A new strategy in root canal therapy: there is a lot going on in endodontic disinfection
van der Waal, S.V.

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Chapter 4

Cytotoxicity, interaction with dentine and efficacy on multispecies biofilms of a modified salt solution intended for endodontic disinfection in a new in-vitro biofilm model.

S.V. van der Waal, N. Scheres, J.J. de Soet, P.R. Wesselink, W. Crielaard

Abstract

Aim To investigate the cytotoxicity of a modified salt solution (MSS) and evaluate its antimicrobial properties on in-vitro biofilm models.

Methodology In a metabolic assay, fibroblasts derived from periodontal ligaments (PDL) of human extracted teeth were cultured and challenged with MSS or controls. Then, in active attachment biofilm models, the efficacy of MSS in the presence of dentine powder and in eliminating mature biofilms was investigated. In the dentine assay, a biofilm of Enterococcus faecalis was employed. For the final assay, microorganisms were retrieved from infected root canals and cultured to produce biofilms. After the treatments with MSS or the controls, the biofilms were collected, serially diluted and plated. The colony-forming units were counted. One-way ANOVA was used to analyse the differences between the groups. A \( P < 0.05 \) was considered significant.

Results The PDL fibroblasts remained metabolically active after challenges with MSS. Dentine powder did not alter the efficacy of MSS \( (P > 0.05) \). In endodontic biofilms, the culturable bacteria were equally reduced by MSS, 2% chlorhexidine (CHX) or 2% sodium hypochlorite (NaOCl) \( (P > 0.05) \).

Conclusions Modified salt solution is noncytotoxic in vitro and has good antimicrobial properties equal to CHX and NaOCl. Although the results are promising, ex-vivo and in-vivo studies are needed before its use as an interappointment root canal dressing can be considered.
Introduction

Apical periodontitis is the inflammatory response to a microbial biofilm infection of the root canal system. Biofilms are clusters of microorganisms attached to a surface. The microorganisms are embedded in a self-produced slimy matrix. Although an infected root canal space is disinfected with irritants such as sodium hypochlorite (NaOCl) or chlorhexidine (CHX), these cannot eradicate all endodontic microorganisms. A prospective study has reported the success rate of root canal treatments to be 80%, which means that one in five AP lesions fails to heal.

Current disinfectants are safe when used within the confinement of the root canal, but are toxic to the periapical tissues after accidental leakage or extrusion. NaOCl is highly cytotoxic, especially in the concentrations which are used for endodontic disinfection, 0.5–5%. Extrusion of NaOCl beyond the apical foramen into the peri-apex, a NaOCl accident, is a dreadful experience for the patient and the operating dentist. Calcium hydroxide (Ca(OH)\textsubscript{2}) causes cell necrosis when directly applied to tissue or cell cultures. Accidental extrusion of Ca(OH)\textsubscript{2} into the peri-apex often results in pain and swelling. Chlorhexidine is cytotoxic to human fibroblasts. Because of the intimate relationship between the periapical tissues and the root canal space, the choice of disinfecting agents is limited. The development of a root canal disinfectant which is safe to the periradicular tissues and yet is still powerful enough to disinfect the root canal system is a challenge.

A previous study showed that biofilms can be eliminated with hypertonic salt solutions. A combination of potassium sorbate and sodium chloride (NaCl) worked synergistically in killing an in vitro Enterococcus faecalis biofilm within 1 h.

Potassium sorbate is the potassium salt of sorbic acid. These results led to the development of a modified salt solution (MSS) whose mode of action is based on a multiple-hurdle strategy. It combines hyperosmotic stress, weak acid stress and the antimicrobial properties of sorbic acid. Both components, potassium sorbate and NaCl, are considered to be generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) of the United States of America and therefore safe for human consumption if the acceptable daily intake (ADI) is not exceeded. In Europe, potassium sorbate has an E-number, E202, which means that it is approved by the European Food Safety Authority.
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Its efficacy should be tested in more robust biofilm models, because root canal medicaments are less effective in vivo than in vitro. One reason is that dentine can inhibit microbial killing. For instance, after pre-incubation with dentine, Ca(OH)$_2$ was ineffective and the efficacy of 0.05% CHX and 1% NaOCl was reduced. On the other hand, 0.5% CHX had no decline in efficacy. Another reason for less optimal results of in-vivo experiments is that biofilms cultured from clinical isolates, multispecies biofilms or old biofilms are less susceptible to disinfecting treatments. No reports are available on in vitro culturing of an existing microbial endodontic infection. Therefore, it will be useful to see whether it is feasible to culture an in-vivo biofilm community in vitro.

Before MSS can be considered for clinical application in endodontic disinfection, more robust data on its properties are needed. The purpose of this study was therefore threefold. First, the cytotoxicity of MSS on fibroblasts from the periodontal ligaments of extracted teeth was explored. Secondly, the effect of dentine on the efficacy of MSS was assessed using a single-species biofilm. Finally, the efficacy of MSS was tested on mature multispecies biofilms grown from isolates collected from infected root canals; for comparison, 2% NaOCl and 2% CHX were included.

**Materials and methods**

**Cytotoxicity assay**

Fibroblasts were obtained from the periodontal ligament of teeth extracted for periodontal reasons. The donors provided their written informed consent. The collection and use of these cells have been approved by the Medical Ethical Committee of the VU University Medical Center, Amsterdam, The Netherlands (#2011/328). Fibroblasts were isolated as previously described. Cells were cultured in Dulbecco minimal essential medium (DMEM; Gibco BRL, Paisley, Scotland) supplemented with 2% antibiotics (antibiotic antimycotic solution: 100 U/mL penicillin, 100 mg/mL streptomycin and 250 ng/mL amphotericin B; Sigma-Aldrich, St. Louis, MT, USA) and 10% foetal bovine serum (FBS; HyClone, Logan, UT, USA) in a humidified atmosphere with 5% CO$_2$ in O$_2$ at 37 °C. Cells were seeded into a 96-well microtiter plate (Cellstar;Greiner Bio-One, Alphen aan den Rijn, The Netherlands) at $3.9 \times 10^4$ cells per well in DMEM with 10% FBS (DMEFs) and incubated in a humidified atmosphere with 5% CO$_2$ in O$_2$ at 37 °C. After 24 h, the cells were washed twice with DMEM. Then, the cells were incubated with solutions of 40 mmol/L or 400 mmol/L MSS and 0.5% NaOCl (60 mmol). All experimental solutions were dissolved in 0.5 DMEMs in demineralised water. Therefore, cells in 0.5 DMEMs served as a negative control. Demineralised water and 0.5%
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NaOCl (60 mmol) served as positive controls. Of each solution, 300 µL was dispersed per well, and immediately after that, 30 µL of the tetrazolium salt WST-1 (Roche Diagnostics Nederland BV, Almere, the Netherlands) was added per well. Cells that are metabolically active convert WST-1 into formazan. This conversion was quantified by measuring the absorbance at 450 nm (Spectramax M2; Molecular Devices, Sunnyvale, CA, USA) and is correlated with the number of viable cells.

Reference measurements at 620 nm were subtracted to correct for any variance of the microtiter plate. The readings were taken every 30 min overnight, 16 h, in a kinetic assay. The experiment was performed thrice in quadruplicate. After subtraction of the reference measurements, the measurements of the blanks were subtracted. Subsequently, the values of challenged groups were calculated as the percentage metabolic activity of the unchallenged controls. These values were averaged and the standard error of the means was calculated.

**In-vitro dentine inhibition assay**

Enterococcus faecalis E2, a clinical isolate, was employed. Dentine powder was obtained by crushing the roots of freshly extracted human third molars in a 6770 Freezer/Mill Cryogenic Grinder (Thermo Fisher Scientific Inc., Asheville, NC, USA). Subsequently, the dentine powder was autoclaved.

The biofilms were grown in semi-defined broth, pH 7.1, containing 0.2% sucrose (BMS). Semi-defined broth contains 76 mmol of K₂HPO₄, 15 mmol KH₂PO₄, 10 mmol (NH₄)₂SO₄, 35 mmol NaCl and 2 mmol MgSO₄·7H₂O and was supplemented with filter-sterilised vitamins (0.04 mmol nicotinic acid, 0.1 mmol pyridoxine HCl, 0.01 mmol pantothenic acid, 1 mmol riboflavin, 0.3 mmol thiamine HCl and 0.05 mmol D-biotin), amino acids (4 mmol L-glutamic acid, 1 mmol L-arginine HCl, 1.3 mmol L-cysteine HCl and 0.1 mmol L-tryptophan) and 0.3% yeast extract.

The ACTA Active Attachment model (AAA-model) was used to grow the biofilms. In brief, 24-well plates (Greiner Bio-One) were inoculated
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with 1.5 mL/well growth medium containing approximately $10^7$ cells/mL. *E. faecalis* E2 and closed with a custom-made stainless steel lid holding 24 glass cover slips. The plates were incubated at 37 °C anaerobically (atmosphere 80% N$_2$, 10% H$_2$ and 10% CO$_2$ with a palladium catalyst). Bacteria attached to the glass cover slips and formed biofilms for 48 h. The medium was refreshed after 8 and 32 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 using a reflected-light microscope (100× magnification; Zeiss Axiolab, Carl Zeiss, Sliedrecht, the Netherlands).

Twenty-four hours prior to the treatment, MSS was incubated with dentine powder (28 mg per 50 µL MSS) at room temperature. The 48-h old biofilms were treated with MSS without or in the presence of dentine powder for 15 min, 30 min or 1 h. Two per cent NaOCl without dentine served as a positive control. The concentration of the stock solution of NaOCl (Boom B.V., Meppel, The Netherlands) was verified with the iodometric titration method and diluted to obtain a 2.0% solution. A treatment with phosphate-buffered saline (PBS) served as a negative control. After the treatments, the biofilms were neutralised for 5 min in 2% buffered peptone water (BPW; Oxoid, Basingstoke, UK) with 1% sodium thiosulphate (Merck, Darmstadt, Germany) and washed thrice in 2 mL PBS. Then, the glass cover slips were removed from the lid using sterile forceps and transferred to a bijou vial containing 2 mL of PBS. The whole biofilm was dispersed by sonication on ice for 45 s at an amplitude of 40 W (Vibra-Cell; Sonics and Materials, Newtown, CT, USA). After vortexing for 5 s, the suspensions were diluted and aliquots were seeded in duplo onto brain–heart infusion agar plates (Bacto, Le Pont-de-Claix, France). The plates were incubated anaerobically at 37 °C for 48 h, and then, the numbers of colony-forming units (CFU) were counted. The biofilms were grown in sextuple and the experiment was repeated once ($n=12$).

**In-vitro endodontic biofilms**

**Handling of endodontic samples**

For the multispecies experiment, untreated root canals of teeth with signs or symptoms of AP and with a radiographic periapical lesion were sampled. The isolation procedure was described previously. After cleaning with pumice, the tooth was isolated with rubber dam. Then, after disinfection with 80% ethanol, the restoration and caries were aseptically removed. The access cavity was disinfected again. Sterility was checked by sampling with a cotton swab over the cavity surface and streaked onto 5% horse blood


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agar plates (Oxoid No. 2; Oxoid) enriched with hemin (5 mg/L) and menadione (1 mg/L). Then, the pulp chamber was accessed with burs and the canal entrances were enlarged with a Protaper SX rotary instrument (Dentsply Maillefer, Ballaigues, Switzerland) and rinsed with reduced transport fluid (RTF). RTF was then introduced in the root canal with a syringe with a 21-mm 30 G Navitip (Ultradent, South Jordan, UT, USA).

The canal was enlarged with a number 20 NiTi file (Dentsply) to the estimated working length as calculated from the preoperative radiograph. The NiTi file was separated under the handle and the file with dentine debris was collected in a sterile tube containing 1 mL RTF. Then, three sterile paper points were placed in the canal for 10 s and transferred to the same tube with RTF. The paper points and the file tip were immediately sonicated on ice for 30 s at an amplitude of 40 W. Five microlitre of the suspension was seeded onto a blood agar plate and incubated anaerobically at 37 °C for 2 weeks to confirm the presence of microorganisms.

One millilitre of 60% glycerol (Merck) was added to the suspension, and the well-mixed suspension was stored at 80 °C until use. Three samples were collected to inoculate the model. The blood agar plates of the sterility controls were incubated for 7 days at 37 °C in air or anaerobically.

**Biofilm growth**

The biofilms were cultivated in a broth, containing amino acids and peptides, which are broken down proteins short of the amino acid stage. This medium had been designed to replicate the conditions found in the root canal. The periodontal version of this medium has shown to stimulate the growth of unculturable species of multispecies periodontal isolates. The periodontal medium contained 0.7% Bacto proteose peptone, 0.3% trypsinase peptone, 0.5% yeast extract, 0.25% KCl, 10 mg/L haemin, 1 mg/L Vit. K, 0.05% cysteine, 50 mmol potassium phosphate, 0.25% pig gastric mucin, 1 mmol urea, 0.1% arginine, 1 mmol lysine, 1 mmol glycine and 10% heat-inactivated FBS. For the endodontic application, porcine mucin was left out and the concentration of the buffer was raised to 50 mmol/L to maintain a constant pH throughout the length of the assay. The pH was set at 7.4, which is the pH measured in human root canals. Again, the AAA-model was used for biofilm culturing.

The microorganisms were thawed at room temperature. The bacteria were dispersed by vortexing for 10 s and vigourously pipetting. Then, the suspension was added to 25 mL of medium and again vortexed, and 1.5 mL/well was suspended. The plates were incubated at 37 °C anaerobically. The biofilms were grown on glass cover slips for 4.5 weeks because in this model, mature biofilms are less susceptible to disinfection. The growth medium was refreshed twice weekly. In the
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final week of incubation, the medium was refreshed once. With each bacterial sample from an infected root canal for this assay, 16 biofilms were produced. Biofilm growth was visible to the naked eye and the parallel biofilms appeared similar. Four biofilms for the negative control group and four biofilms were used per medicament group.

Biofilm treatment
After 4.5 weeks of culturing the biofilms were treated for 1 h with MSS, 2% CHX or 2% NaOCl by transferring the lid with cover slips and attached biofilms to a 24-well plate which had been filled with the medicaments (1.8 mL/well). Again, the plates were incubated at 37 °C anaerobically. Two per cent CHX was prepared from a 20% stock solution chlorhexidine digluconate (Sigma-Aldrich). Biofilms treated with PBS for 1 h served as negative controls. After the treatment, the biofilms and the data were processed for a viability count as described in the previous experiment with one exception: the culture plates were incubated for 2 weeks to allow growth of the slow growing bacteria. All colonies were counted.

Statistical analyses
All data were analysed using statistical analysis software (IBM SPSS Statistics version 21, Chicago, IL, USA). For the dentine inhibition assay and the endodontic biofilm assay, the CFU counts were logtransformed. As the data were normally distributed in all assays, the one-way ANOVA test and post-hoc the Bonferroni and Tukey’s tests were used to evaluate the differences between the groups. A $P < 0.05$ was considered significant.

Results
Cytotoxicity
The cytotoxicity assay was run until a plateau of the conversion of WST-1 into formazan was reached by the unchallenged controls (0.5 DMEMs). This occurred after 4 h. The unchallenged controls showed a linear conversion of WST-1 to formazan in time. Initially, compared with the unchallenged controls, 40 mmol MSS gave rise to an increase in metabolic activity to 140%, which was then followed by a decrease in metabolic activity after 30 min, after which the conversion of WST-1 resumed to 98% after 2 h. The 400 mmol MSS group showed a decrease in metabolic activity at 30 min which resumed to 86% after 1 h. After 90 min, the activity of the cells slowly decreased. There was a significant difference in metabolic activity between MSS and the unchallenged controls at 30 min ($P < 0.05$). From 1 h, the metabolic activity of the MSS groups restored until there was no difference with the unchallenged control ($P >$
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0.05). In the 0.5% NaOCl group, no metabolic activity could be detected throughout the assay ($P < 0.001$; Figure 1).

**Dentine inhibition assay**

All MSS treatments and the 2% NaOCl treatments significantly reduced the bacterial counts compared with the negative controls ($P < 0.001$). Incubation with dentine powder did not influence the efficacy of MSS ($P > 0.05$). There was no difference between the survivals after a 2% NaOCl treatment or an MSS treatment ($P > 0.13$ for MSS without dentine; $P > 0.26$ for MSS with dentine). The average reduction in log CFU after 1 h MSS was 6.8 ±0.7 standard error of the means (SEM). There was a great variation in the survival of the biofilms per group. For instance, after 15 min in the MSS group without dentine, six of 12 biofilms were completely inactivated, whereas the other six biofilms still contained viable cells varying from 2.7 to 5.7 log CFU per biofilm. The 30-min MSS groups showed similar results. In the 1-h MSS groups, three of 12 biofilm still contained viable cells. In the 15-min NaOCl group, two of 12 biofilms still contained viable cells (Figure 2).

**Endodontic biofilm assay**

The endodontic samples contained bacteria. Sample 1 contained approximately $6.4 \times 10^6$ and sample 2 contained approximately $1 \times 10^5$ bacteria. The sterility checks from the sampling procedure did not show growth after incubation. Microorganisms from the sample numbers 1 and 2 attached to the model and grew well (Figure 3). After a 1-h treatment, there was no significant difference between the efficacy of MSS, CHX and NaOCl ($P > 0.05$).

The biofilms contained on average log 6.8 ±0.35 (Figure 3) culturable bacterial cells per biofilm. The average reduction in culturable cells after MSS treatment was log 5.7; SEM 0.4. The average reduction after CHX treatment was log 5.5; SEM 0.6 and after NaOCl treatment was log 6.5; SEM 0.5.

**Discussion**
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Previous findings resulted in the composition of MSS, a modified salt solution which may have a potential future use as a root canal disinfectant. Because few data had been generated on MSS, a series of experiments were conducted: the response of eukaryotic cells to a challenge with MSS and the efficacy of MSS under more stringent conditions. Although a challenge with MSS interrupted the metabolism of PDL cells at 30 min, the metabolism of PDL fibroblasts was restored after a further 30 min. The efficacy of MSS was not altered after pre-incubation with dentine. The culturability of mature 4.5-week-old multispecies biofilms from clinical isolates was reduced by more than log 5 after a 1-h MSS treatment. More familiar antimicrobials were included as a point of reference, that is, NaOCl in the cell safety assay, NaOCl in the dentine inhibition assay, and CHX and NaOCl in the endodontic biofilm assay. The effect of the treatments was assessed with plate counting rather than with vitality stains because of the many drawbacks of vitality staining techniques when used with multispecies biofilms.

When a root canal irrigant is applied, it is contained by the root canal. However, some leakage from the root canal to the periapical tissues can occur. Although the individual components of MSS, NaCl and potassium sorbate are safe for human consumption, the safety of this particular combination has not yet been investigated. Earlier, the biocompatibility of NaCl on cells was demonstrated by who showed that osteoblasts grown on NaCl-coated surfaces did not differ from the controls with regard to morphology, cell number or metabolic activity. The safety of MSS is a consequence of the low quantities that are used for the disinfection of a root canal system. The Dietary Guidelines of the FDA recommend that the general population should consume no more than 2.3 g of sodium daily. The ADI of sorbic acid and its salts is 0–25 mg/kg body weight. The volume of a prepared root canal is about 10–20 μL, which means that per molar no more than 100 mg NaCl and potassium sorbate in MSS will be administered. Consequently, two endodontic treatments of three-rooted molars in one patient will not exceed the ADIs.

In this assay, substantial apical leakage was simulated by the 400-mmol MSS challenge. After an initial decrease in metabolic activity, the cells restored the conversion of WST-1 to formazan to 86% of the unchallenged group. The initial decrease in activity can be explained as follows. A 400-mmol MSS solution is hypertonic and it is known that in the first phase after a hypertonic challenge, cell cycle arrest occurs. The first phase is an important phase, however, because transporters that mediate a rapid uptake of compatible solutes are activated. The uptake of compatible solutes is immediately followed by an osmotic influx of water. This influx restores the cell volume and enables the cell to return to homoeostasis. The current study confirmed the cytotoxic effects on cells of NaOCl. Disinfection
Various properties of the modified salt solution is much more difficult to achieve in vivo than in vitro. Inhibition of the antibacterial activity of compounds by dentine can be one of the reasons. Dentine consists of 90% inorganic calcium phosphate and 10% organic collagenous matter and it seems that the organic part rather than the inorganic part of dentine is responsible for a reduction in antimicrobial activity. Dentine also acts as a buffer and it could have altered the pH of MSS, thus reducing its antibacterial activity. In the present study, MSS was incubated with dentine powder for 24 h prior to the treatment of the biofilms to allow optimal interaction of the dentine with MSS. The efficacy of MSS, however, was the same in both groups, and also, the pH of MSS remained unchanged (van der Waal SV, Scheres N, de Soet JJ, Wesselink PR, Crielard W, unpublished data) which was expected because the sorbic acid, a weak acid, can act as a buffer.

For endodontics, studies on planktonic cultures may well be considered less relevant, because in the root canal system, bacteria prevail in a biofilm configuration. Biofilms can be up to a 1000× less susceptible to disinfection than their planktonic counterparts. Therefore, biofilm studies were employed which may be more laborious to undertake but eventually they give a better insight into the bacteria’s behaviour to disinfecting agents than the planktonic culture-based studies. With the purpose to create a robust and clinically relevant biofilm model, microorganisms were retrieved from infected root canals with signs and symptoms of AP and cultured for 4.5 weeks in vitro. It is noteworthy that 2% NaOCl eliminated a young single-species biofilm within 30 min but failed to eradicate a mature multispecies biofilm after 1 h. This finding is in line with other studies which report that biofilms become less susceptible to disinfection over time and that multispecies biofilms are more resistant than single-species biofilms. There is a great variation in endodontic infections and it was therefore remarkable that the cultured biofilms responded in a similar way to the treatments.
In-vitro culturing of an in-vivo infection is a unique feature of the current study. However, a few issues had to be taken into account. The microorganisms were retrieved by paper point sampling. The drawbacks of paper point sampling are generally acknowledged and will not be mentioned here in detail. Another issue was that little material was available for inoculation of the model.

Finally, it was not possible to predict whether the samples would grow in the AAA-model and in which composition and numbers. After culturing in the AAA-model, the control biofilms contained <10 million culturable bacteria which is a low number. A few explanations are possible. Many bacterial species in a mixed sample and retrieved from an oral infection cannot be cultured in a laboratory. Also, the starvation of the biofilms in the final week of the assay, which was performed to render the biofilms less susceptible to disinfection, can have contributed to a more dormant or nonvital state of a significant portion of the biofilm bacteria. To gain insight into the magnitude of this portion, DNA was extracted from 100 µL of the suspended untreated biofilms and an additional quantitative PCR analysis was performed using a universal primer and probe set which was designed to incorporate the variable region of the 16S rRNA gene. It became apparent that bacterial numbers as determined with qPCR were 10× higher than the CFU counts. Therefore, it can be concluded that at least 90% of the bacteria were dormant, non-viable or could not grow on a culture plate, but did indeed grow in a biofilm configuration in this model.

The AAA-model was developed for culturing biofilms from supra-gingival plaque. This model was adapted for growing more anaerobic and proteolytic biofilms by introducing a growth medium which contains a relatively high concentration of amino acids and peptides and a low concentration of carbohydrates. In this medium, the proteolytic bacteria are favoured and the more glycolytic species cannot easily survive. Consequently, they are eradicated from the biofilm during the 4.5-week growth. This also indicates that the composition of the inoculum is important; too many glycolytic species in a sample will result in poor biofilm growth. This phenomenon may have occurred in one of the samples, not used in this study, where almost no visible biofilm was obtained after 4.5-week incubation (data not shown). After processing of the biofilms, the negative controls had <10³ CFUs per biofilm, whereas the original root canal sample contained approximately 10⁸ culturable cells/mL. Therefore, these data were not included.

Although MSS is a powerful disinfectant, it requires time to eliminate the bacteria in a biofilm. This was confirmed in the current study. In the endodontic biofilm assay, no significant differences were found between the efficacy of NaOCl and MSS. MSS is not a surface disinfectant such as NaOCl or CHX and its mode of action is very different from
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present disinfectants. MSS is a highly concentrated solution of salts and it is therefore expected to diffuse into the biofilm and possibly also into the complexities of the root canal system. This hypothesis needs to be investigated.

**Conclusions**

Modified salt solution is safe for the use as a disinfectant and has a strong antimicrobial property. Its efficacy is not inhibited by dentine. MSS has the potential to be used as an interappointment root canal dressing with the aim to further reduce the microbial load of an infected root canal system. However, *ex-vivo* assays and eventually a clinical trial should be conducted before introduction in endodontics can be considered.

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