A new strategy in root canal therapy: there is a lot going on in endodontic disinfection
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Citation for published version (APA):

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Chapter 3

Sodium chloride and potassium sorbate: a synergistic combination against *Enterococcus faecalis* biofilms: an in vitro study

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Abstract

Aim Incomplete disinfection of the root canal system is a major cause of post-treatment disease. This study aimed to investigate the disinfecting property of organic acid salts and sodium chloride (NaCl), in a double-hurdle strategy, on Enterococcus faecalis biofilms.

Method First of all, the high-throughput resazurin metabolism assay (RMA) was used to test a range of organic acid salts. Then, to gain more insight into the efficacy of sorbate salt solutions, 48-h E. faecalis biofilms were evaluated in colony-forming unit (CFU) assays. Chlorhexidine (CHX) and calcium hydroxide [Ca(OH)\textsubscript{2}] were tested in parallel as controls.

Results Sorbate salt produced the largest and most significant reduction of fluorescence intensity in the RMA assay. Neither NaCl nor potassium sorbate (KS) alone induced a clinically relevant reduction of CFU counts after 1 h. Surprisingly, the combination of the two in a single solution had a synergistic effect on the inactivation of E. faecalis. Potassium sorbate amplified the efficacy of NaCl. Of the salts tested, NaCl with KS eradicated E. faecalis biofilms within 1 h.

Conclusion This study showed that the double-hurdle strategy indeed leads to synergistic efficacy and is a possible next step in the complete disinfection of endodontic infections.
Introduction

As observed with periapical radiographs, 20% of AP cases do not heal after root canal treatment. Given the low sensitivity of periapical radiographs, the occurrence of AP is likely to be even higher. Incomplete disinfection of the root canal system is a major cause of post-treatment disease. After contemporary non-surgical root canal treatment, including sodium hypochlorite (NaOCl) irrigation with or without the use of calcium hydroxide (Ca(OH)₂), biofilms can still be detected in the root canal system. NaOCl is a very powerful disinfectant. In the complexity of the root canal system, however, NaOCl does not remove all biofilm and microorganisms. Probably, this is because of the difficulty of dispersing NaOCl during irrigation into the complete root canal system, especially into the apical part of the root canal system. Furthermore, NaOCl is so reactive that while diffusing into a biofilm, it is reduced and rapidly becomes inactive. Therefore, current disinfection procedures must be improved to promote better healing of AP. If the root canal system cannot be completely cleaned in a mechanical or chemical way, other ways of disturbing the biofilm should be found. Perhaps the introduction of environmental stresses to the biofilm, with the aid of physical phenomena diffusion and osmosis, can enhance disinfection.

Environmental stress can be defined as an external factor that has an adverse effect on the physiological welfare of microbial cells, leading to a reduction in growth rate or, in more extreme circumstances, to cell death. Examples of such stresses include extremes of temperature, pH, hypo-osmotic or hyperosmotic pressure, the depletion of nutrients and the use of preservatives. Hyperosmotic pressure, for instance, can inactivate bacteria in a biofilm because it reduces the amount of water that is available to a micro-organism. A disinfection regime with multiple environmental stresses may result in enhanced disinfection efficacy. The principle of exposing microorganisms to more than one environmental stress factor simultaneously with the purpose to inhibit growth is commonly used in the food industry, where it is referred to as “hurdle technology.” Hurdle technology deliberately combines a series of factors (hurdles) that the microorganisms in question are unable to overcome (jump over). These hurdles are environmental stress factors. While being exposed to such hurdles, the microorganisms try to adapt to the environmental changes. Each adaptation however, demands energy and for this reason, microorganisms can become exhausted once there is more than one hurdle to overcome. The literature is replete on the growth-inhibitory effect of multiple hurdles in sublethal doses. In food preservation, low doses are applied because otherwise the food quality becomes affected.

Some organic sodium and potassium salts are generally recognized as safe (GRAS; a US Food and Drug Administration designation that a chemical
or substance added to food is considered safe by experts \(^5\). These salts are used to prevent microbial growth in food or cosmetic products. When used at high concentrations, a synergistic effect caused by hyperosmotic stress, as well as preservative stress, can be expected. Therefore, the aim of this study was to investigate whether hypertonic solutions of GRAS organic sodium or potassium salts can be used to inactivate existing \textit{in-vitro} grown \textit{Enterococcus faecalis} biofilms. This is a new approach because to our knowledge, the hurdle technology has not been used to challenge existing biofilms. Selection criteria of the salts other than those that were GRAS were high solubility and preservative properties to create at least two hurdles for a double-hurdle strategy.

**Materials and Methods**

This study consists of three parts. In the first part, high-throughput screening was performed with the use of the resazurin metabolism assay (RMA). The second and third parts involved two viability assays that were performed to gain a greater insight into the efficacy of the two most promising salts.

**Bacterial Strains and Growth Conditions**
The \textit{E. faecalis} strain E2, isolated from an oral rinse of an endodontic patient, was kindly provided by Dr. C.M. Sedgley \(^58\). The strain was grown, at 37°C on blood agar in an anaerobic jar with an atmosphere of 80% N\(_2\), 10% H\(_2\) and 10% CO\(_2\). Biofilms were grown in semidefinite broth, pH 7.1, with 0.2% sucrose (BMS). Semidefinite broth contains 76 mM of K\(_2\)HPO\(_4\), 15 mM KH\(_2\)PO\(_4\), 10 mM (NH\(_4\))\(_2\)SO\(_4\), and 35 mM NaCl, 2 mM MgSO\(_4\).7 H\(_2\)O and was supplemented with filter-sterilised vitamins (0.04 mM nicotinic acid, 0.1 mM pyridoxine HCl, 0.01 mM pantothenic acid, 1 mM riboflavin, 0.3 mM thiamine HCl, and 0.05 mM D-biotin), amino acids (4 mM L-glutamic acid, 1 mM L-arginine HCl, 1.3 mM L-cysteine HCl, and 0.1 mM L-tryptophan), and 0.3% [weight by volume (wt/vol)] yeast extract \(^55\).

**Screened Materials**
Sodium acetate, (NaA; Sigma-Aldrich, St. Louis, MO, USA), sodium lactate, (NaL; EMD Chemical Inc., Gibbstown, NJ, USA), sodium sorbate, (NaS; TCI Europe NV, Zwijndrecht, Belgium) and sodium chloride (NaCl; Merck, Darmstadt, Germany) were tested for biofilm-inhibitory properties in a high-throughput screen using the RMA. A 0.2% chlorhexidine solution (CHX; Sigma) served as a positive control. The negative control was phosphate buffered saline (PBS; Oxoid, Basingstoke, UK) and the background fluorescence intensity (FI) was measured in sterile
BMS. Sodium chloride was included to compare the efficacy of osmotic stress alone with that of materials with both osmotic and preservation properties. Stock preparations were freshly prepared before each use and were filter-sterilised with a 0.2-μm syringe filter (VWR International BV, Amsterdam, The Netherlands). The concentration of the experimental compounds was set at 4 M. However, the concentration of a saturated solution of NaS is 2.6 M. For this reason, the analyses included an extra group in which 2 M NaS was supplemented with 2 M NaCl to obtain a 4 M solution. The pH was set at 7.

**Resazurin Metabolism Assay**

The RMA and the model that was used have recently been described. The model consists of a standard 96-well microtitre plate and a lid with an identical number of polystyrene pegs that fit into the wells (TSP; Nunc, Roskilde, Denmark). Overnight culture of *E. faecalis* contained approximately $1.2 \times 10^9$ cells mL$^{-1}$ and was diluted tenfold in BMS. Aliquots (200 μL) of the cell suspension were dispensed into each well, and the plate was covered with an ultraviolet-sterilised TSP-lid. BMS was refreshed after 8 h and biofilms were grown on the pegs for 24 h. (In previous pilots, to confirm biofilm growth on the pegs, the pegs were removed from the TSP-lid. After sonication of the peg in PBS, the suspension was diluted, plated and cultured. The number of colony-forming units (CFU) was counted.) After 24 h of incubation, the pegs with adhered biofilms were transferred to a 96-well plate containing the test materials for 1 h. Biofilms incubated in PBS served as the negative control, CHX-treated biofilms served as positive controls. The treatments were terminated by immersing all biofilms into 2% buffered peptone water (BPW; Oxoid) for 5 min, followed by washing three times in PBS. Then, the pegs were immersed in 0.016 mg mL$^{-1}$ resazurin (Sigma-Aldrich) in BMS and incubated at 37°C in air. Metabolic activity was determined by measuring the 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 after 10 min and 3 h of incubation. Each group consisted of 8 samples, and the assay was conducted twice ($n = 16$).

**Screened Materials in Viability Assays**
Because NaS showed the most promising results in the initial screen, the viability assays were continued with potassium sorbate (KS; Sigma-Aldrich), NaCl and combinations of the two. Sodium chloride was added to KS to raise its osmotic value. (The reasoning underlying the change from NaS to KS is explained in the Results section.) Calcium hydroxide (Merck) was added as a suspension in demineralised water. Chlorhexidine was used as a positive control. The pH was set at 7, except for Ca(OH)$_2$ (pH 12.1) and 3.9 M KS (saturated solution) (pH 8.6).

**Viability Assays**
This model was described previously and is referred to as the ACTA active attachment model (AAA-model). An aliquot (150 µl) of overnight culture of *E. faecalis* was dispersed into wells of a 24-well plate (Greiner Bio One, Frickenhausen, Germany) containing 1.35 mL of BMS per well to obtain a suspension of approximately 1.0 x 10$^8$ cells mL$^{-1}$. Round glass cover slips (12 mm diameter; Thermo Scientific, Braunschweig, Germany) were mounted in a custom-made stainless steel lid and suspended in the medium. The medium was refreshed after 18 h, 26 h and 42 h. Biofilm growth was confirmed after swabbing a cover slip and streaking the biofilm onto a glass microscope slide. After heat fixation of the glass slides, the samples were Gram-stained. Biofilm growth was confirmed after examination of the glass slides with a reflected-light microscope (1000 x magnification; Zeiss Axiolab, Axiolab A, Carl Zeiss B.V., Sliedrecht, The Netherlands). After 48 h, the biofilms were treated by immersion in the test materials for 1 h, after which the treatment was ended by immersing the biofilms in BPW. The biofilms were subsequently washed three times in PBS. Each cover slip was then transferred to a vial containing 2 mL of PBS. Biofilms were dispersed by sonication on ice for 45 s at an amplitude of 40 W (Vibra-cell, Sonics and Materials Inc., Newtown, CT). After vortexing for 5 s, the suspensions were diluted and aliquots were seeded onto brain-heart infusion agar plates (Bacto, Le Pont-de-Clair, France). The plates were incubated anaerobically at 37°C for 48 h; then, the numbers of CFUs were counted and logarithmically transformed. The viability assay was used in two experiments. First, with the following groups: 4 M NaCl; 1 M KS; 3 M NaCl + 1 M KS; 3.9 M KS (saturated solution) and Ca(OH)$_2$ (suspension). CHX served as the positive control, and untreated biofilms and PBS-treatment served as negative controls. Second, to examine the effect of the concentrations of NaCl and KS, these solutes were analysed at 0.5 M – 3 M NaCl in combination with either 0.5 or 1 M KS. Again,
CHX and PBS served as positive and negative controls, respectively. In both experiments, each group contained three samples and the assays were conducted three times ($n = 9$).

**Evaluation of Treatments**
RMA: The background FI (sterile medium) was subtracted from the experimental FI values. Treatment efficacy was calculated as the percentage reduction in FI values after treatment relative to the mean value of the control group.
Viability Assays: The CFUs were counted and the numbers were logarithmically transformed.

**Statistical Analysis**
The data were analysed with SPSS version 18.0 (SPSS, Chicago, IL, USA). One-way analysis of variance was used to evaluate the effects of the treatments on the biofilms (FI and CFU). $\alpha < 0.05$ was considered significant.

**Results**

**RMA**
Variations in the concentration (0.5 – 6.1 M) of the salt solutions and treatment times varying from 15 min to 1 h were examined in earlier pilot studies (data not shown). In a series of cascade experiments, conditions and materials proving unsatisfactory were eliminated sequentially. Concentrations above 4 M did not demonstrate any decrease in metabolic activity relative to 4 M. All materials (4 M NaA, 4 M NaL, 4 M NaCl and 2 and 4 M NaS) induced a significant reduction ($P < 0.001$) in the metabolic activity of the biofilms after 1 h of treatment (Figure 1). The reduction of metabolic activity was reflected in the reduction of FI. The 4 M NaS solution produced the greatest reduction (36%) out of all of the tested materials. The positive control, CHX, caused a 100% reduction of metabolic activity. There was no significant difference in the efficacy of 4 M NaL and 4 M NaCl ($P > 0.05$).

**Viability**
Biofilm formation was reproducible and the negative controls contained an average of $8.0 \times 10^7$ (SD = $3.9 \times 10^7$) cells per biofilm. All groups, except 4 M NaCl, showed significant reductions in the number of CFUs ($P < 0.001$) (Figure 2). However, the CFU reduction by treatment with 1 M KS was considered clinically irrelevant. A solution of 3 M NaCl +1 M KS exhibited a synergistic effect, resulting in a distinct decrease in the
numbers of CFUs. None of the samples in this group showed bacterial growth after treatment. The saturated solution of KS was more effective than CHX. Although CHX was designated as the positive-control group, an average of $2.1 \times 10^4$ (SD = $1.4 \times 10^4$) CFUs per biofilm survived.

In the final part of the study, which focused on the effect on viability of NaCl and KS concentrations (Figure 3), the previously observed efficacy of 3 M NaCl + 1 M KS was confirmed ($P < 0.001$). The addition of 0.5 M KS slightly improved the efficacy of NaCl, and the addition of 1 M KS significantly enhanced the efficacy of 1 M NaCl or higher ($P < 0.001$). A clear dose-response was observed.

Synergy can be defined as the joint action of agents such that their combined effect is greater than the algebraic sum of their individual parts. In this study, synergy was defined as: log reduction (red.) of combination > log red. NaCl + log red. KS. Neither 4 M NaCl alone nor 0.5 M KS had any effect on the numbers of CFUs after 1 h (Figures 2 and 3). Together, however, reductions of log 0.86 (0.5 M KS + 0.5 M NaCl) to log 1.32 (0.5 M KS + 3 M NaCl) were observed. The solution of 1 M KS alone significantly reduced the numbers of CFUs (Figure 2 and Figure 3). When different concentrations of NaCl were added to 1 M KS, an additional reduction was seen at NaCl concentrations ≥ 1 M. The observed reductions varied from log 2.99 (1 M KS + 1 M NaCl) to log 7.41 (1 M KS + 3 M NaCl). According to the definition given above, these reductions can be interpreted as synergistic.

The RMA screening revealed that NaL and NaA were least effective; therefore, these compounds were eliminated. The highest metabolic inhibition occurred with NaS. However, solid NaS is unstable and is rapidly oxidised upon exposure to atmospheric oxygen, which caused problems in achieving reproducibility between experiments. Therefore, for the second and final sets of experiments, NaS was replaced by KS. The active substances of the dissolved salts are identical (sorbic acid and sorbate ions). KS is also highly soluble, GRAS and can act as a preservative.
Discussion

The aim of this study was to investigate whether hypertonic solutions of certain organic sodium or potassium salts can be used in a double-hurdle strategy to inactivate, in vitro, existing *E. faecalis* biofilms. Hyperosmotic stress alone can inactivate biofilm bacteria, but disinfection, defined as a log 5 reduction, takes several days. Hence, novel materials and combinations of materials were sought to enhance hyperosmotic disinfection by addition of a second hurdle. A combined solution of 3 M NaCl + 1 M KS proved to be extremely effective in inactivating an *E. faecalis* biofilm within 1 h. The efficacy of the combination came as a surprise although growth-inhibitory effects of NaCl and KS combined in a low-concentration solution have been previously reported.

The mechanism of the synergy between NaCl and KS is not fully understood, but we can speculate about why this combination acts antimicrobial. In general, hypertonic salt solutions induce a hyperosmotic stress response owing to a loss of turgor as a result of the loss of intracellular water. One universal response to the temporary loss of turgor is accumulation of solutes in the cytoplasm; this increases the internal osmotic value and thus turgor can be restored. Non-ionic uncharged solutes (the so-called compatible solutes) are generally preferred for accumulation because otherwise many enzymes will begin to lose their activity in the presence of high intracellular concentrations of ionic salt. However, when compatible solutes are not available, potassium is accumulated as a first-aid response to the loss of turgor. Low intracellular water levels as a result of hypertonic salt also triggers changes in the membrane lipid composition, which results in loss of membrane integrity.

In hypertonic solutions of NaCl, the cells will plasmolyze. The efficacy of the NaCl + KS combination in particular, may be explained as follows: the hypertonicity of the solution stimulates the cells to store molecules and ions to prevent efflux of water. Moreover, undissociated sorbic acid molecules are hydrophobic and diffuse readily through the plasma membrane but also potassium ions and sorbate ions can be actively accumulated by the cell.
bacteria. However, potassium ions hamper enzyme metabolism due to the increased ionic charge \(^{47}\), and sorbic acid and sorbate ions disturb cell metabolism and de-stabilise cell membranes \(^{39,61,217}\). Thus, the combination of osmotic stress and the presumed subsequent intracellular accumulation of potassium ions, sorbate and sorbic acid results in a cascade of events that is catastrophic to microbial cell viability.

Sorbic acid (trans-trans-2,4-hexadienoic acid) is a six-carbon unsaturated fatty acid that was first isolated from unripe berries of the rowan tree (Sorbus aucuparia). This acid or its anionic salt is used in a variety of food products and has a broad range of antimicrobial activities \(^{23,126}\).

Sodium hypochlorite was not included as positive control in the present study, because in a previous study the efficacy of a 2% NaOCl solution on an E. faecalis biofilm was already demonstrated \(^{228}\). Moreover, because of the evaporation of chlorine, NaOCl solutions should be avoided in high through-put assays such as the peg model used here. Instead, it was chosen to collect more data on the efficacy of a CHX. In the AAA-model, a log reduction of 3.6 was obtained after exposure, for 1 h to 0.2% (0.03 M) CHX. This is in line with the results reported by Zaura-Arite et al. 2001\(^{252}\) who demonstrated that CHX does not penetrate well into a 48-h biofilm. A suspension of Ca(OH)\(_2\) in demi-water was also included as a group, because Ca(OH)\(_2\) is generally accepted as an inter-appointment dressing \(^{117}\). Calcium hydroxide is a slow-release disinfectant, but it was interesting to evaluate its short-term disinfection efficacy. In this model, exposure to Ca(OH)\(_2\) resulted in a reduction of 2.8 reduction after 1 h.

Enterococcus faecalis was chosen as a test organism because of its reported association with persistent peri-apical pathosis \(^{212}\). E. faecalis is a Gram-positive coccus and is able to form biofilms \(^{55}\). It is a tough species to inactivate. Originating from the gut, it can adapt to various environmental changes (pH, osmolarity, competition with other species, nutrient deprivation, or different types of nutrients), which accounts for its resistance \(^{71}\). In previous studies, a single-species biofilm of E. faecalis E2 was found to be more difficult to inactivate than the dual-species biofilm of E. faecalis V583 + Gram-negative Pseudomonas aeruginosa HG 4950 that was described in a previous publication (unpublished data, \(^{228}\)). Therefore the choice was made for this particular strain.

Current root canal disinfection involves copious irrigation with NaOCl solutions, which is a very powerful agent \(^{253}\). However, as a result of the complexity of the root canal system, NaOCl does not inactivate all microorganisms. Because of the physical properties rather than the chemical reactivities, salt solutions may find an application as interappointment root canal dressing to achieve additional reduction of the microbial load or to prevent microbial regrowth between two treatment sessions. Owing
to the high concentrations, diffusion of the ions and molecules into areas beyond the main root canal can be expected. In conclusion, concentrated solutions of NaCl + KS showed highly significant and promising antimicrobial effects. Future studies should focus on the applicability of NaCl and KS as an inter-appointment root canal dressing, as an addition to current disinfecting procedures. For this purpose, a new model is being developed to study whether the hypertonic NaCl and KS solution will diffuse into the complex architecture of the root canal system. Furthermore, the hurdle strategy should be further tested in multispecies biofilms or biofilms grown from bacterial samples retrieved from infected root canals to provide more data on the anti-biofilm efficacy. Although the safety of NaCl, KS and sorbic acid has not been studied yet for endodontic applications, it is not expected that these GRAS-salts will interfere with the positive biological processes of the periapical tissues.