

Effect of 5% Carbohydrate Derived-Fulvic Acid on Smear Layer Removal and Root Dentin Microhardness – An *In Vitro* study

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ABSTRACT

Objective: To compare the ability of 5% carbohydrate derived-fulvic acid (CHD-FA) with 17% ethylenediamine tetraacetic acid (EDTA) on smear layer removal when used as a final irrigant, and to assess their effect on root dentin microhardness.

Methods: A total of 56 single-rooted human mandibular premolars were decoronated to obtain standardized root length of 12 mm. Canal patency was established using 10 size K-file, working length was determined. Based on the irrigation regimen, samples were assigned into three groups; 17% EDTA, 5% CHD-FA and distilled water (control). The canals were instrumented with Protaper till F3 size. A standardized volume of 5 mL of each testing solution was used for 1 min. Roots were longitudinally split into two halves with one half of the samples for SEM analysis to evaluate the smear layer removal at coronal, middle, and apical thirds of root dentin. The other halves of the samples were subjected to Vickers microhardness testing. The data was statistically analyzed using Kruskal Wallis and Post Hoc test ($P < 0.05$).

Results: 5% CHD-FA and 17% EDTA showed significant smear layer removal at all levels compared to distilled water ($P < 0.05$). In the apical third, 5% CHD-FA showed significantly increased smear layer removal than EDTA ($P < 0.05$). Microhardness testing of apical third showed significantly lesser reduction in microhardness for CHD-FA than EDTA ($P < 0.05$).

Conclusion: 5% CHD-FA could be a promising final irrigant for smear layer removal with decreased microhardness reduction on root dentin compared to 17% EDTA.

Keywords: Chelating agents, EDTA, fulvic acid, hardness test, root canal irrigant, smear layer

HIGHLIGHTS

- Carbohydrate derived fulvic acid (CHD-FA) is a pure form of fulvic acid, organic in nature with low-molecular size and weight acid reported to have chelating, anti-inflammatory, antioxidant, antibacterial, antiviral and antifungal properties.
- Use of EDTA has a strong demineralizing effect resulting in greater reduction in microhardness of root dentin.
- CHD-FA a biologic chelator, effectively removed smear layer with minimal reduction in microhardness.


INTRODUCTION

Root canal preparation comprises combined action of endodontic instruments and irrigating solutions for thorough disinfection. The mechanical instrumentation of the canal creates a granular, amorphous smear layer (SL) that contains both organic and inorganic substances which cover the canal wall and occlude the openings of the dentinal tubules. The presence of SL favours the adhesion and colonization of microorganisms and limits effective disinfection by

preventing the irrigants and intracanal medicaments from penetrating into the dentinal tubules. It interferes with adhesion of resin-based sealers and impairs their penetration into the dentinal tubules, compromising the seal of obturating materials and resulting in microleakage (1).

The action of sodium hypochlorite (NaOCl), the most routinely used irrigant is limited to the removal of the organic component of SL when used alone, hence necessitating its association with chelating agents such as ethylenediaminetetraacetic acid (EDTA) as a final irrigant, for removal of SL by acting on its inorganic component as well (2). However, use of EDTA has a strong demineralizing effect on root dentin. This decreases the microhardness and fracture resistance

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of root dentin (2). The intra and peritubular erosion when associated with NaOCl leave the naked collagen fibrils exposed which are prone to bacterial adherence (3). Further EDTA has a cytotoxic effect when extruded beyond the periapical tissues (4), and is considered a weak antibacterial agent.

Various demineralizing agents such as citric acid, maleic acid, apple cider vinegar, chitosan, are being used for their chelating action and relatively low toxicity and anti-microbial properties (5-7). Nevertheless, there is a constant search for natural biocompatible irrigants that can minimize the undesirable changes in dentin microstructure.

Humic substances are natural degradation products of organic matter formed from decomposed plant and animal residues comprising humins, humic acid and fulvic acid. Fulvic acid (FA), is a major constituent of "humic" substances that is water soluble under all pH conditions. FA a biological chelator is lower in molecular size and weight and has lower colour intensity than humic acid. To obtain a pure form, FA was treated with carbohydrate sources such as glucose, sucrose, fructose, starches and cellulose by wet oxidation process to produce a CHD-FA (Carbohydrate-derived fulvic acid) composition (SA Patent 2001/2419). This colloidal organic acid is a cationic, heat stable, low-molecular weight acid reported to have chelating, anti-inflammatory, antioxidant, antibacterial, antiviral and antifungal properties. It has more carboxyl, ester, amide and aliphatic carbons in its molecular structure compared to FA. CHD-FA, is free of heavy metals hence suitable for use in medical and pharmaceutical preparations (8).

There are no studies till date, reporting the use of CHD-FA as irrigating solution in endodontics. Hence, this *in vitro* study was aimed to compare 5% CHD-FA with 17% EDTA for their smear layer removal ability at coronal, middle and apical third, and assess their effect on root dentin microhardness when used as a final irrigant. The null hypothesis proposed was that 5% CHD-FA does not have any significant effect on the smear layer removal at coronal, middle and apical third and does not affect the microhardness of root dentin when compared to 17% EDTA.

MATERIALS AND METHODS

The sample size for each group was set at 25, based on a power calculation (G Power, version 3.1.9.2), which indicated that a sample size of 23 specimens per group would provide 90% power ($\alpha=0.05$, $\beta=0.10$, S.D=7.6) to detect a difference of 10% increase in the microhardness value between 17% EDTA and 5% carbohydrate derived Fulvic acid. A total of 56 human mandibular single canal premolars with mature roots verified radiographically, extracted for orthodontic and periodontal purposes were used for the study after obtaining informed consent from the patients. The study protocol was approved by the Institutional Review Board and Ethical Committee (SRMDC/IRB/2017/MDS/No.301). Teeth with severely curved roots standardised using Schneider technique, calcified canals, morphological defects, caries and fracture were excluded. The collected teeth were cleaned and stored in 0.1% thymol solution at 37°C to maintain hydration until use.

CHD-FA (Mineralife Nutraceuticals, USA) used in this study was diluted to 5% concentration using distilled water. A pilot study was done with different concentrations of CHD-FA for their smear layer (SL) removal ability in which 5% CHD-FA showed the highest efficacy. The pH of 5% CHD-FA was measured using digital pH meter (Orion Star, Thermo Fisher Scientific, USA).

The teeth were decoronated to obtain a standardized root length of 12 mm using a high-speed diamond disc (Axis dental corp, Switzerland) under water coolant. The canal patency was established using 10 size K-file passing through the apical foramen. Working length was determined to be 1mm less than the length of size 15 K-file just exiting the foramen. All the samples were randomly assigned to 3 groups.

Irrigation protocol

The irrigation protocol for group 1 (NaOCl/EDTA; n=23) constituted of using 5 mL of 5% NaOCl for 1 min after each instrument use, followed by a final rinse of 5 mL 17% EDTA for 1 minute. 5 mL distilled water (Shivam Industries, India) rinse was used after active irrigation for 1 minute. In group 2 (NaOCl/CHD-FA; n=23), EDTA was replaced with 5% CHD-FA, while in the control group 3 (distilled water; n=10) both NaOCl and EDTA were replaced with distilled water.

The root canals were prepared with Pro Taper Universal (Dentsply maillefer, USA) rotary instruments using crown-down technique to size F3. The root apices were covered with sticky wax to prevent outflow of the irrigants. The irrigation procedure was done using 30-gauge side vented needle (Biodent Co. Ltd, Korea) placed 1 mm short of working length. After the final rinse with distilled water to remove any residual irrigating solution, the canals were dried using size 30 paper points (Dia Pro T, Diadent group International, Korea). Two longitudinal grooves were made on the outer surface of the roots using a diamond disc (Axis dental corp, Switzerland) at slow speed under water coolant without perforating the canal to facilitate splitting of the roots into two equal halves (n=112). The teeth were split with the aid of a surgical chisel and a mallet (Quinelato, Schobel Industrial Ltd, Rio Claro, SP, Brazil), with one half being subjected to Scanning Electron Microscopic (SEM) (Carl Zeiss Vision GmbH, Hallbergmoos, Germany) analysis for evaluation of smear layer and the other half subjected to Vickers Microhardness Testing (VMT) (Matsuzawa Seiki Co. LTD, Tokyo, Japan) for evaluation of microhardness. To exempt operator bias, the root canal preparation was carried out by one operator, whereas the assessment of smear layer removal was done by another examiner who was blinded to the experimental groups.

SEM evaluation

One half of the samples were delineated into coronal, middle and apical thirds using an indelible marker, mounted on a metallic stub; the prepared root canal surface was gold sputtered using an ion sputter and viewed under SEM at 2000x magnification. The images were scored according to the criteria given by Takeda et al. (9). Score 1 was attributed to the surface completely covered by smear layer; 2, to partially

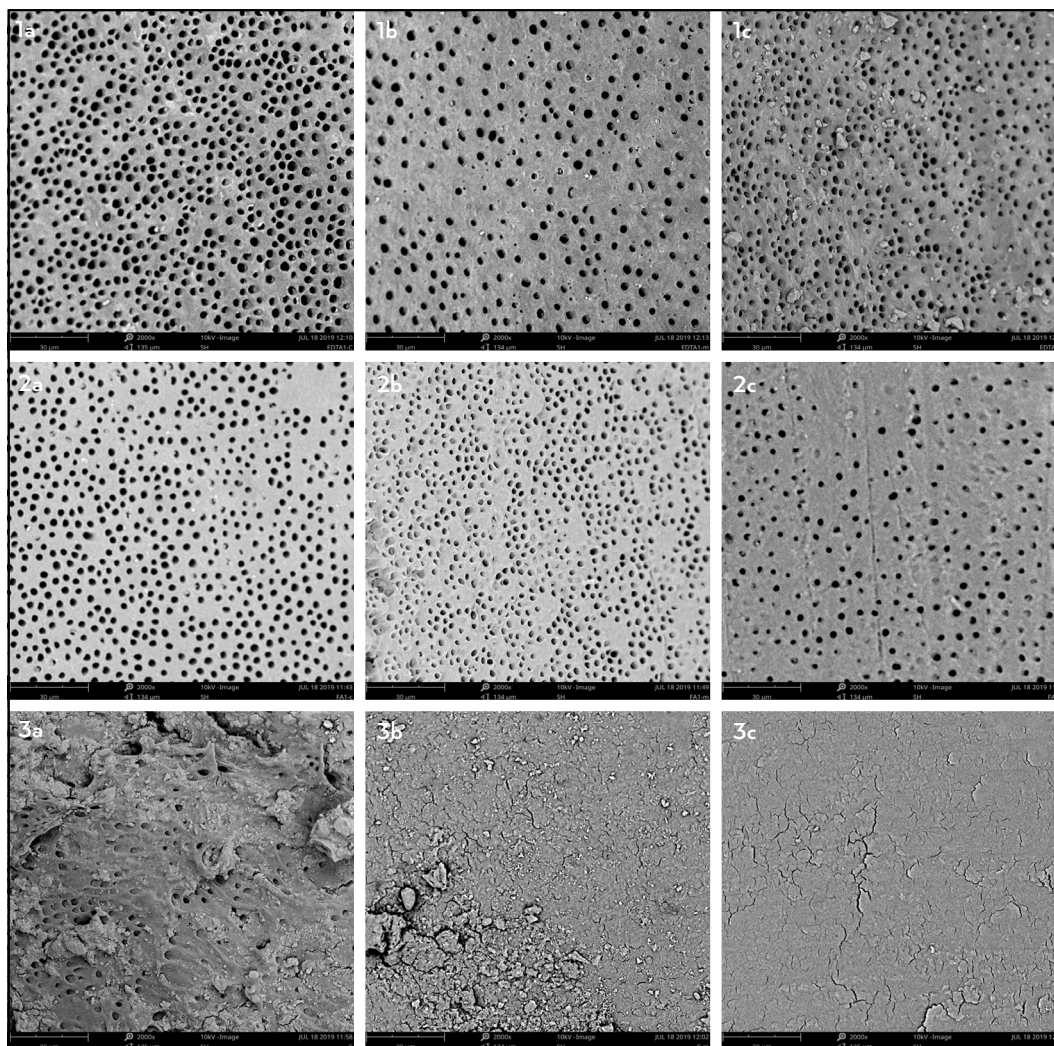


Figure 1. SEM microphotographs of root canal walls instrumented with 17% EDTA (group 1), 5% CHD-FA (group 2), and distilled water (group 3) at (a) coronal, (b) middle and (c) apical thirds at 2000x magnification SEM: Scanning electron microscope, EDTA: Ethylenediaminetetraacetic acid, CHD-FA: Carbohydrate derived fulvic acid

covered surface with few visible dentinal tubules; 3, surface with little smear layer and more tubules visible; and 4, to smear-layer-free surface.

Vickers microhardness testing

The remaining root samples were horizontally mounted in autopolymerizing acrylic resin with the canal lumen exposed. The mounted samples were smoothed with 1500 grit fine emery paper to remove any surface irregularities under distilled water, and polished with a felt cloth impregnated with 0.1 µm alumina particles. Three indentations were made on the apical third (4 mm from apex) of root dentin using Vickers diamond indenter under 300 g load and a dwell time of 20 sec. For each sample, the values were averaged and converted into Vickers hardness number (VHN).

Statistical analysis

Statistical analysis was done using SPSS software version 22.0 (IBM, USA). Both the smear layer removal and the microhardness values for all the groups were compared using non-parametric Kruskal-Wallis test with significance level fixed at 0.05. For pairwise comparison, post-hoc test was done.

RESULTS

The pH of 5% CHD-FA solution measured was 2.7. On SEM analysis (Fig. 1) both 17% EDTA and 5% CHD-FA exhibited a score of 4 in the coronal and middle thirds, indicating complete removal of smear layer, with clean and open tubules. In the apical third, 5% CHD-FA showed complete removal having a score of 4, while 17% EDTA exhibited moderate smear layer removal having a score of 3. Distilled water) which served as a control, showed heavy smear layer completely occluding the dentin surface having a score of 1 at coronal, middle and apical thirds. The mean values of the different groups have been represented graphically (Fig. 2).

VHN values (mean±SD) for different groups are summarised in Table 1. Group 3 showed the highest mean VHN, followed by groups 2 and 1. All the groups exhibited significant difference between each other ($P < 0.05$).

DISCUSSION

The chelating agents improve the chemo-mechanical debridement of the root canal by removing the smear layer (10). Chelators have also been found beneficial in negotiating

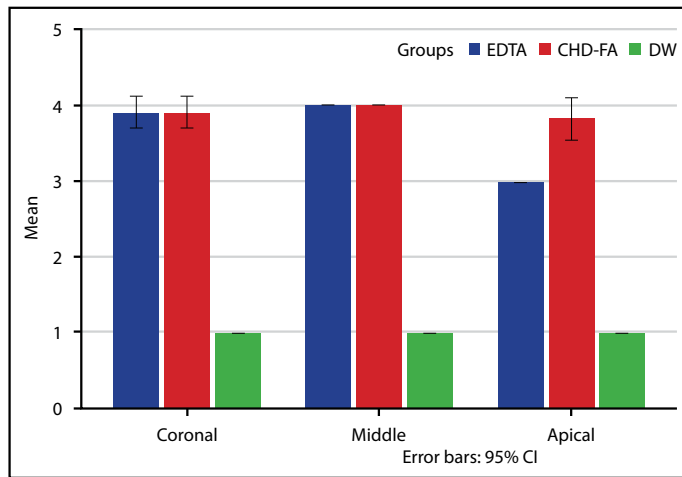


Figure 2. Mean smear layer removal by different irrigants at coronal, middle, and apical levels

EDTA: Ethylenediaminetetraacetic acid, CHD-FA: Carbohydrate derived fulvic acid, DW: Distilled water, CI: Confidence interval

constricted and calcified canals by softening the dentin, however the degree of softening and demineralization induced by these agents can have an adverse effect on dentin leading to reduction in microhardness (11). EDTA, the most widely used chelating agent sequesters calcium ions present in the hydroxyapatite crystals of dentin and can adversely affect the microstructure of the dentin by altering the calcium:phosphorus ratio (12). In order to overcome this adverse effect of EDTA, the present study was conducted to assess the chelating efficacy of 5% CHD-FA and its effect on microhardness when used as a final irrigant.

Microhardness assessment, a simple and non-destructive method, was performed as it provides an indirect evidence of such changes in the mineral content of dental hard tissues (13). The irrigation time for EDTA has been recommended to be 1 minute, as prolonged exposure to chelating agents might weaken the root dentin (14). Also, the paste type chelating agents do not remove the smear layer effectively when compared to liquid EDTA (15). Therefore to maintain standardization 1 minute of irrigation time and chelating agents in solution form was chosen for final irrigation with EDTA and CHD-FA group.

The SEM results indicated that irrigation protocol using 5mL of both group 1 (NaOCl/EDTA) and group 2 (NaOCl/CHD-FA) for 1 min was significantly more effective in the removal of smear layer from coronal and middle third of instrumented root canal surfaces when compared to group 3 (Distilled water). It was observed that 17% EDTA was significantly less effective in removing the smear layer when compared to 5% CHD-FA in the apical third. Studies have mentioned the reduced efficacy of EDTA in the elimination of smear layer in the apical third and thereby reduced bond strength of resin cements (16, 17). This reduced efficacy of EDTA can be ascribed to its increased surface tension and the higher molecular size. Conversely, because of the relatively small size of fulvic acid (FA) molecules CHD-FA can readily enter the apical portion of the canal. The efficiency of irrigating solutions decreases as it descends towards the apical portion of the canal (18, 19). Tubular sclerosis

TABLE 1. Vickers microhardness values (Mean±Standard deviation) of root canal dentin of different groups

Groups	Mean±SD
1 (17% EDTA)	46.42±6.99 ^a
2 (5% CHD-FA)	49.66±2.63 ^b
3 (Distilled water)	75.75±2.29 ^c

Different alphabets denote significant difference between the groups ($P < 0.05$).

and the narrow canal space in the apical third reduces the flow and backflow of the fluid, which can impede the penetration of the irrigants.

The tooth apex of up to 2 mm exhibits marked structural variations which include accessory canals, irregular secondary dentin, cementum-like tissue, low content of non-collagenous proteins (NCPs) and sclerosis which makes it difficult to get accurate microhardness values (12). Keeping this in mind, the microhardness of the apical thirds of the specimens was evaluated at 4 mm from the apex.

Results of microhardness testing exhibited significant reduction in microhardness of root dentin for both NaOCl/EDTA and NaOCl/CHD-FA groups when compared to distilled water. The reduction in dentin microhardness for CHD-FA and EDTA is imputed to their chelating property. In fulvic acid, chelation occurs at salicylate-like bidentate sites for copper (Cu^{2+}) a bivalent ion similar to calcium (Ca^{2+}), by the formation of Ca-fulvic acid complex with hydroxyapatite with the chelate occurring at 1:1 ratio (20). Further, both strong acidic COOH and weak COOH are involved in the formation of complexes and the stability of such complexes is pH dependent (21).

It was noted that CHD-FA group showed significantly lesser microhardness reduction than EDTA group. The pH of CHD-FA as determined by pH meter was 2.7. For fulvic acid, the number of released protons decreases as pH increases and complexation of calcium to fulvic acid involves mainly strong acidic groups. Formation of Ca-fulvic complex is not possible at acidic pH probably due to higher concentration of H^+ compared to that of Ca^{2+} for dissociated COO^- (17). This initial lower pH of CHD-FA may be the reason for lesser reduction in microhardness for CHD-FA when compared to EDTA (pH=7.8). It is also believed that configuration changes in FA occurs at basic pH and allows stability of the complexes formed between fulvic acid and bivalent cations, contributing to the significantly better smear layer removal. Thus the null hypothesis was partially rejected.

CHD-FA, a novel antimicrobial agent was shown to possess antifungal (*C.albicans*) and high activity against oral bacterial biofilms (*A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum*, *S. mitis*), with a MIC of 0.5%. This concentration killed 90% of the multi-species biofilms, and it was proposed that this compound was effective through disruption of the cell membrane and is unaffected by biofilm resistance mechanisms. Furthermore, from the cell culture; pre-treatment of epithelial cells with buffered CHD-FA showed down-regulation of key inflammatory mediators, such as IL-8, after instigation with a multi-species biofilm (22, 23).

Considering the results of the published reports and that of the present study, CHD-FA a naturally derived chelator with supplementary antimicrobial, antioxidant and anti-inflammatory properties can be suggested as a promising final irrigant. Irrigation activation and delivery systems which could influence the effectiveness of the irrigant were not considered in this study. Further, sequential irrigation, instead of continuous chelation was adopted which may need to be considered in future studies.

Ongoing research is focussed on the effect of CHD-FA on intra-tubular sealer penetration and its bond strength to dentin. Further studies should focus on testing the antimicrobial efficacy of this novel material on common endodontic pathogens, biofilms and those involved in recurrent infections.

CONCLUSION

Within the limitations of this study, final rinse with 5% CHD-FA was equivalent to 17% EDTA in smear layer removal at coronal and middle third of the root whereas in apical third CHD-FA removed smear layer more effectively with significantly lesser reduction in microhardness than EDTA.

Disclosures

Conflict of interest: The authors deny any conflict of interest.

Ethics Committee Approval: This study was approved by The Institutional Review Board SRM Dental Collage Ethics Committee (Date: 05/10/2019, Number: SRMDC/IRB/2017/MDS/No.301).

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