Journal of Esthetic and Restorative Dentistry

WILEY

RESEARCH ARTICLE OPEN ACCESS

Advancing Adhesive Strategies for Endodontically Treated Teeth—Part I: Impact of Endodontic Irrigation Protocols on the Chemical Composition and Structural Integrity of Coronal Dentin

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Received: 20 January 2025 | **Revised:** 4 March 2025 | **Accepted:** 6 March 2025

Funding: This study was supported by the CICECO-Aveiro Institute of Materials (UIDB/50011/2020, UIDP/50011/2020, and LA/P/0006/2020) financed by national funds through the FCT/MEC (PIDDAC).

Keywords: chelating agents | dentin | endodontics | root canal irrigants | root canal therapy | sodium hypochlorite

ABSTRACT

Objective: To evaluate the effects of four endodontic irrigation protocols on the chemical composition and ultrastructure of coronal dentin.

Materials and Methods: Coronal dentin fragments were assigned to five study groups: NaOCl (3% sodium hypochlorite), NaOCl/EDTA (3% NaOCl and 17% ethylenediaminetetraacetic acid), NaOCl/EDTA/CHX (3% NaOCl, 17% EDTA and 2% chlorhexidine), NaOCl/HEDP (mixture of 3% NaOCl and 9% etidronic acid), and control (distilled water). Confocal Raman microscopy was employed to analyze the spatial distribution of organic and inorganic components, while attenuated total reflection Fourier transform infrared spectroscopy and energy-dispersive spectroscopy were used to assess the surface composition of dentin. Ultrastructural evaluation was conducted using scanning electron microscopy (SEM). Statistical analysis was performed using a mixed linear model with a significance level of 0.05.

Results: All NaOCl-treated groups showed reduced amide II ($p < 0.001$), indicating protein degradation. Exclusive NaOCl irrigation yielded the lowest amide II, highest mineral content, and increased phosphate/amide II and carbonate/phosphate ratios ($p < 0.05$). Chelators reduced mineral content ($p < 0.001$), with NaOCl/HEDP and NaOCl/EDTA/CHX producing more mineralized surfaces than NaOCl/EDTA and control groups ($p < 0.05$). A general decrease in organic (C and N) and an increase in inorganic (O, P, and Ca) components occurred across treatments, particularly in NaOCl and NaOCl/HEDP groups. EDTA disturbed the Ca/P equilibrium ($p < 0.05$). SEM showed a dense smear layer and mostly obliterated tubules in NaOCl and control samples, while chelators reduced the smear layer, partially opened tubules, and caused erosion.

Conclusions: Exclusive use of 3% NaOCl produces hypermineralized, collagen-depleted coronal dentinal surfaces, with a dense smear layer. Sequential irrigation with 17% EDTA induces stronger demineralization compared to a continuous chelation protocol with 9% HEDP. Both EDTA- and HEDP-treated coronal dentin display partially opened dentinal tubules, signs of erosion, and substantial smear layer reduction.

Clinical Significance: Clinically recommended endodontic irrigation protocols significantly alter the chemical composition and ultrastructural integrity of coronal dentin, the primary substrate for adhesive restorations. These findings enhance the understanding of post-irrigation coronal dentin conditions and their potential implications on the interaction with adhesive restorative materials.

1 | Introduction

Nonsurgical root canal treatment aims to manage or prevent apical periodontitis [1]. The success of this procedure hinges on the eradication or reduction of the microbial load to levels conducive to periapical healing [2, 3]. However, microcomputed tomography imaging reveals that substantial portions of the dentin surface remain untouched by instruments during root canal shaping [4, 5]. These untouched areas may harbor tissue remnants and biofilms, potentially compromising treatment outcomes [5, 6]. Consequently, the intricate anatomy of the root canal system, with its numerous irregularities, lateral canals, isthmuses, and apical ramifications, requires supplementation of mechanical preparation with thorough chemical preparation via irrigation [6–10].

The long-term success of root canal treatment also requires adequate coronal sealing [11–15]. However, restoring endodontically treated teeth presents significant clinical challenges, particularly in establishing reliable adhesion to dentin [16–20]. These teeth often undergo structural, chemical, and mechanical alterations resulting from the procedures involved in root canal treatment, especially irrigation [21]. Factors such as altered collagen structure and changes in the inorganic phase of dentin can compromise the bonding interface [21, 22]. Additionally, different smear layer patterns may further influence adhesion, reinforcing the need to understand how different endodontic irrigation protocols affect the dentin substrate.

The most commonly employed irrigating solutions include sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA) as a chelating agent, and chlorhexidine (CHX) [9, 23–25]. More recently, etidronic acid (HEDP) has gained attention as a promising alternative chelator, with the few available literature attesting to its efficacy in smear layer removal [26, 27]. However, since no single chemical adjunct fulfills all the criteria for optimal root canal disinfection, it is essential to combine different solutions in specific sequences or integrate them into one intracanal irrigant solution [6, 8]. Consequently, clinically recommended irrigation protocols are designed to overcome the shortcomings of individual irrigants and thus achieve the desired chemical preparation outcomes [6, 8].

NaOCl remains the most commonly used endodontic irrigant [8, 9, 23]. NaOCl is an alkaline solution, with a pH of 11–12, and is typically used in concentrations ranging from 0.5% to 6% [8, 9, 23, 28]. Its widespread use is primarily due to its broad-spectrum antimicrobial properties and its ability to dissolve organic tissue [7–10, 23, 25]. The chemical effects of NaOCl are attributed to the presence of free available chlorine, comprising hypochlorite ions (OCl–) and hypochlorous acid (HOCl), both of which are strong oxidizing agents [9]. Since the free chlorine is rapidly consumed in chemical reactions, frequent replenishment of fresh NaOCl solution during chemomechanical preparation is recommended [6, 9, 23]. It has been hypothesized that NaOCl loses most of its activity within 2 min [23, 29]. Despite its powerful oxidizing and proteolytic properties, NaOCl is ineffective at completely removing the smear layer, as it does not act on inorganic components [9, 23].

EDTA and HEDP are chelating agents designed to remove the inorganic phase of the smear layer through calcium chelation [9, 23, 30]. EDTA, generally used in concentrations of 15%–17%, is an effective and biocompatible strong chelator with a neutral or slightly alkaline pH of 7–8 [9, 10, 23]. However, mixing NaOCl with EDTA causes an undesirable chemical reaction, marked by an exothermic process and a pH decrease, which quickly depletes free available chlorine, thereby nullifying NaOCl's activity [9, 19, 26, 31–34]. As a result, EDTA is generally used as an auxiliary irrigant following NaOCl, after root canal instrumentation is completed [6, 9, 23]. Time-dependent erosive effects of EDTA on dentin have been documented, with a 1-min irrigation being recommended to effectively remove the smear layer while preventing severe peritubular dentin erosion [10, 21, 23, 30, 35, 36].

HEDP, also known as etidronate (1-hydroxyethylidene-1,1-disphosphonate), is a more recent addition to the span of endodontic chelating agents [26]. HEDP is a weak chelator with a reported pH range of 10.5–12 [23, 34, 37–39]. Zehnder et al. [26] proposed an irrigation protocol termed soft or continuous chelation, which encompasses combining NaOCl and a weak chelator in a single irrigating solution used throughout the entire mechanical preparation phase. This all-in-one irrigation solution is chairside prepared to preserve its therapeutic properties, as HEDP does not compromise NaOCl's efficacy in the short term [26, 38, 40]. Unlike other chelators, HEDP can be mixed with NaOCl, allowing simultaneous antimicrobial, proteolytic, and demineralizing effects during chemomechanical root canal preparation [26, 40]. Although HEDP exhibits slower demineralization kinetics compared to EDTA, its longer contact time allows it to achieve a comparable chelating effect, effectively removing the smear layer [37].

CHX gluconate is a cationic bisguanide and a strong base with broad-spectrum antimicrobial activity [7, 9, 23]. In root canal therapy, the most commonly employed concentration of CHX is 2% [7, 8, 10, 25, 41]. The optimal pH for CHX solutions ranges

from 5.5 to 7 [23, 41, 42]. A potential added benefit of CHX is its unique substantivity, as it forms a strong electrostatic bond with the phosphate groups in hydroxyapatite, enabling its gradual release from dentin over up to 12 weeks, providing prolonged antimicrobial effects [7, 9, 41, 42]. However, the clinical significance of this substantivity remains questionable [9]. Unlike NaOCl, CHX does not dissolve pulp tissue, precluding its use as the primary endodontic irrigant in root canal treatment [6, 8, 9, 42]. Consequently, CHX has been suggested as a final irrigant in endodontic procedures [6, 8, 9, 23].

Dentin is a complex, moist, heterogeneous, and dynamic substrate. Human dentin is composed of approximately 20% by weight (33% by volume) of organic matrix, 70% by weight (45% by volume) of inorganic phase, with the remainder being water [43]. The extracellular organic dentinal matrix is 90 wt% made up of type I collagen and 10% consisting of non-collagenous proteins [44]. During dentinogenesis, the collagen fibrillar network becomes embedded in a hydroxyapatite crystallite matrix, forming the inorganic phase of dentin [43–45]. A key feature of dentin is its tubularity, which contributes to its permeability [43]. Dentin's ultrastructure encompasses dentinal tubules that decrease in number and diameter from the deeper to the more superficial layers, surrounded by mineralized intertubular dentin and highly mineralized peritubular dentin [43, 46–48]. Despite these average values, regional variations in both composition and structure are observed [43].

Coronal and radicular dentin exhibit substantial morphological and chemical differences, which are crucial in clinical practice. Coronal dentin, with its higher tubular density, is a notably more permeable substrate [47, 49]. In contrast, radicular dentin has a higher degree of fine branching, particularly in areas of low tubule density [47]. Radicular dentin also contains a greater proportion of intertubular dentin, a progressive reduction in tubules from coronal to apical regions, and collagen fibrils that are larger in diameter and exhibit distinct orientations [47]. Chemically, coronal dentin differs by having lower collagen content and a higher mineral-to-collagen ratio [47].

Despite their beneficial role in root canal disinfection, endodontic irrigation solutions can adversely affect dentin [20, 21, 24, 35, 50–57]. NaOCl, as a proteolytic agent, selectively targets organic matter within the root canal space, but it also dissolves both exposed and mineral-bound dentinal collagen [52]. Similarly, chelating agents induce dentinal demineralization, with the extent of deproteinization and decalcification depending on their concentration and exposure time [21].

While the effects of endodontic irrigants on the morphological and chemical features of human root dentin are well documented, the impact of clinically relevant endodontic irrigation protocols on coronal dentin—an important substrate for adhesive restoration—remains underexplored [21, 24, 50, 56–61]. Coronal dentin serves as the primary bonding substrate for the final restoration and is extensively exposed to endodontic irrigants during root canal therapy. Given its susceptibility to chemical agents throughout the procedure, it is essential to investigate its post-irrigation ultrastructural and chemical surface condition to better understand its interaction with adhesive restorative materials. Furthermore, the literature highlights the need for studies specifically focused on coronal dentin, involving adequate sample size calculation, clinically relevant irrigation sequences, and evaluation of novel promising irrigants such as HEDP [21].

The present in vitro study aims to evaluate the effects of four endodontic irrigation protocols on the chemical composition and ultrastructure of coronal dentin.

The research hypothesis posits that the tested endodontic irrigation protocols produce significant differences in the chemical and morphological properties of coronal dentin.

2 | Materials and Methods

This in vitro study followed the Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) guidelines (Supporting Information S1) [62].

2.1 | Sample Size Calculation

Sample size was calculated by simulation in R software (v4.1.2) using a mixed linear model and considering the results of a pilot study. The primary outcome measure was the phosphate/amide II ratio (for attenuated total reflection Fourier transform infrared spectroscopy [ATR-FTIR]) and calcium levels (for energy-dispersive spectroscopy [EDS]), considering five different groups with an equal number of samples. A significance level of 0.05 and a power of 80% were adopted. The assumed group means for ATR-FTIR were 80, 120, 230, 370, and 480, with a standard deviation of 450. The assumed group means for EDS were 17.0, 18.0, 19.0, 20.0, and 21.0, with a standard deviation of 1.5. Under these conditions, the calculated sample size was 22 samples per group for ATR-FTIR and 4 specimens per group for EDS.

2.2 | Specimen Selection

The study was approved by the Ethics Committee of the Faculty of Medicine of the University of Coimbra (CE-042/2021). Twenty-five intact human third molars, extracted for orthodontic or periodontal reasons from 16- to 40-year-old individuals, were collected. Only teeth clinically and radiographically free of caries, cracks, restorations, or other abnormal features, not root-canal treated, were selected. All tooth surfaces were visually inspected under 16× magnification (M300; Leica Microsystems, Wetzlar, Germany), cleaned using hand periodontal scalers, and polished with pumice and water to remove any adherent organic material or calculus. Teeth were then immediately immersed in 1% chloramine-T at 4°C for a maximum of 1 week for disinfection and thereafter stored in distilled water at 4°C, renewed every 2 weeks, for a maximum of 1 month before initiating the experimental procedures (ISO/TS 11405:2015).

2.3 | Dentin Discs Preparation

The apical root third of 23 teeth was embedded in autopolymerizing acrylic resin (Schmidt Laboratory, Madrid, Spain). Two 0.6-mm thick vertical coronal dentin discs (one buccal and one lingual/palatal) were obtained from each tooth by means of crown longitudinal sectioning, as close to the lateral pulp chamber walls as possible. The section was performed with a low-speed (3000 rpm at 0.050 mm/s) diamond saw under continuous water cooling (Accutom-5; Struers, Ballerup, Denmark). Dentin surfaces were then manually prepared using 600-grit silicon carbide (SiC) abrasive paper for 30 s, under constant irrigation, to achieve a final 0.5-mm thickness and create a standardized smear layer. Peripheral enamel was thereafter removed using an orthodontic plier. Each buccal and lingual/palatal dentin disc was divided into three or two dentin fragments, respectively, of approximately 4 mm^2 area. Thus, each tooth provided a total of five dentin fragments, which were randomly assigned to five experimental groups according to the irrigation protocols shown in Table 1. Specimens were kept hydrated with distilled water throughout all dentin discs' preparation procedures.

2.4 | Dentinal Surface Treatment

The dentin surface of specimens from groups NaOCl, NaOCl/EDTA, and NaOCl/EDTA/CHX was firstly immersed in 5 mL of 3% NaOCl (Table 2) at 37°C (Digital Thermostatic Water Bath; Nahita, Beriain-Navarra, Spain) for 30 min. Subsequently, 5 mL of 17% EDTA was applied for 1 min in both NaOCl/EDTA and NaOCl/EDTA/CHX groups. After complete elimination of the chelating agent with copious distilled water irrigation (5 mL) for 1 min, a final rinse with 5 mL of 2% CHX for 2 min was performed in samples from the NaOCl/EDTA/CHX group. Samples from the control and NaOCl/HEDP groups were immersed for 30 min in 5 mL of distilled water or a combination of 3% NaOCl and 9% HEDP at 37°C, respectively. The combined NaOCl/HEDP solution was freshly mixed before initiating the irrigation protocol by using a sterile metallic spatula to mix 10 mL of NaOCl with the powder contained in 1 capsule of Dual Rinse HEDP for 2 min. A new NaOCl/HEDP mixture was prepared 20 min after its preparation. All distilled water, NaOCl, and NaOCl/HEDP solutions were renewed every 2 min. Irrigation procedures were performed using a glass recipient in a 37°C water bath, with a room temperature of 22.3°C and 43% humidity. The exposed dentin was then rinsed with distilled water for 1 min and dried for 30 s using a mild air stream. Finally, specimens were dehydrated in ascending ethanol concentration solutions (25%, 50%, 70%, and 80% for 20 min each; 95%, 100%, and 100% for 30 min each), dried with absorbent paper, and stored at room temperature until further analysis.

TABLE 1 | Study groups.

Study group abbreviation	Irrigation protocol
DW (control)	Distilled water (30 min, renewed every 2 min)
NaOCl	3% NaOCl (37°C, 30 min, renewed every 2 min)
NaOCl/EDTA	3% NaOCl (37°C, 30 min, renewed every 2 min) 17% EDTA (1 min)
NaOCl/EDTA/CHX	3% NaOCl (37°C, 30 min, renewed every 2 min) 17% EDTA (1 min) Distilled water (1 min) 2% CHX (2 min)
NaOCl/HEDP	3% NaOCl/9% HEDP mixture (37°C, 30 min, renewed every 2 min)

Abbreviations: CHX, chlorhexidine; DW, distilled water; EDTA, ethylenediaminetetraacetic acid; HEDP, etidronic acid; NaOCl, sodium hypochlorite.

2.5 | Confocal Raman Microscopy

The spatial distribution of organic and inorganic components within the dentin surface was analyzed using Raman imaging. One randomly selected sample from each experimental group ($n = 1$) was analyzed through the combined Raman-AFM confocal microscope WITec alpha300 RAS$^+$ (WITec, Ulm, Germany). An Nd:YAG laser operating at 532 nm was used as the excitation source, with the power set to 10 mW and a 100× objective (laser spot area ~350 nm). Five randomly selected Raman spectra were acquired 10 times with 2 s for each acquisition within each sample. Raman mappings were performed by raster-scanning the laser beam over the samples, acquiring a complete Raman spectrum at each pixel of the optical image and finally integrating them to generate a color-scale image based on the absolute area underneath specific bands at each pixel (960 cm^{-1} for phosphate, 1070 cm^{-1} for carbonate and 2875 cm^{-1} for organic matrix, namely collagen) [63]. The images were created by the WITec software, WITec Project 5.3$^+$, using 22,500 Raman spectra acquired in 60 × 60 μm^2 areas with 0.05 s for each spectrum.

2.6 | ATR-FTIR

Samples ($n = 22$) were analyzed using an FTIR Spectrometer (InfraRed Bruker Tensor 37; Bruker, MA, USA) with a Diamond ATR accessory (Golden Gate; Specac, Orpington, UK). Spectra were collected in the absorbance mode, 128

TABLE 2 | Irrigation solutions specifics.

Irrigation solution	Manufacturer (City, Country)	Lot number Expiration date
CanalPro NaOCl 3%	Coltène/ Whaledent AG (Altstätten, Switzerland)	20222220 07/2024
CanalPro EDTA 17%		171534 01/2024
CanalPro CHX 2%		171535 01/2024
Dual Rinse HEDP	Medcem GmbH (Weinfelden, Switzerland)	DR210419 03/2024

scans, 4000–500 cm^{-1} wavenumber spectral range, and 4 cm^{-1} resolution. The background spectrum was acquired before each sample analysis. Spectra were acquired in triplicates and normalized based on the amide I band (~1640 cm^{-1}). The area below the curve at the bands of interest was quantified: amide II (~1550 cm^{-1}), phosphate (~1010 cm^{-1}), and carbonate (~871 cm^{-1}). Absorbance data were subsequently processed, and phosphate/amide II and carbonate/phosphate ratios were calculated using Microsoft Excel.

2.7 | EDS

Following ATR-FTIR, four ($n = 4$) randomly selected specimens from each experimental group obtained from the same teeth were submitted to EDS analysis (SU-70; Hitachi, Tokyo, Japan) to determine the surface elemental composition. The selected samples were set up on aluminum stubs using carbon adhesive tape and sputter-coated with 3.8 nm iridium. EDS analysis was performed at 15.0 kV under 10,000× magnification. The atomic percentage of calcium (Ca), carbon (C), nitrogen (N), oxygen (O), and phosphorus (P) was assessed. Three areas were scanned on each sample, and the mean value was obtained for each element. The Ca/P ratio was calculated using Microsoft Excel.

2.8 | Scanning Electron Microscopy (SEM)

Two intact human third molars provided one horizontal deep coronal dentin disc with approximately 0.6 mm thickness. Section was performed with a low-speed (3000 rpm at 0.050 mm/s) diamond saw under continuous water cooling (Accutom-5; Struers, Ballerup, Denmark). The dentin surfaces were then manually prepared using a 600-grit SiC abrasive paper for 30 s, under constant irrigation, to create standardized smear layer. Irrigation procedures were performed as described in Section 2.4. Immediately after irrigation procedures completion, all dentin specimens were firstly immersed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 12 h at 4°C. Following fixation, samples were rinsed with 0.2 M sodium cacodylate buffer (pH 7.4) for 1 h with three changes. The specimens were then dehydrated in ascending ethanol concentration solutions (25%, 50%, 70%, and 80% for 20 min each; 95%, 100%, and 100% for 30 min each), followed by drying through immersion in hexamethyldisilazane (HMDS) for 10 min. Samples were then covered with absorbent paper and stored at room temperature. Subsequently, specimens of each group were fractured into halves to allow transversal and longitudinal SEM visualization of the dentinal surface. Afterward, specimens were set up on aluminum stubs using conductive carbon cement and sputter-coated with gold–palladium for SEM observation (SU-70; Hitachi, Tokyo, Japan) at 15.0 kV to assess dentin ultrastructure. Photomicrographs were obtained under 2500x and 10,000× magnification.

2.9 | Statistical Analysis

Amide II, phosphate, carbonate, phosphate/amide II ratio, carbonate/phosphate ratio, Ca, C, N, O, P, and Ca/P ratio were described for each group using the mean and standard deviation. Box plots were used to observe the different composition profiles of the five study groups. A mixed linear model was used to evaluate the effects of the irrigation protocols, as the same teeth were present in each group. Therefore, the groups were treated as fixed effects, while the individual teeth were treated as random effects. Statistical analysis was carried out in the IBM SPSS v28 platform, with a significance level of 0.05 being adopted.

3 | Results

3.1 | Confocal Raman Microscopy

All samples treated with NaOCl, NaOCl/EDTA, NaOCl/EDTA/CHX, and NaOCl/HEDP were compared to the control sample. Figure 1 shows five random Raman spectra obtained from the dentin surface treated with NaOCl/EDTA. The bands associated with the inorganic component (mineral) appeared at 960 cm^{-1} for phosphate (PO_4^{3-} stretching mode) and 1070 cm^{-1} for carbonate (CO_3^{2-} stretching mode) of the mineral. The bands associated with the organic component (collagen) were observed at 2875 cm^{-1} (CH_2 stretching mode). Both the mineral and the organic matrix appear to be well distributed across the dentin

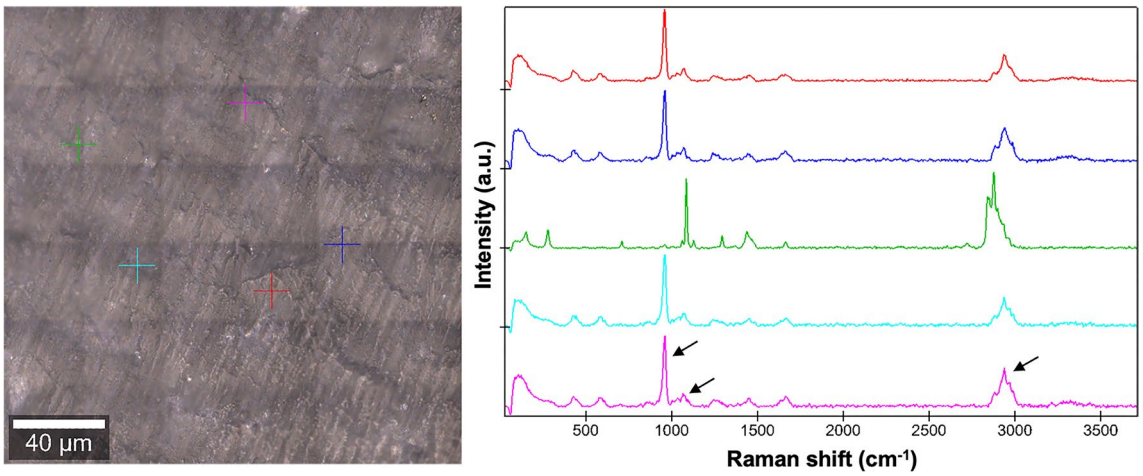

FIGURE 1 | Optical stitching image (left) and the corresponding Raman spectra acquired at random spots on the dentin surface treated with NaOCl/EDTA (right). Arrows depict the bands of interest: Phosphate (~960 cm^{-1}), carbonate (~1070 cm^{-1}), and the organic matrix (~2875 cm^{-1}).

surface. All treated and control samples exhibit the same behavior (data not shown).

To provide more evidence of the distribution of mineral components and the organic matrix on the dentin surface, the samples were further analyzed by Raman imaging (Figure 2). The integration of the absolute area under the phosphate band at 960 cm^{-1}, the carbonate band at 1070 cm^{-1}, and the organic matrix at 2875 cm^{-1} was used to determine the color intensity of the image pixels, thereby creating the Raman maps. In Figure 2, the brighter colors indicate regions with stronger Raman signals for phosphate (left panel), carbonate (middle panel), and the organic matrix (right panel). A homogeneous spatial distribution of phosphate and carbonate is evident in Figure 2 (left and middle panel), while the organic matrix shows a generally even distribution with some small aggregates (right panel). The amount of organic matrix appears to be lower in the samples treated with NaOCl and NaOCl/EDTA/CHX irrigation protocols.

3.2 | ATR-FTIR

The mixed linear model for all variables—amide II, phosphate, carbonate, phosphate/amide II ratio, and carbonate/phosphate ratio—and the experimental groups are statistically significant ($p < 0.001$) (Table 3).

3.2.1 | Amide II

Amide II levels serve as an indicator of protein content, particularly collagen, within dentin (Figure 3A,B). Significant reductions in amide II levels were observed when comparing distilled water (control group) to all treatments involving NaOCl ($p < 0.001$), demonstrating its strong protein-degrading capability (Supporting Information S2). Exclusive NaOCl irrigation resulted in the lowest amide II levels among all groups ($p < 0.001$), indicating maximal protein degradation (Supporting Information S3). A statistically significant difference ($p < 0.001$) between the NaOCl and NaOCl/EDTA groups suggests that EDTA's demineralizing effect exposed additional collagen fibrils that were previously embedded within the inorganic matrix. The NaOCl/EDTA/CHX and NaOCl/HEDP groups exhibited higher collagen content on the dentin surface compared to NaOCl alone ($p < 0.001$). Additionally, these combined treatments produced a chemically similar dentin surface ($p = 0.232$), though both showed lower amide II peaks compared to the NaOCl/EDTA sequence ($p < 0.001$).

3.2.2 | Phosphate and Carbonate

Phosphate and carbonate levels reflect changes in the mineral phase of dentin (Figure 3C,D). NaOCl irrigation alone resulted in a significantly higher mineral content compared to all other treatments ($p < 0.001$), indicating the exposure of the mineral matrix. This outcome was markedly different from combination treatments, where the use of chelating agents appeared to reduce mineral levels ($p < 0.001$). In the NaOCl/EDTA group, the mineral content was similar to that of the control ($p = 0.283$ for phosphate and $p = 0.269$ for carbonate), likely because the prior extensive collagen removal by NaOCl left a mineral-rich surface, and the subsequent EDTA treatment normalized the mineral levels. A significant difference was observed between the NaOCl/EDTA and NaOCl/EDTA/CHX groups, suggesting that using EDTA as a final rinse may be more effective in mitigating the changes induced by NaOCl ($p = 0.003$ for phosphate and $p < 0.001$ for carbonate). The NaOCl/HEDP and NaOCl/EDTA/CHX irrigation protocols resulted in similar phosphate and carbonate levels on the dentinal surface ($p > 0.05$), with both producing a significantly more mineralized surface compared to the NaOCl/EDTA and control groups ($p < 0.05$).

3.2.3 | Phosphate/Amide II Ratio

The phosphate/amide II ratio was extremely high after exclusive NaOCl irrigation, showing a significant difference compared to the control and combination treatments ($p < 0.001$). Although there were variations in the absolute values of phosphate/amide II ratios across the control (60.17 ± 11.19), NaOCl/EDTA (114.05 ± 57.82), NaOCl/HEDP (224.96 ± 73.15), and NaOCl/EDTA/CHX (374.81 ± 358.50) groups, no statistically significant differences were detected in pairwise multiple comparisons ($p > 0.05$).

3.2.4 | Carbonate/Phosphate Ratio

The NaOCl group exhibited subtle but significant changes compared to the control ($p = 0.007$) and all combination treatments ($p < 0.001$). No statistical differences were observed between the control and NaOCl/EDTA groups ($p = 0.069$), though the control group had a statistically higher carbonate/phosphate ratio compared to the NaOCl/EDTA/CHX and NaOCl/HEDP groups ($p = 0.008$ and $p = 0.004$, respectively). However, no statistical differences were found among the combined treatment groups ($p > 0.05$).

3.3 | EDS

The mixed linear model for all elements—C, N, O, P, and Ca—and the experimental groups are statistically significant ($p < 0.001$). Additionally, the mixed linear model for the Ca/P ratio and the experimental groups was also statistically significant ($p = 0.005$).

3.3.1 | Carbon (C)

A general decrease in C content was observed across most treatments, particularly with NaOCl and the NaOCl/HEDP mixture (Table 4 and Figure 4). Although no statistically significant differences were found between NaOCl and NaOCl/HEDP ($p = 0.579$), both treatments significantly reduced C content compared to the control, NaOCl/EDTA, and NaOCl/EDTA/CHX groups ($p < 0.05$) (Supporting Information S4). A smaller decrease in C content was observed in the NaOCl/EDTA group (Supporting Information S5), but this reduction was not statistically significant compared to the control ($p = 0.075$).

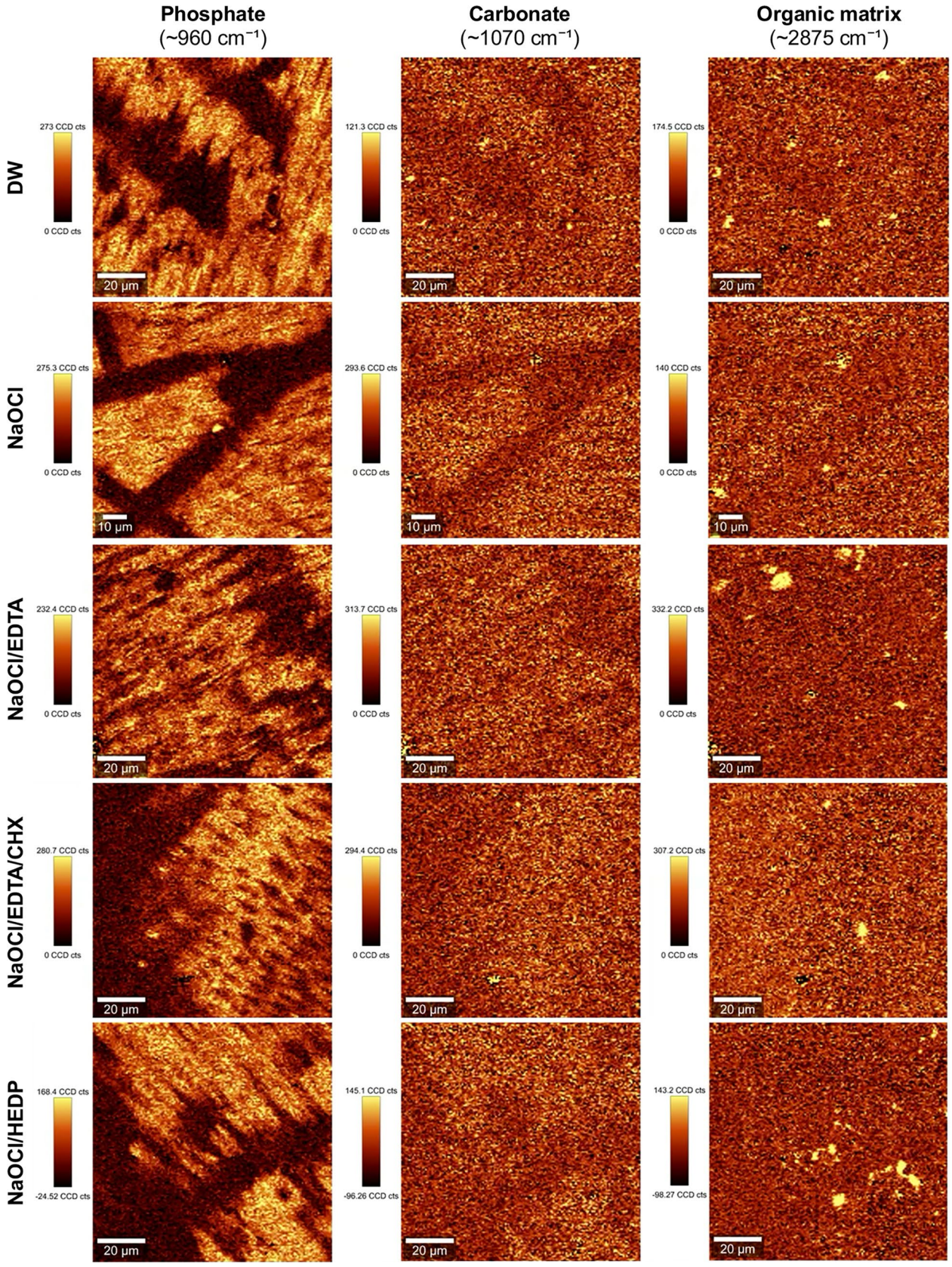

FIGURE 2 | Raman images generated from the integrated intensity of the bands at 960 cm^{-1} for phosphate (left panel), 1070 cm^{-1} for carbonate (middle panel), and 2875 cm^{-1} for collagen (right panel) for the control (DW) and endodontically irrigated samples (NaOCl, NaOCl/EDTA, NaOCl/EDTA/CHX and NaOCl/HEDP). Measurements were taken using 523 nm excitation, 10 mW laser power, with 150 points per line ×150 lines per image, and an acquisition time of 0.05 s per point.

TABLE 3 | Mean ± standard deviation values for amide II, phosphate, carbonate, phosphate/amide II ratio, and carbonate/phosphate ratio.

Study group ($n = 22$)	Amide II	Phosphate	Carbonate	Phosphate/amide II	Carbonate/phosphate
DW	20.39 ± 2.48^{A}	1205.87 ± 130.90^{A}	31.00 ± 2.83^{A}	60.17 ± 11.19^{A}	0.026 ± 0.002^{A}
NaOCl	1.67 ± 0.78^{B}	8980.99 ± 2243.16^{B}	244.11 ± 42.95^{B}	6378.97 ± 2730.61^{B}	0.028 ± 0.003^{B}
NaOCl/EDTA	15.25 ± 3.90^{C}	1557.42 ± 318.27^{A}	38.17 ± 7.88^{A}	114.05 ± 57.82^{A}	0.025 ± 0.002^{AC}
NaOCl/EDTA/CHX	10.47 ± 4.94^{D}	2538.16 ± 1084.06^{C}	60.39 ± 24.63^{C}	374.81 ± 358.50^{A}	0.024 ± 0.002^{C}
NaOCl/HEDP	11.56 ± 2.21^{D}	2491.60 ± 554.21^{C}	58.16 ± 9.30^{C}	224.96 ± 73.15^{A}	0.024 ± 0.003^{C}

Note: Same letters within each column show no statistically significant differences ($p > 0.05$).
Abbreviations: CHX, chlorhexidine; DW, distilled water; EDTA, ethylenediaminetetraacetic acid; HEDP, etidronic acid; NaOCl, sodium hypochlorite.

3.3.2 | Nitrogen (N)

Nitrogen content followed a pattern similar to that of C, with significant decreases observed in NaOCl and NaOCl/HEDP treatments compared to the other experimental groups ($p < 0.05$). The control group exhibited N levels similar to the NaOCl/EDTA/CHX group ($p = 0.081$), and both were statistically higher than those in the NaOCl/HEDP and NaOCl groups ($p < 0.05$), but lower than those in the NaOCl/EDTA group ($p < 0.05$).

3.3.3 | Oxygen (O)

Statistically significant increases in O content were observed in the NaOCl treatment groups ($p < 0.001$), except for the NaOCl/EDTA group, which showed levels similar to the control group ($p = 0.975$). The NaOCl and NaOCl/HEDP groups exhibited the highest O levels, with no statistical differences between them ($p = 0.316$). Both groups, however, showed statistically significant differences compared to all other study groups ($p < 0.05$).

3.3.4 | Phosphorus (P)

Superficial P content generally increases with NaOCl treatment, with slight variations depending on the combination of treatments. The highest P content was observed in the NaOCl and NaOCl/HEDP groups. Although no statistical differences are observed between these two groups ($p = 0.536$), both resulted in significantly higher P levels compared to the control, NaOCl/EDTA, and NaOCl/EDTA/CHX groups ($p < 0.05$). Moreover, the latter three groups exhibited similar P levels ($p > 0.05$).

3.3.5 | Calcium (Ca)

Similarly to P, Ca content increased with NaOCl treatment and varied across different treatment combinations. The highest Ca content was observed in the NaOCl and NaOCl/HEDP groups, with no statistical differences between them ($p = 0.137$). Exclusive NaOCl treatment resulted in significantly higher Ca levels compared to NaOCl/EDTA/CHX ($p = 0.021$), though the latter presented no difference when compared to the NaOCl/HEDP group ($p = 0.333$). Nonetheless, the NaOCl, NaOCl/HEDP, and NaOCl/EDTA/CHX groups all produced significantly higher Ca levels ($p < 0.05$) compared to the NaOCl/EDTA and control groups, which were statistically similar ($p = 0.331$).

3.3.6 | Calcium/Phosphorus Ratio

The Ca/P ratio shows minimal change across different treatments, suggesting a proportional increase or maintenance of Ca and P levels. However, the use of a strong chelating agent disturbed the Ca/P equilibrium, with NaOCl/EDTA and NaOCl/EDTA/CHX groups exhibiting a significantly higher Ca/P ratio compared to all the other groups ($p < 0.05$). This ratio remains relatively stable across the remaining surface treatments (distilled water, NaOCl, and NaOCl/HEDP), with no significant differences between these three groups ($p > 0.05$).

3.4 | SEM

3.4.1 | Control

A cross-section of the control group samples reveals mostly obliterated dentinal tubules, as well as smooth peritubular and intertubular dentin surfaces, showing no signs of degradation or erosion (Figure 5A). A low-magnification longitudinal section shows a rugged, uneven surface, typical of the fracture-based sample preparation method, while also highlighting the natural variations and irregularities of a normal dentin surface. Dentin tubules are seen in the mid-lower part of Figure 5B, exhibiting a standard density and pattern, occluded by the smear layer. At high magnification, the longitudinal section shows dentinal tubules blocked by the smear layer, with normal diameters for untreated dentin (Figure 5C).

3.4.2 | NaOCl

Similar to the control group, the cross section of the NaOCl group exhibits an abundant and slightly more uniform smear layer, with no visible dentinal tubule orifices (Figure 5D). A longitudinal section of a NaOCl group sample, at 2500× magnification, reveals a dense smear layer largely blocking the dentinal tubules, which maintain their normal dimensions (Figure 5E). At 10,000× magnification, the dentin sample clearly shows a fully obliterated dentinal tubule, with the smear layer covering the surface and extending into the tubule's lumen, forming a smear plug (Figure 5F).

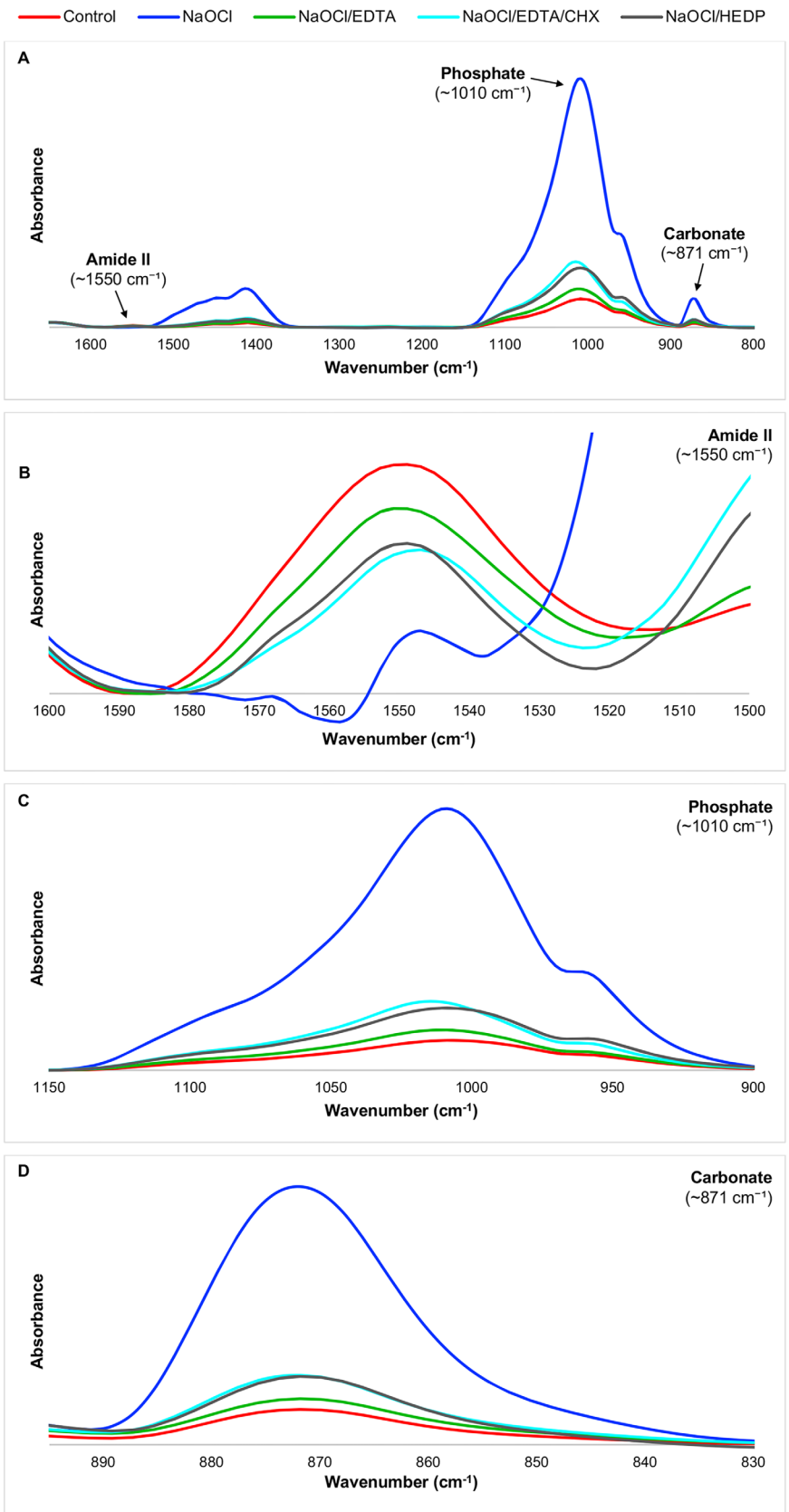

FIGURE 3 | Average ATR-FTIR spectra of the study groups. (A) Overview of the full spectral range. (B) Close-up of the amide II peak, highlighting protein content. (C) Close-up of the phosphate peak, representing mineral content. (D) Close-up of the carbonate peak, representing mineral content.

TABLE 4 | Mean ± standard deviation of atomic percentages (%) from elemental analysis.

Study group ($n = 4$)	C	N	O	P	Ca	Ca/P
DW	19.41 ± 1.40^{A}	5.36 ± 0.58^{A}	47.63 ± 0.97^{A}	9.23 ± 0.55^{A}	17.20 ± 0.36^{A}	1.87 ± 0.08^{A}
NaOCl	9.70 ± 0.87^{B}	2.91 ± 0.26^{B}	54.32 ± 1.33^{B}	10.83 ± 0.72^{B}	20.59 ± 0.86^{B}	1.90 ± 0.07^{A}
NaOCl/EDTA	17.42 ± 1.96^{A}	6.63 ± 0.75^{C}	47.60 ± 1.46^{A}	8.93 ± 0.81^{A}	17.70 ± 1.03^{A}	1.99 ± 0.08^{B}
NaOCl/EDTA/CHX	12.95 ± 2.60^{C}	4.71 ± 0.71^{A}	51.39 ± 1.96^{C}	9.69 ± 0.44^{A}	19.31 ± 0.92^{C}	1.99 ± 0.02^{B}
NaOCl/HEDP	10.29 ± 1.32^{B}	3.62 ± 0.58^{B}	53.53 ± 0.70^{B}	10.59 ± 0.45^{B}	19.81 ± 0.73^{BC}	1.87 ± 0.05^{A}

Note: Same letters within each column show no statistically significant differences ($p > 0.05$).
Abbreviations: CHX, chlorhexidine; DW, distilled water; EDTA, ethylenediaminetetraacetic acid; HEDP, etidronic acid; NaOCl, sodium hypochlorite.

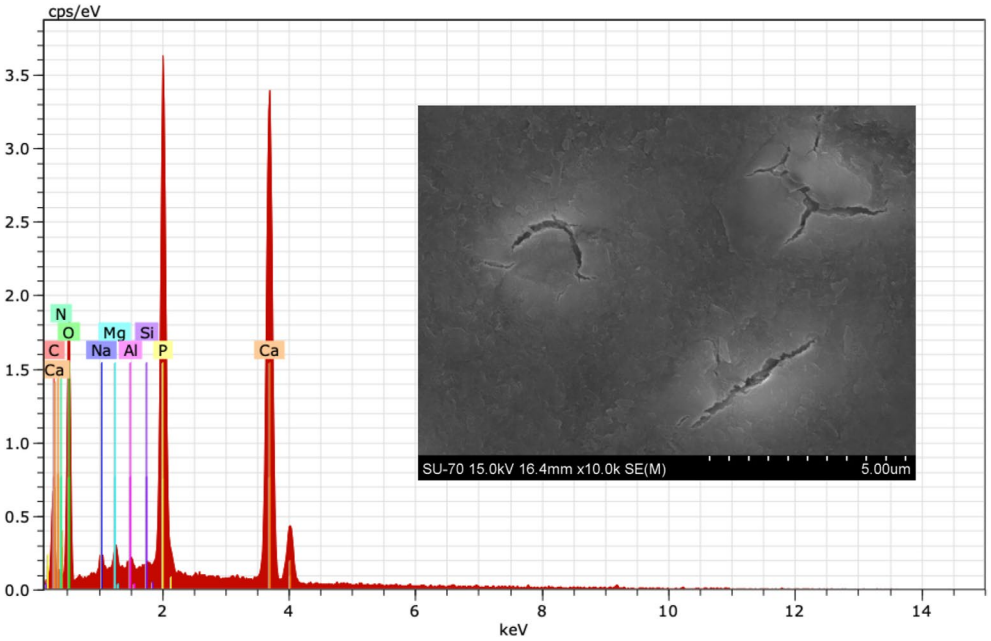

FIGURE 4 | EDS spectrum of a representative sample from the control group, along with the corresponding photomicrograph showing the evaluated area (10,000×).

3.4.3 | NaOCl/EDTA

A cross-sectional view of the NaOCl/EDTA group sample shows a surface with opened or partially opened dentinal tubules. Tubules display round-shaped openings, clear of obstructions in the center, but with slight smear layer accumulation in the peritubular areas, along with signs of erosion or slight degradation (Figure 5G). The longitudinal section of NaOCl and EDTA-treated dentin evinces tubules and surrounding dentin with a rough, irregular surface texture. While the dentinal tubules are opened, debris is visible along the intertubular dentin surface. The edges of dentinal tubules show signs of erosion, with some areas where the dentin appears more severely affected (Figure 5H). At high magnification (10,000×), the opened calyx-shaped tubule orifices are visible in the longitudinal section (Figure 5I).

3.4.4 | NaOCl/EDTA/CHX

Samples from the NaOCl/EDTA/CHX group display a dentin ultrastructure similar to that observed in the NaOCl/EDTA group (Figure 5J–L). Open tubules are evident, with the smear layer predominantly deposited in a centripetal pattern along the tubule periphery, as shown in Figure 5J,L. Surface debris is also evident, as shown in Figure 5K.

3.4.5 | NaOCl/HEDP

The cross-section view from the NaOCl/HEDP group sample (Figure 5M), at 2500× magnification, shows a surface with partially opened dentinal tubules, uniform in size and distribution. Intertubular dentinal erosion is evident, accompanied by clear topographic changes, surface roughening, and an apparent increase in intertubular dentin area, as shown in Figure 5M,O. A thin reedy smear layer surrounds the tubules, with no noticeable modifications in their size, morphology, and opening pattern (Figure 5M–O).

4 | Discussion

The post-irrigation ultrastructural and chemical characteristics of coronal dentin are of paramount importance, as this

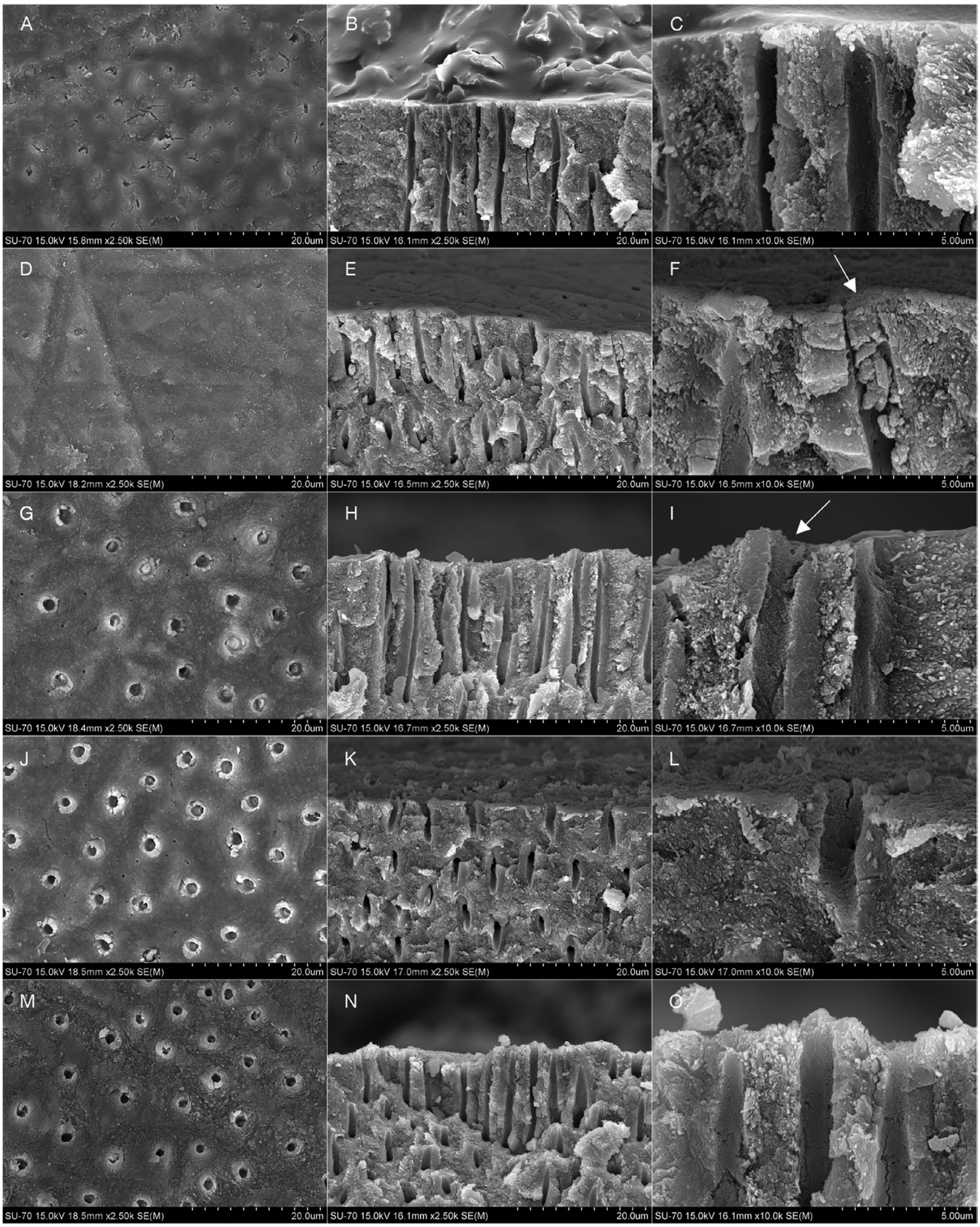

FIGURE 5 | SEM images of coronal dentin after irrigation. Control group: (A) cross section (2500×), (B) longitudinal section (2500×), and (C) longitudinal section (10,000×). NaOCl group: (D) cross section (2500×), (E) longitudinal section (2500×), and (F) longitudinal section (10,000×) highlighting a smear plug (arrow). NaOCl/EDTA group: (G) cross section (2500×), (H) longitudinal section (2500×), and (I) longitudinal section (10,000×) showing an opened calyx-shaped tubule orifice (arrow). NaOCl/EDTA/CHX group: (J) cross section (2500×), (K) longitudinal section (2500×), and (L) longitudinal section (10,000×). NaOCl/HEDP group: (M) cross section (2500×), (N) longitudinal section (2500×), and (O) longitudinal section (10,000×).

tissue serves as the primary substrate for contemporary adhesive restorations. The findings of the present study highlight the significant impact that various endodontic irrigation protocols have on the chemical and structural integrity of dentin, ultimately leading to the acceptance of the original research hypothesis.

While establishing guidelines for irrigation parameters remains an ongoing debate, intermediate irrigant concentrations are generally recommended. These concentrations strike a balance between effective tissue dissolution and antimicrobial properties, while minimizing toxicity, reducing adverse effects on dentin, and ensuring greater clinical safety [18, 21, 64]. Extended contact times, which imply larger solution volumes, have been associated with improved antimicrobial control [23]. However, this approach also intensifies the deleterious effects on dentin [21]. Currently, no consensus exists regarding the optimal exposure time for each NaOCl concentration [9]. Tartari et al. [65] confirmed that higher concentrations and prolonged exposure times increase tissue dissolution and dentin collagen deproteinization. Consequently, in this study, 3% NaOCl was applied for 30 min to reflect a clinically relevant application time in a single-visit root canal treatment [17, 20, 66]. In addition to prolonged exposure times, raising the temperature of NaOCl has been proposed as a way to enhance its efficacy in pulp tissue removal at lower concentrations, while reducing toxicity [7, 23, 67, 68]. Despite the potential advantages of preheating NaOCl to 50°C–60°C to enhance the efficacy of low-concentration solutions, it is important to note that, in vivo, the temperature rapidly drops to 37°C following intracanal delivery, resulting in only a brief effect of uncertain clinical significance [9]. Nonetheless, Tartari et al. [34] reported improved organic tissue dissolution and accelerated smear layer removal when the NaOCl/HEDP mixture was heated, identifying 37°C as the optimal temperature. However, they emphasized the need for frequent mixture refreshments to maintain these effects, as higher temperatures accelerate the chemical reaction between NaOCl and chelators [34, 69]. Therefore, in our study, both NaOCl and the NaOCl/HEDP mixture were preheated to 37°C. Additionally, both irrigation solutions were renewed every 2 min, and the NaOCl/HEDP mixture was freshly prepared every 20 min to ensure its therapeutic properties [69].

Deep dentin was selected as the substrate for the present study, as it represents the tissue encountered in all teeth undergoing root canal treatment. The larger diameter and higher density of dentinal tubules in these deeper layers may enhance the effects of the irrigant by facilitating deeper penetration [66].

4.1 | Chemical Composition

ATR-FTIR is a widely utilized technique for the chemical characterization of dentin due to its non-destructive nature, minimal sample preparation, and ability to provide semi-quantitative analysis [57]. The amide I, II, and III bands, which are directly associated with the polypeptide chains of type I collagen, are commonly used to assess organic modifications, while the phosphate and carbonate bands are indicative of changes in the apatite, reflecting alterations in the inorganic content of dentin [52–57, 65, 70, 71]. Notably, to the best of our knowledge, this is the first study to report the use of Raman imaging to characterize the post-endodontic irrigation condition of coronal dentin. EDS was further used to assess the elemental composition. Carbon (C) and N represent the organic components, while Ca and P correspond to the inorganic content. Oxygen (O) is present in both organic and inorganic phases, but its contribution from the inorganic phase is generally more substantial due to the proportion of inorganic material in dentin. Overall, the EDS results corroborated the ATR-FTIR spectral data, though some discrepancies may be attributed to the distinct sensitivities and specificities of the two analytical techniques [51].

Our FTIR and EDS results are consistent with the majority of the literature, which highlights NaOCl's deproteinization action with no impact on the mineral content [53]. In addition, the reduction in apatite bands intensity following the application of chelators is well documented [54, 55]. Previous studies further demonstrate that the proteolytic action of NaOCl and the decalcifying effects of chelating agents are both concentration- and time-dependent [21, 52, 53].

The control group exhibited the highest collagen content. In contrast, NaOCl treatment left a “ghost mineral layer”, characterized by its mineral-rich and collagen-sparse composition [52], resulting in the lowest collagen content. The application of a chelating agent (EDTA) removed mineral content, exposing the collagen matrix that was previously embedded within the mineralized structure. This exposure led to a significant increase in the amide II peak ($p < 0.001$). However, despite the higher collagen content in the EDTA group, it remained significantly lower than that of the control group ($p < 0.001$), indicating that the chelating agent does not fully restore the surface's organic content to its original dentin condition. The subsequent CHX application resulted in a statistically lower amide II peak compared to EDTA treatment without CHX ($p < 0.001$). This finding is consistent with previous studies suggesting that CHX binds to exposed negatively charged glutamic and aspartic acid residues in collagen, thereby reducing the absorption band heights of amides II and III [72]. These FTIR findings regarding the organic matrix were further corroborated by Raman spectroscopy, which demonstrated that both NaOCl and NaOCl/EDTA/CHX groups exhibited the lowest amounts of the organic matrix. The NaOCl/HEDP mixture produced a surface similar to that observed in the NaOCl/EDTA/CHX treatment ($p = 0.232$), with both yielding intermediate collagen content levels between the NaOCl/EDTA and NaOCl groups. The higher collagen content on the dentin surface after NaOCl/HEDP treatment, compared to NaOCl irrigation alone, may be explained by the simultaneous demineralization and deproteinization processes of NaOCl/HEDP, which continuously expose new dentin layers. In addition, the higher collagen degradation observed after NaOCl/HEDP treatment compared to the control and NaOCl/EDTA groups, was previously demonstrated in a ninhydrin assay, most likely due to the ongoing processes of demineralization and collagen dissolution [56].

In agreement, the reduction in C content may be attributed to the oxidative effects of NaOCl. Nitrogen (N) levels follow a similar pattern to C with only minimal differences, supporting the idea that NaOCl predominantly affects organic components.

In line with FTIR results, the removal of the mineral content by the chelators, especially EDTA, led to an increase in the atomic percentages of both C and N. However, likely due to HEDP's weaker chelating properties, no significant differences were observed between the NaOCl and NaOCl/HEDP groups. Our results align with those of Rath et al. [56] regarding both C and N levels, with the exception that in our study, the NaOCl-EDTA and control groups exhibited similar C levels, whereas Rath et al. reported statistically higher C content in the NaOCl-EDTA group compared to the control. This discrepancy may be attributed to differences in treatment duration: we applied 3% NaOCl for 30 min, whereas Rath et al. [56] used 3% NaOCl for 15 min and applied 17% EDTA for 2 min compared to a shorter 1-min application in our study.

Our findings further demonstrate a higher N over C detection in the NaOCl/EDTA group compared to the control. EDTA's ability to remove Ca exposes the underlying collagen matrix, which is rich in both C and N due to its amino acid composition. However, EDTA may disproportionately expose N-rich collagen fibers over C-rich components, making N more detectable in elemental analyses such as EDS. Furthermore, C in dentin is not only present in collagen but also in other organic molecules and compounds that may be less exposed or affected by the chelation process. Nitrogen groups in amino acids (e.g., amine groups) might react differently to the altered chemical environment post-EDTA treatment compared to C groups, potentially increasing N detectability.

In addition, consistent with FTIR findings, CHX reduced the atomic percentages of C and N compared to the NaOCl/EDTA group ($p < 0.001$), likely due to a similar underlying mechanism.

The observed increase in O content, particularly in the NaOCl and NaOCl/HEDP groups, can be attributed to the oxidizing environment created by the treatments. Oxygen levels were highest in these groups, likely due to NaOCl being the final irrigant applied. On the other hand, O contribution from the inorganic phase is generally more significant. This may explain why, when using EDTA, which primarily removes mineral content, lower O atomic percentages were found compared to both NaOCl and NaOCl/HEDP groups. Indeed, along with the control group, 3% NaOCl followed by 17% EDTA resulted in the lowest O content among all treatment groups. NaOCl's oxidizing nature typically leads to an increase in O content, but when subsequently applied, EDTA binds to Ca ions in the hydroxyapatite, removing mineral content. While EDTA's primary role is not directly linked to altering O content, the removal of these mineral components may lead to the extraction or disruption of structures containing O. Furthermore, the absence of an intermediate rinse between NaOCl and EDTA may have caused chemical interactions leading to the consumption or binding of O in forms that may not be readily detectable by EDS analysis [19]. Our findings regarding the atomic percentages of O, P, and Ca are consistent with those of Pauletto et al. [20], who reported no differences between the control and NaOCl-EDTA groups. Our results also align with Rath et al. [56], where the NaOCl/HEDP group demonstrated the highest O content, followed by the control and, finally, NaOCl-EDTA treated dentin.

Regarding phosphate and carbonate peaks, the data confirm that NaOCl produces a hypermineralized surface by thoroughly removing organic content, leading to statistically significant differences in both inorganic peak levels compared to all other groups ($p < 0.001$). These findings align with those of Rath et al. [56], who reported a sharp phosphate peak in NaOCl-treated dentin, whereas a broadening in the NaOCl–EDTA group indicated a loss of inorganic substance. Similar to our results, EDTA caused greater depletion of the inorganic content compared to HEDP [56]. These observations align with the well-established chelating power of EDTA and HEDP, with EDTA being a strong chelating agent and HEDP a weaker one. Despite EDTA's much shorter contact time with dentin (1 min for EDTA vs. 30 min for HEDP), it still promotes more pronounced demineralization.

A similar trend was revealed in the Raman spectroscopy analysis of both inorganic compounds within each experimental group, suggesting that these are similarly affected by the tested irrigation solutions. In the FTIR analysis, groups that showed a decrease in phosphate levels also exhibited a corresponding decrease in carbonate levels, and vice versa. Moreover, intergroup statistical differences were consistent for both phosphate and carbonate, with the same ranking order observed across the groups regarding the quantities of these elements. However, despite this overall trend, the maintenance of the carbonate-to-phosphate proportionality, as indicated by the carbonate/phosphate ratio, was verified only in the NaOCl/EDTA group, where no statistical difference was observed compared to the control group ($p = 0.069$).

In accordance, both Ca and P contents significantly increased following NaOCl treatments, indicative of deproteinization. In groups treated with EDTA, lower levels of these elements were observed due to the potent demineralization process. The application of CHX positions this group in an intermediate state, likely due to its interaction with the exposed collagen fibrils, as previously discussed. NaOCl, whether used alone or combined with a weak chelating agent, produced the most mineralized surfaces. Rath et al. [56] did not observe significant differences in Ca levels between the control, NaOCl-EDTA, and NaOCl/HEDP groups and found that P levels were significantly lower in the NaOCl/EDTA group compared to the NaOCl/HEDP and control groups. As abovementioned, the discrepancies between our results and those of Rath et al. [56] may be attributed to differences in contact times.

Despite changes in the absolute levels of Ca and P, the stability in the Ca/P ratio suggests that the treatments may alter the mineral composition while preserving a balance that mirrors natural dentin composition. This could be crucial for maintaining the structural integrity and functionality of dentin, which may be compromised in groups treated with EDTA. Notably, NaOCl and the NaOCl/HEDP mixture produced a Ca/P ratio comparable to the control ($p > 0.05$). In agreement with our EDS results, Pascon's micro energy-dispersive X-ray fluorescence spectrometry's findings revealed significantly higher Ca and P content after exclusive NaOCl irrigation compared to both the control and NaOCl-EDTA sequence, with no differences between these two groups [51]. Moreover, pretreatment with NaOCl followed by EDTA led to higher Ca/P

than that observed in the NaOCl group [51]. Conversely, the studies of Pascon et al. [51] and Pauletto et al. [20] reported a significantly lower Ca/P ratio after NaOCl-EDTA sequential irrigation compared to the control. However, they applied 17% EDTA for 5 and 3 min, respectively, which may account for the observed discrepancy.

The phosphate/amide II and carbonate/phosphate ratios are crucial for understanding the relative changes between the organic and inorganic components of dentin. Significant differences in both ratios were observed only in the NaOCl-treated group when compared to the control ($p < 0.05$). Previous studies have similarly reported an increase in the mineral-to-matrix ratio following NaOCl irrigation [52, 53]. However, while Wang et al. [53] found no significant change in the carbonate/phosphate ratio after exclusive NaOCl pretreatment, the absolute values of this ratio in our study were quite similar, which may account for the observed statistical difference. The EDTA and HEDP-treated groups showed lower carbonate/phosphate ratios, with no significant differences between them.

4.2 | Ultrastructure

Based on previous studies, SEM observations agree with the expected intergroup differences [51, 53, 54, 56]. All irrigation protocols caused visible changes in coronal dentin ultrastructure, except for distilled water and NaOCl alone. In both the distilled water and exclusive NaOCl irrigation groups, a dense smear layer and occluded dentinal tubules were observed, occasionally with the formation of smear plugs.

Chelating agents effectively removed the smear layer, resulting in open or partially open dentinal tubules, although peritubular areas with debris accumulation were commonly found. Moreover, signs of erosion and surface roughening were exclusive to chelator-treated groups. These findings agree with those of Rath et al. [56], Gandolfi et al. [54], and Pascon et al. [51]. Although some degree of surface roughening is seen in EDTA-treated dentin, a higher degree of surface roughness was seen in the NaOCl/HEDP group, likely due to the concurrent deproteinizing and demineralizing effects. Although roughness was not quantitatively assessed in our study, previous research has demonstrated that both sequential NaOCl-EDTA irrigation and the continuous chelation protocol produce similar surface roughness [56]. However, it is noteworthy that Rath et al.'s study [56] only evaluated the average roughness (Ra) and lacked comprehensive surface morphology characterization. Furthermore, Rath's protocol involved twice the EDTA exposure and half the NaOCl/HEDP contact time compared to our protocol [56]. Similar to Rath et al.'s [56] findings, we also observed stronger peritubular dentin erosion and consequently wider dentinal tubule openings with the sequential regimen. Although our results diverge from those of Wang et al. [53], who reported varying degrees of erosion based on NaOCl concentration and exposure time, it should be noted that their control group (distilled water-irrigated) lacked a smear layer, likely because the specimens had been polished with 1000- to 5000-silicon carbide abrasive papers, followed by immersion in distilled water and ultrasonic cleaning for 5 min to remove residual particles and the smear layer. This might have facilitated NaOCl's action and does not replicate clinical conditions [53]. The surfaces produced by NaOCl-EDTA and NaOCl-EDTA-CHX groups were structurally similar, which aligns with CHX's well-established lack of tissue-dissolving ability. This aligns with Tartari et al.'s [73] findings that 2% CHX does not induce significant surface alterations compared to NaOCl-EDTA treatment.

4.3 | Limitations and Future Perspectives

Variability in specimen storage conditions, often not reported, small sample sizes, discrepancies in irrigation parameters (e.g., concentration, contact time, renewal, and sequences), and the diversity of evaluation techniques may account for the inconsistencies observed in previously reported findings. Methodological standardization is essential to enable direct comparisons and reliable extrapolation of conclusions [21]. The literature consistently shows that the deproteinizing and demineralizing effects of irrigation solutions are concentration- and time-dependent. In the present study, only one concentration of NaOCl, EDTA, and HEDP was tested. The 30-minute contact time simulates the clinical scenario of a single-session root canal treatment, although multiple sessions are often necessary, which could exacerbate the side effects on dentin. Additionally, agitation methods and preheating are routinely employed to enhance the desired properties of NaOCl but also intensify its adverse effects on dentin. Therefore, future studies evaluating various exposure times, concentrations, temperatures, and adjunct activation techniques are of utmost importance. Additionally, exploring the effects of alternative auxiliary solutions, such as citric acid and various mixtures commonly used worldwide, is also warranted. Finally, gaining a deeper understanding of how the compositional and structural alterations induced by these irrigation protocols impact adhesion and restorative techniques is crucial. Modifications in the collagen network and calcium levels, along with variations in smear layer patterns produced by different irrigation protocols, and the oxygen-rich environment created by some irrigation sequences may compromise bonding to coronal dentin. However, these protocols are necessary and effective in fulfilling the objectives of root canal therapy, making it likely that they will remain the recommended standards. Thus, the findings of the present study underscore the need for research aimed at identifying preventive strategies to mitigate the adverse effects of irrigants on dentin tissue.

5 | Conclusion

The findings of this study highlight the significant impact of various endodontic treatment protocols on the chemical composition and ultrastructural integrity of dentin. The exclusive use of 3% NaOCl results in a hypermineralized, collagen-depleted dentinal surface, with a dense smear layer. Despite the substantially different contact times, sequential irrigation with 17% EDTA induces stronger demineralization compared to the continuous chelation protocol with 9% HEDP. Both EDTA and HEDP-treated dentin display partially opened dentinal tubules, signs of erosion, and substantial smear layer reduction. Additionally, a higher surface roughness is observed in the NaOCl/HEDP group, likely due to the concurrent deproteinizing and demineralizing effects.

Acknowledgments

This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, UIDB/50011/2020, UIDP/50011/2020, and LA/P/0006/2020, financed by national funds through the FCT/MEC (PIDDAC). S.F. thanks FCT for her research contract (REF-069-88-ARH-2018), which is funded by national funds (OE) through, I.P. in the scope of the framework contract foreseen in numbers 4, 5, and 6 of article 23 of Decree-Law 57/2016, of August 29, changed by Law 57/2017, of July 19. P.J.P. thanks project.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.