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A direct comparison between extracted tooth and biofilm models of endodontic irrigation using *Enterococcus faecalis*

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Abstract

Endodontic restorations often fail due to inadequate disinfection of the root canal even though the antimicrobial irrigants used have been shown to be capable of killing the bacterium frequently implicated in this complication, *Enterococcus faecalis* (*Ef*). Extracted human teeth were root-prepared and filled with a liquid culture of *Ef*. Following incubation, the root canals were irrigated with 1% sodium hypochlorite (NaOCl), electrochemically activated water or saline control. Irrigation was modelled using an electronic pipette to deliver the solutions at a reproducible flow velocity. A series of parallel experiments employed a membrane biofilm model that was directly immersed into irrigant. Experimental conditions where contiguous between the extracted tooth model and biofilm model wherever possible. 1% NaOCl effectively sterilised the biofilm model after 60 seconds exposure, whereas log 3.36 viable *Ef* where recoverable from the analogous extracted tooth model. Biofilms of *Ef* were susceptible to concentrations of irrigant that proved ineffective in the tooth model. NaOCl was the most effective biocide in either case. This suggests that the biofilm modality of bacterial growth is not important for the recalcitrance of root canal infections following endodontic irrigation; it is more likely due to the inability of the irrigant to access the infection.

Introduction

Once bacteria become established within the root canal, they cannot easily be reached by the defence mechanisms of the host or by systemic antibiotics. Effective eradication of infection by endodontic irrigation enhances the success rate of root canal treatment (Sjogren et al. 1997).

Enterococcus faecalis is a facultatively anaerobic, Gram-positive coccus that has been implicated in persistent root canal infections and is isolated in 38% of cases with recoverable micro-organisms (Molander et al. 1998). *E. faecalis* has been shown to invade dentinal tubules (Love 2001) which will afford protection from physical removal of the bacteria by mechanical instruments and antimicrobial treatments such as endodontic irrigants and as such it has been isolated from failed endodontic treatments (Zoletti et al. 2006). This bacterium has been used in other studies to test the efficacy of endodontic irrigants since it is extremely robust and exhibits a degree of resistance to sodium hypochlorite (Lima et al. 2001).

Mechanical preparation of the root canal system with hand files or rotary instruments is extremely important but, due to the complexities of the root canal system, mechanical instrumentation will not plane the entire root canal surface. Irrigation helps to flush out the debris created by mechanical intervention, and eases negotiation of the canals by the instruments. In principle, irrigation with an antimicrobial agent also allows disinfection of areas unreachable by instrumentation. The diameter of accessory (lateral) canals range from maxima of 100 µm in permanent molars and 360 µm in primary molars down to a common minima of 10 µm (Dammaschke et al. 2004). Such narrow orifices present a barrier of surface tension to adequate mixing between the irrigant and the liquid within the canal. The narrowing of the root canal apically poses a similar barrier.

Sodium hypochlorite (NaOCl) has been used as an irrigant since its introduction in endodontics by Walker in 1936 (Walker 1936). The cytotoxicity of NaOCl results from the formation of hypochlorous acid (HOCl) by the addition of chlorine to water. HOCl exerts its bactericidal effect by the oxidation of protein sulphydryl groups within bacterial enzyme systems, thereby disrupting the metabolism of the microorganism (Knox et al. 1948) although it also reacts with a variety of biomolecules including DNA, RNA, lipids and protein amino groups. NaOCl fulfils a number of roles during endodontics since it has good tissue solvent action, a broad spectrum of antimicrobial activity, acts as a lubricant for

instrumentation and flushes loose debris from root canals. In principle, irrigation with an antimicrobial agent allows disinfection of areas unreachable by instrumentation (Siqueira et al. 2000).

At the Liverpool University Dental Hospital (LUDH), a 1% NaOCl solution is used as part of a multi-irrigant regime, although it is acknowledged that there are several negative aspects to its use: NaOCl is highly irritant to periapical tissues, cytotoxic if injected into the periapical tissues , does not efficiently remove the smear layer, has a negative effect on the properties of teeth such as reducing the flexural strength and elastic modulus of dentine, can cause hypersensitivity reactions (Kaufman and Keila 1989), has a foul smell and taste, has the ability to bleach clothes and has the potential for causing corrosion. Other reported adverse effects of NaOCl misadventure include paraesthesia, pain, swelling and haematoma.

Chlorhexidine (CHX) has been shown to be ineffective against dental plaque biofilms in an *in vitro* model following 5 minutes exposure, requiring 60 minutes to achieve a 2-$\log_{10}$ to 5-$\log_{10}$ kill (Pratten and Wilson 1999). Confocal laser-scanning microscopy has also been used to visualize changes, in real time, of supragingival plaque biofilm cell vitality during exposure to 0.2% CHX. This study revealed that many bacteria within the biofilm remained viable after 15 minutes exposure (Hope and Wilson 2004). The apparently limited efficacy of CHX is tempered by its long record of safe use and its substantivity in the oral cavity by binding to enamel which leads to a contact times which can effectively be measured in hours (Schiott et al. 1970) and as such it remains a viable alternative endodontic irrigant (Siqueira et al. 2007).

Some irrigants incorporate antibiotics, but this could lead to the proliferation of antibiotic resistance genes throughout the oral microflora since *E. faecalis* has been shown to propensity to acquire new antibiotic resistance genes via mobile genetic elements such as plasmids, transposons and conjugative transposons (Rossi-Fedele and Roberts 2007). A possible alternative to NaOCl based endodontic irrigants is super-oxidised water ($SOXH_2O$) (Sterilox®, Optident, Ilkley, West Yorkshire, UK) - being a clear, colourless, odourless, non-toxic liquid with a pH between 5 and 6.5. $SOXH_2O$ is produced by a proprietary electrochemical cell that produces a super-oxidised solution incorporating electrolysis of a sodium chloride solution using titanium electrodes. $SOXH_2O$ is 99.556% water; other constituents are sodium chloride (0.42%w/v), hypochlorous acid (0.022%w/v) and sodium chlorate (0.002% w/v) (details supplied by Optident). It is currently in use as a broad spectrum biocide for decontamination of dental

unit water lines (Martin and Gallagher 2005) and has also been suggested as a hard surface disinfectant, as a medium for sterilising impression trays. , and it has previously been evaluated as a possible endodontic irrigant (Gulabivala et al. 2004).

Bacteria which are members of a biofilm community can be up to 1000 times more resistant to antimicrobial compounds than their planktonic counterparts (Gilbert et al. 2002). There are two aspects to this phenomenon of recalcitrance; that which is conferred by (direct) and that which is coincidental to (indirect) the biofilm mode of growth. Direct resistance adaptations are acquired by, or activated in response to, cell density such as slower growth rates and the production of persister cells. Such slow growing bacteria are intrinsically less susceptible to the effects of antimicrobial compounds (Gilbert et al. 1990). Indirect resistance to antimicrobials is conferred by the physical properties of the biofilm, including the co-operative adherence to surfaces and the impedance of the penetration of macromolecules to the inner regions of the biofilm by the exopolysaccharide matrix (Thurnheer et al. 2003). Bacteria growing within an infected root canal do so as biofilm (Duggan and Sedgley 2007) in conditions were the availability of carbohydrates is limited.

After conducting a series of experiments using culture of *E. faecalis* within an extracted tooth model and finding that relatively large numbers of survived in the root canal after endodontic irrigation it was decided to conduct a series tangential of experiments with the purpose determining the efficacy of the endodontic irrigants NaOCl, NaOCl with ethanol, CHX and $SOXH_2O$ in a situation where there is effectively no limitation of mass transfer between irrigant and bacteria. An existing model which incorporated nitrocellulose filter-membrane grown biofilms of *E. faecalis (Spratt et al. 2001)* was employed with some minor variations in order to draw comparisons between the two model systems.

Materials and Methods

Bacteriological Culture

Cultures of *E. faecalis* (NCTC 775) were maintained at 37ºC on nutrient agar (NA) (Oxoid, Basingstoke, Hampshire, UK) and under aerobic conditions. Individual colonies of these bacteria were seeded into 10 ml of brain heart infusion (BHI) broth (Sigma Aldrich, Buchs, St. Gallen, Switzerland) at 37ºC overnight shake-cultures. Following incubation, the cultures were centrifuged and the cell pellet washed in PBS (Oxoid) before being re-suspended in fresh BHI. This washing step minimised the carryover of exhausted

culture medium and waste metabolic products into the root canal. The final optical density of the culture was adjusted to 0.1 units (OD 650 nm) with additional BHI; this was subsequently determined as containing 5 x 10^7 cfu ml^{-1}.

Extracted tooth model

Eight extracted, single-rooted teeth were root prepared using a step-back, crown-down method; this being the accepted technique as used by the staff and students at LUDH. Following coronal access, the coronal two-thirds of each canal was prepared using Gates Glidden burs. The apical third was then chemo-mechanically prepared using sodium hypochlorite and K-files to a master apical size 40. Step-back technique ensured maximal flaring of radicular dentine to maximise access to the root canal during the laboratory irrigation experiments. To ensure no leakage of microbial media, the roots were planed, to remove any soft tissue, and sealed using an acid-resistant nail varnish (Revlon, London, UK). The apex was then sealed using composite resin (Spectrum TPH, Dentsply, Weybridge, Surrey, UK).

Each tooth was located in a piece of condensation silicone impression material (Zhermack, Badia Polesine, Veneto, Italy), to maintain it vertically in a glass jar before, being autoclaved at 121ºC for 15 minutes. 20 µl of the washed *E. faecalis* culture was pipetted into the well created by the root preparation procedure. Additionally, 1 ml of distilled water was pipetted into the base of the jar to provide a moist environment for both tooth and culture. The samples were then incubated at 37ºC for 48 hours.

The clinical irrigation procedure was mimicked in the laboratory by using an electronic dispensing pipette (Proline 50 – 1000 µl, Biohit, Torquay, Devon, UK) to supply irrigant in a reproducible manner, being a unique feature of this study. With this particular unit set at output speed setting #3 the flow rate was 0.5 ml s^{-1} (manufacturer's data) which corresponded to a flow velocity at the tip of ~1 m s^{-1}. The pipette tip was loaded with 1 ml of irrigant (1% NaOCl, $SOXH_2O$ or PBS control) and located as far into the root canal as possible. Irrigant was dispensed into the tooth as a series of five consecutive 200 µl aliquots; the final aliquot of irrigant was allowed to remain for one minute. Upon completion of irrigation, the tooth was flushed with casein peptone lecithin polysorbate broth (CPLP) (Fluka, Buchs, St. Gallen, Switzerland) containing 40 ml l^{-1} polyoxyethylene sorbitan monolaurate (Tween® 20, Sigma, Gillingham, Kent, UK) and 0.1 g l^{-1} sodium metabisulfite (Sigma), which was applied in a manner identical to that used during the irrigation procedure. The formulation of the CPLP

mixture is similar in composition to Letheen broth, which is used to neutralise quaternary ammonium compounds (el-Falaha et al. 1987) and also serves to flush residual NaOCl from the root canal in order to minimise its carry-over into the subsequent sampling and dilution phases.

Bacteria were recovered from the teeth using a series of five, ISO 40 paper points (Dentsply) which were in turn rubbed against the walls of the well and allowed to draw up their full capacity of liquid before being transferred to PBS and vortex-mixed for 30 seconds. *E. faecalis* colony forming units were enumerated by performing serial dilutions and growth on NA after 48 hours aerobic incubation at 37ºC.

The same cohort of teeth was used throughout and all irrigation experiments were conducted in triplicate. Differences between irrigants, and between teeth, were tested using a two-way analysis of variance. Post-hoc pair-wise comparisons between irrigants were investigated using Tamhane's adjustment for multiple testing, which does not assume equal variances between groups.

Biofilm model

Nitrocellulose filter-membranes (50 mm diameter, 0.45 µm pore size, Invitrogen Ltd., Paisley, Renfrewshire, UK) were laid onto NA plates and inoculated with 20 µl of *E. faecalis* culture at a cell density of 5×10^7 cfu ml^{-1}. These plates were incubated aerobically at 37ºC. Counts of viable bacteria present on the filter-membranes were determined over a period of 96 hours to construct a growth curve.

To evaluate the efficacy of the irrigants, after 48 hours growth an individual filter-membrane was lifted from the plates and immersed in 25 ml of filter-sterilised irrigant (or PBS control) for 1 minute. Following exposure, the membrane was transferred to 25 ml of PBS for a further minute to immediately dilute any retained irrigant. Finally, the membrane was transferred to 10 ml of CPLP and vortex mixed for 30 seconds to disperse the biofilm. This cell suspension was then serially diluted in PBS before plating onto NA in order to enumerate viable bacteria.

The irrigants tested were NaOCl (0.01%, 0.1%, 0.2%, 0.5%, 1%), NaOCl (0.1% and 1%) with 30% ethanol (EtOH), $SOXH_2O$ and CHX (0.2% and 2%). All irrigant concentrations are expressed as volume / volume (v/v). In addition to the standard 60 second exposure time, additional biofilm samples were exposed to $SOXH_2O$ for 5 minutes. Counts of recovered bacteria were compiled and tested for statistically significant differences using a non-parametric test (Mann-Whitney U test).

The minimum inhibitory concentration (MIC) of NaOCl against *E. faecalis* growing as a planktonic culture in a liquid medium was determined by constructing a low-resolution (i.e. 10-fold) dilution series of NaOCl / BHI from 1% to 0.0001%. After determining that the MIC was between 0.1% and 0.01% NaOCl, a second high-resolution (i.e. 2-fold) dilution series was constructed from 0.4% to 0.0125%. Turbidity after 48 hours incubation at 37ºC indicated a viable culture.

Results

Extracted tooth model

The two irrigants tested were 1% NaOCl and $SOXH_2O$. PBS was used as a negative control to determine the effects of irrigation with a physiologically neutral liquid. The mean log counts of the number of bacteria recovered from the prepared root canals are shown in Table 1.

A pair-wise statistical comparison (Tamhane test) showed that irrigation of the extracted tooth model with 1% NaOCl killed significantly more *E. faecalis* than the PBS control ($p = 0.026$). There was no statistical significance between $SOXH_2O$ and PBS ($p = 0.501$) or 1% NaOCl and $SOXH_2O$ ($p = 0.177$) (Table 2).

Statistical analyses of between subject effects showed no significant difference between individual teeth ($p = 0.384$) (data not shown), whereas the difference between irrigants was significant ($p = 0.026$).

Biofilm model

The *E. faecalis* biofilm cultures reached the stationary phase of growth after approximately 16 hours incubation at 37ºC (data not shown). The stationary phase was maintained beyond 48 hours, the point at which the biofilms were harvested for experimentation.

NaOCl exerted a statistically significant antimicrobial effect at concentrations of 0.1% and above, whilst 0.5% NaOCl killed 99.99% of the bacteria growing as a biofilm with no viable bacteria were detectable at 1% (Table 3). The MIC of NaOCl in overnight planktonic cultures of *E. faecalis* was 0.05%, growth was observed at 0.025% (data not shown). Cultures of *E. faecalis* are of the order of 10 times more resistant to NaOCl when growing in the biofilm modality compared to their planktonic counterparts.

Both 2% CHX and $SOXH_2O$ killed significantly more bacteria than the PBS control ($p<0.001$, $p=0.025$ respectively). However, large numbers of viable bacteria were recovered from the filter membranes following exposure (2.74×10^3 cfu mm^2 and 1.81×10^4 cfu mm^2 for CHX and $SOXH_2O$ respectively).

A comparison of the three primary irrigants; 1% NaOCl, 2% CHX and $SOXH_2O$ revealed that 1% NaOCl killed significantly more bacteria than either 2% CHX ($p=0.001$) or $SOXH_2O$ ($p=0.005$). 2% CHX was more effective than $SOXH_2O$ ($p=0.007$). Increasing the contact time of $SOXH_2O$ from 1 minute to 5 minutes resulted in a significant ($p=0.039$) increase in kill from 51% to 97%.

Discussion

Previous studies of the efficacy of endodontic irrigation have used one of two approaches: clinical trial-type studies, involving endodontic patients, or *in vitro* laboratory models. While clinical trials remain the paradigm for evaluating novel and existing treatment regimes, they are somewhat limited in their scope, controls and treatment parameters, since the safety and well-being of the patient are paramount. However in terms of irrigant testing, the major problem of *in vivo* testing is that follow-up studies to assess success / failure rates can take many years to complete. Whilst the relevance of any laboratory model is dependent upon which aspects of the clinical situation are being evaluated; such relevance can range from "…the sublime to the ridiculous." (Peter Gilbert, Key note lecture, The Biofilm Club workshop on modelling biofilm systems, University of Manchester, November 4th 2004). The key advantage of a laboratory model is its ability to control and, therefore, replicate all parameters of the experiment. Importantly, the user is able to shift these parameters beyond those which would acceptable, or indeed ethical, if patients were involved. In our study, the root canals had previously been root prepared and as such the canals did not require copious flushing to remove infected tissue. A perceived weakness of the study was the use of a standard laboratory pipette tip during the irrigation procedure; however it was felt that the ability to replicate the flow velocity of irrigant was of far greater importance.

E. faecalis was chosen for this study since it is representative of the bacterial contamination found within the root canal, is implicated in the failure of endodontic restorations and has an intrinsic resistance to hypochlorite (Radcliffe et al. 2004). A type strain (NCTC 775; being analogous to ATCC 19433NA) was chosen since this particular strain is readily available and is indeed used in the Royal

Liverpool and Broadgreen University Hospitals Trusts to monitor surface cleansing practices since it exhibits resistance to NaOCl.

One of the perceived advantages of the version of the extracted tooth model presented here is the ability to inoculate a root-prepared tooth with a sufficient microbial load that, theoretically, allows the discrete quantification of antimicrobial irrigant efficacy up to a 4-log reduction. The exposure time of one minute is shorter than that to be expected clinically, but it has already been shown that increasing exposure time from one to five minutes does not significantly increase the percentage of *E. faecalis* that are killed (Dunavant et al. 2006). This model was optimised to maximise the recovery of bacteria from the tooth in an anatomically representative environment. The same cohort of 8 extracted teeth was used throughout the study. However, one of the teeth became unusable after the preliminary trials and control experiments and was not incorporated into any of the irrigant testing experiments. This yielded an effective sample size of 7 teeth, which were tested as a group on three occasions per irrigant (n=21). Statistical analysis revealed that between teeth effects of the seven teeth used in these experiments were not significant. The sequence of irrigants used on the teeth was PBS > $SOXH_2O$ > NaOCl. The variance of the mean log for 1% NaOCl was relatively high (7.02, Table 1) due to a number of samples (33%) from which no viable bacteria were recovered.

During one of the pilot experiments, a mixture of 1% NaOCl with 30% ethanol was employed against the tooth model. This resulted in an increase in the number of viable bacteria recovered from the tooth compared with the PBS control irrigant (data not shown). This was due to the ethanol reducing the surface tension of the irrigant and allowing it to act as a surfactant, thus releasing bacterial cells from the dentine / enamel surfaces. It has been proposed that employing low surface tension irrigants will result in better penetration and delivery of the disinfecting agent into the accessory canals (Cunningham et al. 1982). This effect is an apparent limitation of the model which will require an additional set of controls if irrigants incorporating surfactants are to be assessed effectively since this same NaOCl and ethanol mixture was shown to be an effective antimicrobial agent against biofilms of *E. faecalis*.

It is not easy to ascertain the growth conditions in the root-prepared model after 48 hours incubation, since the small volume (20 µl) of culture inoculated into the tooth and its subsequent flow into the accessory canals is not conducive to recovering a longitudinal sequence of samples. The natural variations in tooth morphology would further complicate the acquisition of a meaningful growth curve.

The resistance of *E. faecalis* against NaOCl has been shown to increase under conditions of carbohydrate starvation (Laplace et al. 1997). It is likely that the samples used in both the tooth and biofilm studies were carbohydrate limited, since they were in the stationary phase, and therefore more resistant to NaOCl than a comparable culture in the logarithmic phase. It was the growth phase, and not biofilm age *per se*, that was considered to be an important factor in these experiments. Following the removal of pulp tissue and sealing the crown, the oxygen tension within the accessory canals surrounding an endodontic restoration will be approaching zero, however; it was not necessary to operate these models under anaerobic growth conditions since it has been previously demonstrated that the susceptibility of *E. faecalis* to NaOCl was indistinguishable following aerobic or anaerobic incubation (Davis et al. 2007).

The results from the PBS control irrigant in the tooth model showed an average *E. faecalis* recovery of 2.5×10^5 cfu, which equals a 24.31% recovery rate of that originally inoculated into the tooth. It should be noted that this figure does not account for any intermediate growth during the 48 hour incubation period. However, due to the lack of agitation of what is essentially a liquid microbial culture, it is unlikely that growth would be exponential (Bergstedt et al. 2004). This suggests that the liquid shear forces of irrigation, in this model at least, removed approximately 75% of the bacteria from the cavity. This percentage would no doubt be higher if the irrigation model were to incorporate aspiration by an endodontic needle rather than the limited access offered by the 1 ml pipette tip.

Using 0.5% NaOCl as an irrigant against the biofilm model ostensibly killed all of the *E. faecalis* within the biofilm, since viable bacteria were only recovered from one of the ten samples. Following exposure to 1% NaOCl, no viable bacteria were recovered from a baseline of 3.69×10^4 cfu mm^2 after a contact time of 1 minute. 1% NaOCl was therefore effectively able to 'sterilise' the filter membrane and its incumbent biofilm of *E. faecalis*.

2% CHX and $SOXH_2O$, although able to kill a significant number of the bacteria, left a considerable microbial load on the filter-membrane following exposure (Table 1). This suggests that 2% CHX and $SOXH_2O$ will not be capable of sterilising the root canal, or even providing adequate disinfection under the conditions modelled in this study. Nevertheless, the antimicrobial efficacy of these agents could be improved *in vivo* by extending the contact time of the irrigant within the root beyond the confines of these models. CHX and $SOXH_2O$ have a distinct advantage over NaOCl as an irrigant in that they present far fewer complications in the event of coming into contact with the patient's soft tissues due

to misadventure during the application of endodontic irrigant. Longer disinfecting times were avoided in this experiment to allow a quantitative assessment of the number of bacteria surviving in the tooth.

The basic methodologies used throughout the biofilm experiments were similar to those successfully employed by Spratt *et al.* (Spratt et al. 2001) and further modified by Sena *et al*. (Sena et al. 2006), with the following variations; the liquid cultures used to inoculate the nitrocellulose filter-membranes in our study were adjusted to a standard optical density and re-suspended in fresh medium, the contact time between biofilm and irrigant in our study was typically 1 minute (as oppose to 15 or 60 minutes) in order to allow a comparison to be made to the extracted tooth model and an additional PBS rinsing step was incorporated prior to re-suspension of the biofilm organisms to minimise the carry-over of irrigant. Although the pore sizes (0.2 µm – 0.45 µm) of the nitro-cellulose filter membranes were different, this factor would be unlikely to affect the results since these pore diameters will neither limit the diffusion of nutrients from the agar to the biofilm, affect the penetration of irrigants nor allow the movement of bacteria since cells of *E. faecalis* have diameters in the range of 0.87 – 1.01 µm (Kokkinosa et al. 1998). The results of our biofilm study agree with the previous findings (Spratt et al. 2001; Dunavant et al. 2006; Sena et al. 2006) in that NaOCl is the most effective endodontic irrigant against biofilms of *E. faecalis* and that 1% NaOCl solution with a contact time of 1 minute is sufficient to kill all the bacteria growing in such experimental biofilms.

The complete eradication (i.e. sterilisation) of the biofilm laden filter-membranes by 1% NaOCl suggests that the failure of root canal disinfection in clinical practice and experimental models is due to mass transfer and mixing limitations during their application and not due to the intrinsic resistance of *E. faecalis* or any deficiency bactericidal properties of the irrigant *per se*. This direct comparison between a biofilm model and an extracted tooth model confirms this. The narrow cross-sectional area of the accessory canals (~100 µm diameter) in relation to their length (up to ~5 mm) provides a barrier of surface tension and edge effects from wall drag (Ciucchi et al. 1995) to the ingress of irrigant. Any fluid flow down the accessory canals from the root canal will be laminar flow; turbulent flow will be not be achievable due to the very low Reynolds numbers inherent at such small 'pipe' diameters, where edge effects and viscosity become the major factors affecting fluid dynamics. At the scale of the accessory canals, diffusion of irrigant down the concentration gradient will be the dominant mechanism by which the agent moves along the canal. The incorporation of 30% ethanol (EtOH) into a mixture containing

0.1% or 1% NaOCl marginally reduced the effectiveness of the irrigant as a biocide; however, the subsequent reduction in the surface tension (23) may outweigh this deficit in practice.

The disparity of the antimicrobial efficacy of NaOCl against *E. faecalis* in a root canal model compared to a biofilm model suggest that disinfection is restricted, during endodontic procedures, by limitations of mixing and mass transfer – the irrigant cannot easily access bacteria dwelling within the accessory canals. It is postulated that improving the irrigant flow / diffusion characteristics would be more beneficial to patients than trying to improve the antimicrobial efficacy of the agent.

Conclusion

The extracted tooth model is capable of evaluating the effectiveness of endodontic irrigants in a system that mimics both the microbiology and physical parameters in an infected tooth. The model also allowed the application of endodontic irrigants in a manner which standardised the microbial inoculum and irrigation procedure, although further improvements could include the incorporation of an endodontic syringe tip to the pipette. The microbial load achieved in this model is theoretically sufficient to detect a 4-log reduction in cell counts of *E. faecalis*. 1% NaOCl was significantly more effective than PBS ($p=0.026$) in the tooth model; however, the differences between $SOXH_2O$ and PBS and between Bleach and $SOXH_2O$ were not significant.

The results of a parallel, filter-membrane study using, biofilms to model the exposure of *E. faecalis* showed that 1% NaOCl resulted in a total kill after 60 seconds and a significant kill was affected as low as 0.1% NaOCl.

This inability of NaOCl to satisfactorily disinfect root canals during endodontic irrigation procedures, both clinically and in extracted tooth models, is not due to the recalcitrance of the infecting organisms growing as a biofilm. The failure of endodontic disinfection is more likely due to the mixing / mass transfer limitations that occur within the root canal system during the irrigation procedure. It is more likely that it is the inability of the irrigant to access the bacteria within the root and accessory canals rather than the recalcitrance of the bacterial cells *per se*.

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CKH wishes to highlight the work of the late Professor Peter Gilbert (1951-2008) as an inspiration to those involved in biofilm modelling.

Table 1. Mean log cfu of *Enterococcus faecalis* recovered from teeth after irrigant experiments.

Irrigant	Number of *Ef* Recoved (mean log cfu)	*n* =	Standard Deviation	Variance
PBS	5.11	20	0.63	0.40
NaOCl (1%)	3.36	20	2.65	7.02
$SOXH_2O$	4.66	21	1.47	2.16

Table 2. Statistical comparison between irrigants pairs (Tamhane's post hoc test).

Irrigant		Mean Difference	Standard	Significance	95% Confidence Interval	
					Lower	Upper
A	B	(A - B)	Error	(p value)	Bound	Bound
PBS	NaOCl	1.76	0.61	0.026*	0.18	3.33
PBS	$SOXH_2O$	0.45	0.35	0.501	-0.44	1.34
$SOXH_2O$	NaOCl	1.30	0.67	0.177	-0.40	3.01

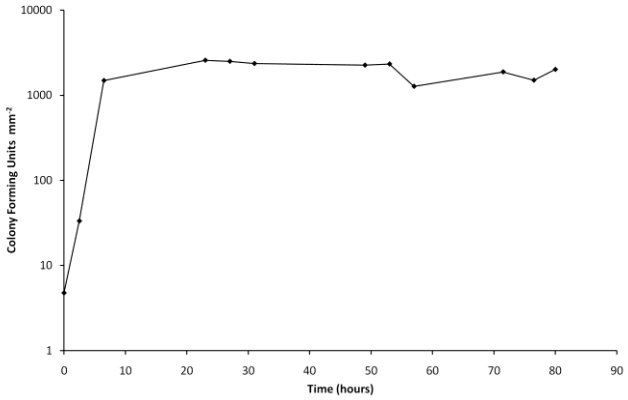

Figure 1. Growth of *Enterococcus faecalis* biofilms on a nitrocellulose filter membrane. The biofilms harvested for this study were in the stationary phase following 48 hours incubation at 37ºC.

Table 3. Number of viable bacteria recovered from biofilms of *Enterococcus faecalis* following exposure to various endodontic irrigants. Exposure time was 1 minute with the exception of a $SOXH_2O$ replicate at 5 minutes. All irrigants yielded a significant kill (i.e. $p < 0.05$) with the exception of 0.01% NaOCl.

Irrigant	**No. of Bacteria Recovered (log cfu mm^{-2})**	**Standard Deviation (log)**	**% Kill (vs PBS)**	**Sample Size (n =)**
PBS (Control)	4.57	4.40	-	25
0.01% NaOCl	4.52	4.48	9.53	6
0.1% NaOCl	4.12	4.66	64.36	6
0.2% NaOCl	0.94	1.18	99.98	3
0.5% NaOCl	0.46	0.96	99.99	10
1% NaOCl	0	-	100	6
0.1% NaOCl +30% EtOH	4.00	3.21	72.64	6
1% NaOCl +30% EtOH	1.07	1.28	99.97	5
0.2% Chlorhexidine	4.21	4.03	56.39	6
2% Chlorhexidine	3.44	3.86	92.58	7
$SOXH_2O$	4.26	4.42	51.00	9
$SOXH_2O$ (5 mins)	3.06	3.25	96.92	5

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