ORİJİNAL ARAŞTIRMA ORIGINAL RESEARCH

DOI: 10.5336/dentalsci.2019-72466

Influence of Chitosan and EDTA Solutions Activated with Sonic and Ultrasonic Systems on the Microhardness of Dentin

Kitosan ve EDTA Solüsyonlarının Sonik ve Ultrasonik Sistemler ile Aktive Edilmesinin Dentin Mikrosertliğine Olan Etkisi

Esin ÖZLEK[a], Hüseyin GÜNDÜZ[a], Elif AKKOL[a], Gizem KADİ[a]

[a]Department of Endodontics, Van Yüzüncü Yıl Faculty of Dentistry, Van, TÜRKİYE

ABSTRACT Objective: The aim of this in vitro study is to assess the effect of activation of EDTA and chitosan by different irrigation activation systems on the microhardness of root canal dentin. **Material and Methods:** A total of 60 single-rooted extracted mandibular premolars were prepared and randomly distributed into two groups (n=30) based on the final irrigant: Group 1, 0.2% chitosan; Group 2, 17% EDTA. Then, specimens of both groups were randomly divided into three subgroups (n=10) based on the irrigant activation; Subgroup A (Sonic), Subgroup B (Ultrasonic) and Subgroup C (Conventional irrigation, control group). The specimens (n=60) were embedded into resin blocks and sectioned horizontally 1 mm thick sections from 2,5 and 8 mm levels from the apex. All samples were used to determine the microhardness of dentin using a Vicker's microhardness tester. The data were analyzed using the three-way analysis of variance (ANOVA) and Tukey post-hoc tests to detect the effects of the independent variables (final irrigation solution, final irrigation techniques, and root canal thirds) on microhardness. **Results:** The three-way ANOVA indicated that chitosan exhibited a significantly lower microhardness value than EDTA ($p<0.001$). Also, regardless of the usage of final irrigation, both PUI and EA had significantly lower microhardness than CI ($p<0.001$). **Conclusion:** Chitosan as compared to EDTA (i), sonic (EA) and ultrasonic (PUI) final irrigation activation methods as compared to the traditional syringe method (CIS) (ii), apical and mid regions of the root as compared to its coronal region (iii), showed lower microhardness values.

Keywords: Chelating agents; chitosan; EDTA; activation; ultrasonic

ÖZET Amaç: Bu in vitro çalışmanın amacı; EDTA ve kitosanın farklı irrigasyon aktivasyon yöntemleri ile aktivasyonunun kök kanal dentin mikrosertliği üzerindeki etkisini değerlendirmektir. **Gereç ve Yöntemler:** Toplam 60 adet tek köklü çekilmiş mandibular premolar diş prepare edildi ve final irrigasyon esas alınarak rastgele iki gruba (n = 30) ayrıldı: Grup 1, %0,2 kitosan; Grup 2, %17 EDTA. Daha sonra, her iki grubun örnekleri, irrigasyon aktivasyon sistemine göre rastgele üç alt gruba (n=10) ayrıldı; Alt Grup A (Sonik), Alt Grup B (Ultrasonik) ve Alt Grup C (Geleneksel irrigasyon, kontrol grubu). Örnekler (n=60) rezin bloklarına gömüldü ve apeksten 2,5 ve 8 mm seviyelerinde yatay olarak 1 mm kalınlıkta kesitler alındı. Tüm örneklerin dentin mikro sertliğini belirlemek için Vicker mikro sertlik test cihazı kullanıldı. Veriler, bağımsız değişkenlerin (final irrigasyonu, final irrigasyon aktivasyon tekniği ve kök kanal üçlüsü) mikrosertlik üzerindeki etkilerini tespit etmek için üç yönlü varyans analizi (ANOVA) ve Tukey post-hoc testleri kullanılarak analiz edildi. **Bulgular:** Üç yönlü ANOVA, kitosanın EDTA'dan anlamlı derecede düşük mikrosertlik değerine neden olduğunu göstermiştir ($p<0,001$). Ayrıca, final irrigasyon kullanımından bağımsız olarak, hem ultrasonik (PUI) hem de sonik (EA), geleneksel irrigasyondan önemli ölçüde daha düşük mikro sertliğe sahipti ($p<0,001$). **Sonuç:** Kitosan ile EDTA karşılaştırıldığında (1), sonik (EA) ve ultrasonik (PUI) final irrigasyon aktivasyon yöntemleri geleneksel iğne irrigasyon (CIS) yöntemiyle karşılaştırıldığında (2) kökün apikal ve orta bölgeleri koronal bölgeleri ile karşılaştırıldığında (3), daha düşük mikro sertlik değerleri göstermiştir.

Anahtar Kelimeler: Şelasyon ajanları; Kitosan; EDTA; aktivasyon; ultrasonik

A successful root canal therapy requires the preparation of root canals chemo-mechanically followed by obstruction of them three dimensionally using biocompatible materials.[1] During chemo-mechanical preparation of root canals a solid tissue debris called "smear layer" is formed. The appearance of smear layer brings about two difficulties. These prevent (i) irrigation solutions and intracanal medica-

Correspondence: Esin ÖZLEK
Van Yüzüncü Yıl Faculty of Dentistry, Department of Endodontics, Van, TÜRKİYE
E-mail: e.ozlek@yyu.edu.tr

Peer review under responsibility of Turkiye Klinikleri Journal of Dental Sciences.

Received: 20 Nov 2019 ***Received in revised form:*** 12 Feb 2020 ***Accepted:*** 11 Mar 2020 ***Available online:*** 20 Apr 2020

ments from penetrating into dentin tubules and (ii) root canal filling from adhering dentin tubules. Moreover, it may play a role as a reservoir for microorganisms.[2] Recent studies have suggested that smear layer should be removed to increase the success of root canal therapy.[3] Despite advances, however, it has been reproductive that smear layer cannot be completely removed. One of the most common clinical practices used for removal of smear layer is to use chelating agents like ethylenediaminetetraacetic acid (EDTA). It is the most commonly applied final irrigation protocol after sodium hypochlorite that solves organic tissue in root canals by its proteolytic action.[4]

EDTA is the first chelator that may soften root canal dentin, solve smear layer, and increase dentin permeability. The chelating effect of EDTA emerges by increasing decalcification of inorganic compounds exposing the dentin collagen network. As a result, dentin microhardness is reduced.[5] As EDTA is both an irritant for periapical tissues and a pollutant for the environment, the search for an alternative chelating agent to remove smear layer has gained pace over years. Recent studies have reported that chitosan with chelating ability may be an alternative to EDTA.[6]

Chitosan is a natural polysaccharide produced by deacetylation of chitin obtained from crab and shrimp shells. As chitin is a natural compound, the use of chitosan is advantageous both ecologically and economically. Chitosan has unique biocompatibility, bio-solubility, superior bioactivity, selecting permeability, antimicrobial efficiency, absorption capacity, and chelating property.[7] Prior studies have indicated that chitosan chelates calcium ions to remove smear layer without increasing dentin erosion.[8] However, its demineralizing effect on dentin tissue has been shown to result in a reduced microhardness similar to EDTA.[6] Moreover, plenty of studies in the literature have reported that it is also very effective as a root canal disinfectant.[7]

Irrıgation solutions need to contact root canal walls maximally in order to remove smear layer and affect microbial biofilm. However, the complex structure of the root canal anatomy prevents irrigants from completely accessing root canal wall and causes untouched areas to remain. Hence, activation of irrigants has been recommended. Such techniques include sonic agitation and ultrasonic activated irrigation.[3]

Ultrasonic activation increases the efficacy of irrigants for removal of organic and inorganic debris from the root canal wall. By means of ultrasonic vibrations, Irrigants reach areas that are inaccessible with the traditional syringe method, providing a more effective cleaning. Ramachondran et al. reported that irrigation with ultrasonic activation reduced the vapor look effect and effectively removed smear layer.[9] Additionally, there are studies reporting that ultrasonic activation may more effectively clean accessory canals which are more numerous in the apical third of the root canal system and may increase irrigants' penetration depth into dentin tubules.[10]

The EndoActivator (Dentsply Sirona Endodontics, York, USA) is a sonic device, which mechanically agitates the irrigating solution to enhance its flow into the root canal system. It uses polymer tips of different sizes, that are operated at speeds of 2000, 6000 and 10,000 rpm. Sonic devices (2-3 kHz) operate at a lower frequency than ultrasonic devices (25-40 kHz). Low frequencies reduce shear stress and cause less changes on dentin surface.[11]

Irrigants used to remove smear layer may cause chemical changes by affecting root dentin surface in a similar manner as smear layer. Collagens are exposed on dentin surface, dentin microhardness is reduced and dentin permeability may increase. As a result, resistance to bacterial entry is reduced, coronal leaks are allowed and adhesion of root canal filling materials to dentin is negatively affected.[12]

While there is a limited number of studies examining the effect of activation (sonic or ultrasonic) of irrigants (EDTA or chitosan) on dentin microhardness, no study has assessed the effect of chitosan activation on dentin microhardness. Thus, the aim of this laboratory study is to assess the effect of activation of EDTA and chitosan by different irrigation activation systems on dentin microhardness. The null hypothesis of the study was that neither the irrigant used nor the activation system has any effect on dentin microhardness.

MATERIAL AND METHODS

The study was realized in accordance with Declaration of Helsinki and it was confirmed by the Non-interventional Clinic Research Ethics Board of Van Yüzüncü Yıl University (2019/04-06) on 22 February 2019. Freshly extracted, single-rooted mandibular human premolars (n=60) with completely formed roots and closed apices, with no cracks or structural anomalies were used for this study. The teeth were extracted for orthodontic and periodontal reasons. The presence of a single root canal was confirmed by taking radiographs in two angulations (mesiodistal and labiolingual). Teeth with cracks or structural anomalies were discarded. The teeth were stored in distilled water with thymol at 4ºC until use. The teeth were cleaned using curettes to remove any attached soft and hard tissue.

ROOT CANAL PREPARATION

All specimens were decoronated with a diamond disc under water cooling to achieve roots with a standardized length of 12 mm. The working length was determined to be 1 mm short of the root apex, using a #15 K-file (Densply, Maillefer). The root canals were instrumented with rotary nickel titanium instruments (ProTaper Universal, Dentsply Maillefer, Ballaigues, Switzerland) up to F3. At every instrument change, the root canals were irrigated with 2 mL of 5.25% NaOCI (Imicrly, Konya,Turkey)

FINAL IRRIGATION AND IRRIGANT ACTIVATION

The samples were randomly divided into two groups (n=30) according to the final irrigant: Group 1, 0.2% chitosan; Group 2, 17% EDTA (Imident, Konya, Turkey). Chitosan solution was prepared by mixing 0.2 g of chitosan (Sigma Aldrich, St.Louis, MO, USA) in 100 ml of 1% acetic acid and stirring in a magnetic stirrer for 2 h.[8] Then, samples of both groups were randomly separated into three subgroups (n=10) according to the irrigant activation:

SUBGROUP A (SONIC)

The red tip (25/04) of the EndoActivator (Denstply-Maillefer, Baillagues, Switzerland) was placed 1 mm short from the working length and moved up and down at 10,000/min cpm without pressure application, as per the manufacturer's recommendation. The procedure was repeated 3 times. The canal was flushed with irrigant using a 27G needle between agitation cycles.

SUBGROUP B (ULTRASONIC)

Ultrasonic activation was performed with IrriSafe ultrasonic tip ISO 25 (Satelec Acteon Group, Merignac, France) mounted on a VDW ultrasonic unit (VDW Ultra, Munih, Germany) with 30 000-Hz frequency. The tip was passively positioned and maintained 1 mm short of the WL and agitated for 20 seconds. The activation was performed at three cycles lasting 20 seconds, according to the manufacturer's recommendation. The canal was flushed with irrigant using a 27G needle needle between agitation cycles.

SUBGROUP C (CONVENTIONAL IRRIGATION) (CONTROL GROUP)

Irrigation was delivered using a 27-G needle (Ays*et*, Adana, Turkey), placed 2 mm short of the working length. The needle was moved in short vertical strokes of 2-3 mm amplitude, at an approximate of 100 strokes/min.[11] This constituted the control group.

A total of 5 mL of EDTA or chitosan was used in each group for 1 minute to ensure standardization. Before the use of EDTA and chitosan solutions, pH was measured and the values obtained were 2.76 for 2% chitosan and 11.83 for 17% EDTA. For the measurement of pH levels, EDTA and chitosan solutions were placed in 1.5 mL Eppendorf tubes in 1 mL volumes. Then, measurements were made with a pH meter (Jenway 3040 Ion Analyzer, Felsted, Essex, UK) calibrated at 25 ºC. Measurements were repeated 3 times for each sample and mean values were recorded. The pH meter was calibrated before each measurement. At the end of the irrigation protocol, all canals were irrigated with 5 mL distilled water for 1 minute to remove the traces of previous irrigants that were used. Then, all root canals were dried with paper points.

PREPARATION OF SAMPLES

The samples (n=60) were embedded into resin blocks and sectioned horizontally using a slow-speed diamond saw to obtain 1 mm thick sections from 2, 5

and 8 mm levels from the apex. The exact thickness of each slice was measured to 0.04 mm accuracy using a digital caliper (Mitutoyo, Tokyo, Japan). Then, the dentin surfaces were polished with silicon carbide abrasive papers (300, 400, 600, 800, 1000 and 1200-grit) under water cooling.

DETERMINATION OF MICROHARDNESS

All samples were used to determine the microhardness of dentin using a Vicker's microhardness tester (Shimadzu, Tokyo, Japan). In this test, the indentations were made with a Vicker's diamond indenter at three different locations. The locations were chosen at the 1 mm level to the root canal wall in the coronal, middle, and apical third of the roots. The indentations were applied on each sample using a 200-g load and a 20-second dwell time.[13] The indentations were measured using an optical microscope with a digital camera and image analysis software. Vickers microhardness number (VHN) was digitally calibrated by applying the following formula; $HV=1854.4´P/d^2$; where, P = force (gf); d = the averaged diagonal length values (d1 and d2).[14] In three samples from chitosan groups random allocation and allocation concealment was made by random selection and were viewed under SEM at 300C magnification.

STATISTICAL ANALYSIS

Normality of the data was checked by Shapiro-Wilk test. The data were analyzed using the three-way analysis of variance (ANOVA) and Tukey post-hoc tests to detect the effects of the independent variables (final irrigation solution, final irrigation techniques, and root canal thirds) on microhardness. Mean and standard deviation values are shown in Table 1. All statistical analyses were performed using IBM SPSS Statistics for Windows Version 23 (IBM Corp. Armonk, NY, USA) at a significance level of 0.05 and a confidence interval of 95%.

RESULTS

The results of the three-way ANOVA test for the microhardness were given (Table 2). Dentin microhardness was affected by final irrigation solution ($p<0.001$) and final irrigation activation method ($p<0.001$) but these methods affected the root canal regions differently ($p<0.001$). Furthermore, the interaction between final irrigation solution and final irrigation activation method ($p<0.001$) exerted a significant effect on dentin microhardness as were the interaction between final irrigation solution and region ($p<0.001$) and the interaction between final irrigation activation method and region ($p<0.001$).

TABLE 1: Mean±standard deviation values.

Final irrigation activation method	Root canal region	Chitosan	EDTA	Total
EndoActivator	Apical	67.16 ± 10.76	99.64 ± 19.19	83.4 ± 22.52
	Middle	61.95 ± 6.03	78.35 ± 14.44	70.15 ± 13.67
	Coronal	70.07 ± 20.62	82.51 ± 17.76	76.29 ± 19.79
	Total	66.39 ± 13.82	86.83 ± 19.1	76.61 ± 19.48
Needle	Apical	80.23 ± 17.46	127.64 ± 41.39	103.93 ± 39.34
	Middle	68.15 ± 6.67	166.73 ± 70.83	117.44 ± 70.39
	Coronal	69.01 ± 4.6	271.2 ± 73.31	170.1 ± 115.39
	Total	72.46 ± 12.1	188.52 ± 86.92	130.49 ± 84.91
PUI	Apical	66.94 ± 8.01	71.64 ± 8.77	69.29 ± 8.52
	Middle	59.36 ± 4.65	64.99 ± 4.12	62.18 ± 5.16
	Coronal	58.28 ± 4.3	69.75 ± 9.99	64.02 ± 9.52
	Total	61.53 ± 6.91	68.79 ± 8.26	65.16 ± 8.39
Total	Apical	71.44 ± 13.8	99.64 ± 34.79	85.54 ± 29.84
	Middle	63.15 ± 6.77	103.36 ± 61.12	83.26 ± 47.64
	Coronal	65.79 ± 13.18	141.15 ± 102.82	103.47 ± 82.01
	Total	66.79 ± 12.07	114.72 ± 73.57	90.75 ± 57.8

TABLE 2: Three-way ANOVA for final irrigation, final irrigation activation method, root canal region and the effect of their interactions on the microhardness of root dentin canal.

Surce of variation	Type III Sum of Squares	df	Mean Square	F	p
Final irrigation solution	103341.077	1	103341.077	130.608	<0.001
Final irrigation activation method	146052.206	2	73026.103	92.294	<0.001
Root canal region	14706.199	2	7353.100	9.293	<0.001
Final irrigation solution * Activation method	105770.329	2	52885.164	66.839	<0.001
Final irrigation solution * Root canal region	18027.887	2	9013.944	11.392	<0.001
Activation method * Root canal region	36497.225	4	9124.306	11.532	<0.001
Final irrigation solution * Activation method*	45415.960	4	11353.990	14.350	<0.001

Statistical analyses also indicated a significant interaction between each of the three parameters.

The mean microhardness scores according to the various final irrigation solution, final irrigation technique and the root canal thirds are presented in Figure 1. Qualitative analysis of the SEM micrographs showed that all chelation solutions removed the smear layer from the middle third of root canals and the orifices of the dentine tubules appeared (Figure 2).

Regardless of the usage of final irrigation techniques, the three-way ANOVA indicated that chitosan exhibited a significantly lower microhardness value than EDTA (p<0.001). Also, independently of the usage of final irrigation, both PUI and EA had significantly lower microhardness than CI (p<0.001). However, there were no statistically significant differences between PUI and EA (p<0.001). The mean microhardness values of the apical (p<0.002) and middle (p<0.001) regions were lower than that of the coronal region whereas there was no significant difference between the mean microhardness values of the apical and middle regions (p<0.001).

The interaction between final irrigation solution and final irrigation activation technique had a significant effect on microhardness (p<0.001). While there was no significant difference between microhardness values of the chitosan solution's PUI, EA, and CI activation (p<0.001), EDTA solution's mean EA and PUI values were lower than CI. EDTA solution's mean value obtained after CI was higher than those obtained by the interactions between other final irrigation solution and final irrigation activation techniques.

The interaction between final irrigation activation technique and root canal region was found statistically significant (p<0.001). No significant difference was found between EA's and PUI's mi-

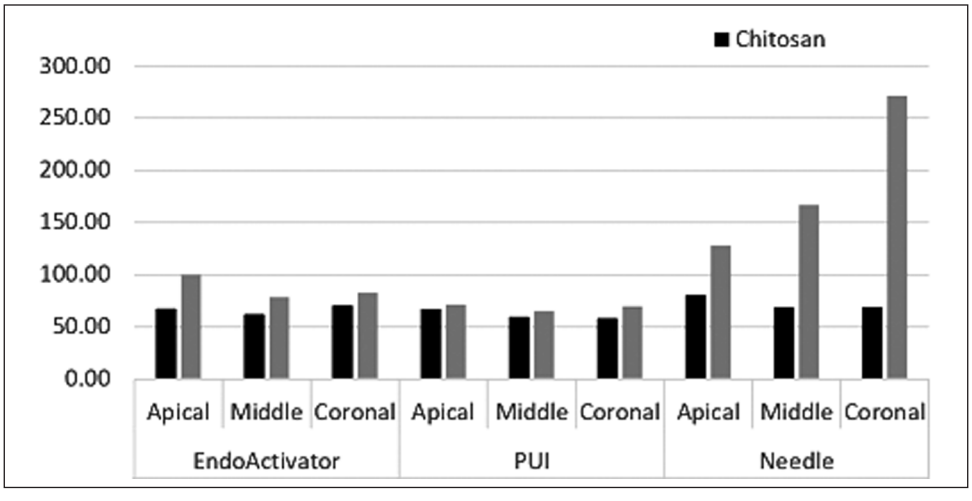

FIGURE 1: The mean of microhardness scores according to the various final irrigation solution final irrigation activation method and the root canal region.

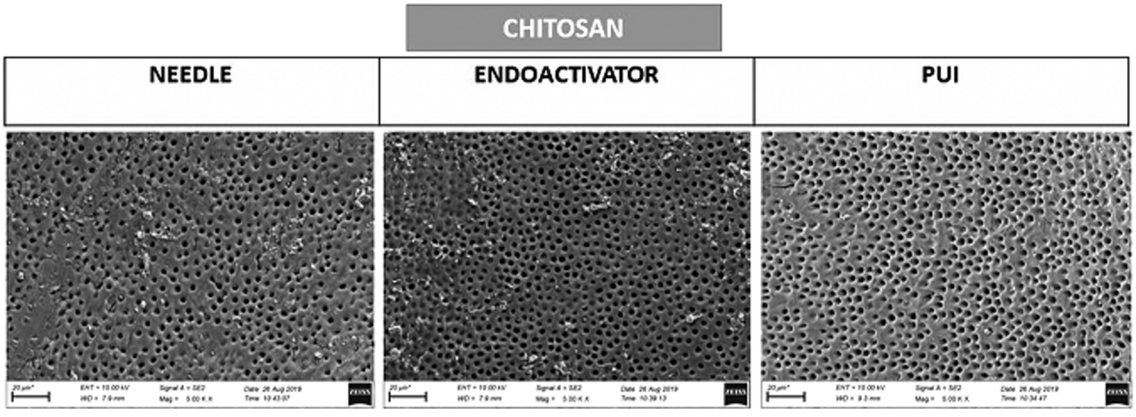

FIGURE 2: SEM micrographs of the middle root canal third (X 300).

crohardness values obtained from the apical, coronal, and middle regions (p<0,001). The mean value of the CIS technique at the coronal region was significantly higher than all other interactions. Additionally, the mean value of the CIS technique at the middle region was higher than all mean values of the PUI technique at all regions.

Final irrigation solution (chitosan or EDTA), final irrigation activation technique (PUI, EA or CI) and root canal region (Coronal, middle or apical) interactions had also significant effects on microhardness (p<0,001). EDTA solution's mean value obtained with the CI technique from the coronal region was significantly higher than all other interactions. EDTA solution's mean values obtained with CI from the apical and middle regions were not significantly different from but were higher than the other mean values. There was no significant difference between the mean values of other interactions.

DISCUSSION

This study investigated the influence of final irrigation solution type and technique on dentin microhardness. The results showed that chitosan exhibited a significantly lower microhardness value than EDTA. Also, both PUI and EA had significantly lower microhardness values than CI and the mean microhardness values of the coronal region were significantly lower than those of the middle and apical regions.

Dentin's mineral content and the amount of hydroxyapatite within the intertubular area are the most important parameters for dentin's inner hardness profile also called as dentin microhardness. Panighi and G'Sell reported a positive correlation between dentin's mineral content and hardness.[15] Irrigants used for root canal therapy may lead to changes in the chemical and structural composition of root canal dentin. Furthermore, these changes may also result in alterations of dentin's solubility properties and applicability. It has been reported that these mineral changes in dentin's content adversely affect the adhesion of resin-based root canal filling materials to root canal dentin.[16] However, there are also several studies reporting a positive correlation between dentin's mineral content and fracture resistance. As a result of a detailed literature review, one may argue that measuring dentin microhardness provides indirect evidence of dental mineral loss.[17]

There are plenty of studies in the literature that have examined the effects of EDTA on dentin microhardness. It has been reported that, with its chelating properties, EDTA removes dentin's calcified components causing it to soften and thus EDTA significantly reduces dentin microhardness.[5,13,14,17] However, although Sayın et al. reported that EDTA reduces dentin microhardness, they specifically stated that EDTA causes a reduction when used in conjunction with NaOCl.[18] It is considered that disruption of collagen in dentin structure results from a hypochlorite effect and it is not related to demineralization induced by final irrigation with EDTA.[15] Zhang et al. in another study, demonstrated that the use of EDTA as a final irrigant had a negligible effect on a well-documented drop in mechanical properties related to

NaOCl use and that mineral changes in dentin were both dependent on both the concentration and timing of NaOCl use.[19]

Recently, Saha et al. investigated the effects of chitosan and EDTA on microhardness and reported that both irrigants showed similar microhardness values.[12] Likewise, our study revealed that chitosan showed lower microhardness values than EDTA irrespective of the final irrigation activation technique used and root canal region. On the other hand, Nikhil et al. in a recent study, reported that EDTA was associated with lower microhardness values than chitosan.[20] Conflicting results have been reported on this subject so far, which is possibly due to the use of varying microhardness parameters, irrigant volumes and irrigant application times (3 min, 5 min or 15 min). There is a need for further studies adequately reflecting clinical conditions.

Root canal dentin mineralization and microhardness values are affected by pH values of irrigants.[17] It is reported that acidic pH facilitates removal of calcium ions from dentin and reduces microhardness values.[21] Sausa and Silva similarly reported that acidic pH removed a greater amount of calcium ions from dentin.[22] In the present study the pH value of 2% chitosan was 2.65 while 17% EDTA had a pH of 11.83. Chitosan was dissolved in acetic acid because it is insoluble in water. The mechanism of effect of chitosan is not entirely clear. Chitosan consists of β-(1–4)-linked 2-acetamido-2- deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D- glycopyranose. The nitrogen atoms in the chitosan polymer contain free electron pairs responsible for ionic exchange between metal and chelating agents. Adsorption, ion exchange, and chelation are the mechanisms responsible for the formation of complexes between chitosan and dentin.[6,20] It is believed that chitosan's polymer is hydrophilic, it supports close contact with root canal dentin and it is absorbed to root canal walls. Moreover, its cationic structure reportedly assists ionic interaction between dentin's calcium ions and chelating agent.[19] The results of the present study indicated that chitosan showed lower microhardness values than EDTA in all groups. We believe that the solvent may have contributed to calcium ion dissolution.

Multiple studies reported to date have shown that irrigation activation methods favorably influenced endodontic treatment success. However, as far as we know, there are insufficient number of studies that specifically investigated the effect of final irrigation activation techniques on dentin microhardness. Thus, the present study examined the effects of different irrigation techniques (sonic or ultrasonic) on dentin's microhardness. Our results indicate that final irrigation activation with EA and PUI resulted in less microhardness values than CI. Arslan et al. reported that final irrigation activation with PUI brought about a drop in dentin's microhardness.[23] Çapar et al. assessed root canal dentin surface alteration with regard to calcium/phosphate ratio; they reported that there was no significant difference between EA and CIS groups although EA and PUI groups had a lower Ca/P ratio than CIS.[24]

On the other hand, although Dinçer et al. reported that there was no significant difference between EA and CIS with respect to dentin microhardness, unlike two other studies and the present study, EA group had a higher microhardness than CIS.[25] We believe that the difference between studies may result from the solution used and differences of its contact time with dentin. Prior studies have reported that root canal dentin's microhardness values are reduced from coronal to apical direction. Recently, Nikvil et al. reported that dentin microhardness decreased from cervical to apical region both before and after irrigation, irrespective of the irrigation solution used (chitosan or EDTA). This study likewise demonstrated a decreasing dentin microhardness from coronal to apical region, irrespective of the final irrigation solution used (chitosan or EDTA) and the final irrigation activation technique (EA, PUI or CIS).[20]

Surface alterations of dental hard surfaces are assessed with Knoop hardness test and Vicker's microhardness test. Former studies have reported that Vicker's microhardness test is more suitable and feasible for assessing the effect of chemical agents on dentin microhardness.[13] In the present study Vicker's microhardness test was used as it is both more feasible and practical. Also, studies in literature reported that dentin microhardness showed variability on the

basis of dentin region and it decreased from pulp to dentin surface.[12] In order to ensure inter-group standardization, samples from the root's coronal, middle and apical sections were measured at areas 100 µm far from the pulp dentin border.

CONCLUSION

Within the limitations of this in vitro study, it may be concluded that all the used irrigating solutions affected the microhardness of root canal dentin. In the present study, it was found that chitosan as compared to EDTA showed lower microhardness values and the null hypothesis was rejected. Hence, it may be concluded that chitosan may serve as an effective alternative to EDTA. However, it is difficult to definitely argue if low dentin microhardness levels are beneficial or harmful under clinical conditions. There is a need for further studies where clinical conditions are entirely reflected.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Esin Özlek;* ***Design:*** *Esin Özlek;* ***Control/Supervision:*** *Esin Özlek;* ***Data Collection and/or Processing:*** *Elif Akyol, Gizem Kadi;* ***Analysis and/or Interpretation:*** *Esin Özlek, Hüseyin Gündüz;* ***Literature Review:*** *Hüseyin Gündüz;* ***Writing the Article:*** *Esin Özlek.*

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