Original Article / Özgün Araştırma

Evaluation of the Efficacy of 2% Chlorhexidine in Combination with Passive Ultrasonic Irrigation on *Enterococcus faecalis* Biofilm

%2'lik Klorheksidinin Pasif Ultrasonik İrrigasyon Eşliğinde Kullanımının Enterococcus faecalis Biyofilmi Üzerine Etkinliğinin Değerlendirilmesi

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Abstract

Objective: This study aimed to evaluate the combined effectiveness of 2% chlorhexidine (CHX) and passive ultrasonic irrigation (PUI) against *Enterococcus faecalis (E. faecalis)* biofilm.

Materials and Methods: The root canals of 66 single-rooted extracted human mandibular premolar teeth (n=66) were enlarged up to a size of 40/0.06 taper and autoclaved. Fifty-nine roots were inoculated with *E. faecalis* and incubated for 4 weeks, whereas 7 roots served as the negative control group and were filled with sterile brain heart infusion broth. The remaining specimens (n=59) were assigned into 4 experimental groups (n=13) and a positive control group (n=7): CHX via standard needle irrigation (SNI), sodium hypochloride (NaOCl) via SNI, CHX via PUI, NaOCl via PUI and non-treated positive controls. Bacteriological samples were collected before and after the intervention, and microbiological analysis was performed by counting the colony forming units. Reduction in colony count (RCC) between before and after the intervention in each group were compared using One-Way ANOVA.

Results: The highest RCC was determined in NaOCl/PUI and the lowest in CHX/SNI ($p<0.05$). Regarding RCC, CHX/PUI showed statistically similar results with NaOCl/PUI and NaOCl/SNI ($p>0.05$).

Conclusion: A PUI of 2% CHX induced a statistically similar amount of RCC with both a PUI of 2.5% NaOCl and SNI of 2.5% NaOCl. PUI combined with 2% CHX can be used in secondary endodontic infections and during routine endodontic treatment due to increased antibacterial efficiency against *E. faecalis*.

Öz

Amaç: Bu çalışmanın amacı %2'lik klorheksidin (CHX) ve pasif ultrasonik irrigasyonun (PUİ) kombine kullanımının *Enterococcus faecalis (E. faecalis)* biyofilmine karşı etkinliğini değerlendirmektir.

Gereç ve Yöntemler: Bu çalışmada 66 adet (n=66) tek köklü insan alt küçük azı dişine ait kök kanalları 40/0,06'lık genişlik ve konisiteye kadar şekillendirildi ve dişler otoklavlandı. Dişlerden 59 tanesi *E. faecalis* suşu ile enfekte edilerek 4 hafta inkübasyona bırakıldı (n=59), kalan 7 adet dişin kök kanalları steril beyin kalp infüzyon sıvı besiyeri ile dolduruldu ve negatif kontrol grubuna alındı (n=7). Elli dokuz adet diş, 4 deney grubu (n=13) ve 1 pozitif kontrol grubuna (n=7) ayrıldı. Standart şırınga irrigasyonu (SŞİ)/CHX, SŞİ/Sodyum hipoklorit (NaOCl), PUİ/CHX, PUİ/NaOCl deney gruplarını oluşturdu ve pozitif kontrol grubuna irrigasyon işlemi uygulanmadı. İrrigasyon işleminden önce ve sonra olmak üzere bakteri örnekleri kök kanallarından toplandı ve kültürde gelişen koloni oluşturan birimler sayıldı. Her grupta irrigasyon işleminin neden olduğu koloni cinsinden azalma (KCA) tek yönlü varyans analizi kullanılarak karşılaştırıldı.
Bulgular: En yüksek KCA, NaOCl/PUİ grubunda saptanırken en düşük KCA, CHX/SNI grubunda tespit edildi ve bu gruplar arasında istatistiksel açıdan anlamlı fark gözlendi (p<0,05). KCA miktarları açısından CHX/PUİ grubunda, NaOCl/PUİ ve NaOCl/SŞİ grupları ile istatistiksel açıdan benzer sonuç elde edildi (p>0,05).
Sonuç: %2'lik CHX solüsyonunun PUİ eşliğinde kullanımı; %2,5'lik NaOCl'nin PUİ ve SŞİ eşliğinde kullanımıyla istatistiksel açıdan benzer KCA'ya neden olmuştur. %2'lik CHX solüsyonunun PUİ eşliğinde kullanımı *E. faecalis*'e karşı üstün antibakteriyel etkinliği nedeniyle ikincil endodontik enfeksiyonlarda ve rutin endodontik tedavi sırasında tercih edilebilir.

Introduction

One of the main actions that affects the success of endodontic treatment is reducing the intensity and diversity of bacterial populations to a threshold level that induces periradicular healing (1) because there is no way to completely eliminate the bacteria from the root canal system (RCS) (2).

Enterococcus faecalis (E. faecalis) is a gram-positive anaerobic cocci that has been associated with endodontic failures in previous studies (3). It can penetrate deep into the dentinal tubules and adhere to the collagen matrix in dentin (4). It can also be located in the isthmus and ramification areas (5), which cannot be adequately reached by some irrigants. After the obturation process, even though its nutrient sources decrease, *E. faecalis* can survive as a single microorganism (6) with prolonged survival capacity (7) that is capable of biofilm formation (8). Bacteria in biofilm are more resistant to antimicrobial agents than bacteria in planctonic form (9). They produce a specific polysaccharide matrix, and in this way, a physical barrier against disinfecting agents can be constructed (10). Moreover, bacterial biofilm provides nutrients to bacteria and enables bypassing of the immune system's defensive mechanisms (11).

A cationic biguanide, chlorhexidine (CHX), shows broad-spectrum antimicrobial activity against endodontic bacteria during root canal treatment (12). It exhibits long-lasting and residual antibacterial effects on dentinal walls (13); its optimum concentration is 2% (14) as a root canal irrigant.

Passive ultrasonic irrigation (PUI), enhances the effectiveness of endodontic irrigants and delivers the irrigants throughout the RCS, including all anatomic irregularities (15). Ultrasound energy inducts acoustic streaming in the root canal and facilitates removal of intraradicular biofilm (16). Moreover, the energy released during ultrasonic movement is converted into heat energy in the root canal space (17), which is expected to increase efficiency when used with sodium hypochloride (NaOCl) (18). To the best of our knowledge, there is a gap in the literature regarding the investigation of the antibacterial activity of 2% CHX via PUI in human root canals infected with *E. faecalis in vitro.* The objective of the present study is to investigate the efficiency of four irrigation protocols in eliminating experimental *E. faecalis* biofilms in root canals.

Materials and Methods

Sample Preparation

G*Power 3.1 software was used to compute the required sample size for One-Way ANOVA testing. The required sample size for experimental groups was calculated to be when the power of the test was 0.80, the effect size was 0.40, the type I error rate was 0.05 and the type II error rate was 0.20. Ethical clearance was obtained from the Ethical Committee of Mersin University, Mersin, Turkey (number: 2018/404, Clinical Research Ethics Committee dated: 17 October, 2018) as an *in vitro* study, no informed consent was required. Human extracted lower premolar teeth with similar root size and anatomy were collected for this study. Sixty-six (n=66) of samples with straight roots and round shaped canals were randomly selected. Mesiodistal and buccolingual dimensions of selected

samples were examined on radiography and round shaped root canals were confirmed. Root canal treated, carious, cracked and calcified teeth are set aside and also teeth with multiple canals and root curvatures were excluded. The coronal portion of the teeth were removed with an Isomet 5.000 saw (Buehler, Lake Bluff, IL, USA) and the length of the root samples was standardized as 14 mm. The working length (WL) was considered 1 mm short of the apical foramen. Root canals were shaped to the WL using ProFile rotary instruments (Dentsply Tulsa Dental, Tulsa, OK, USA). Each canal was shaped to a size 40/0.06 taper. Root canals were irrigated with 2 mL 2.5% NaOCl (Merck KGaA, Darmstadt, Germany) using a 30-gauge endodontic needle (Sybron Endo, Orange, CA, USA) after each instrument. In all experimental groups, root canals were then irrigated with 5 mL 5% ethylenediaminetetraacetic acid (Merck) followed by 5 mL 2.5% NaOCl and 5 mL saline solution. Apical foramina of each root and the root surfaces were covered with 2 layers of a nail varnish. Each root was taken into 1.5-mL Eppendorf tubes filled with sterile brain heart infusion (BHI) broth (Merck KGaA, Darmstadt, Germany) and autoclaved inside these tubes. Teeth were kept in an incubator for two days at 37 °C in order to check for bacterial contamination.

Contamination of the Roots with *E. faecalis*

Seven roots served as a negative control group; and were filled with sterile BHI broth. Fifty-nine roots were infected with *E. faecalis* pure culture, that was cultivated in the BHI agar for 24 hours. A 1 McFarland suspension was prepared in BHI broth and then diluted 30-fold to obtain an initial bacterial suspension of $1x10^7$ colony-forming units (CFUs) per milliliter. Each root canal was completely filled with 10 µL *E. faecalis* suspension using sterile micropipettes; also it was attempted to deliver the bacterial suspension along the entire root canal length with sterile size 15 hand files. Roots were incubated at 37 °C and 95% humidity for 1 month, during this period the BHI was removed and replenished every 48 hours (h) under laminar flow. Two bacterial samples were collected from the root canals, before (S1) and after (S2) final irrigation. Before final irrigation with 2% CHX or 2.5% NaOCl with irrigant delivery techniques, the root canal was rinsed with 1 mL sterile 0.85% saline solution to remove unattached cells, and two sterile size 15 paper points were used sequentially at the WL for 1 minute (min) to soak up the canal contents.

Experimental Groups and Procedures

After 4 weeks, samples were removed from the inoculation tubes that had been placed in biosafety cabinets to prevent sample contamination. The root canals were disinfected using four different irrigation protocols, as described below.

Group 1: CHX via standard needle irrigation (SNI) (n=13): Five mL of 2% CHX irrigation was performed as the final step. A 30-gauge side-vented needle was placed within 2 mm of the WL and moved in a vertical motion to avoid the needle being locked in the canal. To ensure length control, a stopper was placed on the needle at the required length.

Group 2: NaOCl via SNI (n=13): Five mL of 2.5% NaOCl irrigation was performed as the final step. All the procedures, except for the final solution, were the same as for group 1.

Group 3: CHX via PUI (n=13): Root canals were rinsed with 5 mL of 2% CHX. An ultrasonic tip with a noncutting end (Irri-Safe tip K20/21 mm; Acteon, Mt. Laurel, NJ) mounted in a piezoelectric ultrasonic device (P5; Satelec Acteon, Merignac, France) was inserted to 1 mm less than the WL and activated at a power setting of 4 for 20 seconds. The rinsing and ultrasonic activation were repeated for 3 cycles.

Group 4: NaOCl via PUI (n=13): Root canals were rinsed with 5 mL of 2.5% NaOCl. All the procedures, except for the final solution, were the same as for group 3.

Group 5: Negative (sterile) control (n=7): Non-contaminated root canals were irrigated with 5 mL of 2% CHX via SNI technique.

Group 6: Positive (infected) control (n=7): The root canals were infected but received no further treatment.

In each group (except the positive control group), after final irrigation either with CHX or NaOCl via various irrigant delivery techniques, 1 mL 10% sodium thiosulfate was injected into the root canals by a 30-G syringe to neutralize both NaOCl and CHX and remained in the root canals for 30 seconds. Finally, a second bacterial sample from the root canal was taken (S2) with size 25 paper points (Dentsply Sirona), as described previously.

Quantification of the Bacterial Load

The paper points were transferred to the tubes containing 1 mL of 0.85% saline solution and vortexed for 1 min. After 10-fold serial dilutions in sterile

saline, 0.1 mL aliquots of each diluted sample were plated onto BHI agar plates and incubated at 35±2 °C for 24 h. The cultivated CFUs were counted and then transformed into actual counts based on the previously determined dilution factors.

Statistical Analysis

The data were analyzed using SPSS statistics 25.0 software (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY). The normal distribution of the data for quantitative variables was evaluated by the Shapiro-Wilk normality test and Q-Q graphs. The Wilcoxon signed-rank test was used to evaluate the reduction in colony count (RCC) between before and after intervention in each group. Bacterial count comparisons of independent groups with more than two subcategories were evaluated using ANOVA. A Tamhane test was used for multiple comparisons according to the homogeneity of group variance tests. In residual bacterial count (RBC) comparisons, Kruskal-Wallis analysis was used according to the normality test results, and the Dunn-Bonferroni post-hoc method was used for multiple comparisons. Significance level was set at 0.05.

Results

Table 1 represents the RCC based on the mean counts before and after the intervention. The highest percentage RCC was determined in NaOCl/PUI and the lowest in CHX/SNI ($p<0.05$). CHX/PUI showed statistically similar results with NaOCl/PUI and NaOCl/SNI ($p>0.05$). There were no significant differences between CHX/PUI and CHX/SNI ($p>0.05$).

Table 2 shows the logarithmic CFU values of the RBC after intervention in all experimental groups and also in the non-treated (positive control) group. There were significant differences between all experimental groups and the positive control group ($p<0.05$), whereas no statistical difference was observed among experimental groups ($p>0.05$).

Discussion

Today, to achieve predictable results, clinicians and investigators aim to reduce the number of bacteria in RCSs to below the threshold level in order to eliminate pathogen species and remove biofilm associated with the root canal. Ma et al. (19) stated that techniques that should produce at least one logarithmic step decrease in CFUs. *E. faecalis* is a key pathogen identified in failing endodontic cases (20), as this high biofilm producer has the ability to form biofilm within 48-72 h (21), to organize biofilms through its aggregation products and to engage dentine and other bacteria (22). In experimental conditions, the incubation time, which is an important factor for biofilm development, was set to 4 weeks. Stojicic et al. (23) reported that during the first hour, bacteria are mostly planktonic and that, in the first 2 weeks, biofilm bacteria are sensitive to NaOCl (1%), CHX (2%) and iodine (0.2/0.4%); after 3 weeks, however, they become very resistant to the same agents. In the present study, we used *E. faecalis* to evaluate the combined effectiveness of PUI with 2% CHX and 2.5% NaOCl in extracted human teeth.

In the present study, human mandibular premolars with single-rooted, straight and round root canals were standardized to 14 mm root length. Thus, similar dimensions of root canal volumes were incubated with *E. faecalis* strain. In a previous study (24),

Table 1. Tamhane post-hoc Analysis from One-Way ANOVA shows the mean difference, p value, and 95% CI of log (CFU/mL) data between each pair of experimental groups regarding RCC

Group A	Group B	Mean difference (A-B)	p	95% CI	
				Lower bound	Upper bound
NaOCl via SNI	NaOCl via PUI	-0.67	0.342	-1.68	0.34
	CHX via SNI	1.65*	0.043	0.04	3.25
	CHX via PUI	0.22	0.999	-1.26	1.70
NaOCl via PUI	CHX via SNI	2.32*	0.002	0.78	3.86
	CHX via PUI	0.89	0.375	-0.51	2.30
CHX via SNI	CHX via PUI	-1.42	0.190	-3.24	0.39

*indicate that there was statistically difference between values $p<0.05$, NaOCl: Sodium hypochloride, SNI: Standard needle irrigation, PUI: Passive ultrasonic irrigation, CHX: Chlorhexidine, CI: Confidence interval, CFU: Colony-forming unit, RCC: Reduction in colony count

Table 2. The number of residual bacterial count after the intervention (log_{10}) (CFU/mL)				
Groups	**Median (Q_1-Q_3)**	**p**	**Post-Hoc**	**p**
NAOCl via SNI (1)	1 (1-1)	<0.001	1-2:1.000	1-3:1.000
NaOCl via PUI (2)	1 (1-1)		1-4:1.000	**1-5<0.001**
CHX via SNI (3)	1 (1-2,65)		2-3: 1.000	2-4: 1.000
CHX via PUI (4)	1 (1-1)		**2-5<0.001**	3-4: 1.000
Positive control (5)	6.60 (6.47-7.69)		**3-5<0.001**	**4-5<0.001**
NaOCl: Sodium hypochloride, SNI: Standard needle irrigation, PUI: Passive ultrasonic irrigation, CHX: Chlorhexidine, CFU: Colony-forming unit				

variations in root canal size and selection of tooth model were assumed as parameters that had affected the diversity of results in different studies. Samples were more easily collected and better controlled in the straight root canals of single-rooted teeth used in the current study, and sterile paper points were used to collect bacteria with the same technique described in previous studies (25,26).

According to Rôças et al. (27), both 2.5% NaOCl and 2% CHX can be used as root canal irrigant in infected teeth. A few studies (4,28,29) in the literature compared the effectiveness of NaOCl and CHX at various concentrations on *E. faecalis* and reporsted no significant differences. However, in a previous study, Vianna et al. (30) stated that 2.5% NaOCl was significantly more effective than 2% CHX gel in reducing bacterial populations in root canals. In the present study, a higher RCC has been observed in the NaOCl/PUI and NaOCl/SNI groups compared to the CHX/SNI group, while no statistical difference was found between the CHX/PUI and CHX/SNI groups. It is clear that in studies where no difference was found between NaOCl and CHX, different concentrations of NaOCl and CHX were used (28) and that instead of counting bacterial growth in CFU/mL, the minimum contact times for inducing negative cultures were compared (4,29). Although a non-culture dependent methodology was used (30), the same concentrations of NaOCl and CHX were used in the present study and Vianna et al.'s (30) study. Moreover, both of the studies were quantitative, which is why our findings are in accordance with theirs (30).

In previous studies, ultrasonic agitation of irrigants showed better efficacy of cleaning and disinfecting in RCS than in SNI alone (31,32). Nevertheless, Siqueira et al. (33) reported that NaOCl with PUI was not superior to NaOCl with SNI when the efficacy of NaOCl was compared using different delivery techniques. In their study (33), a turbidity test was used to compare the effectiveness of irrigants and delivery techniques, and a number of negative and positive cultures were recorded. In the current study, a quantitative culture-based methodology is used, and it has shown a superior RCC with PUI compared to with SNI, but the difference was not significant. In one study, NaOCl was accepted as the main endodontic irrigant due to its antibacterial properties and its capacity to dissolve organic tissue residues (34). That is why the results in the present study for NaOCl with both PUI and SNI are so similar and are not statistically different. In an *ex vivo* study (35), the sequential use of PUI and a final rinse with CHX was suggested as the best approach, over PUI alone and CHX alone. However, in the current study, no statistical difference was found between CHX/PUI and CHX/SNI, and both NaOCl groups (NaOCl/PUI and NaOCl/SNI) induced a larger RCC than did CHX/SNI. The discrepancy may have occurred because of the usage of NaOCl instead of CHX (35) during the ultrasonication process in the previously mentioned study (35).

According to the results of the current study, in all experimental groups, RBCs (after intervention) were statistically different from the positive control group (Table 2). Ruiz-Linares et al. (36) claimed that 2.5% NaOCl was the most effective irrigant against endodontic pathogens in their examination and evaluation of a multispecies mature biofilm model in human dentine. They found (36) the mean percentage of live cells to be 4.26% in the 2.5% NaOCl group. Although mixed bacterial flora exist in endodontic infections, monospecies biofilm with *E. faecalis* was used in the present study. A higher sensitivity and the almost eradication of biofilm in response to the same

concentration of NaOCl in the second samples may be related with this fact.

As in previous studies (25,26), samples in the present study were taken with the aid of sterile paper points before and after interventions, and cultures were grown for both scenarios. Absorbent paper points can collect bacterial samples from the main canal and from the smooth-surfaced root canal walls but may not be able to pick up bacteria located in more distant areas and in anatomical complexities such as dentinal tubules, isthmuses, recesses and lateral canals. This limitation may be one of the reasons for the small number of bacterial colonies recorded after treatment in all experimental groups. In the future, the results of the present study should be confirmed by a molecular study that provides quantitative results.

Conclusion

A PUI of 2% CHX induces a statistically similar amount of RCC with both a PUI of 2.5% NaOCl and an SNI of 2.5% NaOCl. Thus, we have identified the positive effects of PUI combined with 2% CHX in the elimination of *E. faecalis* in the root canal and recommend it for routine endodontic treatment.

Ethics

Ethics Committee Approval: Ethical clearance was obtained from the Ethical Committee of Mersin University, Mersin, Turkey (number: 2018/404, Clinical Research Ethics Committee dated: 17 October, 2018) as an *in vitro* study.

Informed Consent: No informed consent was required.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: B.K., H.S.T., S.T.Ü., Design: B.K., H.S.T., S.T.Ü., B.S., Data Collection or Processing: B.K., S.T.Ü., N.K., Analysis or Interpretation: B.K., S.T.Ü., H.S.T., G.A., Literature Search: B.K., Writing: B.K., S.T.Ü.

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