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RESEARCH

Effect of ethylenediaminetetraacetic, etidronic, and peracetic acids with different concentrations on the removal of *Enterococcus faecalis* biofilms from root canal walls: an *in vitro* study

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Abstract

E. faecalis is one of the most important microbiological factors responsible for the failures after root canal treatment. The knowledge about the efficacy of HEBP and PAA with different concentrations, when they are used alone or associated with NaOCl on E. faecalis biofilms is limited. In the present study, the efficacy of 17% ethylenediaminetetraacetic acid (EDTA), 9% and 18% etidronic acid (HEBP), and 1% and 2% peracetic acid (PAA), used alone or in combination with sodium hypochlorite (NaOCl), was evaluated on dentine discs experimentally infected with Enterococcus faecalis biofilms using a laser scanning confocal microscope. Irrigation of the samples using HEBP solutions in combination with NaOCl decreased the viability of E.faecalis more effectively compared with the sole use of 9% HEBP and 18% HEBP solutions. The use of two different concentrations of peracetic acid (1–2%) and etidronic acid (9–18%) resulted in similar biofilm elimination when these chelators were used alone or in combination with NaOCl. The sole use of 2% peracetic acid in the experimentally infected samples eliminated more biofilm than the use of 9% and 18% HEBP. Lower concentrations of peracetic acid and etidronic acid can be recommended to remove the E.faecalis biofilms from root canals to decrease the irrigation solutions' potential harmful effects.

Keywords Etidronic acid · E.faecalis · Biofilm · Peracetic acid · Root canal

Introduction

The primary goal of endodontic treatment is to eliminate the microorganisms from the root canal system, provide conditions that will not allow microbial proliferation, and obtain periradicular tissue healing (Siqueira and Roças 2008; Jhajharia et al. 2015). The microorganisms in the infected root canal system exist in planktonic or biofilm formation (Lin et al. 2013). Bacteria resistant to root canal treatment procedures are commonly located in the biofilm structures and firmly adhere to each other and root canal walls (Lin et al. 2013; Sonja et al. 2013). *E. faecalis*, a facultative anaerobic gram-positive coccus, is the most frequent resistant species isolated from root canal biofilms (Liu et al. 2010; Sedgley et al. 2005). This bacteria can survive after conventional chemomechanical procedures due to its adaptation capacity to hard environmental changes and invasion ability to dentinal tubules (Ricucci and Siqueira 2010; Parolia et al. 2021). Therefore, the *E. faecalis* biofilm model is generally used in studies that evaluate the different irrigation and disinfection protocols regarding their antibiofilm capacity (Kishen and Haapasalo 2010).

Irregular areas of the root canal system, such as lateral canals, apical ramifications, and isthmuses that are difficult to access with mechanical instrumentation, are convenient regions for bacterial biofilms (Nair et al. 2005). Therefore, the efficacy of irrigation and disinfection protocols is significant in eliminating biofilms from the root canal system. Sodium hypochlorite (NaOCl) is the most widely used irrigation solution in root canal treatment due to its superior antimicrobial effect

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and organic tissue dissolving ability (Mohammadi 2008). However, due to its limited capacity to remove inorganic compounds from the smear layer from root canal walls, it is recommended to use NaOCl with a decalcifying agent for successful irrigation (Boutsioukis and Ariaz-Moliz 2022). For this purpose, ethylenediaminetetraacetic acid (EDTA) is the most widely used chelating agent combined with NaOCl. However, EDTA has a limited ability to remove the smear layer from the apical third of the root canal, and it decreases the tissue-dissolving and antimicrobial effect of NaOCl in combined use (De-Deus et al. 2008; Nogo Zivanovic et al. 2019). For these reasons, different chelating agents that will not reduce the antibacterial and tissue-dissolving capacities of NaOCl have been investigated.

1-hydroxyethylidine-1, 1-bisphosphonate, also known as etidronic acid (HEBP), is a weak chelating agent. Etidronic acid is a bisphosphonate that does not have toxic effects on tissues and is used in personal cleaning products and swimming pools as a disinfectant due to its antimicrobial properties (Russell and Rogers 1999). In recent years, etidronic acid has been suggested as an alternative chelating agent to EDTA that shows minimal interaction with NaOCl (Arias-Moliz et al. 2016). This solution has also been recommended to be used together with NaOCl as a single irrigation solution (Villalta-Briones et al. 2021). This mixture (NaOCl + HEBP) was suggested to remove the smear layer efficiently and provide sufficient tissue-dissolving and antimicrobial ability in the root canal (Girard et al. 2005).

Peracetic acid (PAA) is another weak chelating agent with antimicrobial activity against spores, viruses and fungi (Lensing and Oei 1985). Acetic acid released from peracetic acid solution can form water-soluble compounds with calcium (Lottanti et al. 2009). It has previously been reported that peracetic acid has a similar smear layer removal ability to EDTA (Ulusoy et al. 2017).

Many methods are being used to examine the antimicrobial effects of the irrigation protocols on in vitro and ex vivo biofilms (Boutsioukis et al. 2022). Confocal laser scanning microscope (CLSM) is a novel technique assessing root canal bacteria's viability, location, and distribution (Flach et al. 2016). There are research studies in the literature evaluating the effect of different irrigation protocols on the *E.faecalis* biofilms (Arias-Moliz et al. 2016; Flach et al. 2016). However, there is limited knowledge regarding the effect of concentrations of the irrigation solutions on their antimicrobial capacity (Briseño-Marroquín et al. 2022). In addition, there is no consensus about the influence of interactions between the irrigants on eliminating biofilms from the root canal system. This study aimed to determine the effects of 17% ethylenediaminetetraacetic acid (EDTA), 9% and 18% etidronic acid (HEBP), and 1% and 2% peracetic acid (PAA) used alone or in combination with NaOCl on *Enterococcus faecalis* biofilms by using a laser scanning confocal microscope. The null hypothesis tested was that there is no difference between the irrigation protocols regarding elimination of *E.faecalis* biofilms from root canal dentinal walls.

Methodology

This study was approved by the institutional ethics committee (No: 21071282-050.99-). Freshly extracted human premolar teeth with a single root and one root canal were selected. Teeth with caries, cracks, fractures, calcified root canals, immature apices, root resorption, and previous endodontic treatment were excluded. The presence of a single canal was confirmed with periapical radiographs. One hundred and fifteen teeth meeting these criteria were stored in sterile water at 4°C before the experiment.

Preparation of dentine discs and generation of *E. faecalis* biofilms

The residual tissues on the root surfaces of the teeth were cleaned using periodontal scalers. The teeth were separated from their crowns under water cooling, remaining with a root length of 13 mm. Each root was sectioned horizontally at the middle third to obtain one slice with a thickness of 3 mm using a precision saw (Isomet, Buehler, NY, USA) (Fig. 1). The thickness of each slice was confirmed using a digital caliper.

The root canal space of the dentine slices was enlarged to a size of 1 mm using a #2 peeso reamer (Mani, Tochigi, Japan). The dentine discs were irrigated using 17% EDTA solution (Wizard, Rehber Chemistry, Turkey) for 1 min to remove the smear layer. They were then washed with 2.25% sodium hypochlorite (NaOCl) (Wizard). The action of NaOCl was neutralized with 5% sodium thiosulfate. The specimens were sterilized using ethylene oxide. *Enterococcus faecalis* ATCC #29,212 standard strain was produced by incubation at 37°C for 24 h in petri dishes containing tryptic soy agar (Merck, Darmstadt, Germany) (TSA). Purity controls were performed on the produced colonies. The produced cell suspension was then adjusted in 100 × 15 mm sterile glass tubes containing 10 mL tryptic soy broth (TSB) (Merck) to match the turbidity of 1.5×10^8 colony forming unit (CFU/mL) equivalent to 0.5 McFarland standards. The determined number of sterile dentine discs in each group was placed on sterile polystyrene

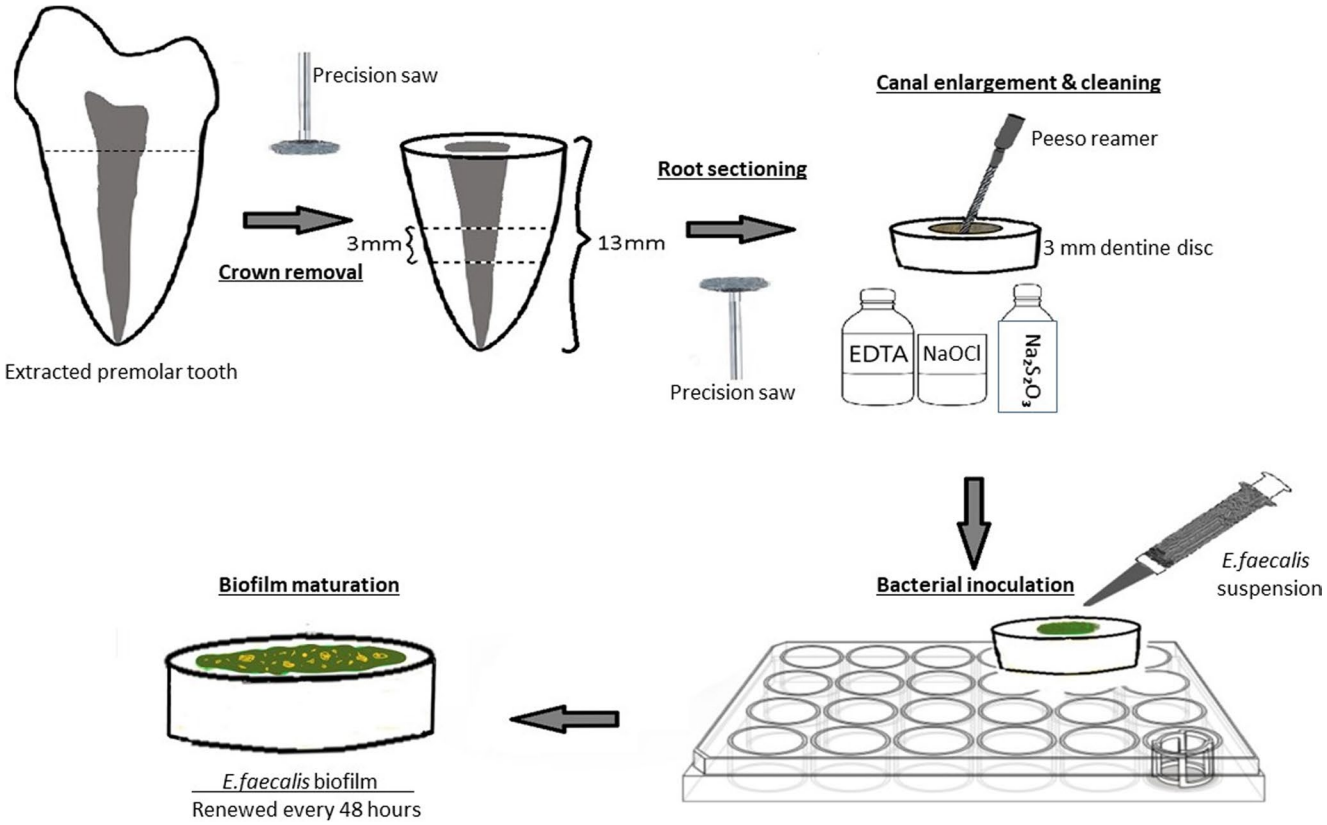


Fig. 1 Schematic illustration summarizing the preparation of dentine discs and biofilm maturation

24-well cell culture plates for biofilm development. Sterile pipettes transferred 100 µL of the *E. faecalis* suspension into the dentine discs. Then, 2 mL of TSB growth culture was added to cover the whole surface of the prepared dentine disc. One hundred µL of 5% sucrose concentration was added to the dentine discs. The dentine specimens were incubated at 37 °C in an aerobic atmosphere for seven days. After 24 h, the samples were checked for bacterial growth. The specimens were replenished with *E. faecalis* biofilm every 48 h to manage biofilm formation and supply nutrients to the bacteria ensuring their survival (Fig. 1).

Treatment of the infected dentine discs with different irrigation protocols

One hundred fifteen infected dentine samples were randomly divided into 11 experimental ($n=10$) and one control group ($n=5$) according to the irrigation regimen:

Group 1 [6 mL 2.5% sodium hypochlorite (NaOCl)]

2.5% NaOCl was obtained by mixing 5.25% NaOCl (Wizard, Rehber Chemistry, Istanbul, Turkey) with water in equal proportions. The solution was applied manually using a disposable dental syringe (Ultradent Products, South Jordan, UT, USA) and a 27-G needle (NaviTip) for 2 min without any activation.

Group 2 [6 mL 17% ethylene diamine tetraacetic acid (EDTA)]

17% EDTA (Wizard, Rehber Chemistry, Istanbul, Turkey) was purchased from a commercial source at the indicated concentrations. The solution was applied manually for 2 min as described above.

Group 3 [6 mL 1% peracetic acid (PAA)]

A 36–40% peracetic acid solution (Code: 433241) supplied from a company (SigmaAldrich, St Louis, MO) was diluted with non-ionized water to obtain a solution with a weight/volume ratio of 1%. This solution was stored in the refrigerator at +4 °C and warmed to room temperature before use. The solution was applied manually for 2 min as described above.

Group 4 [6 mL 2% peracetic acid (PAA)]

A 36–40% peracetic acid solution (Code: 433241) (SigmaAldrich, St Louis, MO) was diluted with non-ionized water to obtain a solution with a weight/volume ratio of 2%. This solution was stored in the refrigerator at +4 °C and warmed to room temperature before use. The solution was applied manually for 2 min as described above.

Group 5 [6 mL 9% etidronic acid (HEBP)]

Aqueous 60% HEBP solution (Code: H6773) was obtained from a commercial market (SigmaAldrich, St Louis, MO). It was mixed with ultrapure water with a weight/volume ratio of 9% and stored at room temperature in a glass bottle until use. The solution was applied manually for 2 min as described above.

Group 6 [6 mL 18% etidronic acid (HEBP)]

Aqueous 60% HEBP solution (Code: H6773) was obtained from a commercial market (SigmaAldrich, St Louis, MO). It was mixed with ultrapure water with a weight/volume ratio of 18% and stored at room temperature in a glass bottle until use. The solution was applied manually for 2 min as described above.

Group 7 [3 mL 2.5% NaOCl- 3 mL 17% EDTA]

3 mL 2.5% NaOCl solution was applied manually using a disposable dental syringe (Ultradent Products, South Jordan, UT, USA) and 27-G needle (NaviTip) for 1 min without any activation. Then, 3 mL of 17% EDTA was applied manually as stated above for 1 min.

Group 8 [3 mL 2.5% NaOCl- 3 mL 1% PAA]

3 mL 2.5% NaOCl solution was applied manually for 1 min as stated above. Then, 3 mL 1% PAA was applied manually for 1 min.

Group 9 [3 mL 2.5% NaOCl- 3 mL 2% PAA]

3 mL 2.5% NaOCl solution was applied manually for 1 min as stated above. Then, 3 mL 2% PAA was applied for 1 min.

Group 10 [6 mL 2.5% NaOCl + 9% HEBP]

A 6 mL 2.5% NaOCl + 9% HEBP single irrigation solution was obtained by mixing 3 mL 5.25% NaOCl solution and 3 mL 18% HEBP solution. This solution was applied manually for 2 min as described above.

Group 11 [6 mL 2.5% NaOCl + 18% HEBP]

A 6 mL 2.5% NaOCl + 18% HEBP single irrigation solution was obtained by mixing 3 mL 5.25% NaOCl solution and 3 mL 36% HEBP solution. This solution was applied manually for 2 min as described above.

Group 12 [Saline (control)]

6 mL saline solution was applied for 2 min as described above.

All the solutions were delivered with the application rate of 1 min/3mL. After the application period, 6 mL of water was added to neutralize the solution.

Investigation of bacterial biofilms with confocal laser scanning microscope (CLSM)

After the irrigation regimen, all the experimentally infected dentine discs were stained with Live/Dead BacLightTM Bacterial Molecular Kit L7012 (Molecular Probes, Invitrogen, Carlsbad, CA, USA), which includes Syto-9 and propidium iodide (PI). Styo-9 is a green fluorescent stain and targets the intact membranes of the live bacteria. Propidium iodide is a red fluorescent stain and marks damaged membranes of the dead bacteria. According to the manufacturer's guidelines, 9 µL dye was added to 3 mL distilled water. The mixture was vortexed for 2 min to obtain homogeneity. Following staining the samples using a 200 µL dye mixture for 15 min, each sample was washed with saline solution. A specialist operator examined each sample under a confocal laser scanning microscope (LSM 510 META, Zeiss, Jena, Germany) at 20x magnification. The recorded 2D images were converted to 3D images using Imaris 9.2.1 software (Bitplane, Oxford Instrument Company, Concord, MA, USA). The biological volumes of the dead and live bacteria were calculated and recorded. Then, the percentage of the dead bacterial biovolume was calculated using the data.

Statistical analysis

The data was statistically analyzed using SPSS (Statistical Package for Social Sciences) version 15. Data were presented as means and standard deviations. The suitability of quantitative variables to normal distribution was investigated by the Shapiro-Wilk test. As the data was normally distributed, the Tukey multiple comparison test with one-way ANOVA was used to determine the significant differences between the groups. A p-value less than 0.05 was considered to be statistically significant.

Results

The mean and standard deviation values of the dead bacteria percentage are shown in Table 1. The use of 2.5% NaOCl-2% PAA resulted in the highest values of dead cell percentage. However, the values obtained from this group are not statistically different from those of the other groups. The percentage of the dead bacterial cells in the samples irrigated using 17% EDTA and saline were significantly lower compared to the samples irrigated with other irrigation regimens ($p<0.001$) (Fig. 2).

The percentage of dead bacteria observed in the samples irrigated using 9% HEBP and 18% HEBP solutions alone is lower compared to the samples irrigated using HEBP in combination with NaOCl ($p<0.001$) (Fig. 3).

There was no statistically significant difference between the two concentrations of peracetic acid (1–2%) and etidronic acid (9–18%) regarding biofilm elimination, when these chelators were used alone or in combination with NaOCl ($p>0.001$). However, the percentage of dead bacteria was lower in the samples irrigated with lower concentrations of PAA and HEBP, compared to the ones irrigated with higher concentrations.

The use of 2% peracetic acid alone in the experimentally infected samples resulted in more biofilm elimination than the use of 9% and 18% HEBP alone ($p<0.001$) (Fig. 4).

Discussion

E. faecalis is one of the most important microbiological factors responsible for the failures after root canal treatment (Molander et al. 1998). There is little evidence in the literature regarding the efficacy of HEBP and PAA with different concentrations, when used alone or associated with NaOCl on *E. faecalis* biofilms (Briseño-Marroquín et al. 2022). In the present study, although the difference is not statistically significant, use of HEBP and PAA with higher concentrations eliminated higher amounts of *E. faecalis* biofilm, when these chelators were used alone or in combination with NaOCl. However, the antibacterial effect is not the only factor to determine the ideal concentration of the irrigation solution when using it in the root canal. It has previously been reported that undesirable effects of the irrigation solutions, such as toxicity, dentine erosion, and excessive decrease in dentin microhardness, are more frequently observed at higher concentrations (Kaya et al. 2011; Silva et al. 2012; Souza et al. 2021). Also, determining the ideal concentration of the irrigant regarding the smear layer removal capacity is another important factor that has to be considered. The lowest concentration that minimizes the potential harmful effects of the irrigation solution, when providing effective elimination of the smear layer and antibacterial capacity, should be preferred to be used in the root canal.

Table 1 Means, standard deviations, minimum and maximum values for the percentages of the dead cells after exposure to different irrigation regimens

Irrigation protocol	Mean±SD	Min-Max	Post-hoc
2.5% NaOCl	0.836±0.096	0.710–0.979	A
17% EDTA	0.190±0.096	0.075–0.367	C
1% PAA	0.729±0.107	0.554–0.873	A, B
2% PAA	0.828±0.138	0.625–0.977	A
9% HEBP	0.599±0.126	0.414–0.818	B
18% HEBP	0.616±0.135	0.461–0.815	B
2.5% NaOCl – 17% EDTA	0.738±0.089	0.560–0.905	A, B
2.5% NaOCl – 1% PAA	0.852±0.166	0.509–0.995	A
2.5% NaOCl – 2% PAA	0.883±0.100	0.693–1.000	A
2.5% NaOCl + 9% HEBP	0.862±0.129	0.540–0.955	A
2.5% NaOCl + 18% HEBP	0.879±0.093	0.686–0.971	A
Saline (Control)	0.057±0.042	0.024–0.124	C

SD standard deviation, *min-max* minimum-maximum, *NaOCl* sodium hypochlorite, *EDTA* ethylenediaminetetraacetic acid, *HEBP* etidronic acid, *PAA* peracetic acid

The different letters indicate statistically significant differences between the groups ($p<0.05$)

The use of 2% PAA provided significantly more effective biofilm elimination than 9% and 18% HEBP in the present research. Thus, the null hypotheses were rejected. This can be explained by the lower antibacterial effect of etidronic acid compared to peracetic acid (Tartari et al. 2018). However, when HEBP solution was used in combination with NaOCl, it showed similar antimicrobial effect with PAA used with NaOCl. This may be because HEBP does not react with NaOCl and does not limit its antibacterial effect (Arias-Moliz et al. 2014). The results of the present study support the findings of the previous ones, which have reported that the combined use of NaOCl and HEBP does not have an undesirable effect on the chemical properties of NaOCl (Tartari et al. 2015). In addition, the present results showed that the use of 9% and 18% HEBP has led to a slight increase in the antibacterial capacity of 2.5% NaOCl when they were used in combination.

There is limited knowledge in the literature about the interaction of peracetic acid with NaOCl that reduces the desirable effects of NaOCl (Arias-Moliz et al. 2015). In the present study, there was no statistically significant difference between the single application of NaOCl and its combined use with PAA in terms of eliminating *E. faecalis* biofilms. However, when both solutions are used together, the percentage of dead bacteria is higher. According to the results of the present study, PAA can also be considered as a promising chelating agent like HEBP, thanks to its adequate antibacterial activity when used alone. In addition, it seems that the use of PAA does not reduce the useful properties of NaOCl when two irrigants are used together. However, the findings of other research evaluating different properties of

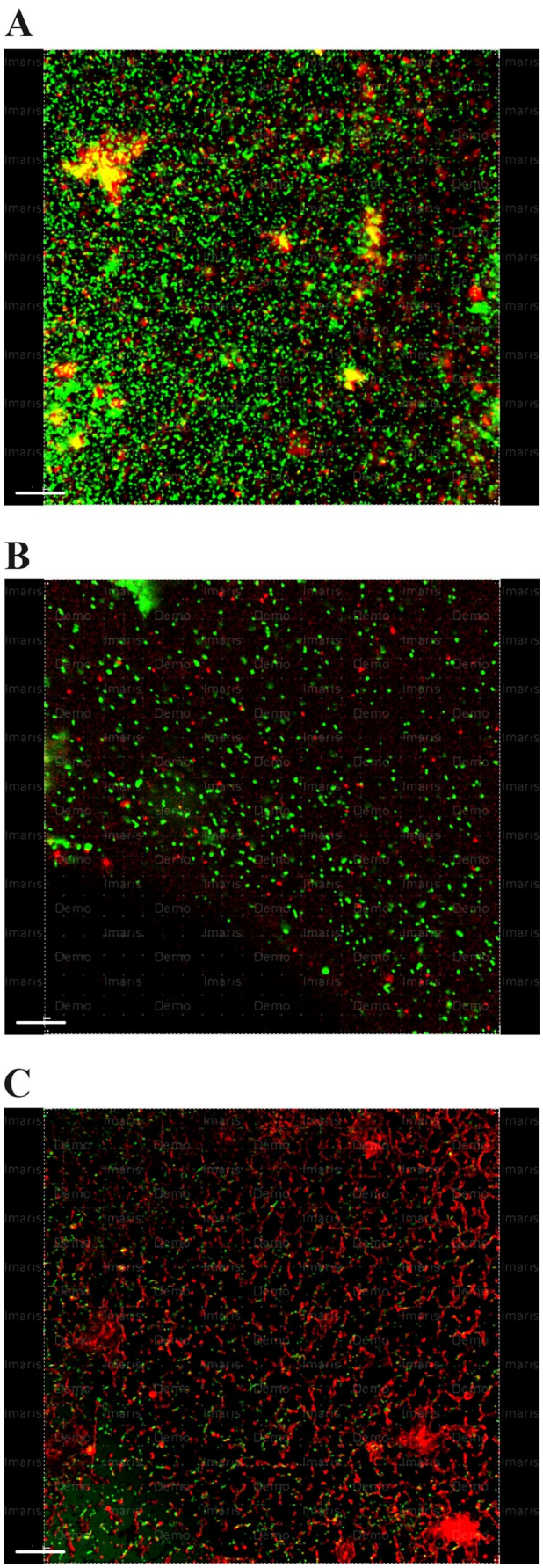


Fig. 2 Representative CLSM photos showing dead and live bacteria within dentine discs after irrigation with (**a**) Saline, (**b**) 17% EDTA, (**c**) 1% PAA. Green color: live cells, red color: dead cells.

PAA such as smear layer removal capacity, biocompatibility should be considered prior to recommend its use for root canal irrigation.

In the present study, CLSM was used to detect the dead and live bacteria in the experimentally developed biofilms. CLSM was suggested to be an effective method for the identification and measurement of viability without disturbing the attached cells (Chávez et al. 2010; Shen et al. 2010). It is also indicated as a suitable tool for understanding the viability profile and spatial distribution in the biofilms (Del Carpio-Perochena et al. 2011; Zaura-Arite et al. 2001). Therefore, studies evaluating the effect of antibiofilm capacity of antibacterial agents in the root canal prefer to use CLSM for three-dimensional imaging of the bacteria (Rodrigues et al. 2018; Fiallos et al. 2020).

In the present study, *the E.faecalis* biofilm model was experimentally developed to investigate the efficacy of irrigation protocols in removing this microbial flora from the root canal system. However, actual endodontic infections are generally resulted from polymicrobial sources, and *E. faecalis* can interact with other microorganisms and change their behavior in real clinical situations (Hasheminia et al. 2013). The root canal microorganism profiles in real endodontic diseases may differ from those that are created experimentally, and they could react differently to the irrigation agents tested here. This issue can be evaluated as a limitation of this in-vitro study.

Considering the findings of this study, peracetic acid and etidronic acid can be evaluated as alternative chelation agents to EDTA due to their less interaction with NaOCl, reducing its antibacterial effect. Previous research has revealed adequate chelation capacity of PAA in removing the smear layer from root canal dentinal walls (Viola et al. 2022). In the present study, a single PAA showed a similar antibiofilm effect to NaOCl. Therefore, the use of PAA as a single final irrigant can be recommended for both the elimination of microorganisms and the removal of the smear layer. Further studies are needed under in-vivo conditions using the same irrigation agents to support the results of the present study.

Conclusion

The antimicrobial capacity of 1–2% peracetic acid is similar to its associated use with 2.5% NaOCl regarding eliminating *E.faecalis* biofilms from root canal walls. Therefore, irrigation of infected root canals with a single use of PAA can achieve an adequate antibacterial effect. Lower

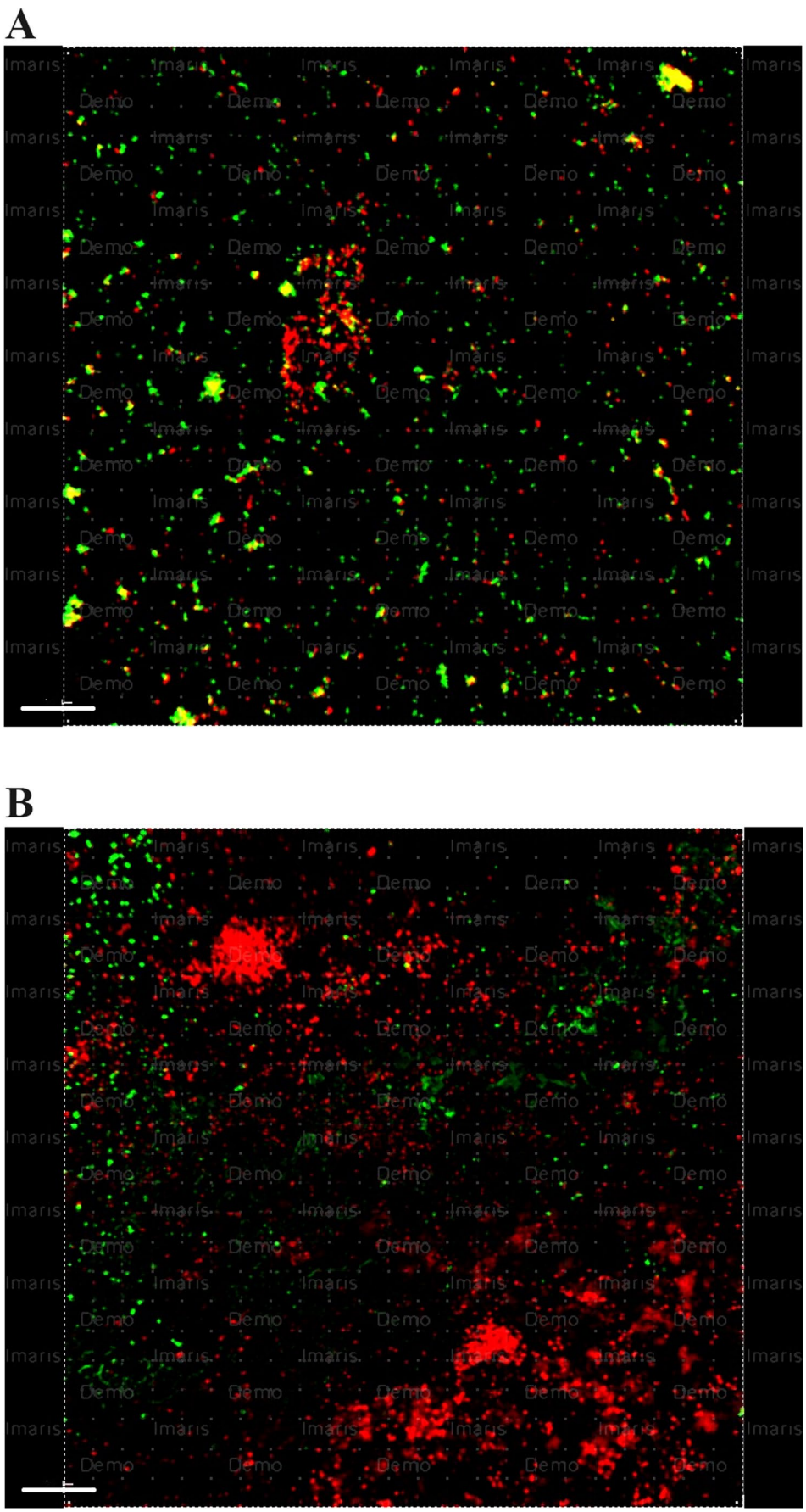


Fig. 3 Representative CLSM photos showing dead and live bacteria within dentine discs after irrigation with (**a**) 9% HEBP, (**b**) 2.5% NaOCl + 9% HEBP. Green color: live cells, red color: dead cells

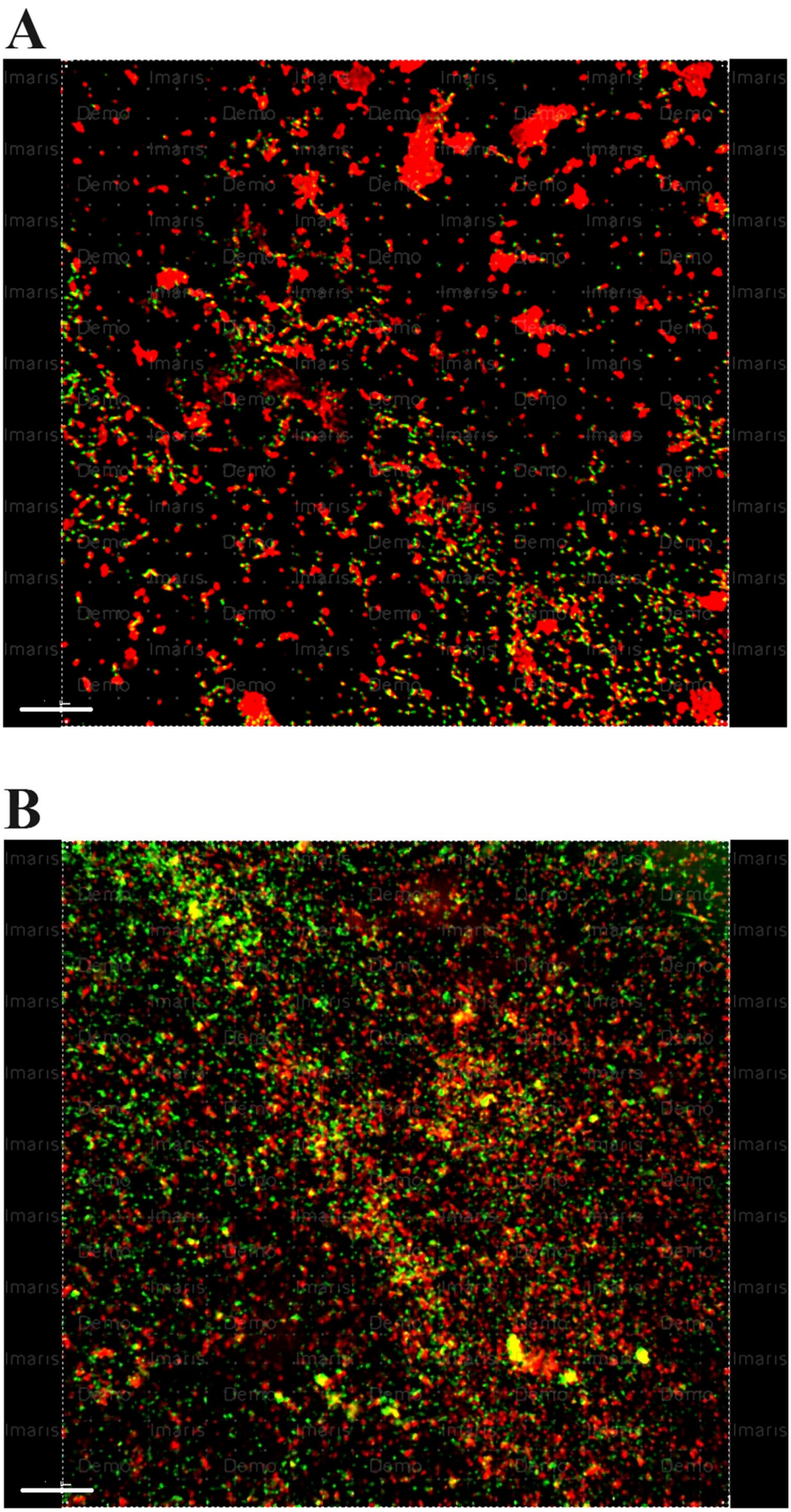


Fig. 4 Representative CLSM photos showing dead and live bacteria within dentine discs after irrigation with (**a**) 2% PAA, (**b**) 18% HEBP. Green color: live cells, red color: dead cells

concentrations of peracetic acid and etidronic acid can be recommended to remove the *E.faecalis* biofilms from root canals to decrease the irrigation solutions' potential harmful effects.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by S.Z., G.A., and İ.G.S. The first draft of the manuscript was written by Ö.İ.U and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval This study was approved by the institutional ethics committee (xxx University No: 21071282-050.99-).

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent to publish No images of human research participants are available in the present paper.

Competing interests The authors declare no competing interests.

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