Calcium hydroxide treatment does not alter the susceptibility of Enterococcus faecalis biofilms to sodium hypochlorite

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ORIGINAL ARTICLE

Calcium Hydroxide Treatment Does Not Alter the Susceptibility of Enterococcus faecalis Biofilms to Sodium Hypochlorite

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ABSTRACT

Objective: To investigate the influence of calcium hydroxide ($Ca(OH)_2$) on susceptibility to disinfection with sodium hypochlorite (NaOCl) of biofilm bacteria.

Methods: Monospecies biofilms of eight *Enterococcus faecalis* strains were subjected to a 2-h challenge with $Ca(OH)_2$. After a recovery phase, the biofilms were treated with a concentration of NaOCl that was lower than the minimum inhibitory concentration. In a metabolic assay, the efficacy of NaOCl disinfection in $Ca(OH)_2$-challenged biofilms and unchallenged biofilms was evaluated. The data were analyzed with Mann-Whitney U and Kruskall- Wallis tests. A P value of less than 0.05 was considered statistically significant.

Results: There were marginal differences in susceptibility to NaOCl among the *E. faecalis* strains. After the $Ca(OH)_2$ challenge, seven strains remained equally susceptible to NaOCl disinfection whereas one strain became more resistant to NaOCl (P= 0.03).

Conclusion: After a $Ca(OH)_2$ challenge, in general *E. faecalis* remained equally susceptible to disinfection with NaOCl.

Keywords: Biofilm, calcium hydroxide, disinfection, endodontic

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HIGHLIGHTS

- *Enterococcus faecalis* is not genetically affected by calcium hydroxide.
- Calcium hydroxide does not induce resistance of *Enterococcus faecalis* against disinfection substances such as sodium hypochlorite.
- It is expected that, after the application calcium hydroxide as a root canal dressing, bacteria can still be disinfected with sodium hypochlorite.

INTRODUCTION

Biofilm infection of the root canal system (RCS) accounts for almost all cases of apical periodontitis. Biofilms are complex aggregates of microorganisms attached to a surface and in which the microorganisms are embedded in a matrix of self-produced extracellular polymeric substances. In most biofilms, the matrix consists of over 90% of the dry mass. The matrix protects organisms against desiccation, biocides, some antibiotics, host immune defenses, etc. (1). These biofilms play an essential role in the creation and maintenance of periapical lesions by both the production of products that are responsible for tissue breakdown and the stimulation of the immune system resulting in an inflammation that needs to be treated.

An important aspect of root canal treatment is to disinfect the RCS by removal of infected tissue using rotating instruments followed by irrigation of an disinfecting agent such as sodium hypochlorite (NaOCl). It is well-known, however, that bacteria remain in the canal space after this instrumentation and irrigation. Thus, many clinicians use a calcium hydroxide ($Ca(OH)_2$) paste as a temporary root canal dressing to gain additional disinfection and to prevent regrowth of remaining bacteria in between two treatment sessions (2, 3).

The use of $Ca(OH)_2$ is somewhat questionable. Bacteria can still be recovered from root canals after endodontic treatment with $Ca(OH)_2$ (4). Also, in *in-vitro* biofilm studies, the disinfecting efficacy

of $Ca(OH)_2$ seems to be limited (5, 6). Moreover, the complex anatomy of the RCS hinders disinfecting agents from penetrating the entire pulpal space and in a histological study residual biofilms were found in niches of the RCS after $Ca(OH)_2$ (7). Therefore, it is interesting to study the effect of $Ca(OH)_2$ on residual biofilms.

Previously, it was shown that $Ca(OH)_2$ causes changes in the configuration of the biofilm matrix (6, 8). *Enterococcus faecalis* has been associated with failed endodontic treatments (9). One study reported that *E. faecalis* still formed biofilms in the presence of $Ca(OH)_2$ and that the bacteria were encompassed in an even denser matrix. Moreover, approximately 90% of these cells were viable (8). Another study showed that a dense flocculation occurred in dual-species biofilms of *E. faecalis* and *Pseudomonas aeruginosa* after a treatment with $Ca(OH)_2$. Also, there was only a factor 100 reduction of viable cells in these biofilms, which contained on average 3×10^8 cells (6). It has also been reported that *E. faecalis* can produce more biofilm mass and protein in response to high pH or sub-inhibitory doses of antimicrobials (10, 11).

From the literature we know that calcium, which is released when $Ca(OH)_2$ is solubilized in water, stimulates the formation of extracellular polysaccharides (EPS) and that it increases the yield strength of biofilms (12, 13). Plus, a high pH favors the uptake of calcium in the EPS (14).

The above-mentioned *in-vitro* observations combined with the clinical findings support the theory that $Ca(OH)_2$ may stimulate the formation of a more dense biofilm of *E. faecalis* that may be less sensitive to disinfection in a subsequent treatment session. The formation of a dense matrix or an increase in the coherence of the biofilm matrix can protect the bacteria from disinfecting measures. Also, the genetic make-up of the bacteria may be altered by $Ca(OH)_2$ (15). Until now, no literature is available on inadequate $Ca(OH)_2$ treatments and the possible effects on the susceptibility to disinfection of the remaining biofilm bacteria. Therefore, the aim of this study was to investigate the influence of calcium hydroxide ($Ca(OH)_2$) on the susceptibility of biofilm bacteria to disinfection with sodium hypochlorite. The null-hypothesis of this study was that $Ca(OH)_2$ treatment of *E. faecalis* strains in an active attachment biofilm model does not induce changes in the sensitivity of these biofilms to NaOCl resulting in a more resistant biofilm.

METHODS

Approval

The ethical committee of Academic Centre Dentistry Amsterdam, ACTA, confirmed that the Medical Research Involving Human Subjects Act (WMO) did not apply to this study and that this study was performed according to the ethical guidelines at ACTA. The reference number is 2017036Waal.

TABLE 1. Origins and sources of the employed *E. faecalis* strains.

Strain name	Origin	Source	Reference
E1	oral rinse	Sedgley	(18, 27)
E2	oral rinse	Sedgley	(18, 27)
E3	oral rinse	Sedgley	(18, 27)
AA-OR34	oral rinse	Sedgley	(18, 28)
ER3/2s	orthograde retreatment	Sedgley	(18)
ER5/1	orthograde retreatment	Sedgley	(18)
OS16	oral rinse	Sedgley	(18, 29)
V583	Lab strain	Sahm	(30)

Medium

Biofilms were grown in modified semi-defined broth, pH 7.1, with 0.2% sucrose (BMS) whose composition has been previously published (16).

Biofilm Growth, Treatment and Resazurin metabolism

The resazurin metabolism assay and the model have been described previously (17). It consists of a standard 96-well microtiter plate and a lid with 96 polystyrene pegs that fit into the wells (TSP; Nunc, Roskilde, Denmark).

The origins of the *E. faecalis* strains can be found in Table 1. *E. faecalis* strains E1, E2, E3, AA-OR34 and OS16 originated from oral rinses and were kindly provided by Christine Sedgley (18). Strains ER3/2s and ER5/1, which have not been published before, also originated from the Sedgley lab and were isolated from orthograde retreatment. Strain V583 (ATCC700802) was used as reference strain. All strains were maintained in stocks at -80°C, and fresh cultures were obtained by the addition of 500 µL of frozen stock culture to 5 mL semi-defined medium supplemented with yeast +0.36% glucose and incubation of 6 h in an anaerobic jar (80% N_2, 10% H_2 and 10% CO_2) at 37°C (16). By initial optical densities at 600 nm (OD_{600}) at culture density 0.8 (Spectramax M2, Sunnyvale, CA, USA) the cultures were in a late log phase and contained approximately $5´10^8$ cells mL^{-1}. After 1:100 dilution with BMS, 200 µL of this inoculant was dispersed per well of a 96-well microtiter plate and the plate was covered with the TSP lid. The medium was refreshed after 18 h. After 24 h, the TSP lid with adhered biofilms was transferred to a 96-well plate with 200 μL well $^{-1}$ BMS or 10% $Ca(OH)_2$ (E. Merck, Darmstadt, Germany) (weight/volume, w/v) in BMS (slurry), pH 12.6 for 2 h. Subsequently, the biofilms were washed twice in 200 μL phosphate buffered saline (PBS) and transferred to BMS for an overnight recovery of the biofilms at 37°C anaerobic. Shortly before the final treatments, the free available chlorine concentration of the NaOCl (Orphi Farma, Lage Zwaluwe, The Netherlands) was verified with an iodometric titration procedure and NaOCl was diluted in demineralized water to obtain 1 or 10 ppm solutions of active chlorine (19). 1 ppm NaOCl appeared to be a sub-bactericidal dose for these particular biofilms and therefore suitable for detecting a possible difference between the groups. When the bio-

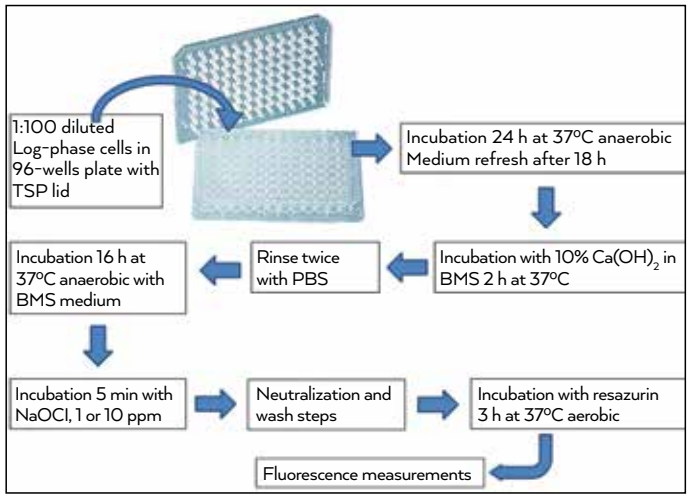

Figure 1. The treatment scheme of the viability assay in which biofilms were exposed to calcium hydroxide ($Ca(OH)_2$) and were subsequently treated with sodium hypochlorite (NaOCl)

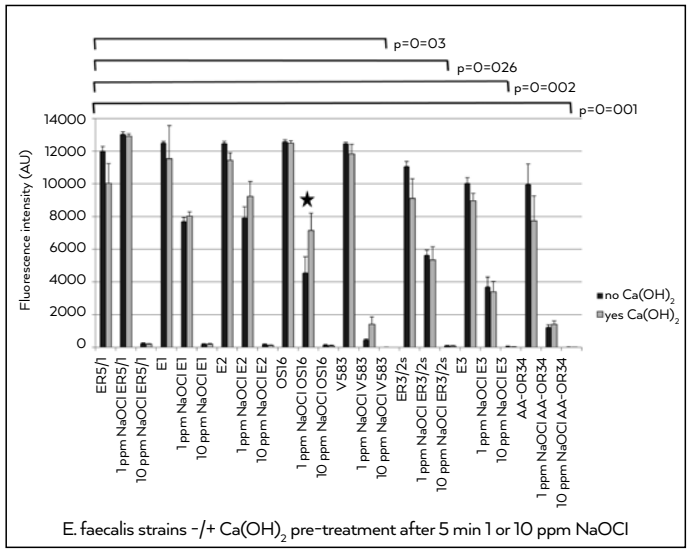

Figure 2. Metabolic activity of various *Enterococcus faecalis* strains without or with pre-treatment with calcium hydroxide and after a 5-min treatment with 1 or 10 ppm sodium hypochlorite. The controls were treated with phosphate buffered saline. Only one strain, *E. faecalis* OS16 (marked with a star), became less susceptible to NaOCl after a pretreatment with Ca(OH)2 (P= 0.03). Error bars represent standard error of the mean. The individual strains are arranged from least susceptible to NaOCl (left) to most susceptible to NaOCl (right). Differences in susceptibility between strains is shown with the bars depicted above the graph.

films were 42-h old, they were treated for 5 min in a 96 wells plate that contained 1 or 10 ppm NaOCl 210 µL in each well. Then, NaOCl was neutralized for 5 min with 1% sodium thiosulphate (Merck) in 2% buffered peptone water (BPW; Oxoid, Basingstoke, UK) after which the biofilms were washed twice in PBS. The negative control group was treated with PBS. Sterile pegs were included to determine the background measurement. Subsequently, the pegs were immersed in 0.016 mg mL^{-1} resazurin (Sigma-Aldrich, St. Louis, MO, USA) in BMS and incubated at 37°C in air. Metabolic activity was determined by measuring the fluorescence intensity (FI) of each well, which was recorded at room temperature in a fluorimeter (Spectramax M2; Molecular Devices, Sunnyvale, CA) using 485-nm excitation and 580-nm emission wavelengths. Readings were taken at 3 h. For an overview of the culturing and handling of the biofilms see Figure 1. Each experiment was performed in duplo and the assay was repeated on two separate occasions. The average background measurement was substracted from the FI readings, which were then averaged.

Statistical analysis

All data were analysed using statistical analysis software (SPSS Version 21, IBM Corp.; Armonk, NY, USA). The data were not normally distributed and therefore were non-parametrically tested. Mann Whitney U tests were used to calculate differences between groups, while a Kruskal-Wallis test was used to test the median of multiple groups. A P value of less than 0.05 was considered significant.

RESULTS

There were significant differences in the susceptibility to NaOCl between the individual strains of *E. faecalis*: strain ER5/1 was the least susceptible, whereas AA-OR34 was the most susceptible (Figure 2). These differences were independent of the use of $Ca(OH)_2$.

On the whole, *E. faecalis* biofilms that had been exposed to $Ca(OH)_2$ were equally susceptible to NaOCl disinfection as the control biofilms (P=0.343). However, when evaluating the strains individually, *E. faecalis* OS16 was less susceptible to NaOCl disinfection (P=0.03) (Figure 2).

DISCUSSION

The aim of the present study was to investigate whether susceptibility to disinfection by NaOCl of *E. faecalis* biofilms would change after treatment with $Ca(OH)_2$. The current results show that, after $Ca(OH)_2$ treatment, the majority of the tested strains remained equally susceptible to disinfection with NaOCl. One strain, OS16, became significantly less susceptible and none of the strains became more susceptible to NaOCl disinfection. The reference strain, V583, was not different from the oral clinical isolates. The null-hypothesis was therefore accepted.

Although, the current model does not resemble the clinical situation, it was specifically designed to investigate our fundamental question about the behavior of *E. faecalis* after $Ca(OH)_2$ treatment. *E. faecalis* is a bacterial species often encountered as a recalcitrant organism in unsuccessful root canal treatments. It is well-studied in laboratory experiments and, because of its tenacity, is often used as a target organism in disinfection experiments. It is known to survive high pH (6, 8, 10, 20). The presence of calcium stimulates the formation of EPS in *E. faecalis* biofilms and this may help bacteria within these biofilms to survive certain antimicrobial conditions (12).

The biofilms were grown on an inert substrate (polystyrene pegs) to exclude any possible interference of dentine. Low sub-inhibitory concentrations of NaOCl were used because the susceptibility of *E. faecalis* before and after $Ca(OH)_2$ application cannot be measured with clinically applicable NaOCl concentrations. Prior to the current study, sub-bactericidal concentrations and incubation-time of $Ca(OH)_2$ and NaOCl were established for these *E. faecalis* biofilms. In a previous study, a saturated slurry of 10% $Ca(OH)_2$ at pH 12.5 was shown to be a non-inhibitory concentration in biofilms. Since the solubility constant of $Ca(OH)_2$ at room temperature is 1.85 g L^{-1}, an active concentration of approx. 0.2% OH^- can be obtained. Commercial $Ca(OH)_2$ pastes/slurries are sold at higher concentrations (30-35%) but will result in a similarly saturated active concentration of 0.2%. The sub-inhibitory treatment of NaOCl was determined at 5 min 1 ppm, which appeared to be a little higher than previously found in similar biofilms (21). If this model is to be employed for investigating the susceptibility of dual- or multi-species biofilms, the concentration of NaOCl will need to be adjusted depending on the type of species and the age of the biofilms (22). It can be argued that these low concentrations of NaOCl are not representative of the clinical situation, but since compounds such as NaOCl strongly react with tissues within the endodontic canals, these low concentrations are likely to be found in the more remote sites of the RCS. This may be one of the reasons that bacteria survive endodontic treatment.

By using a high-throughput system with resazurin, the number of viable cells for the NaOCl sensitivity assay should be roughly similar. This was one of the limitations of the many studies on antimicrobial properties of $Ca(OH)_2$: variation in inoculum size (23). It was therefore necessary to recover the biofilms for 16 hours after the $Ca(OH)_2$ treatment so that the bacterial numbers could be restored to the same number of bacteria in the control biofilms (data not published). A previous study demonstrated that, with $Ca(OH)_2$, the biofilms stayed attached to the surface (6). Therefore, detachment after $Ca(OH)_2$ application, resulting in a lower resazurin signal, was not observed. Also, there was no difference between the negative controls of the non-$Ca(OH)_2$ and $Ca(OH)_2$ groups. Consequently, there were no indications that the results from this study were biased by experimental errors.

Calcium hydroxide is a chemical compound that has one calcium ion and two hydroxide ions (OH^-). These OH^- ions render the environment alkaline in which many bacteria cannot survive. The presence of the free OH- ion as a radical is assumed to have direct effects on the bacterial membrane, protein denaturation and bacterial DNA damage. Certain issues of $Ca(OH)_2$, however, have been largely overlooked. OH^--ions are reducing molecules and are unstable in a solution as they tend to react with CO_2 or other oxides (24). When OH^- is consumed, $Ca(OH)_2$ solubilises and, besides OH^-, Ca^{2+} ions are released. Ca^{2+} ions are divalent cations and as such they bind to the negatively charged surfaces of bacteria, to dentin and to the EPS of the biofilm matrix. This series of events can explain why (traces of) $Ca(OH)_2$ are difficult to remove from the RCS after use. The solubilisation of the depot of $Ca(OH)_2$ in the slurry or paste is a continuous process until the depot has been depleted.

This study was designed for *E. faecalis*. Besides being associated with failed endodontic treatment, *E. faecalis* is easy to culture *in vitro* and therefore is a suitable test microorganism (9). Many properties of *E. faecalis* have already been investigated. For example, a study of the antimicrobial susceptibility of enterococci isolated from root canal showed that *E. faecalis* can be highly resistant to certain antibiotics (25). Our study, however, is the first to show that, despite the changes that occur in the biofilms after the application of $Ca(OH)_2$, *E. faecalis* in general does not become more resistant to disinfection by NaOCl. We should realise though, that the present study was performed on mono-species biofilms and it may be that multispecies interaction, which occurs in endodontic biofilms, can result in more resistance to either $Ca(OH)_2$ or NaOCl (26). It would be interesting to see, in future studies, if our theory holds true when endodontic microcosm biofilms are used. Such a study would possibly also give information about the changes in bacterial composition of the biofilms after the application of $Ca(OH)_2$.

The current findings are clinically relevant because, after the application of $Ca(OH)_2$, there are often residual bacteria. Also, *E. faecalis* is often found in such secondary infections. This study has, however, demonstrated that the implications of an inefficient treatment with $Ca(OH)_2$ may be minor.

CONCLUSION

The current results indicate that, after a challenge with $Ca(OH)_2$, one of eight *E. faecalis* strains became less susceptible to disinfection. However, when using a higher dose of 10 ppm NaOCl, this strain was also inactivated. Seven of eight strains of *E. faecalis* remained equally susceptible to disinfection with NaOCl. Therefore, it appears that, in general, $Ca(OH)_2$ does not affect the susceptibility of *E. faecalis* to NaOCl.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of the Academic Centre Dentistry Amsterdam (ACTA) / 2017036Waal.

Informed Consent: Not applicable as this was a microbiology study with laboratory strains.

Peer-review: Externally peer-reviewed.

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