Evaluation of the Antibacterial Effects of Single and Combined use of Different Irrigation Solutions Against Intracanal Enterococcus Faecalis

Prosudba antibakterijskog učinka pojedinačne i kombinirane uporabe otopina za ispiranje korijenskih kanala na Enterococcus faecalis

Abstract

Objectives: This study assessed the antibacterial activity of both separate and combined uses of 5.25% sodium hypochlorite (NaOCl), 2% chlorhexidine (CHX), 17% ethylenediaminetetraacetic acid (EDTA), 3% hydrogen peroxide (H₂O₂), MTAD, SmearClear (SC) and 13.8% chlorine dioxide (ClO₂) irrigation solutions against Enterococcus faecalis. Material and Methods: Two hundred eighty single rooted human premolars were randomly grouped into 26 test and 2 control (negative and positive) groups and were incubated for 24 h with E. faecalis, except for the negative control group. The tested solutions were as follows: NaOCl; CHX; ClO₂; MTAD; SC; EDTA; H₂O₂; NaOCl + CHX; NaOCl + MTAD; SC + NaOCl; EDTA + NaOCl; H₂O₂ + NaOCl; CIO₂ + CHX; CHX + MTAD; SC + CHX; EDTA + CHX; CHX; CHX + H₂O₂; CIO₂ + MTAD; SC + CIO₂; EDTA + CIO₂; CIO₂ + H₂O₂; SC+MTAD; EDTA+MTAD; MTAD + H₂O₂; SC + H₂O₂; and EDTA + H₂O₂. Optical density values were recorded at 0, 6, 12, 18, 24, 30, 36, 42 and 48 h and bacterial growth curve created for each solution. Results: The CHX, MTAD and ClO₂ showed a high potential for the elimination of E. faecalis, both alone and in all combinations. The EDTA, H₂O₂, NaOCl and SC + NaOCl groups showed less antibacterial activity than the other groups. The SC + CHX group showed the best antibacterial effect against E. faecalis. Conclusion: The SC + CHX combination can be recommended as the most effective irrigation regimen against E. faecalis in persistent endodontic infections.

Introduction

Microorganisms are one of the significant etiological factors in the pathogenesis of periapical tissue diseases. For this reason, it is very important to completely remove microorganisms from the infected root canals during a root canal treatment. Some ex vivo and clinical studies have reported that there are some untouched areas in the root canal walls during mechanical preparation. Therefore, to combine mechanical instrumentation with chemical irrigation is extremely important (1,2,3) in elimination of the microorganisms known to be extremely resistant against antimicrobial agents, such as Enterococcus (E.) faecalis (4).

The relationship of E. faecalis with different forms of periapical infections, including primary and permanent infec-

Uvod

Mikroorganizmi su jedan od važnijih etioloških čimbenika u patogenezi bolesti periapikalnog tkiva. To je razlog da je tijekom endodontskog liječenja vrlo važno potpuno ukloniti mikroorganizme iz zaraženih korijenskih kanala. U nekim istraživanjima ex vivo i kliničkim studijama autori su pokazali da postoje mnoga nedirnuta područja na stijenkama korijenskog kanala tijekom mehaničke pripreme. Zato je kombiniranje mehaničkih instrumenta s kemijskom irrigacijom istekako važno (1, 2, 3) u uklanjanju mikroorganizama za koje se zna da su iznimno otporni na antimikrobnu sredstva, kao što je Enterococcus (E.) faecalis (4).

Odnos bakterije E. faecalis s različitim oblicima periapikalnih infekcija, uključujući primarne i kronične infekcije,
tions, is well known because of its ability to grow in the presence or absence of oxygen. *E. faecalis* has been reported to be associated more with asymptomatic chronic periapical lesions rather than acute periapical periodontitis or acute periapical abscesses in the category of primary endodontic infections (5, 6). It has been confirmed that *E. faecalis* is significantly associated with treatment failures. Where- as this species was detected in 18% of the cases of primary endodontic infections, its prevalence in root-filled teeth was much higher: 67% of the cases (7).

Different endodontic irrigation solutions and disinfection techniques have been introduced to decrease the root canal bacterial count. The main effect of irrigation is to both physically and chemically remove organic and inorganic debris, infected materials and soft and hard tissue residues from the root canals. In this way, these materials are inhibited from accumulating, clogging and becoming inaccessible in the apical section of the root canals. Endodontic irrigation solutions usually have antibacterial properties. It has been proven that a combined use of irrigation materials with a different antimicrobial spectrum can even increase the antibacterial activity provided by a single solution through a synergic and/or additive effect for the elimination of hundreds of types of microorganisms forming on the root canal microflora (5).

There is a limited number of studies in the literature assessing and comparing the combined use of frequently used endodontic irrigation solutions in terms of their antibacterial characteristics. The positive or negative effects of the antibacterial interactions of current and potential irrigation solutions will especially guide the treatment of persistent endodontic infections. Therefore, understanding the interactions between irrigation solutions will be very helpful in implementing the most effective treatment procedure for persistent infections in endodontic cases. This current study aimed to investigate the antibacterial activity of both the single and combined uses of sodium hypochlorite (NaOCl), chlorhexidine (CHX), ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide (H₂O₂), BioPure MTAD (Densply, Tulsa Dental, Tulsa, OK, USA), SmearClear (SC) (Sybron Endo, Orange, CA, USA) and chlorine dioxide (ClO₂) against *E. faecalis* and to compare the antibacterial effects of this study’s solutions against *E. faecalis*.

**Material and Methods**

The current study was started after receiving approval from the Ethical Board of Selcuk University, Faculty of Dentistry (Document # 203). One hundred eighty single-rooted human lower premolars that had been extracted for orthodontic or periodontal reasons were used. The hard and soft tissue residues on the teeth were cleaned with curettes and then the teeth were kept at 4°C in a 100% moist environment until the laboratory procedures were performed.

The teeth were de-coronated with a diamond disk (Ortho Technology Inc., Tampa, FL, USA) under water cooling to a standardized root length of 14 ± 0.5 mm. The canal lengths were standardized with 15 K-File hand devices (Mani Inc., Tochigi, Japan) and enlarged with ProTaper Universal NiTi
dobro je poznat zbog njegovog svojstva rasta kako u prisutnosti kisika, tako i u njegovoj odsutnosti. Za *E. faecalis* dokazano je da je više povezan sa asimptomatskim kroničnim periri- kularnim lezijama, a ne s akutnim peririadikularnim parodon- titisom ili akutnim peririadikularnim apscesima u kategoriji primarnih endodontskih infekcija (5, 6). Potvrđeno je da je ta bakterija značajno povezana s neuspjehom liječenja. Iako je ta vrsta otkrivena u 18 % slučajeva primarnih endodontskih infekcija, njezina prevalencija u endodontski liječenim zubima bila je mnogo veća – 67 % slučajeva (7).

Predstavljene su različite otopine za endodontsku iriga- ciju i tehnike dezinfekcije kako bi se smanjio broj bakterija u korijenskim kanalima. Glavni učinak irrigacije jest fizičko i kemijsko uklanjanje organskih i anorganских nečistoća, zara- ženi materijala i ostataka mekoga i tvrdoga tkiva iz korijen- skih kanala. Na taj se način sprijećava nakupljanje i začeplje- nje tim materijalima koji onemogućuju pristup apikalnom dijelu korijenskih kanala. Otopine za endodontsku irrigaciju obično imaju antibakterijska svojstva. Dokazano je da kombi- binirana upotreba otopina za irrigaciju različitoga antimikrob- nog spektra može čak povećati antibakterijsko djelovanje koje ima pojedinačna otopina sinergijskim i/ili dodatnim učin- kom za uklanjanje stotina vrsta mikroorganizama koji se for- miraju u mikroflori korijenskog kanala (5).

Pregledom dosadašnje literature ustanovljen je ograničeni broj studija u kojima se procjenjuje i uspoređuje kombinirana upotreba često korištenih endodontskih otopina za irrigaciju, kad je riječ o njihovim antibakterijskim svojstvima. Pozitivni ili negativni učinci antibakterijskih interakcija postojećih i potencijalnih otopina za irrigaciju posebno će usmjeravati lije- čenje perzistentnih endodontskih infekcija. Zato će razumi- vanje interakcije između otopina za irrigaciju biti od velike pomoći u provedbi najučinkovitijih postupaka liječenja perzi- stentnih infekcija u endodontskiškim slučajevima. Ova studija imala je za cilj istražiti antibakterijsko djelovanje pojedinač- nih i kombiniranih primjena natrijeva hipoklorita (NaOCl), klorheksidina (CHX), etilendiaminetetraacetne kiseline (ED- TA), vodikova peroksida (H₂O₂), BioPure, MTAD-a (Dens- ply, Tulsa Dental, Tulsa, OK, SAD), SmearClea (SC) (Syb- ron Endo, Orange, CA, SAD) i klorova dioksida (ClO₂) na *E. faecalis* i usporediti antibakterijske učinke svih korištenih otopina u studiji.

**Materijali i metode**

Ovo istraživanje započeto je nakon što ga je odobrilo Etičko povjerenstvo Stomatološkog fakulteta Sveučilišta Sel- cuk (dokument br. 203). Korišteno je stotinu i osamdeset jednokorijenskih ljudskih donjih pretkutnjaka (premolara) izvadjenih iz ortodontskih ili parodontskih razloga. Ostaci tvrdoga i mekoga tkiva na zurima očišćeni su kiretama, a za- tim su do laboratorijskih postupaka zubi čuvani na tempera- turu od 4 °C u 100 % vlažnom okružju.

Zubi su dekoronirani dijamanturnim diskom (Ortho Tech- nology Inc., Tampa, FL, SAD) uz vodenje hlađenja do standar- dizirane dužine korijena od 14 ± 0,5 mm. Dužine kanala standardizirane su ručnim instrumentom K-File #15 (Ma- ni Inc., Tochigi, Japan) i proširene su ProTaper univerzal-
rotary files (Dentsply, Tulsa Endodontics, Tulsa, OK, USA) using the crown-down method. The apical parts of the root canals were finished at F3. During the preparations, the root canals were irrigated with 1 ml of 5.25 % NaOCl solution (Caglayan Kimya San., Konya, Turkey).

In order to remove the smear layer of root canal walls, the roots were exposed to an ultrasonic bath (USG 4000 Ultraschall, Dentaurum, Ispringen, Germany) in 17 % EDTA (AppliChem GmbH, Darmstadt, Germany), 5.25 % NaOCl and distilled water for 10 min in each solution, in that order (8). The samples were then embedded perpendicular to the long axes in a silicone impression material (Zetaplus, Zhermack SpA, Badia Polesine (RO), Italy) and placed in metal plates filled with distilled water and capped, 10 in each group, and sterilized at 121° C for 20 min in an autoclave (Hirayama, Saitama, Japan). The metal plates were then opened inside a Biosafety Level 2 (BSL 2) laminar air-flow cabinet and each sample was coated with two layers of nail polish (Loreal Jet-Set Diamond, Paris, France) in order to prevent bacterial leak during the experimental procedures. In order to contaminate the sterilized root canals with *E. faecalis* for experimental purposes, fresh cultures of *E. faecalis* (ATCC 29212) microorganisms were obtained after 24 h of incubation inside a Brain Heart Infusion Broth (BHI) (bioMérieux sa 69280, Marcy l’Etoile, France). The optical density (OD) of the *E. faecalis* suspension inside BHI was adjusted according to McFarland No: 0.5 standard to approximately 1.5 × 10⁶ colony/ml. Except for the negative control group, the *E. faecalis* suspension was planted to the root canals with the help of a sterile 1 ml tuberculin syringe. Then, the samples in the metal plates were incubated at 37° C for 24 h. After incubation, the metal plates were opened in a BSL 2 air-flow cabin and irrigation was performed with the experimental solutions.

A flow chart of the study design is shown in Figure 1. The basic irrigation solutions and their combinations used in the study are presented in Table 1. All solutions except for EDTA were ready to use. The 17 % EDTA solution was prepared in the laboratory according to the instructions of Sen et al. (9).

Each root canal was irrigated with a single or combined irrigation solution for 5 min using 30-gauge endodontic irrigation needles (KerrHawe SA, Bioggio, Switzerland), according to the irrigation regimens indicated in Figure 1. Sterile F3 paper cones were placed into the root canals for 1 min to allow for complete absorption. The paper cones were then put into sterile tubes containing 1 ml BHI Broth and placed into a vortex device (MS 1 Minishaker IKA®, Darmstadt, Germany) in 10 minutu u svakoj otopini, tim redoslijedom (8). Usorci su zatim ugrađeni okomit na uzdužnu os u silikonski otisni materijal (Zetaplus, Zhermack SpA, Badia Polesine (RO), Italija) te stavljeni u metalne ploče napunjene destiliranom vodom i zatvorene, po 10 u svakoj skupini, i 20 minuta sterilizirane na 121 °C u avtoklavu (Hirayama, Saitama, Japan). Zatim su metalne ploče otvorene u kabinu za laminaciju zraka na razini biološke sigurnosti (BSL 2) i svaki je uzorak premazan dvama slojevima laža za nokte (Loreal Jet-Set Diamond, Pariz, Francuska) da bi se spriječilo istjecanje bakteriija tijekom pokusnog procesa. Kako bi se za pokusne svrhe kontaminirali sterilizirani korijenski kanali bakterijom *E. faecalis*, svježe kulture mikroorganizma *E. faecalis* (ATCC 29212) dobivene su nakon 24-satne inkubacije u juhi od moždane supstancije tj. medicinski Brain Heart Infusion Broth – BHI (bioMérieux sa 69280, Marcy l’Etoile, Francuska). Optička gustoća (OG) suspendije *E. faecalis* unutar BHI-ja podešena je u skladu s McFarlandovim No: 0.5 standardom do približno 1,5 × 10⁶ kolonij/ml. Uz negativnu kontrolnu skupinu, suspendija *E. faecalis* postavljena je u korijenske kanale s pomoću sterilne 1 ml tukurbulinske štrcaljke. Zatim su uzorci u metalnim pločama inkubirani 24 sata na temperaturi od 37 °C. Nakon inkubacije metalne ploče su otvorene u kabinu s protokom zraka BSL 2 i irrigacija je obavljena s pokusnim otopinama.

Shema dijagrama dizajnja studije prikazana je na slici 1. Osnovne otopine za irrigaciju i njihove kombinacije korištene u studiji nalaze se u tablici 1. Sve otopine, osim EDTA-e, bile su spremane za upotrebu. 17-postotna otopina EDTA-e pripremljena je u laboratoriju prema uputama Sena i suradnika. (9).

Svaki je korijenski kanal irrigiran jednom ili kombinirano otopinom u trajanju od 5 minuta s pomoću endodontskih igala izložen medij korijenskih kanala (Kerr-Hawe SA, Bioggio, Švicarska) prema protokolima irrigacije navedenima na slici 1. Zatim su u korijenske kanale jednu minuto stavljeni sterilni papirnati štapići F3 da bi se omogu-

Table 1

| The Basic Irrigation Solutions and manufacturers | Manufacturer | Osnovne irigacijske otopine i proizvođači |
|-----------------------------------------------|-------------|--------------------------------------------|
| 5.25 % Sodium hypochlorite (NaOCl) • 5.25-postotni natrijev hipoklorit (NaOCl) | Caglayan Kimya San., Konya/Turkey • Turska |
| 2 % Chlorhexidine gluconate (CHX) • 2-postotni klorheksidin-glukanat (CHX) | Klorhex, Droguin ikač san., Ankara/Turkey • Turska |
| 13.8 % Chlorine dioxide (ClO₂) • 13.8-postotni cloridi dioksid (ClO₂) | BioLenz, Frontier Pharmaceutical, Melville, NY, USA • SAD |
| BioPure MTAD (MTAD) | Dentsply, Tulsa Dental, Tulsa, OK, USA • SAD |
| SmearClear (SC) | Sybron Endo, Orange, CA, USA • SAD |
| 3 % Hydrogen peroxide (H₂O₂) • 3-postotni vodic peroksid (H₂O₂) | Kimya ikač lab. Ve tic. Ltd. sti.; Istanbul, Turkey • Turska |
| 17 % Ethylene Diamine Tetra Acetic Acid (EDTA) • 17-postotna etilendiamintetraoctena kiselina (EDTA) | Prepared in the laboratory • Pripremljeno u laboratoriju |

The antibacterial effects of irrigation solutions against Enterococcus faecalis

Ozkan et al.
many) for 5 min. A 200 µl sample was taken from the shaken medium and transferred to a well in a 96-well sterile ELISA plate (Costar 3599, Corning, NY, USA). Each sample went through this procedure twice for a total of two wells per sample to get averages for the measurements. The plates were then placed in an ELISA reader (BioTek ELx800, Absorbance Microplate Reader, Winooski, VT, USA) to complete the first optic density (OD) test (hour 0) at a wavelength of 450 nm, and the data were recorded. The data were taken every 6 h in the ELISA reader and repeated twice. The plates were placed in the incubator and kept at 37°C in a 100 % moist environment during the experiment. Data were obtained for each sample at hours 0, 6, 12, 18, 24, 30, 36, 42 and 48. The averages of the data collected at each measurement were calculated separately for each group, and an average OD value was determined for each period. Average OD data obtained for each sample at each time period were used, and a time-dependent OD change graph was created for each experimental group (Figure 2.4–10).

For statistical analysis, the Kruskal–Wallis and Mann–Whitney U tests were used to find any significant differences among the study groups. Significance level was accepted as $p>0.05$.

čila potpuna apsorpcija. Nakon toga su papirnati štapići odloženi u sterilne epruvete s 1 mL medija BHI i stavljeni 5 minuta u vibrirajući uređaj (MS 1 Minishaker IKA®, Darmstadt, Njemačka). Uzorak od 200 ul. uzet je iz protresenog medija i premiješten u jažicu u sterilnoj ELISA ploči s 96 jažica (Costar 3599, Corning, NY, SAD). Svaki je uzorak dvaput prošao taj postupak za ukupno dvije jažice po uzorku da bi se dobili prošjeci za mjerenja. Ploče su zatim stavljene u čitač ELISA-e (BioT ek EL x 800, čitač apsorpcijskih mikroplaća, Winooski, VT, SAD) kako bi se završio prvi test opticke gustoće (OG) (sat 0) pri valnoj dužini od 450 nm. Dobiveni podatci su bilježeni. Iz čitača ELISA-e podatci su uzimani svakih 6 sati i ponovljeni dva puta. Tijekom pokusa ploče su stavljene u inkubator i držane na temperaturi od 37 °C u 100 % vlažnom okružju. Podatci su dobiveni za svaki uzorak u razdoblju od 0, 6, 12, 18, 24, 30, 36, 42 i 48 sati. Prosječni podatci dobiveni pri svakom mjerenju izračunati su zasebno za svaku skupinu, a prosječna vrijednost OG-a utvrđena je za svako razdoblje. Korišteni su prosječni OG podatci dobiveni za svaki uzorak za svako razdoblje, a kreiran je vremenski ovisan grafikon promjene OG-a za svaku pokusnu skupinu (slika 2., 4. – 10.)

Za statističku analizu korišteni su Kruskal-Wallisov test i Mann-Whitneyjev U-test kako bi se utvrdile razlike među ispitivanim skupinama. Razina značajnosti prihvaćena je na $p > 0.05$. 
Results

The post-incubation, time-dependent OD values (at 450 nm) of the samples tested for antibacterial activity following single and combined uses of the root canal solutions are shown in Figure 2.

Overall statistical results showed that significant similarity was found between the OD values of samples taken from root canals irrigated with CHX, NaOCl, MTAD, SC and Rezultati

Postinkubacija i OG vrijednosti uzoraka ovisnih o vremenu (na 450 nm) testiranih na antibakterijsko djelovanje nakon pojedinačnih i kombiniranih primjena otopina korijenskog kanala prikazani su na slici 2.

Ukupni statistički rezultati pokazali su da je pronadena značajna sličnost između OG vrijednosti uzoraka uzetih iz korijenskih kanala irigiranih CHX-om, NaOCl-om, MTAD-
ClO₂ and the negative control group (p>0.05). On the other hand, the positive control group showed significant similarities with OD values of the samples taken from the root canals irrigated with H₂O₂ and EDTA (p>0.05).

CHX and CHX combinations

No statistically significant difference was found between the negative control group and CHX and its combinations at all times, including the NaOCl + CHX group, which showed an increase in OD values (Figure 2, 4) (p>0.05).

NaOCl and NaOCl combinations

No statistically significant difference was found between the NaOCl and NaOCl + MTAD groups and the negative control group at all times (p>0.05). The values of samples irrigated with H₂O₂, NaOCl and EDTA + NaOCl at hours 24, 36 and 48 were found to be similar with the positive control group (p>0.05) (Figure 5). The increase in the OD value of the SC + NaOCl group after hour 6 was found to be significantly different from the negative control group after hour 36 (p>0.05) (Figure 5).

ClO₂ and ClO₂ combinations

ClO₂ and all its combinations were found to be significantly similar with the negative control group at all times (p>0.05) (Figure 6).

S druge strane, pozitivna kontrolna skupina imala je značajne sličnosti s vrijednostima OG uzoraka uzetih iz korijenskih kanala irigiranih otopinama H₂O₂ i EDTA (p>0.05).

CHX i njegove kombinacije

Nije pronađena statistički značajna razlika između negativne kontrolne skupine i CHX-a i njegovih kombinacija u svim razdobljima, uključujući skupinu NaOCl + CHX koja je pokazala porast vrijednosti OG-a (slike 2., 4.) (p>0.05).

NaOCl i njegove kombinacije

Nije nađena statistički značajna razlika između skupina NaOCl i NaOCl + MTAD te negativne kontrolne skupine u svakom trenutku (p>0.05). Vrijednosti uzoraka irigiranih otopinama H₂O₂, NaOCl i EDTA + NaOCl tjekom 24, 36 i 48 sati slične su pozitivnoj kontrolnoj skupini (p>0.05) (slika 5.). Pokazalo se da se porast OG vrijednosti u skupini SC + NaOCl nakon jednog sata značajno razlikuje od negativne kontrolne skupine nakon 36 sati (p<0.05) (slika 5.).

ClO₂ i njegove kombinacije

ClO₂ i sve njegove kombinacije vrlo su slične negativnoj kontrolnoj skupini u svakom trenutku (p>0.05) (slika 6.).
MTAD and MTAD combinations

No difference was found between OD values of MTAD and all its combinations and the negative control group (p > 0.05) (Figure 7).

SC and SC combinations

SC, SC + CHX, SC + MTAD and SC + H2O2 were found to be statistically similar with the negative control group at all times (p > 0.05) (Figure 8). A continuous increase was found in the OD values of the groups irrigated with SC + ClO2 and SC + NaOCl at all time periods (Figure 8). While this increase was not found to be statistically significant in the group irrigated with SC + ClO2 (p > 0.05), in the group irrigated with SC + NaOCl, it was found to be significantly different for the measurements at hours 30, 36 and 48 when compared with the negative control group (p < 0.05).

H2O2 and H2O2 combinations

There were no statistically significant differences between the OD values of the groups irrigated with SC + H2O2, H2O2 + ClO2, H2O2 + CHX and H2O2 + MTAD and those of the negative control group (Figure 9). The group irrigated with H2O2 was found to be statistically significantly similar with the positive control group (p > 0.05). The increase in the first 24-hour period in the groups irrigated with H2O2 + EDTA and H2O2 + NaOCl was not statistically significant when compared with the positive control group (p > 0.05). However, the increase continuing at hours 24 and 48 in the groups irrigated with H2O2 + EDTA and H2O2 + NaOCl was significantly different when compared with the positive control group (p < 0.05) (Figure 9).

EDTA and EDTA combinations

The time-dependent increase in OD values of the groups irrigated with EDTA and EDTA + H2O2 was found to be significantly similar with the positive control group (p > 0.05) (Figure 9). The increase observed for the first 6 hours in the EDTA + H2O2 group was found to be significantly similar with the negative control group (p > 0.05), while that increase was found to be significantly similar with the positive control group at hours 6 and 24 (p > 0.05). In the remaining periods, the increase in the OD values was found to be significantly different from those of the positive control group (p < 0.05). The OD values of the group irrigated with EDTA + ClO2 were found to be similar with the negative control group at all times (p > 0.05) (Figure 10). When the time-dependent OD value of EDTA + NaOCl was compared with the negative control group, it was found to be significantly different after hour 18 (p < 0.05) (Figure 10). However, no statistically significant difference was observed between the groups irrigated with EDTA + CHX and EDTA + MTAD and the negative control group (p > 0.05) (Figure 10).

Discussion

In this study, the bacterial growth in samples from infected root canals irrigated with antibacterial irrigation solutions was compared with normal bacterial growth (positive control group), and bacterial growth from when the sterilized roots were exposed to irrigation solutions (negative control group). The antibacterial effects of irrigation solutions against Enterococcus faecalis Ozkan et al. (2013) were evaluated. The OD values of the group irrigated with EDTA + ClO2 was found to be significantly different when compared with the positive control group (p < 0.05) (Figure 9). In Figure 7, it was significantly different when compared with the negative control group (p < 0.05). The group irrigated with SC + ClO2, H2O2 + EDTA and H2O2 + NaOCl was found to be significantly similar with the positive control group (p > 0.05) (Figure 8). A continuous increase was found in the OD values of the groups irrigated with SC + ClO2 and SC + NaOCl at all time periods (Figure 8). While this increase was not found to be statistically significant in the group irrigated with SC + ClO2 (p > 0.05), in the group irrigated with SC + NaOCl, it was found to be significantly different for the measurements at hours 30, 36 and 48 when compared with the negative control group (p < 0.05).

Rasprava

U ovom istraživanju je rast bakterija u uzorcima iz zaraženih kanala korijena koji se vlaže antibakterijskim otopinama za irrigaciju uspoređen s normalnim rastom bakterija (pozitivna kontrolna skupina) i rastom bakterija nakon inkubacij
were incubated (negative control group). During the incubation period, it was clearly determined in which period the reproduction occurred, slowed down and regressed.

The antibacterial activity of irrigation solutions is known to increase with the increase in volume and application time of the irrigation (10, 11). In this study, the standard irrigation application time was determined to be a total of 5 min of irrigation with a 5 ml solution for all canals. In combined uses, the total volume used was 2.5 ml + 2.5 ml for each solution. For MTAD, the manufacturer recommended an application regimen of 5 ml for each canal. Thus, MTAD also used the same volume and time interval as the other solutions, making all solutions in the experiment comparable. All of the root canals were also irrigated at a post-experiment stage with 5ml saline to achieve maximum dilution of the residual solution. In this way, the impact of the transferred solution on the medium was minimized and the residual antibacterial effect was reduced.

In this study, the order of the solution application recommended by researchers was used (12, 13). It is acknowledged that a residual antimicrobial effect might still be present unless the activity of the solution is neutralized by means of an inactivator (14). However, to be able to standardize the experiments undertaken in this study, neutralization of the test solutions was not carried out. One of the other reasons for this is that some of the test solutions, such as MTAD and SC, are proprietary products and there are no chemical inactivators known to exist for them. Therefore, to achieve uniformity in the experiment methodology and to make it an easier procedure to compare the relatively high number of solutions, none of the test specimens were inactivated.

It has been reported by many researchers that CHX shows antibacterial activity against E. faecalis (15, 16, 17). In accordance with other studies, no significant increase in the OD values of CHX and its combinations (except NaOCl + CHX) were observed in this recent study. However, NaOCl + CHX showed less antibacterial activity with increased OD values. This may be due to the orange-colored residue made of parachlorophenol (PCU) or chloropenfluguanidil-1,6-diguanidil-hexaze (PCGH), which can be obtained when CHX and NaOCl are combined (18, 19). These residues may have had negative effects on the interaction between the root filling and the canal wall dentin by blocking dentin tubules (18, 20). In order to prevent these solution's interaction, it is recommended to irrigate the root canals with saline, sterile distilled water or alcohol before irrigation with CHX, and NaOCl left in the canal can be aspirated with a needle, dried with paper cones or ultrasonic activation with EDTA (20,21,22). However, in the present study, the recommended processes to prevent the interaction of these two solutions mentioned above were not used because of their variations in the experimental procedure. Another important issue noted with the NaOCl + CHX group is that the antibacterial activity did not decrease in the first 12 hours but did decrease after hour 12 (Figure 4). This may be because of the residue that accumulated on the root canal while taking the sample, and/or the possibility of PCU and PCGH being toxic to E. faecalis and the decrease observed in these effects at the end of hour 12, je steriliziranih korijena (negativna kontrolna skupina). Tijekom razdoblja inkubacije bilo je jasno određeno u kojem se razdoblju reprodukcija događala, uspiorila i nazadovala.

Poznato je da se antibakterijska aktivnost otopina za irrigaciju povećava s povećanjem volumena i vremenom primjene irrigacije (10, 11). U ovoj je studiji utvrđeno da standard irrigacije uključuje vrijeme od ukupno 5 minuta s 5 mL otopine za sve kanale. U kombiniranim otopinama ukupni upotrijebljeni volumen bio je 2,5 mL + 2,5 mL za svaku otopinu. Za MTAD je proizvođač preporučio primjenu od 5 mL za svaki kanal. Zato je za MTAD također korišten jednaki interval volumena i vremena kao i za ostale otopine, čineći tako sve otopine u pokušu usporedivim. Svi se korijenski kanali također irrigiraju u fazi nakon pokusa s 5 mL fiziološke otopine kako bi se postiglo maksimalno razrjeđivanje zaostale otopine. Na taj način utjecaj prenesene otopine na medij sveden na minimum, a zaostali antibakterijski učinak je smanjen.

U ovom istraživanju korišten je redoslijed primjene otopina koji su preporučili istraživači (12, 13). Površeno je da je zaostali antimikrobički učinak još prisutan ako se aktivnost otopine ne neutralizira inaktivatorima (14). No kako bi se mogli standardizirati pokusi provedeni u ovom istraživanju, nije obavljena neutralizacija ispitanih otopina. Jedan od razloga jest i to što su neke ispitane otopine, poput MTAD-a i SC-a, zaštićeni proizvodi za koje nije poznato postoje li kemikalni inaktivatori. Zato, kako bi se postigla jedinstvenost u metodologiji pokusa i olakšao proces za usporedu razmjerno velikog broja otopina, nijedan testni uzorak nije bio inaktiviran.

Mnogi su istraživači istaknuti da CHX pokazuje antibakterijsko djelovanje na E. faecalis (15, 16, 17). U skladu s drugim studijama, u ovom nedavnom istraživanju nije uočeno značajno povećanje OG vrijednosti za CHX i njegove kombinacije (osim NaOCl + CHX). No NaOCl + CHX pokazao je manje antibakterijske aktivnosti s povećanim vrijednostima OG-a. To se može dogoditi zbog narančasto obojenih ostataka izgrađenih od paraklorofenola (PCU) ili kloropenfluguanidil-1,6-diguanidil-heksaza (PCGH) koji su mogli dobiti kada se kombiniraju CHX i NaOCl (18, 19). Ti ostaci mogu negativno utjecati na interakciju između korijenskog punjenja i dentinskih stijenki kanala te blokirati dentinske tubule (18, 20). Kako bi se spriječila interakcija tih otopina preporučuje se neutralizacija ispitanih otopina fiziološkom otopinom, sterilnom destiliranom vodom ili alkoholom prije irrigacije CHX-om, a NaOCl koji je ostavljen u kanalu može se aspirirati iglom, osušiti papirnatim štapićima ili ultrazvučnom aktivacijom.

Kako bi se spriječila interakcija tih otopina preporučuje se neutralizacija ispitanih otopina fiziološkom otopinom, sterilnom destiliranom vodom ili alkoholom prije irrigacije CHX-om, a NaOCl koji je ostavljen u kanalu može se aspirirati iglom, osušiti papirnatim štapićima ili ultrazvučnom aktivacijom. No u ovoj studiji preporučeni postupci za spriječavanje interakcije spomenutih otopina nisu korišteni zbog njihovih varijacija u pokusnom postupku. Drugo što je važno, a zabilježeno je kod skupine NaOCl + CHX je da se antibakterijska aktivnost nije smanjila u prvih 12 sati, nego nakon 12 sati (sljedeća figura 4.). Razlog mogu biti ostaci koji su nakupljeni u korijenskom kanalu tijekom uzimanja uzorka i/ili mogućnosti da su PCU i PCGH toksični za E. faecalis te smanjenje opaženo nakon 12 sati.

Kako nije utvrđeno povećanje OG vrijednosti u skupini EDTA + CHX tijekom vremena (p > 0,05), ta kombinacija može učinkovito antibakterijski djelovati na E. faecalis. Liu
Since no increase was found in the EDTA + CHX group's OD values over time (p<0.05), this combination has the potential to show effective antibacterial activity against *E. faecalis*. Liu et al. (23) reported that the combined use of CHX and EDTA had antibacterial activity and it was also better than MTAD and EDTA + NaOCl. The present study also supports these results. Gonzalez-Lopez et al. (24) observed a pink-colored residue when CHX and EDTA were mixed. In their study, Rasmick et al. (25) showed a white-colored salt when these two materials were mixed. Later, Prado et al. (21) examined the interaction of CHX and EDTA and observed a milky residue and concluded that this was a result of an acid-base reaction of the combination. The results of the present study confirmed that the white and/or salt residue formed by these two materials does not negatively affect the antibacterial activity of CHX in infected root canals.

No studies have been conducted so far on whether ClO₂ + CHX shows antibacterial activity against *E. faecalis*. According to the results of the present study, ClO₂ + CHX is effective in the elimination of *E. faecalis*. These two agents do not form any reactions nor do they negatively influence each other (26, 27) and they maintain their antibacterial activity at all times when used in combinations (Figure 4,6).

Although it has been reported in a great number of studies that MTAD has antibacterial activity against *E. faecalis* (23, 28), no studies have been found comparing the antibacterial characteristics of CHX + MTAD to MTAD. The results of this study showed that the CHX + MTAD and MTAD groups have similar antibacterial activities against *E. faecalis* (p>0.05) (Figure 7). The CHX + MTAD combination did not cause any negative change in the antibacterial characteristics of either solution. This important characteristic can be evaluated with further studies not only on *E. faecalis* but also on the elimination of other bacteria that are also responsible for endodontic infections.

In this study, an increase was found in the OD value of the H₂O₂ + NaOCl group after 12 hours, and this increase was found to be different from the negative control group's (p<0.05) (Figure 5,9). The combined use of these two solutions has been recommended to ease organic and inorganic debris from the root canal, and it has disinfecting and whitening properties through its foaming effect (29). The antibacterial activity of an irritant increases as its volume increases (30). Since, NaOCl's volume in combined use is less than MTAD and EDTA + NaOCl, the antibacterial activity in the EDTA + NaOCl group was found to be less than the activity in the SC + NaOCl group. The reason for the decrease in antibacterial activity may be suradnici (23) pokazali su da kombinirana primjena CHX-a i EDTA-e ima antibakterijsko djelovanje, te da je također uspješnija od kombinacije MTAD-a i EDTA + NaOCl. Ova studija također podupire te rezultate. Gonzalez-Lopez i suradnici (24) uočili su ostatak ružičaste boje kada su se pomijesali CHX i EDTA-a. U svojoj studiji su Rasmick i suradnici (25) istaknuli da su dobili sol bijele boje kada su mijesali ta dva materijala. Poslije su Prado i suradnici (21) ispitali interakciju CHX-a i EDTA-e i uočili mlijecnio ostatak te zaključili da je to rezultat kiselo-bazne reakcije kombinacije. Rezultati ove studije potvrdili su da bi biljkast ili sol stvorena s tim dvama materijalima ne utječe negativno na antibakterijsko djelovanje CHX-a u zaraženim korijskim kanalima.

Do sada nisu provedena ispitivanja djeluje li ClO₂ + CHX antibakterijski na *E. faecalis*. Prema rezultatima ove studije, ClO₂ + CHX učinkovito uklanjanju tu bakteriju. Ta dva sredstva ne stvaraju nikakvu reakciju, niti negativno utječu jedno na drugo (26, 27) i zadržavaju svoje antibakterijsko djelovanje u svakom trenutku kada se koriste u kombinacijama (slika 4., 6.).

Iako je u mnogim studijama objavljeno da MTAD antibakterijski djeluje na *E. faecalis* (23, 28), nisu pronađena istraživanja u kojima su autori uspoređivali antibakterijsku svojstva CHX + MTAD-a i MTAD-a. Rezultati ove studije pokazali su da skupine CHX + MTAD i MTAD imaju slične antibakterijske aktivnosti kad je riječ o *E. faecalis* (p > 0,05) (slika 7.). Kombinacija CHX + MTAD nije uzrokovala niskove negativne promjene u antibakterijskim karakteristikama ni u jednoj otopini. Ovo važno svojstvo može se procijeniti u daljnjim istraživanjima ne samo na *E. faecalis*, nego i u uklanjanju drugih bakterija koje su također odgovorne za endodontske infekcije.

U ovom istraživanju prikazano je povećanje vrijednosti u skupini OG H₂O₂ + NaOCl nakon 12 sati, a utvrđeno je da se taj porast razlikuje od onoga u negativno kontrolnoj skupini (p < 0,05) (slike 5., 9.). Preporučuje se kombinirana upotreba tih dviju otopina za ublažavanje organskih i anorganskih nečistoća iz korijenskog kanala, a kako se ipak, imaju svojstva dezinfekcije i izbjeljivanja (29). Antibakterijsko djelovanje irigansa povećava se s povećanjem volumna (30). Budući da je volumen natrijeva hipoklorita u kombiniranoj uporabi manji negoli kada se koristi sam, očekuje se smanjenje antibakterijske aktivnosti. U ovoj studiji također je utvrđeno nedovoljno antibakterijsko djelovanje H₂O₂. Za to se nagadalo da kombinirana primjena tih dviju otopina ne pridonosi pozitivno antibakterijskim svojstvima otopina. Drugi razlog za smanjene antibakterijske aktivnosti može biti smanjenje hidrokislinskih radikala, što zapravo stvara antibakterijsko djelovanje reakcijom dviju otopina (31).

U skupinama EDTA + NaOCl i SC + NaOCl utvrđeno je vrhunski ovisno povećanje u mjerenjima nakon 6 sati (slika 5.). U usporedbi s negativnom kontrolnom skupinom, istaknuto je da je antibakterijska aktivnost u skupini EDTA + NaOCl manja od one u skupini SC + NaOCl. Razlog za to može biti negativni učinak EDTA-e na tkivno otapanje natrijeva hipoklorita i smanjenje količine aktivnoga klora u kombinaciji (32).
related to EDTA's negative effect on the tissue-dissolving capacity of NaOCl and decrease in the amount of active chlorine in the combination (32).

It has previously been reported that NaOCl + MTAD is successful in E. faecalis elimination (33). Tay et al. (34) reported a brown liquid developed when NaOCl and MTAD were combined and that this combination decreased the dentin substantivity of MTAD (35). For these reasons, it has been reported that the canals need to be irrigated in intervals with saline. In the present study, no decrease was found in the combination's antibacterial activity. Thus, the results of the current study support Shabahang and Torabinejad's study (13), which states that the antibacterial effect of the combination of 1.3% NaOCl and MTAD is an effective solution in eradicating E. faecalis.

ClO₂ is a strong oxidizing agent, and it effectively kills pathogenic microorganisms (36). ClO₂ actually has smear layer removing characteristics and the capacity to dissolve organic tissue (37–42). In this study, it was found that when used alone and in combination with other solutions, ClO₂ showed similar antibacterial characteristics with NaOCl and its combinations against E. faecalis. However, ClO₂ and the SC + ClO₂, EDTA + ClO₂, H₂O₂ + ClO₂, and ClO₂ + CHX combinations showed relatively higher antibacterial characteristics compared to the NaOCl combinations (Figure 6). These results are parallel to Eddy et al.'s findings (37), which stated 10 % and 13.8 % Chlorine dioxide and 5.25 % NaOCl were both effective in eliminating E. faecalis from the dentinal disks within 30 min.

According to the results of the present study, a fair amount of increase was found in the OD values of the EDTA + ClO₂ and SC + ClO₂ groups; however, this increase was found to be statistically similar to the negative control group (p>0.05) (Figure 6). This increase in the OD levels of the EDTA + ClO₂ group may be due to the decrease in antibacterial activity as a result of the ClO₂-oxidizing effect against EDTA (26). In addition, this increase in the OD values of the samples treated with SC+ClO₂ was found to be lower than that of EDTA + ClO₂ (Figure 6). This may be due to the antibacterial characteristic of the surface-active agent (cetrimide) in SC (43). Since there are no studies researching the antibacterial activity of SC + ClO₂ combinations, further studies are needed to explain the reasons for this increase.

There was a continuous increase in the OD values of the SC + ClO₂ and SC + NaOCl groups in the OD measurements after hour 6 (Figure 8). While the SC + ClO₂ group's increase was found to be significantly similar to the negative control group's (p>0.05) at all hours (Figure 8), the measurements at hours 30, 36 and 48 in the SC + NaOCl group were found to be significantly higher than those of the negative control group's (p>0.05) (Figure 8). Thus, SC + ClO₂ showed better antibacterial activity than SC + NaOCl. This may be lower inhibitory effect of SC on ClO₂ than the inhibitory effect of NaOCl.

A time-dependent increase was found in the EDTA + H₂O₂ group. Although the increase in the OD values of EDTA + H₂O₂ within the first 18 hours was significantly similar to the positive control group (p>0.05), it was significant.

Prieve is objavljeno da je NaOCl + MTAD uspješan u uklanjanju E. faecalis (33). Tay i suradnici (34) ističu da je smeh da tekućina nastala kada su kombinirani NaOCl i MTAD te da je ta kombinacija smanjila dentinsku vezu MTAD-a (35). Iz tih je razloga objavljeno da se kanali trebaju u intervalima irrigirati fiziološkom otopinom. U ovoj studiji nije uočeno smanjenje antibakterijskog djelovanja te kombinacije. Dakle, rezultati ove studije podupiru Shabahangovu i Torabinejadovu studiju (13) u kojoj se ističe da je antibakterijski učinak kombinacije 1,3-postotnoga natrijeva hipoklorida i MTAD-a učinkovito rješenje u iskorjenjivanju E. faecalis.

ClO₂ snažno je oksidacijsko sredstvo i učinkovito ubija patogene mikroorganizme (36). Zapravo ima svojstva uklanjanja zaostatog sloja i otapanja organskoga tkiva (37 – 42). U ovoj studiji ustanovljeno je da, kada se koristi sam i u kombinaciji s drugim otopinama, ClO₂ ima slična antibakterijska svojstva kao i NaOCl i njegove kombinacije kad je riječ o E. faecalis. No kombinacije ClO₂ i SC + ClO₂, EDTA + ClO₂, H₂O₂ + ClO₂ i ClO₂ + CHX pokazale su razmjerno bolja antibakterijska svojstva u usporedbi s kombinacijama u koji-ma je NaOCl (slika 6.). Ti su rezultati jednaki kao i u izve-sčima Eddyja i ostalih (37) koji su izjavili da su 10-postotni i 13,8-postotni klorov dioksid te 5,25-postotni NaOCl dje-lovorni u uklanjanju E. faecalis iz dentinskih diskova u ro-ku od 30 minuta.

Prema rezultatima dobivenima u ovoj studiji, ustanov-ljen je priličan porast vrijednosti OG-a u skupinama EDTA + ClO₂ i SC + ClO₂ no ustanovljeno je da je taj porast statistički sličan negativnoj kontrolnoj skupini (p > 0.05) (slika 6.). To povećanje razine OG-a u skupini EDTA + ClO₂ može biti posljedica smanjenja antibakterijskih aktivnosti kao rezultat učinka oksidacije ClO₂ na EDTA-u (26). Uz to, utvrđeno je da je to povećanje OG vrijednosti uzorak tretiranih s otopinom SC + ClO₂ niže od onih tretiranih otopinom EDTA + ClO₂ (slika 6.). To može biti posljedica antibakterijskih svoj-stava površinski aktivnoga agensa (cetrimida) u SC-u (43). Budući da ne postoje studije u kojima je istraženo antibakte-rijsko djelovanje kombinacija SC + ClO₂, potrebne su dodat-na istraživanja za objašnjenje toga porasta.

Zabilježeno je kontinuirano povećanje OG vrijednosti u skupinama SC + ClO₂ i SC + NaOCl u OG mjerenjima nakon 6 sati (slika 8.). Dok je uočeno da je porast u skupini SC + ClO₂ vrlo sličan negativnoj kontrolnoj skupini (p > 0.05) u svim satima (slika 8.), rezultati mjerenja poslije 30, 36 i 48 sati u skupini SC + NaOCl bili su značajno veći od onih u negativnoj kontrolnoj skupini (p < 0.05) (slika 8.). Zato je kombiniranje SC + ClO₂, pokazalo bolje antibakterijsko djelovanje od onoga u skupini SC + NaOCl. To može biti posljedica nižega inhibicijskog učinka SC-a na ClO₂ od inhibi-cijskog učinka natrijeva hipoklorita.

Nadalje, utvrđeno je povećanje ovisno o vremenu u sku-pini EDTA + H₂O₂. Iako je porast OG vrijednosti EDTA + H₂O₂ u prvih 18 sati bio sličan pozitivnoj kontrolnoj skupini (p > 0.05), značajno se razlikovalo od pozitivne kontrolne skupine nakon 30 sati (p < 0.05) (slika 9.). Razlog za porast u toj skupini može biti medij stvoren kombinacijom tih oto-pina jer je bio pogodan za rast E. faecalis. No potrebne su do-datne studije za potvrdu tih rezultata.
ly different from the positive control group after hour 30 (p<0.05) (Figure 9). The reason for the increase in this group may be because the medium created with the combination of these solutions was a suitable medium for E. faecalis to grow. However, further studies are needed to confirm these results.

Conclusions

Within the limitations of this study, the following conclusions can be drawn:

EDTA is not a suitable solution for providing antibacterial activity in the irrigation process of root canals. Although it did not have any negative effects in combinations with CHX and MTAD, combined uses of EDTA and H₂O₂ solutions may be harmful for disinfecting the root canals since they can potentially provide a medium for bacteria reproduction; Chelation agents, such as EDTA and SC, can be used for the removal of the smear layer from the root canal walls. However, their application together with NaOCl reduces the antibacterial effect of these agents. For these reasons, additional irrigation with another irrigation solution and/or solution combination may be suggested; ClO₂ is chemically similar to NaOCl and when used in combination with H₂O₂, CHX, EDTA or SC, its antibacterial activities are less affected than with combinations of NaOCl. Therefore, ClO₂ with its positive properties, may be considered as an alternative to NaOCl and suitable for routine clinical use; Overall it can be concluded that the SC + CHX combinations can be recommended as the most effective irrigation regimen against E. faecalis in persistent endodontic infections.

Conflict of Interest

The authors report no conflict of interest

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Contribution to the paper

H. B. O. Carried out the study experiments, collected the data, wrote and edited the manuscript; F. K. C. Contributed to experimental design of the study, helped with analysis of the data, interpreted and discussed the results; Z. S. Contributed to experimental design of microbiological part of the study, commented on the manuscript at the microbiological stages; F. O. Contributed to research idea, commented on the manuscript at all stages, critically proof read the manuscript and gave final approval for publication.

Zaključci

Uz ograničenja u ovoj studiji mogu se izvući sljedeći zaključci:

EDTA nije prikladna otopina za postizanje antibakterijske aktivnosti pri irrigaciji korijskih kanala. Iako nije imala negativnih učinaka u kombinaciji s CHX-om i MTAD-om, kombinirana uporaba otopina EDTA i H₂O₂ može štetiti u dezinfekciji korijskih kanala zato što mogu stvoriti medij za razmnožavanje bakterija; Sredstva za kelaciju, poput EDTA-e i SC-a, mogu se upotrijebiti za uklanjanje zaostatnog sloja sa židova korijskog kanala. No njihova primjena zajedno s natrijevom hipokloritom smanjuje njihov antibakterijski učinak. Zato se može predložiti dodatna irrigacija drugom otopinom i/ili kombinacija otopina; ClO₂ je kemijski sličan natrijevom hipokloritom i kada se koristi u kombinaciji s H₂O₂, CHX-om, EDTA-om ili SC-om, a njihovo antibakterijsko djelovanje manje je negoli kod kombinacija s natrijevim hipokloritom Zato se ClO₂, sa svojim pozitivnim svojstvima, može smatrati alternativom natrijevom hipokloritu i prikladnim za rutinsku kliničku upotrebu; Sveukupno se može zaključiti da se kombinacija SC + CHX može preporučiti kao najbolji alternativ za razboro endodontskih infekcija.

Sukob interesa

Autori ističu da nisu bili u sukobu interesa.

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Doprinosi autora

H. B. O. – obavljala je eksperimente, prikupljala podatke te napisala i uredila rukopis; F. K. C. – pridionila je eksperimentalnom oblikovanju studije, pomagala je pri analizi podataka, interpretirala i raspravljala o rezultatima; Z. S. – pridonio je eksperimentalnom oblikovanju mikrobiološkog dijela studije, komentirao rukopis u mikrobiološkoj fazi; F. O. – surađujući u istraživačkoj ideji komentirao je rukopis u svim fazama, kritički je pročitao rukopis i dao konačno odborenje za objavljivanje.
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