



SATHYABAMA INSTITUTE OF SCIENCE & TECHNOLOGY

(Deemed to be University)
Jeppiar Nagar, Chennai



EFFECT OF SODIUM HYPOCHLORITE COMBINED WITH DIFFERENT CHELATING AGENTS ON ULTRASTRUCTURE OF DENTIN: AN *IN VITRO* STUDY

By

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Dissertation submitted to
Sathyabama Institute of Science & Technology
(Deemed to be University) Chennai, Tamil Nadu.

In partial fulfillment of the requirements for the degree of

Master of Dental Surgery

in the speciality of

CONSERVATIVE DENTISTRY AND ENDODONTICS

under the guidance of

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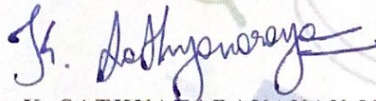
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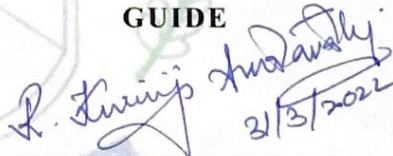
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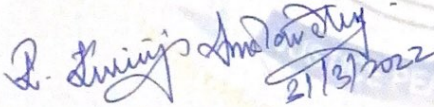
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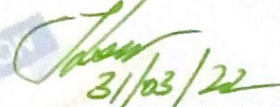
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ACKNOWLEDGEMENT

This thesis project has been the biggest and most important team effort I have been a part of during post-graduation. Therefore, I would like to take this opportunity to credit and thank all the individuals who have helped me along the way.

*I would like to begin by thanking my respected Professor, **Dr.R.Kurinji Amalavathy**, M.D.S, Head of the Department of Conservative Dentistry and Endodontics, Sathyabama Dental College and Hospital, Chennai. I am incredibly grateful for her expertise, advice, effort and time spent in order to help me complete the thesis. She has always gone above and beyond what is needed and I sincerely appreciate everything she has done for me over the past 3 years. I feel privileged to have been under her guidance. It has enriched my education and contributed immensely to both my personal and professional growth during post-graduation. I would like to express my gratitude to my respected teacher and co-guide, **Dr.K.Sathyanarayanan**, M.D.S, Reader, Department of Conservative Dentistry and Endodontics, Sathyabama Dental College and Hospital, Chennai, for his*

valuable guidance, suggestions, support and constant motivation during this period.

I would like to express my sincere thanks to **Dr. Arun. D. R**, M.D.S, Senior lecturer, Department of Conservative Dentistry & Endodontics, Sathyabama Dental College and Hospital, Chennai for graciously dedicating his time to proof-read the thesis and his helpful recommendations and advice along the way.

My sincere and respectful thanks to **Dr. Murali Sivakumar**, M.D.S Reader, Department of Conservative Dentistry & Endodontics, **Dr. Sushmita. S**, M.D.S, Reader, Sathyabama Dental College and Hospital, Chennai, for their constant support and encouragement.

I would like to express my gratitude to **Dr. Thayumanavan. B**, M.D.S, Dean, Sathyabama Dental College and Hospital for always supporting me in all my academic endeavours over the years and giving me the freedom to utilize college facilities.

My heartfelt thanks and respect to **Dr. Purushotham.M**, **Dr. Megavarnan.R**, **Dr. Hasini.V.R. R**, **Dr.Aravinthan.S.**, **Dr. K. Hari Krishna Reddy**, **Dr. Hrudi Sundar Sahoo**, **Dr. Sonali S**, **Dr. Shalini.S**, **Dr. Pavani** for their guidance

and support throughout the course. I am deeply indebted to them for all their contributions to my clinical and academic knowledge and growth.

*I would like to thank **Dr. Jayalakshmi**, M.D.S and **Dr. Vaishnavi**, B.D.S of White Lab, Saveetha Dental College, Chennai for co-ordinating appointments and sectioning dentin blocks for use in my experiment.*

*I thank **Dr. Devendiran.M**, Scientific Officer and **Mr. Manikandan** from Central Instrumentation Library, VISTAS, Pallavaram, Chennai for performing the atomic force microscopy and ATR-FTIR analyses respectively, and helping with post-processing analysis and interpretation of the results.*

*I sincerely thank **Dr.P. Malar**, PhD, Research Associate Professor, **Mr.M.G. Srinivasan** and **Mr. Thatheyus Peter**, research scholars from the department of Physical sciences at SRMIST, Kattankalathur, Chennai for their help with Vicker's microhardness testing.*

*I thank **Mr. Maheshkumar J** and **Mrs. R. Gowsalya** from CATERS Lab, Central Leather Research Institute, Adyar, Chennai for their timely help with scanning electron microscopy and elemental analysis portion of my study.*

*I thank **Dr. Vennila Anand** for helping me with the statistical analysis for my study.*

*I would like to acknowledge the constant motivation, support received and above all, friendship of my batchmates and colleagues, **Dr. Brindha Jain R**, and **Dr. Deepika R**, which have helped me sail through post-graduation smoothly. I would also like to acknowledge my seniors, **Dr.N. Akshaya**, **Dr. S. Aravinthan**, and **Dr. Dhanireddy.V. Padmaja** for their incredible help and advice throughout my course of study. I thank my juniors **Dr. Angel Mariyam John**, **Dr. R. Adisree**, **Dr. Jayakrishnan.P**, **Dr. Anjana Mathew**, **Dr. Pavitra.P. S** and **Dr. Neela Tarun** for their help.*

*Last, but certainly not least, I would like to express my sincere gratitude, respect and love for my family. I wholeheartedly thank my parents, **Mr.V.P. Ravichandiran** and **Mrs. Anupama Bugatha**, my brother **Venkatesh. R**, my grandmother, **Mrs.Pragna Paramitha**, my paternal aunt, **Mrs.N. Ramathal** and my maternal uncle, **Dr.B.S. Anil Chandra**. None of it would have been possible without their unwavering love and support.*

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INTRODUCTION

Endodontic success relies on the elimination of microbes from infected root canal systems and prevention of future infection.⁽¹⁾ This goal is achieved by shaping and cleaning of the root canal systems. Although instrumentation is known to reduce the microbial load by disrupting the biofilms formed on dentin walls ⁽²⁾, studies have shown that significant portions of root canal walls are untouched and are inaccessible to mechanical instrumentation alone emphasizing the need for chemical disinfection. ⁽³⁻⁵⁾ Furthermore, this step produces debris of infected organic and inorganic substrates that is smeared on the instrumented surfaces of the root dentin. ^(6,7) which has also been confirmed by micro-CT studies. ^(8,9) The presence of such a layer physically impedes penetration of intracanal medicaments, adaptation of sealers and filling material resulting in microleakage and further tissue irritation. ^(6,10) Despite these limitations, instrumentation remains an indispensable step in root canal treatment as it improves access of root canal irrigants, increases the volume of irrigant contacting the infected root canal system and facilitates obturation.

Sodium hypochlorite (NaOCl) is the recommended irrigant of choice as it satisfies most of the core requirements of an ideal irrigant. ^(1,11) Its unique tissue dissolution and antimicrobial capabilities have rendered it irreplaceable as the main root canal

irrigant.^(12,13) However, its effects are limited to organic portions of the smear layer.⁽¹⁴⁾ Therefore, additional chelators such as 17% ethylenediaminetetraacetic acid (EDTA) or 10% citric acid (CA) are employed to remove inorganic portions.⁽¹⁵⁾ The “***sequential irrigation***” protocol is the gold standard and achieves optimal disinfection and smear layer removal, but it is not without limitations.^(14,16) Negative interactions occur between irrigants when used simultaneously or consecutively resulting in an instant loss of free available chlorine (FAC) which reduces the efficacy of NaOCl but with slower, time-dependent effect on the chelator. Hence, it is advisable to flush out remnants in between uses with saline which makes the entire procedure more time consuming and necessitates additional armamentarium.^(17,18)

A novel protocol combining sodium hypochlorite with a weaker chelator, 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) also known as etidronic acid (EA), was introduced as a potential all-in-one irrigant to be used during the entire duration of canal preparation.⁽¹⁹⁾ It was termed “continuous chelation” in 2012 due to the sustained simultaneous action of the chelator.⁽²⁰⁾

Combining 0.5-5.25% NaOCl and 18% HEBP leads to a stable mixture that is still capable of tissue dissolution, with no immediate loss of chlorine levels.^(19,21) However, the therapeutic window ranges from 20 minutes to an hour and is dependent on

concentration, time and temperature. ^(22–26) This mandates chairside preparation of the solution just before use. The antimicrobial, anti-biofilm and dentinal tubule disinfection efficacy of this mixture was comparable to that of standard irrigation protocols in-vitro. ^(27–32) The formation of a smear layer is said to be minimised while using this mixture due to continual complexing of calcium throughout the irrigation cycle. ^(19,20,33) Smear layer removal efficacy of etidronic acid has been extensively studied using different methods. ^(15,19,34–36) Studies which utilised the mixture in intended continuous chelation mode (combination mixture throughout irrigation) demonstrated comparable smear layer removal to standard sequential irrigation protocols. ^(15,37) However, when used only as a final rinse, authors have reported inferior performance when compared to stronger chelators like EDTA, maleic acid. ^(38–43) The proponents of continuous chelation claim that the root dentin walls are spared from aggressive decalcification on account of it being a softer chelator. ^(19,20) The erosive effect of these mixtures was found to be much lower than that of standard practice.

Recently, another weak calcium chelator called clodronate disodium, a newer generation bisphosphonate, was investigated for its potential use in a continuous chelation protocol. ⁽⁴⁴⁾ This chelator performed in a similar manner to etidronic acid with respect to tissue dissolution, smear layer removal and has the added benefit of an increased therapeutic window of 180 minutes. ^(24,26,44)

Dentin contributes to the majority of the bulk of a tooth and can be likened to a biological composite. The components of dentin influence biomechanical properties of teeth. Inorganic components of dentin contribute to the tooth's fracture strength, i.e., stiffness and modulus of elasticity.⁽⁴⁵⁾ Organic matrix and the water content dictate fracture toughness. Changes in these properties may in turn affect long term survival and prognosis of a restored/endodontically treated tooth.^(46,47) Each irrigant has its own mechanism of action which may impact differently on different portions of dentin.⁽¹⁷⁾ The sequence of irrigation and the choice of final irrigant have also been shown to influence biomechanical properties of dentin.⁽⁴⁸⁾ NaOCl causes deproteinization of dentin walls, and the subsequent use of decalcifying agents exacerbate this effect which may be perceived as erosion in SEM studies.^(15,16,43,49) Prolonged contact time with the chelators have also led to tubule erosion.^(38,50,51) When used as a final rinse after EDTA, NaOCl has resulted in widening of the dentinal tubules and dentin erosion.⁽⁴⁹⁾ This finding has been reaffirmed by numerous other studies.^(52–55)

Evidence, thus far, remains limited, on continuous chelation mixtures' influence on dentin properties. The vast majority of literature has assessed the effects of these solutions on root dentin on a surface level employing scanning electron microscopy (SEM).^(15,20,38,43) To the best of our knowledge, only one study has performed multiple advanced analyses such as Atomic Force

Microscopy (AFM), Fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), ninhydrin assay and attempted to correlate the ultrastructural changes.⁽⁵⁵⁾ Therefore, we intended to study the effect of sodium hypochlorite and potential chelators on ultrastructure of dentin in blocks using AFM, Attenuated Total Reflectance-FTIR(ATR-FTIR), Vicker's microhardness tester and in root canal samples using SEM and Energy Dispersive X-ray Spectroscopy(EDS).

AIM AND OBJECTIVES

AIM:

The aim of this study was to compare the effect of sodium hypochlorite combined with different chelating agents, namely ethylenediaminetetraacetic acid, etidronic acid, and clodronate disodium on ultrastructure of dentin.

OBJECTIVES:

Dentin blocks:

1. To measure surface roughness using Atomic Force Microscopy (AFM) after exposure to various irrigation regimens.
2. To measure micro-hardness with Vicker's microhardness tester after exposure to various irrigation regimens.
3. To evaluate compositional changes by measuring carbonate-phosphate ratio and amide-phosphate ratio using Attenuated Total Reflectance-Fourier Transform Infrared spectroscopy (ATR-FTIR) after exposure to various irrigation regimens.

Root canal samples:

4. To evaluate the percentage area fraction of open dentinal tubules using Scanning Electron Microscope (SEM) in prepared root canals after exposure to various irrigation regimens.
5. To evaluate changes in atomic weight percentage of carbon, oxygen, nitrogen, phosphorus, and calcium using Energy Dispersive X-ray Spectroscopy (EDS) after exposure to various irrigation regimens.

HYPOTHESES

NULL HYPOTHESIS

The null hypothesis was that there would be no significant difference in the effects of sodium hypochlorite combined with different chelators on the ultrastructural properties of dentin.

ALTERNATE HYPOTHESIS

The alternate hypothesis was that there would be a significant difference in the effects of sodium hypochlorite combined with different chelators on the ultrastructural properties of dentin.

REVIEW OF LITERATURE

Effects of irrigants on compositional and mechanical properties of dentin

Rath et al., 2020⁽⁵⁵⁾ characterized and compared the effects of continuous chelation (DualRinse HEDP (6% NaOCl/18% HEDP), MedCem, Vienna, Austria) (1-hydroxyethylidene-1,1-diphosphonate) and sequential chelation (3% NaOCl/17% EDTA) protocols on the ultrastructure of dentin blocks, dentin powder, and root canals of mandibular premolars. Ultrastructural characteristics of the treated dentin were investigated in detail using scanning and transmission electron microscopy and light microscopy. Surface roughness was analyzed using atomic force microscopy. Chemical compositional changes were evaluated using Fourier transform infrared spectroscopy (FTIR) and energy-dispersive-X-ray spectroscopy (EDS). Collagen degradation was determined using ninhydrin assay. Continuous chelation resulted in partially degraded collagen fibres that were still encapsulated by hydroxyapatite, with minimal alteration to the subsurface matrix. Sequential irrigation completely dissolved the mineral layer, exposing thick collagen fibre bundles. There was no significant difference in the surface roughness or smear layer removal between the two protocols. It was concluded that the 3% NaOCl /9% HEDP protocol resulted in a more homogenous distribution of organic and inorganic components on

treated dentin surfaces, whereas sequential irrigation resulted in dentin surfaces rich in the organic phase.

Wright et al., 2020⁽⁵⁶⁾ investigated whether mixtures of weak chelators etidronate or clodronate and NaOCl could adversely affect the mechanical strength of teeth compared to the standard sequence of 5%NaOCl /17 %EDTA/ 5% NaOCl. Forty-five matched pairs of bovine teeth (n=15) were divided into 3 groups: one tooth per pair was irrigated using the standard sequence, and the other tooth was treated with either demineralized water, 0.26 mol/L etidronate, or 0.26 mol/L clodronate. Irrigation was carried out for over 60 minutes. Fracture testing was performed in a universal testing machine, and the compressive load at fracture was recorded. No statistically significant differences in load at fracture were observed between either mixture and the standard sequence. However, loads were higher in the teeth irrigated with water compared to the experimental solutions ($P<0.0001$). It was concluded that the continuous chelation mixtures did not alter tooth mechanical properties compared to the standard sequence.

Tartari et al., 2017⁽⁵⁷⁾ studied the effect of different chelators (9 and 18% HEDP, 5 and 10% tetrasodium EDTA, 17% EDTA, 0.5 and 2% peracetic acid (PAA)) alone and in combination with NaOCl on the organic and inorganic components of dentin at different time intervals using ATR-FTIR (attenuated total reflectance in Fourier

Transform infrared spectroscopy). Bovine dentin slices were used and subjected to irrigation with only chelators or combinations of chelators and NaOCl. The slices were analyzed using ATR-FTIR before and after exposure to irrigants, specifically for amide III/phosphate and carbonate/phosphate ratios. HEDP (9 and 18%) did not significantly alter the amide/phosphate ratio and only caused minor demineralization, whereas peracetic acid and EDTA produced dramatic changes. This study reinforced the weaker chelating ability of etidronic acid and its gentleness on the root canal dentin. It was also concluded that chelators have different degrees of action on the mineral phase of dentin, and the action is dependent on the time of contact, concentration, and chelation kinetics of the solution ($P < 0.05$). The solution used as the final rinse dictates the type of surface and the functional group exposed, which may play a role in adhesion to dental materials.

Wagner et al., 2016⁽⁵⁸⁾ studied the effects of different irrigation protocols on bovine incisors using scanning electron microscopy and transmission electron microscopy. Thirty-nine bovine incisors were decoronated and divided into 13 groups ($n=3$) based on the irrigation protocol followed: Group 1-distilled water, Group 2-0.9% saline; group 3-saline+17% EDTA, Group 4-saline+passive ultrasonic irrigation (PUI), Group 5-Saline+17% EDTA+PUI, Group 6-9 received same protocols as Group 2-5 with 2.5% NaOCl replacing saline while in Groups 10-13, 2% chlorhexidine replaced

saline. Group 1 teeth were hemisected and ultrasonicated in distilled water for 5 minutes. All other teeth were instrumented using Gates-Glidden drills until the working length of 15 mm was reached. Teeth were then hemisected similarly to Group 1 but ultrasonicated in 17% EDTA bath followed by distilled water. This was done to remove the smear layer. The tooth roots were all reassembled before exposure to the various protocols. One-half of each sample was assessed under SEM, and the other through TEM. It was found that the use of 2.5% NaOCl resulted in disinfection but also deleterious ultrastructural changes in collagen fibrils, such as thinning and erosion of the dentin walls. These changes were exacerbated by activation of the irrigant using PUI or following up with 17% EDTA.

Ozdemir et al., 2012⁽⁵⁹⁾ tested the time-dependent and ultrastructural changes caused by 17% EDTA & 2.5% NaOCl in young and old dentin. Sixty-four extracted teeth were classified as young and old based on the age of patients at the time of extraction (>30 or <30). After access cavity preparation in 48 molars, enamel and cementum layers were stripped, and the dentin was ground to a powder. The powder was divided into two subgroups to test the 1- or 10-minute exposure effects of irrigants using X-ray diffraction. Sixteen single-rooted teeth (n=8) were prepared to #60 K-file. Mid-root sections were obtained and bisected longitudinally. Each half was exposed to irrigants for either 1 or 10 minutes & observed

under SEM to calculate the area of opened tubules using Image J software. XRD analysis revealed a statistically significant decrease in hydroxyapatite intensity in old dentin after 10 minutes with EDTA & EDTA/NaOCl ($P<0.05$), whereas young dentin showed a decrease only with EDTA after 10 minutes ($P<0.01$). SEM results showed that time did not affect the area or diameter of exposed dentinal tubules in young dentin ($P<0.05$), but prolonged contact time in old dentin resulted in erosion.

Cobankara et al., 2011⁽³⁴⁾ evaluated the effect of various chelators (17% EDTA, 10% citric acid, 18% etidronate, 2.25% peracetic acid) on the mineral content of root dentin. Sixty mandibular incisors were prepared using K-files up to #35 with 2mL of deionized water between instrument changes and divided into five experimental groups ($n=12$). Dentin chips obtained during preparation were used to calibrate the ICP-AES (inductively coupled plasma-atomic emission spectrometry) machine and check levels of 8 elements-Ca, K, P, Mn, Zn, Na, S, Mg. After exposure to 5 mL of the different experimental solutions for 5 minutes, the test was repeated. Results revealed that peracetic acid was highly aggressive on the mineral content compared to EDTA, etidronate, and citric acid ($P<0.05$).

Zhang et al., 2010⁽⁵³⁾ evaluated the effects of varying concentrations and contact times of NaOCl on organic phase of dentin and canal wall erosion before and after use of EDTA as the

final irrigant. Dentin powders obtained from 60 third molars were divided into 16 groups of 50 milligrams each and immersed in 5.25% or 1.3% NaOCl for varying contact times of 10,20,30,60,120,240 minutes and then rinsed with 17% EDTA for 2 minutes. The powders were examined using ATR–FTIR spectroscopy before and after rinsing with EDTA to analyse the relative loss of organic and inorganic components (Amide I/ ν 3PO₄ ratio). Scanning and transmission electron microscopy were used to examine the erosion of 16 instrumented canal walls irrigated with 5.25% NaOCl/17% EDTA or 1.3% NaOCl/17% EDTA (n=8). Although both NaOCl concentrations caused a time-dependent, linear increase in removal of the organic phase from mineralized dentin, the rate and extent of removal were considerably more severe with the use of 5.25% NaOCl as the initial irrigant (p<0.001). Erosion was only observed in <20% of canal walls in the 5.25% NaOCl group and not observed at lower concentrations. It was concluded that the superficial erosive effect of NaOCl is irreversible and is present regardless of EDTA use. EDTA removed the collagen-depleted apatite phase to expose the underlying cause of destruction that is morphologically perceived as canal wall erosion.

Marending et al., 2007⁽⁶⁰⁾ assessed the impact of different irrigation sequences of 2.5% NaOCl and 17% EDTA on the elastic modulus and flexural strength of standardized human root dentin

bars from third molars. Fifty-five samples were divided among 5 groups (n=11) and immersed in 5mL of solutions in tubes in a water bath for 30 minutes. Groups were as follows: Group I- NaOCl/EDTA/NaOCl/H₂O; Group II-NaOCl/EDTA/H₂O; Group III- NaOCl; Group IV-EDTA; Group V-H₂O. The specimens were subjected to 3-point bending tests after different exposure times. Results showed that 24-minute exposure to NaOCl caused a significant drop in flexure strength when compared to other groups. Flexural strength was not affected by the use of EDTA with NaOCl. Elastic modulus remained unaffected. A 3-minute exposure to EDTA did not significantly affect mechanical properties when compared to water ($P>0.05$). Based on the results, shorter exposures to EDTA are clinically recommended as it did not negatively affect the mechanical dentin parameters, regardless of the irrigant sequence used.

Marending et al., 2007⁽⁶¹⁾ studied the mechanical, chemical and structural alterations of human root dentin bars after being exposed to increasing concentrations of NaOCl (1,5 and 9%). Eight maxillary third molars were used to obtain 32 dentin bars (n=8 per group) and immersed in either 5 mL of water (control) or the test solutions at 37 °C for 1 hour. Three-point bending tests were done to evaluate flexural strength and modulus of elasticity; and micro-elemental analysis was performed to assess carbon and nitrogen content. Additional samples (n=3) were studied using light

microscopy and scanning electron microscopy. Immersion in 1% NaOCl did not reveal a significant reduction in mechanical properties or element content when compared to water but immersion in 5 and 9% NaOCl reduced values by half ($P < 0.05$). 3-dimensional SEM reconstructions of partially demineralized samples showed concentration-dependent deterioration of the organic matrix. The inorganic components of dentin remained unaffected at all concentrations.

Smear layer removal

Kfir et al., 2020⁽³⁷⁾ compared the cleanliness and erosion of root canal walls using a HEDP-based irrigant (DualRinse HEDP, MedCem, Vienna Austria) with conventional irrigation protocol of 3% NaOCl/17% EDTA. Sixty single-rooted teeth were prepared using ProTaper Next system (Dentsply Maillefer, Switzerland) till size F3 and randomly assigned into 2 experimental groups ($n=20$) based on irrigation protocol followed; an additional 10 teeth were used as controls. Group 1: 2mL of 3% NaOCl per instrument followed by 2mL of 17% EDTA; Group 2: 2 mL of DualRinse HEDP per instrument; Control: 2 mL of deionised water per instrument. Teeth were longitudinally split and observed under SEM to check for presence of a smear layer, debris, and erosion of the root canal wall in coronal, middle and apical thirds. Smear layer removal efficacy was not statistically significant between the experimental groups ($P=0.545$). It was observed that smear layer removal was less

effective in the apical third than the coronal third in both the experimental groups. Debris was absent in almost all cases($p=0.342$). Moderate erosion was found in 10–26% of the cases in both groups and one case of severe erosion was reported in both groups ($p=0.606$). It was concluded that the HEDP-based irrigation solution did not differ significantly from sequential irrigation in terms of cleanliness or erosion of the canal wall.

Emre Erik et al., 2019⁽⁶²⁾ examined the smear layer removal with various concentrations of etidronate solutions using scanning electron microscopy. Seventy-eight teeth with single root canals were instrumented upto size X4 using ProTaper Next system (Dentsply Maillefer, Switzerland). Two millilitres of 2.5% NaOCl was used between file changes. The teeth were divided into 6 groups ($n=13$) based on final irrigation (5 mL for 3 minutes): saline, 17% EDTA, 9% HEBP, 18% HEBP , 1% NaOCl + 9% HEBP , 2% NaOCl + 18% HEBP. The teeth were split longitudinally and observed for smear layer under 2000x magnification and scored according to Torabinejad et al criteria⁽⁶³⁾. There were no significant differences among the treatment groups in the smear layer scores of the coronal and middle thirds. Overall, the 2% NaOCl + 18% HEBP group had the lowest scores ($P<0.05$). The clinical recommendation made was to use 18% HEBP along with an oxidizing agent such as NaOCl to ensure optimum smear layer removal.

Wright et al., 2019⁽⁴⁴⁾ investigated chelators which would be stable when mixed with NaOCl and exhibit minimal loss of free available chlorine (FAC) over a period of 80 minutes. They also investigated potential mixtures by further assessing FAC levels over 18 hours and the capacity to remove smear layer. Of the chelators tested, alkaline 0.5 mol/L clodronate mixed equally with 10% NaOCl had potential for use in continuous chelation. The mixture lost no FAC over 18 hours. The pH remained above 12 only in the clodronate mixture throughout the experiment. Although smear layer removal was achieved, the percentage area fraction (%AF) removed by the 0.5mol/L clodronate mixtures was lesser when compared to 0.5 mol/L etidronate + 10% NaOCl and sequential irrigation protocol groups.

Deari et al., 2018⁽⁴¹⁾ compared calcium chelating ability and smear layer removal of solutions of di- and tetrasodium EDTA and HEDP. Solutions were prepared by dissolving di- and tetrasodium salts of HEDP or Na₄EDTA in deionized water to achieve same molarity as 17% EDTA. Root dentine disks from human third molars were prepared to form a smear layer and then immersed in the experimental solutions or phosphate-buffered saline for 1 minute. Dissolved calcium levels were estimated by atomic absorption spectroscopy. Patent dentinal tubules were assessed by laser scanning microscopy. Results revealed that acidic disodium salts of HEDP dissolved more calcium than alkaline tetrasodium salts of

HEDP ($P<0.05$). EDTA solutions dissolved more calcium than both di- and tetrasodium solutions of HEDP($P<0.05$). The number of open tubules was directly related to the pH of the solution. Therefore, it was concluded that chelation and smear layer removal ability were influenced by pH of the solution.

Patil et al., 2018⁽⁴²⁾ compared smear layer removal efficacy of saline, 5.25% NaOCl & 17% EDTA with surfactant (Cerkamed, Poland), MTAD (Dentsply Sirona) & Chloroquick (18% etidronic acid+5% NaOCl) in the apical third of 40 single rooted mandibular premolars using scanning electron microscopy. After tooth preparation, teeth were randomised into 4 groups($n=40$), subjected to 5 mL of the respective final irrigants for 3 minutes. Teeth were split longitudinally and observed at 500x and scored according to Hulsmann criteria. They concluded that sequential use of NaOCl followed by EDTA was more efficient than MTAD or Chloroquick ($P<0.001$). Chloroquick was cited as having less efficacy due to its weaker chelating ability and lower contact time with root dentin as it was used only as a final rinse.

Ulusoy et al., 2017⁽⁴⁰⁾ investigated smear layer removal by different irrigants and marginal adaptation of a resin-based sealer to root canal dentine treated with the irrigants. Seventy instrumented root canals were divided into 7 groups ($n=10$) according to the final irrigant employed: 9% and 18% HEBP; 0.5, 1 and 2% peracetic acid,

17% EDTA or saline. After longitudinal splitting, root halves were evaluated for amount of smear layer using scanning electron microscope at 2000x and scored according to Hulsmann criteria. Eighty-two root canals were instrumented and obturated with gutta percha and AH Plus sealer (Dentsply Maillefer, Switzerland). Slices obtained from apical third of each specimen were used to assess marginal adaptation of root canal sealer under SEM. Use of 9% and 18% HEBP resulted in more efficient smear layer removal in the apical third compared to the other chelators ($P<0.05$). Eighteen percent HEBP group showed the lowest gap size values and better marginal adaptation of the sealer to root dentin walls ($P<0.05$).

Yadav et al., 2017⁽³⁹⁾ compared smear layer removal efficacy of 9 and 18% etidronic acid, SmearClear (Kerr Dental, USA), MTAD (Dentsply Sirona) in 50 mandibular premolars. The teeth were standardised and instrumented upto size ProTaper F5 (Dentsply Maillefer, Switzerland). They were then randomly divided into 5 groups based on the chelator used ($n=10$). 5mL of the chelating solutions were used for 5 minutes following instrumentation and irrigation with 1% NaOCl. The samples were split longitudinally and observed under SEM for smear layer. It was found that etidronic acid was inferior to both MTAD and SmearClear, probably due to its weaker chelating ability ($P<0.05$).

Wang et al., 2016⁽⁶⁴⁾ examined the level of erosion in root dentin caused by different irrigation methods and protocols. 35 extracted maxillary molars were instrumented and divided into 7 groups (n=5) according to the irrigation methods employed. The root canal surface was observed by scanning electron microscopy, and the dentin composition was analysed by continuous line scanning for 300 µm into dentin using energy dispersive X-ray spectroscopy. Both GentleWave cleaning and syringe needle irrigation with 3% and 5% NaOCl followed by final rinse with 8% or 17% EDTA cause insignificant changes in the relative proportions of C, O, Ca, and P in the circumpulpal dentin. On the contrary, additional final irrigation with 3% and 5% NaOCl following EDTA caused a rapid decline in Ca and P content of dentin, atleast to a depth of 300 µm in dentin. It was concluded that final irrigation with NaOCl following 17% EDTA should be avoided to reduce weakening of the root.

Kuruvilla et al., 2015⁽⁶⁵⁾ used scanning electron microscopy to compare the efficacy of 17% EDTA, 18% etidronic acid and 7% maleic acid on smear layer removal. Thirty mandibular premolars were prepared upto MAF #40 K-file with 2.5% NaOCl as main irrigant. Samples were divided into 3 groups (n=30) according to chelator used. 5 mL of the chelators were in contact with root canal dentin for 1 minute. SEM analysis of split teeth was done at 2000x magnification. Scoring of smear layer was done according to

Torabinejad et al. ⁽⁶³⁾ It was found that 7% maleic acid performed better than 17% EDTA and 18% etidronic acid, attributed to its lower surface tension and acidity ($P=0.007$).

Yadav et al., 2015⁽³⁶⁾ quantified the amount of calcium ions removed from root canals by 9% etidronic acid (HEBP), BioPure MTAD (Dentsply Sirona), and SmearClear (Kerr Dental, USA) using atomic absorption spectrophotometer. Fifty extracted human mandibular premolar teeth were instrumented and divided into 5 groups based on chelators used-9% HEBP, 18% HEBP, SmearClear, MTAD, saline and subjected to atomic absorption spectrophotometry. 18% HEBP removed more calcium than 9% HEBP from the root canal, but was relatively weaker than SmearClear and BioPure MTAD due to the lack of surfactants which enhanced their diffusion inside dentinal tubules ($P<0.001$).

Ashraf et al., 2014⁽⁶⁶⁾ compared smear layer removal in apical thirds of 50 premolars ($n=15$) by 17% EDTA, 18% etidronate and Er:YAG laser. Canals were instrumented using HERO 642 rotary files and 1% NaOCl as main irrigant before experimental interventions. Group 1: 2 minutes of laser irradiation; Group 2: 2 minutes with 5 mL of 17% EDTA irrigation followed by 5 minutes with 5 mL of 5.25% NaOCl; Group 3: 5 minutes with 5 mL of 18% etidronate followed by 5 minutes with 5 mL of 5.25% NaOCl. Scanning electron microscopy was used to obtain photos at 2000x

magnification and images were scored using a 5-point scoring system by Hulsmann et al. Scores 1& 2 were deemed acceptable and 3&4 were unacceptable. Results showed statistically significant differences ($P<0.05$) among all groups, with EDTA showing most effective smear layer removal.

Paque et al., 2012⁽³³⁾ compared the reduction of hard-tissue debris accumulation during root canal instrumentation in 60 mandibular molars with isthmuses in the mesial root canal system when irrigated with either 2.5% NaOCl or 2.5% NaOCl + 9% etidronic acid (HEBP) using micro-CT. Two millilitres of irrigants were used after each instrument and 5 mL as a final rinse. Micro-CT scans were taken at high resolution before and after exposure to the irrigants to calculate accumulated hard-tissue debris as percentage volume of the original canal anatomy. The debris accumulated was 5.5 ± 3.6 vol% vs. 3.8 ± 1.8 vol% for NaOCl and NaOCl/HEBP respectively ($P<0.05$). It was concluded that an etidronate mixture could reduce but not fully prevent the formation of hard tissue debris.

Paque et al., 2011⁽⁹⁾ evaluated the effect of sequential irrigation with 1% NaOCl/17% EDTA activated by passive ultrasonic irrigation (PUI) on accumulated hard tissue debris (AHTD) in 20 mandibular molars with isthmuses in the mesial root canal system using micro-CT. Pre-operative scans were taken. Teeth were

prepared using ProTaper rotary system (Dentsply Maillefer, Switzerland) till size F3 with 1 mL of NaOCl in between files and 5 mL as final rinse and scanned again. Teeth were scanned for a third time following irrigation with 5mL of 17% EDTA over 2 minutes. PUI was done thrice over 20 seconds and teeth were rescanned, resulting in 4 scans per tooth. AHTD was calculated as percentage volume of the original canal anatomy. Sequential irrigation protocol of NaOCl followed by EDTA significantly reduced AHTD from $6.9 \pm 4.2 \text{ vol\%}$ to $4.9 \pm 3.6 \text{ vol\%}$ ($P < 0.01$). PUI further reduced AHTD to $3.7 \pm 2.8 \text{ vol\%}$ ($P < 0.01$). It was concluded that sequential irrigation and activation reduced AHTD significantly but around 50% still remained within root canal systems.

Qian et al., 2011⁽⁵⁴⁾ quantitatively analysed the erosion of root canal dentin by different irrigation regimens using 5.25% NaOCl and chelators such as 17% EDTA, 10% citric acid and 17% ethyleneglycoltetraacetic acid (EGTA). Eighty-four single rooted teeth were used for the experiment and divided into nine groups based on the time and sequence of irrigants used after preparation. Time of exposure to irrigants varied from 1-10 minutes and the sequences were varied. After the experimentation, blocks were cut from the samples and viewed under SEM at 2000x until an area of minimum 500 tubule openings were seen. The area of the orifices was auto-calculated using Image-Pro 5.0 software. To assess the reproducibility of the method, calibrated examiners repeated the

analyses. Results showed that tubule openings were larger when NaOCl was used after chelators ($p < 0.05$). There was no significant difference among chelators ($p > 0.05$). EGTA caused less severe erosion than the other two chelators.

Mello et al., 2010⁽⁶⁷⁾ assessed the influence of final rinse technique on the ability of 17% EDTA to remove smear layer. Sixteen single-rooted anterior human teeth were instrumented upto #20 MAF using hand files and 1% NaOCl. They were then divided into 2 groups ($n=14$) depending on the technique of final rinse step: continuous rinse of 5 mL EDTA for 3 minutes or rinse and soak over 3 minutes. Specimens were split longitudinally and observed under scanning electron microscope at 500x magnification and scored on an ordinal scale. The continuous rinse group presented more debris-free surfaces when compared with the rinse and soaking group; and control group ($P < 0.01$). It was concluded that a continuous rinse with 5 mL of EDTA for 3 minutes removed smear layer more efficiently from root canal walls.

Lottanti et al., 2009⁽¹⁵⁾ evaluated the effects of chelators used with 1% NaOCl on calcium chelation and smear layer removal from canals. Forty-eight premolars were divided into 4 groups ($n=12$) and instrumented to size 45/0.04 using ProFile system (Dentsply Maillefer, Switzerland). Irrigant volumes and contact times were standardised as 10 mL over a period of 15 minutes during

instrumentation and 5 mL over 3 minutes for the final irrigation. The protocols were as follows: Group 1-1% NaOCl + deionised water; Group 2-1% NaOCl +17% EDTA; Group 3- 1:1 mix of 2% NaOCl with 18% etidronic acid; Group 4-1% NaOCl+2.25% peracetic acid. Eluted calcium was assessed using atomic absorption spectroscopy while smear-layer removal was studied using scanning electron microscopy. Most canal walls exposed to chelators were free of smear layer with no statistically significant difference among them($p=0.95$). EDTA and peracetic acid showed marked demineralization patterns while HEBP was milder on root dentin. It was concluded that all the decalcifying agents evaluated were able to remove as well as prevent smear layer formation.

De-Deus et al., 2008⁽³⁸⁾ compared smear layer removal kinetics of 9 and 18% etidronate(HEBP), 17% EDTA longitudinally and quantitatively on human dentin using a single-tooth comparative model. Dentin disks from the middle third of crown of 3 maxillary molars were used and a smear layer was produced on the pulpal side. This surface was divided into 3 equal areas, one for each solution. The other 2 areas would be masked with tape during exposure to a particular chelator, hence facilitating single-tooth comparison. A motorised microscope of 0.1 micron/pixel resolution was focused at 1000x magnification and coupled to a digital camera. Chelators could be applied without disturbing the sample under the microscope. Co-site image sequences of these areas were obtained

after multiple exposures, providing 16 images from each area of each tooth for each experimental time. Percentage area fraction (% AF) for thousands of tubules over time was obtained which allowed quantification of the effect of the chelators. EDTA samples were found to be completely free of smear layer after 60 seconds with enlargement of the tubule diameter over time. HEBP specimens at both concentrations were completely smear-free only after 300 seconds of etching. EDTA exposed more tubules than 18% and 9% HEBP at all experimental times. Overall, demineralization kinetics of HEBP solutions were significantly slower than those of 17% EDTA ($P < 0.05$).

Mello et al., 2008⁽⁶⁸⁾ analyzed the effect of different volumes of 17% EDTA as a final rinse on smear layer removal in different areas of root canals. Forty single-rooted anterior teeth were instrumented and divided into 3 test groups according to the volume of 17% EDTA used for final rinse (5, 10, 15 mL) and 1 control group (10 mL of 1% NaOCl). The roots were axially split and the smear layer removal was determined under scanning electron microscope. Control group samples exhibited statistically significant amount of smear layer compared to test groups ($P < 0.01$). No statistically significant difference was found among test groups or across root thirds ($P > 0.05$). It was concluded that a final rinse with 5 mL of EDTA was sufficient for good smear layer removal.

Crumpton et al., 2005⁽⁶⁹⁾ quantified the volume of 17% EDTA needed to efficiently remove the smear layer after instrumentation with ProFile GT rotary system. Forty teeth with a single canal were instrumented, irrigated with 1mL of 5.25% NaOCl between files and divided into 4 groups. The control group didn't receive any further action. The 3 experimental groups received either 1, 3, or 10 ml of 17% EDTA for 1 minute, followed by a final rinse with 3 ml of 5.25% NaOCl. Roots were split buccolingually and photos taken using a digital camera were evaluated for debris using Adobe Photoshop 7.0. Four random samples from each group were scored for smear layer removal using the criteria proposed by Torabinejad et al⁽⁶³⁾ under SEM. Results showed no significant differences among groups with respect to debris remaining or quality of smear layer removal ($P < 0.05$). It was concluded that a volume greater than 1 ml of EDTA did not significantly improve debris or smear layer removal.

Girard et al., 2005⁽⁷⁰⁾ tested an aqueous gel containing 18% HEBP as a potential decalcifying agent by evaluating its interactions with 1% NaOCl. Two commercial paste-type chelators containing 15% EDTA and urea peroxide (RC-Prep and Glyde) were used as controls. Hundred milligrams of the chelators was mixed with 1mL of 1% NaOCl. Iodometric titration was done after 5 minutes and 1 hour to measure free available chlorine; and UV spectrometry was performed to test interactions with 1% NaOCl. Forty-eight single

rooted premolars were assigned to 3 groups (n=16), control group with no chelator, RC-prep and experimental gel group. Teeth were instrumented to size 45/0.04 with 10 mL of 1% NaOCl solution between file changes; the files were dipped in the experimental gel, paste or nothing and rinsed off after preparation. Longitudinal grooves placed buccally and lingually helped split the teeth which were then assessed using scanning electron microscopy at 500x for smear layer removal. Scores were given based on a pre-determined scale. Results from the iodometric titration showed that none of the gel components had short-term interactions with NaOCl, whereas RC-Prep and Glyde caused an immediate reduction in free available chlorine. The gel chelated more calcium and had significantly lower smear scores than RC-Prep in the coronal and middle root thirds, whereas no differences were observed in apical root thirds ($P < 0.05$).

Zehnder et al., 2005⁽⁷¹⁾ evaluated the effect of reducing surface tension of chelators on their ability to remove calcium from instrumented root canals. Aqueous solutions of 15.5% EDTA, 10% citric acid, 18% HEBP were prepared with and without 1% polysorbate (Tween 80) and 9% propylene glycol. Surface tension in these solutions was measured using the Wilhelmy plate method. Sixty-four single-rooted human teeth were instrumented using ProFile system upto 45/0.4 and were divided into 8 groups(n=8) which received 5 mL of respective chelators for 1 minute. Calcium concentration in eluates was measured using atomic absorption

spectrometry. Addition of surfactants caused a reduction of surface tension values by approximately 50% in all tested solutions but none of these solutions chelated more calcium from canals than their pure forms. Citric acid removed more calcium than HEBP and EDTA($P<0.05$), but there was no significant difference between EDTA and HEBP.

Scelza et al., 2004⁽⁷²⁾ tested 3 different time periods of irrigation (3,10,15 minutes) with 17% EDTA, EDTA-T (EDTA+1.25% sodium lauryl ether sulfate), 10% citric acid on smear layer removal efficacy in root canal dentin using scanning electron microscopy. Ninety canines ($n=10$) were divided among 9 groups according to time and solution. 20 mL of the chelating solution was used as final irrigant before subjecting the samples to SEM analysis. The 3-minute exposure duration exposed most dentinal tubules in all groups. A statistically significant difference was observed between 3- and 15-minute exposure to EDTA($P<0.05$), with the shorter duration exhibiting greater number of open tubules. It was concluded that short exposures were effective and increasing the duration of exposure to chelators did not improve results.

Calt and Serper 2002⁽⁷³⁾ evaluated the effects of EDTA on smear layer removal and dentin after 1 and 10 minutes of exposure. Six single-rooted teeth were instrumented to #60 using K-files. A 5 mm segment from the middle third was cut longitudinally into two

halves. Halves belonging to the same root were irrigated for either 1 or 10 minutes using 10 ml of 17% EDTA, followed by 10 ml of 5% NaOCl. The specimens evaluated under scanning electron microscope at 2500-4000x. Results showed that 1-minute EDTA irrigation effectively removed the smear layer but a 10-minute application caused excessive peritubular and intertubular dentinal erosion.

Niu et al., 2002⁽⁴⁹⁾ studied the erosive effects of final irrigation with 15% EDTA and/or 6% NaOCl on root dentin when exposed for different time periods (1-3 minutes) under a scanning electron microscope. Instrumented samples were randomised into 5 groups according to the irrigation protocols followed. Roots were split longitudinally and observed under SEM at 3000x magnification. Images were evaluated for remaining debris and erosion were assessed at 1, 3 and 6 mm away from the apex. Results showed that final rinses with EDTA resulted in smooth surfaces with well separated open tubules. Final irrigation with 6% NaOCl after 15% EDTA resulted in peri- and intra-tubular erosion with rough surfaces and enlarged tubule orifices ($P < 0.05$).

Effect of root canal irrigants on dentin hardness

Ulusoy et al., 2020⁽⁴³⁾ evaluated the nanohardness values and erosion of root canal dentine after exposure to various chelators, namely, 17% EDTA, 9% etidronic acid and 2% peracetic acid either

alone or in combination with 2.5% NaOCl. For nanohardness evaluation, mid-root dentine blocks were obtained from 162 single rooted teeth, embedded in epoxy resin blocks exposing the root canal walls (n=10). Nanohardness values were measured using a nanoindenter with a Berkovich tip before and after exposure to irrigants. Eighty instrumented root canals (n=80) were used to assess erosion using scanning electron microscope. Both concentrations of etidronic acid, when used alone or in combination with 2.5% NaOCl significantly reduced nano-hardness values and caused pronounced peritubular and intertubular erosion of root dentin compared to other groups ($P<0.05$).

Grinkeviciute et al., 2019⁽⁷⁴⁾ evaluated the effect of different irrigant solutions on the microhardness of root canal dentin and surface erosion. Twenty-eight single rooted teeth were decoronated and sectioned longitudinally to produce 56 halves which were embedded in resin. They were then divided into 4 groups (n=14) as per final irrigation solution used-control: saline for 1 minute, E group: 17% EDTA for 1 minute, ES group: 17% EDTA for 1 minute+2.5% NaOCl for 1 minute, H group: DualRinse HEDP(MedCem, Vienna Austria) for 3 minutes. Vicker's microhardness test was performed and results showed that DualRinse caused the largest reduction in microhardness ($P<0.05$), possibly due to the longer exposure time of 3 minutes.

Bhagwat et al., 2016⁽⁷⁵⁾ compared the effect of 17% EDTA, 2% chlorhexidine, 18% etidronic acid and 4% propolis on the microhardness of root dentin. 100 longitudinally sectioned halves of single - rooted teeth embedded in acrylic resin were divided randomly into five groups (n=20). The halves were first exposed to 1.3% NaOCl for 20 minutes followed by 17% EDTA for 1 minute or the other irrigants for 5 minutes, according to the groups. Following this, all specimens were placed on the Vickers hardness tester and three readings were taken. 18% HEBP had the least effect on the root dentin microhardness, followed by 4% propolis and 2% CHX. 17% EDTA showed maximum effect on the microhardness of the dentin.

Tartari et al., 2013⁽⁷⁶⁾ investigated the effect of 2.5%NaOCl, 17% EDTA, 9% etidronic and 10% citric acid in different irrigation regimens on root dentin microhardness. Teeth that met inclusion criteria were decoronated, sectioned apically and longitudinally split to obtain 45 root halves. These were then split horizontally based on root thirds and embedded in resin. Surfaces were polished and prepared for Knoop's indentation. Samples were randomly divided into 9 groups: G1-saline; G2-5% NaOCl+18% HEBP for 30 minutes, G3-2.5% NaOCl for 30 minutes (n=27); After microhardness measurements, G3 was subdivided (n=9) based on the chelating agent to form G4-17% EDTA for 3 minutes;G5-10% Citric acid for 3 minutes,G6-9% HEBP for 5 minutes. Following new

microhardness measurements, G4,G5 and G6 received a final flush with 2.5% NaOCl for 3 minutes forming G7,G8 and G9 respectively. All tested irrigation regimens significantly decreased the microhardness except the control($P<0.05$). None of the root thirds exhibited statistically significant differences in microhardness values within the groups.

Ulusoy et al., 2013⁽⁷⁷⁾ compared the effects of different irrigants on root dentin microhardness, smear layer removal and erosion. Seventy-two single rooted teeth were instrumented to size F4(ProTaper) with saline as working solution. Slices measuring 5 mm were obtained from mid-root area and further sectioned buccolingually to produce root halves. One half of each sample was embedded in resin and divided into 6 groups according to irrigant used ($n=12$). The groups were as follows:17% EDTA+2.5% NaOCl; 7% Maleic acid+2.5% NaOCl; 1.3% NaOCl +BioPure MTAD; SmearClear; 5%NaOCl and 0.9% saline. The samples were then subjected to microhardness tests and SEM analysis. EDTA, maleic acid and MTAD caused significant reductions in microhardness ($P<0.05$), with maleic acid exhibiting the greatest reduction ($P<0.05$). There was no significant difference in smear layer removal in coronal and middle thirds but maleic acid performed better in the apical third ($P<0.05$).

Dineshkumar et al., 2011⁽³⁵⁾ evaluated the effect of 17% EDTA, MTAD (Dentsply Sirona), and 18% HEBP on the microhardness of human root canal dentin using Vickers microhardness test. Forty human single-rooted teeth were sectioned mesiodistally and the split halves were divided into four groups (n=20) based on final rinse. 1.3% NaOCl was used as working solution for 20 minutes during preparation. All final irrigants were used for 5 minutes, except 17% EDTA, which was used for 1 minute. 18% HEBP showed the highest dentin microhardness values while least microhardness values were found with MTAD ($P < 0.001$).

Saghiri et al., 2009⁽⁷⁸⁾ investigated the relation between erosion and microhardness of root canal dentin after irrigation with different agents. Seventy-two human premolars with single canals were instrumented and the middle part of each root was transversely sectioned to obtain 4-mm slices. The specimens were divided into 6 groups based on irrigation regimens. G1-2.6% NaOCl for 5 minutes; G2-17% EDTA for 5 minutes + 2.6% NaOCl for 5 minutes; G3-17% EDTA for 1 minute + 2.6% NaOCl for 1 minute; G4-MTAD for 5 minutes; G5-2% Chlorhexidine for 5 minutes; G6-saline. Vickers microhardness tester was used to measure microhardness value before and after exposure to the irrigants. Specimens were also prepared for SEM analysis to assess dentin erosion. Samples irrigated with 17% EDTA and then 2.6% NaOCl showed the most erosive effect on dentin along with the least reduction of dentin

microhardness at depth of 100 microns ($P < 0.0001$). MTAD showed the most reduction of dentin microhardness and less erosive effect on dentin. It was concluded that erosion is not a main factor in decreasing the dentin microhardness, whereas the amount of irrigant penetration might be the main cause. MTAD, by virtue of its lower surface tension was able to penetrate better and hence reduced dentin microhardness.

Sayin et al., 2007⁽⁷⁹⁾ evaluated the effect of application of various chelators alone (17% EDTA, 15% EDTAC [EDTA+0.1%Cetavlon], 17% EGTA, 1% Tetracycline-Hydrochloride) or followed by 2.5% NaOCl on root canal dentin microhardness. Thirty premolars were longitudinally sectioned and embedded in resin and subjected to Vicker's microhardness test before and after exposure to irrigants ($n=60$ [10 per group]). Of the tested solutions, use of 17% EDTA alone or 17%EDTA followed by 2.5%NaOCl resulted in the largest reduction in microhardness ($P < 0.05$). Combination treatment protocols using both a chelator and 2.5% NaOCl led to a significant reduction in microhardness compared to exposure to chelators alone ($P < 0.05$). The effects of NaOCl on dentin were dependent on the region of the root canal tested and the material it was paired with.

Slutzky-Goldberg et al., 2004⁽⁸⁰⁾ evaluated the effect of 2.5% and 6% NaOCl solutions on root dentin microhardness when used for 5,10 and 20 minutes. Forty-two bovine roots were divided into

seven groups (n=6). The control group was irrigated with saline. Microhardness was measured using Vicker's microhardness tester at depths of 500,1000 & 1500 microns from the lumen. The reduction in microhardness at 500 microns distance for samples irrigated with 6% NaOCl (P=0.352) and 2.5% NaOCl (P=0.084) was statistically insignificant after 5 minutes, but statistically significant after 10 and 20 minutes of exposure (P<0.001). At all distances, the decrease in microhardness was marked after irrigation with 6% NaOCl than 2.5% NaOCl (P=0.031). It was concluded that exposure of bovine dentin to NaOCl solutions for more than 10 min decreased dentin microhardness significantly. Therefore, it was advised to avoid higher concentrations of NaOCl for root canal irrigation.

Effect of final irrigant on sealer penetration

Gandhi et al., 2020⁽⁸¹⁾ evaluated the effect of various chelators on wettability of AH Plus(Dentsply Sirona) and BioRoot RCS sealers(Septodont, USA) on root dentin. Fifty single rooted premolars were split longitudinally into 100 sections, flattened and polished to obtain flat surfaces for analysis. The sections were randomised into 5 groups (n=20), based on the final irrigant used: 17% EDTA, 7% maleic acid, SmearOFF, DualRinse HEDP (MedCem, Vienna Austria) (9% HEDP) and distilled water. 5 mL of the final irrigants were used for 1 minute before rinsing with distilled water. After drying the specimens, contact angle measurements of both sealers on treated dentin was carried out.

Results showed that wettability of both sealers was highest when maleic acid was used. BioRoot RCS exhibited better wettability with SmearOFF than 17%EDTA ($P<0.001$). It was concluded that final irrigation with 7% maleic acid led to better wettability of both sealers.

Neelakantan et al., 2012⁽²⁰⁾ tested dentin bond strength of AH Plus sealer (Dentsply Sirona) to root canal walls after using a sequential/continuous chelation protocol. One hundred maxillary canines were recruited and prepared using MTwo instruments (VDW GmbH, Munich) upto 35/0.06 taper and randomly divided based on irrigation protocol ($n=20$): Group 1-2.5% NaOCl; Group 2- 2.5% NaOCl+17% EDTA; Group 3-1:1 mix of 5% NaOCl+18% HEBP as both working solution and final rinse; Group 4-1:1 mix of 5% NaOCl+18% HEBP followed by 17% EDTA; Group 5- 1:1 mix of 5% NaOCl+18% HEBP followed by distilled water. Roots were obturated with AH Plus sealer & embedded in resin molds. Twelve slices per root were cut using a saw and 3 were subjected to push-out test. Results showed a statistically significant difference in bond strength depending on the root third examined ($P=0.0009$). Continuous chelation protocols showed higher bond strength than traditional protocols in all root thirds; Usage of 17% EDTA after continuous chelation increased bond strength further ($P<0.05$) whereas usage of NaOCl alone led to least bond strength($P<0.05$).

De-Deus et al., 2008⁽³⁸⁾ compared the effects of a strong and soft chelating agent (MTAD (Dentsply Sirona) and 18% HEBP) on the

bond strength of Resilon/Epiphany root fillings. 17% EDTA and a group with no chelator were used as controls. Forty maxillary lateral incisors were randomised into the 4 experimental groups. After standardisation of working length, root canal preparation was performed using K3(Kerr Dental) instruments upto 35/0.06 with 0.5 mL of 1.25% NaOCl in between files. Group 1 was not flushed with chelating solution; Group 2 samples received 3 mL flush of 17% EDTA for 3 minutes; Group 3 received 5mL of MTAD for 5 minutes; Group 4 received 0.25 mL of 18% HEBP mixed with 0.25% NaOCl during instrumentation and a final flush with 2mL of 18% HEBP for 2 minutes. Prepared teeth were obturated using Resilon core with Epiphany sealer. The teeth were then decoronated and the roots were stored for a week at 100% humidity. Each root was horizontally sectioned to create 9 slices measuring 1 mm per root. Micropush-out assessment was performed on a universal testing machine. Bond strength was highest in HEBP samples; intermediate in EDTA, MTAD groups and least in NaOCl group ($P < 0.05$). It was concluded that HEBP optimised the bonding quality of Resilon/Epiphany fillings.

Fracture resistance

Ulusoy et al., 2021⁽⁸²⁾ evaluated the effects of 9% etidronic acid (HEBP) & 17% EDTA used with either passive ultrasonic (PUI) or conventional syringe irrigation (CSI) on fracture resistance of roots of varying thickness. One hundred and thirty mandibular premolars

were decoronated and standardised to 13 mm lengths, of which 120 were instrumented to size F3 and progressively widened using burs to result in various thicknesses of root dentin. These were divided into 3 groups (n=40) based on thickness 0.75, 1.5 and 2.5 mm and further subdivided into 4 groups based on final irrigation and activation regimen. The roots were then obturated and subjected to vertical compressive force in a universal testing machine. Results indicated that irrigant used, irrigation method and dentin thickness were all significant predictors of fracture resistance of roots ($P<0.05$, $P<0.001$, $P<0.001$). 9%HEBP was associated with higher fracture resistance values compared to 17%EDTA.

Gonzalez et al., 2020⁽⁸³⁾ evaluated the effects of 9% etidronic acid (EA), 2 different EDTA preparations (17% EDTA, SmearClear), alternated with 2.6% NaOCl when used as a final rinse, on fracture resistance of human roots. Seventy-two single rooted premolars were decoronated, embedded in wax to create customised receptacles, randomised into 4 groups (n=16-21). Canals were prepared using M-Two file system (VDW GmbH, Germany) upto size 40/0.04 with 2.5% NaOCl as working solution for the EDTA groups; 5% NaOCl was combined with 18% EA for the EA group. After irrigant activation and drying, the roots were embedded in cylindrical moulds with coronal 4mm exposed and mounted on a universal testing machine. The roots were subjected to vertical compressive force until fracture. Results indicated lowest values for

SmearClear group and highest for control group, with no significant difference between the groups ($P=0.82$)

Dominguez et al., 2018⁽⁸⁴⁾ evaluated the effects of 2.5% NaOCl, 17% EDTA, 2% CHX and 18% HEBP on root fracture resistance in root canal treated human teeth. Seventy extracted single-rooted teeth were decoronated and dentin thickness was standardised before randomly dividing them among 4 groups ($n=16$). Canals were prepared using ProTaper Next rotary system (Dentsply Maillefer, Switzerland) upto size X3 with 2 mL of irrigant between files. Group 1 samples were treated with saline; 17% EDTA was used as final irrigant in groups 2,3 whereas group 4 samples were exposed to mixture of 18% HEBP and 5% NaOCl for 25 minutes. They were subjected to axial forces by mechanical compression testing in a universal testing machine after 1 week in an incubator. The control group presented greatest resistance to root fracture. Results indicated that all tested irrigation regimens reduced fracture resistance to some degree with a significant reduction observed in the continuous chelation group ($P<0.05$). The time-dependent action of 9%HEDP combined with 2.5% NaOCl has been claimed as the reason for this finding.

Uzunoglu et al., 2016⁽⁴⁸⁾ compared the effect of different final irrigants on the fracture resistance of root filled teeth. Eighty mandibular incisors were prepared and divided into 8 groups (2

control, 6 experimental) (n=10). The final irrigants tested were saline, 17% EDTA, REDTA, 2% chlorhexidine, QMix, BioPure MTAD (Dentsply Sirona). All irrigants had a contact time of 1 minute before obturation except BioPure MTAD which had a 5-minute exposure time. The samples were then vertically fractured in a universal testing machine. Results showed that endodontic preparation as well as the final irrigant used contributed significantly to reduced fracture resistance values ($P < 0.05$). The MTAD group, by virtue of its longer exposure time, had the lowest fracture resistance scores; the REDTA and QMix groups had higher scores attributed to the presence of surfactants and hence, shorter exposure times.

Interactions between irrigants

Wright et al., 2020⁽²⁶⁾ evaluated the stability of mixtures of 0.26M clodronate with 5% NaOCl at room temperature (23°C), root canal temperature (34–35°C) and in refrigerated storage temperatures (2–4°C). FAC levels were measured using iodometric titration for clodronate–hypochlorite mixtures at all 3 temperatures. The solutions were stable for 180 min at 34–35°C, ($p < 0.05$), for 3 months at 4–5 °C, they maintained 95% of the FAC. It was concluded that the therapeutic window of clodronate–hypochlorite mixtures is unaffected at root canal temperature.

Wright et al., 2019⁽²⁴⁾ compared organic tissue dissolution, residual FAC and tissue dissolution capacity of 0.26M etidronate and 0.26 M clodronate mixtures with 5% NaOCl after 6 hours of mixing. Immediately after mixing, porcine palatal mucosa samples were added to 32°C solutions containing chelator-NaOCl mixtures, NaOCl alone or phosphate-buffered saline. Weights and FAC, were recorded initially and again after 15 minutes. FAC was measured by iodometric titration. 6 hours after mixing, mucosa was again added to the same mixtures. Sample weights at 0, 5, 10, and 15 minutes were recorded. Immediately after mixing, clodronate-5% NaOCl mixture dissolved mucosa just as well as 5% NaOCl alone and better than etidronate-5% NaOCl($P<0.05$) At 6 hours after mixing, clodronate-2.5% NaOCl dissolved organic material just as well as 2.5% NaOCl alone. However, at doubled concentrations clodronate mixtures dissolved less mucosa than 5% NaOCl alone. Hence, it was concluded that soon after mixing, clodronate mixtures better dissolve organic material than etidronate mixtures and have higher residual FAC. Six hours from mixing, 0.26 mol/L clodronate-2.5% NaOCl mixtures dissolved organic material similar to controls.

Zollinger et al., 2017⁽²²⁾ assessed the short-term storage stability of NaOCl solutions when combined with Dual Rinse HEDP (MedCem, Vienna Austria). The mixtures were prepared in a way that they initially contained varying concentrations of NaOCl (5.0%, 2.5%, or 1.0% NaOCl) and always 9.0% of dissolved Dual Rinse HEDP

powder. Plain NaOCl solutions were used as controls. Stability of these solutions over 8 hours was assessed in transparent borosilicate glass bottles at 23°C. The effects of heating to 60°C or storing the solutions at 5°C were studied in polypropylene syringes. Concentrations were measured by iodometric titration. At 23°C, the 5.0% NaOCl/9.0% Dual Rinse HEDP solution lost 20% of the available chlorine after 1 hour whereas the corresponding 2.5% NaOCl and 1.0% NaOCl solutions retained this relative amount of available chlorine for 2 and 4 hours, respectively. Heating of the mixtures had an adverse effect on available chlorine, with a complete loss after 1 hour. Storing the mixtures at 5°C kept the available chlorine high for 7 hours. It was concluded that initial NaOCl concentration and temperature both affected short-term storage stability of combined solutions containing Dual Rinse HEDP.

Biel et al., 2017⁽⁸⁵⁾ assessed interactions between tetrasodium salts of EDTA and HEDP with 1 and 5% NaOCl over the course of 1 hour. The compatibility of these salts with NaOCl was tested by measuring the free available chlorine (FAC) levels using iodometric titration over 60 minutes. A calcium complexing experiment was performed using a calcium-selective electrode. Results showed that the salts dissolved in 1 minute. HEDP mixtures lost 16% FAC after 1 hour whereas EDTA mixtures lost 95% FAC within 1 minute. HEDP mixtures exhibited 24% loss in calcium complexing ability

after 1 hour compared to 34% by EDTA mixtures. It was concluded that tetrasodium salts of HEDP were more compatible with NaOCl than EDTA and may be used as the sole endodontic irrigant for upto 1 hour.

Tartari et al., 2014⁽²¹⁾ evaluated the effect of individual and combined use of 2.5% NaOCl, 18% etidronate and 17% EDTA in tissue dissolution. Sixty fragments of bovine muscle tissue were prepared and their weights determined on a precision scale. The specimens in each group were immersed in the solutions for 5, 10 and 15 min and reweighed at each time period. NaOCl and NaOCl/etidronate groups significantly reduced the weights of specimens at all periods. The only solution capable of dissolving organic matter was NaOCl. In the mixtures analysed, this ability was arrested by EDTA; however, it was minimally affected by the HEDP, proving that this combination, if used during the biomechanical preparation, will be able to dissolve organic matter.

Prado et al., 2013⁽⁸⁶⁾ studied the by-products formed on mixing commonly used irrigants in a 1:1 ratio through electrospray ionization quadrupole time-of flight mass spectrometry analyses. Varying concentrations of sodium hypochlorite (0.16%, 1%, 2.5%, and 5.25%) was mixed with 2% chlorhexidine (CHX) solution and gel, 17% EDTA, 10% citric acid, 37% phosphoric acid, saline solution, ethanol and distilled water. When NaOCl was mixed with

EDTA, citric acid, and phosphoric acid, chlorine gas formation occurred in an exothermic reaction. Mixing NaOCl with saline or distilled water did not produce any changes but saline with chlorhexidine produced a salty precipitate. Hence, it was concluded that intermediate flushes with distilled water may prevent or reduce formation of by-products.

Clarkson et al., 2011⁽⁸⁷⁾ tested time-related effects on active chlorine content of different concentrations (1,1.5,4 and 16.5%) of commercial NaOCl solutions available for domestic use after dilution with 17% EDTA in varying proportions. Five NaOCl solutions were mixed with EDTA in the following proportions: 90:10, 75:25, and 50:50. Changes in active chlorine content were determined at intervals of 5,9,13 and 18 minutes. It was found that EDTA caused substantial and rapid reduction in available chlorine even in small proportions, with the loss being extreme initially and tapering gradually. The authors concluded by saying that NaOCl and EDTA should not be used simultaneously if the therapeutic benefits of NaOCl are to be optimised.

Grande et al., 2006⁽⁸⁸⁾ demonstrated the negative interactions between 5% NaOCl and 17% EDTA powder by monitoring the oxidation process through nuclear magnetic resonance analysis. The peaks relative to the protons and ¹³C isotopes of EDTA obtained showed that the oxidation of EDTA by NaOCl is a complex process

that takes time and also yields unknown by-products. They concluded that a final flush of NaOCl to remove EDTA remnants was unnecessary and would only accelerate erosion of dentin.

Zehnder et al., 2005⁽¹⁹⁾ assessed interactions between conventional chelators like 17% EDTA and 10% citric acid with 1% NaOCl. Alternate chelators were also simultaneously tested for the same: sodium triphosphate (STP), amino tris methylenephosphonic acid (ATMA), 1- hydroxyethylidene-1, 1-bisphosphonate (HEBP). Free available chlorine (FAC) and pH levels were measured for these chelator-NaOCl mixtures. It was found that when mixed with citric acid, FAC decreased to 0 in less than a minute, whereas EDTA required between 1 and 60 minutes to reduce the FAC to the same level. HEBP and STP did not affect FAC levels for 1 hour and 21 days respectively. Atomic absorption spectrometry and SEM evaluation of root canal walls was performed on 60 instrumented teeth (n=6) which were divided into groups based on whether they were exposed to chelators mixed with water or NaOCl in a 1:1 ratio. Results indicated that NaOCl had no negative effect on calcium-complexing ability of chelators. HEBP exhibited calcium chelating ability that was 3 times weaker than EDTA & 5 times weaker than citric acid ($P<0.05$). The mixtures were also evaluated for their antimicrobial effectiveness against *E.faecalis* through culture method . As expected, EDTA and citric acid negatively interfered with the action of NaOCl, while HEBP did not.

Saquy et al., 1994⁽⁸⁹⁾ demonstrated through 3 different experimental methods that the addition of 0.5% NaOCl to EDTA does not alter EDTA's ability to chelate metallic ions, decalcify human dentin (calcium ions) using flame spectrophotometry and Vickers microhardness test. Five maxillary central incisors were sectioned transversely at the cervical third to obtain 3 sections. Only the middle section was used for testing. Samples were exposed to distilled water (DW), NaOCl alone (Dakins solution), 17% EDTA+DW (1:1) or EDTA+NaOCl for 7 minutes. There was a statistically significant difference in microhardness only when samples were exposed to EDTA in combination with NaOCl or distilled water. It was therefore concluded that addition of NaOCl to EDTA did not affect its chelating ability.

Volume of sodium hypochlorite

Gazzaneo et al., 2019⁽⁹⁰⁾ conducted an ex-vivo study to compare bacterial reduction by a single file technique VS a multiple file system while varying volume, concentration and time of exposure to NaOCl in the canals. The study was done in palatal roots of 96 maxillary molars which were matched anatomically using micro-CT. Canals were contaminated with mixed bacterial culture obtained from a tooth diagnosed with apical periodontitis. Samples were taken before canal preparation (S1) and analysed using qPCR. The roots were divided among 4 groups (n=24) on the basis of the file system Reciproc (VDW) or BioRaCe (FKG Dentaire) used and the

volume of irrigant (high/low). Samples in the high-volume group were exposed to 13 mL of 2.5% NaOCl over a period of either 4.5 minutes or 9 minutes depending on the file system used; Samples in low volume groups were exposed to 8mL of 6% or 2.5 % NaOCl over a period of 4.5 minutes. Bacteriological samples were collected again for qPCR analysis (S2). Overall, disinfection was best achieved when multiple instruments were used with high volumes of irrigant with longer exposure times.

Time of contact of sodium hypochlorite

Souza et al., 2019⁽⁹¹⁾ evaluated whether increasing the volume and/or time of contact of NaOCl affected the fracture strength of endodontically treated bovine teeth. Four bovine incisors from 10 animals were allocated into 4 separate groups using a split-mouth design (n=40). The root lengths were standardised and instrumentation was done using a sequence of stainless-steel hand files with 5.25% alkalized NaOCl as the working solution. Grouping according to the total volume and time of irrigation, was as follows: 15 mL/11.5 min; 30 mL/11.5 min; 15 mL/19 min; 30 mL/19 min. Samples were later subjected to compressive force in a universal testing machine until fracture. Results showed that variations in time, volume of irrigation and the combination of both, negatively influenced the fracture resistance (P=0, P=0, P=0.038 respectively). Control group with standard volume and time of irrigation showed the highest fracture strength whereas increasing volume or time of

contact or both led to reductions in fracture strength by 25%, 37%, 47% respectively. Effect size estimation revealed that the time of contact had a greater influence than the volume. It was concluded that raising the volume and/or time of a 5.25% alkalized NaOCl solution reduces the fracture resistance of endodontically treated bovine teeth.

Retamozo et al., 2010⁽⁹²⁾ determined the concentration and time needed for NaOCl to disinfect dentin cylinders infected with *Enterococcus faecalis*. Four hundred and fifty dentin cylinders from bovine incisors were obtained. Dentinal tubules were opened using 17% EDTA-5.25% NaOCl over 4 minutes and then infected with *E. faecalis* (ATCC 4082) for 3 weeks in brain-heart infusion broth (BHI). The cylinders were divided into 3 groups based on NaOCl concentrations (1.3, 2.5 or 5.25%) and subdivided based on time intervals (5-, 10-, 15-, 20-, 25-, 30-, 35-, and 40-minute) to achieve 30 subgroups. Each sample was incubated for 72 hours in 2mL of BHI broth. Absence of turbidity was taken to indicate no bacterial growth, whereas turbidity indicated presence of remaining viable bacteria. Non-turbid samples were vortexed, plated onto BHI plates and further incubated for a day to check for signs of growth. It was found that the most effective irrigation regimen to disinfect dentinal tubules was 5.25% NaOCl at 40 minutes, whereas irrigation with 1.3% and 2.5% NaOCl for the same time interval proved to be ineffective.

Teixeira et al., 2005⁽⁹³⁾ verified under the scanning electron microscope, the influence of irrigation time with 15% EDTA and 1% NaOCl on intracanal smear layer removal. 21 extracted human permanent teeth with single straight root canals were used. The root canals of the teeth were instrumented and, at the end of preparation, were irrigated with 3 mL of 15% EDTA, followed by 3 mL of 1% NaOCl for 1,3 or 5 minutes. The teeth were sectioned longitudinally and prepared for an SEM. The dentinal wall of cervical, middle and apical thirds was graded according to the amount of debris and smear layer remaining on the walls. The association of EDTA and NaOCl solutions proved effective in removing the smear layer from the cervical and middle thirds for all times of application (1, 3 and 5 min). In the apical third the efficacy of the smear layer removal was decreased.

MATERIALS AND METHODOLOGY

ARMAMENTARIUM

➤ MATERIALS [Figure 1]:

1. Endodontic access burs (Endo Access bur, EX-24) (Dentsply Sirona, Mani) (LOT NO: D18C256800)
2. K-files (Mani, Japan) (LOT NO: R111019600, R110789900, R120947700)
3. Endobloc (Dentsply Sirona)
4. ProTaper Gold rotary file system (PTG; Dentsply Sirona) (LOT:1706725,1715747)
5. X-smart plus (Dentsply Sirona; REF NO. U1058131002,08899161,08516818)
6. 2 mL and 5mL disposable syringes (Unolok, Faridabad,India; batch no. 915023USF2)
7. Side-vented irrigation needle (RC Twents, Prime Dental Products Pvt Ltd; Batch no. 2002-01)
8. Borosilicate beakers (100 mL) and bottles (500 mL) (Borosil, India)
9. Paper points (Dentsply Sirona) (LOT 020416)
10. Diamond disc and mandrel 0.25 mm (Nexus Medodent)
11. Micromotor (Marathon,India) (NO.796651)
12. Silicon carbide abrasive papers (400,600,1000,1500 grit) (Carborundum Universal, India)
13. Nail varnish

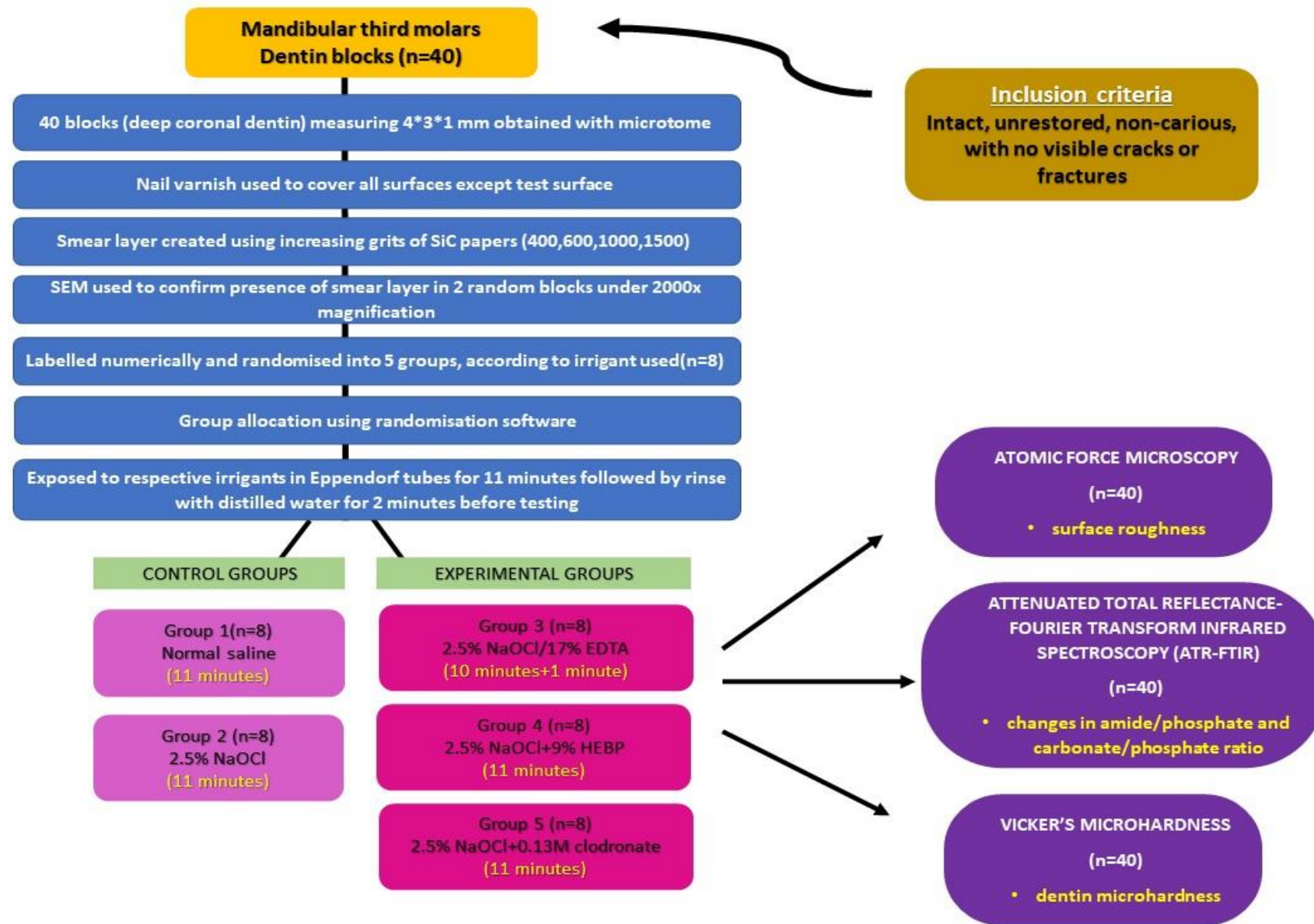
14. Plastic droppers (Sri Hari Scientific, Pallikaranai, Chennai)
15. Absorbent filter paper
16. Micro-centrifuge tubes (5 mL) (Tarsons Micro centrifuge tube, REF NO.500050)

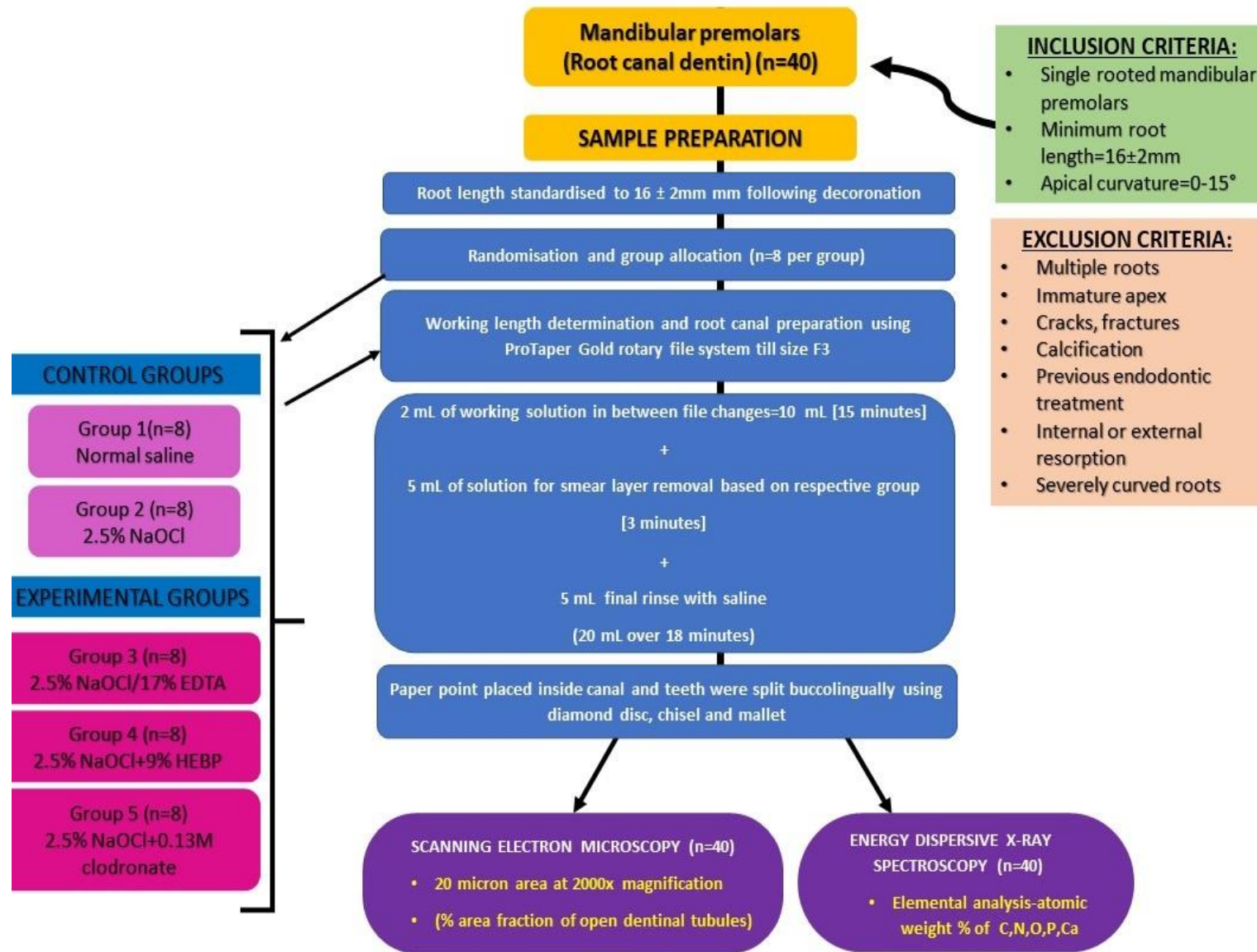
Reagents purchased were of reagent grade or commercially available. The concentrations and source are mentioned below:

➤ **REAGENTS [Figure 1]:**

17. Normal saline (0.9% NaCl, Fresenius Kabi India pvt.Limited)(BATCH NO:93QE204001)
18. Sodium hypochlorite 5% (NaOCl) (VIP, India)
19. 17% EDTA solution (Waldent) (LOT NO. 99003,99002)
20. 60% etidronic acid (HEBP) (TCI chemicals, Hyderabad) (lot no. HJRYK-TD)
21. Disodium clodronate tetrahydrate (TCI chemicals, Hyderabad) (Lot OFEPJ-GN) (LOT FQNUM-PM)
22. Distilled water (Krishna Aqua, Chennai)
23. 10% Phosphate buffered saline (Sisco Research Laboratories Pvt. Ltd) (LOT NO.9695087)

11. ImageJ bundled with 64-bit Java 1.8.0_172
(<https://imagej.nih.gov/ij/download.html>)
12. G-Power version 3.1.9.3
13. EPI-INFO (version 7.2.2.6, CDC license: Atlanta, Georgia US:
public domain) software.





STUDY APPROVAL

This study was approved by the Institutional Review Board and Institutional Biosafety and Ethical Committee (IBEC), SIST (IRB Ref. number: 174/IRB-IBSEC/SIST).

STUDY DESIGN

In-vitro quantitative and qualitative analyses.

SAMPLE SIZE CALCULATION

The sample size was estimated using a study by Rath et al.⁽⁵⁵⁾, using G*power 3.1.9.3 software. With given effect size of 1.8, an alpha error of 0.05 and a power of 0.90 into an a priori sample test from the F tests family, the sample size was estimated to be 40 with 8 samples per group.

PREPARATION OF STOCK SOLUTION OF CHELATORS

60% etidronic acid (HEBP) solution was diluted to 18% using distilled water and stored in airtight amber bottles until use. Commercially procured disodium clodronate tetrahydrate powder was combined with distilled water to produce a 0.26M solution and stored in amber bottles. Just before use, these chelators were mixed with 5% NaOCl in 1:1 ratio to achieve a final concentration of 2.5% NaOCl+9% HEBP and 2.5% NaOCl+0.13M clodronate, respectively.

(44,57)

SAMPLE RECRUITMENT

Twenty mandibular third molars [Figure 2] and forty single-rooted mandibular premolars [Figure 3] that met the inclusion criteria were collected from the university tooth bank and stored in saline with 0.5% thymol solution (mixed in 1:1 ratio) until use.

INCLUSION CRITERIA

Single rooted mandibular premolars without caries, internal or external resorption, calcifications, cracks or fractures, previous endodontic treatment with minimum root length of 16 ± 2 mm, apical curvature of $0-15^\circ$ and initial apical file size not exceeding #15 size K-file.

EXCLUSION CRITERIA

Multiple roots, immature apices, caries, resorption, cracks or fractures, previous endodontic treatment, severely curved roots

Radiographs were taken in mesiodistal and buccolingual directions to exclude teeth with more than one root canal [Figures 4,5]. Premolars were examined under stereomicroscope (Leica M205A) at 12x to exclude teeth with visible cracks on the root surface [Figures 6,7].



FIGURE 2: Mandibular third molars for preparation of dentin blocks



FIGURE 3: Mandibular premolars for preparation of root canal dentin samples



FIGURE 4: Mesiodistal view of radiograph of premolar samples (excluded samples circled in red)

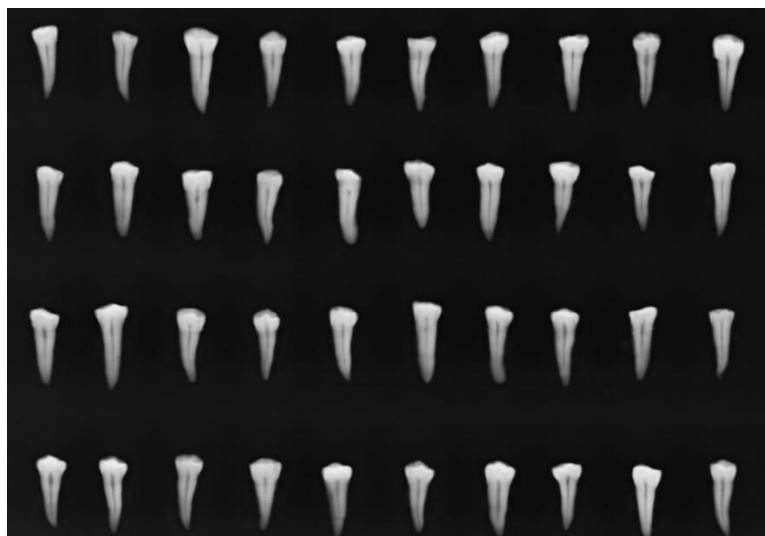


FIGURE 5: Buccolingual view of radiograph of premolar samples (excluded samples circled in red)



FIGURE 6: Examination of premolars under stereomicroscope



FIGURE 7: Example of an excluded sample after stereomicroscopic evaluation (Red arrow indicates cracks and fractures)

SAMPLE PREPARATION

➤ Dentin blocks

Twenty mandibular third molars were embedded in acrylic resin, and 42 deep coronal dentin blocks measuring 4*3*1 mm were obtained using a microtome [Figure 8-10]. The blocks were labeled numerically, and all surfaces except the one to be treated were covered by nail varnish [Figure 11,12]. A smear layer was created on the uncoated surface of the dentin blocks using silicon carbide abrasive papers in increasing order of grit, namely 400, 600, 1000, 1500, mounted with a mandrel in a straight handpiece attached to a micromotor at a speed of 3000rpm with water coolant. Two random blocks were used to confirm the presence of a smear layer under a scanning electron microscope (TESCAN 2000x) [Figure 13]



FIGURE 8: Third molars mounted in resin before placement in microtome

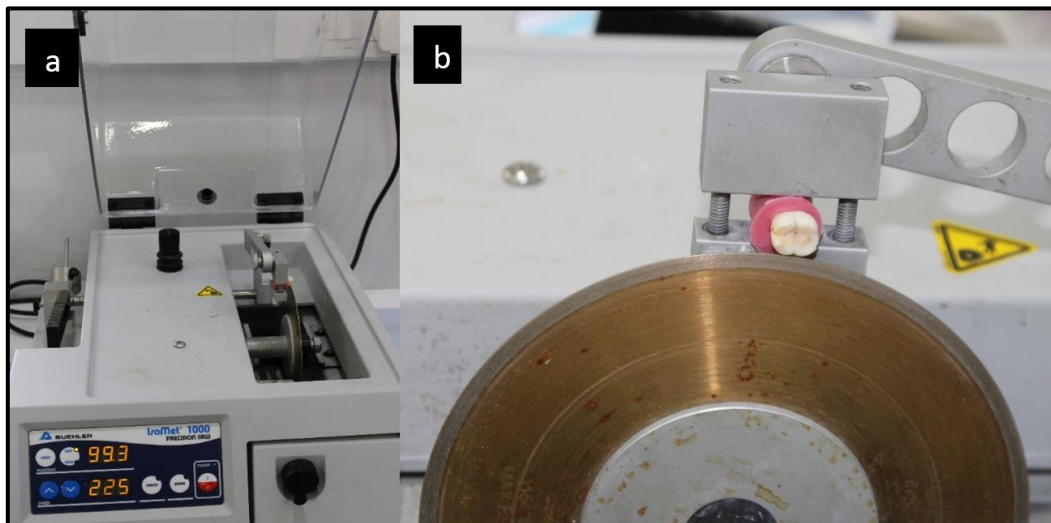


FIGURE 9: a) IsoMet 1000; b) Sectioning to obtain dentin blocks

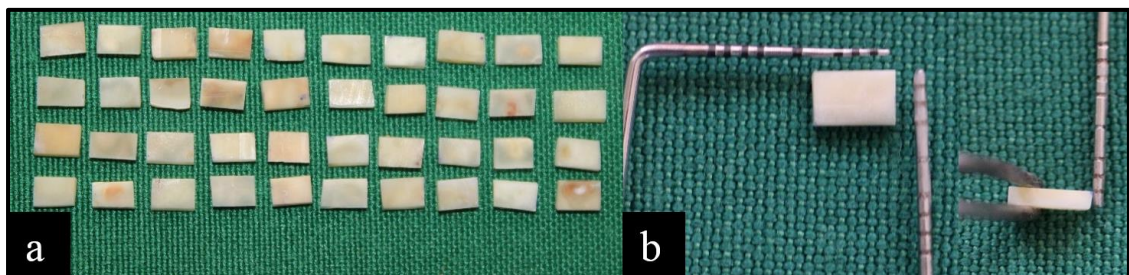


FIGURE 10: a) 40 dentin blocks obtained; b) Blocks measured 4*3*1mm



FIGURE 11: All surfaces except one coated by nail varnish

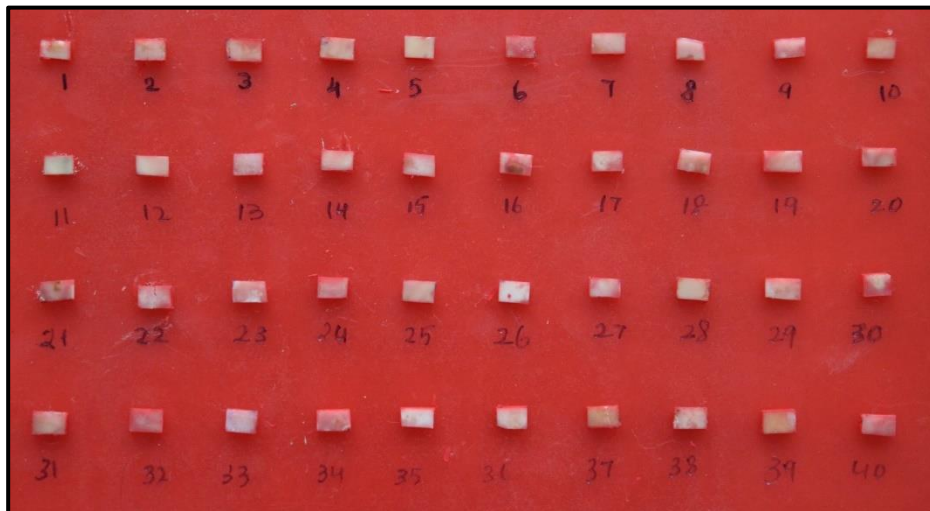


FIGURE 12: Smear layer created on blocks, labelled and arranged before randomisation into respective groups

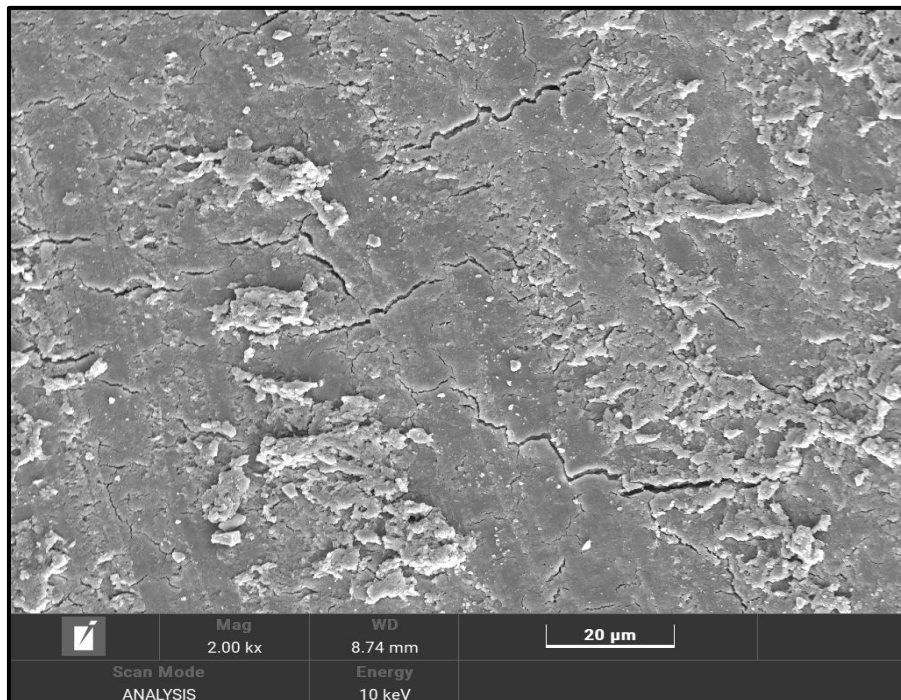


FIGURE 13: Scanning electron microscopic image confirming presence of smear layer on dentin blocks (TESCAN CLARA FESEM-2000x magnification)

The remaining 40 blocks were divided randomly among 5 groups (n=8) using www.random.org as follows:

- a) *Group 1(negative control): normal saline*
- b) *Group 2(positive control): 2.5% NaOCl*
- c) *Group 3: 2.5% NaOCl followed by 17% EDTA*
- d) *Group 4:2.5% NaOCl+ 9% Etidronic acid (5% NaOCl+18% HEBP)*
- e) *Group 5: 2.5% NaOCl+ 0.13M Clodronate disodium (5% NaOCl+0.26 M clodronate)*

The dentin blocks were then placed in Eppendorf tubes [Figure 14a-14e] containing 5 mL of the irrigants according to the groups as mentioned above for 11 minutes and rinsed with 2 mL of distilled water for 2 minutes before subjecting to experimentation, except Group 3. In Group 3, the blocks were removed from the Eppendorf tubes containing 2.5% NaOCl after 10 minutes, rinsed off using 2 mL of distilled water, blot dried and immersed in fresh Eppendorf tubes containing 5 mL of 17% EDTA for 1 minute. The period of exposure and volume of irrigants used in this study was based on a protocol modified from previous studies. ^(41,55,57)

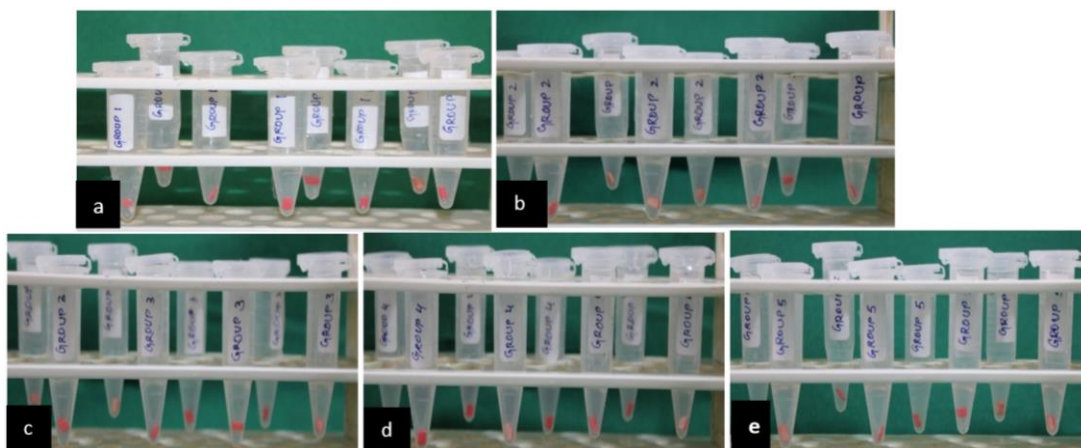


Figure 14 a-e: Dentin blocks randomly divided amongst the 5 groups (n=8) and placed in 5 mL Eppendorf tubes containing respective irrigating solutions

Forty premolars that met inclusion criteria were selected, and root length was standardized to 16 ± 2 mm following decoronation [Figure 15a]. A #10 K file was inserted until it appeared at the apical foramen, and the working length was determined as 1 mm short of this length. ⁽⁶⁶⁾ Teeth with an initial apical size not exceeding #15 K-file were included in this study. The premolars were labelled [Figure 15b] and divided among the five groups after randomization using www.random.org, as previously mentioned (n=8). Root canals were prepared in a crown-down manner using Protaper Gold rotary file system (Dentsply Maillefer, Switzerland) with an X-Smart endo motor upto size F3 following built-in programmed speed and torque settings for each file [Figure 16]. Each file was used for 5 canals and discarded.

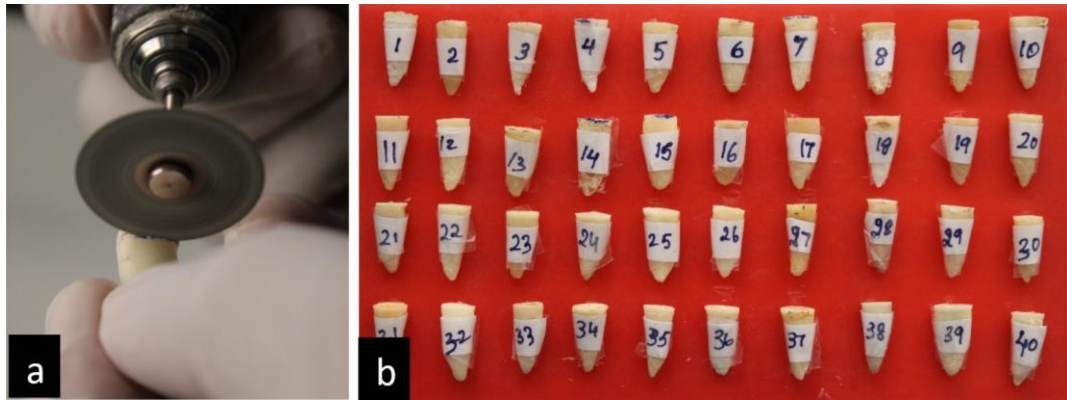


Figure 15: a)Decoronation of premolars using diamond disc to standardise root length to 16 ± 2 mm; b)Decoronated premolars

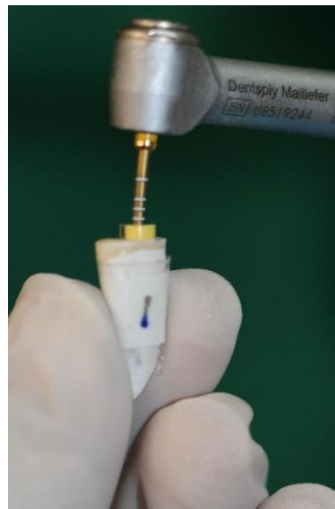


Figure 16: Root canal preparation done using ProTaper Gold system

IRRIGATION PROTOCOL

Two millilitres of the assigned irrigant was delivered using 30-G side-vented irrigation needles between each file change [Figure 17] ⁽⁵⁵⁾. A final rinse with 5 mL saline was employed & the canals were dried with corresponding paper points [Box 1].

BOX 1: Irrigation protocol followed

GROUPS	WORKING SOLUTION (2 mL between instruments)	SMEAR LAYER REMOVAL	FINAL RINSE	TOTAL VOLUME	TIME SPLIT UP
1 (Negative control)	Normal saline 10 mL 15 minutes	Normal saline 5 mL 3 minutes	Normal saline 5 mL	20mL	18 minutes
2 (Positive control)	2.5% NaOCl 10 mL 15 minutes	2.5% NaOCl 5 mL 3 minutes	Normal saline 5 mL	20mL	18 minutes
3 (2.5% NaOCl+17% EDTA)	2.5% NaOCl 10 mL 15 minutes	17% EDTA 5 mL 3 minutes *Intervening rinse with 2 ml saline to prevent negative interactions	Normal saline 5 mL	20mL	18 minutes
4 (2.5% NaOCl+9% HEBP)	2.5% NaOCl+9% HEBP 10 mL 15 minutes	2.5% NaOCl+9% HEBP	Normal saline	20mL	18 minutes
5 (2.5% NaOCl+0.13 M clodronate)	2.5% NaOCl+0.13 M clodronate 10 mL 15 minutes	2.5% NaOCl+0.13 M clodronate	Normal saline	20mL	18 minutes

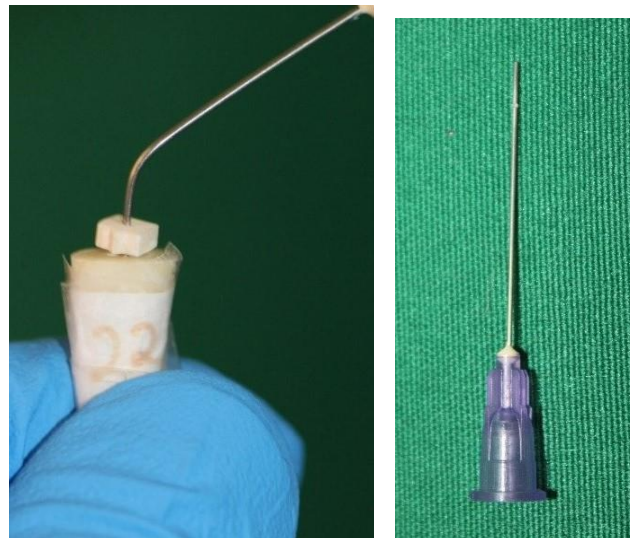


Figure 17: Root canal irrigation done using 30 G side-vented needles

The time of exposure to irrigant solutions overall was kept constant at 18 minutes while the volume was adjusted across various groups to result in 20 mL of irrigant per canal. After canal preparation, longitudinal cuts were placed on the buccal and lingual surface using a diamond disc under constant water irrigation with a F3 paper point (Dentsply Malleifer, Switzerland) placed inside the canal to prevent debris from entering the canal [Figure 18a]. The canals were then split longitudinally using a chisel-mallet and stored in suitable containers containing phosphate-buffered saline until analysis.

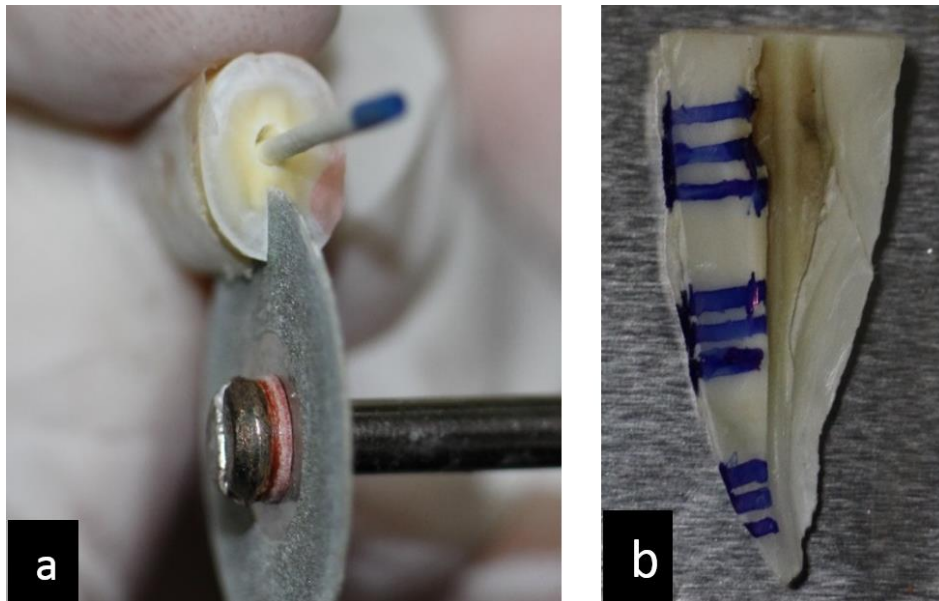


FIGURE 18: a) Longitudinal cuts along buccal and lingual surface made using diamond disc with paper points blocking the canals; b) Root canals divided into 3 thirds for observation under scanning electron microscope

ANALYSIS OF DENTIN ULTRASTRUCTURE

1. Surface characterisation

Atomic force microscopy (AFM) was used to study the surface topography of treated dentin blocks. Surface roughness (Ra -arithmetic roughness average) was calculated using an atomic force microscope (Park system XE7, Korea) in non-contact mode with a tip radius of <10nm (PPP NCHR), scan size of 20 μm at a resonating frequency of 0.040 Hz. XE software (version 7.0) was used for post-processing analysis. [Figure 19].

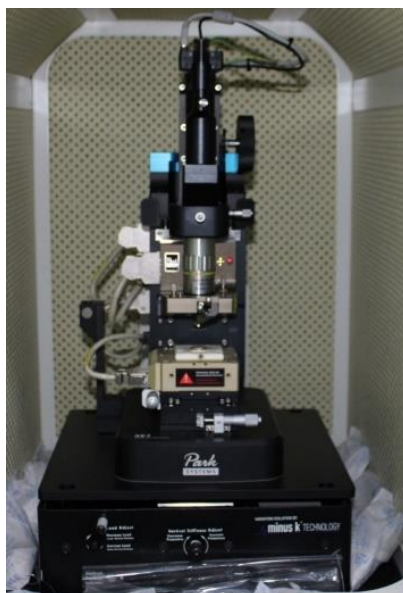


FIGURE 19: Atomic Force Microscope

2. Chemical compositional analysis

2.1 Attenuated Total Reflectance-Fourier Transform Infrared spectroscopy

Attenuated Total Reflectance -Fourier Transform Infrared Spectroscopy (ATR-FTIR) was used to study changes in chemical composition of dentin blocks (n=40). Spectra were collected from each specimen using a FTIR spectrometer (Perkin Elmer Spectrum

Two spectrometer, USA) between 400 and 4000 cm^{-1} using 10 scans at 4 cm^{-1} resolution [Figure 20a, b].⁽⁵⁵⁾ Peak values were selected based on previous literature^(57,94,95). Quantitative assessment of compositional changes in dentin blocks was done by converting percentage transmittance (%T) values obtained from scans for the selected reference peaks to percentage absorbance (%A) values using the formula:

$$\%A = 2 - \log_{10}[\%T] \quad (\text{Beer's law})^{(96)}$$

Changes in amide I/phosphate, amide III/phosphate and carbonate/phosphate ratios were calculated from these absorbance values by dividing either amide or carbonate values by the phosphate values respectively. The qualitative FTIR spectra for each group and inter-group comparisons were plotted using Origin software (OriginPro 8.5.1).



FIGURE 20: a) Spectrum 2 spectrometer b) Sample scanned in ATR mode

2.2 Elemental analysis of root canal samples

The samples were dehydrated with increasing concentrations of ethanol and sputter-coated with a gold-palladium mixture [Figure 22 a,b] (Quorum Q150T ES Plus) for 90 seconds and subjected to Energy-Dispersive X-ray Spectroscopy (Octane elect plus, Ametek, USA). The atomic weight percentages of different elements (C, N, O, P, Ca) were recorded to assess compositional changes at selected sites in root canal samples of different groups. ⁽⁵⁵⁾

3. Dentin microhardness

Vicker's microhardness test was performed on treated dentin blocks (n=40) to measure dentin microhardness. Indentations were made at three random locations with a 300g load and a dwell time of 10 seconds. ⁽⁷⁵⁾ [Figure 21a, b] An average of the three readings was calculated to arrive at a mean hardness value for each block which was expressed in Vicker's hardness number (VHN).

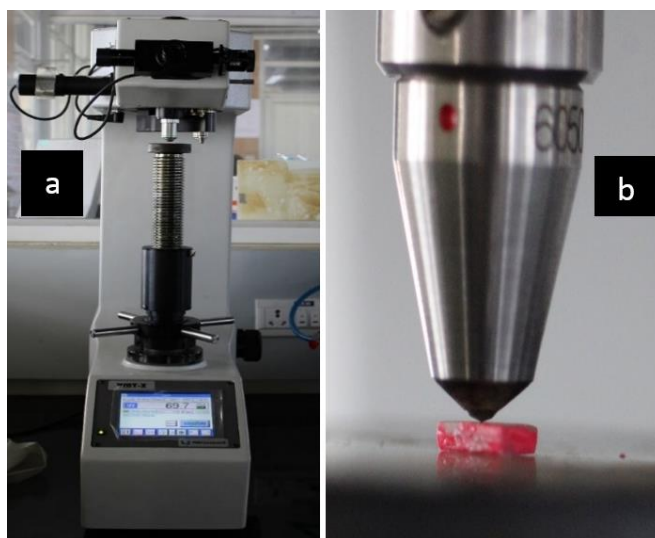


FIGURE 21: a) Vickers microhardness tester; b) Sample being indented

4. Percentage area fraction of open dentinal tubules

Prepared root canal specimens (n=40) were used to evaluate the percentage area fraction of open dentinal tubules (%AF) using scanning electron microscopy (TESCAN CLARA FESEM). The root was divided into equal thirds measured from the apex (coronal, middle, and apical) and sites for observation under SEM were standardised by horizontal markings 1 mm apart made along the lumen wall using a felt tip pen [Figure 20b]. The samples were dehydrated with increasing concentrations of ethanol and sputter-coated with a gold-palladium mixture [Figure 22 a, b] (Quorum Q150T ES Plus) for 90 seconds. A total of six images were obtained at 2000x magnification from each sample, two from each third. Image J- software auto-calculated the percentage area fraction of open dentinal tubules.^(15,44) [Refer Appendix B]



FIGURE 22 a) Sputter coater (QUORUM 150 ET PLUS) b) Sputter coated samples

STATISTICAL ANALYSES

Data that was statistically analysed included surface roughness values from atomic force microscopy in microns, ratios of carbonate/phosphate and amide/phosphate ratios as obtained through ATR-FTIR, atomic weight percentages of carbon, nitrogen, oxygen, calcium and phosphorus from EDS spectra, Vicker's hardness values (VHN) from microhardness test and percentage area fraction (%AF) of open dentinal tubules obtained from scanning electron microscopy.

Statistical analysis was performed using EPI-INFO (version 7.2.2.6, CDC license: Atlanta, Georgia US: public domain) software. The data were not normally distributed (Kolmogorov-Smirnov test < 0.005). Kruskal-Wallis test followed by Mann-Whitney U test was used to analyse the differences in the two control and three experimental groups to know the effects of sodium hypochlorite combined with different chelators on dentin. Significance level was fixed at 5% ($\alpha = 0.05$).

RESULTS

1. Surface characterisation

Roughness average (R_a) of treated surface of the dentin blocks was evaluated using Atomic Force Microscopy. The mean and standard deviation of R_a values in micrometers of all groups are listed in Table 1A. A statistically significant difference was observed between groups, as shown by Kruskal-Wallis test ($P=0.04$) [Table 1B]. Intergroup comparison using Mann-Whitney U test is tabulated in Table 1C. Surface roughness increased by 2-fold in Group 4 ($P=0.001$) and 3-fold in Group 2, Group 3, and Group 5 ($P=0.001$) compared to Group 1. Whereas, Group 4 showed a significant reduction in surface roughness compared to Group 2 ($P=0.012$), Group 3 ($P=0.001$), and Group 5 ($P=0.001$), respectively. Figures 23 a-e depict representative AFM images of treated dentin block surfaces.

TABLE 1A: Mean and standard deviation of surface roughness values of dentin blocks in micrometers

GROUPS	MEAN R_a (μm)	SD
1(Negative control)	0.07	0.02
2(Positive control)	0.21	0.05
3(2.5%NaOCl/17%EDTA)	0.23	0.02
4 (2.5%NaOCl+9% HEBP)	0.15	0.03
5(2.5% NaOCl+0.13 M clodronate)	0.23	0.02

TABLE 1B: Kruskal-Wallis analysis of variance test for surface roughness between the groups

GROUP	N	MEAN RANK	P-VALUE
1	8	4.50	0.004*
2	8	24.44	
3	8	30.13	
4	8	13.50	
5	8	29.94	

***Statistically significant difference $P < 0.05$**

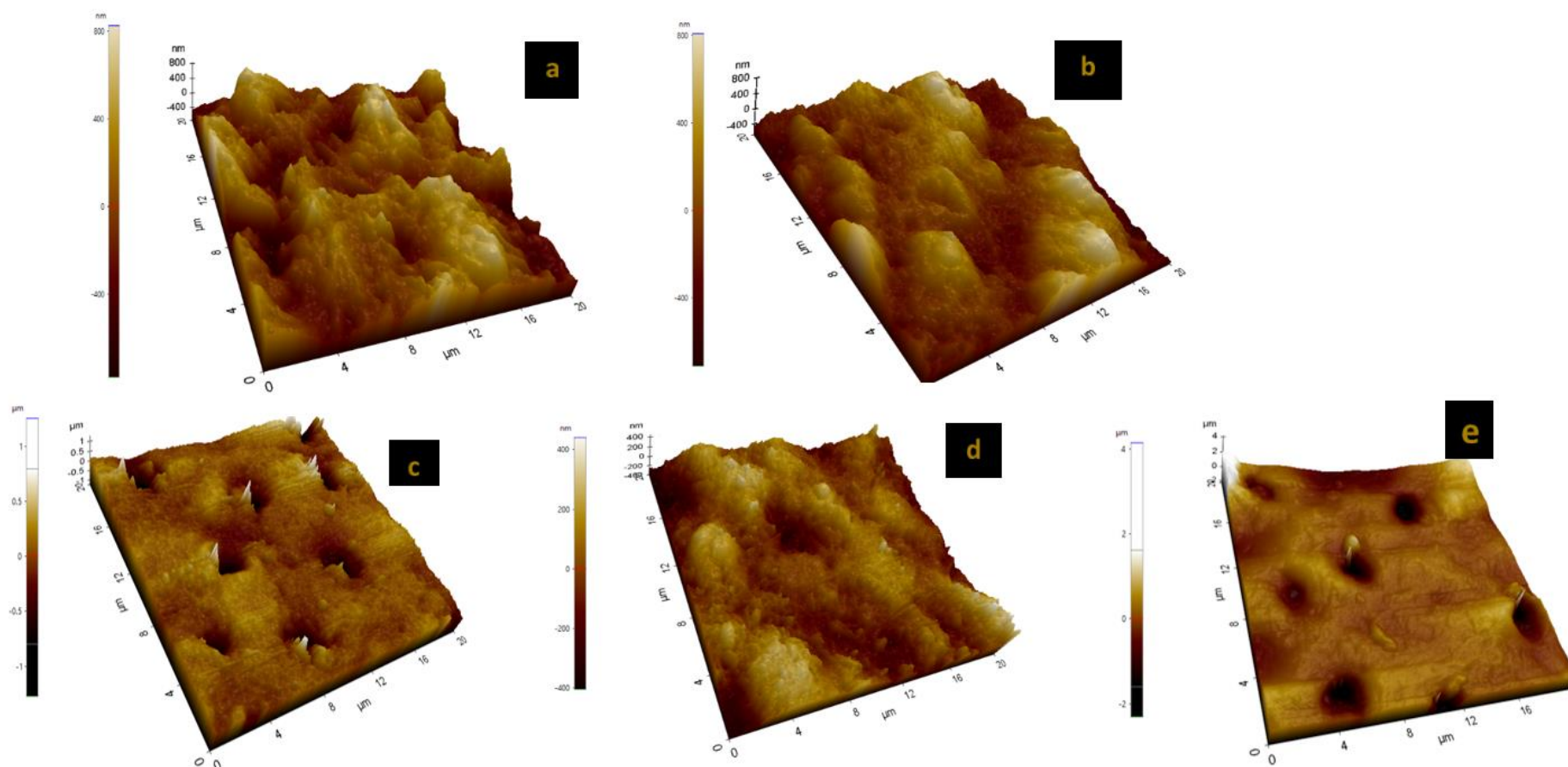
TABLE 1C: Mann-Whitney U inter-group comparison test of surface roughness values

GROUPS (A)	GROUPS (B)	P VALUE
1(Negative control) (saline)	2	0.001*
	3	0.001*
	4	0.001*
	5 (2.5% NaOCl+0.13 M clodronate)	0.001*
2(Positive control) (2.5%NaOCl)	3	0.318
	4	0.012*
	5	0.115
3(2.5%NaOCl/17%EDTA)	4	0.001*
	5	0.713
4 (2.5%NaOCl+9% HEBP)	5	0.001*

***Statistically significant difference $P < 0.05$.**

FIGURE 23: Representative non-contact mode AFM images.

a) Group 1-Saline; b) Group 2-2.5% NaOCl; c) Group 3-2.5% NaOCl/17% EDTA; d) Group 4- 2.5% NaOCl+9% HEBP; e) Group 5-2.5% NaOCl+0.13M clodronate



***All images depict a 20*20 micrometre scan area (x and y axis); Degree of surface roughness changes are reflected in differences in height of peaks & valleys (z axis)**

2. Chemical compositional changes

2.1.ATR-FTIR analysis

Spectra obtained through ATR-FTIR analyses of treated dentin blocks were compared to assess changes in carbonate/phosphate and amide/phosphate ratios [Figure 24]. Spectral peak values for amide I, amide III, carbonate and phosphate were fixed based on literature as 1643,1242,871,1026 cm^{-1} respectively. ^(57,94,95) The percentage absorbance values at these peaks were used to compare changes in compositional ratios to describe effects of different irrigants on dentin. [Table 2A,2B].

Figure 24: Stacked ATR-FTIR spectra of treated dentin blocks depicting % transmittance of different groups at inspected wavelengths of 871,1026,1242,1643 cm^{-1}

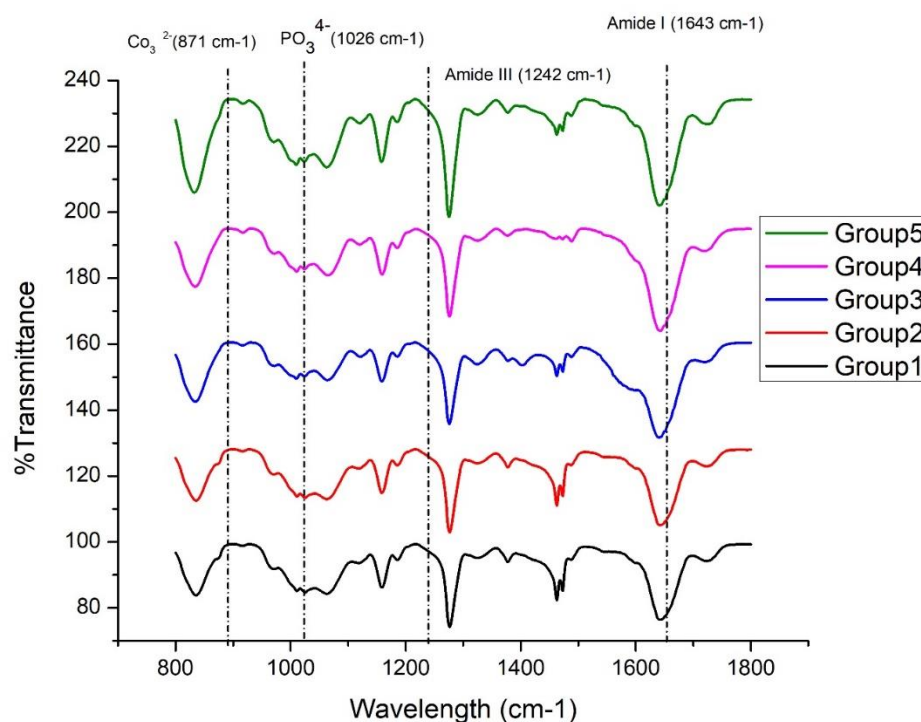


TABLE 2A: Mean and standard deviation values for the ratio of Carbonate/ Phosphate, Amide I/ Phosphate and Amide III/Phosphate on surface of treated dentin blocks

GROUPS	Carbonate/ Phosphate Mean ratio $\pm$ SD	Amide I/ Phosphate Mean ratio $\pm$ SD	Amide III/ Phosphate Mean ratio $\pm$ SD
1(Negative control)	0.34 $\pm$ 0.03	1.41 $\pm$ 0.15	0.23 $\pm$ 0.03
2(Positive control)	0.37 $\pm$ 0.12	1.50 $\pm$ 0.25	0.26 $\pm$ 0.06
3(2.5% NaOCl/17% EDTA)	0.36 $\pm$ 0.07	2.13 $\pm$ 1.36	0.25 $\pm$ 0.10
4 (2.5%NaOCl+9% HEBP)	0.34 $\pm$ 0.04	1.71 $\pm$ 0.50	0.28 $\pm$ 0.03
5(2.5% NaOCl+0.13 M clodronate)	0.34 $\pm$ 0.02	1.44 $\pm$ 0.20	0.25 $\pm$ 0.03
P-VALUE	0.971	0.240	0.209

***Statistically significant difference P<0.05**

TABLE 2B: Kruskal-Wallis comparison of the ratio of Carbonate/Phosphate, Amide I/Phosphate and Amide III/Phosphate among groups

	GROUP	N	MEAN RANK	P-VALUE
Carbonate/Phosphate ratio	1	8	21.50	0.971
	2	8	18.63	
	3	8	22.38	
	4	8	19.50	
	5	8	20.50	
Amide I/phosphate ratio	1	8	16.00	0.240
	2	8	19.63	
	3	8	27.13	
	4	8	23.63	
	5	8	16.13	
Amide III/Phosphate ratio	1	8	14.50	0.209
	2	8	21.63	
	3	8	19.88	
	4	8	28.13	
	5	8	18.38	

2.2 Elemental analysis

Elemental analysis of root canal dentin samples was performed using Energy Dispersive X-ray Spectroscopy to compare atomic weight percentages of Carbon(C), Nitrogen(N), Oxygen(O), Phosphorus(P) and Calcium (Ca). The mean atomic weights of individual elements among the groups are tabulated in Table 2C and plotted in Graphs 1-5. Kruskal-Wallis test revealed statistically significant differences in atomic weight percentages of all elements among groups [Table 2D]. Group 1 samples treated with saline were used as reference. There was a significant drop in carbon, nitrogen, oxygen and phosphorus levels in Group 3, Group 4, and Group 5 when compared to Group 1($P<0.05$). Group 2 showed an overall significant increase in levels of calcium, phosphorus and significant reduction in levels of nitrogen and carbon, ($P=0.001$) [Graph 2]. Group 3 had the most significant effect on both organic and inorganic component of root dentin when compared to the control groups, i.e., carbon, nitrogen, calcium and phosphorus levels as depicted in Graph 1-5 [Table 2E]. Nitrogen levels were significantly higher and the phosphorus levels were significantly reduced in Group 3 compared to Group 4 and Group 5. Group 4 and Group 5 did not show any significant differences in calcium levels compared to Group 1. There were no significant differences between Group 3 and Group 4($P=0.128$), but both groups had significantly lower calcium levels compared to Group 5. [Table 2E] Appendix A depicts EDS graphs showing varying percentages of tested elements in all groups.

Table 2C -Mean and standard deviation of atomic weight percentages of carbon, oxygen, nitrogen, calcium and phosphorus of all groups

GROUPS	CARBON		NITROGEN		OXYGEN		CALCIUM		PHOSPHORUS	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
1 (Negative control)	21.72	2.13	7.56	1.28	42.88	3.64	14.93	4.00	12.92	1.95
2 (Positive control)	13.43	1.80	3.37	1.02	42.01	3.26	24.21	4.71	16.61	2.25
3 (2.5%NaOCl/ 17%EDTA)	34.45	3.38	17.23	4.04	32.49	2.33	8.83	1.08	7.00	0.81
4 (2.5%NaOCl +9% HEBP)	33.29	2.58	11.57	1.92	35.37	3.38	11.92	4.10	7.85	0.81
5 (2.5% NaOCl+0.13 M clodronate)	31.06	1.92	10.44	1.92	31.83	1.73	16.68	3.26	10.00	2.37

Table 2D: Kruskal-Wallis comparison of atomic weight percentages of different elements among groups

Variable	Group	N	Mean Rank	P-value
Carbon atomic weight	1	8	12.50	.001*
	2	8	4.50	
	3	8	31.75	
	4	8	29.69	
	5	8	24.06	
Nitrogen atomic weight	1	8	13.25	.001*
	2	8	4.50	
	3	8	35.00	
	4	8	26.44	
	5	8	23.31	
Oxygen atomic weight	1	8	32.38	.001*
	2	8	31.31	
	3	8	10.75	
	4	8	18.81	
	5	8	9.25	
Phosphorus atomic weight	1	8	27.88	.001*
	2	8	35.63	
	3	8	7.00	
	4	8	12.75	
	5	8	19.25	
Calcium atomic weight	1	8	21.00	.001*
	2	8	35.25	
	3	8	7.69	
	4	8	14.31	
	5	8	24.25	

***Statistically significant difference $P < 0.05$**

Graph 1-5: Mean plot of atomic weight percentage across groups. 1-Carbon; 2-Nitrogen; 3-Oxygen; 4-Phosphorus; 5-Calcium

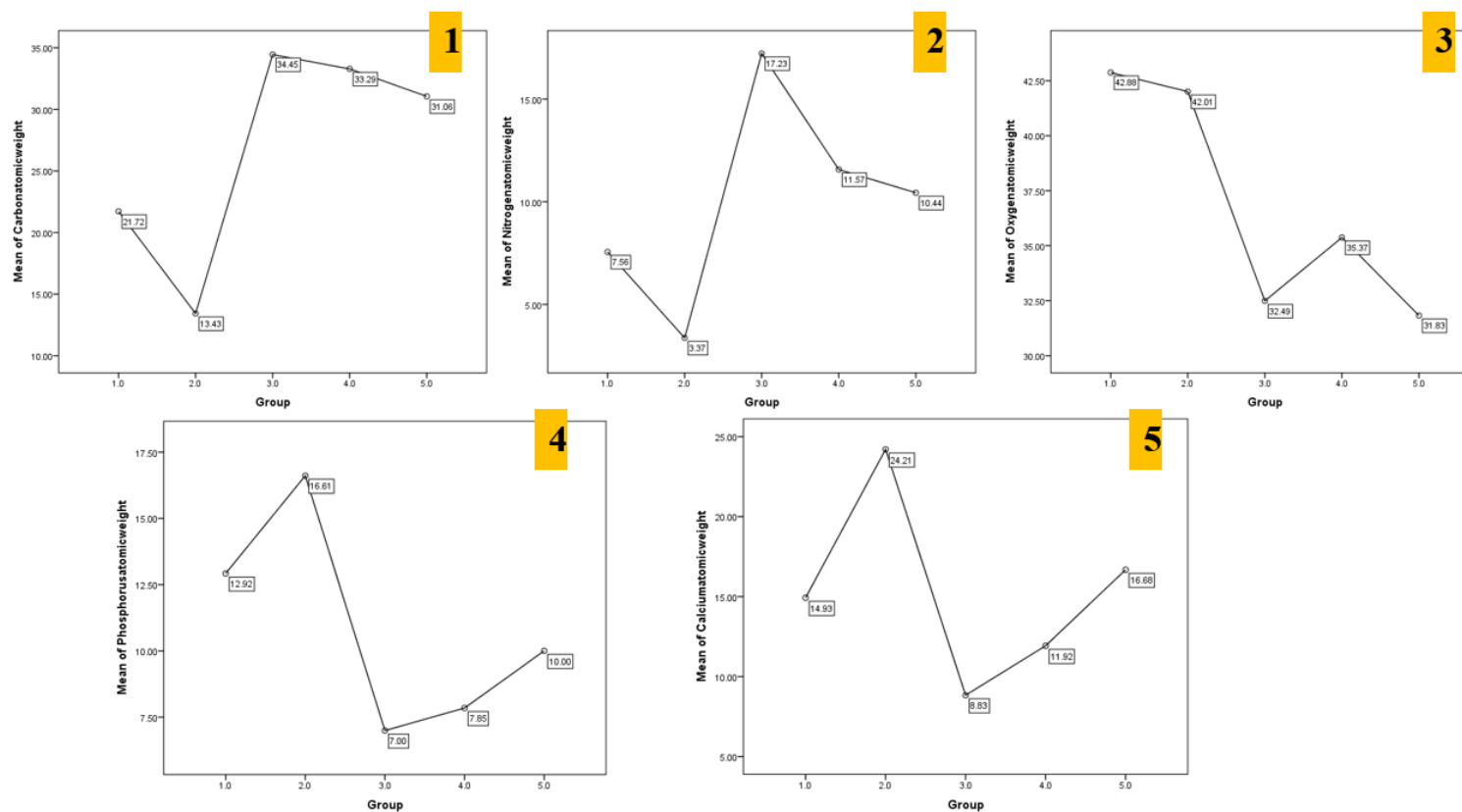


Table 2E: Mann-Whitney U inter-group comparison of mean atomic weight percentages of different elements

GROUP (A)	GROUP (B)	ATOMIC WEIGHT %				
		CARBON	NITROGEN	OXYGEN	PHOSPHORUS	CALCIUM
1	2	0.001	0.001	0.713	0.006	0.004
	3	0.001	0.001	0.001	0.001	0.012
	4	0.001	0.001	0.004	0.001	0.141
	5	0.001	0.005	0.001	0.027	0.462
2	3	0.001	0.001	0.001	0.001	0.001
	4	0.001	0.001	0.001	0.001	0.001
	5	0.001	0.001	0.001	0.001	0.003
3	4	0.462	0.012	0.059	0.046	0.128
	5	0.046	0.003	0.674	0.009	0.001
4	5	0.083	0.372	0.021	0.074	0.046

***Statistically significant difference $P < 0.05$**

3. Dentin Microhardness

Vicker's microhardness testing was done on treated dentin blocks to assess changes in microhardness values after exposure to irrigants [Table 3A]. Kruskal-Wallis test showed a statistically significant difference between all groups ($P=0.04$) [Table 3B]. Group 2, Group 3, Group 4 and Group 5 had significantly decreased dentin microhardness values when compared to Group 1 ($P < 0.05$) [Table 3C]. Group 4 caused the highest reduction in microhardness of dentin when compared to Group 2, Group 3 and Group 5 ($P < 0.05$) [Table 3B,3C].

Table 3A: Mean and standard deviation of dentin microhardness values of all groups expressed in Vicker's hardness number (VHN)

GROUP	MEAN VHN	SD
1(Negative control)	108.43	56.28
2(Positive control)	58.19	8.41
3(2.5%NaOCl/17%EDTA)	60.76	13.47
4 (2.5%NaOCl+9% HEBP)	42.07	17.41
5(2.5% NaOCl+0.13 M clodronate)	64.35	15.09

TABLE 3B: Kruskal-Wallis comparison of VHN among groups

GROUP	N	MEAN RANK	P-VALUE
1	8	31.56	.004*
2	8	19.38	
3	8	19.69	
4	8	8.88	
5	8	23.00	

***Statistically significant difference $P < 0.05$**

TABLE 3C: Mann-Whitney U inter-group comparison of mean VHN values

Intergroup comparison		P VALUE
1(Negative control)(saline)	2	0.027*
	3	0.024*
	4	0.003*
	5 (2.5% NaOCl+0.13 M clodronate)	0.05*
2(Positive control) (2.5%NaOCl)	3	0.834
	4	0.027*
	5	0.462
3(2.5%NaOCl/17%EDTA)	4	0.027*
	5	0.401
4 (2.5%NaOCl+9% HEBP)	5	0.016*

***Statistically significant difference $P < 0.05$**

4. Percentage area fraction of open dentinal tubules

ImageJ software was used to calculate percentage area fraction (% AF) of dentinal tubules in coronal, middle and apical levels from SEM images obtained from prepared root canals at 2000x magnification. No open dentinal tubules were observed in any of the thirds evaluated from Group 1 (saline) and Group 2 (2.5% NaOCl) [Figure 25,26]. The samples treated with chelating agents showed varying degrees of tubular opening across all thirds of the root canal [Table 4A]. Percentage area fraction of open tubules was highest in coronal thirds, followed by middle and apical thirds in Group 3, Group 4 and Group 5 [Figure 27-29; Graphs 6-8]. A statistically significant difference in %AF of open dentinal tubules was observed between all groups across all thirds of the canal ($P < 0.001$) using Kruskal-Wallis test [Table 4B]. Mann-Whitney U inter-group comparison revealed that Group 3 had a significantly higher %AF of open tubules than Groups 4 in coronal, middle and apical thirds of root canals ($P < 0.001$, $P < 0.012$, $P < 0.02$) as well as Group 5 ($P < 0.001$, $P < 0.012$, $P < 0.09$) respectively [Table 4C]. No statistically significant difference was observed between Group 4 and Group 5 ($P > 0.05$) Graphs 6-8 depict comparison of means of %AF in coronal, middle and apical third of the three experimental groups.

Table 4A-Mean and standard deviation of percentage area fraction (%AF) of tubular openings in coronal, middle and apical third of all groups

ROOT THIRD	Group	Mean (%AF)	SD
Coronal area fraction	1(Negative control)	0	0
	2(Positive control)	0	0
	3(2.5%NaOCl/17%EDTA)	42.12	5.49
	4 (2.5%NaOCl+9% HEBP)	18.05	2.05
	5(2.5% NaOCl+0.13 M clodronate)	16.27	5.64
Middle area fraction	1(Negative control)	0	0
	2(Positive control)	0	0
	3(2.5%NaOCl/17%EDTA)	14.23	4.16
	4 (2.5%NaOCl+9% HEBP)	7.64	1.11
	5(2.5% NaOCl+0.13 M clodronate)	7.38	3.63
Apical area fraction	1(Negative control)	0	0
	2(Positive control)	0	0
	3(2.5%NaOCl/17%EDTA)	1.81	0.61
	4 (2.5%NaOCl+9% HEBP)	0.78	0.25
	5(2.5% NaOCl+0.13 M clodronate)	0.77	0.64

Table 4B- Kruskal-Wallis comparison of %AF at all thirds among groups

VARIABLE	GROUP	N	MEAN RANK	P-VALUE
Coronal third % area fraction	1	8	8.50	0.001*
	2	8	8.50	
	3	8	36.50	
	4	8	26.00	
	5	8	23.00	
Middle third % area fraction	1	8	8.50	0.001*
	2	8	8.50	
	3	8	34.50	
	4	8	26.00	
	5	8	25.00	
Apical third % area fraction	1	8	8.50	0.001*
	2	8	8.50	
	3	8	35.38	
	4	8	26.00	
	5	8	24.13	

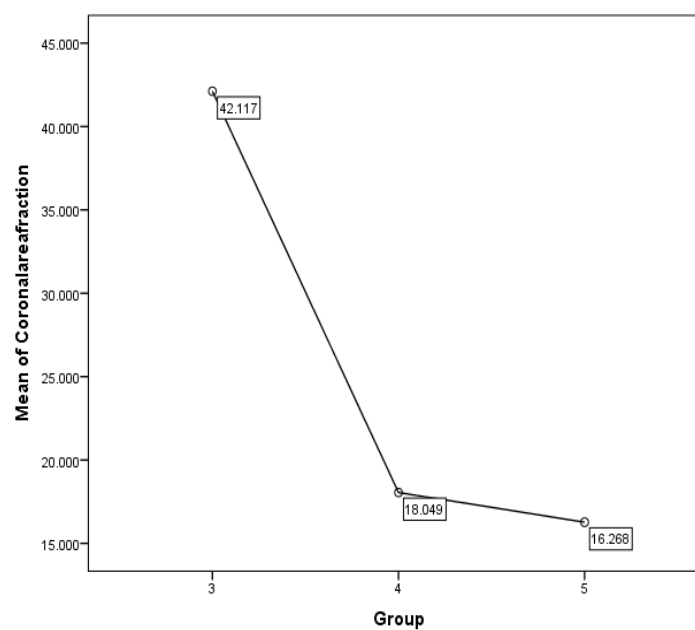
***Statistically significant difference $P < 0.05$**

Table 4C: Mann-Whitney U inter-group comparison of mean % AF

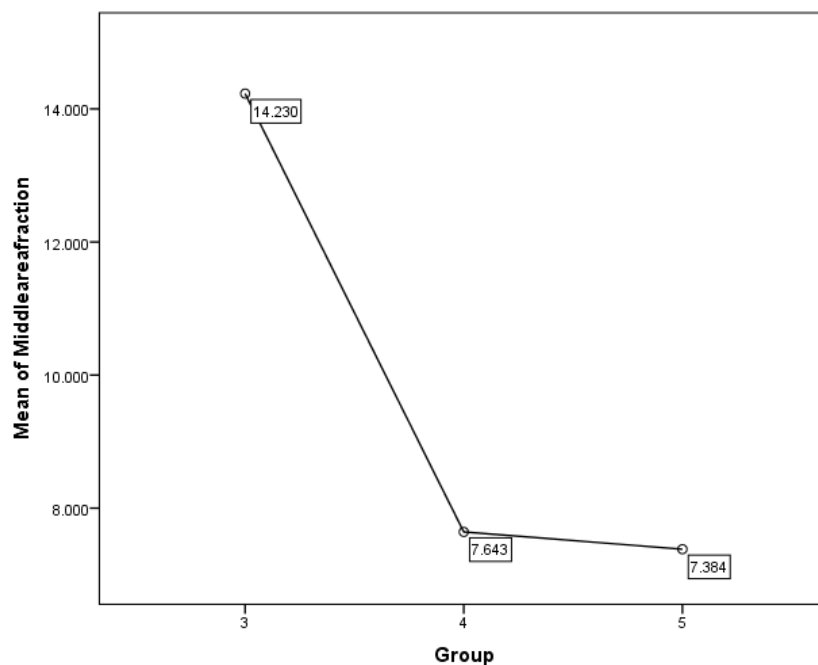
GROUPS (A)	GROUPS (B)	P VALUE		
		Coronal third	Middle third	Apical third
1 (Negative control) (saline)	2	0.001*	0.001*	0.001*
	3	0.001*	0.001*	0.001*
	4	0.001*	0.001*	0.001*
	5 (2.5%NaOCl+0.13 M clodronate)	0.001*	0.001*	0.001*
2(Positive control) (2.5%NaOCl)	3	0.001*	0.001*	0.001*
	4	0.001*	0.001*	0.001*
	5	0.001*	0.001*	0.001*
3 (2.5%NaOCl/17%EDTA)	4	0.001*	0.012*	0.02*
	5	0.001*	0.012*	0.09*
4 (2.5%NaOCl+9% HEBP)	5	0.208	0.674	0.293

***Statistically significant difference $P < 0.05$**

Graph 6: Comparisons of mean of percentage area fraction of open dentinal tubules in coronal third of experimental groups- Group 3, Group 4 and Group 5



Graph 7: Comparison of mean of percentage area fraction of open dentinal tubules in middle third of experimental groups- Group 3, Group 4 and Group 5



Graph 8: Comparison of mean of percentage area fraction of open dentinal tubules in apical third of experimental groups- Group 3, Group 4 and Group 5

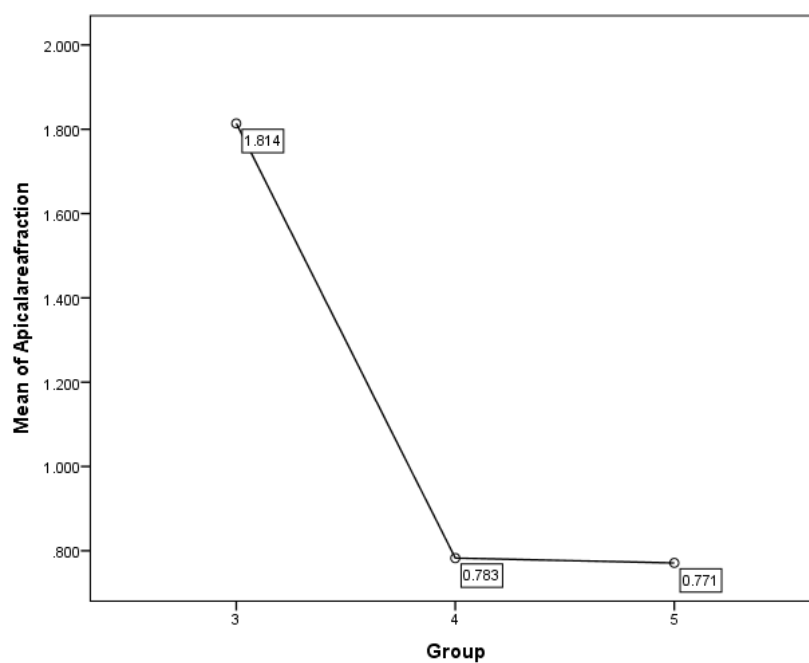
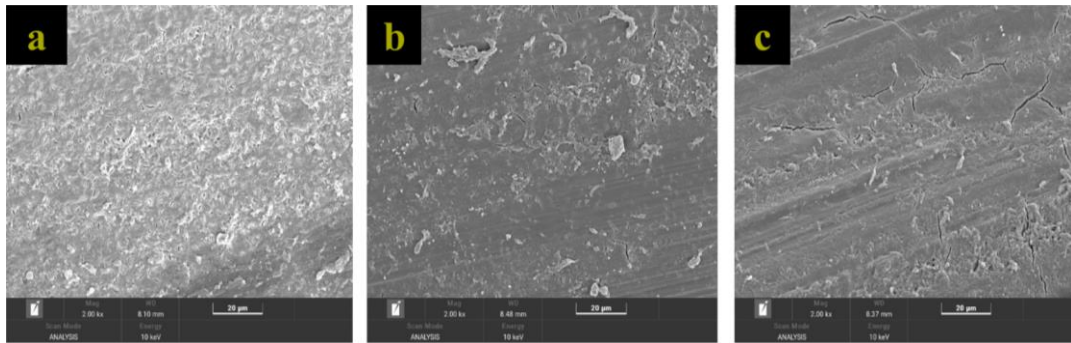
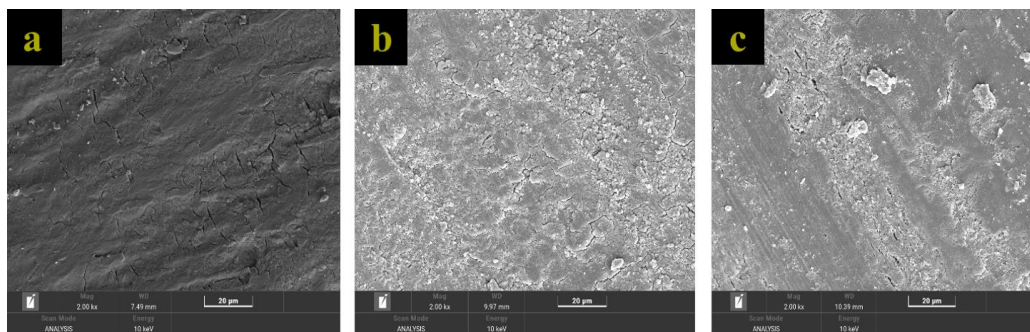
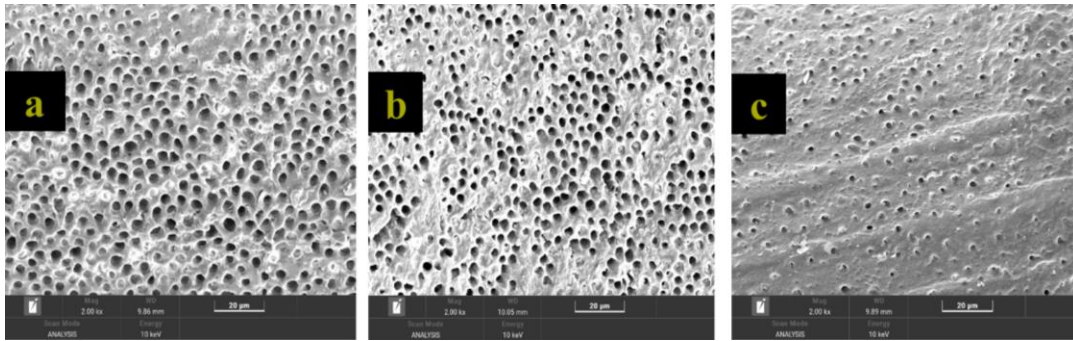
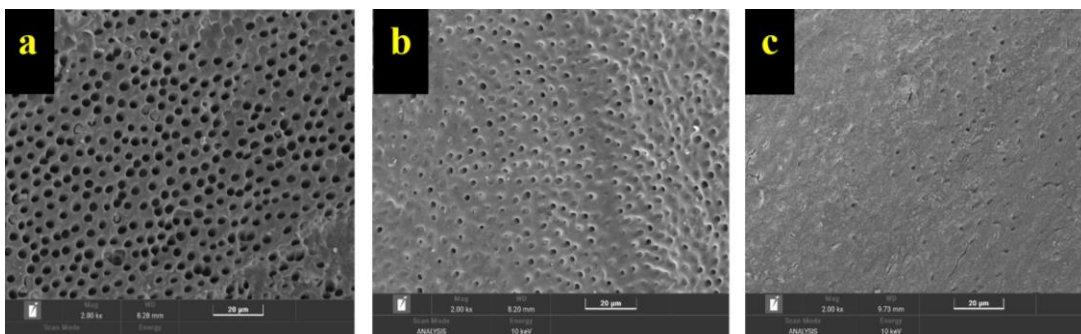
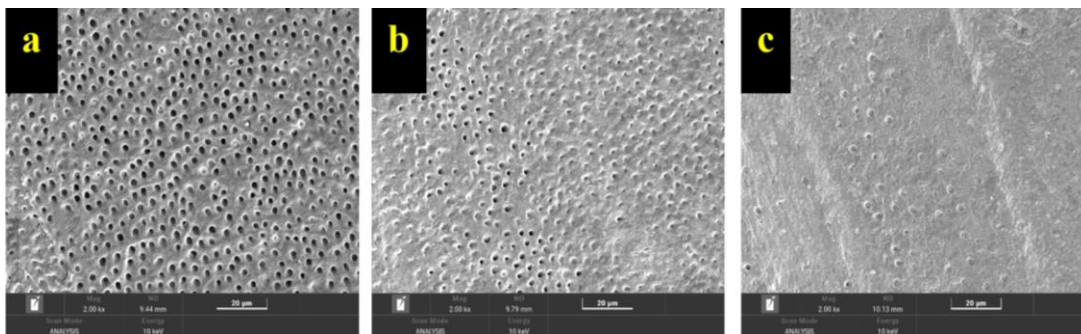


FIGURE 25: Representative scanning electron microscope images of Group 1**FIGURE 26: Representative scanning electron microscope images of Group 2**

a) Coronal third; b) Middle third; c) Apical third

****Scanning electron microscope images of root canal wall at 2000x magnification from a 20µm field of view. Images depict absence of open dentinal tubules in all root thirds in Group 1 [Fig 25] and Group 2 [Fig 26].**

FIGURE 27: Representative scanning electron microscope images of Group 3**FIGURE 28: Representative scanning electron microscope images of Group 4****FIGURE 29: Representative scanning electron microscope images of Group 5**

a) Coronal third; b) Middle third; c) Apical third

******Scanning electron microscope images of root canal wall at 2000x magnification from a 20µm field of view. Images depict highest %AF of open dentinal tubules in coronal third (a) followed by middle (b) and apical third (c) in Group 3 [Fig 28], Group 4 [Fig 29] and Group 5 [Fig 30]. The images also depict highest %AF of open dentinal tubules at pre-determined sites at all 3 levels in Group 3 [Fig 28] compared to Group 4 [Fig 29] and Group 5 [Fig 30].

DISCUSSION

The primary objectives of endodontic therapy include biofilm eradication and prevention of reinfections.⁽⁹⁷⁾ Non-instrumentation techniques to achieve root canal disinfection may be the ideal, but it is still a distant goal. Until then, endodontists are still reliant on instrumentation to aid in the disinfection of root canal systems. Therefore, irrigants and intracanal medicaments are needed to complement the instrumentation of root canals to aid in smear layer removal and to achieve adequate disinfection. Currently, there is no “one-size fits all” approach to irrigation, and a combination of 2 or more irrigants are employed to achieve the desirable effects. Of the many available irrigants, sodium hypochlorite is the universally accepted working solution. It is capable of tissue dissolution, and its antimicrobial action is undisputed even in concentrations as low as 0.5% .^(12,13) However, it is ineffective in removing inorganic portions of the smear layer. The American Association of Endodontists (AAE) recommend using a decalcifying agent for a minimum of 2 minutes to assist in smear layer removal.^(11,18) The two most commonly used irrigants, NaOCl and EDTA, are incompatible. Therefore, the two solutions cannot be mixed and used together, or an intermediate rinse with saline or distilled water is advocated when used sequentially.⁽¹⁸⁾

The term “continuous chelation”⁽²⁰⁾ describes a regimen where a chelator is mixed with NaOCl, resulting in a single mixture

that is capable of achieving all core functions of an ideal irrigant. Etidronic acid (EA) or HEBP ⁽¹⁹⁾ and disodium clodronate ⁽⁴⁴⁾, both belonging to the bisphosphonate group of drugs, are weaker calcium chelators with potential to be used in continuous chelation with NaOCl.

Dentin is a biological composite which is structurally complex and compositionally heterogenous.⁽⁴⁶⁾ The organic matrix is comprised predominantly of type I collagen, with non-collagenous proteins such as glycoproteins and proteoglycans. ⁽⁹⁸⁾ The inorganic component of dentine consists predominantly of calcium hydroxyapatite containing calcium (Ca^{2+}), phosphate (PO_4^{3-}), and hydroxyl (OH^-) ions. The hydroxyapatite nanocrystals are irregularly shaped, and are located both within and external to the collagen fibrils. The water component binds to both the collagen and inorganic components, and is also found unbound in the dentinal tubules. ⁽⁴⁶⁾⁽⁹⁹⁾ Both components of dentin contribute to the overall mechanical properties exhibited by dentin. The inorganic component is responsible for dentin stiffness and elasticity. ^(45,46) The collagen absorbs strain and is responsible for fracture resistance and toughness. Water content also affects fracture strength, with dehydrated dentin being considerably more prone to fracture.⁽⁹⁹⁾

Irrigants have the potential to affect mechanical properties of dentin because different solutions exert different chemical actions on the organic and inorganic portions of dentin. Such irreversible damage to dentin caused by irrigants may act as secondary causes for potential fractures in endodontically treated teeth.⁽⁴⁶⁾ While the smear layer removal capacity of etidronic acid has been shown to be comparable to conventional irrigation protocols (15,19,43,100), the assumption has always been that HEBP would be less aggressive on dentin walls due to its weaker stability constant. The literature review suggests mixed opinions in this regard. Some authors have found it to be milder ^(35,55,57,75), while others have demonstrated deleterious effects on mechanical and ultrastructural properties of dentin. ^(43,84) These variations may have been caused due to differences in methodologies, techniques, source, concentrations, and time of exposure to irrigants. To the best of our knowledge, no study has compared the effects of etidronic acid and clodronate in continuous chelation mode against sequential irrigation protocol on the ultrastructure of dentin. Therefore, the present study aimed to compare the effects of sodium hypochlorite combined with different chelating agents on the ultrastructure of dentin.

The use of extracted human teeth and deep coronal dentin ensured that conditions were as close as possible to root dentin (101,102) and allowed for comparison to previous studies based on similar protocols. ^(55,57) The selection of dentin blocks for studying

the effects of irrigants cannot be expected to simulate a clinical condition, but they were used nonetheless because the methodology dictated that the sample dimensions be completely flat and relatively homogenous.^(55,57) Therefore, in the present study, dentin blocks from deep coronal dentin of human mandibular third molars measuring 4*3*1mm were obtained using a microtome according to a modified protocol by Rath et al.⁽⁵⁵⁾ Single-rooted mandibular premolars that met all inclusion criteria were chosen to minimize anatomical variations and facilitate easy splitting of teeth for percentage area fraction of open dentinal tubules and elemental analysis study under SEM.

There is still no consensus regarding the choice of irrigant, concentrations, duration, and sequence of the final irrigation protocol for non-surgical root canal therapy (NSRCT). Numerous options exist for the choice of decalcifying agents to be used as final irrigants, including pure chelators and ones with additives such as surfactants or antimicrobials.⁽¹⁷⁾ However, the most popular sequences followed are NaOCl/EDTA or NaOCl/EDTA/NaOCl. The sequence of NaOCl/EDTA/NaOCl has been proven to cause marked dentinal wall erosion^(1,16,49,52). Hence, it is no longer considered best practice even though it may provide cleaner canals. Therefore, we used NaOCl/EDTA sequence as a standard against which continuous chelation groups were compared. Irrigation protocol for the root canal samples and time of exposure to irrigants was based

on a study by Lottanti et al ⁽¹⁵⁾ as it closely matched clinical exposure time. For the dentin blocks, the protocol was further modified from previous studies (57,103), to optimize volume and time of exposure to irrigants to suit the smaller surface area of the dentin blocks.

Surface roughness is a parameter that may help evaluate the depth of penetration/action of irrigants into/on dentin. It also helps assess how an irrigant may affect the dentin surface, which may have implications for the adhesion of sealers. It may be evaluated using optical profilometry or AFM (55,104). AFM was chosen for this study due to the higher resolution offered compared to a profilometer or digital tester. It also has the added advantages of providing a 3-D pictorial representation of the surface tested (105), minimal sample preparation of non-conducting materials like teeth, and non-destructive nature. (106) Atomic surface microscopy of the treated dentin blocks in non-contact mode revealed varying degrees of surface roughness between groups. Surface roughness was significantly increased in Group 2 (2.5% NaOCl), Group 3(2.5% NaOCl/17% EDTA), Group 4(2.5% NaOCl+9% HEBP) and Group 5(2.5% NaOCl+0.13M clodronate) when compared to Group 1(saline), suggestive of active smear layer removal by the components ($P<0.05$). The results are in agreement with Hu et al⁽⁹⁴⁾, Rath et al ⁽⁵⁵⁾, who found increased surface roughness values in experimental groups treated with 17% EDTA and 9% HEDP

compared to control groups. Group 4 showed significantly lower surface roughness than Group 2, Group 3 and Group 5 ($P=0.012, 0.001, 0.001$), respectively. This could be the result of increased exposure time to NaOCl and HEBP, which in turn may have resulted in shortening and thinning of collagen fibrils as described by Rath et al.⁽⁵⁵⁾ in their TEM analysis; and dentin erosion as observed by Ulusoy et al.⁽⁴³⁾

To study the chemical compositional changes in dentin, techniques such as Attenuated total reflectance of Fourier transform infrared spectroscopy (ATR-FTIR) or Fourier transform infrared spectroscopy (FITR), elemental analysis using micro elemental analysis⁽⁶¹⁾ or energy-dispersive X-ray spectroscopy (EDS)⁽⁶⁴⁾, have been employed.^(15,55,57,94,95) Although FTIR has traditionally been used as a qualitative measure of changes in the chemical composition of dental tissues, it is currently being used to quantitatively measure the changes in ratios of organic and inorganic components, which may accurately reflect the dynamics of interactions between irrigants and the substrate upto a depth of 300 μm . This technique is non-destructive and also does not require additional surface preparation of the samples, which allowed us to measure multiple parameters in the same dentin blocks.^(57,94,95) ATR-FTIR analysis was done quantitatively to evaluate changes in carbonate/phosphate and amide/phosphate ratios. Smaller amide/phosphate ratios indicate deproteination, whereas smaller

carbonate/phosphate ratios indicate demineralization.⁽⁵⁷⁾ These can reflect changes in collagen and apatite contents, respectively, both of which contribute to biomechanical properties of dentin.^(46,47) But in the present study, no statistically significant differences were observed between groups in carbonate/phosphate ratios ($P>0.05$). Group 1 and Group 2 did not receive any treatment with chelating agents, and therefore, the carbonate/phosphate ratios remained unaltered. Group 3 samples also did not exhibit any changes in carbonate/phosphate ratio, probably due to the limited duration of exposure to EDTA of 1 minute and the lack of any activation or replenishment of the irrigant. The lack of difference in Groups 4 and 5 could be due to prolonged simultaneous exposure of dentin blocks to both the chelator and NaOCl in continuous chelation mode which may have led to the emergence of a fresh dentin surface, thereby restoring the carbonate/phosphate ratio. These findings are supported by Tartari et al.⁽⁵⁷⁾ Similarly, no statistically significant difference was observed in Amide I/phosphate and amide III/Phosphate ratios among all groups ($P>0.05$). However, a numerical increase in amide I/phosphate ratio was observed in Group 3 and Group 4 compared to Group 1, Group 2, and Group 5, indicating a relatively higher degree of demineralization by EDTA exposing the collagen fibrils on treated dentin block surfaces.

Elemental analysis of the prepared root canal samples revealed varying percentages of atomic weights of the elements

carbon, nitrogen, oxygen, calcium, phosphorus among all groups. [Table 2C] The significant drop in carbon, nitrogen, oxygen and phosphorus levels in Group 3, Group 4, and Group 5 when compared to Group 1 ($P < 0.05$) confirms deproteinization by the sodium hypochlorite and demineralization effects of all the chelators in these groups. The overall significant increase in levels of calcium, phosphorus and significant reduction in levels of nitrogen and carbon in Group 2, highlight the effect of NaOCl on the organic amide portion ($P = 0.001$). Group 3 (2.5% NaOCl/17% EDTA) revealed significant alterations in both organic and inorganic components, with the highest nitrogen content and least phosphorus levels. [Graph 3]. This is suggestive of aggressive demineralisation and exposure of collagen on root canal walls. These findings are in corroboration with that of Rath et al. ⁽⁵⁵⁾ Between Group 4 and Group 5, there was a significant finding only in relation to calcium content. Group 4 showed a higher calcium chelating ability than Group 5 and no significant changes were observed in percentages of other elements indicating that the chelator acts independently of NaOCl and that HEBP is a more potent calcium chelator than clodronate. [Graph 4,5]

The hardness of dentin may be assessed using methods such as micro-or nano-hardness testing with Knoop or Vicker's microhardness testers ^(75,107) or, more recently, using nano indenters ⁽⁴³⁾. Microhardness testing is considered the gold standard for

observation of hard tissues and is a strong predictor of Young's modulus and yield stress ⁽¹⁰⁸⁾ which are linked to the fracture strength of a material. Although nanoindentation allows operators to choose the indentation location, its use may be limited to superficial layers. ⁽¹⁰⁹⁾ Hardness testing helps to indirectly evaluate the ultrastructural changes caused by different chelator groups ^(45,110). A significant drop in dentin microhardness may be caused by both the deproteinization due to NaOCl as well as demineralization ^(55,111). This may be suggestive of a considerable change in crystallinity of the surface being tested. The loss of hydroxyapatite crystals is also associated with the loss of tightly bound water, which is responsible for the viscoelastic behaviour of dentin. ⁽⁴⁷⁾ In this study, Group 2, Group 3, Group 4, and Group 5 exhibited a significant decrease in dentin microhardness compared to Group 1 ($P < 0.05$), indicating that exposure to sodium hypochlorite, alone or in combination with chelators leads to decrease in dentin microhardness values. These results were similar to those observed by Tartari et al ⁽⁷⁶⁾, Bhagwat et al ⁽⁷⁵⁾, Ulusoy et al ⁽⁴³⁾, Dineshkumar et al. ⁽³⁵⁾ Group 4 (2.5% NaOCl+9%HEBP) exhibited the least dentin microhardness values when compared to Group 2(2.5% NaOCl), Group 3(2.5% NaOCl/17% EDTA) and Group 5 (2.5%NaOCl+0.13M clodronate) ($P < 0.05$). This too, may be attributed to the prolonged contact time of the dentin blocks with the irrigants in continuous chelation mode. Grinkeviciute et al ⁽⁷⁴⁾ also observed a significant reduction in dentin microhardness in the

etidronic acid group when compared to NaOCl/17%EDTA when used in continuous chelation mode.

Due to dentin's tubular structure, it can be compared to a porous material. Studies have demonstrated correlations between its porosity and mechanical properties.^(112,113) Fracture toughness of a tooth, which governs viscoelastic behaviour, can be a function of lumen area fraction (ϵ) or porosity as described in a formula by Balshin in 1949⁽¹¹⁴⁾, where K_{ic} is fracture toughness of dentin with specific porosity and K_{ic0} is fracture toughness of sound dentin and m is a stress dependent constant.

$$K_{ic} = K_{ic0}(1-\epsilon)^m$$

Therefore, this study aimed to calculate the percentage area fraction of open dentinal tubules observed from SEM images as a surrogate measure of ultrastructural properties of root dentin. To the best of our knowledge, this is the first study to use the area fraction of open dentinal tubules obtained through image analysis as a criterion to study the ultrastructural properties of root canal dentin which may influence fracture toughness. Digital image analysis of scanning electron microscope images has been proven to be a valuable alternative to study the microstructure of dentin^(54,115,116). Various softwares such as ImageJ, ImagePro, Adobe Photoshop can be used to auto-calculate open dentinal tubules. This method minimizes subjectivity and allows for a quantitative

comparison of findings, which lends itself to statistical testing. Groups 1 and 2 (controls) exhibited a total absence of tubular openings in all root thirds due to incomplete removal of the smear layer ($P < 0.001$). Group 3 (2.5% NaOCl/17% EDTA), Group 4 (2.5% NaOCl+9% HEBP) and Group 5 (2.5% NaOCl+0.13M clodronate), however, displayed varying degrees of tubular opening that may be correlated to their demineralizing efficacy and by extension, their effect on fracture toughness of a tooth. All samples from Group 3, Group 4, and Group 5 displayed the highest percentage area fraction of open dentinal tubules in the coronal third, followed by the middle third and then the apical third ($P < 0.001$). The gradient can be attributed to the inherent decrease in the number of tubules from the coronal end towards the apex, sclerosis and aging of the dentin, apical vapor lock effect, lack of irrigant activation ^(117–120). This finding is in accordance with studies by Lottanti et al ⁽¹⁵⁾, Ulusoy et al ⁽⁴³⁾, Kfir et al ⁽³⁷⁾, Wright et al ⁽⁴⁴⁾ Percentage area fraction of open tubules is not just a function of sodium hypochlorite action but a function of its activity in unison with that of the different chelators. ^(55,111) EDTA has higher stability constant for calcium chelation when compared to HEBP or clodronate ⁽¹⁹⁾. This may be the reason why Group 3 samples exhibited the highest %AF of open tubules in all thirds. The values were also significantly higher than Group 4 and Group 5 ($P < 0.001$). This confirms weaker chelation capacity of etidronic acid and clodronate compared to EDTA, which was also observed in the EDS results. Although no significant

differences were observed between Group 4 and Group 5 in %AF of open dentinal tubules at all levels of the root canals ($P>0.05$), indicating that HEBP and clodronate are comparable in action, the results of EDS indicate that etidronate is a stronger chelator for calcium than clodronate [Graph 4,5; Table 2E].

The chemical constituents of irrigants have different effects on dentin properties, as demonstrated by this study. When considering continuous chelating solutions, it is important to understand that these chelators, though weaker in calcium complexing abilities, are in contact with dentin for much longer than traditional chelators because they are mixed with NaOCl during entire irrigation cycles. Continuous chelation with 2.5% NaOCl/9% HEBP and 2.5% NaOCl+0.13M clodronate in instrumented root canals appears to be less aggressive than 2.5% NaOCl/17% EDTA sequence from SEM analysis. But these results were not reflected in the analysis of ultrastructural properties in dentin blocks as well as EDS of prepared root canal samples. In dentin blocks, continuous chelation with 2.5% NaOCl/9% HEBP showed significantly more deleterious effects on ultrastructural properties such as surface roughness and dentin microhardness, unlike EDTA and clodronate. Similarly, elemental analysis of root canal samples using EDS showed that calcium chelation capacity of etidronic acid was comparable to EDTA and significantly higher than clodronate. Therefore, the null hypothesis was partially rejected.

Limitations of this study include:

- The differing nature of dentin and preparation in dentin blocks and root canal samples did not allow us to make a direct correlation between the results obtained in their respective tests
- Duration of irrigation was not standardised between dentin blocks and root canals.
- Transmission electron microscopy, SEM and EDS analysis of treated dentin blocks may have helped describe and understand deeper effects of irrigants on the ultrastructure of dentin.

Within the limitations of this study, continuous chelating solutions may offer a potential alternative to the conventional irrigation regimen. Still, the claims of them having milder effects on dentin ultrastructure remains debatable. Clodronate has shown comparable efficacy to etidronate with minimal change in the dentin ultrastructure. Future studies that standardize time, volume, chemical composition, the effect of activation, and sealer interaction of these weaker chelators are required to devise standardized protocols and recommendations for clinical use.

SUMMARY

This quantitative study compared ultrastructural changes caused by the effects of sodium hypochlorite combined with different chelating agents in dentin blocks and root canal dentin and correlated the findings. Forty dentin blocks were obtained from deep coronal dentin using a microtome and randomly divided among 5 groups depending on the irrigation protocol used (n=8): Group 1- saline (negative control); Group 2-2.5% NaOCl (positive control); Group 3-2.5%NaOCl/17% EDTA; Group 4-2.5% NaOCl+9% HEBP; Group 5-2.5% NaOCl+0.13M clodronate. Dentin blocks were exposed to irrigants for varying time periods based on the irrigation protocol of the respective groups. Atomic force microscopy was performed to evaluate surface roughness. Quantitative ATR-FTIR analysis was done to determine compositional changes in carbonate/phosphate and amide/phosphate ratios of the dentin blocks. Vicker's microhardness testing was performed using a load of 300g with a dwell time of 10 seconds to determine changes in dentin microhardness.

Forty single-rooted mandibular premolars were decoronated, and the working length was standardized to 16 ± 2 mm. They were then randomly divided among the five experimental groups and instrumented using ProTaper Gold rotary system to size F3 following irrigation regimens of their respective groups. The teeth were then split longitudinally and subjected to analysis under a

scanning electron microscope at 2000x. The percentage area fraction of open dentinal tubules was auto-calculated using ImageJ software. Statistical analysis using the Kolmogorov-Smirnov test showed that data was not normally distributed. Therefore, non-parametric tests were performed. Kruskal-Wallis and Mann-Whitney U tests were used to detect statistically significant differences among groups. Tests conducted on dentin blocks revealed that treatment with sodium hypochlorite combined with different chelating agents caused ultrastructural changes. Group 4 (2.5% NaOCl+9% HEBP) caused significant reductions in surface roughness and dentin microhardness than Group 2(2.5% NaOCl), Group 3(2.5% NaOCl/17% EDTA) and Group 5(2.5% NaOCl+0.13M clodronate). ATR-FTIR analysis did not reveal any statistically significant differences among groups, but a numerical increase in amide I/phosphate ratio was detected in Group 3. SEM analysis of instrumented root canals revealed varying percentage area fractions of open tubules in groups treated with chelators. The coronal third showed the highest percentage area fractions of open tubules, followed by middle and apical thirds in all samples of those groups. Statistically significant differences were observed at all thirds of the root canals for Group 3 compared to Group 4 and Group 5. However, Group 4 and Group 5 did not differ significantly from each other. Elemental analysis of root canals revealed significantly different effects among groups on the atomic weight percentages of carbon, nitrogen, oxygen, calcium, and phosphorus. While Group 2

had pronounced effects on the organic portions, Group 3, Group 4, and Group 5 affected inorganic portions. Group 3 and Group 4 had comparable calcium chelation efficacy which was significantly higher than that of Group 5. Overall, it was found that irrigation protocols employing sodium hypochlorite along with different chelators caused ultrastructural changes in dentin. Of the tested continuous chelation regimens, Group 4 caused significantly more harmful effects on dentin ultrastructure than Group 5. .

CONCLUSION

Within the limitations of this ultrastructural study, it is concluded that,

1. Dentin blocks treated with NaOCl in combination with different chelating agents revealed changes in dentin's ultrastructure.
2. Dentin blocks in Group 4(2.5% NaOCl+9% HEBP) exhibited reduced surface roughness and dentin microhardness when compared to Group 2 (2.5% NaOCl), Group 3(2.5% NaOCl/17%EDTA) and Group 5 (2.5% NaOCl+0.13M clodronate).
3. No statistically significant changes were observed in the chemical composition of dentin blocks, although Group 3 showed a numerical increase in Amide I/Phosphate ratios.
4. Root canal samples treated with NaOCl combined with different chelating agents revealed varying percentage area fractions of tubular openings.
5. In all groups treated with a chelating agent, the coronal third revealed the highest percentage area fraction of open dentinal tubules, followed by the middle third and apical third.
6. Group 3 (2.5% NaOCl/17%EDTA) revealed a significantly higher percentage area fraction of open dentinal tubules in all root canal thirds when compared to Group 4 (2.5% NaOCl+9% HEBP) and Group 5(2.5% NaOCl+0.13M clodronate). No

statistically significant difference was observed between Group 4 and Group 5.

7. Elemental analysis of root canal samples revealed that Group 2(2.5% NaOCl) had significant effects on root canals' carbon and nitrogen content. In contrast, groups containing a chelating agent had a substantial impact on calcium and phosphorus portions of dentin to varying degrees.
8. Calcium chelation efficacy of Group 4 (2.5% NaOCl+9% HEBP) was comparable to that of Group 3(2.5% NaOCl/17%EDTA) and was significantly higher than that of Group 5(2.5% NaOCl+0.13M clodronate).

All irrigation regimens combining NaOCl with chelators in sequence or continuous chelation modes lead to ultrastructural changes in the dentin. Among the weaker chelators, Clodronate exhibited a milder effect on dentin ultrastructure. Future studies are required to confirm its superiority over other chelators.

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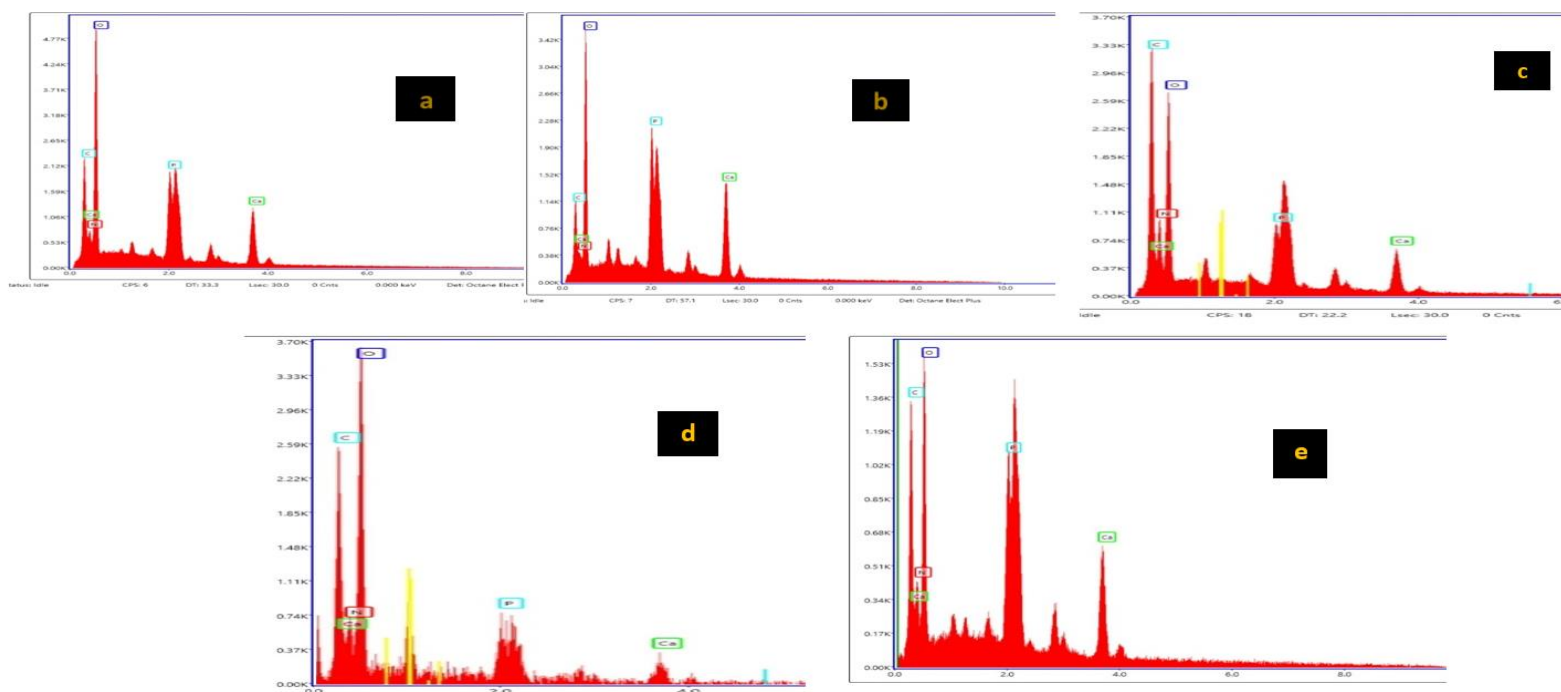
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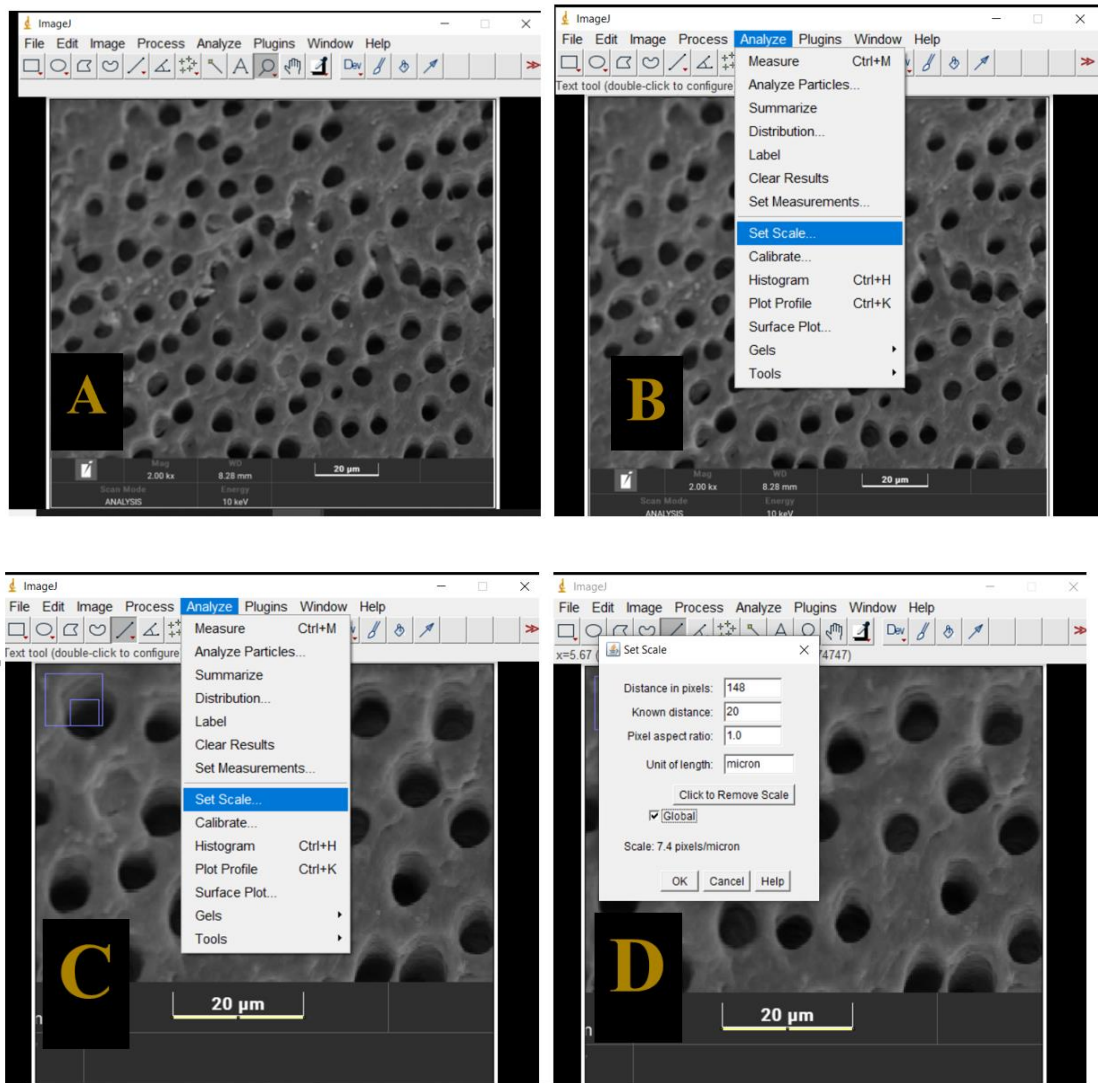
APPENDIX

APPENDIX A: Representative EDX graphs of all groups depicting amounts of various elements- C,N,O,P,Ca. a) Group 1-saline; b) 2.5% NaOCl; c) 2.5% NaOCl/17% EDTA; d) Group 4- 2.5% NaOCl+9% HEBP; e) Group 5- 2.5% NaOCl+0.13 M clodronate



***EDS spectra of all groups shows varying peak heights for different elements in different groups, indicating varied effects of irrigants on ultrastructure of dentin. Reduction in calcium and phosphorus peak heights relative to negative control indicate effect on inorganic portions (demineralisation) whereas reduction in carbon, nitrogen peak heights indicate effect on organic portions (deproteinisation).**

APPENDIX B: Image series depicting process of image thresholding of SEM images to drive % area fraction of open dentinal tubules

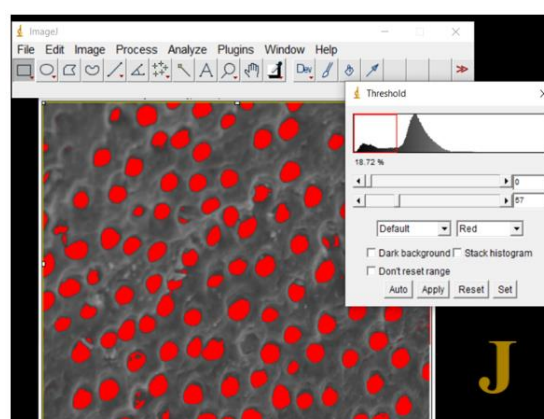
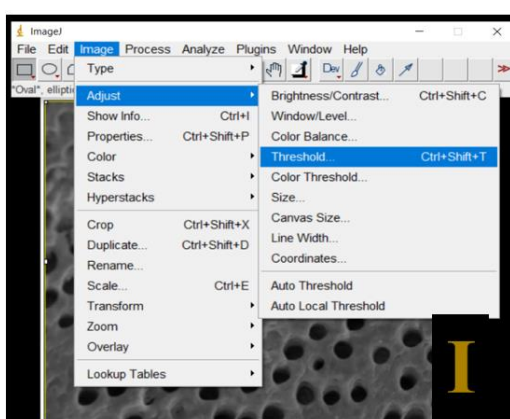
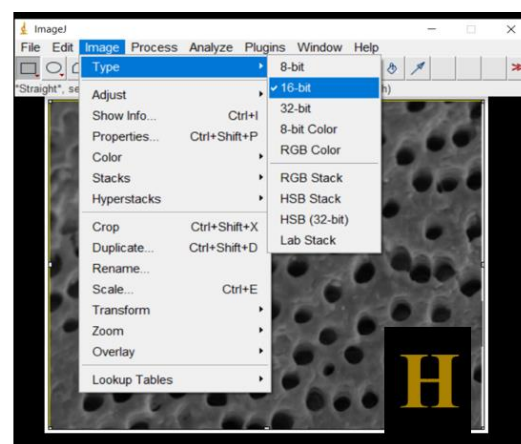
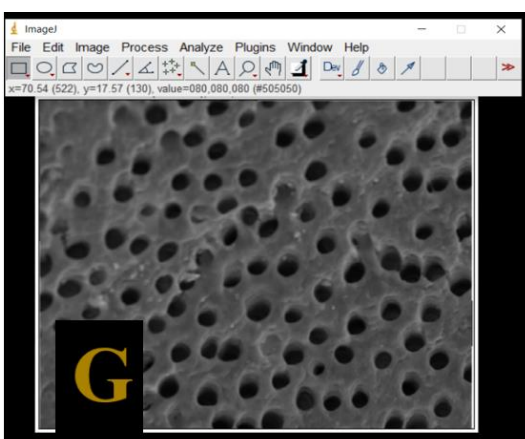
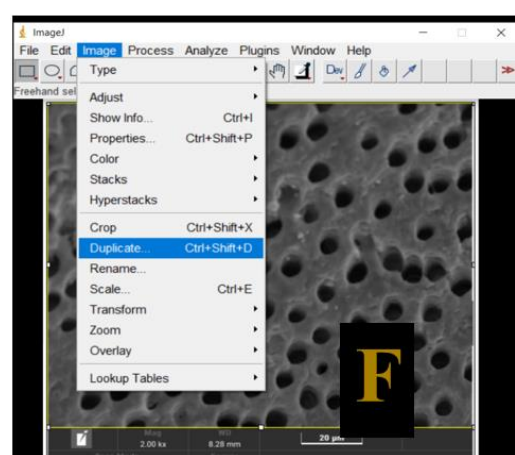
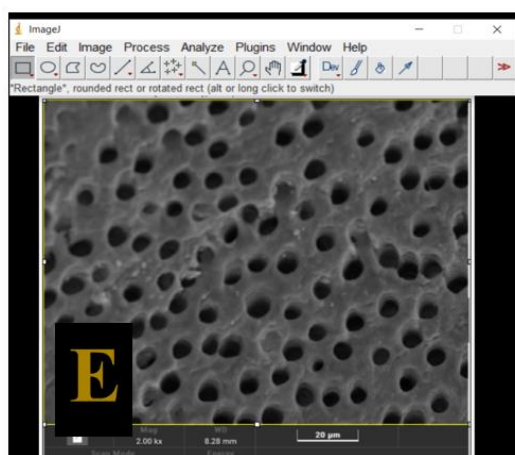


A-Open SEM image in ImageJ either by drag and drop or opening file location

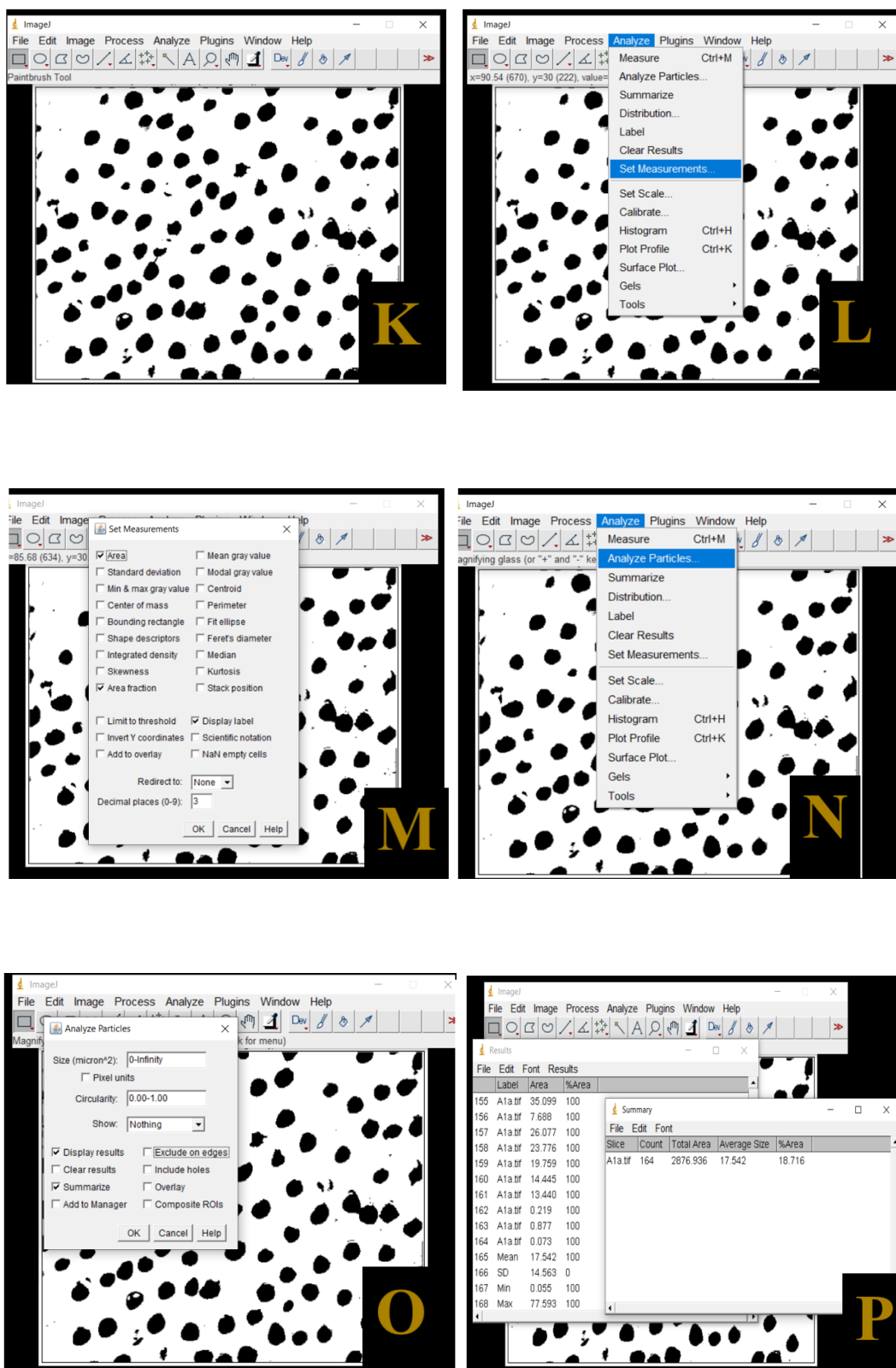
B-Set known measurements by setting scale

C-Drag a line over the scale provided at the bottom of the SEM image

D-Enter “known distance” according to SEM scale



E-Select area required for analysis; F,G-Duplicate the image; H-Convert duplicated image to 16 bit image for thresholding; I,J-Image thresholding; Adjust scroll bar if needed to capture all required areas for analysis



K-Apply changes; **L**-Set required measurements needed; **M**-In this instance, area and area fraction were selected; **N**-Analyze particles; **O**-Choose required data to be displayed; **P**-Final calculations which may be exported and saved in other file formats