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Characterisation of the efficacy of endodontic medications using a three-dimensional fluorescent tooth model: An ex vivo study

Author

Chen, Emily W, Carey, Alison J, Ulett, Glen C, George, Roy

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Introduction

Our understanding of microbial ecology and bacterial fitness dynamics within the root canal is fundamental to the management of endodontic disease (Svensäter & Bergenholtz 2004). The success of endodontic treatment lies in the elimination of bacteria and their by-products from the canal spaces, as well as the establishment of a 3-dimensional seal (Sundqvist 1994; Walker 1996). Bacterial biofilms within the root canal are a major challenge for endodontic therapy because they protect microorganisms from adverse environmental challenges including antimicrobial action (Costerton Lewandowski et al. 1994; Svensäter & Bergenholtz 2004). Re-infection of endodontically treated teeth may stem from persistence of bacterial biofilms in unprepared locations including in the main canal, isthmuses, and accessory canals, thus threatening the success of endodontic therapy (Oguntebi 1994; Siqueira & de Uzeda 1996; Nair 1999).

Several models have been used for evaluating the efficacy of medications for root canal therapy (Haapasalo & Orstavik 1987; Orstavik & Haapasalo 1990; Siqueira & de Uzeda 1997; Norrington Ruby et al. 2008). Each of these methods has provided insight into the efficacy of various treatments and conditions that influence bacterial viability on teeth. Past models such as the simple plate count method is limited in that it cannot replicate the biological complexity of bacterial biofilms in which there is continual cell growth and death, and persistent cell turn-over (Costerton Lewandowski et al. 1994; Lui Sae-Lim et al. 2004; Chavez de Paz 2007). The frequently used agar diffusion test is dependent on the ability of the medications to diffuse in agar (Siqueira & de Uzeda 1997). Calcium hydroxide's poor diffusion into agar and the buffering action of agar makes this model unsuitable for testing its

efficacy (Siqueira & de Uzeda 1997; Estrela Bammann et al. 2000; Estrela Bammann et al. 2001; Haenni Schmidlin et al. 2003). The use of scanning electron microscopy (SEM) as a possible method to evaluate efficacy of medicament (Haapasalo & Orstavik 1987; Peters Wesselink et al. 2001; Clegg Vertucci et al. 2006; Norrington Ruby et al. 2008) is limited by not only the susceptibility of bacteria to its preparation techniques but also its inability to visualize all sections of teeth with hidden microorganisms (Orstavik & Haapasalo 1990). Dentine block model, created by Haapasalo and Orstavik (Haapasalo & Orstavik 1987; Orstavik & Haapasalo 1990), involves the sealing of one end of the dentinal tubules with nail polish, which prevents the evaluation efficacy of medicament across tubules. The use of dentine scrapings to evaluate disinfection on selected walls of the root canal does not provide a 3-dimensional profile of disinfection along the length and thickness of the root dentine (Portenier Haapasalo et al. 2001).

The viability of microorganisms may vary throughout tooth surface biofilms (Netuschil Reich et al. 1998). Recently a 3D dentine infection models was proposed by (Ma Wang et al. 2011). The model allowed for quantitative assessment of bacterial viability for short-term disinfection by irrigants in sectioned roots that predictable, dense, and deep penetration of bacteria. This model however does not allow for assessing the uniformity of bacterial kill of medicaments over time points or the survival dynamic of the bacterial biofilms over a time period; Further the study design allowed for evaluating only a small section of the root dentine.

The aim of the current study was to establish a novel *ex-vivo* model for the evaluating the uniformity of bacterial kill in dental bacterial biofilm by medicaments over a period an extended time period of 72 hours. This model assess the efficacy of treatment using a dynamic *E. faecalis* and *S. mutans* biofilm grown on the external

root surface rather than within the root canal.

Materials and Methods

Sample Preparation

Thirty-two single-rooted extracted human teeth, with intact crowns were collected from adult patients attending dental clinics. This study was approved by the Griffith University Human Research Ethics Committee. Prior to commencing this study, all crown and root surfaces of each tooth were debrided with an ultrasonic scaler (Satalec/Acteon; Niort, France) to remove residual soft tissue and calculus and then sterilized in an autoclave (121°C for 15 min). A sterile carbide bur was used to establish access to the pulp chamber and canals. The working length of each tooth was established 1 mm short of the apical foramen by inserting a size 10 K-file (Dentsply; Maillefer, Ballaigues, Switzerland) past the apex until the tip was visible, and then withdrawing the file by 1 mm. ProTaper^{TM} files (Dentsply; Maillefer, Ballaigues, Switzerland) were used to enlarge the root canals. All canals were prepared using ProTaper^{TM} files (Dentsply;) according to manufacturer's instruction to a final file size corresponding to an F3 instrument. The canals were copiously irrigated alternatively with 1% NaOCl (Dentalife; Ringwood, VIC, Australia) and 15% EDTA/C (EDTA/C 15%; Endoprep solution; Bayswater, VIC, Australia, Milton's solution) during the mechanical preparation. The preparation was followed with a rinse of NaOCl for 2 minutes and EDTA/C for 2 minutes, to ensure complete removal of smear layer and finally flushed with de-ionized water.

Establishment of biofilm on the surface of the root

To prepare the model, a hole was initially bored into a coverlid of a sterile cylindrical 5ml vial. The tooth was then inserted into the lid to the cemento-enamel junction (CEJ) and stabilized and sealed with sticky wax (Kemdent; Brisbane, QLD, Australia). The set-up enables the crown of the tooth to protrude above the lid, and the root of the tooth to be suspended in the center of the vial (Figure. 1). To prevent bacteria from the culture broth entering the canals through the apex of the roots, the apex of the each tooth was sealed with wax, and a paper point was placed into the chemo-mechanically prepared canal to indicate effectiveness of the seal.

To support biofilm growth a salivary pellicle was first generated around the surface of the root by submerging teeth in fresh, unstimulated saliva, pooled from two healthy volunteers, up to the level of the CEJ for 24h at 37°C statically (Clegg Vertucci et al. 2006). Teeth with salivary pellicle were then submerged into 5ml of freshly diluted THB (1/10 in water) with inoculate of five microliters of an overnight broth culture of *S. mutans* or *E. faecalis*. The *S. mutans* and *E. faecalis* used in this study were originally isolated at the University of Alabama, Birmingham Hospital (Ulett Benjamin et al. 2009; Tan Ulett et al. 2012), and were grown in Todd Hewitt broth (THB; Oxoid, North Ryde, NSW, Australia) for 18h at 37°C shaking at 180 rpm. The teeth set-up in the vials were incubated at 37°C to mimic the oral environment. At 48h, fresh media was added by removing three quarters of the original suspension from each vial and replacing this with fresh, diluted THB. Preliminary experiments demonstrated that establishment of *S. mutans* or *E. faecalis* surface biofilm on the root required 4-days, which we regarded as time zero; medicament was then applied at this time point (t=0 hours).

Experimental groups

Once biofilm formation is confirmed, each experimental root canal was treated as per Table 1. Paper points were removed from each of the sample teeth. The root canal of each sample tooth of the positive control groups (n=2 x 7 for *S. mutans* and *E. faecalis*) was sealed at the coronal part of the tooth with cavit G (3M; North Ryde, NSW, Australia). For the calcium hydroxide treatment groups (n=9 each for *S. mutans* and *E. faecalis*), calcium hydroxide paste (Henry Schein; Melville, NY, America) was placed in the root canal and sealed with Cavit G®.

To analyze bacterial survival dynamics following treatment with calcium hydroxide on the surface of biofilm, we captured representative images at 0h, 1h, 3h, 6h, 24h, 48h, and 72h. Between each time point, it was ensured that each tooth root was fully submerged in PBS in order to prevent the bacterial biofilm on the root surface from drying out.

Bacteria and fluorescent staining techniques

For staining of bacterial biofilms, teeth were submerged in 3ml of BacLight stain for 15 minutes in the dark (Viable Technologies, Mulgrave, VIC, Australia). The stain is comprised of SYTO9, which stains viable bacteria (emitting green fluorescence), and propidium iodine, which stains non-viable bacteria (emitting red fluorescence) and was prepared according to the manufacturer's instructions(Lehtinen Nuutila et al. 2004). The excitation/emission wavelengths for SYTO9 were 480/500 nm and propidium iodide were 490/635 nm, with GFPHQ and RFP1 filters used respectively.

The teeth were immersed briefly in a Phosphate Buffered Saline (PBS) series following BacLight staining to remove residual stain. The staining procedure was repeated before each time point prior to microscopic analysis.

For visualization of viable and non-viable bacterial cells in biofilms images were acquired using a DP72 Olympus digital camera fitted to an Olympus 3D SZX16 fluorescent microscope. Viability of biofilm was measured by calculating the total pixel area of green (viable bacteria) and red (non-viable bacteria) for each image. ImageJ 1.45s software (National Institutes of Health, USA) was used to measure pixel densities for the green and red channels in each image. All data generated were assessed for normality using Kolmogorov-Smirnoff test and then analyzed using unpaired Mann Whitney t-test. Scanning electron microscopic image of an untreated tooth with E. *faecalis* biofilm was obtained (at zero time point) to visualize bacterial presence (Figure 2, black arrow). The image was obtained using a Zeiss Sigma VP Field Emission Scanning Electron Microscope at low-vac. Further visualization was obtained in fluorescent images that were acquired with a Nikon R microscope to visualize the bacterial kill (Figure 5 panel band c) following development of *E. faecalis bacterial biofilm on the surface of the tooth.*

Results

The results of this study showed viability of bacterial biofilm on the surface of the roots over 72 hours and its growth patterns when subjected to calcium hydroxide (Figure 3 and 4). The control groups showed that the growth of both *S. mutans* and *E. faecalis* biofilm were unstable in the first few hours as the viability of both types of biofilms decreased sharply at 3 hours. Both biofilm models tested in this study showed consistent growth after three hours (Figure 3).

Following the first 3 hours of $Ca(OH)_2$ treatment, the viability of *E. faecalis* biofilm showed a gradual decrease when compared to the control group for the first 24 hours, however this was not statistically significant. The viability of the *E. faecalis* biofilm after 24 hours showed gradual increases, reaching to a similar level of biofilm viability as the control group over 72 hours.

S. mutans biofilm in the $Ca(OH)_2$ treatment group exhibited a decrease in viability in the first 6 hours. The viability continued to decrease over time with significant differences ($p=0.042$) being noticed to the control group at 48 hours and the end of the study period.

Discussion

Current approaches to study the efficacy of medicament towards surface bacterial biofilms on teeth are largely based on those involving agar diffusion (Siqueira & de Uzeda 1997; Estrela Bammann et al. 2000; Haenni Schmidlin et al. 2003), infected human and bovine teeth in *ex vivo* using microscopy (Haapasalo & Orstavik 1987; Norrington Ruby et al. 2008) limited in efficacy of medicaments and assessing 3-dimensional penetration. Thus, it is important to determine if medicaments can penetrate to sufficient depth into the dentinal tubules and effectively reach the external surface to be effectual against bacterial biofilms.

Calcium hydroxide has been considered the gold standard for medication of the root canal (Siqueira & Lopes 1999). The strong alkalizing effects of $Ca(OH)_2$ stemming from the generation of hydroxyl ions are directly antibacterial and have

broad-spectrum activity against different microbes (Siqueira & Lopes 1999). Calcium hydroxide can diffuse up to 500μm into dentinal tubules and is reported to more than halve the viable bacteria within 7 days (Lin Tsesis et al. 2005). Clinical studies of the efficacy of calcium hydroxide on previously untreated bacterial biofilms have shown more than 90% elimination of bacteria (Sjogren Figdor et al. 1991; Shuping Orstavik et al. 2000). Studies have revealed that hydroxyl ions derived from $Ca(OH)_2$ diffuse through root dentine and have greater penetration into biofilm (Chai Hamimah et al. 2007; Norrington Ruby et al. 2008). *E. faecalis* is most commonly associated with failure of root canal treatment (Orstavik & Haapasalo 1990; Estrela Pimenta et al. 1998; Molander Reit et al. 1998; Lima Fava et al. 2001; Pinheiro Gomes et al. 2003), however, $Ca(OH)_2$ is not known to be effective against *E. faecalis* biofilms (Estrela Pimenta et al. 1999). In our study, $Ca(OH)_2$ was used as the prototypic medicament for testing our model of both *E. faecalis* and *S. mutans* dental biofilm as the former is a recognized bacteria seen in infected root canals, while the latter is known to be susceptible to $Ca(OH)_2$ and helps provide validity to this model (Pinheiro Gomes et al. 2003; Gangwar 2011). The present study showed that there was an increase in viability of bacterial biofilms from hour 3 and peaking at hour 24 where it remained much more stable for both bacterial biofilms. This trend persisted for both bacterial biofilms and subsequently decreased for the remainder of the assay as broth was not replenished during the 72 hours. The initial significant decrease in viability of bacterial biofilms on the surface of teeth (at hours 0,1 and 3) could be attributed to constant handling and disturbance to the bacterial biofilms (Figure 2).

The study model showed for the first time notably different survival dynamics of *S. mutans* and *E. faecalis* biofilm on tooth following $Ca(OH)_2$ treatment compared to the overall root surface. Thus this model offers a unique advantage for concurrent

monitoring of the patterns of live and dead bacteria in dental biofilm and the variations of survival of various bacterial biofilms in response to different medicaments. Finally, the use of saliva to promote colonization in the current model mimics the poly-microbial nature of surface bacterial biofilms on human teeth (Rosan & Lamont 2000).

Root surface images captured prior to bacterial staining revealed either auto-fluorescence of teeth or fluorescence of the epithelial cells with the SYTO9 stain from the salivary pellicle (Larrosa Truchado et al. 2012). Consequently, the results may have shown an under-representation of the amount of bacteria kill. A counter stain that has no reaction with the viability of the bacterial biofilm or sterifiltration of the saliva may be required to minimize green-fluorescence of the tooth. As samples were reused for each time point, the fluorescent stains may have influences on the viability of bacteria. It may also be possible with the use of advanced fluorescence microscopes to evaluate the amount of auto-fluorescence's and hence enabling better evaluation of the bacterial cells using the BacLight live dead cells. An additional advantage of this model is that the external root surface of the teeth can be ground to different fixed dimensions from the internal root canal wall to standardize the assessment of depth of kill, uniformity of kill over a different time points.

Conclusion

The novel 3D fluorescent tooth model described here reflects the *in vivo* environment with an adequate human root canal including variations in anatomic complexity. The proposed biofilm model will be useful for studies on antimicrobial efficacy of

endodontic materials under clinically relevant conditions. Future studies investigating the effects of medicaments other than $Ca(OH)_2$ on biofilms would benefit from the standardized biofilm tooth model as described in this study.

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Tables and figure legends

Table 1. Treatment of tooth samples

Bacteria	**Treatment**	**Number of Samples**
Enterococcus faecalis	Positive control (no treatment)	7
	Calcium hydroxide	9
Streptococcus mutans	Positive control (no treatment)	7
	Calcium hydroxide	9

Figure 1. Diagrammatic representation of the 3D fluorescent tooth model.
A. Root of tooth suspended in pooled fresh human saliva for 24h to allow for the formation of pellicle. B. Root of the tooth suspended in a 5ml vial containing the bacterial growth media (Todd Hewitt Broth) inoculated with 10µl of overnight broth culture of *S. mutans*. C. Root with sample set up incubated for 4days to allow for growth of bacterial biofilm. D. Root of tooth suspended in Phosphate Buffered Saline (PBS) with bacterial biofilms on the surface of the root and calcium hydroxide medicament placed in the root canal and sealed using Cavit G. At this stage, the model is established and is considered as time zero.

Figure 2: SEM Image of for *E. faecalis* at 5000x magnification in the no treatment group. Note the presence of *E. faecalis* on the surface and embedded in the thick layer of biofilm at time zero.

Figure 3. Top panel (A) show the viability of *Enterococcus* biofilm on the root surface following CaOH, Calcium hydroxide treatment; compared to no treatment Bottom panel (B) show the viability of *Streptococcus mutans* biofilm on the apical region of root surface shown following CaOH treament; compared to no treatment.

Figure 4: Panels A, B, C show *S. mutans* biofilm on the external surface of a tooth root treated with calcium hydroxide at time points 0 hours, 24 hours and 72 hours, respectively. Panels D, E, F show equivalent images for *E. faecalis*. Note the intensity of green (Live bacteria) in panels D, E, F Vs. panels A, B, C. Scale bars are 1000 µm.

Figure 5: Panel showing proof of concept of bacterial kill using fluorescent imaging (B and C) after development of *E. faecalis* bacterial biofilm on the external root surface.(A)