Arch Microbiol (2010) 192:775–781
DOI 10.1007/s00203-010-0604-6

ORIGINAL PAPER

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

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Received: 6 April 2010 / Revised: 21 June 2010 / Accepted: 3 July 2010 / Published online: 23 July 2010

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. After 60 s of exposure, 1% NaOCl effectively sterilised the biofilm model, whereas log 3.36 viable *Ef* where recoverable from the analogous extracted tooth model, the other irrigants proved ineffective. 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 may not be the most important factor for the recalcitrance of root canal infections during endodontic irrigation; it is more likely due to the inability of the irrigant to access the infection.

Communicated by Jorge Membrillo-Hernández.

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Keywords *Enterococcus faecalis* · In vitro modelling · Hypochlorous acid · Sodium hypochlorite

Introduction

Once bacteria become established within the root canal of the teeth, especially within the smaller accessory canals, they cannot easily be reached by the defence mechanisms of the host or by systemic antibiotics. Flushing the root canal with an antimicrobial solution, typically sodium hypochlorite (NaOCl), is termed endodontic irrigation, and its successful application enhances the success rate of root canal treatment (Sjogren et al. 1997). *Enterococcus faecalis* (*Ef*) 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). 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 NaOCl (Lima et al. 2001) and can be isolated as a monoseptic culture from infected root canals (Fabricius et al. 1982). A possible confounding factor is that the bacteria growing within an infected root canal do so as biofilm (Duggan and Sedgley 2007) in conditions where the availability of carbohydrates is limited. Bacteria that are members of a biofilm community can be up to 1,000 times more resistant to antimicrobial compounds than their planktonic counterparts (Gilbert et al. 2002), which may affect the success of endodontic irrigation.

In principle, endodontic irrigation with an antimicrobial agent allows disinfection of areas of the root canal that are unreachable by instrumentation (Siqueira et al. 2000). Accessory (lateral) canals branch radially from the main root canal with diameters ranging from maxima of 100 μm

in permanent molars to 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 (towards the root) poses a similar barrier.

NaOCl has been used as an irrigant since its introduction in endodontics by Walker (1936). 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, although it is acknowledged that there are several negative aspects to its use (Kaufman and Keila 1989). Chlorhexidine (CHX) has been shown to be relatively ineffectual against dental plaque biofilms in an in vitro model following 5-min exposure, requiring 60 min to achieve a 2-$\log_{10}$ to 5-$\log_{10}$ kill (Pratten and Wilson 1999). 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 that can effectively be measured in hours (Schiott et al. 1970) and as such it remains a viable alternative endodontic irrigant (Siqueira et al. 2007). A possible alternative endodontic irrigant is super-oxidised water ($SOXH_2O$), which is a clear, colourless, odourless and non-toxic liquid with a pH between 5 and 6.5. $SOXH_2O$ is produced by a proprietary electrochemical cell 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, Ilkley, West Yorkshire, UK). It is currently in use as a broad spectrum biocide for the 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 been shown to be have limited potential as an alternative endodontic irrigant (Gulabivala et al. 2004).

In order to elucidate the importance of the biofilm modality of growth of *Ef* with respect to its recalcitrance during endodontic irrigation, a series of experiments were planned in which a clinically relevant extracted tooth was compared to those from a variation of an existing model, which incorporated nitrocellulose filter-membrane–grown biofilms of *Ef* (Spratt et al. 2001).

Materials and methods

Bacteriological culture

Cultures of *Ef* (NCTC 775) were maintained at 37°C 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) and grown overnight at 37°C with agitation. Following incubation, the cultures were centrifuged, and the cell pellet was washed in PBS (Oxoid) before being re-suspended in fresh BHI. This washing step minimised the carry-over of exhausted culture medium and waste metabolic products into the root canal/filter-membrane models. 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 5×10^7 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 Liverpool University Dental Hospital prior to endodontic irrigation. Following coronal access, the coronal (upper) two-thirds of each canal was root-prepared using Gates Glidden burs. The apical (lower) third was then chemo-mechanically prepared using sodium hypochlorite and K-files to a master apical size 40 (i.e. the final ISO size of the modified canal). Step-back technique ensured maximal flaring of radicular dentine to maximise access to the root canal during the laboratory irrigation experiments (see Fig. 1, inset), whilst root preparation removed pulp material. 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 121°C for 15 min. Twenty microlitres of the washed *Ef* 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 37°C for 48 h.

The clinical irrigation procedure was mimicked in the laboratory by using an electronic dispensing pipette (Proline 50–1,000 μl, Biohit, Torquay, Devon, UK) to supply irrigant in a reproducible manner. 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 $\sim$1 m s^{-1}. The pipette tip was loaded with 1 ml of irrigant, 1% NaOCl, $SOXH_2O$ (Sterilox®, Optident) 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

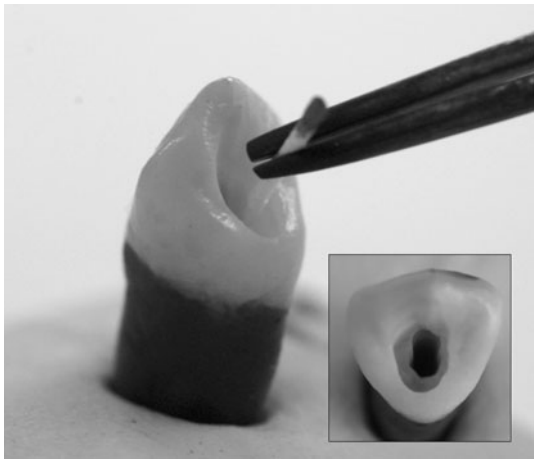

Fig. 1 Sampling viable bacteria from the root canal of an extracted human tooth with a sterile paper point

of irrigant was allowed to remain for 1 min. 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 root canal using a series of five sterile paper points (size ISO 40, Dentsply), which were in turn rubbed against the walls of the root canals (Fig. 1), and allowed to draw up their full capacity of liquid before being transferred to PBS and vortex-mixed for 30 s. *Ef* colony forming units were enumerated by performing serial dilutions and growth on NA after 48 h aerobic incubation at 37°C.

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.

Filter-membrane 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 *Ef* culture at a cell density of 5×10^7 cfu ml^{-1}. These plates were incubated aerobically at 37°C. Counts of viable bacteria present on the filter membranes were determined over a period of 96 h to construct a growth curve.

To evaluate the efficacy of the irrigants, after 48 h of growth, an individual biofilm-laden, filter membrane was lifted from the plate and immersed in 25 ml of filter-sterilised irrigant (or PBS control) for 1 min. 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 mixture and vortex-mixed for 30 s 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-s exposure time, additional biofilm samples were exposed to $SOXH_2O$ for 5 min. 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 *Ef* growing as a planktonic culture in a liquid medium was determined by constructing a low-resolution (i.e. tenfold) 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-h incubation at 37°C 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's test) showed that irrigation of the extracted tooth model with 1% NaOCl killed significantly more *Ef* 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 ($P = 0.384$;

Table 1 Mean log cfu of *Enterococcus faecalis* recovered from an extracted tooth model of endodontic irrigation

Irrigant	Bacteria recovered (mean log cfu)	% Kill (vs PBS)	*n*	Standard deviation	Variance
PBS	5.11	–	20	0.63	0.40
NaOCl (1%)	3.36	98.22	20	2.65	7.02
$SOXH_2O$	4.66	64.52	21	1.47	2.16

Table 2 Statistical comparison between irrigants pairs (Tamhane's post hoc test) in an extracted tooth model of endodontic irrigation

Irrigant		Mean difference (A–B)	Standard error	Significance (*P* value)	95% confidence interval	
A	B				Lower bound	Upper 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

* Significant at 0.05 level (i.e. $P < 0.05$)

data not shown), whereas the difference between irrigants was significant ($P = 0.026$).

Filter-membrane biofilm model

The *Ef* filter-membrane biofilm cultures reached the stationary phase of growth after approximately 16-h incubation at 37°C (Fig. 2). The stationary phase was maintained beyond 48 h, the point at which the biofilms were harvested for experimentation.

PBS was used as a negative control to determine the effects of immersing the biofilm in a physiologically neutral liquid. 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 *Ef* was 0.05%, and growth was observed at 0.025%. Cultures of *Ef* NCTC 775 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 $SOXH_2O$ ($P = 0.005$). Compared to $SOXH_2O$ ($P = 0.007$), 2% CHX was more effective. Increasing the contact time of $SOXH_2O$ from 1 to 5 min resulted in a significant ($P = 0.039$) increase in kill from 51 to 97%.

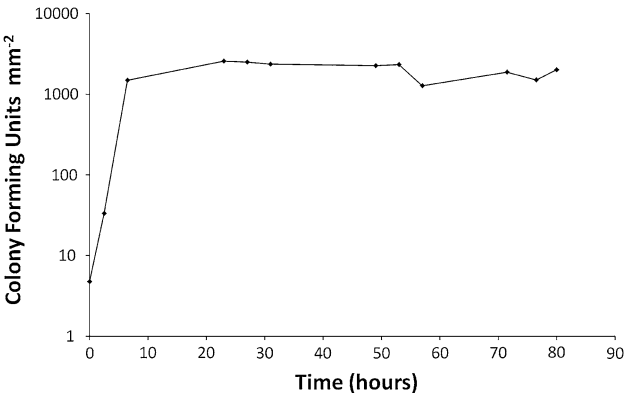

Fig. 2 Growth of *Enterococcus faecalis* biofilms on a nitrocellulose filter membrane. The biofilms harvested for this study were in the stationary phase following 48-h incubation at 37°C

Discussion

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 be acceptable, or indeed ethical, if patients were involved.

A pure culture of *Ef* was chosen for this study since it is representative of the bacterial contamination found within the root canal (often as a monoseptic infection), 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 it is used in the Royal

Table 3 Number of viable bacteria recovered from filter-membrane biofilms of *Enterococcus faecalis* following exposure to various endodontic irrigants

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[a]	–	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 min)	3.06	3.25	96.92	5

Exposure time was 1 min with the exception of a $SOXH_2O$ replicate at 5 min. All irrigants yielded a significant kill (i.e. $P < 0.05$) with the exception of 0.01% NaOCl

[a] No viable bacteria were recovered from any of the six samples; this is the minimum bactericidal concentration (MBC)

Liverpool and Broadgreen University Hospitals Trusts to monitor surface cleansing practices as it exhibits resistance to NaOCl. Whilst academically interesting and challenging, a model that used mixed cultures of root canal infections would unnecessarily introduce a highly variable dynamic.

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 1 min is shorter than that to be expected clinically, but it has already been shown that increasing exposure time from 1 to 5 min does not significantly increase the percentage of *Ef* that are killed (Dunavant et al. 2006). Statistical analysis revealed that between-teeth effects of the seven teeth used in these experiments were not significant. 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, although microbial counts typically have a high intrinsic variability (Jannasch and Jones 1959).

It is not easy to ascertain the growth conditions in the extracted tooth model after 48-h 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 resistance of *Ef* 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 membrane 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 *Ef* 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 *Ef* 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-h 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 was to incorporate aspiration by an endodontic needle rather than the limited access offered by the 1-ml pipette tip.

Although able to kill a significant number of the bacteria, 2% CHX and $SOXH_2O$ 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 these experiments. 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 laboratory 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 filter-membrane biofilm experiments were similar to those successfully employed by Spratt et al. (2001) and further modified by 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 experiments was typically 1 min (as opposed to 15 or 60 min) 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 (0.2–0.45 μm) of the nitrocellulose 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 *Ef* 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 *Ef* and that 1% NaOCl solution with a contact time of 1 min 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 *Ef* or any deficiency of the bactericidal properties of the irrigant per se. The narrow cross-sectional area of the accessory canals ($\sim$100 μm diameter) in relation to their length (up to $\sim$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 (Cunningham et al. 1982) may outweigh this deficit in practice.

Conclusion

This inability of NaOCl to satisfactorily disinfect root canals during endodontic irrigation procedures, in both clinically relevant and extracted tooth models, is not entirely due to the recalcitrance of the infecting organisms growing as a biofilm. A significant aspect responsible for the failure of endodontic disinfection is due to the mixing/mass transfer limitations that occur within the root canal system during the irrigation procedure. In summary, 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, which is responsible for the failure of endodontic irrigation.

Acknowledgments The extracted tooth model experiments, by SGG, were funded by the School of Dental Sciences, University of Liverpool, as a 4th year undergraduate Bachelor of Dental Sciences elective project, whilst the filter-membrane biofilm models were undertaken as part of a self-funded post-graduate degree by QW who is in turn partly supported by a University of Liverpool Graduates Association (Hong Kong) Scholarship. The authors would like to thank Dr Fadi Jarad BDS PhD MDFS RCS (Eng) for editorial comments. CKH wishes to highlight the work of the late Professor Peter Gilbert (1951–2008) as an inspiration to those involved in modelling microbial biofilms.

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