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ORIGINAL ARTICLE

Comparative Evaluation of the Effect of EDTA, Chitosan, Etidronic Acid, and Silver Citrate on the Mineral Content of Root Canal Dentine Using Energy Dispersive X-ray Spectroscopy: An *In-Vitro* Study

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ABSTRACT

Objective: Sodium hypochlorite is a common irrigant in endodontics, used to eliminate microorganisms and dissolve pulpal tissue. However, adjunctive chelating agents, while aiding in smear layer removal, can reduce dentinal wall microhardness, affecting dentine permeability, solubility, and the sealing ability of root canal sealers. This study aims to evaluate the impact of newer chelating agents—Silver Citrate, Chitosan, HEBP, and EDTA—on the mineral composition of root canal dentine using energy-dispersive X-ray Spectroscopy (EDXS).

Methods: The root canals of 120 freshly extracted human mandibular premolars with single straight canals and intact, mature apices were prepared following standard endodontic procedures using Rotary Pro Taper nickel-titanium files. Final irrigation was conducted with 5 mL of the respective chelating solution. Changes in the mineral composition of the root canal dentine were analyzed using EDXS. Descriptive statistics were presented as means and standard deviations. The impact of chelating solutions on the mineral content of root canal dentine at the cervical, middle, and apical thirds was compared between groups using One-Way ANOVA followed by a post hoc Bonferroni test.

Results: The highest mean concentrations of minerals such as Mg, Ca, and P at both the coronal and apical levels ($p<0.05$), along with the Ca/P ratio in the coronal third, were observed with distilled water, followed by HEBP, Chitosan, EDTA, and Silver Citrate, which showed the lowest values. In the middle third, the concentrations of Ca and P, as well as the Ca/P ratio in the apical and middle thirds, followed the sequence: Distilled Water>EDTA>HEBP>Chitosan>Silver Citrate. The difference in the levels of all minerals was statistically significant ($p<0.05$) at the coronal, middle and apical third levels.

Conclusion: Among the tested solutions, 9% HEBP caused the least alteration in the mineral content of root canal dentine when compared to 0.2% Chitosan, 17% EDTA, and Silver Citrate. This suggests that HEBP may be a preferable choice in endodontic procedures where minimal alteration of dentine mineral content is desired.

Keywords: Chelating agents, chitosan, HEBP, silver citrate

HIGHLIGHTS

- Among all tested agents, 9% HEBP showed the least alteration in dentine minerals, making it a safer choice for preserving dentine integrity during root canal treatments.
- Silver Citrate caused the most mineral loss in dentine, suggesting it may need cautious use to avoid weakening the root canal walls.
- Mineral changes were most noticeable in the coronal part of the root canal and least in the apical part, highlighting that irrigant flow and effectiveness vary along the root length.
- Using gentler agents like HEBP can help maintain dentine strength, which is crucial for the long-term success of root canal treatments.

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INTRODUCTION

Root canal treatment aims to eliminate debris or microorganisms from the canal as well as prevent recontamination in the future (1). Studies have demonstrated that bacterial penetration into the dentinal tubules may extend up the depth of 2100 µm (2, 3). Furthermore, root canal anatomy is frequently complicated by anatomical variations such as lateral canals, fins, webs, apical deltas, or transverse anastomoses and is seldom straight/conical. Cleaning them fool-proof using only instruments is impossible, warranting the use of additional chemical means for thorough disinfection (1, 4, 5).

Irrigating solutions facilitate the disinfection of canals while removing the smear layer, and organic and inorganic matter in the root canal dentine (6). An ideal irrigating solution should have a broad anti-bacterial spectrum and the ability to inactivate endotoxins. (7). There is no single irrigating solution that alone sufficiently meets all the criteria required for an ideal irrigating agent and thus a sequence of two or more irrigants is usually used.

One of the most common irrigating solutions used in endodontics since 1920, is sodium hypochlorite (NaOCl) known for its tissue dissolving ability and antibacterial activity (8). At all concentrations, it alters the micromechanical characteristics of dentine (9). However, one of the major disadvantages is that it is ineffective in removing the inorganic portion of the smear layer (10). The sequential chelation protocol, involving the use of sodium hypochlorite along with a chemical chelating agent for the complete removal of the smear layer is routinely employed in endodontic practice (11, 12).

Chelating agents improve the efficiency of canal preparation by demineralizing and subsequently, softening the dentine (13). These agents remove the inorganic portion of the smear layer. There are various chelating agents like Ethylenediaminetetraacetic acid (EDTA), Chitosan, Etidronic acid (HEBP), and citric acid used in endodontics. The most common and widely accepted combination is the use of EDTA along with the use of NaOCl (11, 14–16).

EDTA can form chelates by reacting with calcium ions in root canal dentine and is generally used in 17% concentration. The reaction can achieve decalcification of dentine at approximate depths of 20–30 µm within five minutes (17). However, its interaction with NaOCl decreases the antimicrobial effect of the latter by reducing the free available chlorine and also causes excessive erosion of peritubular and intertubular dentine leading to a decrease in microhardness of dentinal walls (18).

Chitosan is a biocompatible natural glucosamine polysaccharide that has a broad-spectrum antimicrobial activity and is also able to remove the smear layer effectively. The material has gained popularity in endodontics by virtue of its biodegradability, bio-adhesion property, and low toxic profile (19). The material is preferred at a concentration of 0.2% at which it performs satisfactory chelation while causing minimal dentinal erosion.

Citric acid (pH 1.8) causes optimal chelation of the calcium and phosphorus ions of the smear layer, although it lacks a good antibacterial effect. To overcome this drawback, it is used in combination with silver nanoparticles to achieve comparable chelating and antimicrobial properties to the gold standard irrigants while maintaining biocompatibility and non-toxicity (20). HEBP (9–18%) also known as Etidronate, a weak biocompatible chelator (21), is used in combination with NaOCl as a continuous chelation protocol as it does not interfere with antimicrobial or dissolution activity of the latter (22).

A collateral effect of the action of chelating agents is the reduction in the surface microhardness of the root canal wall dentine. This affects the sealing ability of root canal sealers and the adhesion of resin-based cement (23, 24). It is, thus, essential to study the effect of irrigants on the mineral content of the dentinal walls. Various techniques such as Energy Dispersive X-ray spectroscopy (EDXS), Atomic Absorption Spectrometry, Flame photometry, Raman spectroscopy, and Secondary ion mass spectrometry have been employed to this effect (25, 26). EDXS offers an efficient and non-invasive approach to estimate quantitatively the mineral composition of the given tooth sample.

Very few studies have comparatively evaluated the effect of various chelating agents on the mineral content of the root canal dentine when used in conjunction with NaOCl. This present study was conducted to compare the effect of EDTA, Chitosan, HEBP, and Silver Citrate irrigants on the mineral composition of root canal dentine when used along with NaOCl using Elemental analysis of root canal dentine.

MATERIALS AND METHODS

The present *in-vitro* study was conducted in the Department of Conservative Dentistry and Endodontics at Dr. G. D. Pol Foundation's YMT Dental College and Hospital. The study was performed in accordance with the Declaration of Helsinki and ethical approval of the study protocol was obtained by the institutional ethical review board (Reference Letter No.: YMTD-C&H/IEC/2021/0104).

Sample Size Estimation

Sample size was determined using the mean and standard deviation values from literature using the formula

$n=2 (Z\alpha+ Z\beta)^2 [s]^2 /d^2$

where Zα is the z variate of alpha error i.e. a constant with value 1.96, Zβ is the z variate of beta error i.e., a constant with value 0.84.

Approximate estimates: 80% power, type I error to be 5%, type II error to be 20%, true difference of at least 3.01 units between the groups, and pooled standard deviation of 2.78 .

Substituting the values, $n=2 (2.8)^2 [2.78]^2 /(3.01)^2$, we obtained n=13.37.

It was, thus, determined that a minimum of 13 specimens per group was required for completion of the study. To increase the power of the study, a sample size of 24 specimens per group was decided mutually by the investigators and a statistician.

Selection of the Samples

A total of 120 freshly extracted human mandibular single-rooted first premolars of adolescent patients undergoing orthodontic treatment where 1st mandibular premolar was

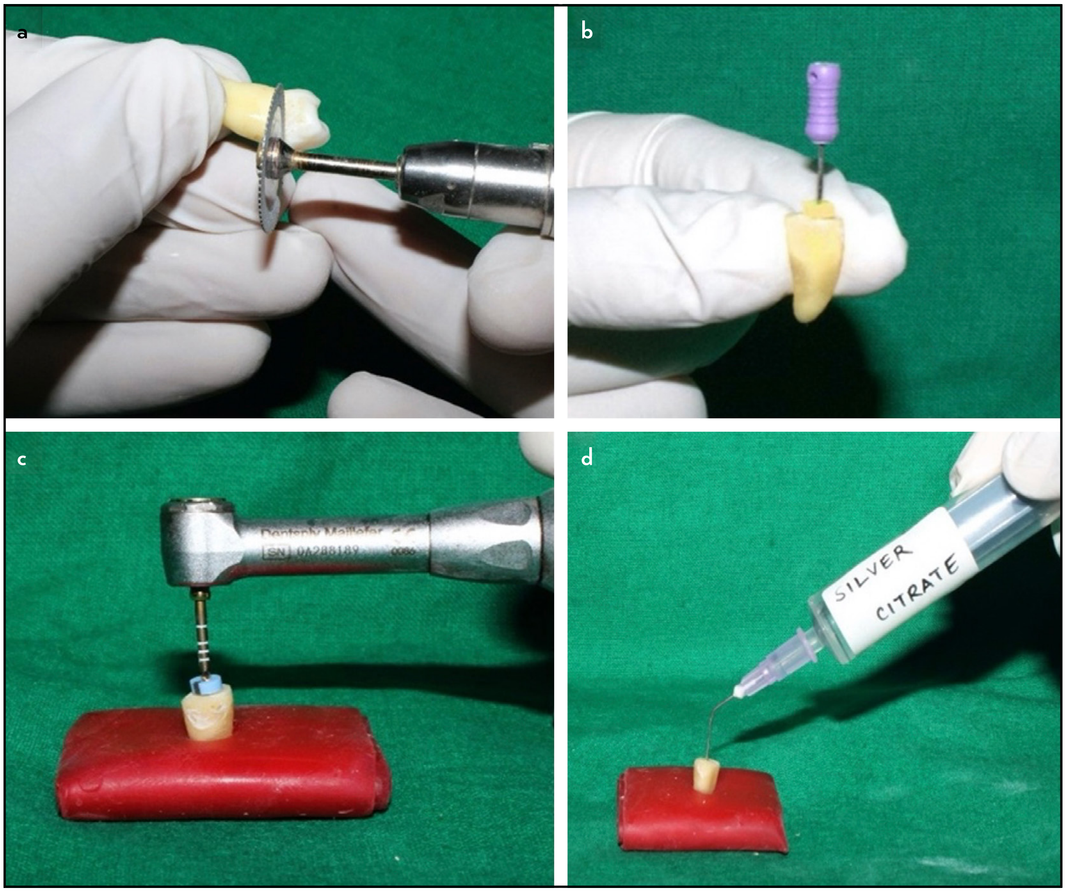

Figure 1. (a) Decoronation of the tooth, (b) Establishment of working length by K-file; (c) Canal preparation by Rotary Pro Taper nickel-titanium file; (d) Irrigation by labeled syringes

extracted for orthodontic purposes was chosen for the study. Further, mandibular first premolars with single straight intact canals and mature root apices were selected.

Teeth having a root curvature greater than 5 degrees (Schneider's method) were excluded from the study. Deciduous teeth, teeth with caries, defects, complex canal morphology, regressive alterations, hypoplastic defects, cracks, fractures, open apex, and internal and external root resorption were excluded. Endodontically treated teeth were also excluded.

The selected specimens were randomly assigned to one of the five groups, depending on the agent used in combination with NaOCl (CanalPro Coltene, Mumbai, Maharashtra, India): distilled water (R. K Laboratories Pvt. Ltd.. Baddi, Himachal Pradesh, India), 9% HEBP (Twin Kleen, MAARC, Mumbai, Maharashtra, India), 0.2% Chitosan solution (Swakit Biotech Pvt. Ltd., Bangalore, Karnataka, India), 17% EDTA solution (Prime Dental Products Pvt. Ltd. Mumbai, Maharashtra, India), and 4.8% Silver Citrate solution (BioAKT Endo, New Tech Solutions, Italy).

Sample Preparation

Soft tissue and calculus were carefully removed from freshly extracted human mandibular first premolars. Following this, mesiodistal projection radiographs were taken, and mandibular teeth with circular canals were selected for further analysis. To ensure sterility, the specimens were immersed in 5.25% sodium hypochlorite for one hour, after which they were stored in sterile normal saline at room temperature until use. The crowns of the teeth were removed using a low-speed diamond disc (Golden Nimbus India Pvt. Ltd., Mumbai, Maharashtra, India) with water cooling, achieving a standardized root length of 16 mm as measured by a digital Vernier Caliper.

A size #10 K-file was inserted into the canal until the tip was visible at the apex, and the working length was determined by subtracting 1 mm from this length. The glide path was prepared up to a #15 K-file (Mani™, Tochigi, Japan). Each canal was irrigated with 2 ml of 5.25% NaOCl for 15 seconds following the establishment of the glide path. Canal preparation was carried out using Rotary Pro Taper nickel-titanium files (Dentsply™ Maillefer, Charlotte, North Carolina, USA) in the sequence of SX, S1, S2, F1, F2, and F3. After each file, the canals were irrigated with 2 ml of 5.25% sodium hypochlorite for 15 seconds using a syringe and a 30-gauge needle, with the needle tip positioned 2 mm short of the working length.

To remove loose dentinal debris and residual NaOCl and to avoid interference with the final irrigating solutions, the canals were flushed with 20 ml of distilled water for 60 seconds. The canals were then dried using paper points (DiaDent™, Gurgaon, India) in preparation for final irrigation. Apical patency was rechecked with a #10 K-file after completing the shaping process. Depending on the assigned group, 5 ml of the respective chelating solution was introduced into the canal for 60 seconds. The preparatory procedures are depicted in Figure 1.

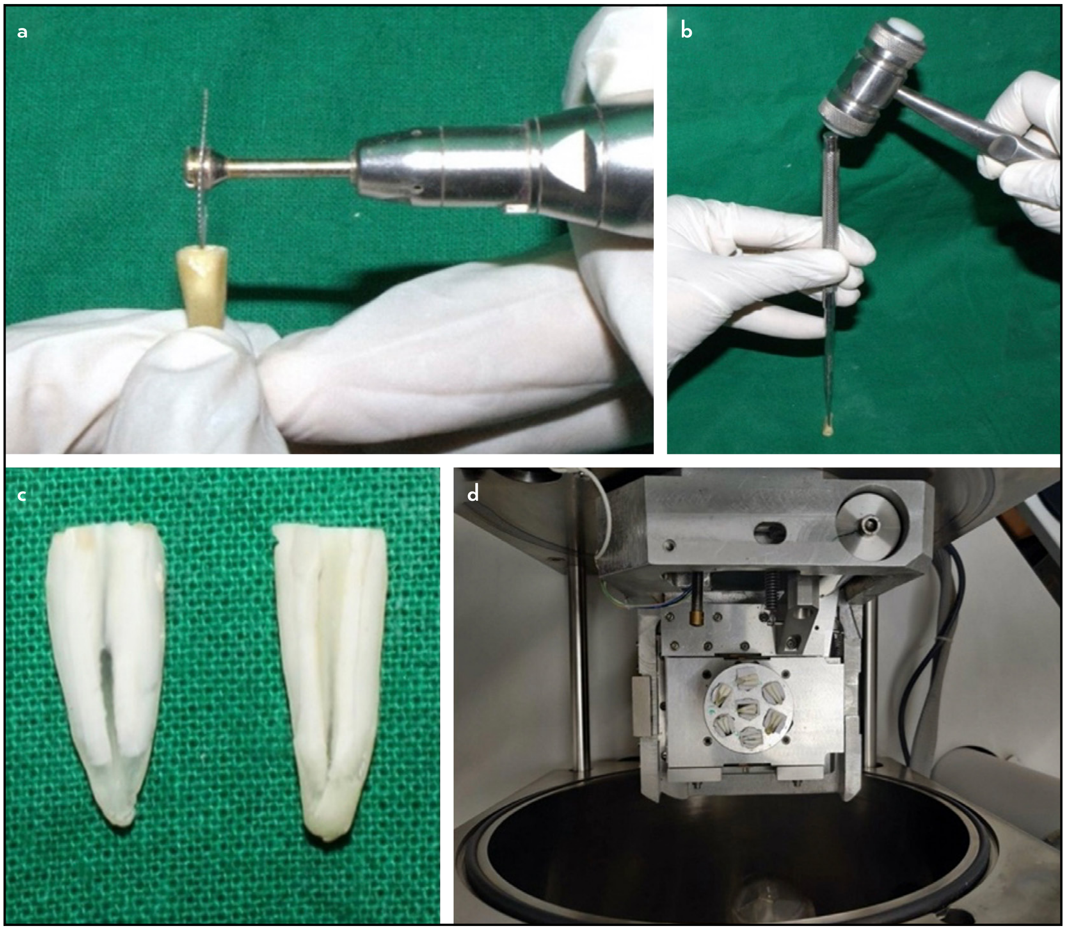

Figure 2. (a) Longitudinal sectioning of the root; (b) separation of the fragments by chisel and mallet; (c) split root fragments; (d) specimens placed in Energy Dispersive X-Ray Spectroscopy Unit

Evaluation of Mineral Content of Root Dentine Using EDXS

Following final irrigation, two grooves were made on each tooth, diametrically opposite each other, using metallic discs under cooling. The teeth were then split longitudinally into two halves using a chisel. The specimens were examined using an Energy Dispersive X-Ray Unit (Icon Analytical Instruments Pvt. Ltd., Mumbai, Maharashtra, India), and the images were analyzed to assess the mineral content of the root canal dentine using the appropriate imaging system. The levels of calcium, phosphorus, and magnesium were measured and compared at the cervical, middle, and apical thirds of the root canal dentine. The testing procedures are depicted in Figure 2.

Statistical Analysis

Descriptive statistics were presented as means and standard deviations. The impact of chelating solutions on the mineral content of root canal dentine at the cervical, middle, and apical thirds was compared between groups using One-Way ANOVA followed by a post hoc Bonferroni test. A p-value of less than 0.05 was considered statistically significant in these tests. All statistical analyses were conducted using SPSS software version 20, with a 95% Confidence Interval and 80% power for the study.

Evaluating the Ca/P ratio is crucial, as changes in this ratio can alter the balance of organic and inorganic components, potentially affecting the dentine's micro-hardness, solubility, and permeability. These changes may increase the risk of fracture in endodontically treated teeth.

RESULTS

The mean values of concentrations of the minerals are summarized in Table 1. The highest mean values of concentrations for all the minerals Mg, Ca, and P as well as the Ca/P ratio at the coronal levels were noted for distilled water followed by HEBP, chitosan, EDTA, and were least in the Ag citrate group. The difference between the values was statistically significant ($p<0.05$). The mean values of Calcium, phosphorous and the Ca/P ratio were in the order Distilled water> EDTA> HEBP> Chitosan>Ag Citrate in the middle level and the difference as statistically significant ($p<0.05$). At the apical level, the concentrations again followed the order as in the coronal level for Calcium and Phosphorus, however, the Ca/P ratio was in the order Distilled water>EDTA>HEBP>Chitosan>Ag citrate. For Magnesium concentration at the apical level, the order was distilled water>HEBP>EDTA>Chitosan>Ag citrate.

A pairwise comparison of the minerals at the coronal, middle, and apical levels is summarized in Table 2.

DISCUSSION

The rationale behind the present study was to analyze the alteration in mineral content of the dentinal walls achieved by the use of different chelating agents along with NaOCl. Ca, P,

TABLE 1. Overall comparison at coronal level between five groups

Groups	Mg		Ca		P		Ca/P	
Coronal level								
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DW	1.22	0.35	19.11	1.25	10.73	0.83	1.79	0.18
HEBP	1.18	0.37	18.18	0.97	10.68	0.74	1.71	0.14
Chitosan	1.05	0.38	14.18	1.40	8.99	0.87	1.59	0.21
EDTA	0.90	0.37	13.21	1.13	8.84	0.98	1.50	0.11
Ag Citrate	0.71	0.21	9.56	1.33	6.66	0.98	1.46	0.25
p	<0.001*		<0.001*		<0.001*		<0.001*	
Middle level								
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DW	1.02	0.28	20.05	0.89	11.86	0.69	1.70	0.12
HEBP	0.97	0.33	17.00	0.83	10.51	0.82	1.63	0.10
Chitosan	0.93	0.35	14.86	2.19	10.14	1.06	1.59	0.22
EDTA	0.91	0.34	13.23	1.18	9.73	1.04	1.54	0.15
Ag Citrate	0.61	0.11	6.06	1.12	9.35	1.48	1.46	0.37
p	<0.001*		<0.001*		<0.001*		0.003*	
Apical level								
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DW	0.80	0.54	22.02	3.99	11.96	3.03	2.02	0.85
HEBP	0.74	0.47	20.16	3.89	11.17	2.64	1.93	0.67
Chitosan	0.47	0.28	18.93	3.85	10.65	2.36	1.92	0.85
EDTA	0.51	0.28	17.98	3.76	10.16	2.70	2.02	1.41
Ag Citrate	0.42	0.27	16.79	4.00	9.91	3.00	1.89	0.90
p	0.002*		<0.001*		0.080		0.984	

One-way ANOVA test; *: Indicates significant difference. Mg: Magnesium, Ca: Calcium, P: Phosphorous, SD: Standard deviation, DW: Distilled water, HEBP: Chitosan, Etidronic acid, EDTA: Ethylenediaminetetraacetic acid

TABLE 2. Pairwise comparison at coronal level between five groups

Pair	Coronal level				Middle level				Apical level			
	Mg	Ca	P	Ca/P	Mg	Ca	P	Ca/P	Mg	Ca	P	Ca/P
DW vs. HEBP	1.000	0.097	1.000	0.331	1.000	<0.001*	1.000	0.909	1.000	1.000	1.000	1.000
DW vs. Chitosan	0.775	<0.001*	<0.001*	<0.003*	1.000	<0.001*	<0.001*	0.144	0.036*	0.070	1.000	1.000
DW vs. EDTA	0.015*	<0.001*	<0.001*	<0.001*	1.000	<0.001*	<0.001*	1.000	0.095	0.005*	0.254	1.000
DW vs. Ag Citrate	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	0.002*	0.010*	0.001*	0.112	1.000
HEBP vs. Chitosan	1.000	<0.001*	<0.001*	0.285	1.000	0.022*	1.000	1.000	0.173	1.000	0.112	1.000
HEBP vs. EDTA	0.056	<0.001*	<0.001*	0.002*	1.000	0.169	1.000	1.000	0.400	0.544	1.000	1.000
HEBP vs. Ag Citrate	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	0.104	0.341	0.059	0.033*	1.000	1.000
Chitosan vs. EDTA	1.000	0.070	1.000	1.000	1.000	<0.001*	0.140	1.000	1.000	1.000	1.000	1.000
Chitosan vs. Ag Citrate	0.007*	<0.001*	<0.001*	0.157	0.003*	<0.001*	1.000	1.000	1.000	0.590	1.000	1.000
EDTA vs. Ag Citrate	0.492	<0.001*	<0.001*	1.000	0.001*	<0.001*	0.002*	0.062	1.000	1.000	1.000	1.000

*: Statistically significant.

and trace levels of Mg influence the mineralization process in dental hard tissues and thus, these were selected for assessment of the impact of their irrigants on the mineral levels of the dentine. The mechanical properties of dentine such as its micro-hardness and permeability are greatly influenced by drastic alterations in the Ca/P ratio of its constituent crystals (23, 24). This, in turn, may place the endodontically treated tooth at a higher risk for fracture.

The irrigation technique was standardized for all the groups by using a conventional syringe irrigation technique with a 30 G side vented needle with a 5 ml syringe which enables to achieve flow rates of 0.20– 0.25 mL/s on an average (27, 28). Side-vented needles create more pressure on the walls of the root canal thereby improving the hydrodynamic activation of an irrigant and reducing the chance of apical extrusion (27). The EDXS used in the present study enabled an efficient and high-resolution analysis of the samples without damaging them (25, 26).

The result showed a significant difference amongst all the groups with Distilled water showing the least alteration in the mineral content followed by 9% HEBP, 0.2% Chitosan, 17% EDTA, and the highest alteration was noted in silver citrate. This implies that the chelating action of silver citrate was more potent as compared to other agents, although an alteration of a larger effect size may compromise the mechanical properties of the dentinal walls.

Citric acid is able to achieve decalcification of hard tissues of the teeth by chelation of the calcium ions in an acidic environment (29). Citric acid solutions with a concentration ranging from 25% to 50% have also been used as endodontic irrigants. However, Machado et al. (30) demonstrated that the concentration of citric acid does not affect its decalcification ability wherein concentrations as low as 10% were able to achieve results comparable to 17% EDTA. On the other hand, another research demonstrated that citric acid-based agents are able to release calcium cations at a considerably higher rate as compared to EDTA (31).

The lower pH of the citric acid solutions (pH<2), increased the removal of inorganic components from the hydroxyapatite crystals which resulted in lower values of Ca, P, and Ca/P ratio in the present study. Silver Citrate has pH-based chelation action, wherein its chelation is not influenced by the change in calcium ion concentration thereby it's not self-limiting and hence has more progressive and erosive action on radicular dentine (20, 32). Additionally, the viscosity of Silver Citrate is lower than that of EDTA. The molecular weight of irrigating agents can influence their viscosity, flow, and ability to penetrate the intertubular spaces of dentine (33). Therefore, silver citrate having lower molecular weight can easily penetrate deeper into dentinal tubules than EDTA and therefore, bind to more calcium ions in dentine.

Due to neutral pH, the action of EDTA is self-limiting thus it is less effective than citric acid for smear layer removal. Since 99% of EDTA is present in the form of trisodium EDTA, the action of EDTA reduces as acidity rises because H^+ ions will exchange Ca^{++} from dentine, causing the pH to drop (34). When the disodium salts of EDTA are added to the equilibrium, calcium ions are removed from the solution. Thus, in order to maintain a constant solubility product, there is further dissolution of ions from the dentine. This results in a low Ca/P ratio of the dentine after using 17% EDTA in comparison to 9% HEBP, 0.2% chitosan, and distilled water. Unlike EDTA, the demineralization actions of acidic chelators are dependent on the hydrogen ion concentration.

Likewise, HEBP also has a self-limiting weak action of chelation when equilibrium of calcium ions is obtained (35). In the present study, HEBP (9%) showed a better preservation Ca/P ratio along with other mineral content at all levels compared to silver citrate,17% EDTA, and 0.2% chitosan. It is a weak calcium-complex agent that causes fewer changes in dentine than other chelating agents. The use of 7–10% HEBP has been recommended as a less aggressive calcium complex agent to prevent erosive dentinal change. Thus, being a soft chelating agent, the 9% concentration of HEBP used in the present study caused minimal compositional changes in the dentinal structure. The advantage of continuous chelation with the HEBP-NaOCl combination, over the use of a strong chelating agent as only a final irrigation protocol, is that less smear layer and dentine debris is generated and prolongs the duration of action of sodium hypochlorite in relatively inaccessible areas (36).

Concentrations of chitosan solution as low as 0.2% were able to remove the smear layer and provide similar outcomes to other agents of higher concentrations (15% EDTA and 10% citric acid) (37, 38). The probable mechanisms for the formation of chitosan complexes include Chelation, adsorption, and ionic exchange. The present study found that the calcium ion chelation of 0.2% chitosan was less as compared to that of 17% EDTA and silver citrate.

Maximum alteration in Ca/P ratio along with alteration in mineral content of root canal dentine was seen in the coronal third of the root canal followed by the middle and least in the apical third across all groups irrespective of the chelating agent. This could be attributed to various factors such as the coronal third providing better flow and movement of the irrigating solutions producing greater hydrodynamic forces in the coronal and middle third as compared to the apical third of the root canals (39, 40). The constant replenishment of fluid also provides improved contact of the irrigating solution in the coronal and middle third and the least replenishment in the apical third of the root canal.

In the present study, the effect of chelating agents on the mineral content of root canal dentine in the apical third of all the experimental groups was non-significant compared to the control group. Compared to the coronal and middle third, the apical third of the canals exhibit a smaller cross-sectional size, curvatures, and deposition of sclerotic dentine causing inefficient irrigation (41). As a result, the volume of the irrigant replenished is less causing sluggish flow of the solution and less clearance of debris from the apical third of the root canal. The apical third of the root canal has a complex anatomy that can affect the hydrodynamics of the irrigating agent (42). For this purpose, various stimulation methods such as ultrasonic activation or heating are employed to augment the flow of the irrigant into areas otherwise difficult to reach. The protocol in the present study did not include any stimulation method, which may also be a confounding factor accounting for the observed lack of effectiveness in the apical third.

In this study, all the parameters were standardized an *in vitro* system may not be able to completely simulate conditions of a clinical scenario that could influence the loss of minerals. Canals with a complex morphology, which were excluded from the present study, are often encountered in endodontics which may affect the demineralization rate. Additionally, studies have supported the use of soft chelating agents such as 9% HEBP as continuous irrigating agents. However, to standardize the methodology for obtaining valid comparisons, only the effect as the final irrigant was considered in the present study. Future studies can incorporate designs to compare continuous versus final irrigation protocols for different agents.

Additionally, while EDS is an effective method for studying quantitative changes in the mineral content of dentine, it is unable to provide any information on the impact of the mineral variations on crystallin conformation. The incorporation of X-ray diffraction or scanning electron microscopy in the study design would allow a more detailed study of the mineral crystals and ultrastructure. Future studies could take into account these modalities when studying the mineral structure alterations in dentine induced by different irrigants.

CONCLUSION

Based on the results obtained and taking into account the limitations of this study, it could be concluded that among the tested materials, comparing the alteration in Ca/P ratio and other mineral content of root canal dentine that 9% HEBP showed the least alteration in the mineral content of root canal dentine as compared to 0.2% chitosan, 17% EDTA, silver citrate.

Disclosures

Ethics Committee Approval: The study was approved by the Dr. G. D. Pol Foundation's YMT Dental College Ethics Review Board (no: YMTDC&H/IEC/2021/0104, date: 08/01/2021).

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