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Original research

In vitro antimicrobial activity of different electrochemically-activated solutions on enterococcus faecalis

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Purpose

The aim of this *in vitro* study was to assess and compare the antimicrobial efficacy of different electrochemically-activated solutions (ECA) and contemporary irrigants, in root canals infected with *Enterococcus faecalis*, used with or without EndoActivator (EA).

Materials and methods

A hundred single-rooted human teeth were prepared. Ninety of the root segments were infected with *E. faecalis* for four weeks, and divided into eight test groups (n = 10) (four with and four without EA sonication) and a positive control (n = 10). The irrigants tested were electrochemically-activated solutions produced by the Medilox® (ECA-MX) and Envirolyte® devices (ECA-EN), 2% CHX and 2.5% NaOCl. The root specimens were irrigated with 5 mL of the test solution, with additional sonic agitation applied to the EA groups. The dentine samples that were obtained from the walls were cultured, and the antibacterial efficacy was evaluated by counting the colony-forming units.

Results

The ECA-EN, 2.5% NaOCl and 2% CHX were more effective than the ECA-MX ($p < 0.05$) with the addition of EA sonication, showing no statistical difference in the elimination of *E. faecalis*.

Conclusion

The ECA-EN shows potential as an endodontic irrigant, while EA usage gives no benefit in reducing bacteria from root canals.

Keywords: *Electrochemically-activated solution; EndoActivator; endodontics; Enterococcus faecalis; root canal irrigants*

Introduction

Microorganisms and their products play substantial roles in pulpal and periapical diseases (1,2). Therefore, bacterial elimination in the root canal system is the primary measure to prevent major periapical diseases, like apical periodontitis (3). This can be achieved by chemo-mechanical preparation, including root canal irrigation and inter-appointment medication (3). Ideally, effective endodontic irrigants should exhibit antimicrobial activity, and an absence of toxicity toward the periapical tissues (4).

Sodium hypochlorite (NaOCl) has been widely used as a root canal irrigant, due to its strong antimicrobial properties and tissue dissolving ability (4). Other advantages of NaOCl include its low price, easy accessibility and long shelf life (5). However, the cytotoxicity of NaOCl when it comes in contact with periapical or oral mucosal tissues is an undesirable characteristic (6). Chlorhexidine gluconate (CHX) has also been suggested as both an irrigant and intracanal medicament (7,8). CHX exhibits broad spectrum

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antimicrobial activity, as well as substantivity, but lacks tissue dissolving capacities in the root canals (9).

Electro-chemically activated solutions (ECA), denominated super oxidized water, are electrochemically processed aqueous solutions generated from tap water, using low concentration salt solutions. Their usage in endodontic therapy has been investigated in several studies, which evaluated the ECA solutions with regard to their antibacterial activity (10,11), tissue dissolving capacity (12), ability to debride root canals and smear layer removal (6,13). Recently, Medilox® (SOOSAN E&C CO., LTD., Seoul, Korea) and Envirolyte® (Envirolyte Industries International Ltd., Tallinn, Estonia) electrolysis devices have been introduced in medicine to manufacture ECA solutions endowed with a disinfecting capacity. The ECA solution produced by the Medilox® device (ECA-MX) contains about 50-80 ppm of hypochlorous acid (HOCl), with a pH of 5.0-6.5 and an oxidation-reduction potential (ORP) of 800-1000 mv, according to its manufacturer. The ECA solution generated by the Envirolyte® device (ECA-EN) has a greater concentration of HOCl (500-700 ppm), with a pH range of 7.0-7.5 and an ORP of 700-900 mv, according to its manufacturer. To date, no studies have compared the antimicrobial effects of different ECA solutions as root canal irrigants, other than the present study.

Attempts at the complete elimination of bacteria from the root canal system have resulted in the usage of adjunct devices to improve irrigation efficiency. One recent system, the EndoActivator (EA) (Dentsply/Tulsa Dental Specialties, Tulsa, OK), has received substantial attention because of its proposed properties. The EA is a cordless, battery-operated hand piece with a sonic motor, and it has been designed to enhance the cleaning efficacy of the irrigation of the root canal system. The EA system uses non-dentine cutting polymer tips in three different sizes (small/yellow, medium/red, large/blue) and the sonic motor provides 3-speed options including 2.000, 6.000 and 10.000 cpm. The EA has been recommended to activate EDTA and NaOCl solutions (14). For example, Pasqualini et al. (15) revealed increased antibacterial activity with the sonic activation of NaOCl. Considering application time, irrigant activation for 30 seconds during a 60-second period of QMix application has been proposed to enhance debris and smear layer removal potential of the EA (16).

The purposes of this study were to determine and compare the ex vivo susceptibility of *Enterococcus faecalis* to 2.5% NaOCl (Caglayan Kimya, Turkey), 2% CHX (Drogsan, Turkey) and two different ECA solutions (ECA-MX and ECA-EN), and to evaluate whether the addition of EA to the standard irrigation protocol results in a greater elimination of *E. faecalis* from the root canals. The null hypothesis tested were: 1) there are no differences among tested irrigant groups, and 2) EA sonic activation does not improve antibacterial effectiveness of irrigation solutions.

Materials and methods

Root dentine specimen preparation

One-hundred single-rooted intact human teeth, extracted for orthodontic or periodontal reasons, were used in this study. They were stored in 1.3% NaOCl for <3 months to disinfect the surface and to remove organic debris before use. The calculus was removed with periodontal cretuars; then, the crown was cut off and the root were shortened apically, leaving a root segment with a length of 10 mm. The root cementum was not removed.

The root canal instrumentation was performed by using ProTaper files (Dentsply Maillefer, Ballaigues, Switzerland) to size #F3, under irrigation of 1 mL of 2.5% NaOCl (Caglayan Kimya, Turkey) between each file. The removal of the smear layer was carried out in an ultrasonic bath (Bandelin Sonorex, Berlin, Germany) with the sequential use of 17% EDTA (pH 7.3) (Merck KGaA, Darmstadt, Germany) and 5.25% NaOCl (Caglayan Kimya, Turkey), for 5 min each (17). The root specimens were then placed in test tubes containing phosphate buffered saline (PBS) (Sigma-Aldrich, Germany), and autoclaved for 20 min at 121°C. Each sterile test specimen was incubated in 2 mL of tryptic soy broth (bioMerieux, France) for 24 hours at 37°C, to confirm the sterility.

Infection of root specimens

A clinical strain of E. faecalis organisms (A197A) was grown on tryptic soy agar (TSA) (BioMerieux, France) for 24 hours, at 37°C (18). A 24-hour-old E. faecalis suspension was adjusted spectrophotometrically to $OD_{600} = 0.6$.

Ninety root segments were infected with the *E. faecalis* strain for 4 weeks at 37°C. During the infection period, the media was changed every second day. The purity of the cultures was controlled once per week, based on the colony morphology on the TSA plates and cellular characteristics. Bacteria penetration into dentinal tubules was checked using scanning electron microscope. Ten samples from the negative control group were used to check the sterility, and submerged in PBS before sampling.

Irrigation

The teeth were randomly divided into eight experimental groups (n = 10) (four without the EndoActivator combination and four with) and two control groups (n = 10). The four irrigants tested were ECA-MX, ECA-EN, 2% CHX and 2.5% NaOCl.

Following the contamination period, the apical portions of the root segments were sealed with ethanol sterilized sticky wax to prevent flow of the solution, and then fixed on sterile glass petri dishes. Each root canal in the experimental groups was treated with 5 mL of each test solution, using a 27-gauge syringe. In the EA groups, the test solutions were left in the root canal, and the sonic activation was applied for 60 seconds, by inserting the red 25/04 polymer EA tip into the root canal at the highest speed (10,000 cpm). The infected teeth that served as the positive control were rinsed with 5 mL of PBS solution, while the sterilized teeth of the negative control group were left untreated.

Sampling procedure

At the end of the irrigation period, the root canals were immediately dried with sterile paper points, and stored in a freezer for 1 hour at -27°C. The dentine samples were obtained from the canal walls with Gates Glidden burs (# 3, 4 and 5) under aseptic conditions (Figure 1), and the burs never made contact with the outer surfaces of the root segments while sampling. The dentine chips obtained were transferred to glass vials containing PBS/glass beads, and vortexed for 30 seconds (19). The PBS with the re-suspended enterococci was then diluted

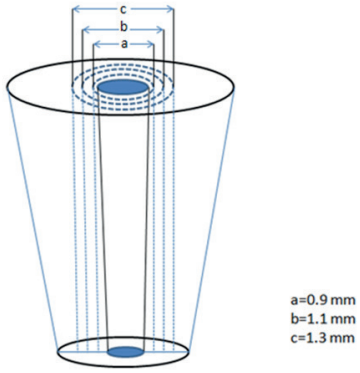

Figure 1. *Schematic view of sampling procedure.*

to 10-fold. Then, 25 µL droplets from each of the four parallel dilutions were inoculated on TSA plates, and incubated at 37°C for 48 h. The visible colonies from the appropriate dilutions yielding 5-50 colonies were counted, and the colony forming unit (CFU) mL^{-1} was calculated and transformed to log_{10}.

Scanning electron microscopy (SEM)

One extra sample from each test and positive control group was incubated with *E. faecalis* and treated as described above, to illustrate the colonization of the bacteria, and show the efficiency of the disinfection methods with the SEM. The samples were fixed in a 2.5% glutaraldehyde solution, and then evaluated (EVO LS10, Zeiss, Oberkochen, Germany). The SEM micrographs were taken from representative areas at various magnifications (×2000 and ×15000).

Statistical analysis

The data was analysed by using the SPSS (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp, USA). The statistical analyses were performed on log_{10} converted data. The data were nonparametric, because of the absence of normal distribution; therefore, the Kruskal-Wallis test and Mann-Whitney test were used to compare the different groups, with the significance level at $p< 0.05$.

Results

The *E. faecalis* growth after sampling on the TSA plates and the CFUs are listed in Table 1. All of the specimens in the positive control group showed the presence of growth (Figure 2a) in the agar plates, whereas all of the negative control specimens remained free of growth. The ECA-EN, 2.5% NaOCl and 2%

Table 1. *E. faecalis growth after sampling and mean values and standard deviations (SD) of colony-forming units (CFU).*

Groups (n=10)	Samples with CFU-negative	Samples with CFU-positive	Log10 CFU (mean ± SD)
ECA-MX	0	10	3.75 ± 0.65
ECA-EN	10	0	0 ± 0
2% CHX	8	2	0.47 ± 0.95
2.5% NaOCl	10	0	0 ± 0
ECA-MX+EA	1	9	3.27 ± 0.17
ECA-EN+EA	10	0	0 ± 0
2% CHX+EA	9	1	0.35 ± 0.70
2.5% NaOCl+EA	10	0	0 ± 0
Co-p	0	10	4.34 ± 0.25
Co-n	10	0	0 ± 0
Co-p: Positive control, Co-n: Negative control			

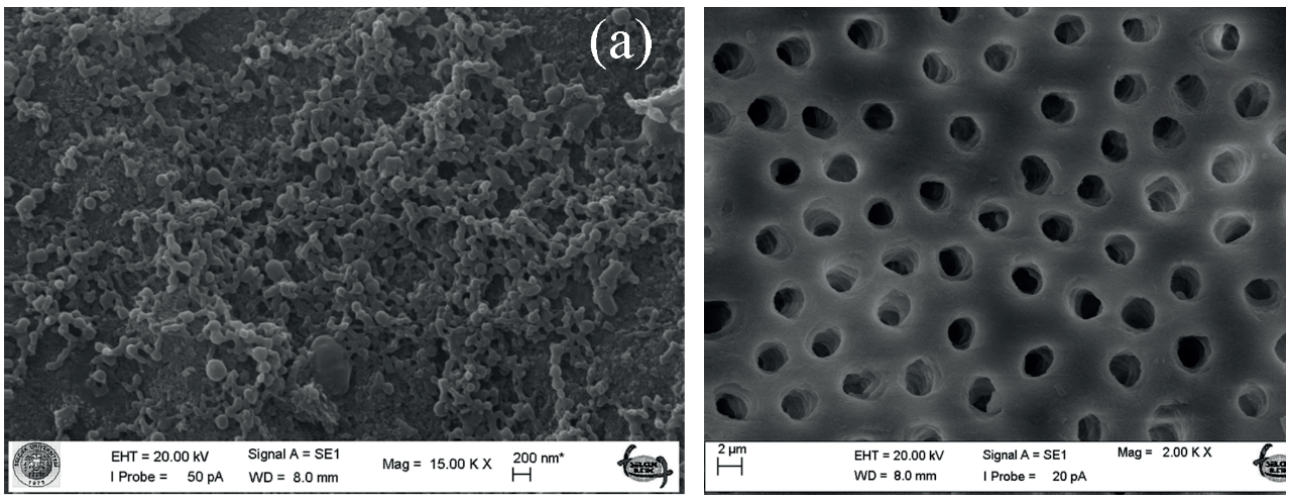

Figure 2. *(a) Scanning electron micrograph showing colonization of the root canal walls by E. faecalis A197A after a four-week infection period (original magnification, ×15.000). (b) Scanning electron micrograph showing open dentinal tubules without enterococcus species.*

Table 2. *Statistical comparison between test group pairs in an extracted tooth model*

Test groups		Significance (p value)
A	B	
ECA-MX	ECA-EN	0.000*
ECA-MX	2% CHX	0.000*
ECA-MX	2.5% NaOCl	0.000*
ECA-EN	2% CHX	0.436
ECA-EN	2.5% NaOCl	1.000
2% CHX	2.5% NaOCl	0.436
ECA-MX	ECA-MX+EA	0.077
ECA-EN	ECA-EN+EA	1.000
2% CHX	2% CHX+EA	0.863
2.5% NaOCl	2.5% NaOCl+EA	1.000

*Significant at 0.05 level ($p<0.05$)

CHX showed stronger bactericidal effects, and there were no statistically significant differences among these groups ($p > 0.05$) (Table 2). Both the ECA-EN (Figure 2b) and 2.5% NaOCl groups, and their EA combinations, completely eliminated the *E. faecalis*. Moreover, the statistical analyses indicated significantly lower bacterial reduction ($p<0.05$) in the ECA-MX group, when compared with the other tested irrigation solutions. Overall, there was no significant difference between the irrigant groups and irrigant groups combined with the EA.

Discussion

The findings of this study revealed that ECA-EN, NaOCl and CHX showed better antibacterial activity against *E. faecalis* than ECA-MX. Therefore, our first hypothesis was rejected. The secondary result of the present study showed no significant difference between irrigant groups and irrigant + EA groups. Consequently, our second hypothesis was accepted.

The infected tooth model used in this study was a modification of the one previously described by Haapasalo and Qrstavik (20), and this method has the advantage of simulating clinical conditions. However, variables such as root canal morphology, density of dentine, degree of calcification in dentine, content of dentinal tubules and amount of dentine chip samples are difficult to standardize in this dentine block model (21). The dentine block model appears to be more appropriate for this study since dentine and dentine components inhibit the antimicrobial activity of various root canal medicaments (22). In the present study, the root canals were contaminated with *E. faecalis* for 4 weeks. *E. faecalis* is a facultative bacteria frequently isolated in endodontic infections, especially in retreated cases (23). Its resistance to antimicrobial agents and ability to invade the dentinal tubules are possible reasons for the presence of *E. faecalis* in the microflora of persistent apical periodontitis (20). In order to provide sufficient time for the *E. faecalis* to penetrate into the dentinal tubules, a 28-day infection period was selected (20). The eradication of this bacterium from the root canals is important; therefore, strong antibacterial agents and effective methods are required for the killing of enterococci in infected root canals and dentine.

Neutralization of the irrigant to decrease possible substantive effects was not performed due to the absence of standard deactivating agent proper for all endodontic irrigants used. Also, neutralizing step does not take part in the clinical use of irrigants. Additionally, some of the components in neutralizing agents may possess antibacterial action on the biofilms therefore may lead to deceptive results (24). Dentine powder analysis was immediately carried out subsequent to irrigation under same experimental conditions to eliminate prolonged contact time of irrigation solutions (25).

In the present study, various ECA solutions demonstrated different effects on *E. faecalis*. The results suggest that ECA-EN, when used alone or combined with EA, has greater antibacterial activity (100% bacterial reduction) against *E. faecalis* than ECA-MX. Different ECA solutions can be produced by a similar electrolysis process, but the product can have a different antimicrobial activity due to the differences in the ORP values and the pH of the solutions (26). These two ECA solutions had similar ORP values, while the ECA-EN had a neutral pH, and the ECA-MX was slightly more acidic. It has been proposed that the neutral pH of the ECA solutions might be responsible for the longer shelf-life and perpetuation of the microbicidal activity (27). Therefore, the more potent bacterial reduction observed in the ECA-EN group could be explained by the neutral pH of this solution.

The main biocidal reagents in ECA solutions are chlorine related substances, such as chlorine (Cl_2), hypochlorous acid ions (ClO^-) and hypochlorous acid ($HClO^-$). The active component of ECA solutions is predominantly HOCl, which is known to be biocompatible and antimicrobial against a broad spectrum of microorganisms (28). Therefore, when comparing ECA solutions, it is necessary to take into account the difference in the HOCl concentration. ECA-EN has an approximately ten times higher concentration of HOCl than ECA-MX, which might be another explanation for the strong antimicrobial activity of ECA-EN.

The basic materials in ECA solutions are purified water and a small quantity of sodium chloride (NaCl). Unlike ECA-EN, the ECA-MX is generated at the point of use by passing Annexol solution over the electrodes, instead of NaCl. Annexol, which contains hydrochloric acid (HCl), NaCl and water, provides stability to the solution, according to the manufacturer. Although the manufacturer alleges improved antibacterial efficacy by means of Annexol, it appears that the Annexol had no substantial effect on the antimicrobial properties of the ECA-MX. On the other hand, the use of either NaCl or Annexol in the electrochemical procedure is not the only difference between the two ECA solutions. The HOCl concentration may influence the antimicrobial capacity more than the input material used in the electrolytic process.

NaOCl has extensive uses in endodontic treatment, but its potential toxicity on vital tissue has led to further investigations for alternative irrigants. One of the known advantages of the ECA solution is its non-toxicity when in contact with vital biological tissues (29). A novel ECA solution (Aquatine EC; Sterilox, PuriCore, Malvern, PA, USA) has been proposed as a highly biocompatible irrigating solution that permits the pulp stem cells to survive and attach to the root canal dentine, in regenerative endodontic therapy (30). Gomi et al. (31) reported that ECA solutions had mild cytotoxicity

on pulp cells when compared to NaOCl. In addition, Gonzales-Espinoza et al. (32) revealed that a pH neutral super-oxidized solution is less cytotoxic than antiseptic hydrogen peroxide concentrations. ECA-EN could have major advantages over NaOCl with regard to biocompatibility, and further studies are required to evaluate its cytotoxic effects.

Based on the results of the present study, ECA-EN is equally as effective as NaOCl, because both the ECA-EN and 2.5% NaOCl killed 100% of the bacteria in the root canal dentine. Our results corroborate the findings of previous studies that demonstrated the comparable antimicrobial activity of ECA solutions with NaOCl (31,33). In contrast, the findings of the present study are not in accordance with the findings of Marais & Williams (11), who showed no antibacterial activity with ECA solutions having pHs of 7.0 and 9.0, whereas the ORP levels and HOCl concentrations, which are considered to be important parameters for antimicrobial action, were not reported in that research. One recent study showed that although super oxidized water has the ability to prevent the growth of *E. faecalis*, NaOCl exhibited better antimicrobial action (34). The differences amongst the results of these studies and the present study could be attributed to the differences in the chemical properties of the ECA solutions used.

Hypochlorous acid (HOCl) and hypochlorite (OCl^-) are chlorine by-products, and in chlorine containing aqueous solutions, hypochlorous acid is the predominant form below a pH of 7.6; above this value, the predominantly active form is hypochlorite (35). The sodium hypochlorite solution that was used in the present study had a pH of 11-12; thus, the entire available chlorine content was in the form of hypochlorite. However, the germicidal activity of hypochlorous acid is superior to that of hypochlorite (36). ECA-EN has been confirmed to have good antimicrobial properties through the prepotency of hypochlorous acid, and based on the results of this study, it may be an attractive alternative to NaOCl.

Chlorhexidine (CHX) is a potent broad-spectrum antimicrobial agent that has substantive characteristics (9); therefore, it has been suggested for a final irrigation due to its residual antimicrobial activity (4). In addition, CHX has been found to be more effective against gram-positive bacteria than gram-negative bacteria (4). CHX effectively killed *E. faecalis* in the present study, which is in accordance with previous studies showing that CHX is an effective antibacterial agent (9,37). In the CHX with EA combination group, only one sample resulted in positive cultures on the plates, and in the CHX group, two samples showed *E. faecalis* growth on the agar plates. In a recent extracted tooth and membrane biofilm study, NaOCl, CHX and super oxidized water were investigated with regard to their antibacterial activities. The NaOCl was shown to be the most effective endodontic irrigant, while the 2% CHX and super oxidized water could not provide adequate disinfection (38). However, no other published study has compared the ECA solutions and CHX under the same experimental conditions as the present study.

The findings of the present study did not reveal a significant difference between the intracanal microbial reduction obtained by standard irrigation alone, and the reduction obtained with standard irrigation and EA sonication. This is consistent with the results of a recent in vivo study that evaluated the effects of EA on bacterial elimination (39) and the report of Ordinola-Zapata et al. (40) who showed that biofilm removal with EA was similar to conventional needle irrigation. This also corroborates the findings of an *in vitro* study which indicated that the sonic activation of EDTA and NaOCl with EA after chemomechanical preparation did not lead to better antibacterial activity (41). However, it was not the same as the results of Bago et al. (42), who reported that EA usage with NaOCl provides better antimicrobial activity when compared with NaOCl irrigation alone. This could be attributed to the differences in the infection period, since they used 7 days instead 28 days, which we used in the present study, and bacteria in young biofilms are more susceptible to endodontic irrigants than bacteria in old biofilms (43). Our results are also contradictory to the results of Shen et al. (44), who stated that sonic agitation improves the effectiveness of chlorhexidine. The previous study used an in vitro biofilm model on sterile hydroxyapatite discs, so the methodological differences may also be responsible for the different results.

In another *in vitro* study, sonic and ultrasonic activation were found to provide superior penetration of sodium hypochlorite at the apical third of the root, compared to traditional needle irrigation alone (45). When comparing these results, it should be considered that the apical 3 mm part of the roots was removed in the present study, in order to prevent deterioration of the results due to anatomical variations, and EA may show its real contribution to the conventional chemo-mechanical preparation on the apical region of the roots. The mechanism of action for EA involves acoustic microstreaming inside of the root canal system, and EA has been proposed to produce cavitation bubbles. A recent study evaluating the cleaning mechanisms of sonic and ultrasonic activated irrigation showed that cavitation was not found during sonic agitation, unlike the cavitation shown to arise during ultrasonic activation (46). One reason for the inefficiency of EA might be the non-occurrence of cavitation. Further *ex vivo* and *in vivo* studies evaluating the contemporary irrigants, together with extended EA sonication times, are needed.

Conclusion

ECA-EN can be considered to be as effective as 2.5% NaOCl in the elimination of *E. faecalis*. The addition of EA to standard irrigation was not effective in the further reduction of viable bacteria that resides within the root canal system. The use of ECA-EN could be recommended for endodontic irrigation procedures since it showed good antibacterial activity and ECA-EN may be a promising alternative to the relatively more cytotoxic NaOCl.

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Author contributions: AUE participated in designing and generating the data for the study. MBA gathered and analyzed the data. MBA wrote the majority of the original draft. MBA and AUE participated in writing the paper. All authors approved the final version of this paper.

Conflict of interest: The authors have no conflicts of interest to declare.

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Türkçe öz: *Farklı elektrokimyasal olarak aktive edilmiş solüsyonların Enterococcus faecalis'e karşı in vitro antimikrobiyal etkinliği. Amaç: Bu in vitro çalışmanın amacı, EndoActivator (EA) ilavesiyle ya da yalnız başına kullanılan farklı elektrokimyasal olarak aktive edilmiş solüsyonlar ve geleneksel yıkama solüsyonlarının, Enterococcus faecalis ile enfekte edilmiş kök kanalları üzerindeki antimikrobiyal etkinliğini değerlendirmek ve karşılaştırmaktır. Gereç ve yöntem: Bu çalışma için 100 adet tek köklü insan dişi hazırlandı. Doksan adet kök segmenti E. facealis ile 4 hafta boyunca enfekte edildi ve bir pozitif kontrol grubu (n=10) ve 8 deney grubuna ayrıldı (n=10) (4 gruba ilave EA aktivasyonu uygulandı). Test edilen irrigasyon solüsyonları; Medilox® cihazından (ECA-MX) ve Envirolyte® cihazından (ECA-EN) elde edilen elektrokimyasal olarak aktive edilmiş solüsyonlar, %2'lik CHX ve %2.5'luk NaOCl'dir. Kök örnekleri 5ml deney solüsyonuyla yıkandı, EA gruplarında ilave olarak sonik ajitasyon uygulandı. Kök kanal duvarlarından elde edilen dentin örneklerinin kültürü yapıldı, koloni oluşturan birimler sayılarak antibakteriyel etkinlik değerlendirildi. Bulgular: ECA-EN, %2.5'luk NaOCl ve %2'lik CHX, E. faecalis'in eliminasyonunda ECA-MX'ten daha etkili bulundu (p < 0.05). İlave EA sonik aktivasyonu istatistiksel olarak anlamlı antibakteriyel etkinlik göstermedi.Sonuç: ECA-EN, endodontik irrigasyon solüsyonu olarak potansiyel taşırken EA kullanımı kök kanallarından bakteriyi azaltmada ekstra fayda sağlamamıştır. Anahtar kelimeler: Elektrokimyasal olarak aktive edilmiş solüsyon; EndoActivator; Enterococcus faecalis; kök kanal yıkama solüsyonları; endodonti*

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