

In Vitro Evaluation of Smear Layer and Debris Removal and Antimicrobial Activity of Different Irrigating Solutions

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ABSTRACT

Objective: The aim of this *in vitro* study was to compare the smear layer and debris removal and antimicrobial activity of two dual-action irrigating solutions for continuous chelation (Triton; Brasseler, Savannah, USA and Dual Rinse HEDP; Medcem GmbH, Weinfelden, Switzerland) with a dual step irrigation protocol with sodium hypochlorite (NaOCl) followed by ethylenediaminetetraacetic acid (EDTA).

Methods: Thirty single-rooted single-canal teeth were divided into three groups (n=10) and irrigated with Triton, Dual Rinse HEDP mixed with 6% NaOCl and 6% NaOCl/17% EDTA. The teeth were observed under a scanning electron microscope (SEM) to assess the canal wall cleanliness. In addition, 80 dentine discs were contaminated with *Candida albicans* and 80 discs with *Enterococcus faecalis* and irrigated with Triton, Dual Rinse HEDP mixed with 6% NaOCl and 6% NaOCl/17% EDTA or not treated (n=20). Fifteen discs were used to evaluate colony-forming units, while 5 discs were analysed by SEM. Data were analysed using the Shapiro-Wilk, Kruskal-Wallis and One-Way ANOVA tests.

Results: Triton was statistically more effective than Dual Rinse HEDP and NaOCl/EDTA in removing debris (p<0.05), except with NaOCl/EDTA in the coronal third. Triton was more effective than Dual Rinse HEDP in removing the smear layer from the apical and middle thirds (p<0.05). All the irrigation protocols significantly reduced the number of *E. faecalis*. The Triton group showed the lowest number of remaining *C. albicans* (p<0.05).

Conclusion: Triton was the most effective irrigation solution in removing debris and as effective as NaOCl/EDTA in removing the smear layer. Triton showed the highest efficacy against *C. albicans*. New irrigating solutions that provide continuous chelation may provide an alternative to current irrigation protocols.

Keywords: Antimicrobial activity, continuous chelation, root canal irrigants, scanning electron microscopy

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HIGHLIGHTS

- Alternate use of NaOCl and EDTA may reduce the antibacterial effect of NaOCl and may result in intertubular and peritubular dentine erosion and, consequently, in a reduction of the flexural strength of dentine.
- The continuous chelation concept has been introduced to combine the advantages of NaOCl with those of chelating solutions.
- Triton was the most effective irrigation solution in removing debris and against *C. albicans*.
- Continuous chelation could be an alternative to current irrigation protocols.

INTRODUCTION

The goal of endodontic treatment is to eliminate bacterial biofilm attached to the root canal walls to achieve healing of the periapical tissues or to prevent apical periodontitis (1, 2). During root canal treatment, the anatomical complexity of many roots does not allow mechanical instrumentation to sufficiently achieve bacterial eradication (3). Antibacterial irrigation solutions are used to improve disinfection. Sodium hypochlorite (NaOCl), used in concentrations between 0.5 and 6%, is the most important irrigating solution in root canal therapy (4, 5). NaOCl is used throughout the root canal preparation phase, effectively removing organic tissue and bacteria (6). However, the formation of a smear layer and hard tissue debris during instrumentation requires the use of chelating agents for removing inorganic tissues (7). The smear layer can decrease antimicrobial activity and diffusion of NaOCl inside the dentinal tubules (8). Ethylenediaminetetraacetic acid (EDTA) has been widely recommended as a final irrigating solution to solubilise and remove the inorganic aspects of the smear layer and debris (7). During root canal treatment, the alternating use of NaOCl and EDTA should be avoided as this may reduce the antibacterial effect of NaOCl and may result in intertubular and peritubular dentine erosion (9) and consequently in a reduction of flexural strength of dentine (10, 11).

The concept of continuous chelation has been introduced to combine the advantages of NaOCl with those of chelating solutions to remove inorganic debris while disinfecting and dissolving organic tissues (12). A soft chelator can be mixed with NaOCl to provide a dual-action endodontic solution that can be used during the instrumentation phase of the root canal and also as a final irrigant. The Dual Rinse HEDP (Medcem GmbH; Weinfelden, Switzerland) is an etidronic acid powder (HEBP) studied to be mixed with NaOCl to obtain mild chelation (13), while Triton (Brasseler; Savannah, US) is an all-in-one dual-action endodontic irrigant launched in 2022, obtained by mixing two solutions (Part A and Part B) before use (14). Part A consists of novel patented gentle chelators, surfactants, PH modifiers, stabilizers and water, while Part B consists of 8% sodium hypochlorite, pH modifiers and water. The Dual Rinse HEDP contains etidronic acid powder, while Triton has 2-phosphonobutane-1,2,4-tricarboxylic acid (PBTC) as the chelating agent.

Rath et al. (10) have shown that the continuous chelation protocol resulted in a more homogenous organic and inorganic composition of the dentine surface than sequential chelation with NaOCl/EDTA. To our knowledge, there are no current studies on Triton.

This *in vitro* study aimed to compare the smear layer and debris removal and the antimicrobial activity of two dual-action irrigating solutions for continuous chelation (Triton and Dual Rinse HEDP) with dual-step NaOCl and EDTA irrigation.

MATERIALS AND METHODS

Smear Layer and Debris Removal

All procedures were carried out in accordance with the ethical rules and the principles of the 1964 Helsinki Declaration

and its later amendments or comparable ethical standards. Permission for the use of the biological material was obtained from each patient.

A total of 30 human maxillary and mandibular premolars, straight single-rooted single-canal teeth with similar root canal length and shape, were included and divided into three groups (n=10), according to the irrigation protocol used. Each tooth was radiographed in mesiodistal and bucco-lingual directions to determine the shape of the root canal. Only roots that demonstrated one round canal (radiographic buccolingual/mesiodistal ratio <2:1) were included (15). The curvature of the root canals was assessed according to Schneider's method (16). Permanent teeth with intact apices, without previous root canal treatment or restorations were included. Teeth with cracks, fractures, root caries, internal or external root resorption, calcification or apical diameters larger than size .30 were excluded. An access cavity was created, and the working length was measured using a K-file #10 advanced under a stereomicroscope (Zeiss AxioPhot; Carl Zeiss Jena GmbH, Zeiss Group, Jena, Germany) at 30× microscopic magnification until the tip was visible at the apical foramen. To better simulate the clinical conditions, the apex was protected with PTFE tape and the tooth was embedded in a siloxane putty material (DuoSil Putty Set; Bukwang, Busan, Korea).

Root canal instrumentation and irrigation

All root canals were instrumented using Mtwo system (VDW, Munich, Germany) up to size 40/.04 and irrigated using a syringe with a 30-gauge endodontic needle (NaviTip; Ultradent, UT, USA). Irrigation during instrumentation was performed using 14 ml of irrigant solution (2 ml after each instrument for 30 seconds). In the Triton group, Triton irrigating solution was used immediately after mixing the two liquids in a syringe directly from the bottles, as indicated by the manufacturer. In the Dual Rinse HEDP group, Dual Rinse HEDP was mixed with 6% NaOCl (Vista Dental Products, Racine, WI) and used immediately after mixing. For the NaOCl/EDTA group, irrigation in this phase was accomplished using 6% NaOCl. In the final irrigation rinse, the root canals were irrigated with 6 ml of the specific irrigating solution used in each group for two minutes. In the NaOCl/EDTA group, additional irrigation was performed with 6 ml of 17% EDTA (Vista Dental Products, Racine, WI) for two minutes. All irrigants were rinsed with 5 ml of distilled water for 2 minutes. The needle of the syringe was inserted into the root canals up to 1 mm short of the working length.

SEM analysis

After the intracanal procedures, the teeth were cut in two parts, making two longitudinal grooves with a diamond-coated high-speed bur, as reported by Wu & Wesselink (17). Care was taken to ensure that the grooves did not penetrate the canal. The teeth were then split with a mallet and chisel, made with an adapted cementum spatula, resulting in a mesial and distal half of the root canals. Both halves were prepared and observed under a scanning electron microscope (SEM, Supra 25; Zeiss) to evaluate the cleanliness of

the canal walls in the coronal, middle and apical thirds. The SEM used had a resolution of 1.7 nm at 15 KV and 3.5 nm at 5 KV. The area with the most smear layer and debris was photographed for each third of the canal at 1000× magnification. The presence of smear layer and debris was evaluated using Gutmann et al. (18) grading score system. To evaluate debris, the following score system was used: score 1, none to slight presence of superficial debris covering up to 25% of the dentinal surface; score 2, little to moderate presence of debris covering between 25 and 50% of the surface; score 3, moderate to heavy presence of residual debris covering between 50 and 75% of the surface; score 4, heavy amount of aggregated or scattered debris covering over 75% of the surface. The following score system was used to evaluate smear layer: score 1, little or no smear layer, covering <25% of the specimen with tubules visible and patent; score 2, little to moderate or patchy amounts of smear layer, covering between 25 and 50% of the specimen with many tubules visible and patent; score 3, moderate amounts of scattered or aggregated smear layer, covering between 50 and 75% of the specimen with minimal to no tubules visible or patent; score 4, heavy smear layer covering over 75% of the specimen with no tubule orifices visible or patent. The evaluators were not aware of the treatment done on the specimens. The analysis was performed by two different operators.

Antimicrobial Activity

Root canal preparation

A total of 90 straight single-rooted single-canal teeth with similar root canal length and shape as previously selected were included in the study. All root canals were instrumented using Mtwo system up to size 40/.04 and irrigated using a 30-gauge endodontic needle. Irrigation was performed using 14 ml of 6% NaOCl irrigant solution during the instrumentation (2 ml after each instrument for 30 seconds), 6 ml of 6% NaOCl at the end of the instrumentation for 2 minutes and 5 ml of 17% EDTA for 2 minutes as a final rinse. All irrigants were rinsed with 5 ml of distilled water for 2 minutes.

The teeth were cut with a 0.2 mm low-speed diamond disc under water cooling. The cuts were made from the CEJ to the apex, and 2 discs with a 3 mm thickness were obtained from the middle third of each root, for a total of 180 discs. Every disc was evaluated using a caliper to obtain standardised samples, which were stored in saline solution (NaCl 0.9%) and then sterilised using Steris V-PRO® maX Low Temperature Sterilization System (Steris, Mentor, OH, USA).

Sample infection

Eighty dentine discs were contaminated with *Candida albicans* biofilm, and another 80 discs with a biofilm of *Enterococcus faecalis* ATCC 29212. In the negative control group, 20 additional discs were not contaminated; 15 were used for a colony-forming unit (CFU) study, and 5 were used for SEM analysis.

C. albicans was cultivated in RPMI media at 37% for 72 hours. The yeast suspension was diluted in saline solution at a final concentration of 0.5 McFarland, corresponding to 10^7 CFU/ml,

and the solution was slowly added to the top of the discs. The yeasts were kept in contact with the dentine disc surface for 72 hours to allow the formation of biofilm. After 72 hours of incubation, the suspension was removed, and the discs were gently washed with sterile 0.9% NaCl.

Biofilm formation by *E. faecalis* followed the method above with the modification of Mohamed et al. (19). *E. faecalis* isolates were cultivated in Brain Heart Infusion media (BHI, Sigma), adding 1% fetal bovine serum (FBS, Sigma) and 0.25% glucose at 37% in ambient air for 10–12 hours. Afterwards, 1.25 ml of *E. faecalis* suspension adjusted at 107 CFU/ml concentration was added on the top of the dentine discs (1 McFarland) allocated in 24-wells Microtiter Plate (Costar3527, Corning). Specimens were incubated for 72 hours at 37°C to allow the formation of biofilm and then washed with sterile 0.9% NaCl to remove not adhered bacteria (20).

Irrigation protocol

Forty-five discs contaminated with *C. albicans* and 45 discs contaminated with *E. faecalis* were divided into 3 groups (n=15) for microbial analysis. Samples were continuously flushed with a syringe equipped with a 30-gauge needle for 3 minutes using 10 ml of Triton (Triton group), Dual Rinse HEDP mixed with 6% NaOCl (Dual Rinse HEDP group) and 6% NaOCl (NaOCl/EDTA group). In the NaOCl/EDTA group, an additional continuous flush with 5 ml 17% EDTA was performed for 1 minute. Five additional discs for each group were similarly treated for SEM analysis.

In the positive control groups, 20 additional discs contaminated with *C. albicans* and 20 with *E. faecalis* were not subjected to further treatment. Fifteen positive control discs were used for the CFU valuation assay and five for the SEM analysis.

Colony forming units (CFU) and SEM analysis

Dentine discs were washed carefully to remove not adhered bacteria. After washing the samples, the biofilm was detached from the surfaces by bath sonication. Using a sterile technique, dentine discs were put into a single sterilised 1.5 ml tube (Eppendorf, Thermofisher), to which 500 ml of PBS solution was added; the container was processed as shown in Figure 1, and the sonicate fluid was cultured as described. Bacterial cell suspensions were then serially diluted and spread on ESA plates (ESA, Sigma, St. Louis, MO, USA) and yeasts on Can BCG and then incubated at 37°C overnight. After 24 h, microbial counts were expressed as CFU per ml. Sonicate fluid was stored at 4°C for 24 hours for a second test.

During the sonication procedure, the container was vortexed for 30 seconds (Vortex Genie, Scientific Industries Inc., Bohemia, NY) and then subjected to sonication in an Aquasonic Model 750T ultrasound bath (VWR Scientific, West Chester, PA) for 5 minutes, followed by additional vortexing for 30 seconds. Microorganisms were enumerated and identified using routine techniques.

The dentine discs examined with SEM (500× and 2000× magnification) were fixed with 2.5% glutaraldehyde in phosphate buffer and put in 1.33% osmium tetroxide. After fixation, sam-

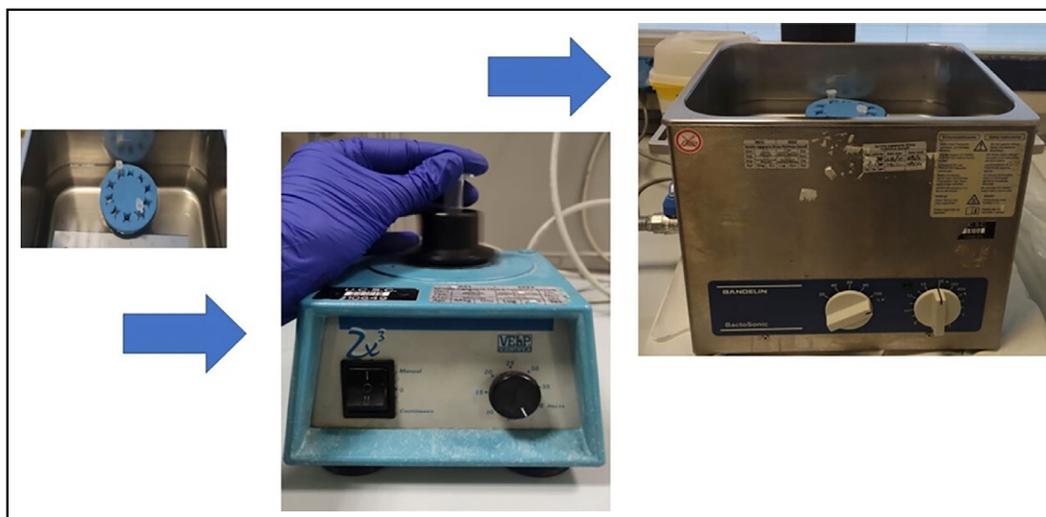


Figure 1. The sonication procedure of the container

ples were dehydrated, mounted on stubs, sputter-coated with gold (Balzers Union-SCD 040), and observed by two operators.

Statistical Analysis

The debris and smear layer score data were analysed using the Shapiro-Wilk test to verify the assumption of normality. For inter-group comparison, the Kruskal-Wallis test and the Dunnett test with Benjamin Hochberg correction were used to analyse the data that were not normally distributed. All analyses were performed using RStudio software (RStudio Inc., Boston, MA, USA). Statistical analysis for the antimicrobial activity (CFU) was performed using the One-Way ANOVA test (GraphPad 8.0, GraphPad Software, San Diego, CA, USA). The statistical significance level was set at 0.05. The sample size was initially calculated based on previous studies (21). After the experiments, the power analysis for the ANOVA test was performed using Person and Hartley's graphs, corresponding to a power of 0.99.

RESULTS

The results for the smear layer and debris removal are shown in Table 1 and Table 2. Triton was statistically more effective than Dual Rinse HEDP and NaOCl/EDTA in removing debris from all root canal thirds ($p < 0.05$), except with NaOCl/EDTA in the coronal third, and it was statistically more effective than Dual Rinse HEDP in removing smear layer from apical and middle thirds ($p < 0.05$). There was a significant difference between Dual Rinse HEDP and NaOCl/EDTA only in the removal of debris from the coronal third and in removing the smear layer from the middle third ($p < 0.05$) (Fig. 2).

All the solutions used significantly reduced the number of *E. faecalis* (CFU 0) compared with the untreated group ($p < 0.05$). The results of CFU of *C. albicans* are shown in Figure 3. The Triton group showed the lowest number of remaining yeasts compared to other groups ($p < 0.05$), and the Dual Rinse group reduced *C. albicans* more than NaOCl/EDTA group ($p < 0.05$). SEM analysis showed the presence of *C. albicans* and *E. faecalis* biofilm on untreated dentine discs while confirming the absence of microorganisms or a reduced number of microorganisms in the treated groups.

DISCUSSION

The ideal endodontic irrigating solution should have a strong antibacterial effect and, at the same time, remove the organic and inorganic debris and smear layer from the root canal walls without damaging root dentine. Several studies have evaluated the properties of new irrigating solutions (22–24). The concept of continuous chelation involves using a soft chelating agent throughout all of the instrumentation phase. Continuous chelation protocols showed a better adhesion of epoxy resin-based sealers to root dentine (25) and dentinal penetration of bioceramic root canal sealers (26). Dual Rinse HEDP has low cytotoxicity and no genotoxicity, while when mixed with NaOCl, it demonstrated the same toxicity as NaOCl alone (27). Furthermore, in a randomised clinical trial, Dual Rinse HEDP did not alter the clinical efficacy of NaOCl (28).

In the present *in vitro* study, a new irrigating solution (Triton) made by NaOCl and mild chelators has been compared with

TABLE 1. Mean score registered for each group in the different root canal thirds in evaluating residual smear layer

Smear layer	Triton group	Dual rinse group	NaOCl/EDTA group
Apical	1.25 ^a	1.80 ^b	1.50 ^{ab}
Middle	1.05 ^a	1.80 ^b	1.00 ^a
Coronal	1.00 ^a	1.20 ^a	1.00 ^a

Different superscript letters indicate a statistical difference at a 5% significance level ($p < 0.05$). NaOCl: Sodium hypochlorite, EDTA: Ethylenediaminetetraacetic acid

TABLE 2. Mean score registered for each group in the different root canal thirds in evaluating superficial debris

Debris	Triton group	Dual rinse group	NaOCl/EDTA group
Apical	1.20 ^a	1.70 ^b	1.90 ^b
Middle	1.10 ^a	1.50 ^b	1.80 ^b
Coronal	1.10 ^a	1.60 ^b	1.00 ^a

Different superscript letters indicate a statistical difference at a 5% significance level ($p < 0.05$)

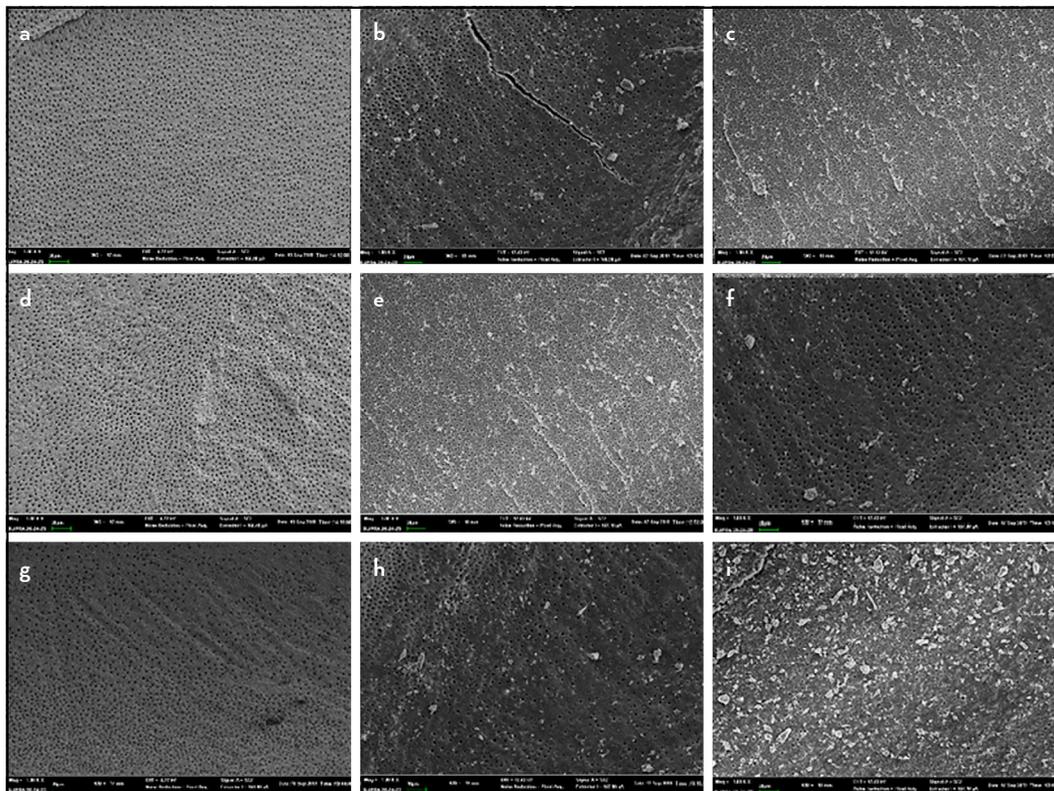


Figure 2. Smear layer and debris in the coronal, middle and apical parts of root canals after treatments at SEM examination: (a) Triton Group coronal 1000x, (d) Triton Group middle 1000x, (g) Triton Group apical 1000x, (b) Dual Rinse HEDP Group coronal 1000x, (e) Dual Rinse HEDP Group middle 1000x, (h) Dual Rinse HEDP Group apical 1000x, (c) NaOCl/EDTA Group coronal 1000x, (f) NaOCl/EDTA Group middle 1000x, (i) NaOCl/EDTA Group apical 1000x

SEM: Scanning electron microscope, HEDP: 1-hydroxyethane 1,1-diphosphonic acid, NaOCl: Sodium hypochlorite, EDTA: Ethylenediaminetetraacetic acid

Dual Rinse HEDP and NaOCl/EDTA. Triton was statistically more effective than Dual Rinse HEDP and NaOCl/EDTA in removing debris from all root canal thirds ($p < 0.05$), except with NaOCl/EDTA in the coronal third. Triton was more effective than Dual Rinse HEDP in removing the smear layer from the apical and middle third and showed the same efficacy as 6% NaOCl and 17% EDTA in removing the smear layer. Dual Rinse HEDP showed similar results to NaOCl/EDTA, but it was less effective in removing debris from the coronal third and the smear layer from the middle third. These results agree with those of Kfir et al., (29) who showed Dual Rinse HEDP did not differ from NaOCl followed by EDTA regarding canal cleanliness. Giardino et al. (30) reported that Dual Rinse HEDP increased the surface tension of NaOCl, which could limit the penetration of the solution into the apical third, isthmuses, fins and dentinal tubules. The differences observed in the present study could also be due to a difference in surface tension and penetration ability of the solutions, but further studies are needed to investigate the surface tension of Triton.

The microbiota associated with primary endodontic infection and endodontic failures is characterised by the predominance of facultative anaerobes and Gram-positive species (31). *E. faecalis* is a facultatively anaerobic, Gram-positive coccus associated mainly with asymptomatic cases of primary endodontic infections and failed endodontic cases (32). On the other hand, *C. albicans* is the fungal species most frequently isolated from

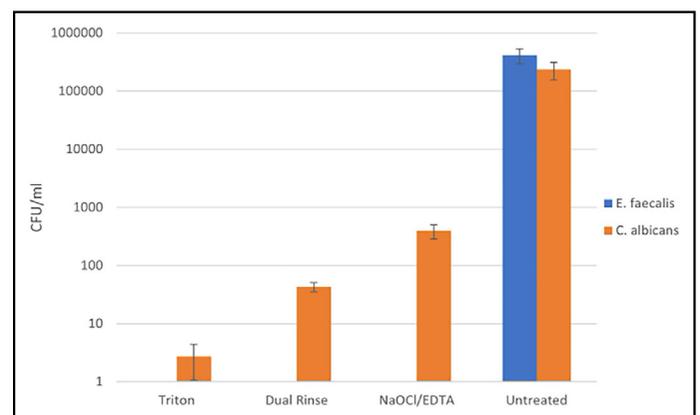


Figure 3. Mean CFU results and standard deviations of discs infected with *E. faecalis* and *C. albicans* untreated, treated with Triton, Dual Rinse HEDP and NaOCl/EDTA

CFU: Colony-forming unit, NaOCl: Sodium hypochlorite, EDTA: Ethylenediaminetetraacetic acid

persistent endodontic infections (33, 34). In the present study, dentine discs were contaminated with *E. faecalis* and *C. albicans* biofilms to test the different irrigating solutions. All tested irrigating solutions killed 100% of *E. faecalis*, and SEM evaluation confirmed the absence of bacteria or the presence of small amounts of bacteria not possible to detect by CFU anal-

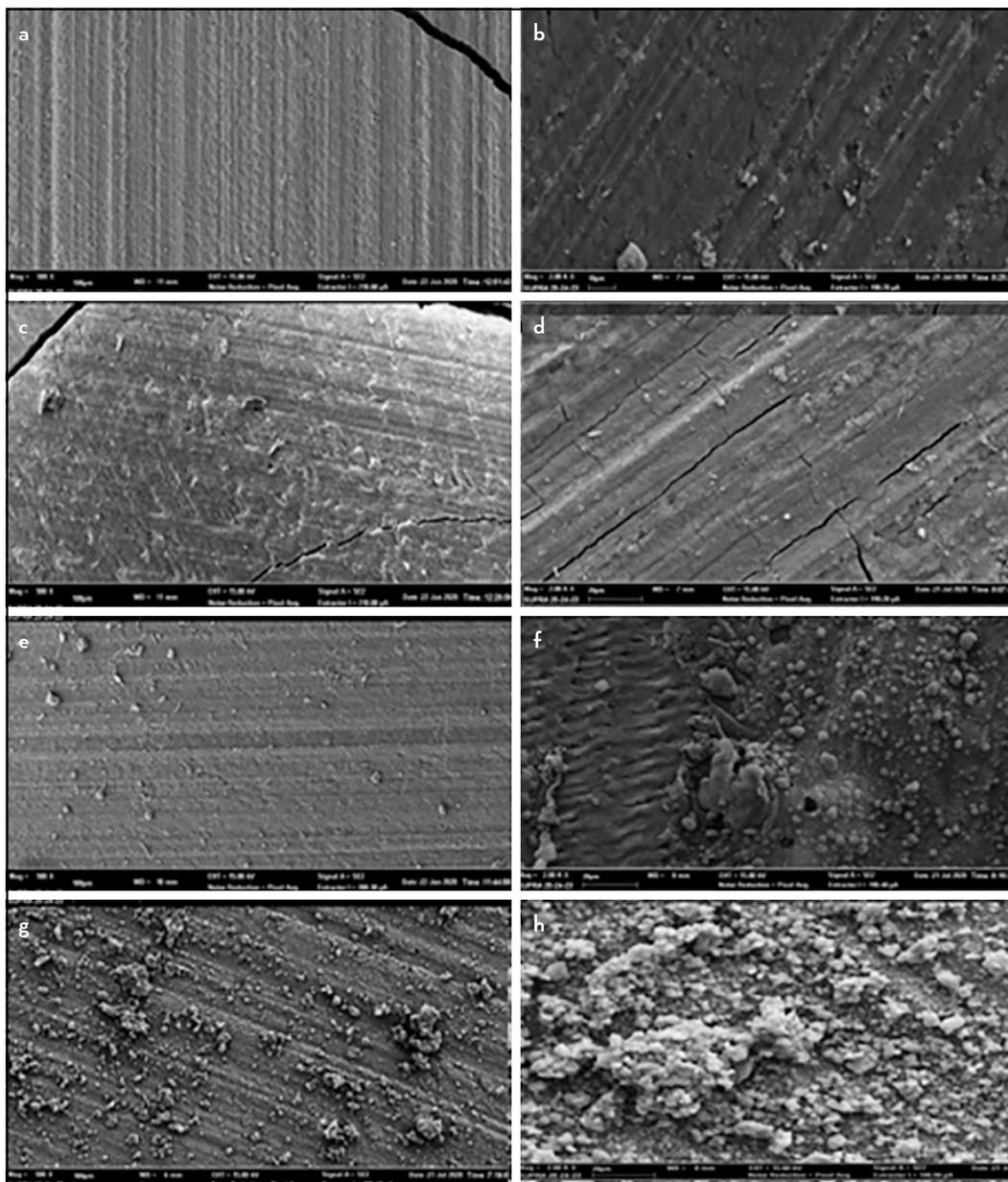


Figure 4. Dentine discs contaminated with *E. faecalis* after treatments at SEM examination: (a) Triton Group 500 \times , (b) Triton Group 2000 \times , (c) Dual Rinse HEDP Group 500 \times , (d) Dual Rinse HEDP Group 2000 \times , (e) NaOCl/EDTA Group 500 \times , (f) NaOCl/EDTA Group 2000 \times , (g) untreated Group 500 \times , (h) untreated Group 2000 \times SEM: Scanning electron microscope, NaOCl: Sodium hypochlorite, EDTA: Ethylenediaminetetraacetic acid

ysis (Fig. 4). These results are in agreement with those of Aris-Moliz et al., (35) who evaluated the antimicrobial activity of 2.5% NaOCl and 2.5% NaOCl/9% HEDP and reported that both solutions killed 100% of the *E. faecalis* biofilms. In contrast with these results, Giardino et al. (30) have shown that Dual Rinse HEDP had higher efficacy in killing *E. faecalis* than NaOCl and EDTA. However, both studies evaluated bacterial tubule penetration through confocal laser scanning microscopic analysis. Baumgartner et al. (36) reported that teeth contaminated with *E. faecalis* and treated with 5.25% NaOCl/15% EDTA produced 0 CFU/ml and showed no growth in any sample.

In the present study, Triton irrigating solution showed the highest efficacy against *C. albicans*, and Dual Rinse HEDP showed better results than NaOCl and EDTA (Fig. 5). The etidronic acid

(HEDP) alone demonstrated a weak antimicrobial effect on *C. albicans* (37); when mixed with NaOCl solution, it achieved a better antimicrobial efficacy than NaOCl and EDTA without compromising its antimicrobial property (12). The results of the present study should be confirmed by further *in vitro* studies using contaminated root canals.

Furthermore, this study is another step toward understanding the antimicrobial activity, smear layer, and debris removal of these all-in-one dual-action endodontic irrigating solutions. However, further studies are needed to investigate their performance in other conditions, such as with ultrasonic activation or in a clinical scenario. The semi-qualitative grading score to evaluate smear layer and debris removal, the use of dentine discs, and the mono-species biofilm are the limitations of this *in vitro* study.

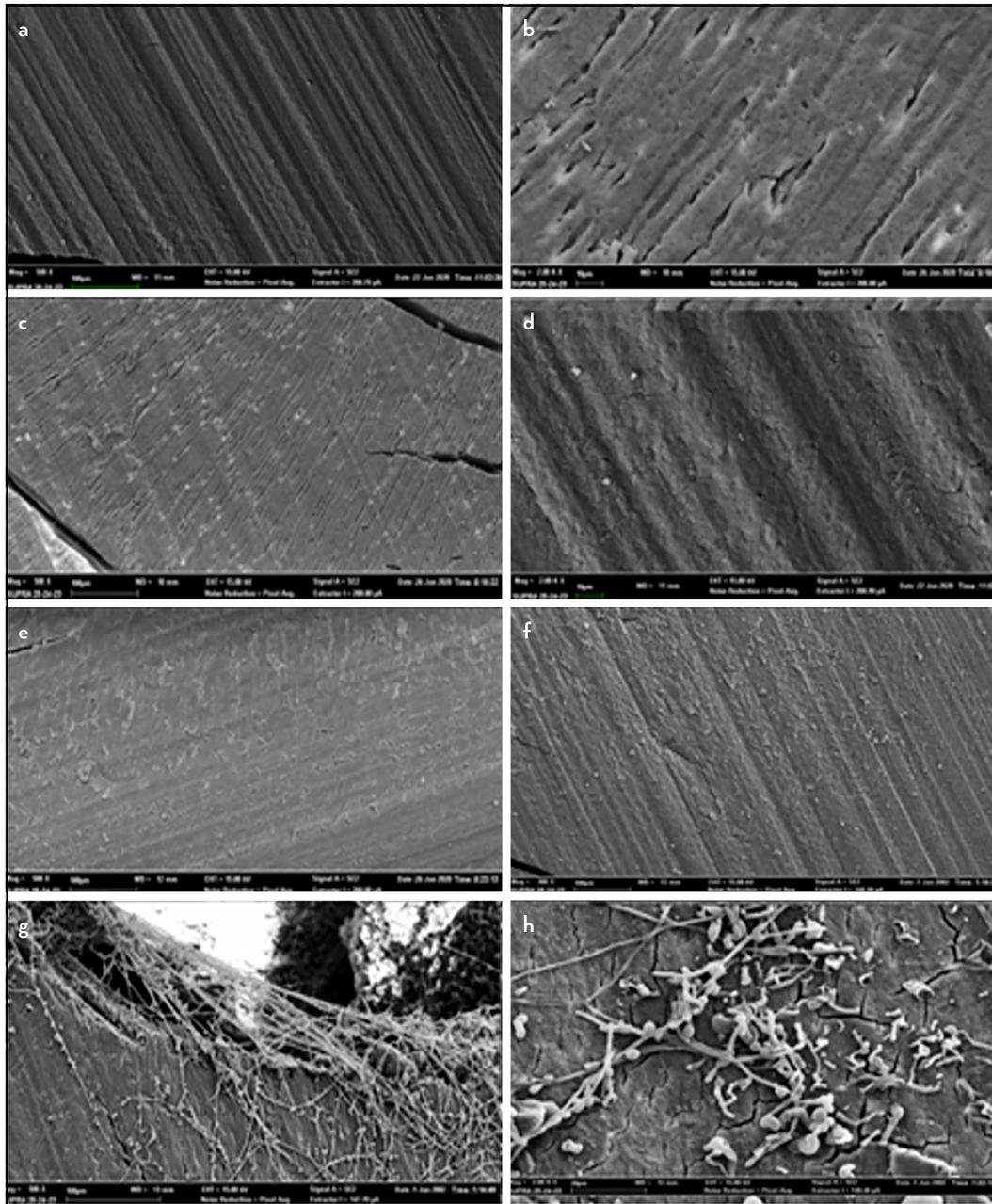


Figure 5. Dentine discs contaminated with *C. albicans* after treatments at SEM examination: (a) Triton Group 500 \times , (b) Triton Group 2000 \times , (c) Dual Rinse HEDP Group 500 \times , (d) Dual Rinse HEDP 2000 \times , (e) NaOCl/EDTA Group 500 \times , (f) NaOCl/EDTA 2000 \times , (g) untreated Group 500 \times , (h) untreated Group 2000 \times

SEM: Scanning electron microscope, NaOCl: Sodium hypochlorite, EDTA: Ethylenediaminetetraacetic acid

CONCLUSION

Despite the limitations of this *in vitro* study, it can be concluded that Triton was effective in removing debris from all root canal thirds, it was more effective than Dual Rinse HEDP in removing the smear layer from the apical and middle thirds and as effective as NaOCl/EDTA in removing smear layer. In addition, Triton showed the highest antimicrobial efficacy against *C. Albicans* compared to the other groups, and Dual Rinse HEDP reduced *C. albicans* more than NaOCl/EDTA. All the irrigation protocols significantly reduced the number of *E. faecalis*. This study has shown continuous chelation can be a good alternative to the current irrigation protocol regarding antimicrobial activity and removal of smear layer and debris.

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