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ORIGINAL ARTICLE

Effect of DNase Treatment on Adhesion and Early Biofilm Formation of *Enterococcus Faecalis*

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ABSTRACT

Objective: Extracellular DNA (eDNA) has been shown to be important for biofilm stability of the endodontic pathogen *Enterococcus faecalis*. In this study, we hypothesized that treatment with DNase prevents adhesion and disperses young *E. faecalis* biofilms in 96-well plates and root canals of extracted teeth.

Methods: *E. faecalis* eDNA in 96-well plates was visualized with TOTO-1®. The effect of DNase treatment was assessed in 96-well plates and in extracted single-rooted premolars (n=37) using a two-phase crossover design. *E. faecalis* was treated with DNase (50 Kunitz/mL) or heat-inactivated DNase for 1 h during adhesion or after 24 h of biofilm formation. In 96-well plates, adhering cells were quantified using confocal microscopy and digital image analysis. In root canals, the number of adhering cells was determined in dentine samples based on colony forming unit counts. Data from the 96-well plate were analyzed using one-tailed t-tests, and data from extracted teeth were analyzed using mixed-effect Poisson regressions.

Results: eDNA was present in wells colonized by *E. faecalis* after 1 h of adhesion and 24 h of biofilm formation; it was removed by DNase treatment, as evidenced by TOTO®-1 staining. DNase treatment reduced the area covered by cells in 96-well plates after 1 h ($P<0.05$), but not after 24 h ($P=0.96$). No significant differences in the number of adhering cells were observed in extracted teeth after 1 ($P=0.14$) and 24 h ($P=0.98$).

Conclusion: DNase treatment does not disperse endodontic *E. faecalis* biofilms. The sole use of DNase as an anti-biofilm agent in root canal treatments is not recommendable.

Keywords: Adhesion, biofilm; DNase, *Enterococcus faecalis*, endodontic biofilms, extracellular DNA

HIGHLIGHTS

- *E. faecalis* releases extracellular DNA, which can be removed by DNase treatment, during bacterial attachment and early biofilm formation.
- DNase treatment reduces the amount of *E. faecalis* cells adhering to 96-well plates, but it does not significantly reduce the amount of bacteria adhering to root canals of extracted teeth.
- DNase treatment does not disperse 24-h-old biofilms of *E. faecalis* formed in 96-well plates or in root canals of extracted teeth and is, thus, unlikely to be a valuable adjunct to endodontic therapy.

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INTRODUCTION

Bacterial biofilms tolerate high concentrations of biocides; this property is partly attributed to the protective matrix in which bacterial cells are embedded. Therefore, treatment of bacterial biofilms with matrix-degrading enzymes has become a field of increasing research interest in the past years. Extracellular DNA (eDNA) entered in the list of potential targets in the matrix when Whitchurch et al. (1) first demonstrated its importance for the mechanical stability of young *Pseudomonas aeruginosa* biofilms. eDNA is present in biofilms formed by a wide range of different species, including *Bordetella pertussis*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Staphylococcus epidermidis*, *Streptococcus mutans* and *Streptococcus pneumoniae* (2–7), where it plays a role in the initial attachment of bacterial cells to the substratum, but also serves as a scaffold for biofilm architecture (8). Enzymatic digestion of eDNA with DNase has been shown to effectively disperse biofilms, particularly during initial stages of biofilm growth (9). For treatment of cystic fibrosis, a lung disease caused by P. aeruginosa biofilms, treatment with DNase has become the standard therapeutic approach to suppress biofilm formation (10).

Biofilm formation of *Enterococcus faecalis*, frequently associated with failed root canal treatment, also depends on the production of eDNA. Bacterial DNA is released by the action of the secreted metalloprotease gelatinase (GelE) on the major autolysin (AtlA), but only in a subpopulation of

cells (ca 15%) that do not, concomitantly, express serine protease (SprE) for preventing autolysis. The released chromosomal eDNA then facilitates initial attachment and microcolony formation (11, 12).

In addition to this fratricidal mechanism of release, lysis-independent secretion of eDNA from metabolically active cells during early stages of biofilm formation has been described (13). As *E. faecalis* is highly prevalent in endodontic retreatment cases (14), rinsing with DNase might serve as a therapeutic adjunct, provided that it prevents bacterial adhesion to the canal wall and/or disperses established biofilms of *E. faecalis*.

It has previously been shown that DNase treatment reduces the amount of cells recovered from *E. faecalis* biofilms grown on dentine blocks (15), but thus far, no data are available on the effect of DNase treatment on adhering cells of *E. faecalis* in root canals. This study hypothesized that DNase treatment reduced adhesion and dispersed young biofilms of *E. faecalis*, in 96-well plates and extracted teeth. In this study, we, therefore, aimed a) to determine the effect of DNase treatment on *E. faecalis* in 96-well plates after 1 h of adhesion and after 24 h of biofilm formation using a combination of confocal microscopy and digital image analysis and b) to determine the effect of DNase treatment on the number of attached *E. faecalis* cells to the root canal wall of extracted teeth after 1 h of adhesion and 24 h of biofilm formation.

MATERIALS AND METHODS

Bacterial cultures

E. faecalis DSM 20478^T was cultivated on blood agar plates (SSI, Copenhagen, Denmark) at 35°C under aerobic conditions and grown in tryptic soy broth (TSB, Scharlab, Barcelona, Spain) for 18 h. Prior to experimental use, cells were washed with fresh TSB and adjusted to an optical density of 0.7 (550 nm) for adhesion experiments (2×10^8 cells/mL) and of 0.4 (550 nm) for biofilm experiments (10^8 cells/mL).

Effect of DNase treatment on bacterial adhesion and biofilm dispersal in 96-well plates

For adhesion experiments, *E. faecalis* suspensions were mixed with equal volumes of DNase I (Sigma Aldrich, Brøndby, Denmark; final concentration of 50 Kunitz/mL, 5 mM $MgCl_2$ in 0.9% NaCl) or heat-inactivated DNase I (100°C, 30 min), injected in 96-well plates (Ibidi µ-plate uncoated, Planegg/Martinsried, Germany) in triplicates, and incubated for 1 h at 35°C in jars containing anaerobic gas generating sachets (Anaerogen; Thermo Fisher Scientific, Copenhagen, Denmark). Thereafter, wells were washed five times with 0.9% NaCl for removing non-adherent cells. For biofilm experiments, *E. faecalis* was injected in 96-well plates, incubated for 24 h at 35°C under anaerobic conditions, and then washed five times with 0.9% NaCl. Triplicate biofilms were thereafter incubated for 1 h at 35°C in anaerobic jars with DNase I or heat-inactivated DNase I and washed five times with 0.9% NaCl to remove unbound cells. Bacterial cells were stained with SYTO® 9 (Thermo Fisher Scientific, Copenhagen, Denmark; 3.3 µM in 0.9% NaCl) and imaged using a confocal laser scanning microscopy (Zeiss LSM 510 META, Jena, Germany) equipped with a 20×/0.8 NA objective (Plan-Apochromat, Zeiss, Jena, Germany) and a 488-nm laser line. Detection was performed from 501 to 554 nm; three images (450×450 µm) per well were acquired with the following settings: Pinhole 3 Airy units (optical slice, 117 µm), pixel dwell time 3.29 µs, line average 2, and resolution 0.22 µm/pixel. Images were then exported to the software daime (16), segmented with appropriate brightness thresholds, and the area covered by bacteria was determined. All experiments were performed in biological triplicates.

Visualization of eDNA

Cells of *E. faecalis* were allowed to adhere or form biofilms, as described above. Non-adherent cells were removed by washing five times with 0.9% NaCl. eDNA and dead (permeable) cells were visualized by staining with the membrane-impermeant nucleic acid stain TOTO®-1 (2 µM; 15 min incubation time; Thermo Fisher Scientific, Copenhagen, Denmark), whereas remaining cells were stained with the membrane-permeant nucleic acid stain SYTO® 60 (10 µM; incubation time, 15 min; Thermo Fisher Scientific, Copenhagen, Denmark). Excitation was performed with 514- (TOTO-1®) and 633-nm (SYTO® 60) laser lines. Detection was performed from 522 to 597 nm (TOTO-1®) and from 640 to 704 nm (SYTO® 60). Images were acquired using the 20×/0.8 NA objective (450×450 µm image size; 1 Airy unit pinhole (optical slice, 60 µm), pixel dwell time 1.6 µs, line average 2, and resolution 0.22 µm/pixel) or a 63×/1.2 NA water immersion objective (C-Apochromat, Zeiss; 143×143 µm image size; 1 Airy unit pinhole (optical slice, 0.9 µm), pixel dwell time 1.6 µs, line average 2, and resolution 0.07 µm/pixel). Experiments were performed in biological duplicates.

Selection and preparation of teeth

Teeth were obtained from a pool of extracted teeth stored at −4°C immediately after extraction. Informed consent was obtained from the donors. According to the local ethics committee, an approval for irreversibly anonymized use of these bypass products was not needed. Single-rooted maxillary and mandibular permanent premolars were chosen for the study. Inclusion criteria were a single root with a mature apex and a single round-to-oval root canal; exclusion criteria were an existing root canal filling, deep coronal caries, obvious translucency of the root, a pronounced root curvature, and an immature apex.

The teeth were defrosted for 4 h at 4°C. They were then stored in 3% (w/v) sodium hypochlorite for 1 h for removing the periodontal tissue. After access cavity preparation, the working length was established using a size 10.02 file and was defined to be 0.5 mm shorter than the length after first apical visibility of the instrument. After establishing a glide path, canals were negotiated with rotating instruments up to size 40 (ProTaper Universal F4, Maillefer, Ballaigues, Switzerland). The irrigation solution used during instrumentation was 3% sodium hypochlorite, followed by a final rinse with 2 ml 17% (w/v) EDTA. After closing the apex with glass ionomer cement (Chemfil Rock, Dentsply, Tulsa, OK, USA), teeth were stored in deionized water until further use.

Effect of DNase treatment on bacteria adhered to the extracted teeth

Effect of DNase treatment in extracted teeth was assessed using a two-phase crossover design. For adhesion experiments, 28 teeth were randomly assigned to a treatment order and

autoclaved. Root surfaces were sealed with nail varnish, and canals were dried with sterile paper points (Protaper Universal F4, Maillefer, Ballaigues, Switzerland). *E. faecalis* suspensions were mixed with equal volumes of DNase I (final concentration 50 Kunitz/mL; 5 mM $MgCl_2$ in 0.9% NaCl) or heat-inactivated DNase I (100°C, 30 min) and injected into the root canals (n=56) using sterile syringes (gage 27, BD Microlance, Franklin Lakes, NJ, USA). Teeth were incubated for 1 h at 35°C under anaerobic conditions in a humid chamber. Then all teeth were thoroughly rinsed with 5 mL of sterile 0.9% NaCl using sterile syringes for removing non-adherent cells. Circumferential filing was performed (1 cm height of stroke, 2 strokes/s) with Hedström files (30.02, VDW, Munich, Germany) for 30 s. Thereafter, 10 µl of 0.9% NaCl was sampled from the canals using sterile paper points (Protaper Universal F4) that were then transferred to 1 mL of 0.9% sterile NaCl and vortexed for 2 min. The resulting suspension was serially diluted and plated on blood agar plates in sextuplicates. After 2 days of incubation at 35°C, colony forming units (CFU) were determined. Between two experimental phases, teeth were cleaned thoroughly, autoclaved again, and the nail varnish was applied again. Sterility after autoclaving was assured by incubating teeth other than the ones used for the experiment with sterile TSB and cultivating the broth on blood agar plates.

Effect of DNase treatment on biofilm dispersal in extracted teeth

For biofilm experiments, nine teeth were randomly assigned to a treatment order and autoclaved. Suspensions of *E. faecalis* were injected into the canals (n=18), as described above, and incubated at 35°C for 24 h. Thereafter, teeth were carefully dried with sterile paper points, and DNase I or heat-inactivated DNase I (50 Kunitz/mL; 5 mM $MgCl_2$ in 0.9% NaCl) was injected with sterile syringes and left to incubate for 1 h at 35°C under anaerobic conditions. Teeth rinsing, circumferential filing, sampling, serial dilution, and *E. faecalis* cultivation were performed as described in the adhesion experiments.

Statistical analysis

For the experiments in 96-well plates, average areas covered by bacteria were calculated for DNase- and heat-inactivated DNase-treated wells. Normal distribution was verified using QQ-plots and Shapiro–Wilk normality tests. One-tailed t-tests were performed for assessing whether the area was lower after DNase treatment. Calculations were performed using R (www.r-project.org). Based on results on bacterial adhesion in 96-well plates, a sample size of 28 was calculated for detecting a 25% treatment difference (α=0.05; 1-β=0.8) in teeth. Because no effect of DNase was observed on established biofilms in 96-well plates, only nine teeth were included in biofilm dispersal experiments. CFU data from the experiments involving teeth were analyzed using mixed-effect Poisson regressions with counts as the dependent variable, treatment and date of experiment as fixed effects, and the teeth as random effects. The logarithm of the dilution factor was included as an offset. For each tooth and treatment, logarithms of mean CFU counts, adjusted for respective dilution factors and date, were calculated. To allow for overdispersion relative to the Poisson variance, robust standard errors were used. Stata version 13.1 (StataCorp LLC, College Station, TX, USA) was used for statistical calculations. For all calculations, p-values<0.05 were considered to be statistically significant.

RESULTS

After 1 h of adhesion in 96-well plates, most but not all *E. faecalis* cells showed overlapping signals from TOTO®-1 (green) and SYTO® 60 (red). In some areas, eDNA was observed as cloud-like structures without SYTO® 60 signals (white arrowheads; Fig. 1a). If both stains have equal access to DNA targets, TOTO®-1 outcompetes SYTO® 60 because of its higher binding affinity. Therefore, double staining of cells with both stains can be explained by the fact that the cell membrane is partially permeable to TOTO®-1 or it can be an indication of eDNA being present on the bacterial cell surface. As no green fluorescence was observed on DNasetreated cells (Fig. 1b), the signal is probably due to eDNA. Cloudlike eDNA structures were also present in 24-h-old biofilms of *E. faecalis*, but only few cells showed distinct cell surface staining (Fig. 1c). In DNase-treated 24-h-old biofilms, TOTO®-1 staining revealed considerably fewer cloud-like eDNA patches (Fig. 1d).

To test whether eDNA present on and in between cells of *E. faecalis* had an impact on bacterial attachment, the area of adhering cells in the presence of DNase or heat-inactivated DNase was quantified. The area covered by cells after 1 h of incubation with DNase was 54.6%±5.9% (SD) lower that that

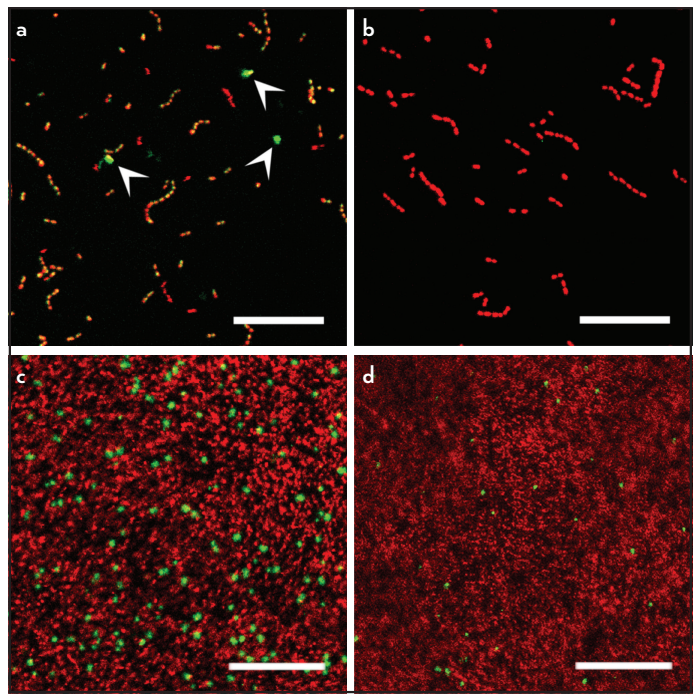

Figure 1. (a-d) Visualization of eDNA. Cells of *E. faecalis* were inoculated into 96-well plates and allowed to adhere for 1 h (a and b) or to form biofilms for 24 h (c and d). eDNA was stained with TOTO®-1 (green), and bacteria were counterstained with SYTO® 60 (red). eDNA was present on the surface of most cells and as cloud-like patches (arrowheads) after 1 h of adhesion (a). After DNase treatment for 1 h, eDNA was removed (b). After 24 h, eDNA could not be visualized on cell surfaces, but was observed as numerous cloud-like patches in the biofilms (c). DNase-treated 24-h-old biofilms displayed considerably fewer structures stained with TOTO®-1 (d). Bars=20 µm

by cells under control treatment with heat inactivated DNase (P<0.05). Treatment with DNase, thus, hampered adhesion of *E. faecalis* to 96-well plates (Fig. 2a and b). In extracted teeth, treatment with DNase resulted in fewer cells of *E. faecalis* adhering to the dentine wall after 1 h of adhesion, but the difference with the control treatment with heat-inactivated DNase was not statistically significant (P=0.14; Fig. 3).

The area covered by bacteria in 24-h-old biofilms in 96-well plates did not decrease as a result of DNase treatment (−15.0%±9.8%; P=0.96). Representative images are shown in Fig. 2c and d. Likewise, DNase treatment of 24-h-old *E. faecalis* biofilms did not reduce the number of CFU recovered from dentine samples (P=0.98; Fig. 3). As the recovered CFU were nearly identical for DNase and heat-inactivated DNase treatments, the experiments were stopped after nine teeth had undergone both treatments.

DISCUSSION

The importance of eDNA for bacterial biofilm formation, including biofilms of *E. faecalis*, is uncontested; dispersing biofilms by enzymatic treatment of matrix components may be a promising therapeutic approach in different medical fields (9). Our data, however, do not support the sole use of DNase as an anti-biofilm agent during root canal treatment.

It has previously been shown that the number of *E. faecalis* cells adhering to dentin blocks is reduced in the presence of DNase (15), albeit with less than 1 log reduction. In this study, we found that DNase treatment decreased adhesion of *E. faecalis* to the polymer surface of microwell plates (P<0.05), but not to the surface of root canals (P=0.14; Fig. 2 and 3). Root canal anatomy is complex, and although treatment was performed in singlerooted premolars with one canal, it is conceivable that the enzyme did not exert its action in all sampled areas or that the rinsing procedure was less effective compared with treatment on dentine blocks. Furthermore, differences in the choice of strains (*E. faecalis* DSM 20478 vs. ATCC 29212), the employed medium (TSB vs. brain heart infusion), the concentration of DNase (50 Kunitz/mL vs. 220 Kunitz/mL), and in the sampling procedure (30 s of circumferential filing vs. 5 min of ultrasonication) may explain the different results observed by Li et al. (15). In the present study, root canals were irrigated with sodium hypoclorite and EDTA during instrumentation, and no inactivation of sodium hypoclorite with sodium thiosulfate was performed prior to bacterial inoculation and DNase treatment. While both irrigation solutions can compromise enzyme activity of DNase, an inhibition was unlikely in the present setup because the teeth were stored in demineralized water for several weeks and autoclaved before DNase treatment. Finally, differences in root canal anatomy between individual teeth are unlikely to influence the treatment results as all teeth were treated both with active and heat-inactivated DNase. Changes in the canal morphology produced by 30 s of manual circumferential filing in between treatments may be considered negligible.

After 24 h of undisturbed biofilm formation, no effect of a 1 h DNase treatment could be observed in 96-well plates and extracted teeth (Fig. 2 and 3). Similarly, Li et al. reported lower effects of DNase treatment after 12, 24, and 48 h compared to 1 h, although DNase was present during the entire experiment (15). These results are in accordance with those of other studies that have demonstrated that the contribution of eDNA

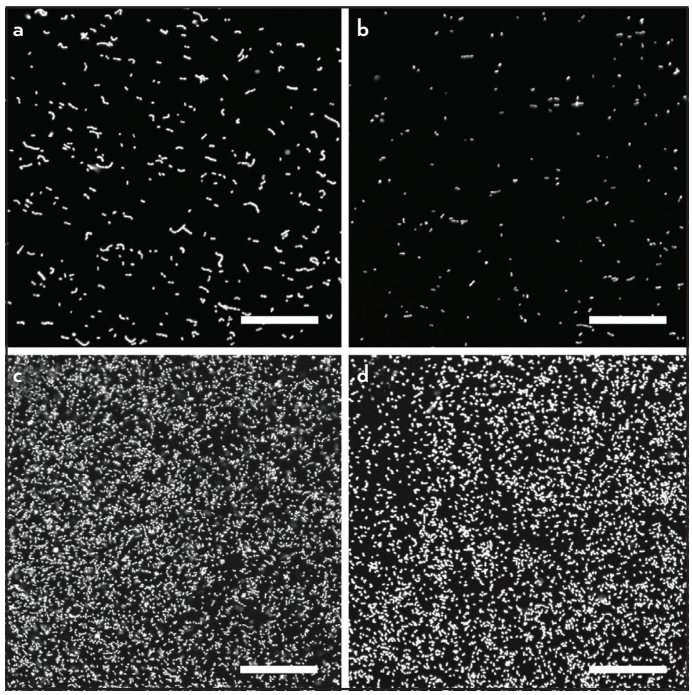

Figure 2. (a-d) Effect of DNase treatment on *Enterococcus faecalis* in 96-well plates. Cells of *E. faecalis* were inoculated into 96-well plates and treated for 1 h with heat-inactivated DNase (HI DNase) (a and c) or DNase (b and d) during adhesion (a and b) or after 24 h of biofilm formation (c and d). Bacteria were stained with SYTO® 9 and quantified in confocal microscopy images. DNase treatment hampered adhesion of *E. faecalis* after 1 h compared with that observed with the control treatment (P<0.05), but did not reduce the amount of biofilm grown after 24 h (P=0.98). Bars=100 µm

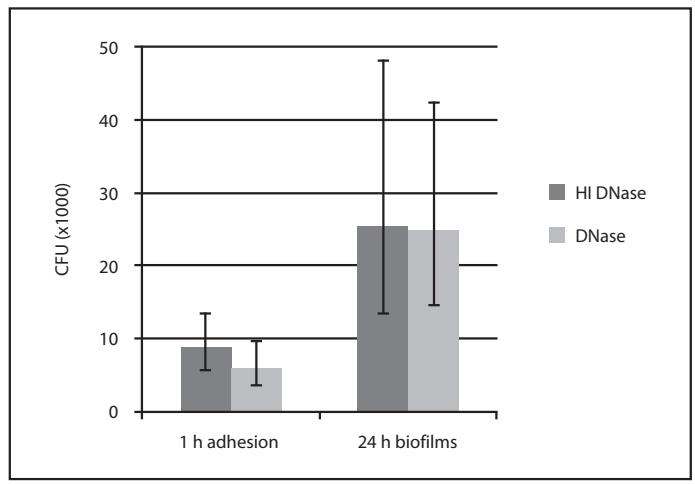

Figure 3. Effect of DNase treatment on *Enterococcus faecalis* in teeth. Cells of *E. faecalis* were inoculated into extracted teeth and treated for 1 h with heat-inactivated DNase (HI DNase) or DNase during adhesion or after 24 h of biofilm formation. The number of adhering cells was quantified using CFU counts. No statistically significant difference was found between DNase and control treatment. Error bars=95% confidence intervals

to biofilm stability is predominantly observed in the initial stages of biofilm formation (1, 4, 11, 17). Staining with TOTO®-1 showed that DNase treatment of 24-h-old biofilms in 96-well plates removed most of the cloud-like eDNA patches in the biofilms (Fig. 1). It is, thus, unlikely that the treatment effect ceased after 24 h because of penetration problems or because eDNA was protected from enzymatic digestion. Most likely, matrix components other than eDNA secure biofilm stability at this stage of biofilm development (18).

The present study mimicked a clinical setting only to some degree. Single-rooted teeth with straight and thus easy-to-treat canals were chosen, and monospecies biofilms were grown for no more than 24 h. In a clinical setting, and particularly in retreatment cases, biofilms are mature and typically comprise multiple species (19); often, the intricate root anatomy renders mechanical and chemical debridement more difficult. Even under the chosen simplified conditions, though, DNase treatment had little impact on adhesion and biofilm stability of *E. faecalis*. Prior to sampling, the root canals were flushed with copious amounts of NaCl to remove planktonic organisms and predominantly sample bacteria that adhered firmly to the canal walls (20, 21). Sampling was, however, limited to areas that were accessible to mechanical debridement with 30.02 Hedström files and may not have reached all parts of the root canal (22). Still, there is little reason to believe that the effect of DNase treatment is more pronounced in sites inaccessible to mechanical cleaning. It is conceivable that the matrix-degrading effect of DNase enhances the penetration of antiseptic agents into the biofilm, as shown by Li et al. for chlorhexidine (15), but the combined application of DNase and NaOCl or Ca(OH)2 is precluded because a strongly alkaline environment would inactivate the enzyme (23).

CONCLUSION

In conclusion, this study shows that DNase treatment hampers adhesion of *E. faecalis* under in vitro conditions. However, DNase has no effect, as a therapeutic agent to disturb *E. faecalis* colonization in an ex vivo model.

Disclosures

Conceived and designed the experiments: S.S., J.G., K.W.N., R.L.M.

Performed the experiments: S.S., K.W.N., J.G.

Analyzed the data: S.S., M.V.

Wrote and approved the paper: S.S., J.G., K.W.N., M.V., R.L.M.

Conflict of interest: The authors deny any conflict of interest.

Ethics Committee Approval: Not required.

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Authorship contributions: Concept – S. S., K. W. N.; Design – S. S., K. W. N., J.G.; Supervision – S. S., R. L. M.; Fundings – S. S., K. W. N., R. L. M.; Materials – S.S.; Data collection &/or processing – S. S., J. G., M. V.; Analysis and/or interpretation – S. S., K. W. N., M. V.; Literature search – S. S., J. G.; Writing – S. S., K. W. N.; Critical review – S. S., K. W. N., R. L. M., J. G., M. V.

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