The Effect of the Enzymes Trypsin and DNase I on the Antimicrobial Efficiency of Root Canal Irrigation Solutions

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Abstract

Objectives

The purpose of this study was to use a confocal laser scanning microscope (CLSM) to evaluate the antibacterial efficacy of using 2.5% sodium hypochlorite (NaOCL), 2% chlorhexidine (CHX), Irritrol, and chitosan-coated silver nanoparticles (AgCNPs) alone or in combination with deoxyribonuclease I (DNase I) and trypsin pre-enzyme applications in dentin samples contaminated with Enterococcus faecalis (E. faecalis).

Materials and Methods

144 dentin blocks with confirmed E. faecalis biofilm formation were divided randomly according to the irrigation protocol to be tested (n = 12): NaOCl, CHX, Irritrol, AgCNPs, Trypsin/ NaOCl, Trypsin/ CHX, Trypsin/ Irritrol, Trypsin/ AgCNPs, DNase I/ NaOCl, DNase I/ CHX, DNase I/ Irritrol, and DNase I/ AgCNPs. Dentin blocks were stained with the Live/Dead BacLight Bacterial Viability Kit and viewed with CLSM after irrigation applications. The percentage of dead and viable bacteria was calculated using ImageJ software on CLSM images. At a significance level of p < 0.05, the obtained data were analyzed using One-Way Anova and Post-Hoc Tukey tests.

Results

In comparison to NaOCl, CHX had a higher percentage of dead bacteria, both when no pre-enzyme was applied and when DNase I was applied as a pre-enzyme (p < 0.05). There was no difference in the percentage of dead bacteria between the irrigation solutions when trypsin was applied as a pre-enzyme (p > 0.05). AgCNPs showed a higher percentage of dead bacteria when trypsin was applied as a pre-enzyme compared to other irrigation solutions (p < 0.05), while the pre-enzyme application did not affect the percentage of dead bacteria in NaOCl, CHX, and Irritrol (p > 0.05).

Conclusions

No irrigation protocol tested was able to eliminate the E. faecalis biofilm. While the application of trypsin as a pre-enzyme improved the antimicrobial effect of AgCNPs, it did not make any difference over other irrigation solutions.

Clinical Relevance:

Pre-enzyme treatment with trypsin improved the antimicrobial activity of AgCNPs. The combined use of these two solutions may be useful as a treatment support in endodontic practice, as it helps to separate bacteria from the biofilm matrix.

Introduction
Microbial elimination of root canals is an important factor for successful endodontic treatment [1]. However, despite the variety of chemical irrigation solutions and mechanical strategies available today, it has been reported that no disinfection procedure has been able to eliminate microorganisms in the root canal. Some microorganisms in the polymicrobial flora of endodontic infections show more resistance to antimicrobial applications. *E. faecalis*, one of these microorganisms, has been reported to be isolated from 18% of primary endodontic infections and 67% of unsuccessful endodontic treatments [2]. *E. faecalis*, as a single organism, has root canal survival, biofilm formation, long-term resistance to food starvation, natural antibiotic resistance, the ability to penetrate dentinal tubules, and virulence factors that facilitate tissue invasion. The elimination of *E. faecalis* from root canals during endodontic treatment is therefore difficult, and research continues to find the best irrigation protocol for endodontics to improve root canal disinfection [3].

Because of its capacity to dissolve organic tissue and broad-spectrum antibacterial properties, sodium hypochlorite (NaOCl) is the most often used irrigation solution in endodontics [4]. However, contact with NaOCl in especially high concentrations with periapical tissues may cause cellular destruction of tissues, a delay in healing, and many potential complications, such as pain [5]. Chlorhexidine (CHX), another irrigation solution widely used in endodontics, has advantages such as broad-spectrum antimicrobial activity, substantivity, and low toxicity [6]. CHX has reportedly not been shown to be sufficient in removing the biofilm and smear layer from the root canal system [7]. Irritrol (Essential Dental Systems, S. Hackensack, New Jersey, USA), which was released to overcome these disadvantages of CHX, is a solution that is a combination of CHX, EDTA, and surfactants. The manufacturer claims that thanks to its composition, Irritrol dissolves the smear layer less aggressively than other irrigation solutions, which prevents dentin from becoming demineralized [8]. To overcome the disadvantages of conventional antimicrobial agents, there is growing interest in innovative and alternative approaches to endodontic treatment [9]. The effectiveness of combining conventional irrigation solutions with different enzymes and nanoparticles, both of which are included in these approaches, was evaluated in the present study. Because of its physiochemical properties, including sensitivity, antimicrobial activity, and bioavailability, chitosan (CNPs), a commonly used nanoparticle in endodontics, has been suggested as an alternative antimicrobial application against intracanal infections [10]. However, because of their antimicrobial properties, metallic nanoparticles such as silver (Ag), copper (Cu), and zinc (Zn) have also received a lot of interest [11]. It has been reported that trypsin, which is a proteolytic enzyme, can cleave lysine and arginine peptide bonds in the EPS matrix of bacteria, thereby reducing the cohesion of the biofilm [12]. However, exogenous DNases, which are used to control biofilm growth and therefore have the potential to improve the antimicrobial activity of irrigants, are shown to have an effect on eDNA, which maintains the biofilm structure [13].

There are many studies evaluating the antimicrobial activities of silver and chitosan nanoparticles separately in the literature, but no study evaluating the antimicrobial effect of chitosan-coated silver nanoparticles has been found (AgCNPs) [14, 15]. In this study, CLSM was used to evaluate the antimicrobial effects of Irritrol and AgCNPs on dentin blocks contaminated with *E. faecalis*, both while
used alone and after pre-enzyme was applied with trypsin and DNase I. The study's null hypothesis is that all irrigation protocols that will be tested on *E. faecalis* biofilms will have the same antimicrobial effect.

**Materials And Methods**

**Determining the number of specimens**

The design of this study was approved by the XX Ethics Committee (XX). The sample size was calculated based on a study [16] with similar methodology in the literature. As a result of the power analysis performed using the variance statistical test (G*Power 3.1 Software, Heinrich Heine University, Düsseldorf, Germany; $\alpha = 0.05$ and $\beta = 0.95$), it was determined that statistically, the number of samples in each group should be at least 12. Therefore, 152 dentin blocks from 76 teeth were used in the study.

**Preparation of dentin blocks**

The study included teeth that were extracted for periodontal or orthodontic reasons, were free of caries, had closed root tips, had a single root and a single canal, and had straight root structures [17]. Teeth with any fracture, crack, root canal obliteration, complete or partial root canal filling, and internal or external resorption were not included in the study. Hard and soft tissue attachments were mechanically removed from the study teeth using a periodontal curette. First, the teeth were soaked in a 0.5% Chloramine-T (Sigma-Aldrich, Missouri, USA) solution for 48 hours for disinfection. It was stored at +4°C in a 0.5% Thymol solution (Acros Organics, Thermo Fisher Scientific, USA) until use.

The procedure described by Ruiz-Linares et al. [18] was used to obtain the dentin blocks. The crowns of the included teeth were removed using a diamond fissure bur (G&Z Instrumente, Lustenau, Austria) under water cooling at the enamel-cement level. The middle and apical thirds of the root were removed, leaving 4 ± 1 mm cervical thirds of the tooth roots. To achieve and standardize a diameter of 1.5 mm, root canals of dentin blocks were enlarged with a full pass of gates glidden burs 1, 2, 3, 4, 5, and 6, respectively (Proud, London, UK) at 300 rpm under water cooling. The dentin blocks were then divided into two longitudinally semi-cylindrical equal parts with an endless diamond separator (Sunshine Diamonds, Langenhagen, Germany). For sample standardization, cement was removed from the outer surfaces of 152 semi-cylindrical dentin blocks using an endless diamond separator, with the dimensions of each block being approximately 4 mm x 4 mm x 0.7 mm. For the final shaping of the dentin blocks and to obtain a better appearance in CLSM, the dentin surfaces were sanded using 2000-grit silicon carbide water abrasives (Atlas Brand, Gebze, Kocaeli) adhered with double-sided silicone tape on a manual polishing device (Presi, Minitech 233, France). The thickness of the dentin blocks was checked by measuring with a digital caliper (Mitutoyo Absolute, Kanagawa, Japan).

5% NaOCl (Wizard, Istanbul, Turkey) and 17% EDTA were used, respectively, for 4 min in an ultrasonic bath to remove the smear layer from the dentin blocks. Dentin blocks were rinsed with sodium thiosulfate for 5 min and then with distilled water for 10 min to eliminate any remaining EDTA and NaOCl on the dentin surfaces. The dentin blocks were sterilized in an autoclave at 121°C and 15 psi for 20 min after
being placed in 1.5 mL Eppendorf tubes containing PBS. After sterilization, the dentin blocks were kept in an oven for 2 hours at 37°C. 4 randomly selected dentin blocks were examined using SEM (Carl Zeiss Sigma 300VP, Oberkochen, Germany) to confirm that the smear layer was removed and sterilized. Confirmed samples were accepted as a negative control.

**Dentin infection with E. Faecalis biofilm**

This study used *E. faecalis* ATCC 29212, the most common enterococcal reference strain in lab-based endodontic research [19]. One colony from the +4°C bacterial culture was inoculated into 50 mL of brain heart infusion (BHI; Difco Laboratories, Detroit, MI, USA) and 0.25% glucose and incubated at 37°C for 24 hours. Bacterial culture inoculation in BHI medium with 148 dentin blocks adjusted to McFarland (1) turbidity (3×10^8 cells/mL) was performed under the same incubation conditions. Each dentin block was contaminated with *E. faecalis* for 3 weeks using a sterilized 0.1–10 µl pipette tip and 5 mL of sterilized BHI and bacterial inoculum. At 48-hour intervals, 24-hour pure cultures were freshly prepared and adjusted to the McFarland standard for these procedures. Dentin blocks were stored at 37°C and 95% humidity. All experiments were done aseptically. To remove loosely adherent planktonic bacteria, dentin blocks were aseptically removed from each tube and gently washed with sterile phosphate-buffered saline for 1 min [16].

**SEM examination of dentin blocks**

To control the formation of *E. faecalis* biofilms, 4 randomly selected dentin blocks were kept in 10% buffered formalin for 1 week for SEM examination. The samples were gradually dehydrated with ethanol solutions (70%, 95%, and 100%) and dried at room temperature for 1 hour. Then, dentin blocks were coated with gold-palladium, and each dentin block was examined with a SEM device under 5000x, 10000x, and 20000x magnifications.

**Biofilm antimicrobial activity test**

Dentin blocks with confirmed *E. faecalis* biofilm formation were washed with saline for 1 min and randomly divided according to the irrigation solutions to be tested (n = 12): NaOCl, CHX, Irritrol, AgCNPs, Trypsin/ NaOCl, Trypsin/ CHX, Trypsin/ Irritrol, Trypsin/ AgCNPs, DNase I/ NaOCl, DNase I/ CHX, DNase I/ Irritrol, and DNase I/ AgCNPs.

In our study, trypsin (Sigma-Aldrich, Missouri, USA), a proteolytic enzyme obtained from pig pancreas, and DNase I (Sigma-Aldrich, Missouri, USA), an EPS-degrading enzyme obtained from bovine pancreas, were used. Concerning similar studies in the literature, 100 µg/mL concentrations of trypsin and DNase I were prepared according to the manufacturer’s instructions [12, 20–23]. AgCNPs used in the present study was synthesized according to the method reported by Bin Ahmad et al. [24], and it was confirmed by SEM that the obtained solution gained nanoparticle properties. The size of AgCNPs synthesized from SEM images was observed to vary between 50–82 nm (Fig. 1).
All dentin blocks were irrigated with 2 mL of NaOCl followed by 2 mL of distilled water to mimic the standard irrigation procedure before the irrigation solutions were tested. Then, 6 mL/5 minutes of irrigation were done with the relevant solutions in the groups. Then irrigated with 2 mL of distilled water. After all irrigation procedures, dentin blocks were inactivated for 5 minutes with 5% sodium thiosulfate to prevent interference between the vitality dye and the irrigation solutions. Then the dentin blocks were rinsed with 0.9% saline.

Confocal laser scanning microscopic analysis

Fluorescent LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR, ABD) (lot: 1919628), consisting of green and red fluorescent nucleic acid dyes (SYTO 9 and propidium iodide), respectively, was used for staining dentin blocks for CLSM imaging. The manufacturer reported that after staining, bacteria with intact cell membranes will be green, and bacteria with damaged membranes will be red. The dye prepared according to the manufacturer’s instructions was applied to each dentin block with 5µl with a micropipette tip and the dyes were kept in the dark for 15 minutes for the penetration of bacteria. Dentin blocks were rinsed with PBS for 1 min.

After staining, dentin blocks were placed in a glass-bottomed petri dish (Ibidi 81218-200, Munich, Germany) in a CLSM lens with the canal cavity facing down and visualized in 1 mL of PBS (pH 7.4). Imaging was performed with an inverted confocal microscope (Carl Zeiss LSM 800, Jena, Germany) and excitation lasers (SYTO 9-488nm and propidium iodide-543nm) at a wavelength suitable for dyes. 3D images (z-stacks) were taken with a x20/0.8 NA dry objective in 2 different channels (SYTO 9-green and PI-red), 1024x1024 pixel lateral (xy) resolution, and 1 µm z-plane steps in 5 randomly selected areas on each sample surface. Obtained 7–20 slices (scan area) of z-stack images were combined in a single plane with the “maximum intensity projection” method with ZEN lite software (ZEISS, Oberkochen, Germany). Thus, two-dimensional images representing 7–20 scan areas were obtained. 12 dentin blocks were used for image analysis in each group. The density of both live (green fluorescence) and dead (red fluorescence) bacteria in each sample was automatically calculated using ImageJ 1.8 software (Wayne Rasband, NIH, USA). Then, the calculated values and the percentages of dead bacteria were calculated according to the formula below [25].

Statistical analysis

The Shapiro-Wilk test was applied to confirm the normality of the obtained data. Since the data showed a normal distribution, One-Way ANOVA and Post-hoc Tukey tests were used to compare the percentages of dead bacteria calculated on the images obtained by CLSM imaging after the irrigation protocols were tested. SPSS software version 17 (IBM, Armonk, NY, USA) was used at a significance level of p < 0.05.

\[
\text{Red fluorescence} \times 100 \\
\text{Green and Red fluorescence}
\]

Results
In the current study, the absence of any microbial contamination in 4 randomly selected dentin blocks belonging to the negative control group before bacterial contamination was confirmed in the images obtained by SEM (Fig. 2a). Biofilm formation was confirmed by SEM images in 4 dentin blocks of the randomly selected positive control group after 21 days of the incubation period of *E. faecalis* (Fig. 2b).

As a result of the study, it was determined that none of the irrigation protocols tested could eliminate the *E. faecalis* biofilm in the root canal (Fig. 3). The mean and standard deviation (SD) values of the percentages of dead bacteria were calculated and compared to the total percentage of bacteria calculated on the images obtained because of CLSM imaging of the irrigation protocols tested (Table 1). After the irrigation protocols were tested, a significant difference was determined in terms of the percentage of dead bacteria calculated on the CLSM images of *E. faecalis* biofilms in dentin samples (P < 0.05).

<table>
<thead>
<tr>
<th>Enzyme Ø</th>
<th>NaOCl (Mean ± SD)</th>
<th>CHX (Mean ± SD)</th>
<th>Irritrol (Mean ± SD)</th>
<th>AgCNPs (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Ø</td>
<td>29.6 ± 15.6 AbAa</td>
<td>52.6 ± 19.3 AbBa</td>
<td>48.6 ± 17.9 AbBa</td>
<td>31.3 ± 10.1 AbBa</td>
</tr>
<tr>
<td>Trypsin</td>
<td>47.9 ± 20.3 AbAa</td>
<td>44.8 ± 20.4 AbBa</td>
<td>60.8 ± 26.4 AbBa</td>
<td>61.1 ± 18.8 Ab</td>
</tr>
<tr>
<td>DNase 1</td>
<td>31.9 ± 29.4 AbAa</td>
<td>61.6 ± 16.1 AbBa</td>
<td>51.6 ± 21 AbBa</td>
<td>39.1 ± 15.1 AbBa</td>
</tr>
</tbody>
</table>

*Significant differences between rows are indicated by A, B, C in the superscript, and significant differences between the columns are indicated by a, b, c in the superscript.

In irrigation applications no pre-enzyme applied, a higher percentage of dead bacteria was obtained in CHX compared to NaOCl (p < 0.05), and there was no difference between other irrigation solutions (p > 0.05). No difference found between irrigation solutions when trypsin applied as a pre-enzyme (p > 0.05). When DNase I was applied as the pre-enzyme, a higher percentage of dead bacteria was observed in CHX than in NaOCl (p < 0.05), and there was no significant difference between other irrigation solutions (p > 0.05).

Whether pre-enzyme was applied in NaOCl, CHX, and Irritrol solutions, there was no statistical difference (p > 0.05). Compared to other irrigation solutions, only AgCNPs had a higher percentage of dead bacteria with the trypsin pre-enzyme application (p < 0.05).

**Discussion**

In the present study, the antibacterial efficacy of NaOCl, CHX, Irritrol, and AgCNPs alone or in combination with DNase I and trypsin enzymes on *E. faecalis* biofilm was evaluated by CLSM. The null hypothesis
was rejected as there was a difference in the antimicrobial efficiency of the tested irrigation protocols on *E. faecalis* biofilms.

Endodontic success requires root canal microbial load removal. Unlike planktonic bacteria, *E. faecalis* can form a biofilm that protects it from antibodies, phagocytosis, and antimicrobials. [26]. Seneviratne et al. [27] reported that strain ATCC 29212 has clinical isolate-like CFU counts, biofilm growth, and architecture. Swimberghe et al. [19] reported that *E. faecalis* was the most commonly used test bacteria and human dentin was the most frequently used substrate in a study in which they examined laboratory-based root canal biofilm models described in the endodontic literature. Therefore, human dentin was used as a substrate, and the *E. faecalis* ATCC 29212 strain was used in the contamination of these dentin blocks to mimic clinical conditions. Stojicic et al. [28] reported that after a 3-week incubation period of polymicrobial biofilms, the bacteria in the biofilm were less sensitive to disinfecting agents. For these reasons, clinical difficulty was simulated in the present study by inoculating *E. faecalis* with a 3-week incubation period.

The search for new treatment strategies continues to overcome the emerging resistance to antimicrobial procedures. In recent years, it has been reported that dextran and extracellular DNA (eDNA) in the matrix of *E. faecalis* biofilms play an important role in the resistance of bacterial communities to antimicrobial applications [29]. The sensitivity of *E. faecalis* biofilms to DNase has been previously reported, especially in the early stages of growth [30]. Tetz et al. [31] reported that in *Escherichia coli* and *Staphylococcus aureus* biofilms formed in the presence of DNase I, eDNA separation from the cell caused an increase in antibiotic penetration and a decrease in biofilm biomass and CFU counts. Niazi et al. [12] reported that proteolytic enzymes can also degrade the extracellular matrix produced from proteins secreted by bacteria, thus reducing the cohesion of the biofilm. Considering all this information, the effects of trypsin, a proteolytic enzyme, and DNase I, an EPS-degrading enzyme, on the antimicrobial efficiency of irrigation solutions were tested in the current study.

In studies investigating the capacity of various endodontic procedures to eliminate biofilm from root canals, many methods have been used for qualitative and quantitative analysis [32]. Zapata et al. [33] used CLSM to quantify and visualize microorganisms in dentin and dentinal tubules. Unlike other microscopic systems, the CLSM can control depth of field by eliminating or reducing background information from the focal plane and obtain serial optical sections from thick samples [34]. CLSM helps visualize live and dead bacteria and can penetrate 10 µm below the sample surface [20]. Kishen et al. [35] reported that CLSM analysis with viability dyes is a reliable method to evaluate bacterial biofilm formation in dentinal tubules after incubation. By evaluating all this information, the antimicrobial efficacy of the irrigation protocols tested in the current study was evaluated by CLSM analysis, which provides three-dimensional visualization of dentinal tubules and obtains quantitative data.

Similar to many studies in the literature, LIVE/DEAD BacLight bacterial viability dye with fluorescent properties was used to ensure the visibility of both dead and live microorganisms under CLSM in the current study [18]. The fluorescent LIVE/DEAD BacLight bacterial viability dye can accurately measure the
number and viability of bacterial cells on a surface. [28, 36]. However, the use of viability dyes (STYO9 and PI) has its inherent disadvantages. Netuschil et al. [37] reported that cells with intact membranes (green stain) may be metabolically active and may not be cultured.

According to the results of this study, the microbial load in the root canal system has not been completely removed in many studies that used irrigation protocols and other protocols that have been tested in the literature [14, 16, 18]. On the other hand, Gomes et al. [6] found that using the plaque culture method with 5.25% NaOCl, 1% CHX, and 2% CHX solutions killed 100% of *E. faecalis* bacteria in less than 30 seconds. We think that the reason for the differences between the study results is due to methodological differences, such as using different evaluation methods, different incubation times, and different solution concentrations.

As a result of the current study, CHX without pre-enzyme treatment showed a higher percentage of dead bacteria compared to NaOCl. Similarly, Menezes et al. [38] reported that in the culture method, 2.5% NaOCl could not completely eliminate *E. faecalis*, and 2% CHX showed a better antibacterial effect than 2.5% NaOCl. Dametto et al. [39] reported that 2% CHX provided better antibacterial properties than 5.25% NaOCl in 7-day CFU counts. Contrary to these findings, Hope et al. [40] reported that 1% NaOCl provided better antimicrobial activity than 2% CHX with the culture method. Rodrigues et al. [14] reported that 2.5% NaOCl showed a better antibacterial effect than 2% CHX on *E. faecalis* biofilm in bovine dentin blocks by CLSM analysis. In addition, Ruiz-Linares et al. [18] reported that NaOCl provided better antimicrobial activity against bacteria in human dentin samples with the application of 2.5% NaOCl, 2% CHX, 2% alexidin and 0.2% cetrimidine alone or in combination with KLTM analysis. On the other hand, Ma et al. [41] reported that there was no difference in the percentage of dead bacteria between 1% NaOCl, 2% NaOCl, and 2% CHX by CLSM analysis. We presume that the differences in the study's results are due to differences in the irrigation solutions’ concentration, volume, and application time, as well as differences in the evaluation method and substrate used.

In the literature review conducted by us, we did not find any study evaluating the antibacterial activity of AgCNPs as an irrigation solution in the field of endodontics. Similarly, although the effect of Irritrol on dentin tubule penetration, adhesion of filling materials, and its effectiveness in removing the smear layer was investigated in the literature, it was determined that there was no study on its antibacterial activity. Therefore, the results of the current study on AgCNPs and Irritrol were not directly compared with any other study. In this study, for the first time, the antimicrobial efficacy of AgCNPs and Irritrol, both alone and in combination with enzymes, was tested on *E. faecalis*, an endodontic pathogen. As a result of this study, no significant difference was found between NaOCl, Irritrol, and AgCNPs and between CHX, Irritrol, and AgCNPs in terms of the percentage of dead bacteria after the irrigation protocol without pre-enzyme application. Similar to our study, Moghadas et al. [42] reported that ethanol and sodium hydroxide containing AgNP were as effective as 5.25% NaOCl in controlling intracanal bacterial growth against *E. faecalis* and *S. aureus*. However, Jaiswal et al. [43] reported that 5% NaOCl showed equal antimicrobial efficacy with the combined use of 2% chitosan and 2% CHX. Contrary to the findings of our study, Rodrigues et al. [14] reported that AgNP showed less antibacterial activity compared to 2.5% NaOCl at all
time intervals tested by CLSM analysis. However, Afkhami et al. [44] reported that AgNPs had better antimicrobial activity than 2.5% NaOCl by the culture method in the disinfection of inoculated root canals of *E. faecalis*. We think that the differences between the study results are due to the concentration of the irrigation solutions tested, the evaluation method, and the differences in the synthesis procedures of the nanoparticles. In addition, because of our study, the antimicrobial activity of Irritrol and AgCNPs, as well as CHX and NaOCl, which have gained routine use in clinical practice in endodontics, showed that the use of these two solutions in endodontics is promising.

There was no difference in the percentage of dead bacteria between NaOCl, CHX, Irritrol, and AgCNPs used after trypsin application as a pre-enzyme. Contrary to these findings, Niazi et al. [22] in their study using CLSM analysis, reported that trypsin added to the growth media of multi-species biofilms made the biofilms more sensitive to CHX. Contrary to our study protocol, differences between the study results found by Niazi et al. [22] may be due to the fact that trypsin was added to the growth media during biofilm formation, causing a more limited volume and looser biofilm formation, making the biofilm more sensitive to the applied irrigation solutions.

CHX, which was used after DNase I administration as the pre-enzyme, showed a higher percentage of dead bacteria than NaOCl. No significant difference was found between NaOCl, Irritrol, and AgCNPs and between CHX, Irritrol, and AgCNPs. Consistent with the results of the present study, Li et al. [21] reported that DNase I reduced the adhesion of *E. faecalis* and increased the sensitivity of biofilms to CHX in their study, in which they evaluated the effects of DNase I, which they added to the biofilm growth medium, on the biofilm with CFU counts. Contrary to the results of the present study, Ganesh et al. [20] reported that the addition of DNase I with or without Tween 80 to 2% CHX by CLSM analysis did not make any difference in antibacterial activity on *E. faecalis* biofilm. The differences between the study results can be attributed to the different bacterial incubation times, the addition of DNase I to the biofilm growth medium, and the different route of administration of DNase I.

Pre-enzyme application to NaOCl, CHX, and Irritrol did not make any difference in the percentage of dead bacteria. Similarly, Ganesh et al. [20] reported that the addition of DNase I to 2% CHX did not make a difference in its antibacterial activity on *E. faecalis* biofilm. In contrast to our findings, Niazi et al. [22] found that adding trypsin to growth media made biofilms more sensitive to CHX. Yu et al. [45] reported that DNase added to *E. faecalis* medium during 2 days of culturing or for 1 hour after biofilm formation reduced biofilm formation and increased the sensitivity of *E. faecalis* to NaOCl even at low concentrations (0.5%), by removing eDNA in the biofilm. Differences between study results may be due to the addition of enzymes to the medium and the different concentrations of irrigation solutions.

In this study, only trypsin pre-enzyme improved AgCNPs antimicrobial activity. These two solutions help separate bacteria from the biofilm matrix, making them a potential treatment supplement. Although root canals contain many types of biofilms clinically, the use of a single biofilm model in the current study is an important limitation. Therefore, there is a need to investigate the effectiveness of the irrigation protocols tested in this study on multi-species biofilm models, teeth with different root morphologies, and
different segments of roots. In addition, it is thought that the realization of randomized controlled clinical studies is important in terms of providing clinical data to the literature.

**Conclusion**

Within the limitations of this study, it was determined that the use of NaOCl, CHX, Irritrol, and AgCNPs alone and in combination with both DNase I and trypsin enzymes could not provide complete bacterial elimination in dentin blocks contaminated with *E. faecalis*. In the current study, CHX with and without DNase I pre-enzyme application demonstrated superior antimicrobial activity to NaOCl. However, there was no difference in antimicrobial activity between NaOCl, Irritrol, and AgCNPs as well as between CHX, Irritrol, and AgCNPs. The use of trypsin as a pre-enzyme improved AgCNPs antimicrobial activity, whereas the use of DNase I had no effect on AgCNPs.

**Declarations**

**Acknowledgement**

The authors deny any conflicts of interest related to this study.

**Author Contributions**

**TDC:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing- Original Draft, Review & Editing, Visualization, Project administration. **ZUA:** Conceptualization, Formal analysis, Data Curation, Review & Editing. **DE:** Methodology.

**Ethics Approval**

The design of this study was approved by the XX Ethics Committee (XX).

**Consent to Participate**

Not Applicable

**Funding**

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**Conflict of Interests**

The authors deny any conflicts of interest related to this study.

**References**


Figures

Figure 1
SEM image of chitosan; 20000X (a), SEM image of chitosan with added silver nanoparticles; 20000X (b), SEM image of chitosan with added silver nanoparticles; 100000X (c)

Figure 2

Confirmation of smear layer removal and sterility of specimens by SEM; 20000X (a). SEM micrograph of *E. faecalis* biofilm covering the dentin surface; 20000X (b)
Figure 3

Representative three-dimensional (3D) CLSM images of dentin specimens from the groups. Scale bar= 30 µm