIM CHRISOSTOMO

Estudo *in vitro* de técnicas de instrumentação mecanizada e medicações intracanais como propostas de protocolos para pulpectomia em dentes decíduos

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Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, como parte dos requisitos para obtenção do título de Doutora em Ciências, área de concentração Saúde Bucal da Criança.

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“Não sabendo que era impossível, foi lá e fez.”

Jean Cocteau

CHRISOSTOMO, DA. **Estudo *in vitro* de técnicas de instrumentação mecanizada e medicações intracanais como propostas de protocolos para pulpectomia em dentes decíduos.** 2023. 50 f. Tese (Doutorado) – Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2023.

RESUMO GERAL

Esta tese foi dividida em 2 artigos científicos. O artigo 1 teve como objetivo comparar dois protocolos de instrumentação endodôntica com limas rotatórias ou com limas reciprocantes, realizados em protótipos de molares decíduos por meio de microtomografia computadorizada (micro-CT); e o artigo 2 teve como objetivo avaliar o efeito de duas propostas de medicações intracanais: combinação triantibiótica (TRI) de metronidazol, ciprofloxacina e fosfomicina (FOSFO), e a associação de epigalocatequina-3-galato (EGCG) com FOSFO, em comparação com os controles pasta de hidróxido de cálcio (CH) ou clorexidina (CHX), todos dissolvidos em polietilenoglicol-400 (PEG) em biofilmes multiespécies, toxicidade em fibroblastos e atividades colagenolíticas e gelatinolíticas detectadas na dentina radicular. Para a metodologia do artigo 1 foram utilizados 24 protótipos impressos em 3D de molares decíduos artificiais que foram distribuídos em dois grupos de 12 cada, de acordo com o tipo de instrumentação: com limas rotatórias Sequence Baby File NiTi© (SBF) ou limas reciprocantes com limas X1-Blue File NiTi© (XBF). Foram avaliados os parâmetros: volumes do canal radicular e da dentina, transporte e centralização do canal, risco de perfuração radicular e tempo de instrumentação. Para a metodologia do artigo 2, o efeito antibiofilme e a citotoxicidade de medicamentos contendo combinação de antibióticos, EGCG+FOSFO ou CH foram avaliados em biofilmes multiespécies formados em amostras de dentina radicular por microscopia confocal e em células de fibroblastos por ensaios de resazurina, respectivamente. A inibição da atividade das proteases por medicamentos foi medida por ensaios de atividade enzimática colagenolítica (ELISA) e atividade gelatinolítica por metaloproteinases - MMP com zimografia *in situ* em espécimes de dentina radicular. Os resultados do artigo 1 mostraram que o SBF promoveu menor alargamento do canal radicular e consequentemente removeu menos dentina quando comparado ao sistema reciprocante. De modo geral, ambos os protocolos de instrumentação não apresentaram diferença estatística entre si considerando o transporte do canal radicular e a capacidade de centralização. Não houve diferença na espessura da

dentina, fraturas e fissuras comparando os momentos pré e pós-operatórios para ambos os sistemas endodônticos. O tempo de instrumentação foi menor para XBF. Os resultados do artigo 2 mostraram que EGCG+FOSFO, combinação de antibióticos e CH em PEG 400 reduziram significativamente as bactérias dos biofilmes multiespécies nos túbulos dentinários e em concentrações mais baixas não foram tóxicos para os fibroblastos. Todos os medicamentos apresentaram atividade anticolagenolítica em comparação ao PEG e à água. PEG+CH e PEG+CHX diminuíram a atividade gelatinolítica *in situ* da MMP quando comparado ao controle sem tratamento. PEG+TRI aumentou a atividade gelatinolítica na dentina radicular. Concluindo, ambos os protocolos de instrumentação endodôntica foram seguros para serem utilizados em pulpectomia de dentes decíduos, pois promoveram mínima redução do volume dentinário, transporte e descentralização do canal, e consequentemente amenizaram alterações na espessura dentinária e as possibilidades de fraturas. Embora o tempo de instrumentação tenha sido menor para o sistema reciprocante, ele causou maior aumento no volume do canal. EGCG+FOSFO contidos no PEG-400 apresentou propriedades antibiofilme e anticolagenolíticas, além de baixa citotoxicidade em concentrações mais baixas, podendo ser uma alternativa de medicação intracanal para fins endodônticos.

Palavras-chaves: Pulpectomia. Instrumentos odontológicos. Antibacterianos. Anti-infecciosos. Biofilme. Metaloproteinases. Dentes decíduos.

CHRISOSTOMO, DA**. *In vitro* study of mechanized instrumentation techniques and intracanal medications as protocols proposed for pulpectomy in primary teeth.** 2023. 50 f. Tese (Doutorado) – Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2023.

GENERAL ABSTRACT

This thesis was divided into 2 scientific articles. The article 1 aimed to compare two endodontic instrumentation protocols with rotary or reciprocating files, performed on prototypes of primary molars using microcomputed tomography (micro-CT); and article 2 aimed to evaluate the effect of two proposed intracanal medications: triantibiotic combination (TRI) of metronidazole, ciprofloxacin and fosfomycin (FOSFO) and the association of epigallocatechin gallate (EGCG) with FOSFO, in comparison with the controls calcium hydroxide paste (CH) or chlorhexidine (CHX), all dissolved in polyethylene glycol 400 (PEG) in multispecies biofilms, toxicity in fibroblasts and collagenolytic and gelatinolytic activities detected in root dentin. For the methodology of article 1, 24 3D printed prototypes of artificial deciduous molars were used, which were distributed into two groups of 12 each, according to the type of instrumentation: with Sequence Baby NiTi files© (SBF) rotary files or reciprocating files with X1-Blue File NiTi© (XBF). The following parameters were evaluated: root canal and dentin volumes, canal transportation and centralization, risk of root perforation and instrumentation time. For the methodology of article 2, the antibiofilm effect and cytotoxicity of drugs containing a combination of antibiotics, EGCG+FOSFO or CH were evaluated in multispecies biofilms formed in root dentin samples by confocal microscopy and in fibroblast cells by resazurin assays, respectively. Inhibition of protease activity by drugs was measured by collagenolytic enzyme activity assays (ELISA) and gelatinolytic activity by metalloproteinases - MMP with *in situ* zymography in radicular dentin specimens. The results of article 1 showed that SBF promoted less enlargement of the root canal and consequently removed less dentin when compared to the reciprocating system. In general, both instrumentation protocols showed no statistical difference between them considering root canal transport and centralization capacity. There was no difference in dentin thickness, fractures and fissures comparing the pre- and postoperative moments for both endodontic systems. Instrumentation time was shorter for XBF. The

results of article 2 showed that EGCG+FOSFO, combination of antibiotics and CH in PEG 400 significantly reduced bacteria from multispecies biofilms in dentinal tubules and at lower concentrations were not toxic to fibroblasts. All drugs showed anticollagenolytic activity compared to PEG and water. PEG+CH and PEG+CHX decreased the *in situ* gelatinolytic activity of MMP when compared to the untreated control. PEG+TRI increased gelatinolytic activity in root dentin. In conclusion, both endodontic instrumentation protocols were safe to be used in pulpectomy of primary teeth, as they promoted a minimum reduction in dentin volume, transport and decentralization of the canal, and consequently mitigated changes in dentin thickness and the possibility of fractures. Although instrumentation time was shorter for the reciprocating system, it caused a greater increase in canal volume. EGCG+FOSFO contained in PEG-400 showed antibiofilm and anticolagenolytic properties, in addition to low cytotoxicity at lower concentrations, and could be an alternative intracanal medication for endodontic purposes.

Keywords: Pulpectomy. Dental Instruments. Antibacterial. Anti-infectives. Biofilm. Metalloproteinases. Deciduous teeth.

LISTA DE FIGURAS

Article 1: Root canal instrumentation of primary teeth with rotary and reciprocating files: a micro-computed tomography analysis

LISTA DE TABELAS

Article 1: Root canal instrumentation of primary teeth with rotary and reciprocating files: a micro-computed tomography analysis

LISTA DE FIGURAS

Article 2: Antibiofilm properties, cytotoxicity, and effect on protease activity of antibiotics and EGCG-based medications for endodontic purposes

SUMÁRIO

Introdução Geral

Embora no contexto de saúde bucal, a educação e a prevenção são prioritárias na Odontologia Contemporânea, as alterações pulpares que acometem os dentes decíduos em função de lesões de cárie ou por motivos traumáticos ainda são muito frequentes, necessitando, muitas vezes, de tratamento endodôntico. O principal objetivo da endodontia na Odontopediatria é manter o dente decíduo na cavidade bucal até o momento de sua esfoliação fisiológica, retornando à sua função biológica, ou seja, mastigatória, fonética, estética, entre outras, com a regressão dos sinais e sintomas clínicos e radiográficos (AAPD, 2014; Smaïl et al., 2018) ressaltando ainda que a permanência destes também atuará como guia para a erupção dos dentes permanentes sucessores (Nascimento et al., 2014).

A pulpectomia é o procedimento endodôntico mais indicado em Odontopediatria para o tratamento dos tecidos pulpares infectados, ou seja, para dentes com diagnóstico de pulpite irreversível. Consiste na remoção total do tecido pulpar coronário e radicular por meio do preparo biomecânico (instrumentação e irrigação), uso de medicação intracanal e posterior obturação dos canais radiculares (AAPD, 2014). Entretanto, a instrumentação dos canais de dentes decíduos é considerada um desafio por estes dentes serem anatomicamente mais complexos do que os de dentes permanentes pela presença de áreas de rizólise, menor espessura de dentina em área de furca, curvatura acentuada das raízes, principalmente em molares superiores, além da questão do tempo de colaboração das crianças ser bem mais reduzido que em adultos (Duque et al., 2013; Ozcan et al., 2016).

Dessa forma, novos métodos de preparo dos canais radiculares em dentes decíduos estão sendo estudados visando reduzir o número de limas, bem como o tempo do procedimento de instrumentação em si (Prabhakar et al., 2016). Alguns pesquisadores estudaram a endodontia mecanizada direcionada ao tratamento de crianças, porém a maioria dos estudos se concentra ainda em experimentos laboratoriais (Kummer et al., 2008; Moghaddam et al., 2009; Katge et al., 2016; Kaya et al., 2017; Moraes et al., 2019; Souza et al., 2023). Kummer et al. (2008) compararam limas manuais e rotatórias com objetivo de verificar o desgaste de dentina, risco de perfuração e tempo do preparo dos canais. De acordo com os dados obtidos, o desgaste em dentina no terço coronal e médio foi maior com as limas manuais, e o tempo de instrumentação foi menor para as limas rotatórias e estas também promoveram maior regularidade nos canais radiculares. Quanto

às perfurações radiculares devido à instrumentação, não houve diferença significante entre os grupos.

O principal objetivo do preparo biomecânico deve ser a eliminação dos microrganismos e a prevenção de reinfecção nos canais radiculares. Para atingir esse objetivo, deve-se fazer a combinação da instrumentação com limas mais rápidas e seguras e a irrigação com soluções antimicrobianas efetivas. Diversos estudos têm avaliado a capacidade desinfetante de agentes químicos altamente concentrados, como hipoclorito de sódio ou associação de agentes como hipoclorito de sódio e ácido cítrico como soluções irrigantes em endodontia para dentes decíduos (Salama et al., 1994; Götze et al., 2005; Tannure et al., 2011). Entretanto, devido à complexidade da malha de canais secundários e acessórios, ainda há persistência de algumas espécies microbianas após o preparo químico-mecânico impossibilitando a total desinfecção do sistema de canais radiculares, o que leva à necessidade do uso de medicação intracanal (Chávez et al., 2003). Idealmente, a medicação intracanal deve apresentar uma boa ação antibacteriana, para que a sua permanência no interior do canal radicular tenha maiores chances de atingir áreas não alcançadas pela instrumentação, como istmos, ramificações, reentrâncias e túbulos dentinários; potencializando assim a sanificação do sistema de canais e túbulos dentinários e impedindo que os microrganismos proliferem no intervalo entre as sessões de tratamento (Byström et al., 1985, 1987; Sjögren et al., 1991).

Bactérias gram-negativas, membros comuns das infecções primárias, são geralmente eliminadas após procedimento de tratamento químico-mecânico. Exceções podem incluir alguns bacilos anaeróbios como *Fusobacterium nucleatum*, *Prevotella* species e *Campylobacter rectus*, que estão entre as espécies encontradas em amostras após instrumentação/medicação endodôntica. Entretanto, a maioria dos estudos tem revelado que as bactérias gram-positivas são as mais frequentemente presentes nestes casos, incluindo estreptococos (*S. mitis, S. gordonii, S. anginosus, S. sanguinis, S. oralis*), *P. micra*, *Actinomyces* species (*A. israelii* e *A. odontolyticus*), *Propionibacterium* species (*P. acnes* e *P. propionicum*), *P. alactolyticus*, lactobacilos (*L. paracasei, L. acidophilus*), *Enterococcus faecalis*, entre outros (Siqueira e Rôças, 2009).

Dentre as medicações intracanais frequentemente utilizadas destaca-se a pasta de hidróxido de cálcio, indicada por seu efeito antimicrobiano e potencial para estimular o reparo dos tecidos periapicais. A pasta Calen® (SS White, RJ, Brasil) que apresenta em sua composição 2,5g de hidróxido de cálcio, 0,5g de óxido de zinco, 0,05g de colofônia

hidrogenada, 1,75mL de polietilenoglicol 400 (PEG 400, como veículo) tem sido usada para diversas finalidades, como curativos de demora entre sessões de pulpectomia e inclusive como material obturador em dentes decíduos por sua boa tolerância pelos tecidos, natureza hidrossolúvel e baixa solubilidade (Ranly e Garcia, 2000).

Antimicrobianos naturais ou sintéticos estão sendo empregados como alternativas às medicações intracanais tradicionais, com o intuito de eliminar as bactérias e suas toxinas e não agredir os tecidos periapicais adjacentes (Iglesias et al., 2013; Moreno et al., 2014). Sato *et al.* (1993) e Hoshino et al. (1996) foram os primeiros investigadores a avaliarem a pasta triantibiótica contendo metronidazol, ciprofloxacina e minociclina. Estudo analisou a eficácia antibacteriana destes antibióticos isoladamente e em combinação contra bactérias isoladas de dentina infectada, polpas infectadas e periodontite apical e observou que nenhum deles sozinho eliminou todas as bactérias, mas em combinação foram suficientemente potentes para erradicar as bactérias em todas as amostras (Hoshino et al., 1996). O metronidazol é um composto nitroimidazol que exibe ampla atividade antiprotozoária, antibacteriana contra bacilos Gram-negativos anaeróbios, bacilos Gram-positivos esporulados e cocos anaeróbios. A ciprofloxina é uma fluoroquinolona que apresenta ação bactericida, principalmente contra Gram-negativas (Vijayaraghavan et al., 2012). A minociclina, um antibiótico da família das tetraciclinas, apesar do seu grande potencial bactericida, pode causar alergias e manchamento da coroa dentária (Kim et al., 2010). Essas limitações da minociclina levou ao estudo de novas alternativas para a combinação da pasta triantibiótica, como a fosfomicina, um potente inibidor da proteína MurA, envolvida na biogênese da parede celular, com amplo espectro de ação contra bactérias Gram positivas e Gram negativas (Trope, 2010). Estudo apontou que a combinação de metronidazol, ciprofloxicina e fosfomicina apresentou efeito inibitório *in vitro* contra diversas cepas microbianas, incluindo *E. faecalis*, e atividade antibiofilme simples e misto de *E. faecalis e C. albicans* (Machado, 2016)

Entre os antimicrobianos naturais que vêm sendo muito estudados em Medicina e Odontologia está o epigalocatequina-3-galato (EGCG), um importante flavonoide extraído das folhas de chá verde (Reygaert WC, 2018; Steinmann et al., 2013). Além de apresentar propriedades antioxidantes, antitumorais e anti-inflamatórias, o EGCG também é eficaz contra bactérias Gram-positivas e Gram-negativas (Steinmann et al, 2013). Também se mostrou eficaz contra bactérias multirresistentes, como *Pseudomonas aeruginosa* e *Escherichia coli* (Jeon et al., 2014). EGCG demonstrou atividade inibitória

contra *Enterococcus faecalis*, tanto em células planctônicas quanto em biofilme (Lee e Tan, 2015) e inibiu a adesão de *Streptococcus mutans* de maneira dose-dependente por meio da supressão de genes relacionados à formação de biofilme (Xu et al., 2012). Estudos demonstraram que baixas concentrações de EGCG mostram sinergismo com antibióticos β-lactâmicos sobre bactérias multirresistentes, provavelmente porque EGCG atua direta ou indiretamente no mesmo local dos antibióticos, o peptidoglicano nas paredes celulares, aumentando a atividade antimicrobiana (Zhao et al., 2001). Além disso, a combinação de antibióticos com EGCG aumentou o efeito sobre biofilmes orais (Hu et al., 2001; Duque et al., 2023). Ferreira (2013) avaliou *in vitro* uma medicação intracanal com EGCG associado ao PEG 400 em dentes decíduos e permanentes e notou que EGCG não mudou suas propriedades físico-químicas, sua estabilidade, além de não alterar a coloração do dente até 56 dias após sua aplicação nos canais desses dentes.

A otimização do tratamento endodôntico em dentes decíduos combinando a instrumentação com o uso de limas mecanizadas e medicações intracanais com melhor efeito antimicrobiano é de extrema relevância em Odontopediatria, pois poderia diminuir o tempo clínico e melhorar os resultados clínicos do procedimento. Inúmeros sistemas de limas endodônticas mecanizadas estão disponíveis no mercado, porém, a maioria deles foram desenvolvidos para a instrumentação de dentes permanentes. Assim, estudos *in vitro* ainda são necessários para determinar o efeito desses sistemas no desgaste de dentina nos terços radiculares dos dentes decíduos a fim de aumentar a segurança na sua indicação clínica. Paralelamente, a literatura ainda é bastante divergente sobre qual medicação intracanal seria ideal para dentes decíduos que pudesse ter o efeito antimicrobiano desejado, sem afetar o dente permanente subjacente, auxiliando na manutenção dos dentes decíduos no arco até a época mais próxima possível da sua esfoliação fisiológica. Dessa forma, os objetivos da presente tese serão: 1) comparar dois protocolos de instrumentação endodôntica: um sistema rotatório e um sistema reciprocante, realizados em protótipos de molares decíduos por meio de microtomografia computadorizada (micro-CT); e 2) avaliar o efeito de duas propostas de medicações intracanais: uma combinação triantibiótica (TRI) de metronidazol, ciprofloxacina e fosfomicina (FOSFO) e a associação de EGCG com FOSFO, em comparação com pasta de hidróxido de cálcio (CH) ou clorexidina (CHX), todos dissolvidos em polietilenoglicol 400 (PEG) em biofilmes multiespécies, a toxicidade em fibroblastos e atividades colagenolíticas e gelatinolíticas detectadas na dentina radicular.

Root canal instrumentation of primary teeth with rotary and reciprocating files: a micro-computed tomography analysis*

Short title: Endodontic instrumentation in primary teeth

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*This article followed the guidelines of the International Endodontic Journal (ANEXO 4).

Abstract

Aim: to compare the outcomes of two protocols of endodontic instrumentation with rotary and reciprocating files, in 3D-printed prototypes of upper and lower primary molars using micro-computed tomography (micro-CT).

Methodology: Twenty-four 3D-printed prototypes of upper and lower artificial primary molars with coronal and root pulp were distributed into two groups of 12 each (6 upper and 6 lower primary molars), according to the type of instrumentation – with rotary files (Sequence Baby NiTi Files©) and with reciprocating files (X1-Blue File NiTi files©). The following parameters were evaluated: root canal and dentin volumes, canal transportation and centralization, risk of root perforation and time of instrumentation.

Results: The results showed that the rotary files promoted a smaller enlargement of the root canal, and consequently removed less dentin when compared with the reciprocating files. In general, both protocols of instrumentation did not present statistical difference compared to each other considering root canal transportation and ability of centralization. There was no difference on dentin thickness, fractures and cracks comparing pre and pos-operatory time-points for both endodontic files. Time of instrumentation was shorter for reciprocating files.

Conclusion: In conclusion, both protocols of endodontic instrumentation presented general safety to be used for the pulpectomy in primary teeth, since they promoted minimal decrease in dentin volume, canal transportation and decentralization, minimizing the reduction of dentin thickness and fractures. Although time of instrumentation was shorter for reciprocating files, it causes higher increase in canal volume compared to rotary files.

Keywords: pulpectomy, dental instruments, micro-computed tomography, primary teeth.

1. Introduction

Pulpectomy is an endodontic treatment indicated for primary tooth with irreversible pulpitis or pulp necrosis when roots present minimal or no resorption and there are adequate bone support and tooth structure for posterior restoration (AAPD, 2022). It consists of the total removal of the coronal and root pulp tissue through chemical-mechanical preparation, inter-appointment intracanal medication and subsequent filling of the root canals (AAPD, 2014). However, the root canal instrumentation of deciduous teeth is considered a challenge due to their complex anatomy including thinner dentin thickness in the furcation area, accentuated curvature of the roots, areas of physiological resorption, in addition to shorter time of collaboration during the treatment in comparison to adults (Duque et al., 2013, Ozcan et al., 2016).

New methods for debriding and shaping root canals have been studied to simplify and reduce the time of the instrumentation in primary dentition (Prabhakar et al., 2016). Some *in vitro* and *in vivo* studies have evaluated the efficiency of rotary endodontic files compared to conventional files aimed at treating children (Kummer et al., 2008; Moghaddam et al., 2009; Katge et al., 2016; Kaya et al., 2017; Moraes et al., 2019, Manker et al., 2020, Souza et al., 2023). For *in vitro* studies, some parameters have been used to evaluate the efficiency of biomechanical preparation of primary or permanent teeth such as shaping characteristics, canal transportation, amount of dentine removal, untouched canal surface area and preparation time (Moraes et al., 2019; Manker et al., 2020). Most of investigations have demonstrated better root canal shaping, lower dentin wear and shorter working time using rotary files compared to manual files (Kummer et al., 2008; Moraes et al., 2019; Manker et al., 2020). Comparing rotary to reciprocating files, it has been shown that instrumentation's systems with reciprocating movement in permanent teeth lead to faster mechanical preparation of the root canal and a greater amount of dentin debris (Caviedes-Bucheli et al., 2016; Toyoğlu and Altunbaş, 2017; Uslu et al., 2018). Those debris eliminated from reciprocating files probably occur due to their cross-sectional design, more cutting capacity, and more dentin removal (Eshagh Saberi et al.,2020). In primary teeth, few *in vitro* studies have compared rotary and reciprocating files, and the results were divergent, possibly due to methodological differences (Prabhakar et al., 2016; Ramazani et al., 2016; Moraes et al., 2019, 2021). Some studies have showed more canal transportation and accumulated debris for reciprocating instrumentation (Moraes et al., 2019, 2021) and other studies have no found difference

between endodontic file systems (Ramazani et al., 2016) or detected more efficiency for reciprocating files considering canal transportation and mean of instrumentation time (Prabhakar et al., 2016).

Cone beam computed tomography (CBCT) has been used in endodontics to generate three-dimensional images of teeth helping clinicians to analyze root canal morphology with more accuracy and without the superimposition of anatomical structures (Acar et al., 2015). Ultrahigh resolution 3D images can be obtained by another more advanced technique, the microcomputed tomography (micro-CT), which is able to obtain very thin sections (reaching to 1μm) and provide the anatomical parameters of the root canal to allow appropriate group comparisons in *in vitro* studies (Peters et al., 2001). An *in vitro* study demonstrated more detailed and accurate data regarding root canal systems from primary teeth obtained by micro-CT compared to CBCT (Acar et al., 2015). The authors pointed out that micro-CT can be used only for laboratory-based studies, and it is not suitable for clinical uses since extreme high radiation, not compatible with humans (Acar et al., 2015).

Studies comparing the results of different root canal instrumentation files in primary teeth are extremely restricted, considering the difficulty in collecting natural teeth with adequate root length (Moraes et al., 2021). To overcome this limitation, artificial 3D-printed polymer prototypes of primary teeth with coronary and root pulp have been used to evaluate the effectiveness of new endodontic rotary files in primary teeth (Hecksher et al., 2019; Moraes et al., 2021, Souza et al., 2023). Considering the anatomical specificities of primary teeth and large variety of endodontic systems, including a new generation of heat-treated nickel-titanium (NiTi) files available in the market, new studies are relevant to determine protocols of root instrumentation dedicated to primary dentition. The objective of this study was to compare the outcomes of two protocols of endodontic instrumentation with rotary and reciprocating files, both with heat-treated nickel-titanium technology, in 3D-printed prototypes of upper and lower primary molars using micro-computed tomography (micro-CT). The null hypothesis is that there will not be difference among the two protocols of endodontic instrumentation, considering the following parameters: root canal and dentin volumes, canal transportation and centralization, risk of root perforation and time of instrumentation.

2. Material and methods

2.1. Teeth preparation and instrumentation methods

Sample size calculation was undertaken using data from a previous study by Barasuol et al. (2021) and Souza et al. (2023) using G*Power (v3.1.9.2, University of São Paulo, Brazil), resulting in 26 canals per group, and to account for possible losses, 42 canals per group were used (n=12 teeth per group). Twenty-four 3D-printed prototypes of upper and lower artificial primary molars with coronal and root pulp (DENARTE, São Paulo, SP) were selected and distributed into two groups of 12 each (6 upper and 6 lower primary molars), according to the type of instrumentation. Initially, a pilot study was carried out including 8 artificial deciduous molars, 4 for each group of files. Coronary access to the root canals was prepared by a single operator with high-speed water-cooled diamond bur (#FG1012, KG Sorensen Indústria e Comércio, São Paulo, SP, Brazil), followed by conical steel bur with inactive tips (Endo Z, Maillefer Instruments, Ballaigues, Switzerland) (Pinto et al., 2011). The working length of each canal by introducing a size 10 K-file (Dentsply-Maillefer, Ballaigues, Switzerland) 1 mm shorter than tooth length (measurements determined by the manufacturer). The first group of artificial molars (n=12) was instrumented with the SBF-Sequence Baby NiTi files© (SBF) (MKLife, Porto Alegre, RS, Brazil) following the sequence determined by the manufacturer: #17/08 (11 mm), #20/04, #25/04 and #30/04 (16 mm) with 350 rpm and 1.5N torque. The second group of artificial teeth was instrumented initially with X1-Blue File Glide Path© (MKLife, Porto Alegre, RS, Brazil) #15/04 (25 mm) and after that with X1-Blue File© (MKLife, Porto Alegre, RS, Brazil) reciprocating NiTi files (XBF), according to the canal width: #20/06 or #25/06 (25 mm) with 3 pecking movements. Each instrument was used to prepare a maximum of 3 root canals and then discarded. All files were connected in an endodontic motor (E-connect Pro©, MKLife) and the teeth were irrigated with 3mL of saline solution between the different files, using a 20G NaviTip needle (Ultradent, Indaiatuba, Sao Paulo, Brazil) inserted up to 2 mm with simultaneous aspiration. The canals were dried with sterile absorbent paper points. The instrumentation time (including time needed for instrumentation, instrument change and irrigation) was recorded in seconds, from the start of cleaning and shaping until the completion of biomechanical preparation and was recorded using a chronometer. (Manker et al., 2020; Ramazani et al., 2016).

2.2. Microcomputed Tomography (micro-CT) acquisition

Micro-CT analyses were conducted as described in Pinto et al. (2019) and de Camargo et al. (2021) and performed by the same operator. Briefly, the teeth were scanned before and after mechanical preparation with high-energy microcomputed tomography (SkyScan 1176; Bruker Micro-CT, Kontich, Belgium) using the following acquisition parameters: 70 kV, 142 uA, 14 μm pixel size, 1344 × 896-pixel matrix, 0,5 mm-thick AI filter, 880 ms exposure, resulting in a total scan time of about 23 minutes. The images obtained before and after preparation, were reconstructed using NRecon software (NRecon v.1.6.3; Bruker-microCT) and a data viewer software (Data Viewer v.1.5.1, Bruker, Kontich, Belgium) was used to pair and standardize the same position of the sample for all the analyses. Quantitative analyses were then made using CTAn software (CTAn v.1.14.4, Bruker, Kontich, Belgium), by applying task lists with arithmetic and logic operations between the superimposed sections. The total length of each canal was measured and divided in three equal parts corresponding to the thirds of the canals (coronal, middle and apical). Figure 1 shows micro-CT reconstructions and cross-sections at all levels of representative samples evaluated before and after instrumentation, which were instrumented with protocols of instrumentation using rotary and reciprocating files.

2.3. Measurement of root canal and dentin volume

In this study, although the 3D printed prototypes of primary molars are made by resin (their composition was not disclosed by the manufacturer), the term “dentin” was kept facilitating the comparison with other previous studies. Root canal initial and final volume, dentin initial and final volume, were obtained for each canal third, before and after preparation. Based on these values, the percentage of volumetric increase of root canal (% Volumetric increase) and the percentage of volumetric decrease of root dentin (% Volumetric decrease) were calculated using the following formulas (Pinto et al., 2019; Neves et al.,2015):

$$\%\ \text{Volumetric increase root canal} = \left(\frac{\text{Final volume of the root canal} \times 100}{\text{Initial volume of root canal}} \right) - 100$$

$$\%\ \text{Volumetric decrease dentin} = \left(\frac{\text{Initial volume of dentin} \times 100}{\text{Final volume of dentin}} \right) - 100$$

Each parameter was evaluated for each canal (mesial, distal, and palatal for maxillary teeth and mesial/distal for mandibular teeth).

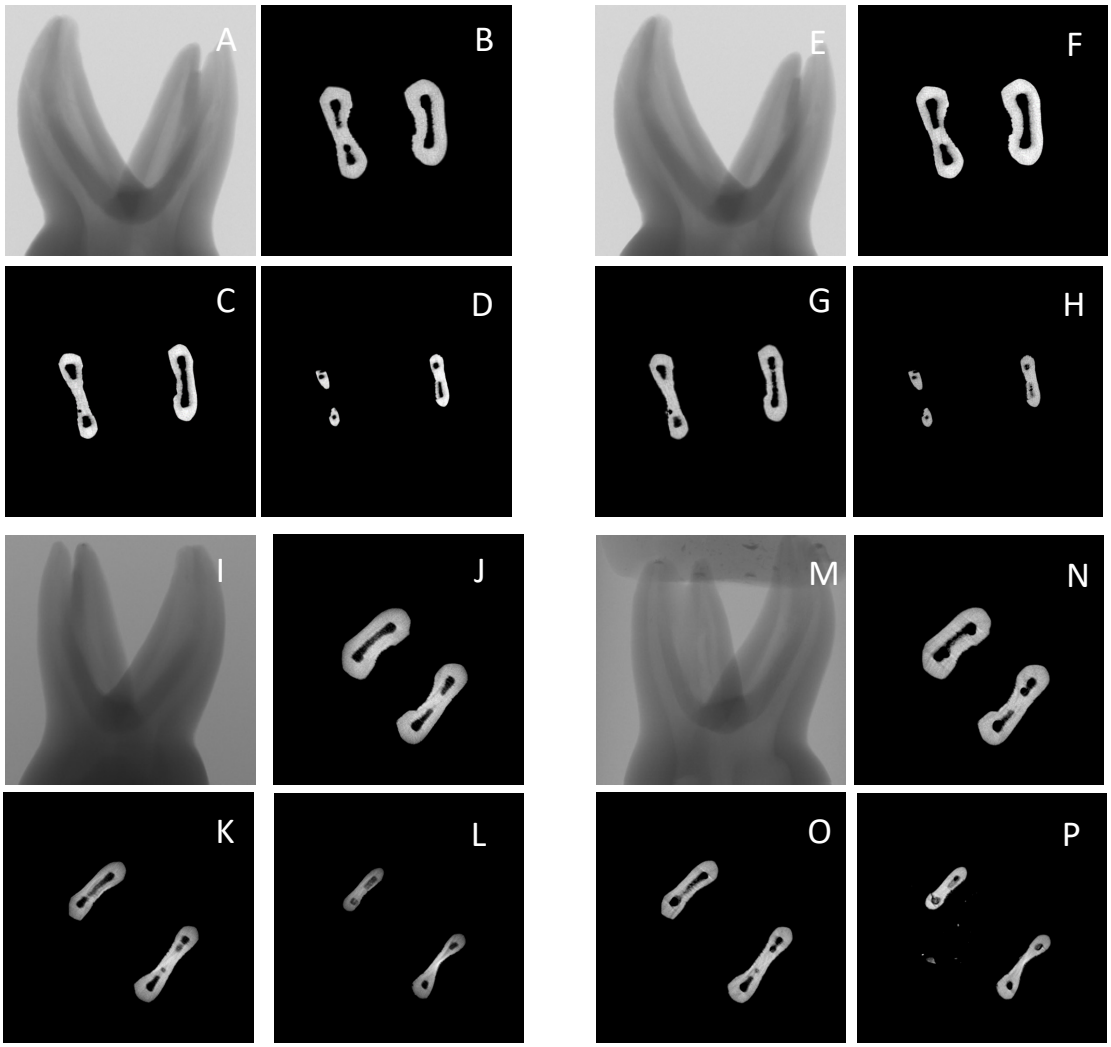

Figure 1. Representative micro-CT images of teeth before and after instrumentation with rotary and reciprocating files. A,E – Lower second molar before and after rotary instrumentation; B, F – cervical third of the same tooth before and after rotary instrumentation; C,G – middle third of the same tooth before and after rotary instrumentation; D,H - apical third of the same tooth before and after rotary instrumentation; I,M – Lower second molar before and after reciprocating instrumentation; J, N – cervical third of the same tooth before and after reciprocating instrumentation; K,O – middle third of the same tooth before and after reciprocating instrumentation; L,P - apical third of the same tooth before and after reciprocating instrumentation.

2.4. Measurement of root canal transportation (RCT) and ability of centralization (CA)

Analyses of root canal transportation and ability of centralization were performed from the superimposed images, using the CTAn software. The root canal transportation was calculated according to the following formula suggested by Gambill et al. (1996): (X1-X2)-(Y1-Y2), where X1 and Y1 measurements represented the shortest distance from the external surface of the root to the periphery of the uninstrumented root canal before the preparation, for the mesial and distal side, respectively; the X2 and Y2 measurements represented the shortest distance from the external surface of the root to the periphery of the instrumented canal after the preparation, for the mesial and distal side, respectively (Figure 2) (Pinto et al., 2019). A result of 0 from the canal transportation formula indicated no canal transportation. A positive value indicated that transportation occurred to mesial side of the root, which characterized the side towards the furcation area (inner curve), while the negative value showed that transportation occurred to the distal side which characterized the side opposite to furcation area (outer curve) (de Camargo et al., 2021).The ability of instrument centralization was calculated using the following ratio suggested by Gambil et al. (1996): (X1-X2)/(Y1-Y2) or (Y1-Y2)/(X1-X2). The lower value was considered the numerator of the formula, if the number was unequal and a result of 1 indicated perfect centering (de Camargo et al., 2021). Negative numbers represented deviation in the mesial direction, and positive numbers, in the distal direction (Pinto et al., 2019). Each parameter was evaluated for each canal (mesial, distal, and palatal for maxillary teeth and mesial/distal for mandibular teeth) and canal thirds (cervical, middle, and apical).

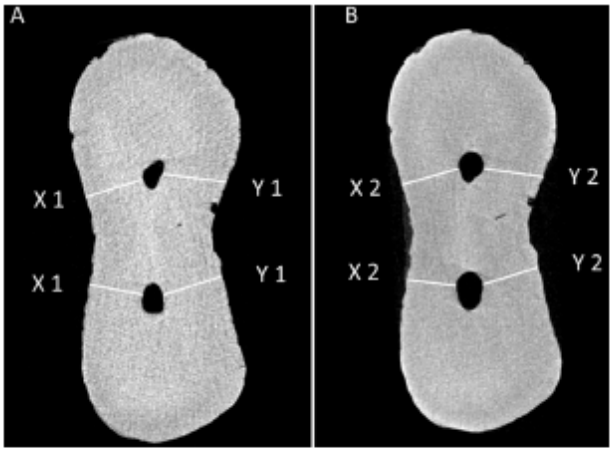

Figure 2. Representative micro-CT cross sections of the middle third of the root, taken from the mesial root canals of mandibular molar, showing the shortest distance between the edge of the root and the canal,

determined to perform the analyses of transportation and centralization in each canal. A. Preoperative root canal and B after root canal preparation. (figures from Pinto et al., 2019).

2.5. Measurement of risk of root perforation (dentin thickness,cracks and perforations)

To analyze the risk of root perforation, the smallest dentin thickness, in the cervical, middle, and apical thirds, after each instrumentation procedure was obtained. In the CTAn software (CTAn v.1.14.4, Bruker, Kontich, Belgium), the thickness of the dentin between the root canal and the external surface was mapped and quantified in millimeter. The amount of dentine removal in each third was defined by subtracting the thicker dentine thickness from the thinner dentine thickness before and after instrumentation (de Camargo et al., 2021). Cracks and perforations were recorded by checking the 2D slices for each of the instrumented stacks of images and if detected compared to baseline specimens (Moraes et al., 2021).

2.6. Statistical analysis

The Shapiro-Wilk test was applied to verify the normality of the data. All data were submitted to ANOVA and Tukey tests at the 5% of significant level. JAMOVI statistical software version 2.3.19.0 was used for running the statistical analyses.

3. Results

Table 1 shows the analysis of root canal volume and dentin volume for the entire root canal considering each canal separately: mesial, distal, and palatal canals. Independent on the molar and canal analyzed, the results revealed a significant difference between the rotary (SBF) and reciprocating (XBF) files comparing the increase in root canal volume, indicating that the rotary files promoted a smaller enlargement of the root canal ($p < 0.05$), and consequently removed less dentin when compared with the reciprocating files. The decrease in dentin volume was also higher for canals treated with XBF, although difference was not detected for distal canals when all molars were analyzed together. When molars were analyzed separately, same differences were observed between the endodontic files for root canal volume for all canals, except by mesial canal in maxillary molars. Comparing the root canals, the highest increase in the canal volume was observed for distal canals for maxillary molars and mesial canal for mandibular molars in XBF. For rotary files, the highest increase in canal volume was observed for both mesial and distal canals of maxillary molars, without difference between them. No difference was noted

between mesial and distal canals of mandibular molars treated with SBF. When the molars were analyzed separately, XBF promoted significantly decrease in dentin volume only for distal canal of mandibular molars, when compared to SBF.

Table 1. Increase in root canal volume (%) and decrease in dentin volume (%) after instrumentation with rotary (SBF) and reciprocating (XBF) files.

Teeth	Canals	Increase in canal volume (%)		Decrease in dentin volume (%)	
		SBF	XBF	SBF	XBF
Maxillary molars	Mesial	39.53(11.54)^{Aa}	**41.5 (12.9)^{Aa}**	5.24 (2.7)^{Aa}	5.75 (2.62)^{Aa}
	Distal	**32.96(24.05)^{Aa}**	**55.42(5.25)^{Bb}**	7.7(4.1)^{Aa}	9.8 (7.26)^{Aa}
	Palatal	**4.23 (10)^{Ba}**	**14.08 (10)^{Cb}**	3.19(1.3)^{Aa}	5.04 (3.6)^{Aa}
Mandibular molars	Mesial	**14.65(5.29)^{Aa}**	**35.79 (6.27)^{Ab}**	4.26(2.52)^{Aa}	11.38 (8.81)^{Aa}
	Distal	**9.35(5.42)^{Aa}**	**24.15 (2.97)^{Bb}**	**3.36(0.89)^{Aa}**	**5.41 (3.9)^{Ab}**
All molars	Mesial	**27.1 (15.4)^{Aa}**	**38.6(10.4)^{Ab}**	**4.8(2.6)^{Aa}**	**8.6(6.9)^{Ab}**
	Distal	**21.2(20.9)^{Aa}**	**39.8(29.2)^{Ab}**	5.5(3.7)^{Aa}	7.6(6.1)^{Aa}

[A] Superscript uppercase letters in the same column indicate statistical difference among the canals for each endodontic file system.

[a] Different superscript lowercase letters in the same line indicate statistical difference between the endodontic files. Values are presented in means/standard deviations (ANOVA and Tukey tests, $p<0.05$).

SBF – Sequence Baby File NiTi©; XBF – X1-Blue File NiTi©

Table 2 presents the analysis of root canal transportation and ability of centralization evaluated after rotary and reciprocating instrumentation. In general, both protocols of instrumentation did not present statistical difference compared to each other considering root canal transportation and ability of centralization. They promoted negative values or transportation to the opposite side of the furcation in canals, however, most of them were close to zero. Considering the ability of instrumentation, most of results for both endodontic files showed deviation to the opposite side of furcation area for both protocols of instrumentation. Statistical differences among the canal thirds in the canal transportation were evidenced comparing both cervical and middle thirds with apical thirds, especially for specimens treated with rotary files. These differences can be seen for all canals of maxillary molars and distal canals of mandibular molars. During the shaping procedure, no files were fractured or deformed.

Table 2. Root canal transportation and ability of centralization after instrumentation with rotary (SBF) and reciprocating (XBF) files.

Teeth	Canals	Thirds	†Root canal transportation		‡Ability of centralization	
			SBF	XBF	SBF	XBF
Maxillary molars	Mesial	Cervical	**-0.15^{Aa} (-0.23; -0.05)**	-0.14^{Aa} (-0.96; -0.02)	0.09^{Aa} (-0.6;0.26)	0.2^{Aa} (-0.019;0.65)
		Middle	**-0.02^{ABa} (-0.24;0.09)**	-0.002^{Aa} (-1.03;0.16)	0.47^{Aa} (-0.97;0.9)	0.33^{Aa} (-2.33;0.96)
		Apical	**-0.008^{Ba} (-0.07;0.10)**	-0.039^{Aa} (-0.59;0.18)	0.33^{Aa} (-0.23;0.8)	-0.06^{Aa} (-5.2;0.87)
	Distal	Cervical	**-0.14^{Aa} (-0.19;-0.08)**	**-0.2^{Ab} (-0.3;-0.15)**	**0.2^{Aa} (-0.1;0.5)**	**0.053^{Ab} (-0.21;0.23)**
		Middle	**-0.004^{ABa} (-0.21;0.09)**	**-0.02^{ABa} (-0.3;0.04)**	0.49^{Aa} (-0.7;1)	0.25^{Aa} (-1.23;2.74)
		Apical	**0.06^{Ba} (-0.07;0.18)**	**-0.01^{Ba} (-0.10;0.58)**	0.27^{Aa} (-0.4;0.84)	0.2^{Aa} (-1.04;0.97)
	Palatal	Cervical	**-0.14^{Aa} (-0.23;0.01)**	-0.15^{Aa} (-0.31;0.08)	0.19^{Aa} (-0.9;0.62)	-0.33^{Aa} (-0.83;0.04)
		Middle	**-0.064^{ABa} (-0.09;011)**	-0.03^{Aa} (-0.38;0.25)	-0.12^{Aa} (-1.72;0.5)	-0.23^{Aa} (-2.79;1.88)
		Apical	**0.13^{Ba} (-0.04;0.88)**	0.01^{Aa} (-0.97;0.10)	-0.04^{Aa} (-0.3;0.01)	-0.06^{Aa} (-0.6;1.5)
Mandibular molars	Mesial	Cervical	-0.07^{Aa} (-0.19;0.05)	-0.04^{Aa} (-0.17;0.08)	-0.21^{Aa} (-2.23;1.8)	0.11^{Aa} (-1.14;1.24)
		Middle	0.026^{Aa} (-0.11;0.19)	-0.05^{Aa} (-0.17;0.11)	0.08^{Aa} (-1.4;1.97)	-0.21^{Aa} (-3.07;2.11)
		Apical	-0.007^{Aa} (-0.07;0.09)	-0.01^{Aa} (-0.08;0.07)	0.32^{Aa} (-1.5;2.42)	0.29^{Aa} (-0.19;1.4)
	Distal	Cervical	**-0.12^{Aa} (-0.23;0.84)**	-0.046^{Aa} (-0.39;0.16)	-0.17^{Aa} (-3.1;2)	0.069^{Aa} (-2.1;0.90)
		Middle	**-0.03^{ABa} (-0.12;0.10)**	**-0.11^{Ab} (-0.18;0.01)**	-0.48^{Aa} (-4.2;0.3)	-0.38^{Aa} (-2.32;0.66)
		Apical	**0.05^{Ba} (-0.10;0.18)**	-0.4^{Aa} (-0.06;0.13)	-0.2^{Aa} (-1.5;2.3)	0.14^{Aa} (-1.76;1)
All molars	Mesial	Cervical	**-0.09^{Aa} (-0.23;0.05)**	-0.06^{Aa} (-0.96;0.08)	**0.021^{Aa} (-2.2;1.8)**	**0.21^{Ab} (-1.14;1.24)**
		Middle	**-0.011^{ABa} (-0.24;0.19)**	-0.037^{Aa} (-1.03;0.16)	0.11^{Aa} (-1.4;1.9)	0.13^{Aa} (-3.07;2.11)
		Apical	**-0.008^{Ba} (-0.07;0.10)**	-0.023^{Aa} (-0.59;0.18)	0.33^{Aa} (-1.5;2.4)	0.16^{Aa} (-3.4;1.4)
	Distal	Cervical	**-0.14^{Aa} (-0.23;0.84)**	**-0.18^{Aa} (-0.39;0.16)**	0.05^{Aa} (-0.7;0.5)	0.06^{Aa} (-2.1;0.9)
		Middle	**-0.008^{Aa} (-0.21;0.10)**	**-0.06^{ABb} (-0.31;0.04)**	-0.035^{Aa} (-4.28;1)	0.07^{Aa} (-2.3;2.74)
		Apical	**0.05^{Ba} (-0.10;0.18)**	**-0.03^{Ba} (-0.10;0.58)**	-0.12^{Aa} (-1.5;2.3)	0.21^{Aa} (-1.76;1)

[A] Superscript uppercase letters in the same column indicate statistical difference among the thirds for each canal and endodontic file system, separately. [a] Different superscript lowercase letters in the same line

indicate statistical difference between the endodontic files. Values are presented in medians (minimum; maximum) (Kruskal Wallis and Dunn, $p<0.05$)

† Regarding root canal transport, negative values indicate transportation to the opposite side of the furcation area in canals; positive values indicate transportation towards the furcation area in canals. Zero indicates that no canal transportation occurred.

‡ Regarding ability of centralization, negative numbers represented deviation to the furcation area, and positive numbers, to the opposite direction. Number 1 indicates the perfect centering ability (Carvalho et al., 2021). SBF – Sequence Baby File NiTi©; XBF – X1-Blue File NiTi©

Table 3 shows the thickness of the root dentin in the inner side of canals before and after instrumentation with the rotary and reciprocating files, considering the cervical, middle, and apical thirds separately. Considering all molars together, there was no difference on dentin thickness comparing pre and pos-operatory time-points for both endodontic files, except by cervical third from mesial canals of molars. Statistical differences among the thirds were observed for all canals treated at both time-points, independent on the group, except by mesial canals for SBF. For maxillary molars, statistical differences between pre and pos-operatory times were observed for all thirds of mesial canals and cervical thirds of distal and palatal canals only for SBF. There were statistical differences among the thirds for most of canals treated at pre and pos-operatory time-point for both files. For mandibular molars, statistical differences between pre and pos-operatory times were not observed for any group. There were statistical differences among the thirds for all canals of mandibular molars treated at both time-points, independent on the group, except by mesial canals for rotary files.

Table 3. Dentin thickness (mm) in the inner side of canals before and after instrumentation with rotary (SBF) and reciprocating (XBF) files.

Teeth	Canals	Thirds	SBF		XBF	
			Pre-operatory	Pos-operatory	Pre-operatory	Pos-operatory
Maxillary molars	Mesial	Cervical	**0.55^{Aa} (0.08)**	**0.39^{Ab} (0.10)**	**0.60 ^{Aa} (0.11)**	**0.44 ^{Ab} (0.15)**
		Middle	**0.44^{Aa} (0.13)**	**0.36^{Ab} (0.08)**	**0.43 ^{Ba} (0.12)**	0.40 ^{Aa} (0.15)
		Apical	**0.33^{Ba} (0.10)**	**0.34^{Ab} (0.08)**	**0.32^{Ca} (0.11)**	0.32 ^{Aa} (0.11)
	Distal	Cervical	**0.56^{Aa} (0.12)**	**0.36^{Ab} (0.13)**	**0.72 ^{Aa} (0.08)**	**0.47 ^{Ab} (0.10)**
		Middle	**0.31^{Ba} (0.15)**	**0.23^{Ba} (0.13)**	**0.47 ^{Ba} (0.08)**	**0.44 ^{Aa} (0.07)**

Mandibular molars	Palatal	Apical	**0.26^{Ba} (0.11)**	**0.24^{Ba} (0.09)**	**0.26 ^{Ca} (0.04)**	**0.24 ^{Ba} (0.05)**
		Cervical	**0.62^{Aa} (0.06)**	**0.49^{Ab} (0.08)**	**0.86 ^{Aa} (0.14)**	**0.75 ^{Ab} (0.05)**
		Middle	**0.34^{Ba} (0.25)**	**0.35^{Ba} (0.21)**	**0.56 ^{Ba} (0.19)**	**0.55 ^{Ba} (0.11)**
		Apical	**0.44^{Ba} (0.28)**	**0.47^{Aa} (0.24)**	**0.40 ^{Ca} (0.22)**	**0.44 ^{Ca} (0.29)**
	Mesial	Cervical	0.59^{Aa} (0.17)	0.55^{Aa} (0.16)	**0.74 ^{Aa} (0.18)**	**0.72 ^{Aa} (0.15)**
		Middle	0.41^{Aa} (0.13)	0.44^{Aa} (0.14)	**0.52 ^{A,Ba} (0.14)**	**0.51 ^{Ba} (0.10)**
		Apical	0.43^{Aa} (0.14)	0.42^{Aa} (0.14)	**0.45 ^{Ba} (0.11)**	**0.47 ^{Ba} (0.07)**
	Distal	Cervical	**0.73^{Aa} (0.08)**	**0.67^{Aa} (0.19)**	**0.84 ^{Aa} (0.19)**	**0.77 ^{Aa} (0.19)**
		Middle	**0.43^{Ba} (0.09)**	**0.41^{Ba} (0.14)**	**0.53 ^{Ba} (0.15)**	**0.44 ^{Ba} (0.14)**
		Apical	**0.27^{Ca} (0.07)**	**0.32^{Ca} (0.06)**	**0.39 ^{Ca} (0.06)**	**0.35 ^{Ba} (0.08)**
All molars	Mesial	Cervical	0.55 ^{Aa} (0.08)	0.39 ^{Aa} (0.10)	**0.60 ^{Aa} (0.11)**	**0.44 ^{Ab} (0.15)**
		Middle	0.44 ^{Aa} (0.13)	0.36 ^{Aa} (0.08)	**0.43^{Ba} (0.12)**	**0.40 ^{A,Ba} (0.15)**
		Apical	0.44 ^{Aa} (0.10)	0.44 ^{Aa} (0.10)	**0.32 ^{Ca} (0.11)**	**0.31 ^{Ba} (0.11)**
	Distal	Cervical	**0.56 ^{Aa} (0.12)**	**0.36 ^{Aa} (0.13)**	**0.72 ^{Aa} (0.08)**	**0.47 ^{Aa} (0.10)**
		Middle	**0.31^{A,B,a} (0.15)**	**0.23 ^{Aa} (0.13)**	**0.47 ^{Ba} (0.08)**	**0.44 ^{Aa} (0.07)**
		Apical	**0.26 ^{Ba} (0.11)**	**0.24 ^{Ba} (0.09)**	**0.26 ^{Ca} (0.04)**	**0.24 ^{Ba} (0.05)**

[A] Superscript uppercase letters in the same column indicate statistical difference among the thirds for each canal and endodontic files, separately.

[a] Different superscript lowercase letters in the same line indicate statistical difference between operatory and pos-operatory time-points. Values are presented in means(standard deviations) (ANOVA and Tukey tests, $p<0.05$).

SBF – Sequence Baby File NiTi©; XBF – X1-Blue File NiTi©

Table 4 reports the working time (in seconds) evaluated after instrumentation and the total number of fractures and cracks in the root canal. There was statistically significant difference between the groups in the instrumentation working time ($p<0.05$) with the shortest time for reciprocating files. Regarding to the total number of fractures and cracks, there was no statistical difference between the groups ($p>0.05$). Root fractures were only observed in three specimens treated with SBF (total of 5 fractures) and in three specimens

treated with the XBF (total of 5 fractures), with more than 1 type of fracture in some of them. Cracks were observed in only 1 specimen treated with XBF. **Figure 3** illustrates fractures and cracks detected after instrumentation with rotary and reciprocating files.

Table 4. Time of Instrumentation, fractures and cracks detected after instrumentation with rotary (SBF) and reciprocating (XBF) files.

	SBF	**XBF**
Time of instrumentation (s)	399.25 (38.12)*	232.5 (23.07)
Total of fractures and cracks (n of fractures/n of cracks)	Mesial – 3C, 1M, 1A/0 Distal - 0/0 Palatal - 0/0	Mesial – 1C, 2M, 1A /0 Distal – 1C/1A Palatal – 0/0

Cervical (C), middle (M), and apical (A)

*Statistical difference between the endodontic files according to t Student test ($p<0.05$). SBF – Sequence Baby File NiTi©; XBF – X1-Blue File NiTi©

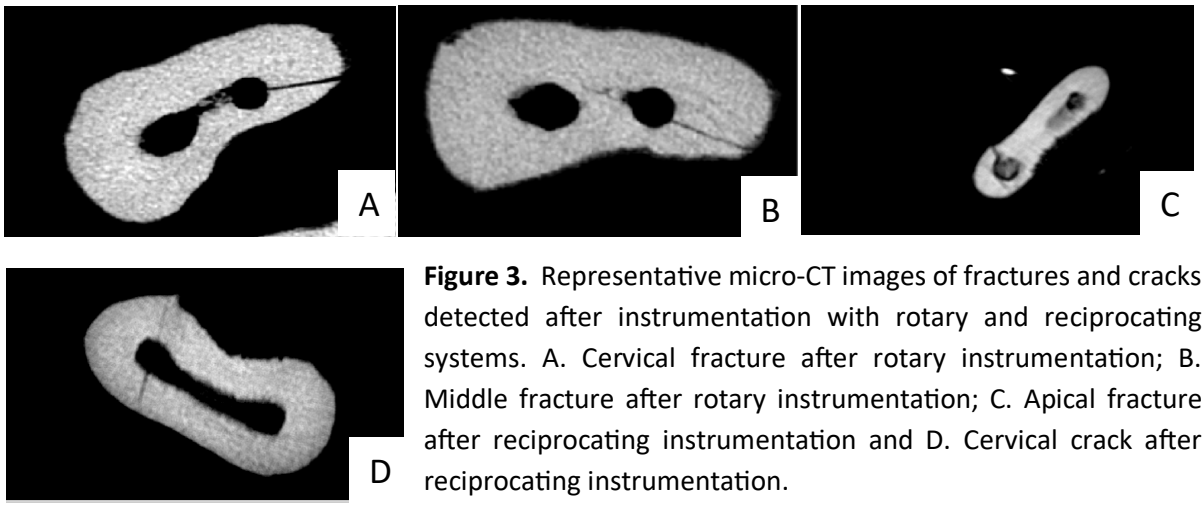

Figure 3. Representative micro-CT images of fractures and cracks detected after instrumentation with rotary and reciprocating systems. A. Cervical fracture after rotary instrumentation; B. Middle fracture after rotary instrumentation; C. Apical fracture after reciprocating instrumentation and D. Cervical crack after reciprocating instrumentation.

4. Discussion

There are few *in vivo* studies evaluating protocols of instrumentation in primary teeth (Boonchoo et al., 2020; Tyagi et al., 2021; Elheeny et al., 2022) especially because of the cost-effectiveness of pulpectomy procedures in children in some countries, children's behavior, anatomical difficulties, and different stages of physiological or pathological root resorption (Manker et al., 2020; Moraes et al., 2021). *In vitro* studies have been conducted to evaluate new techniques of root instrumentation in primary teeth and evaluate some parameters related to efficacy, shaping characteristics and canal

transportation with rotary and reciprocating files using micro-CT analyzes (Manker et al., 2020; Barasuol et al., 2021; Moraes et al., 2019, 2021). Only one *in vitro* study evaluated specific rotary files for primary teeth - Sequence Baby NiTi files© (SBF) and observed statistically greater weariness and shorter working time than manual instrumentation (Souza et al., 2023).

Considering the difficulty in collecting natural teeth with adequate root length (Moraes et al., 2021), 3D-printed polymer prototypes of teeth with root canals have been used in some recent studies to test contemporary instrumentation systems, their application, and their effectiveness on primary teeth (Hecksher et al., 2019; Moraes et al., 2019; 2021). In this present study, artificial teeth were chosen because of the benefits of having roots and canals with standardized length and width reducing the variables inherent to natural primary teeth that could interfere in the results when different endodontic files are compared.

In the present study, null hypothesis was not accepted since some differences were detected between rotary and reciprocating files considering the parameters analyzed in micro-CT images before and after instrumentation. Independent on the molar and canal analyzed, the protocol of instrumentation using rotary files (SBF-Sequence Baby NiTi files©) promoted a smaller enlargement of the root canal and removed less dentin when compared with the reciprocating files (X1-Blue File reciprocating NiTi files©). In contrast with our results, Tavares et al. (2019) did not find difference in volume increase for root canals prepared by rotary or reciprocating files (Tavares et al., 2019). No guidelines suggesting an allowable amount of dentin removal in root canal preparation have been established, but excessive dentin removal can lead to failure and weakening of the remaining tooth structure (Manker et al., 2020). In the present study, the highest volume of dentin removed by the reciprocating files (XBF) may be a result of the instrument design which has a convex triangular cross-section with a larger taper (#25/06). Comparing the root canals, the highest increase in the canal volume was observed for distal or mesial canals comparing to palatal canals of maxillary molars for both endodontic files. Studies have showed that the amount of dentin removed can be influenced by the taper and size of the file and the anatomy of the root canal of primary teeth (Kaya et al., 2017; Radhika et al., 2017). In this study, it is probably that the dentin removal was less effective in the palatal canals due to their larger volume compared to other canals of maxillary molars.

Canal transportation indicates excessive dentin removal from the canals and the formation of gaps/perforations, and the choice of the ideal file can minimize this effect (Peters, 2004; Manker et al., 2020). Nickel-titanium (NiTi) rotary instruments, such as those used in our study, have become popular owing to their superior flexibility (Ebihara et al., 2011) and canal-centering ability (Gambill et al., 2996) compared with stainless steel instruments, following the original anatomy of the curved canals of primary teeth, and minimizing the risk of procedural errors (Guelzow et al., 2005). In particular, heat treatment changes the phase transformation temperature of the NiTi alloy, which results in the growth of soft and ductile phases, i.e., the martensite phase and R-phase, thereby enhancing the fracture resistance and flexibility (Zupanc et al., 2018). In the current study, statistical difference was not observed between the endodontic files regarding the canal transportation and ability of centralization (Table 2). Similar results between rotary and reciprocating files were also observed by Nazari Moghadam et al. (2019) and different results between those instruments were observed by de Camargo et al., (2021) and Gergi et al. (2015). In the present study, statistical differences among the canal thirds in the canal transportation were evidenced comparing both cervical and middle thirds with apical thirds, especially for specimens treated with rotary files, different from the results obtained by Nazari Moghadam et al. (2019). These findings can be explained by the fact that rotary files – Sequence Baby NiTi Files© - involves a sequence of 4 files with different conicities #17/08 (11 mm), #20/04, #25/04 and #30/04 (16 mm) for instrumentation. Shaikh and Goswami (2018) compared the cleaning and shaping effectiveness of rotary, sonic, and conventional instruments in primary root canals and found greater deviations at cervical third, followed by middle and apical thirds. In the current study, both files caused higher transportation to the opposite side of the furcation in canals and most of them were close to zero, which is in accordance with the studies by Dhingra et al. (2014) and Nazari Moghadam et al. (2019). According to Peters (2004), canal transportation at 0.1 mm or less is clinically acceptable. In summary, both endodontic files used during root canal preparation showed general safety, because neither group excessively removed dentin in the cervical, middle, or apical thirds.

In general, in the present study, there was no difference on dentin thickness in the inner side of canals comparing pre and pos-operatory time-points for both endodontic files, however, differences among the thirds were observed for most of canals treated at both time-points, with the lowest results for apical third, as expected. Previous study

showed that a limit of 0.3 mm dentin thickness for root canals instrumented with stainless steel files demonstrated general safety and minimized risks of cracking and perforations (Lim and Stock, 1987). In this current study, root fracture was only observed in three specimens treated with SBF and in three specimens treated with XBF, with more than one type of fracture in some of them. It is important to mention that the prototypes used in the present study and in a previous study (Moraes et al., 2021) did not share the same mechanical properties as the dentin of primary teeth. The difference in elastic modulus between the resin prototype material (2GPa) (Moraes et al., 2019) and primary root dentin (11.6GPa) (Angker et al., 2003) indicates that the prototype can likely possess higher deformation elastic than the dentin of the primary root. The formation of fractures and cracks in the dentin of the primary root, in response to mechanical instrumentation, may occur more rapidly than recorded in artificial teeth (Moraes et al., 2021).

In Pediatric Dentistry clinical practice, pulpectomy should be an uncomplicated procedure with a short duration to increase the patient cooperation and the success of the treatment (Manker et al., 2020). In the present study, as expected, the instrumentation time was shorter with reciprocating files compared to rotary files, as observed in other previous studies (Paqué et al. 2011; Katge et al., 2014; Ramazani et al., 2016; Manker et al., 2020; Barasuol et al., 2021). We used only two files (one for glide path and another for root canal preparation) in the protocol of reciprocating group different from rotary files which uses four files and requires more time for the instruments to be changed. Considering the clinical relevance, there are several advantages related to the use of these mechanized files in primary teeth, especially the reduction of dental chair time, increased patient cooperation and better preparation of root canals for uniform obturation and rapid tissue reabsorption. Due to the scarcity of studies on endodontics in primary teeth in the literature, this study serves as a basis for future clinical studies.

The limitations of this study can be related to possible small defects caused by 3D printing process in both internal root canal surfaces and external surfaces; however, we used the same molars to evaluate pre- and post-operatory time-points and the same type of maxillary and mandibular molars to compare the endodontic files to minimize the impact of those limitations in the results. Another limitation is the difference between file size and taper from each instrumentation system, however, we focused more on “cleaning” than on “shaping” to minimize excessive dentin removal and used the

appropriate movement amplitude inside the root canal, as proposed by Moraes et al. (2021).

In conclusion, both protocols of endodontic instrumentation with rotary files - Sequence Baby NiTi Files© and reciprocating files - X1-Blue File Glide Path and X1-Blue File reciprocating NiTi files© - presented general safety to be used for the pulpectomy in primary teeth, since they promoted minimal decrease in dentin volume, canal transportation and decentralization, minimizing the reduction of dentin thickness and fractures. Although time of instrumentation was shorter for reciprocating files, it causes higher increase in canal volume compared to rotary files.

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Antibiofilm properties, cytotoxicity, and effect on protease activity of antibiotics and EGCG-based medications for endodontic purposes*

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*This article followed the guidelines of the journal Dental Materials (ANEXO 5).

Abstract

Objective: To evaluate the effect of two medications: a triantibiotic paste (TRI) of metronidazol, ciprofloxacin and fosfomycin and the association of epigallocatechin-3-gallate (EGCG) with Fosfomycin (FOSFO), compared to controls calcium hydroxide paste (CH) or chlorhexidine (CHX), all dissolved in polyethylene glycol 400 (PEG) on multispecies biofilms, murine fibroblast (3T3) cytotoxicity and on collagenolytic and gelatinolytic activities detected in radicular dentin.

Methods: The antibiofilm effect and cytotoxicity of medications containing triantibiotic combination, EGCG + FOSFO or CH were evaluated on multispecies biofilms formed in root dentin specimens by confocal microscopy and on fibroblast cells by resazurin assays, respectively. Inhibition of protease activity by medications was measured by collagenolytic enzyme activity (Enzyme-linked immunosorbent assay – ELISA) assays and gelatinolytic activity by metalloproteinases (MMPs) with *in situ* zymography in radicular dentin specimens.

Results: EGCG+FOSFO, TRI, and CH significantly reduced the growth of multispecies biofilms in dentin tubules and at concentrations that were found to non-cytotoxic to 3T3 cells. Additionally, all medications presented antiproteolytic activity reducing telopeptide fragments released from radicular dentin compared to PEG and water controls. PEG+CH and PEG+CHX decreased the *in situ* gelatinolytic activity of MMPs when compared to control. Interestingly, PEG+TRI was found to increase gelatinolytic activity in radicular dentin.

Significance: EGCG+FOSFO in PEG-400 presented both antibiofilm and antiproteolytic properties at concentrations that elicited minimal cytotoxicity of 3T3 cells and could be used as an alternative intracanal medication for endodontic purposes.

Keywords: endodontics, antibiotics, antimicrobial, biofilms, collagen degradation, metalloproteinases.

1. Introduction

Dental pulp is a sterile and highly vascularized tissue enclosed within the mineralized enamel and dentin of health dentition. Following dental caries progression or trauma, oral microorganisms can penetrate thorough dentin tubules and form clusters of multispecies biofilms on the dentinal walls of root canal space – leading to progressive decay and damage. Even following endodontic treatment, such biofilms can persist especially in areas of ramifications and isthmus [1]. During the inflammatory process, pulp cells that include fibroblasts and macrophages, are activated by bacterial toxins and metabolites, and release a variety of inflammatory mediators which are capable of regulating pro- and anti-inflammatory immune responses [2]. Additionally, enzymes such as metalloproteinases (MMPs) that are responsible for catalyzing the turnover of extracellular matrix macromolecules such as collagen, decorin, biglycans, fibromodulins and fibronectin are involved in the pathogenesis of inflammation, stimulating both immunity mechanisms and tissue destruction [3,4]. In low pH environments, such as infected endodontic space, can activate exposed dentinal MMPs (especially MMP-2 and MMP-9) and cysteine cathepsins (CT-B, CT-K, CT-L) which have previously been established to both contribute to the degradation of the dentin-pulp complex [5,6].

MMPs are organized into groups of collagenases, gelatinases, stromalisins, among others, and degrade native collagen at specific sites in ¾ of the N-terminal fragment and ¼ of the C-terminal [7] which can be effectively quantified *in situ* (via ICTP, type I collagen crosslinked carboxyterminal telopeptide) [8]. Previous studies have also effectively quantified the release of C-terminal telopeptides from CT-K cleaved type I collagen (via CTX, cross-linked C-terminal telopeptide from type I collagen) [9]. However, in addition to ICTP and CTX, another important macromolecular product of collagen degradation associated with CT-K activity [10] is cross-linked N-telopeptide (NTX) [11].

To effectively control microbial proliferation within the root canal system and inflammation in the periapical or interradicular areas, the use of an intracanal medication (IM) between sessions is an essential facet of endodontic treatment to ensure favorable prognosis in both primary and permanent teeth [12]. However, there is currently no consensus on the ideal IM for such treatment [13]. Aqueous calcium hydroxide paste (CH) is frequently used as an IM for its biocompatibility and potential to stimulate periapical repair [14]. Despite its extensive and historical use, CH paste has displayed limited

antimicrobial activity and low solubility, which hinders the diffusion of hydroxyl ions to more distant and frequently infected areas, such as the periapical region [15]. To overcome these disadvantages, alternative non-aqueous solvents, such as polyethylene glycol (PEG-400), have been studied to prolong the functional properties of CH and reduce the number of appointments of IM, particularly in cases of periapical infection [16]. Studies have shown enhanced clinical efficiency of CH pastes associated with PEG-400 for different applications in endodontics [17,18].

Considering the polymicrobial nature of endodontic infections and the difficulty of eradicating microorganisms, studies have investigated the therapeutic potential of intracanal antibiotics to increase antimicrobial effects [19,20]. Metronidazole, ciprofloxacin, and minocycline are components of a commonly used triantibiotic paste (TRI), that was previously shown to be effective in reducing bacteria isolated from infected dentin, infected pulps, and apical periodontitis, where their combination was potent enough to eradicate bacteria in all samples [21]. TRI has since been the subject of clinical studies, mainly in young permanent teeth with pulp necrosis [20,22]. Despite its antimicrobial effectiveness, modern TRI face clinical challenges in widespread adoption. For example, minocycline has been found to cause tooth staining and is no longer indicated to be used in TRI [19] which has resulted in investigations into alternative antibiotics [23]. Fosfomycin, a potent inhibitor of the MurA protein involved in cell wall biogenesis, is a broad-spectrum antibiotic that has been suggested as a potential substitute for minocycline in TRI [24]. However, studies are still investigating the ideal concentrations of alternative antibiotics that conserving their antimicrobial efficacy without causing cytotoxicity and suitable drug delivery systems to facilitate the application of the TRI and prolong its therapeutic effect [23].

Among natural products, epigallocatechin gallate (EGCG), a phenolic compound extracted from green tea leaves, has been recognized for having a broad spectrum of therapeutic action, including antimicrobial, anti-inflammatory, antioxidant properties, among others [25], in addition to an inhibitory effect against MMPs [26] and cysteine cathepsins [6]. Considering the high cost of polyphenols, studies have associated EGCG with antibiotics and observed an increase in the effect on oral biofilms at lower concentrations [27,28]. Additionally, an *in vitro* study evaluating the effect of EGCG (delivered with PEG-400) as an IM found maintained physicochemical properties and no staining in deciduous and permanent teeth up to 56 days after its application [29]. EGCG

also demonstrated a suppression of NF-κb (nuclear factor Kappa b) activation, leading to inhibition of pro-inflammatory cytokine release in bacteria-exposed dental pulp cells [30]. A recent study also illustrated the synergistic antimicrobial potential of EGCG combined with fosfomycin against multispecies biofilms associated with endodontic infections at concentration causing limited cytotoxicity [31]. Despite these promising preliminary evaluations of EGCG as an IM, future studies are necessary to evaluate other therapeutic properties of antibiotic and EGCG combinations and vehicles to effectively delivery such compounds.

The aim of this study was to evaluate the effect of two medications: a triantibiotic paste (TRI) composed of metronidazol, ciprofloxacin and fosfomycin and the combination of EGCG with Fosfomycin (FOSFO), compared to clinical medicaments calcium hydroxide paste (CH) and chlorhexidine (CHX), all dissolved in PEG-400, on multispecies biofilms, fibroblast viability and collagenolytic and gelatinolytic activities presented in radicular dentin. The null hypothesis is that TRI and EGCG+FOSFO will not provide an antimicrobial effect against multispecies biofilms without cause cytotoxicity, and, that the combination of EGCG will not present an additional inhibitory effect on collagenolytic and gelatinolytic activities detected in radicular dentin.

2. Materials and methods

The following antimicrobial agents were evaluated: epigallocatechin gallate (EGCG, #E4143), metronidazole (METRO, #M3761), ciprofloxacin (CIPRO, #17850), fosfomycin (FOSFO, #34089), obtained from Sigma-Aldrich (St. Louis, MO, USA). The compounds were all solids, of analytical standard, >90% (thin layer chromatography). Stock solutions were prepared in deionized water for all compounds. Chlorhexidine gluconate (CHX, Manipullis Pharmacy, Araçatuba – SP/Brazil) and calcium hydroxide (CH, Biodinâmica, Ibiporã, PR, Brazil) diluted in sterile water were used as control. The compounds were filtered on a 0.22 μm membrane filter. All following assays were performed in triplicate on three independent days.

2.1. Preparation of IMs

The concentrations for antibiotic, EGCG+FOSFO combinations and controls (CH and CHX) were based on microbiological results obtained by previous studies [31,32]. encompassing minimal bactericidal concentration -MBC [10x minimum inhibitory

concentration (MIC)] and fractional inhibitory concentration (FIC) (ANEXO 1). The combinations of EGCG (at 0.625mg/mL) and FOSFO (at 0.156mg/mL), triantibiotic paste (TRI) with METRO, CIPRO and FOSFO (all at 0.625 mg/mL) and controls CH (at 1 mg/mL) or CHX (at 0.5 mg/mL) were mixed with 1mL PEG-400 for subsequent assays. Water and/or PEG-400 were used as negative controls.

2.2. Biofilm assays in radicular dentin and confocal microscopy

2.2.1. Microbial strains and growth conditions

To evaluate the antimicrobial activity of the compounds, the following standard strains were used: *Enterococcus faecalis* (ATCC 51299), *Actinomyces israelii* (ATCC 12102), *Streptococcus mutans* (ATCC 25175), and *Fusobacterium nucleatum* (ATCC 25586). The strains were donated by the Oswaldo Cruz Foundation – FIOCRUZ, Rio de Janeiro. The culture media used for each bacterial species were Mitis Agar Salivarius Agar (Difco Laboratories, Kansas City, MO, USA) with 0.2 U/mL bacitracin for *S. mutans*, Brain Heart Infusion Agar – BHIA (Difco) for *A. israelii* and *E. faecalis*, and BHIA containing 5mg/mL of hemin, 5mg/mL of menadione, 0.5% of yeast extract and 5% of defibrinated sheep blood for *F. nucleatum*. The microorganisms were incubated at 37 °C in an atmosphere of 5% CO_2, except for *F. nucleatum* which was grown in jars with an anaerobic system (Anaerogen, Oxoid^{TM}, Thermo Fisher Scientific, Waltham, MA, USA) containing 5% CO_2, 10% H_2 and 85% N_2.

2.2.2. Biofilms assays and confocal laser scanning microscopy (CLSM)

The effect of IMs on multispecies biofilms formed in bovine dentin roots were evaluated by CLSM according to methodology proposed by Ma et al. (2011) [33] and Duque et al. (2023) [31]. After approval of the Ethics Committee on the Use of Animal (0078-2022) (ANEXO 2), bovine roots (n=3/group) were sectioned horizontally 1mm below the cementoenamel junction to obtain a dentin block with a length of 4mm. Root canals were enlarged with Gates Glidden drill #6 (1.5mm diameter) and fractured into two semi-cylindrical halves. The outer surfaces of halves were ground by 600-grit silicon carbide paper and the end block size was 4mm x 4mm x 2mm. The blocks were cleaned in ultrasonic bath using 17% EDTA solution for 3 min and distilled water for 5 min. After cleaning, the blocks were dried, sterilized in autoclave and inserted in a microtube with the canal side up and gaps sealed with composite resin. Bacterial suspensions of *E.*

faecalis, S. mutans, A. israelii and F. nucleatum (all at 1-5x10^3 CFU/mL) were mixed in equal aliquots in BHI broth containing 1% glucose and added to each microtube with dentin blocks inside. The microtubes were harvested at 1400g, 2000g, 3600g and 5600g in a sequence twice each for 5 minutes and a fresh solution of bacteria was inserted between every centrifugation and the last solution was discarded.

Dentin blocks were incubated individually in 24-wells plates in BHI broth for 14 days, replacing culture medium every 48h. After this period, blocks were washed twice with sterile saline and transferred, under aseptic conditions, to a new plate and exposed to the following medications: PEG+EGCG/FOSFO, PEG + TRI, PEG+CH, and PEG (for concentrations see item 2.1) for 48h and 7 days. After that, dentin blocks were washed, cut into 2 new halves, and stained with 100μL of fluorescent LIVE/DEAD BacLight Bacterial Viability stain (L13152, Molecular Probes, Eugene, OR) containing SYTO 9 and propidium iodide, according to the manufacturer's instructions. Two additional uninfected specimens were stained using the same protocol as negative controls. Fluorescence from the stained cells was viewed using CLSM (Leica TCS SP5, Microsystems GmbH) and images were acquired using software (LAS AF Leica Microsystems) at a resolution of 1024 by 1024 pixels. The quantification of red fluorescence ratio (dead cells) in relation to green-and-red (total cells) fluorescence was determined using Image J software and the results were expressed in percentage.

2.3. Effect of medications on fibroblast viability

Murine fibroblast cells (NHI-3T3, CRL 1658) were maintained in Dulbecco's Modified Eagle's medium (DMEM; with high glucose, L-glutamine, and sodium pyruvate) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), with 100 IU/mL of penicillin, 100 mg/mL of streptomycin and 0.25 mg/mL of fungizone (Gibco). Cells were subcultured every 48h and allowed to grow in a humidified incubator at 37°C with 5% CO2 (Isotemp; Fisher Scientific, Pittsburgh, PA, USA).

After 48h of growth in DMEM, fibroblasts were seeded in DMEM in a 96-well plate at 2× 10^4 cells/200 μL/well and incubated for 24h. Then, the cells were exposed to serial dilutions of the medications (1:25, 1:50, 1:100, 1:150, 1:200): 1) PEG+EGCG/FOSFO, 2) PEG + TRI (METRO+CIPRO+FOSFO), 3) PEG+CH, 4) PEG, at concentrations showed in item 2.1, for 24h. The metabolic activity of the cells was evaluated by resazurin reagent (Thermo Fisher Scientific) at 550 and 600nm and

determined in percentage in relation to the control (DMEM, 100% cell viability). [34] Results above 80% demonstrate cytocompatibility of the compounds [35].

2.4. Collagenolytic enzyme activity assays

2.4.1. Preparation of demineralized dentin samples

After approval of Research Ethics Committee (protocol nº 72332323.3.0000.5420) (ANEXO 3), 20 recently extracted sound human third molars were cleaned, stored in 1% NaCl at 4 °C and sterilized with gamma radiation at a dosage of 2.5 MRad before use [36]. Discs were obtained from the middle third root dentine of human third molars by cutting 0.5 ± 0.1 mm slabs with diamond saw under water cooling (Isomet, Buehler Ltd, Lake Bluff, IL). The dentin discs were submerged in 10% H_3PO_4 (LabChem, Zelienople, PA, USA) for 40h at room temperature under agitation to completely demineralize dentin [37]. After that, the dentin discs were rinsed with deionized water for 5 min and pulverized to yield dentin powder using a freezer/mill machine (SPEX Sample Prep, Metuchen, NJ). 20 mg of dentin powder was placed in 5 microtubes and treated with 500 μL of 0.1 M lactic acid (LA) pH 5.5 for 30 min [37]. Subsequently, the dentin powder was washed and centrifuged, and each of the new subgroup had the dentin powder treated for 1 h with one of the following solutions: PEG+EGCG/FOSFO, PEG + TRI (METRO+CIPRO+FOSFO), PEG+CH, PEG and Water (see item 2.1). Then, the dentin powder of each group was centrifuged and incubated in 1 mL of buffer medium at 37 °C for 7 days [38].

2.4.2. Total protein and solubilized telopeptide assays

Aliquots of the buffer media were used for total protein and for solubilized telopeptides analysis. The Pierce Bicinchoninic Acid Assay (Thermo Scientific) was used to measure the total protein concentration at 562 nm. Dentin collagen degradation was determined by measuring the quantities of solubilized C-terminal telopeptides ICTP (#MBS040005) and CTX (#MBS731778C) and N-terminal telopeptide NTX (#MBS2700252) in the incubation media using respective ELISA kits (MyBioSource Inc., San Diego, CA, USA) (n=6). Concentrations of solubilized telopeptides were normalized to total protein (TP) concentration (ng/mg) [39].

2.5. Analysis of MMPs activity *in situ* zymography

2.5.1. Samples and root canal preparation

After approval of Research Ethics Committee (protocol nº 72332323.3.0000.5420) (ANEXO 3), 12 recently extracted healthy human third molars were stored in 1% NaCl at 4 °C and sterilized with gamma radiation before use [36]. The crown of each tooth was transversely cut at the cement–enamel junction (CEJ) with diamond saw (Buehler Ltd., Lake Bluff, IL, USA) under water cooling. All root canal treatments were performed by a single operator (DAC), following the criteria described by Baruwa et al. (2022) [40]. The root canals were instrumented according to the instrumentation technique of Dia-X rotary files (DiaDent) #18.02, #18.04, #20.06, #25.07. This instrumentation was performed using an E-connect Pro endodontic motor (MKLife, Porto Alegre, RS, Brazil) and irrigation with 1mL of water using a 27-gauge irrigation needle (Henry Schein, New York, NY, USA) in a 5mL syringe. After irrigation, the canals were dried with sterile absorbent paper points. The samples were distributed equally into 6 groups (n = 2), according to the IMs: PEG+EGCG/FOSFO, PEG + TRI (METRO+CIPRO+FOSFO), PEG+CH, PEG+CHX, PEG and Water (see item 2.1). After IM insertion, the canal orifice was sealed with utility wax and restored with Z250 composite resin (3M ESPE, St. Paul, USA). The roots were wrapped in gauze wet and stored at 37 °C for 14 days until preparation for zymography [40].

2.5.2. *In situ* zymography

After 14 days, the teeth were cut to obtain longitudinal slices of 200 μm thickness using a water-cooled low speed (Buehler Ltd., Lake Bluff, IL, USA). The slices were immersed in 10% phosphoric acid for 30 seconds and then washed with deionized water for 1 minute. *In situ* zymography was performed using a quenched fluorescein-conjugated gelatin as substrate (E-12055, Molecular Probes, Eugene, OR, USA), as described by Mazzoni et al. (2012) [41]. The gelatin stock solution was diluted 1:8 in a buffer (NaCl 150 mM, $CaCl_2$ 5 mM, Tris-HCl 50 mM, pH 8.0) with 10 μL of anti-fading agent (Mounting Medium with Dapi H-1200, Vectashield, Vector Laboratories LTD, Cambridgeshire, UK) [42].

Each dental slice was placed on the glass slide into a small Petri dish and immersed in 80 μL of fluorescent gelatin mixture, prior to being covered with a coverslip sealed with a self-cure nail varnish. Thereafter, all slides were stored at 37 °C, and protected

from light for 24 h. The enzymatic gelatin activity of each sample was assessed based on hydrolysis of the quenched fluorescein-conjugated gelatin as the MMP substrate (E-12055, Molecular Probes, Eugene, OR, USA), and evaluated under a multi-photon confocal laser scanning microscope (LSM 5 Pa: Carl Zeiss, Oberkochen, Germany) with excitation and emission wavelengths of 488 nm and 530 nm, respectively [40,42]. Multiple optical sections of 85 μm-thick were acquired from different focal planes from each root section, which were stacked, quantified, and processed using ZEN 2010 software (Carl Zeiss, Oberkochen, Germany) before analysis. The enzymatic activity of each group was quantified by fluorescence in the radicular dentine adjacent to IM. Five measurements at different sites on each image was recorded using a standardized rectangular selection and then the mean of intensity (pixels/area μm^2) of the green fluorescence was determined using Image J software (National Institutes of Health, Bethesda, MD, USA) [40].

2.6. Statistical Analysis

Data from assays were expressed in means/standard deviations and submitted to ANOVA and Tukey's tests, considering each compound or IM separately. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to run the statistical analysis considering $p<0.05$.

3. Results

3.1. Inhibition of multispecies biofilms in radicular dentin by medications

Figure 1A-E show representative confocal laser scanning microscopy images of bovine root dentin specimens contaminated for 14 days with multispecies biofilms and treated for 48 h with PEG+EGCG/FOSFO; PEG+TRI (METRO+CIPRO+FOSFO); PEG+CH and PEG. In **Figures 1A to 1C**, high predominance of red-stained cells was observed for all experimental medications containing EGCG+FOSFO, TRI and CH. More than 80% of dead cells were detected in multispecies biofilms for all those groups, without statistical difference among them, showing short-term antibiofilm effect of medications, as we can see in **Figure 1F**. **Figure 2A-E** show representative confocal laser scanning microscopy images of bovine root dentin specimens contaminated for 14 days with multispecies biofilms and treated for 7 days with PEG+EGCG/FOSFO; PEG+TRI (METRO+CIPRO+FOSFO); PEG+CH and PEG. In **Figures 2A to 2B**, groups PEG+EGCG/FOSFO and PEG+TRI (METRO+CIPRO+FOSFO) presented the highest

predominance of red-stained cells compared to other groups. 82.94%, 64.88% and 54.45% of dead cells were counted for multispecies biofilms treated for 7 days with PEG+EGCG/FOSFO, PEG+TRI and PEG+CH, respectively, showing long-term antibiofilm effect of medications, as we can see in **Figure 2F**.

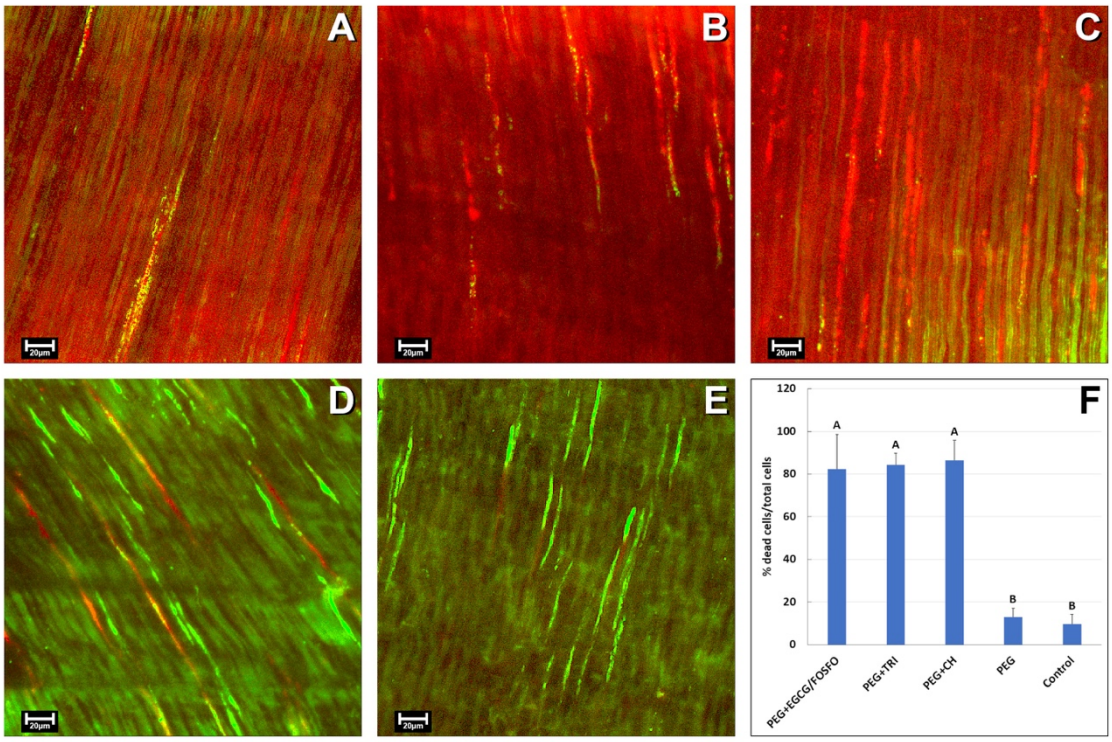

Figure 1. Representative confocal laser scanning microscopy images of bovine root dentin specimens contaminated for 14 days with multispecies biofilms and treated for 48 h with the following groups with A: PEG+EGCG/FOSFO; B – PEG+TRI (METRO+CIPRO+FOSFO); C – PEG+CH; D – PEG; E - Control – Bacterial growth without antimicrobial agents. F. Mean (SD) of the percentages of dead cells of multispecies biofilms formed in root dentin of bovine teeth, after 48 h of treatment with antimicrobial agents and controls.

[A]Different letters show statistical difference between the antimicrobial groups, according to One-Way ANOVA and Tukey test ($p<0.05$).

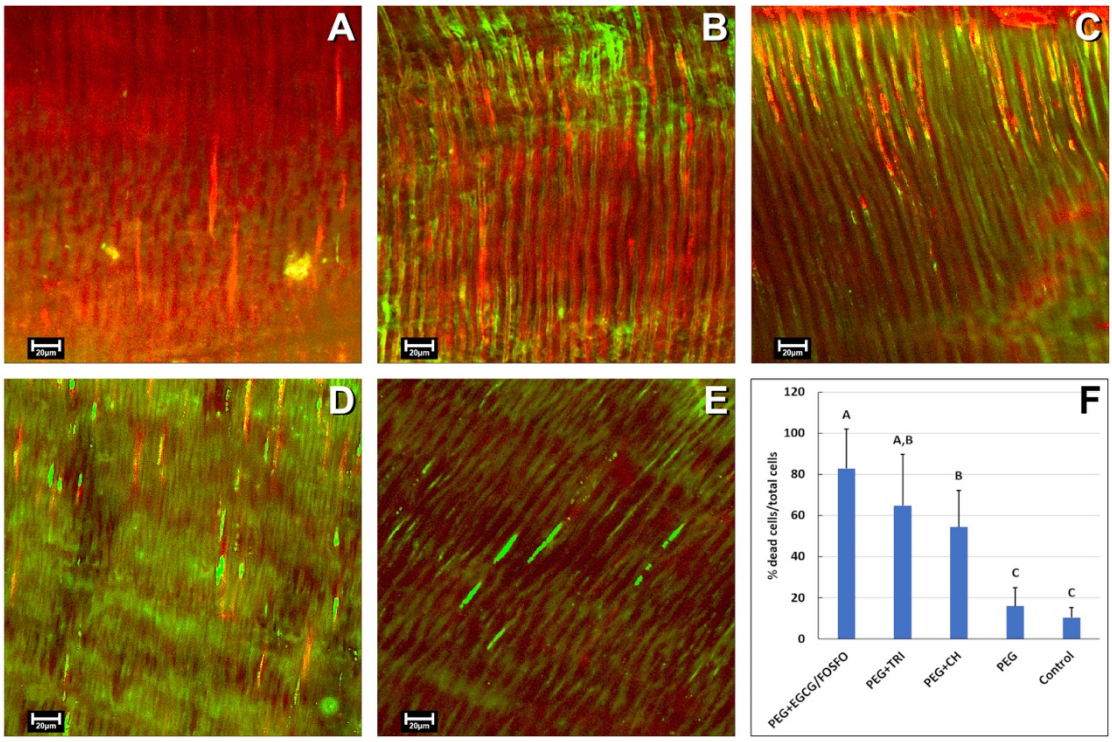

Figure 2. Representative confocal laser scanning microscopy images of bovine root dentin specimens contaminated for 14 days with multispecies biofilms and treated for 7 days with the following groups with A: PEG+EGCG/FOSFO; B – PEG+TRI (METRO+CIPRO+FOSFO); C – PEG+CH; D – PEG; E - Control – Bacterial growth without antimicrobial agents. F. Mean (SD) of the percentages of dead cells of multispecies biofilms formed in root dentin of bovine teeth, after 7 days of treatment with antimicrobial agents and controls.

[A]Different letters show statistical difference between the antimicrobial groups, according to One-Way ANOVA and Tukey test ($p<0.05$).

3.2. Effect of medications on fibroblast viability

The effect of the medications on 3T3 viability after 24 h of exposure to their dilutions can be seen on **Figure 3**. All medications at D1:25 and D1:50 were not cytocompatible promoting a reduction of more than 30% of cell viability, without statistical difference among the groups. At D1:100, cell viability was superior to 80% for all groups (80.71%, 93.61% and 93% for PEG+EGCG/FOSFO, PEG+TRI and PEG+CH, respectively), showing cytocompatibility of medications at lower concentrations. All medications at concentrations above D:1:100 were cytocompatible.

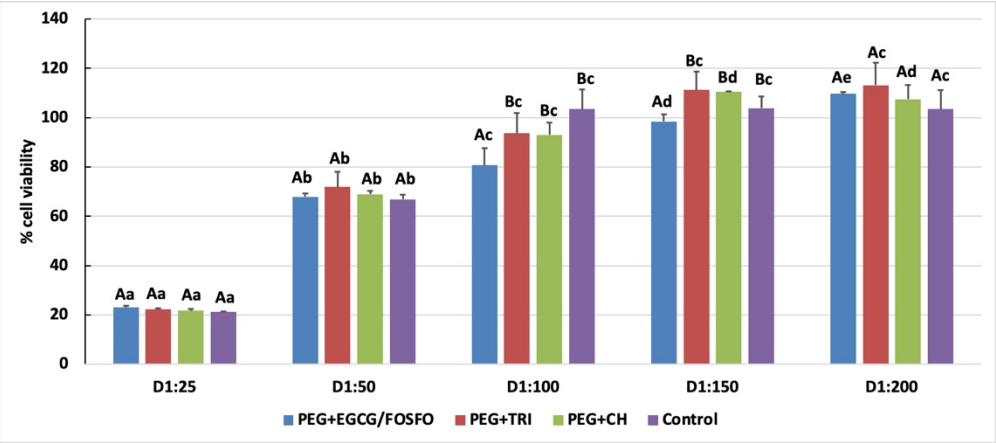

Figure 3. Fibroblast viability after 24h of exposure to dilutions (D1:25, D1:50, D1:100, D1:150 and D1:200) of medications.

[A] Different upper-case letters show statistical differences among the groups, in each dilution, according to ANOVA and Tukey, considering $p<0.05$.

[a] Different lower-case letters show statistical differences among the concentration, considering each group of medication separately, according to ANOVA and Tukey, considering $p<0.05$.

3.3. Effect of IMs on collagenolytic activity

Figure 4 shows the effect of medications on dentin collagen degradation which was determined by the quantities of solubilized C-terminal collagen telopeptides ICTP and CTX and solubilized N-terminal collagen telopeptide NTX in incubation media using respective ELISA methods. Medications of EGCG+FOSFO, TRI and CH in PEG 400 had antiproteolytic activity reducing the quantity of collagen telopeptides (ICTP, CTX and NTX) released from radicular dentin, when compared to controls (water and PEG). The lowest levels of ICTP and NTX were observed in samples from groups PEG+CH, followed by PEG +EGCG/FOSFO. For CTX, the lowest levels were detected by samples treated with PEG +EGCG/FOSFO.

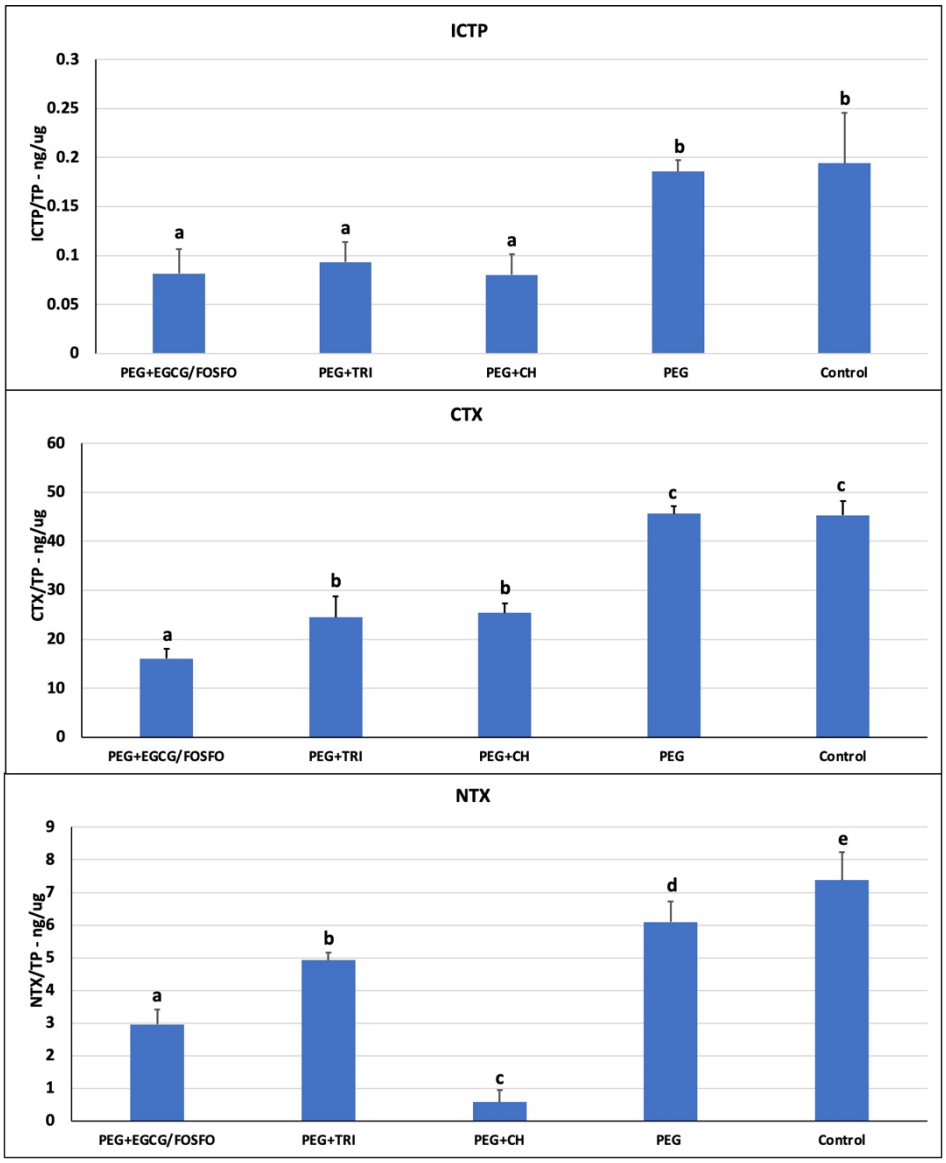

Figure 4. Dentin collagen degradation determined by quantification of solubilized C-terminal telopeptides (ICTP and CTX) and N-terminal telopeptides (NTX). The values are expressed in proportion of telopeptides (ICTP, CTX and NTX in µg/mL) under total protein content (TP in µg/mL).

[a] Different lowercase letters show statistical difference among the groups, according to ANOVA and Tukey, considering $p<0.05$.

3.4. Effect on MMPs activity by IMs

Figure 5 shows the representative images of *in situ* zymography of dentin slices treated with IMs. Gelatinolytic activity of MMPs was determined by the intensity of fluorescence by hydrolyzation of fluorescein conjugated gelatin in the dentin interface between medication and root canal walls. PEG+CH and PEG+CHX presented the lowest means of fluorescein intensity, indicating inhibition of gelatinolytic activity in dentin. Although

lower levels of fluorescein intensity were observed for PEG+EGCG/FOSFO specimens compared to PEG+TRI, however, there was no significant difference compared to PEG and control. PEG+TRI increases gelatinolytic activity expressed in dentin radicular specimens.

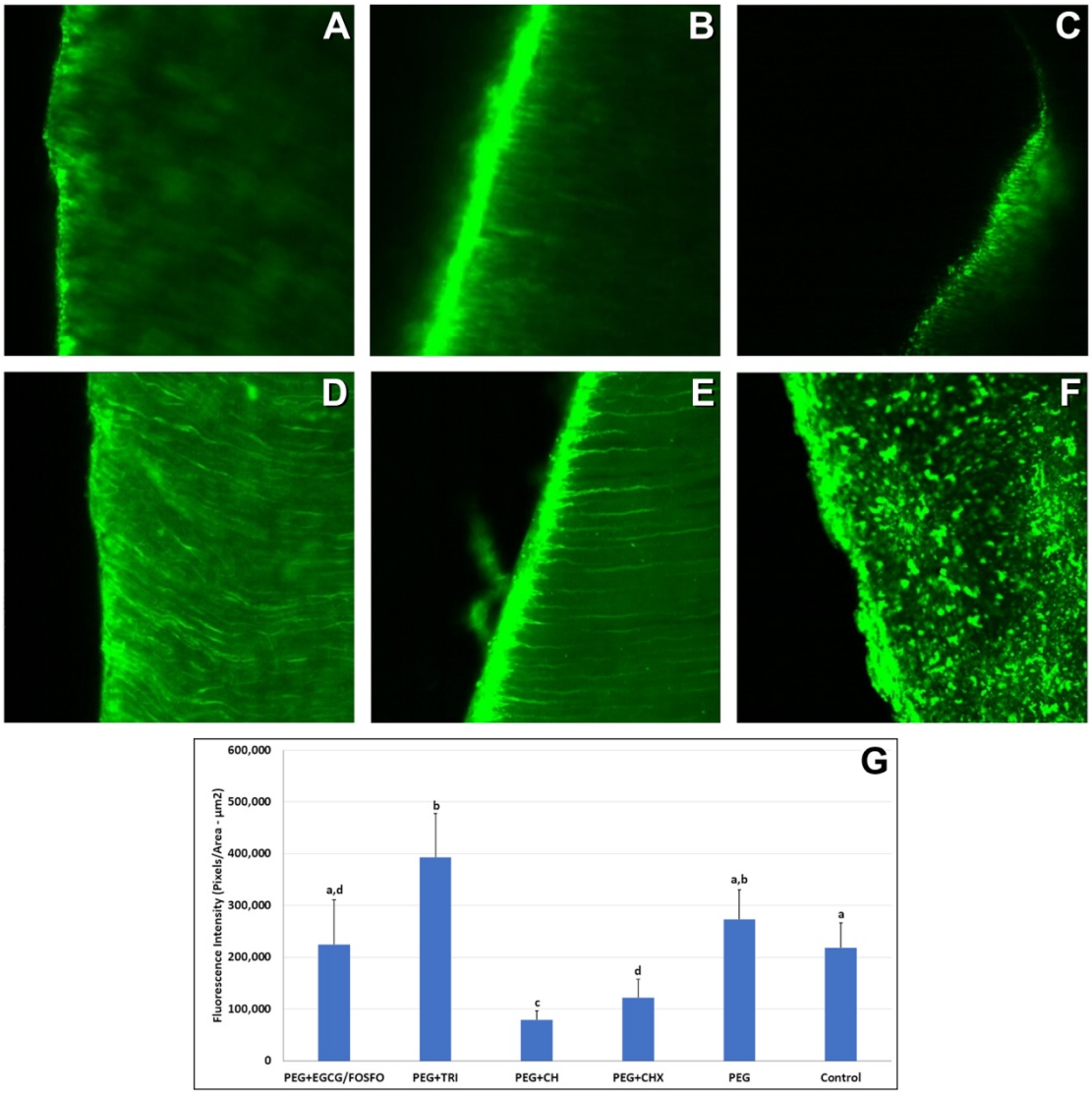

Figure 5. Effect of IMs on dentin MMP inhibition by *in situ* zymography. **A-F.** Representative images of confocal microscopy showing dentin slices after treatment with the experimental groups A: PEG+EGCG/FOSFO; B: PEG+TRI; C: PEG+CH and D: PEG+CHX compared to controls (E: PEG or F: water) and incubation for 24h with quenched fluorescein-labeled gelatin. **G.** Quantitative analysis of fluorescence observed *in situ* zymography of samples treated with the same medications. The values are means /standard deviation of fluorescence intensity which represents hydrolyzation of fluorescein by dentin gelatinases.

[a]Different letters show statistical differences among the groups according to ANOVA and Tukey, considering $p<0.05$.

4. Discussion

Endodontic treatments, in addition to the complexity of the root canal system, present clinical challenges due to the polymicrobial nature of intracanal biofilm. Other limitations for clinical success include the natural root resorption in primary teeth and incomplete root formation in young permanent teeth and the persistence of the inflammatory processes inside root canals that increases cytokine and MMP release, leading to collagen degradation and bone resorption in the inter-radicular and periapical space [2,5].

In this study, EGCG+FOSFO delivered with PEG-400 presented an inhibitory effect on multispecies biofilms and provided antiproteolytic properties, while causing low cytotoxicity, rejecting partially the hypothesis proposed. TRI used in the study had both a similar antibiofilm and antiproteolytic efficacy reducing the quantity of collagen telopeptides, however, did not display effectiveness against MMPs activity. The combination of conventional antibiotics with natural compounds such as EGCG has emerged as a strategy to control bacterial resistance and reduce the antimicrobial concentration of natural compounds, which are expensive to synthesize and purify [43]. In a previous study, EGCG+FOSFO demonstrated synergism against planktonic oral bacteria at planktonic concentrations that elected no cytotoxic effects. Additionally, EGCG+FOSFO has also been shown to reduce monospecies and multispecies biofilms formed in microplates and inside dentin tubules, displaying superior performance to chlorhexidine [31]. In the present study, the antibiofilm effect of EGCG+FOSFO loaded in PEG-400 (represented by 82.29 e 82.94% of dead cells after 48h and 7 days of treatment) was similar to the effect of EGCG+FOSFO solution alone (represented by 84.85% of dead cells after 48h of treatment in a previous study [31], showing that PEG-400 allowed the release of EGCG+FOSFO in both moments and the bactericidal effect was sustained for 7 days. The antibacterial mechanism of EGCG involves damage to bacterial cell membranes, inhibiting the bacteria's ability to adhere to host cells, and down-regulating enzymes involved in fatty acid biosynthesis to reduce the production of toxic metabolites [44]. Fosfomycin interferes with the first stage of bacterial cell wall biosynthesis, specifically the formation of the UDP N-acetylmuramic peptidoglycan precursor (UDPMurNAc), interrupting bacterial proliferation [45]. The combination of different mechanisms of action can be a strategy to increase the antibacterial activity and reduce the bacterial resistance. In the current study, the antibiofilm effect of CH, after 7

days of treatment, was lower than that of the other groups, with a statistical difference in relation to the EGCG+FOSFO group. This finding may have occurred due to a possible drying out of the CH during the experiment, damaging its antimicrobial properties.

Cytocompatibility is a crucial property for any medication recommended for endodontic treatment, particularly in primary teeth and young permanent teeth with incomplete root formation [31] in order to maintain periapical cells viable. In this study, all medications delivered with PEG-400 at dilutions 1:100 or below did not affect cell viability (> 80% compared to control culture). Although all medications were found to be toxic to cells at the highest test concentrations, there was no difference among the groups, indicating cytocompatibility of the compounds and the potentially added toxicity related to PEG-400. In a previous study, EGCG at 0.5 mg/mL and FOSFO at 0.125 mg/L, alone or in combination, had a minimal effect on fibroblast viability, showing more than 80% cell viability [31]. PEG-based monomers have showed toxicity on human cervical cancer cells (HeLa) and L929 fibroblast cells, with PEG-400 shown to be cytocompatible up to 20mg/mL for both cell lines [46]. In this study, PEG-400 was used to dissolve all medication and was used at a concentration of 1% (10mg/mL) that did not present cytotoxic effect.

The extracellular matrix (ECM) of dentin is composed primarily of type I collagen and non-collagenous proteins, such as the matrix metalloproteinases (MMPs) and cysteine cathepsins (CT), which are proteolytic enzymes secreted as latent proenzymes and trapped within dentin after dental development. During caries process, microorganisms promote a drop in pH in the dental microenvironment that induces proteases exposure and activation that is thought to contribute to dentin degradation [39]. Endogenous proteases with telopeptidase activity for type I collagen are present in dentin and can result in three major macromolecular degradation products either carboxyl (ICTP and CTX) or amino (NTX) [11]. Some studies have determined the release and quantification of these telopeptides (ICTP, CTX and NTX) from demineralized dentin to measure the effect of dental materials in reducing collagen degradation [8-10,39]. The present study validates the antiproteolytic activity of medications in a model of demineralized dentin. According to our findings, medications with EGCG+FOSFO, TRI and CH significantly decreased the amounts of ICTP, CTX and NTX fragments, in relation to the control group, suggesting a decrease in collagen degradation. In a previous

study, EGCG was also found to protect dentin against degradation measured by the reduction of solubilized telopeptides in demineralized dentin [39].

Some studies have shown that MMPs are relevant to the pathogenesis of pulpal and peri-radicular tissue damage, in particular MMP-2 and MMP-9, which could be acting in the degradation of the ECM, favoring the progression of the lesion and, together with cytokines, contributing to the bone destruction resulting from endodontic infection [47,48]. In this study, only medications with CH and CHX were found to inhibit the activity of MMPs by as analyzed by *in situ* zymography. The protective effect of CHX on collagen degradation and inhibition of endogenous proteases such as MMPs has been widely reported by other studies [49-51] and can be explained by the CHX calcium-chelating mechanism that affects the requirement of MMPs for metal ions for their catalytic activity [52]. CH medications (with sterile saline or associated with 2% CHX gel) effectively reduced the levels of MMP-1, MMP-2 and MMP-9 in periapical tissues after 14 days of root canal treatment [53]. While EGCG+FOSFO did display an effect on MMP expression there were no statistical differences between the control group (without intraconal treatment). Other studies have shown the ability of EGCG to inhibit the recombinant activities of human MMPs and cysteine cathepsins [6,54]. EGCG-treated collagen can effectively resist the degradation of collagenase [55–57] and maintain three-helix structure in the 37°C. However, mechanisms explaining that EGCG can stabilize the collagen and resist collagenase are still in a controversy. Madhan et al. (2007) found that, after EGCG treatment, 95% collagen can resist the degradation of collagenase. They suggested that hydrogen bonding between EGCG and collagenase and hydrophobic interaction inhibit the activity of collagenase, and they observed second structure change of collagenase with round two dichroism spectropolarimeter [56]. Another study observed that EGCG closed collagenase-active site of collagen by being closely conjugated with collagen, which was examined by high performance liquid chromatography and consequently resist hydrolysis of collagenase [57].

In this study, TRI was found to increase the gelatinolytic activity by MMPs in dentin specimens. To the best of the authors knowledge, this is the first study investigating the effect of antibiotics on MMP activity. However, the investigations in this study were conducted *in vitro* that have limitations in reproducing all intraoral conditions, such as the diversity of oral microorganisms present in endodontic infections or the presence of saliva or collagenolytic and gelatinolytic agents that could interact with medications and

dentin [31,58]. Hence, future *in situ* endodontic treatment study models and clinical studies are recommended to better elucidate the effect of medications on the IMs. However, within the limits of this *in vitro* study, it can be concluded that EGCG+FOSFO in PEG-400 can effectively elicit both antibiofilm and antiproteolytic properties, while maintaining high cytocompatibility and could be an alternative IM for endodontic purposes.

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Conclusão Geral

Conclui-se que ambos os protocolos de instrumentação endodôntica foram seguros para serem utilizados na pulpectomia em dentes decíduos, pois promoveram mínima diminuição do volume dentinário, transporte e centralização do canal, minimizando a redução da espessura dentinária e fraturas. Embora o tempo de instrumentação tenha sido menor para as limas reciprocantes, houve maior aumento no volume do canal em comparação às limas rotatórias. Em relação às medicações, EGCG+FOSFO contidos no PEG-400 apresentou propriedades antibiofilme e antiproteolítica, além de baixa citotoxicidade em concentrações mais baixas, podendo ser uma alternativa de medicação intracanal para fins endodônticos.

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ANEXOS

ANEXO 1 – Minimal inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) values

Table 1. Minimal inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) values (in µg/mL) obtained by compounds and control (CHX) after 24 h of exposure, in planktonic conditions.

	MIC (MBC) µg/mL			
	E. faecalis	***A. israelii***	***S. mutans***	***F. nucleatum***
Metronidazol* (METRO)	> 1	0.5 (0.5)	0.5 (1)	0.25 (0.5)
Ciprofloxacin* (CIPRO)	0.015 (0.0625)	0.0019 (0.0039)	0.01562 (0.03125)	0.125 (0.25)
Fosfomycin* (FOSFO)	0.03125 (0.0625)	0.0019 (0.00078)	0.0625 (0.125)	0.0097 (0.0097)
EGCG*	0.25 (0.5)	0.03125 (0.0625)	0.25 (0.5)	0.0625 (0.0625)
EGCG+FOSFO* (FIC)**	0.062 EGCG 0.078 FOSFO	0.0039 EGCG 0.00049 FOSFO	0.062 EGCG 0.0156 FOSFO	0.00156 EGCG 0.0001 FOSFO
Chlorhexidine* (CHX)	0.0078 (0.0078)	0.0019 (0.0019)	0.0097 (0.0019)	0.0078 (0.0078)

These results were obtained by previous studies (Machado, 2016; Duque et al., 2023)

**FIC – Fractional inhibitory concentration - The concentrations of compounds were derived from the highest dilution of the non-growth antimicrobial combination (synergy or additive effect).

ANEXO 2 - CEUA - Ethics Committee on the Use of Animals

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Estudo in vitro de técnicas de instrumentação mecanizada e medicações intracanais como propostas de protocolos para pulpectomia em dentes decíduos"**, Processo FOA nº 0078-2022, sob responsabilidade de Cristiane Duque apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 28 de Março de 2022.

VALIDADE DESTE CERTIFICADO: 31 de Julho de 2023.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 31 de Agosto de 2023.

CERTIFICATE

We certify that the study entitled **"In vitro study of mechanized instrumentation techniques and intracanal medications as proposed protocols for pulpectomy in primary teeth"**, Protocol FOA nº 0078-2022, under the supervision of Cristiane Duque presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on March 28, 2022.

VALIDITY OF THIS CERTIFICATE: July 31, 2023.
DATE OF SUBMISSION OF THE FINAL REPORT: August 31, 2023.

Prof. Dr. João Carlos Callera
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
Rua José Bonifácio, 1193 – Vila Mendonça - CEP: 16015-050 – ARAÇATUBA – SP
Fone (18) 3636-3234 Email CEUA: ceua.foa@unesp.br

ANEXO 3 – Research Human Ethics Committee

UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Citotoxicidade e efeito de medicações baseadas em EGCG sobre o perfil de citocinas em fibroblastos e atividade de metaloproteinases

Pesquisador: DANIELA ALVIM CHRISOSTOMO

Área Temática:

Versão: 3

CAAE: 72332323.3.0000.5420

Instituição Proponente: Universidade Estadual Paulista Júlio de Mesquita Filho

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 6.465.214

Apresentação do Projeto:

Trata-se de reapresentação de projeto de pesquisa, intitulado: Citotoxicidade e efeito de medicações baseadas em EGCG sobre o perfil de citocinas em fibroblastos e atividade de metaloproteinases.

Objetivo da Pesquisa:

Avaliar o efeito da associação de EGCG com fosfomicina em comparação com hidróxido de cálcio e pasta triantibiótica contendo metronidazol, ciprofloxicina e fosfomicina, todos dissolvidos em polietinoglicol 400 (PEG 400), sobre o perfil de citocinas em fibroblastos e sobre a atividade getinolítica de metaloproteinases.

Avaliação dos Riscos e Benefícios:

Riscos Mínimos.

Comentários e Considerações sobre a Pesquisa:

Reapresentação de projeto sem pendências.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos foram apresentados, de acordo com a a Resolução 466 de dezembro de 2012.

Endereço: JOSE BONIFACIO 1193
Bairro: VILA MENDONÇA **CEP:** 16.015-050
UF: SP **Município:** ARACATUBA
Telefone: (18)3636-3234 **Fax:** (18)3636-3203 **E-mail:** cep.foa@unesp.br

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Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

Projeto reapresentado sem pendências, de acordo com as recomendações e normas deste CEP.

Considerações Finais a critério do CEP:

Salientamos que, de acordo com a Resolução 466 CNS, de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, devendo o primeiro relatório ser enviado até 01/04/2024.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_2171064.pdf	04/10/2023 21:32:06		Aceito
Folha de Rosto	Folha_de_Rosto.pdf	04/10/2023 21:31:37	DANIELA ALVIM CHRISOSTOMO	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TERMO_DE_CONSENTIMENTO_LIVRE_E_ESCLARECIDO.docx	04/10/2023 21:31:23	DANIELA ALVIM CHRISOSTOMO	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.docx	04/10/2023 21:31:07	DANIELA ALVIM CHRISOSTOMO	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Endereço: JOSE BONIFACIO 1193
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UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

Continuação do Parecer: 6.465.214

ARACATUBA, 27 de Outubro de 2023

Assinado por:
André Pinheiro de Magalhães Bertoz
(Coordenador(a))

Endereço: JOSE BONIFACIO 1193
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ANEXO 4 - Author Guidelines – International Endodontic Journal – Article 1.

INTERNATIONAL ENDODONTIC JOURNAL
The official journal of the British Endodontic Society and the European Society of Endodontology

Author Guidelines

Submission and Peer Review Process

Once the submission materials have been prepared in accordance with the Author Guidelines, manuscripts should be submitted online at https://wiley.atyponrex.com/journal/IEJ.

This journal does not charge submission fees.

Article Preparation Support

Wiley Editing Services offers expert help with English Language Editing, as well as translation, manuscript formatting, figure illustration, figure formatting, and graphical abstract design – so you can submit your manuscript with confidence.

Also, check out our resources for Preparing Your Article for general guidance about writing and preparing your manuscript.

Free Format submission

International Endodontic Journal now offers Free Format submission for a simplified and streamlined submission process. Before you submit, you will need:

- Your manuscript: this should be an editable file including text, figures, and tables, or separate files – whichever you prefer. All required sections should be contained in your manuscript, including abstract (which does need to be correctly styled), introduction, methods, results, and conclusions. Figures and tables should have legends. Figures should be uploaded in the highest resolution possible. References may be submitted in any style or format, as long as it is consistent throughout the manuscript. Supporting information should be submitted in separate files. If the manuscript, figures or tables are difficult for you to read, they will also be difficult for the editors and reviewers, and the editorial office will send it back to you for revision. Your manuscript may also be sent back to you for revision if the quality of English language is poor.
- An ORCID ID, freely available at https://orcid.org. (Why is this important? Your article, if accepted and published, will be attached to your ORCID profile. Institutions and funders are increasingly requiring authors to have ORCID IDs.)

- The title page of the manuscript, including:
    - data availability statement
    - funding statement
    - conflict of interest disclosure
    - ethics approval statement and document
    - patient consent statement
    - relevant reporting guidelines paperwork (see 5.2 Reporting Guidelines below)
    - permission to reproduce material from other sources
    - clinical trial registration
    - Your co-author details, including affiliation and email address. (Why is this important? We need to keep all co-authors informed of the outcome of the peer review process.)
    - Statements relating to our ethics and integrity policies, which may include any of the following (Why are these important? We need to uphold rigorous ethical standards for the research we consider for publication):

Important: the journal operates a double-blind peer review policy. Please anonymise your manuscript and supply a separate title page file.

Open Access

This journal is a subscription journal that offers an open access option. You will have the option to choose to make your article open access after acceptance, which will be subject to an APC, unless a waiver applies.

Preprint policy:

This journal accepts articles previously published on preprint servers.

International Endodontic Journal will consider for review articles previously available as preprints. You may also post the submitted version of a manuscript to a preprint server at any time. You are requested to update any pre-publication versions with a link to the final published article.

This journal operates a double-blind peer review process. Authors are responsible for anonymizing their manuscript in order to remain anonymous to the reviewers throughout the peer review process (see "Main Text File" above for more details). Since the journal also encourages posting of preprints, however, please note that if authors share their manuscript in preprint form this may compromise their anonymity during peer review.

Data Sharing and Data Availability

This journal encourages data sharing. Review Wiley's Data Sharing policy where you will be able to see and select the data availability statement that is right for your submission.

Data Protection

If you are submitting a manuscript to the journal, or alternatively if you are reviewing for the *International Endodontic Journal*, your name, email address, and affiliation, and other contact details might be required for the regular operations of the publication. Please review Wiley's Data Protection Policy to learn more.

Funding

You should list all funding sources in the Acknowledgments section or in abstract of systematic review. You are responsible for the accuracy of their funder designation. If in doubt, please check the Open Funder Registry for the correct nomenclature.

Authorship

All listed authors should have contributed to the manuscript substantially and have agreed to the final submitted version. Review editorial standards and scroll down for a description of authorship criteria. An author contribution section must be completed for a submitted manuscript.

Conflict of Interest

The journal requires that all authors disclose any potential sources of conflict of interest. Any interest or relationship, financial or otherwise that might be perceived as influencing an author's objectivity is considered a potential source of conflict of interest. These must be disclosed when directly relevant or directly related to the work that the authors describe in their manuscript. The existence of a conflict of interest does not preclude publication. If the authors have no conflict of interest to declare, they must also state this at submission. It is the responsibility of the corresponding author to review this policy with all authors and collectively to disclose with the submission ALL pertinent commercial and other relationships. Please fill out the below form if any authors have a conflict of interest.

Style and Formate

While this journal operates free format, this is the preferred style:

- Double space the text;
- Blank out the funding information at this stage of the review process;
- Remove the keywords from the abstract they should be on the title page only;
- Put a page break between the abstract and the introduction;
- Remove the numbering of the sections in the main document;

Title Page

The title page should contain:

i. A brief informative title containing the major key words. The title should not contain abbreviations;
ii. A short running title of less than 30 characters;
iii. The full names of the authors;
iv. The author's institutional affiliations where the work was conducted, with a footnote for the author's present address if different from where the work was conducted;
v. Acknowledgements.

Important: the journal operates a double-blind peer review policy. Please anonymise your manuscript and prepare a separate title page containing author details.

Main Text File

Please ensure that all identifying information such as author names and affiliations, acknowledgements or explicit mentions of author institution in the text are on a separate page.

All manuscripts submitted to the *International Endodontic Journal* should include Title Page (separate file), Abstract, Main Text, References, Acknowledgements, Funding, Conflict of Interest, Tables, Figures, Checklists and Figure Legends as appropriate.

Manuscripts can be uploaded either as a single document (containing the main text, tables and figures), or with figures and tables provided as separate files. Should your manuscript reach revision stage, figures and tables must be provided as separate files. The main manuscript file can be submitted in Microsoft Word (.doc or .docx) or LaTex (.tex) format.

Reference Style

This journal uses Harvard reference style; as the journal offers Free Format submission, however, this is for information only and you do not need to format the references in your article. This will instead be taken care of by the typesetter.

It is the policy of the journal to encourage reference to the original papers rather than to literature reviews. Authors should therefore keep citations of reviews to the absolute minimum.

Tools such as EndNote or Reference Manager for reference management and formatting can be useful. The EndNote reference style can be obtained upon request to the editorial office (iej.office@wiley.com). Reference Manager reference styles can be searched for here: www.refman.com/support/rmstyles.asp. You may also wish to review your reference style guidelines prior to submission.

Figures and Supporting Information

Figures, supporting information, and appendices should be supplied as separate files. You should review the basic figure requirements for manuscripts for peer review, as

well as the more detailed post-acceptance figure requirements. View Wiley's FAQs on supporting information.

Peer Review

This journal operates under a double-blind peer review model. Except where otherwise stated, manuscripts are peer reviewed by at least two anonymous reviewers and an Associate Editor. Papers will only be sent to review if the Editor-in-Chief determines that the paper meets the appropriate quality and relevance requirements.

In-house submissions, i.e. papers authored by Editors or Editorial Board members of the title, will be sent to Editors unaffiliated with the author or institution and monitored carefully to ensure there is no peer review bias.

Refer and Transfer Program

Wiley believes that no valuable research should go unshared. This journal participates in Wiley's Refer & Transfer program. If your manuscript is not accepted, you may receive a recommendation to transfer your manuscript to another suitable Wiley journal, either through a referral from the journal's editor or through our Transfer Desk Assistant.

Guidelines on Publishing and Research Ethics in Journal Articles

The journal requires that you include in the manuscript (and submit a hard copy) details of IRB approvals, ethical treatment of human and animal research participants, and gathering of informed consent, as appropriate. You will be expected to declare all conflicts of interest, or none, on submission. Please review Wiley's policies surrounding human studies, animal studies, clinical trial registration, biosecurity, and research reporting guidelines.

This journal follows the core practices of the Committee on Publication Ethics (COPE) and handles cases of research and publication misconduct accordingly (https://publicationethics.org/core-practices).

All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study. The authors MUST upload a copy of the ethical approval letter when submitting their manuscript and a separate English translation.

Author Contributions

For all articles, the journal mandates the CRediT (Contribution Roles Taxonomy)—more information is available on our Author Services site.

2. Article Types

Original Articles (includes Clinical Research (randomised control trials, cohort studies, case control studies, cross sectional studies, case series), Basic Research – Biological, Basic Research — Technical, and Education):

These must describe significant and original experimental observations and provide sufficient detail so that the observations can be critically evaluated and, if necessary, repeated. Original Articles must conform to the highest international standards in the field.

Article Type	Structure Description	Abstract / Structure
Original Articles	Main text includes: Introduction, Materials and Methods, Results, Discussion and Conclusion.	Structured: Aim, methodology, results, conclusions, funding, conflict of interest. No more than 350 words
Systematic, Scoping and Narrative Review Articles	Main text includes: Introduction, method, results, discussion, conclusions, funding, Conflict of Interest	Structured: Background, Objectives, Method, Results, Conclusion, Funding, Conflict of interest, Registration No more than 350 words.
Case Reports and Clinical Commentaries	Main text includes: Introduction, report, discussion and conclusion	Structured: Aim, summary, key learning points
Supporting Information	Supporting information, such as data sets or supplemental figures or tables, that will not be published in the print edition of the journal, but which will be viewable via	

	the online edition, can be submitted. It should be clearly stated at the time of submission that the supporting information is intended to be made available through the online edition. The availability of supporting information should be indicated in the main manuscript by a paragraph, to appear after the References, headed 'Supporting Information' and providing titles of figures, tables, etc. In order to protect reviewer anonymity, material posted on the authors Web site cannot be reviewed. The supporting information is an integral part of the article and will be reviewed accordingly.	
Perspective/Leading Opinion	Leading opinion or perspective papers that explore important clinical and scientific issues that are designed to stimulate further discussion and development of Endodontology by a subject expert. The subject expert should have an established publication and academic record with regards to the particular topic. Opinionated or perspective papers should identify major questions, challenges or propose new hypothesis testing, as well as attempt to resolve controversies in Endodontology. Manuscripts submitted for “Opinion” are designed to be stimulating and potentially provocative. Manuscripts are to be submitted by invitation only by the Editor-in-Chief or Associate Editor responsible for this section and should be clearly demarcated on submission; these manuscripts will still undergo a peer review process.	In general, these papers should not exceed 5,000 words and should contain illustrations and recent papers.

3. After Acceptance

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When an accepted article is received by Wiley’s production team, the corresponding author will receive an email asking them to login or register with Wiley Author

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The journal offers Wiley's Accepted Articles service for all manuscripts. Manuscripts accepted 'in press' are published online shortly after acceptance, prior to copy-editing or typesetting and appear in PDF format only. After the final version article is published (the article of record), the DOI remains valid and can still be used to cite and access the article.

Accepted Articles will be indexed by PubMed; submitting authors should carefully check the names and affiliations of all authors provided in the cover page of the manuscript so it is accurate for indexing. The final copyedited and proofed articles will appear in an issue on Wiley Online Library; the link to the article in PubMed will update automatically.

Examples of correct forms of reference

Standard journal article

Jakovljevic, A., Duncan, H.F., Nagendrababu, V., Jacimovic, J., Milasin, J. & Dummer, P.M.H. (2020) Association between cardiovascular diseases and apical periodontitis: an umbrella review. *International Endodontic Journal,* 53, 1374–1386.

Selman, P. (2016) The global decline of intercountry adoption: what lies ahead? Social Policy and Society, 11(3), 381–397.

Corporate author

British Endodontic Society (1983) Guidelines for root canal treatment. *International Endodontic Journal*16, 192-5.

Department of Health. (2009) Living well with dementia: a national dementia strategy.

Journal supplement

Frumin AM, Nussbaum J, Esposito M (1979) Functional asplenia: demonstration of splenic activity by bone marrow scan (Abstract). Blood 54 (Suppl. 1), 26a.

Holding, M.Y., Saulino, M.F., Overton, E.A., Kornbluth, I.D. & Freedman, M.K. (2008) Interventions in chronic pain management. 1. Update on important definitions in pain management. Archives of Physical Medicine and Rehabilitation, 89 (3, Supplement 1), S38–S40.

Books and other monographs

Personal author(s)

Gutmann J, Harrison JW (1991) Surgical Endodontics, 1st edn Boston, MA, USA: Blackwell Scientific Publications.

Barnes, R. (1995) Successful study for degrees, 2nd edition, London: Routledge.

Chapter in a book

Wesselink P (1990) Conventional root-canal therapy III: root filling. In: Harty FJ, ed. Endodontics in Clinical Practice, 3rd edn; pp. 186-223. London, UK: Butterworth.

Partridge, H. & Hallam, G. (2007) Evidence-based practice and information literacy. In: Lipu, S., Williamson, K. & Lloyd, A. (Eds.) Exploring methods in information literacy research. Wagga Wagga, Australia: Centre for Information Studies, pp. 149–170.

Published proceedings paper

DuPont B (1974) Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. Proceedings of the Third Annual Meeting of the International Society for Experimental Rematology; pp. 44-46. Houston, TX, USA: International Society for Experimental Hematology.

Wittke, M. (2006) Design, construction, supervision and long-term behaviour of tunnels in swelling rock. In: Van Cotthem, A., Charlier, R., Thimus, J.-F. and Tshibangu, J.-P. (Eds.) Eurock 2006: multiphysics coupling and long term behaviour in rock mechanics: proceedings of the international symposium of the international society for rock mechanics, EUROCK 2006, 9–12 May 2006, Liège, Belgium. London: Taylor & Francis, pp. 211–216.

Agency publication

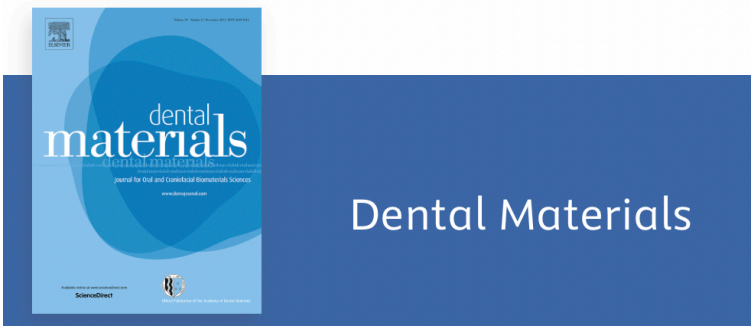
Ranofsky AL (1978) Surgical Operations in Short-Stay Hospitals: United States-1975. DHEW publication no. (PHS) 78-1785 (Vital and Health Statistics; Series 13; no. 34.) Hyattsville, MD, USA: National Centre for Health Statistics.8.

Wittke, M. (2006) Design, construction, supervision and long-term behaviour of tunnels in swelling rock. In: Van Cotthem, A., Charlier, R., Thimus, J.-F. and Tshibangu, J.-P. (Eds.) Eurock 2006: multiphysics coupling and long term behaviour in rock mechanics: proceedings of the international symposium of the international society for rock mechanics, EUROCK 2006, 9–12 May 2006, Liège, Belgium. London: Taylor & Francis, pp. 211–216.

Dissertation or thesis

Saunders EM (1988) *In vitro* and *in vivo* investigations into root-canal obturation using thermally softened gutta-percha techniques (PhD Thesis). Dundee, UK: University of Dundee. The University Encyclopedia (1985) London: Roydon.

ANEXO 5 - Author Guidelines – Dental Materials – Article 2.



Preparation
Peer review

This journal operates a double anonymized review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. Editors are not involved in decisions about papers which they have written themselves or have been written by family members or colleagues or which relate to products or services in which the editor has an interest. Any such submission is subject to all of the journal's usual procedures, with peer review handled independently of the relevant editor and their research groups. More information on types of peer review.

Double anonymized review

This journal uses double anonymized review, which means the identities of the authors are concealed from the reviewers, and vice versa. More information is available on our website. To facilitate this, please include the following separately:
Title page (with author details): This should include the title, authors' names, affiliations, acknowledgements and any Declaration of Interest statement, and a complete address for the corresponding author including an e-mail address.
Anonymized manuscript (no author details): The main body of the paper (including the references, figures, tables and any acknowledgements) should not include any identifying information, such as the authors' names or affiliations.
Use of word processing software
It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
This must be presented in a structured format, covering the following subjects, although actual subheadings should not be included:
• succinct statements of the issue in question;
• the essence of existing knowledge and understanding pertinent to the issue (reference);
• the aims and objectives of the research being reported relating the research to dentistry, where not obvious.
Materials and methods
• describe the procedures and analytical techniques.
• only cite references to published methods.
• include at least general composition details and batch numbers for all materials.
• identify names and sources of all commercial products e.g.
"The composite (Silar, 3M Co., St. Paul, MN, USA)..."
"... an Au-Pd alloy (Estheticor Opal, Cendres et Metaux, Switzerland)."
• specify statistical significance test methods.
Results
• refer to appropriate tables and figures.
• refrain from subjective comments.
• make no reference to previous literature.
• report statistical findings.
Discussion
• explain and interpret data.
• state implications of the results, relate to composition.
• indicate limitations of findings.
• relate to other relevant research.
Conclusion (if included)
• must NOT repeat Results or Discussion
• must concisely state inference, significance, or consequences

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• Title. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• Author names and affiliations. Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.
• Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Highlights

Highlights are mandatory for this journal as they help increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the example Highlights.
Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Abstract (structured format)

• 250 words or less.
• subheadings should appear in the text of the abstract as follows: Objectives, Methods, Results, Significance. (For Systematic Reviews: Objectives, Data, Sources, Study selection, Conclusions). The Results section may incorporate small tabulations of data, normally 3 rows maximum.
Graphical abstract
Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site.

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include

3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See https://www.elsevier.com/highlights for examples.

Keywords
Up to 10 keywords should be supplied e.g. dental material, composite resin, adhesion.

Abbreviations
Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources
List funding sources in this standard way to facilitate compliance to funder's requirements:
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