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Impact of different activation procedures on sodium hypochlorite penetration into dentinal tubules after endodontic retreatment via confocal laser scanning microscopy

Betul Gunes[1], Kübra Yeşildal Yeter[1*] and Yasin Altay[2]

Abstract

Background Infected dentinal tubules are a possible source of bacteria that are responsible for the failure of root canal treatment. Therefore, disinfection of dentinal tubules by increasing the penetration of the irrigation solution is important for success in retreatment cases. This study utilized confocal laser scanning microscopy (CLSM) to assess and compare the impact of XPR, ultrasonic irrigation (UI) and sonic activation (SA) on NaOCl penetration into dentinal tubules following endodontic retreatment.

Methods A total of forty mandibular premolars were enrolled in this investigation. Following root canal preparation up to ProTaper X3 file (30/0.07), root canals were obturated with gutta-percha and bioceramic root canal sealer with single cone technique. The root canal filling materials were removed using ProTaper nickel-titanium rotary retreatment files until the working length was reached. The retreatment procedure was finalized using the ProTaper Next X4 (40/0.06). The teeth were divided into four groups based on the irrigation activation technique: control (conventional needle irrigation), SA, UI and XPR. During the final irrigation procedure, Rhodamine B dye was introduced to 5% NaOCl for visualization via CLSM. Subsequent to image acquisition, the maximum penetration, penetration percentage, and penetration area were calculated. Data were statistically analyzed using the Kruskal-Wallis, Friedman, and Bonferroni Dunn multiple comparison tests through R software ($p < 0.05$).

Results In the middle third, UI yielded a significantly higher penetration percentage than the control group ($p < 0.05$). The UI and XPR groups showed increased penetration percentages in the coronal and middle thirds compared with the apical third ($P < 0.05$). Maximum penetration was notably reduced in the apical third than in comparison with the coronal and middle thirds in all groups ($p < 0.05$). In the control, SA and XP groups, the penetration area was ranked in descending order as coronal, middle and apical ($p < 0.05$). Conversely, in the ultrasonic group, the penetration area was significantly lower in the apical third than in the middle and coronal thirds ($p < 0.05$).

Conclusions UI enhanced the penetration percentage in the middle third of the root compared with that in the control group. XPR and SA showed no significant effect on NaOCl penetration following retreatment.

*Correspondence:
Kübra Yeşildal Yeter
kubrayesildal@gmail.com

Full list of author information is available at the end of the article

Keywords Confocal laser scanning microscopy, Endodontic retreatment, Irrigation penetration, Ultrasonic irrigation, Sonic activation, XP-endo finisher R

Introduction

Endodontic treatment failures can result from various factors, including coronal leakage, missed canals and procedural errors; a systematic review was reported that the success rate of primary root canal treatment ranged between 68% and 85% [1]. In such cases, clinicians must select an appropriate retreatment option to ensure long-term success [2]. The primary choice for addressing endodontic failures is non-surgical endodontic retreatment, involving the complete removal of existing filling materials and the eradication of microorganisms from the root canal system [3]. However, the complete removal of root canal filling materials is the most challenging step in non-surgical retreatment. Residual root canal filling materials can impede the penetration of irrigation solutions and root canal sealers into dentinal tubules [4–7], potentially leading to incomplete healing or new inflammation. Numerous methods have been proposed for root canal retreatment, using hand and/or rotary instruments with or without different solvents. However, neither instruments nor solvents can entirely eliminate root canal filling material [8–10]. Therefore, supplementary procedures are recommended to reduce the remaining root canal filling material [11–14].

Infected dentinal tubules are a possible source of bacteria responsible for post-treatment diseases [15]. Therefore, the complete removal of root canal filling material and the disinfection of dentinal tubules are vital for long-term success in retreatment cases. Previous studies have primarily focused on the impact of supplementary procedures such as sonic, ultrasonic and laser assisted irrigation activation techniques, as well as specially designed instruments (XP Endo Finisher R), on the removal of root canal filling material from the root canal walls [9–14].

Ultrasonic irrigation (UI) generates cavitation and acoustic streaming that enhances cleaning of the root canal wall surface by increasing the shear stress [16]. Prior researches have investigated the effectiveness of UI in removing root canal filling materials during retreatment [17–19] and enhancing the penetration of irrigation solutions into dentinal tubules during primary root canal treatment [12, 20, 21]; these studies suggest that UI is a viable method for both procedures. Notably, irrigation activation using sonic devices operates at lower frequencies than that using UI devices. Nevertheless, existing studies examining the efficacy of sonic activation (SA) in promoting the penetration of irrigation solutions during primary root canal treatment have produced conflicting results [18, 19].

Recently, the XP-endo Finisher R (XPR) (FKG, La Chaux-de-Fonds, Switzerland) file, crafted from a MaxWire alloy with a core diameter of ISO #30 (distinct from its precursor, the XP-endo Finisher (XPF), which has an ISO #25 core diameter) and featuring no taper, has been introduced. XPR can alter its shape by adjusting its temperature during the transition from the martensite to the austenite phase. At body temperature, XPR adopts a spoon-shaped configuration, enabling it to eliminate debris and root canal filling material from otherwise inaccessible regions without modifying the canal shape [22, 23]. A recent systematic review and meta-analysis of in vitro studies involving XPF and XPR concluded that both files offer benefits in removing root canal filling materials from the root canal walls [24]. Notably, to the best of our knowledge, no previous study has examined the impact of XPR on the penetration of sodium hypochlorite (NaOCl) following the removal of root canal filling materials.

The objective of this in vitro study was to compare the effects of XPR, UI, and SA on the penetration of NaOCl into the dentinal tubules after retreatment using confocal laser scanning microscopy (CLSM). The null hypothesis proposed that there would be no significant difference in the penetration of NaOCl into the dentinal tubules between the groups.

Materials and methods

Ethical compliance of this in vitro study was granted by the University Ethics Committee (18/24.09.2019). The sample size was calculated based on a previous study [25] with a similar methodology in the literature. As a result of the power analysis performed using G*Power 3.1 Software (Heinrich Heine University, Düsseldorf, Germany), following these input conditions: effect size = 0.58; α err = 0.05; power = 0.95, total sample size was determined to be at least 33. Forty freshly extracted human mandibular premolars with a single root and root canal, confirmed through radiographs, fully developed roots and no signs of root cracks or resorption, were selected for this study. The teeth were preserved in a saline solution at room temperature until they underwent the experimental procedure. Prior to root canal preparation teeth were decoronated at 16 mm from the apex for standardization.

Preparation and obturation of the root canals

A #10 K-file was introduced into the root canal until the tip of the file was visible from the apex; then, 1 mm was subtracted from this length to determine the working length for each specimen. Root canals were prepared using X1, X2 and X3 ProTaper Next rotary system files (Dentsply Maillefer, Balleigues, Switzerland) following the manufacturer's recommendations. During the preparation, the root canals were irrigated with 5% NaOCl. Final irrigation was carried out using 17% ethylenediaminetetraacetic acid (EDTA), 5% NaOCl and distilled water. After the final irrigation procedure, the root canals were dried with paper points and filled with a bioceramic-based root canal sealer (BioRoot RCS; Septogon, Saint Maur Des Fosses, France) and #25/0.06 tapered ProTaper X3 gutta-percha (Dentsply Maillefer, Ballaigues, Switzerland) using the single-cone obturation technique. Coronal access to the root canal was sealed using temporary filling material (Cavit G; 3 M Espe, Seefeld, Germany). Specimens were stored at 37 °C under 100% humidity for 2 weeks to allow the sealer to completely set.

Retreatment of root canal filling materials

After removing the temporary filling material, the coronal 3 mm of the root canal filling was removed using a Gates-Glidden #2. Retreatment of the root canal filling materials was performed using D1, D2 and D3 ProTaper retreatment files until the working length was reached. The retreatment procedure was finalized using the ProTaper Next X4 (40/0.06). No solvent was used during retreatment procedure. Root canals were irrigated with NaOCl during the retreatment procedure. The specimens were randomly divided into four groups ($n=10$) according to the final irrigation activation procedure.

Conventional needle irrigation (control)

The root canals were irrigated with 17% EDTA and 5% NaOCl solutions for 1 min each. For irrigation, a 31-gauge closed end, two-sided vented irrigation needle (NaviTip Sideport; Ultradent Products. Inc., South Jordan, UT, USA) was placed in the canal 2 mm short of the working length and used in a back-and-forth motion.

SA

The root canals were irrigated with 17% EDTA and activated with a sonic irrigation activation device (EndoActivator, Dentsply Advanced Endodontics, Santa Barbara, CA, USA) using a 25/0.04 polymer tip placed 2 mm short of the working length. Activation was performed for 20 s at 10,000 cpm according to the manufacturer's recommendations. This procedure was repeated three times for a total of activation time 1 min. The same procedure was repeated to activate the 5% NaOCl solution.

UI

Root canals were irrigated with 17% EDTA and then activated using an ultrasonic device (Newtron P5 XS BLED, Satelec/Acteon, Merignac, France) with an Irrisafe 20/0.01 tip (Satelec/Acteon, Merignac, France) placed 2 mm short of the working length. Activation was carried out for 20 s at a power setting of 6, following the manufacturer's recommendations. This procedure was repeated three times for a total of activation time of 1 min. The same procedure was repeated to activate the 5% NaOCl solution.

XPR

Root canals were irrigated with 17% EDTA and then activated with the XP Endo Finisher R (30/0.00) retreatment file at 800 rpm and with a torque of 1 Ncm. Activation involved a 20-s up-and-down motion over a range of 7–8 mm, following the manufacturer's recommendations. This procedure was repeated three times for a total of activation time of 1 min. The same procedure was repeated to activate the 5% NaOCl solution. Notably, the XPR file is activated at body temperature, and preparations for this group was carried out in a 37 °C water bath.

A 0.1% rhodamine fluorescent dye was added to the NaOCl solution before the irrigation procedure to enable the examination of solution penetration using CLSM. During the final irrigation procedure, each solution was applied at a volume of 2.5 ml for each specimen. At the conclusion of the final irrigation procedure, the root canals were irrigated with 2.5 ml of distilled water. All endodontic procedures were performed by an experienced endodontist.

Assessment of penetration of NaOCl

Following the final irrigation procedure, the specimens were embedded in auto-polymerizing acrylic, and ~1 mm thick horizontal sections were obtained at 3 mm (apical), 8 mm (middle) and 12 mm (coronal) from the apex using a diamond cutting disc (Isomed1000, Buehler, Lake Bluff, IL) under water cooling. The apical, middle and coronal thirds were scanned using CLSM at x5 magnification with a laser wave-length of 561 nm (LSM 800; Zeiss, Jena, Germany) (Fig. 1). Subsequently, after acquiring the images, the following three parameters were calculated for each third.

1) Maximum penetration depth: This parameter was defined as the distance from the root canal wall to the deepest point of penetration at four standardized points with 90° angles. The total value of these four measurements was divided by 4 to calculate the mean maximum penetration depth (μm) [26] (Fig. 2a).

2) Penetration percentage: The penetration percentage was calculated by dividing the length to which the irrigation solution penetrated the dentinal tubules along the root canal walls by the circumference of the root canal wall and multiplying this result by 100 (as %) [25] (Fig. 2b).

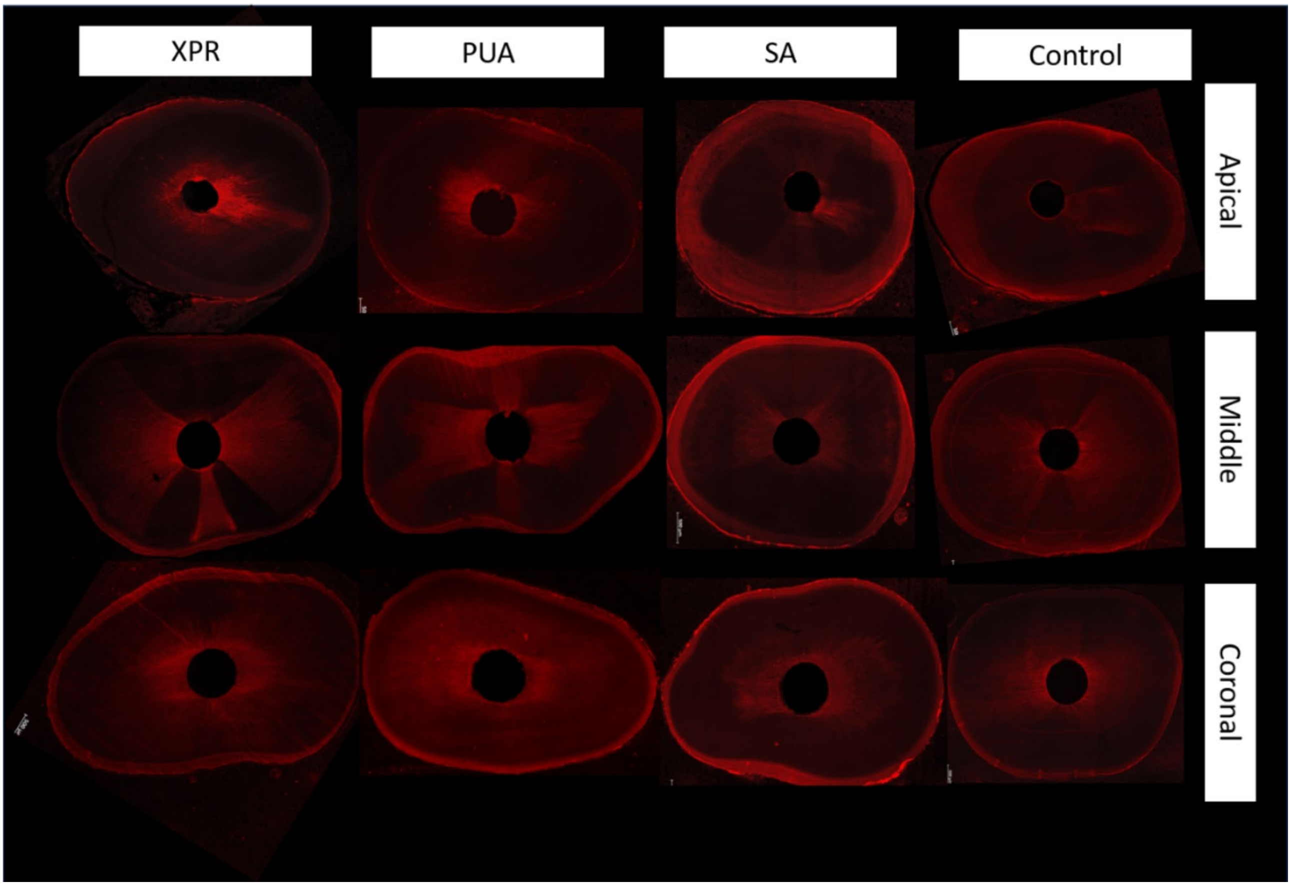

Fig. 1 Representative confocal laser scanning images of the coronal, middle and apical root thirds of each experimental group

3) Area of penetration: To calculate the area of penetration, the total area covered by penetration of the irrigation solution was measured (μm^2) [27] (Fig. 2c).

The investigator who performed the measurements on the CLSM images was blinded to the treatment groups. The images were analyzed using the Zeiss Zen software 2.3 (Carl Zeiss).

Statistical analysis

The study was conducted with an average of 10 replicates, following a 4×3 factorial experimental design. The analysis aimed to determine whether a statistical difference existed between the means of the activation methods (Control, SA, UI and XPR) and the root thirds (apical, middle and coronal). Statistical analysis was performed using the Anderson Darling test to assess normal distribution and Levene test to evaluate the homogeneity of group variances. However, these tests did not meet the prerequisites for parametric tests ($P<0.005$). The Kruskal–Wallis test was employed to compare the medians of the activation methods in each root third, whereas the Friedman test was used for the median comparisons of the root third for each activation method. Statistical analyses were performed using R software (R Core Team, 2020). To determine which activation method and root third exhibited statistically significant differences between medians, the Bonferroni–Dunn multiple comparison test was applied at a significance level of 5%.

Results

The mean, standard error and *p-values* obtained by comparing the data of root regions for each experimental group are shown in Table 1. When examining each activation method for the different root thirds, the SA and control groups had no significant impact on the penetration percentage in all root thirds ($p>0.05$). In contrast, the UI and XPR groups displayed a higher penetration percentage in the coronal and middle thirds than in the apical third ($p<0.05$). The maximum penetration value was significantly lower in the apical third than in the coronal and middle thirds in all groups ($p<0.05$). In the control, sonic and XP groups, the irrigation solution penetration area was ranked from highest to lowest as coronal, middle and apical, respectively ($p<0.05$). However, in the ultrasonic group, the penetration area was significantly smaller in the apical third than in the middle and coronal thirds ($p<0.05$).

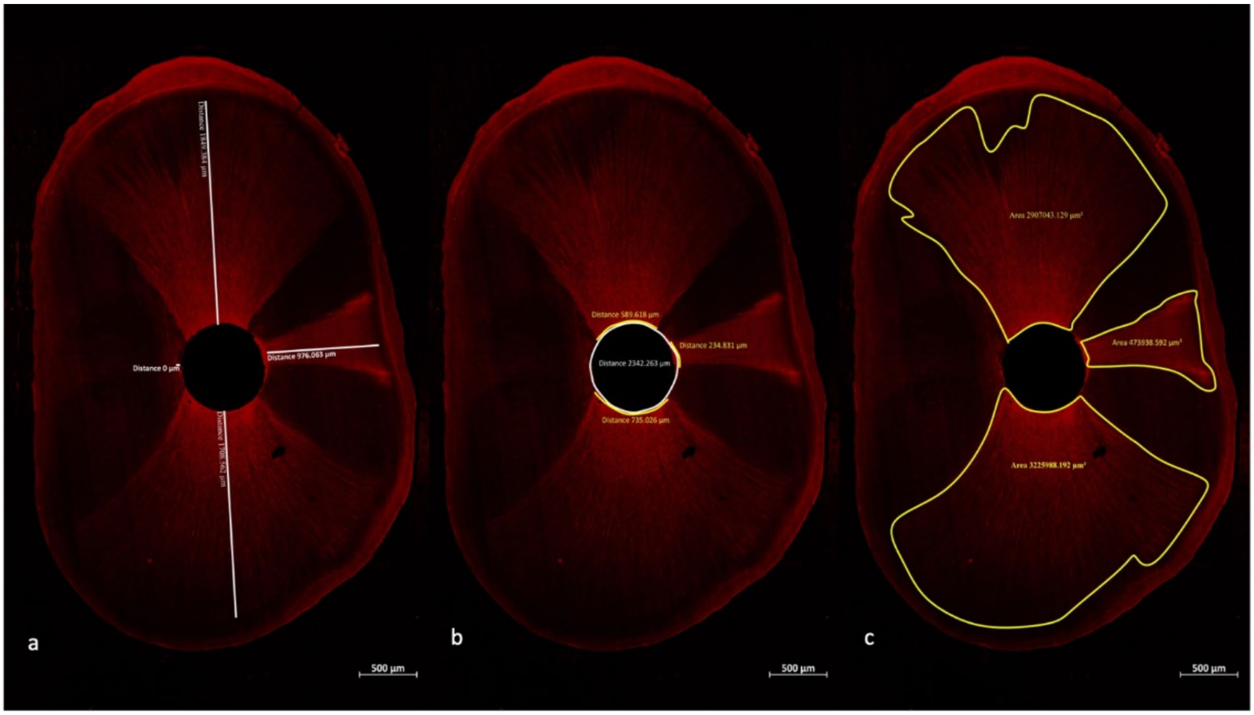

Fig. 2 (**a**) Measurement of maximum dentinal tubule penetration at four standardized points. (**b**) Measurement of sealer penetration (yellow lines) and circumference of the root canal wall (white line) to calculate percentage of sealer. (**c**) Measurement of the area of penetrations

The mean, standard error and *p-values* obtained by comparing the data of experimental groups for each root region are shown in Table 2. When comparing the irrigation activation methods for each root third, no statistically significant differences were observed in the penetration area, penetration percentage, or maximum penetration parameters in the apical and coronal thirds ($p>0.05$). In the middle third, the UI group exhibited a higher percentage of penetration than the control group ($p<0.05$), whereas the penetration area and maximum penetration parameters were statistically similar for all experimental groups ($p>0.05$).

Discussion

After primary root canal therapy, the presence of bacteria within the dentinal tubules is considered as a primary cause of apical periodontitis [15]. Thus, effective irrigation during and after the removal of root canal filling materials is crucial to achieving optimal disinfection in retreatment cases. In this study, we evaluated the impact of various final irrigation activation techniques on the penetration of NaOCl into the dentinal tubules using CLSM. According to our results, UI demonstrated a significantly higher penetration percentage in the middle third of the root than in the control and SA group, where the UI and XPR performed similar. In the apical root third, NaOCl exhibited less penetration than in the coronal and middle thirds, except for the SA and control groups, where these changes remained statistically insignificant. As a result, we partially rejected null hypothesis.

It has been reported that the failure of endodontic treatment may be due to the persistence of microorganisms in the root canal system, especially in the apical part and even in well-treated teeth [28]. The persistence of microorganisms in the root canal system after retreatment may cause periradicular inflammation to continue. Therefore, optimum disinfection of the root canal system is important to increase the success rate of retreatment. In a previous study examining bacterial penetration in root filled teeth, bacteria were most frequently detected in the cervical third and inner dentin adjacent to the root canal [29]. In our study, we aimed to evaluate the penetration of NaOCl after retreatment by calculating the penetration percentage parameter around the root canal circumference and the maximum penetration and penetration area parameters in the region between the root canal and edge of the root in the horizontal root section for the coronal, middle and apical thirds.

Previous studies examining the effects of different activation systems on irrigation penetration solution into dentinal tubules primarily focused on primary root canal treatment [17, 19, 30–32]. These studies varied in terms of specimen type (human or bovine teeth), irrigation solution (NaOCl, CHX, Irritol), irrigation activation system (UI, SA, Laser-assisted activation) and observation method (Stereomicroscope, CLSM), making direct

Table 1 Mean, standard error (SE) and *p-values* for maximum penetration depth, percentage of the penetration, area of the penetration of different root regions for each experimental group

Variables	Irrigation activation	Root sections	Mean ± SE	p-value
Maximum penetration depth (μm)	Control	Coronal	1092.3 ± 75.8[a]	< 0.001
		Middle	832 ± 102[b]	
		Apical	333.7 ± 69.7[c]	
	SA	Coronal	934.6 ± 87.7[a]	0.008
		Middle	779.9 ± 80.7[a]	
		Apical	319.8 ± 59.8[b]	
	UI	Coronal	944.5 ± 84.8[a]	0.005
		Middle	800 ± 100[a]	
		Apical	435.4 ± 76.9[b]	
	XPR	Coronal	1097.7 ± 75.6[a]	< 0.001
		Middle	1001.8 ± 90.4[a]	
		Apical	377.2 ± 58[b]	
Percentage of the penetration (%)	Control	Coronal	95.66 ± 2.97	0.078
		Middle	74.1 ± 6.21	
		Apical	57.1 ± 12.7	
	SA	Coronal	97.48 ± 2.52	0.127
		Middle	90.49 ± 5.26	
		Apical	66.6 ± 13.4	
	UI	Coronal	90.04 ± 9.05[a]	0.04
		Middle	94.66 ± 1.95[a]	
		Apical	67.17 ± 7.47[b]	
	XPR	Coronal	98.33 ± 1.67[a]	0.001
		Middle	88.14 ± 3.74[b]	
		Apical	48.48 ± 7.56[c]	
Area of the penetration ($μm^2$)	Control	Coronal	9,241,573 ± 835,020[a]	< 0.001
		Middle	4,692,941 ± 811,729[b]	
		Apical	134,522 ± 338,281[c]	
	SA	Coronal	7,779,318 ± 963,772[a]	0.008
		Middle	4,857,883 ± 694,355[b]	
		Apical	1,362,494 ± 328,714[c]	
	UI	Coronal	8,244,414 ± 999,220[a]	0.008
		Middle	5,729,646 ± 102,681[a]	
		Apical	2,574,752 ± 817,479[b]	
	XPR	Coronal	8,767,562 ± 704,961[a]	< 0.001
		Middle	6,559,919 ± 717,202[b]	
		Apical	1,174,996 ± 287,736[c]	

Different letters show significant difference among root sections for each irrigation activation technique separately. ($p < 0.05$)

Table 2 Mean, standard error (SE) and *p-values* for maximum penetration depth, percentage of the penetration, area of the penetration of experimental groups for different root regions

Variables	Root sections	Irrigation activation	Mean ± SE	p-value
Maximum penetration depth (μm)	Coronal	Control	1092.3 ± 75.8	0.596
		SA	934.6 ± 87.7	
		UI	944.5 ± 84.8	
		XPR	1097.7 ± 75.6	
	Middle	Control	832 ± 102	0.310
		SA	779.9 ± 80.7	
		UI	800 ± 100	
		XPR	1001.8 ± 90.4	
	Apical	Control	333.7 ± 69.7	0.666
		SA	319.8 ± 59.8	
		UI	435.4 ± 76.9	
		XPR	377.2 ± 58	
Percentage of the penetration (%)	Coronal	Control	95.66 ± 2.97	0.11
		SA	97.48 ± 2.52	
		UI	90.04 ± 9.05	
		XPR	98.33 ± 1.67	
	Middle	Control	74.10 ± 6.21[a]	0.04
		SA	90.49 ± 5.26a[a]	
		UI	94.66 ± 1.95[b]	
		XPR	88.14 ± 3.74[ab]	
	Apical	Control	57.1 ± 12.7	0.744
		SA	66.6 ± 13.4	
		UI	67.17 ± 7.47	
		XPR	48.48 ± 7.56	
Area of the penetration ($μm^2$)	Coronal	Control	9,241,573 ± 835,020	0.596
		SA	7,779,318 ± 963,772	
		UI	8,244,414 ± 999,220	
		XPR	8,767,562 ± 704,961	
	Middle	Control	4,692,941 ± 811,729	0.226
		SA	4,857,883 ± 694,355	
		UI	5,729,646 ± 1,026,815	
		XPR	6,559,919 ± 717,202	
	Apical	Control	1,345,822 ± 338,281	0.646
		SA	1,362,494 ± 328,714	
		UI	2,574,752 ± 817,479	
		XPR	1,174,996 ± 287,736	

Different letters show significant difference among irrigation activation techniques for each root section separately. ($p < 0.05$)

comparisons challenging. To the best of our knowledge, this study is the first to evaluate the penetration of irrigation solutions into dentinal tubules after retreatment. Therefore, directly comparing the results of our study with those reported in the literature is not appropriate.

According to our results, the only difference between the activation methods was observed between the percentage of penetration of the irrigation solution in UI and control groups in the middle third of the root. UI increased the penetration percentage of NaOCl compared with that in the control group. This result is in accordance with the results of the study of Akcay et al. [19] that observed irrigation penetration during primary root canal treatment. The UI provides cavitation and acoustic streaming [16] and produces sufficient shear forces to dislodge debris in instrumented canals [33]. Greater cleanliness of the canal walls may result in increased irrigation penetration. The effect of SA on the penetration of irrigation solutions has been studied, but the results are controversial [19, 31, 33]. Some studies reported that SA was superior to conventional needle irrigation, but these studies evaluated different types of irrigation solutions [31, 33]. Similar to our study, Akcay et al. [19] reported no significant difference between SA and conventional needle irrigation and the same irrigation solution was used in our study. SA does not have the ability to allow sufficient streaming because of its low energy level and long wavelength [34]. The efficacy of XPR in the removal of root canal filling materials has been previously studied [11, 23, 35, 36]. The results of these studies demonstrated that XPR was more effective than UI and SA for the removal of root canal filling materials. However, our results do not directly correlate with those of the aforementioned studies. In this study, no statistically significant differences were observed among XPR, UI and SA.

While bacterial penetration can be seen in every region of the root canal, more bacterial presence can be seen in the apical third because it is more difficult to reach this region via preparation or irrigation procedures [37]. The importance of bacterial presence in the apical region in root canal treatment failure was previously emphasized [28]. Studies that evaluated the penetration of irrigation solution into dentinal tubules showed lower penetration values for the apical third than for the middle and coronal thirds [19, 31]. This result can be explained by the presence of narrower, sclerotic and fewer dentinal tubules at the apical root third [38]. Furthermore, the flow of the irrigation solutions to the apical third may be inefficient at removing the smear layer and debris [39]. Consistent with these studies, our results showed less penetration for the apical third than for the middle and coronal thirds for the UI and XPR groups for all of the parameters. In the SA and control groups, the apical root third showed less penetration than the middle and coronal thirds in terms of the penetration area and maximum penetration parameters. However, the SA and control groups showed similar percentages of penetration in all of the root thirds. The percentage of penetration, a circumferential measurement of the root canal wall, may be more affected by dentin tubule density. The inability to establish standardization in specimens may have revealed this contrasting result. In terms of clinical significance, future studies on the penetration of irrigation solution penetration should focus on alternative methods to enhance the penetration of NaOCl in the apical region.

The penetration of irrigation solutions into the dentinal tubules was observed using various microscopy techniques, including stereomicroscopy, scanning electron microscopy (SEM) and CLSM [17, 19, 31, 33, 40, 41]. Detailed images of dentinal tubules can be obtained using CLSM, providing observations and measurements of penetration into the dentinal tubules in a single image along the circumference of the root canal walls [41]. Moreover, CLSM is a non-destructive method and does not dehydrate the specimen [42]. In contrast, SEM can visualize the dentinal tubules in only one plane, necessitating the further reconstruction of high magnification images to obtain a detailed final image. Acquiring images via SEM involves additional procedures, such as coating the specimens with gold and working under a vacuum, which can be time-consuming and can introduce artefacts during the preparation of the specimens [43]. Stereomicroscopy, on the other hand, is not capable of providing sufficiently detailed images of the dentinal tubules. Given these considerations, CLSM imaging was chosen as the preferred method for evaluating NaOCl penetration into the dentinal tubules in this study.

Every effort was made to select teeth that met the inclusion criteria. Notably, aging can lead to dentinal tubule sclerosis, especially in the apical third. This sclerosis can alter the number and diameter of the dentinal tubules, directly influencing the penetration of the irrigation solutions into them [44]. This variation can introduce considerable variability in experimental results and should be recognized as a limitation. Furthermore, to maintain standardization, the number and volume of irrigants, as well as the duration of the irrigation and activation procedures, were kept consistent for all experimental groups.

Conclusions

In this in vitro study, the following conclusions were drawn within the established limitations: in the middle third of the root, UI increased the penetration percentage compared with the control group, while XPR and SA showed no notable effect on NaOCl penetration following retreatment. Future studies are needed to explore the impact of various irrigation activation techniques on penetration of irrigation solutions into dentinal tubules after the retreatment procedure to provide definitive insights.

Abbreviations

CLSM	Confocal laser scanning microscopy
EDTA	Ethylenediaminetetraacetic acid
NaOCl	Sodium hypochlorite
SA	Sonic activation
SEM	Scanning electron microscopy
UI	Ultrasonic irrigation
XPF	XP-endo Finisher
XPR	XP endo Finisher R

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None.

Author contributions

B. G.: Conceptualization, Investigation, writing- reviewing&editing. K. Y. Y.: Conceptualization, writing-original draft preparation, writing-reviewing&editing, visualization, project administration. Y. A.: Formal analyses, writing- reviewing&editing.

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Data availability

The data of this research are available upon reasonable request from the corresponding author.

Declarations

Ethical approval and consent to participate
Ethical compliance of this in-vitro study was approved by the Eskişehir Osmangazi University Ethics Committee (18/24.09.2019). All participants gave informed consent to participate.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Faculty of Dentistry, Department of Endodontics, Eskisehir Osmangazi University, Eskişehir 26040, Turkey
[2]Faculty of Agriculture, Department of Animal Science Biometry and Genetic, Eskisehir Osmangazi University, Eskişehir, Turkey

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