Contemporary Root Canal Irrigants Are Able to Disrupt and Eradicate Single- and Dual-Species Biofilms

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Abstract

Introduction: Clinical/microbiological studies have consistently revealed the persistence of some bacteria after conventional root canal debridement. Although this was originally attributed to the complexity of the root canal anatomy and the difficulty of delivering antibacterial agents effectively, it has emerged that the biofilm encasement of bacterial cells may confer a further mechanism of resistance. The purpose of this study was to investigate the relative disruption and bactericidal effects of root canal irrigants on single- and dual-species biofilms of root canal isolates. **Methods:** Biofilms of *Streptococcus sanguinis, Enterococcus faecalis, Fusobacterium nucleatum*, and *Porphyromonas gingivalis* were grown on nitrocellulose membranes for 72 hours and immersed in NaOCl, EDTA, chlorhexidine, and iodine for 1, 5, or 10 minutes. The number of viable and nonviable bacteria disrupted from the biofilm and those remaining adherent were determined by using a viability stain in conjunction with fluorescence microscopy. **Results:** Gram-negative obligate anaerobe species were more susceptible to cell removal than gram-positive facultative anaerobes. The majority of cells were disrupted after the first minute of exposure; however, the extent varied according to the agent and species. The most effective agent at disrupting biofilms was NaOCl. Iodine was generally effective at bacterial killing but not disruption. **Conclusions:** Biofilm disruption and cell viability were influenced by the species, their coassociation in dual-species biofilms, the test agent, and the duration of exposure. The effectiveness of NaOCl as an endodontic irrigant was reinforced. *(J Endod 2009;35:1243–1248)*

Key Words

Biofilm, disruption, *Enterococcus faecalis*, Fusobacterium nucleatum, irrigant, *Porphyromonas gingivalis*, root canal, *Streptococcus sanguinis*, susceptibility

Bacterial colonization of the pulp-dentine complex and the root canal system has been definitively implicated as the primary etiologic factor in pulpal and periapical disease (1, 2). Elimination of such infections has been the empirical cornerstone of root canal treatment for over a century. Although the techniques and materials used have changed and probably improved, the success rates of root canal treatment do not appear to have increased over this time (3, 4). Root canal preparation and irrigation reduce the bacterial load to varying degrees but fail to achieve negative cultures universally (5, 6). Even contemporary techniques seem unable to eliminate the bacteria completely, although the most accessible parts of the root canal system seem better debrided and enable periapical healing (7, 8), despite the presence of residual biofilm-encased bacteria in the complex apical anatomy (9, 10). This residual biofilm infection in the apical part of the root canal system appears to influence root canal treatment failure, but it has proved difficult to track the presence of residual bacteria clinically in real time to predict the possible outcome (11, 12). Elucidation of the mechanisms involved in bacterial resistance to treatment requires the *in vitro* investigation of the relative effects of biofilm encasement, bacterial species and their interaction, and antibacterial agents and their duration of action (13, 14). Since this early work, a number of studies have evaluated the influence of irrigants on endodontic-related biofilms (15–17). To date, little work has been performed investigating the disruption of the endodontic biofilms. Therefore, the purpose of this study was to assess the effectiveness of irrigants to disrupt and hence aid in biofilm removal from the root canal. This was evaluated by determining both the disruptive and bactericidal effect of various root canal irrigants on single- and dual-species biofilms.

Materials and Methods

The bacterial isolates used in the study were *Streptococcus sanguinis* E01-07-001, *Enterococcus faecalis* E01-07-048, *Porphyromonas gingivalis* E01-07-035, and *Fusobacterium nucleatum* E01-07-13. All four strains had previously been isolated from infected root canal systems in teeth associated with periapical disease.

The following test solutions selected were irrigants commonly used during root canal treatment: 1% NaOCl (5% Teepol Bleach; Teepol Products, Egham, UK) diluted in water, 0.2% chlorhexidine gluconate (Corsodyl; GlaxoSmithKline Ltd, Brentford, UK), 10% povidone-iodine (Betadine; Seton Health Care PLC, UK), and 17% EDTA (AnalaR grade; Merck BDH, Poole, UK). Reduced transport fluid (RTF) prepared according to Syed and Loesche (18) was used as a control.

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TABLE 1. Means and Standard Deviations of Live or Dead Bacteria per View (n = 5) of All Bacteria Recovered from Single-species Biofilms (n = 3) at Baseline and after Exposure to Test Agents for Various Durations

			1 minute				5 minutes				10 minutes			
Agent			Ss	Ef	Fn	Pg	Ss	Ef	Fn	Pg	Ss	Ef	Fn	Pg
Baseline	Live	Mean	428.7	898.5	480.0	343.3	428.7	898.5	480.0	343.3	428.7	898.5	480.0	343.3
		SD	376.5	493.8	292.4	545.2	376.5	493.8	292.4	545.2	376.5	493.8	292.4	545.2
	Dead	Mean	15.0	66.5	141.5	708.9	15.0	66.5	141.5	708.9	15.0	66.5	141.5	708.9
		SD	31.7	61.0	140.7	510.3	31.7	61.0	140.7	510.3	31.7	61.0	140.7	510.3
RTF	Live	Mean	227.3	446.7	141.3	43.3	232.7	453.3	100.7	2.7	155.3	453.3	104.7	6.7
		SD	45.7	230.1	123.7	38.8	96.7	83.3	59.0	4.6	78.6	458.8	56.9	7.0
	Dead	Mean	0	273.3	160.0	113.3	0	206.7	305.3	47.3	0	113.3	310.7	61.3
		SD	–	80.8	63.9	26.6	–	64.3	88.1	24.2	–	23.1	48.4	26.9
NaOCl	Live	Mean	18.0	0	4.0	0.7	0	0	0	0	0	0	0	0
		SD	4.0	–	5.3	1.2	–	–	–	–	–	–	–	–
	Dead	Mean	28.7	31.3	81.3	94.7	21.3	16.7	44.7	90.0	9.3	10.0	36.0	26.7
		SD	11.5	9.5	31.9	31.0	3.1	5.0	15.5	49.9	5.8	5.3	26.2	7.6
Iodine	Live	Mean	0.7	0.7	23.3	0.7	0.3	0	9.3	0	0.2	0	9.3	0
		SD	0.7	1.2	6.4	1.2	0.6	–	4.6	–	0.2	–	4.6	–
	Dead	Mean	135.1	188.7	77.3	178.7	135.4	335.3	58.7	144.7	87.7	252.0	62.7	208.0
		SD	54.9	55.9	35.6	40.5	62.9	46.9	13.3	27.3	34.1	20.9	32.6	118.1
CHX	Live	Mean	216.0	38.7	7.3	0.7	82.0	19.3	8.7	0	38.0	7.3	16.0	0
		SD	68.4	18.6	7.0	1.2	32.7	18.6	4.2	–	29.9	7.0	8.0	–
	Dead	Mean	46.0	414.0	27.3	52.7	93.3	407.3	24.7	52.7	84.0	386.0	35.3	56.0
		SD	11.1	41.3	6.1	21.4	27.2	31.9	6.1	11.4	21.2	83.0	8.1	14.0
EDTA	Live	Mean	19.8	37.4	10.0	0	48.0	32.0	19.3	0	11.4	20.7	16.0	0
		SD	6.5	16.9	5.3	–	33.0	17.8	9.9	–	6.9	5.0	8.0	–
	Dead	Mean	4.0	30.0	34.7	112.7	72.7	24.7	32.7	93.3	2.8	34.7	35.3	48.0
		SD	4.6	33.6	8.1	8.1	52.1	23.7	18.0	81.6	0.5	16.0	8.1	5.3

CHX, chlorhexidine; EDTA, ethylenediaminetetraacetic acid; Ef, *Enterococcus faecalis*; Fn, *Fusobacterium nucleatum*; NaOCl, sodium hypochlorite; Pg, *Porphyromonas gingivalis*; RTF, reduced transport fluid; SD, standard deviation; Ss, *Streptococcus sanguinis*.

Biofilm Generation

Single-species biofilms were generated on nitrocellulose membranes following a previously described protocol (13). Briefly, 20-μL aliquots of a standardized culture (absorbance of 0.4 at 540 nm) were inoculated onto sterile membrane filters (0.2-μm pore size, 5-mm diameter; Whatman International Ltd, Maidstone, UK) and placed on Blood Agar plates (Oxoid, Basingstoke, UK). *S. sanguinis* and *E. faecalis* were incubated at 37°C in air supplemented with 5% CO_2 for 48 hours, whereas *P. gingivalis* and *F. nucleatum* were incubated in an anaerobic cabinet (Don Whitley Scientific Ltd, Shipley, UK) at 37°C for 72 hours. Dual-species biofilms were generated by using two of the test species (*S. sanguinis* and *F. nucleatum*). The ratio of each organism was 1:2 (absorbance of 0.2 and 0.4 at 540 nm) for the *S. sanguinis* and *F. nucleatum,* respectively, and these were incubated anaerobically at 37°C for 72 hours.

Disruption Assay

After incubation of the single- and dual-species biofilms, each membrane filter was immersed into 1 mL of the selected test or control solution. Single or dual-species biofilms were exposed to the test agent or the control (RTF) at room temperature under aerobic conditions for 1, 5, or 10 minutes.

After the designated contact time, each membrane filter was removed and placed into 1 mL of neutralizing broth (BD Difco, Oxford, UK). To determine the number of cells disrupted, the filter was then vortexed at 200 rpm for 20 seconds to resuspend those cells sufficiently loosened by the effect of the test agent from the biofilm. Each membrane filter was then transferred to a sterile 3-mL bottle containing 1 mL of RTF and vortexed at full speed for 1 minute to remove those bacteria that remained attached to the membrane.

Mean cell counts of the disrupted or attached cells were performed as described earlier using a viability stain (LIVE/DEAD *Bac*Light; Invitrogen, Paisley, UK). The bacterial samples were diluted 1:10 and 5 μL was placed on a microscope slide and overlaid with a coverslip (22 mm × 22 mm). A five-point cell count (viable and nonviable cells) was conducted following the method of Defives et al (19) using a microscope with a fluorescent light source (Olympus, Watford, UK). The mean of the five-point cell count was used as a representation of the mean total number of cells on each membrane filter. The control specimens (n = 3 per species) were exposed to RTF to enumerate baseline counts, which were used to compare with the number of bacteria remaining on the membranes after test agent exposure. Each experiment was performed in triplicate and a statistical software package (STATA version 9.2; STATA Corporation, College Station, TX) was used to apply linear regression to investigate the influence of time and test agent on the single- and dual-species biofilms.

Confocal Laser Scanning Microscopy

To visualize the disruptive effect of the irrigants, confocal laser scanning microscopy (CLSM) was performed by using a Bio-Rad MRC 2000 (Hemel Hempstead, UK). Briefly, membrane filters containing treated biofilms were attached to the base of a 5-cm Petri dish (Sterilin, Caerphilly, UK) using a small drop of adhesive (Loctite; Winsford, Cheshire, UK). The filters were then covered with 5 mL of *Bac*Lightviability stain and left to incubate for 10 minutes and viewed with a water-immersion objective (Olympus ×40). These images were then constructed and manipulated using a software program (Image J, http://rsb.info.nih.gov/ij/).

Results

The mean data (together with standard deviations) relating to the numbers of live or dead cells per slide view (five fields) of all cells harvested from single-species biofilms (n = 3) at baseline and after exposure to test or control (RTF) agents for 1, 5, and 10 minutes are

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