Influence of irrigant needle depth in removing bioluminescent bacteria inoculated into instrumented root canals using real-time imaging *in vitro*

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Abstract

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Aim To test the hypothesis that the mechanical efficacy of irrigation in reducing bacteria in the root canal is dependent on depth of placement of the irrigation needle.

Methodology The root canals of 30 permanent cuspids were instrumented to apical size 60 using a crown-down technique. A suspension of the bioluminescent reporter strain *Pseudomonas fluorescens* 5RL was inoculated into each canal of sterilized teeth. Emission of bioluminescence (photons s^{-1}) from each tooth was quantified on four sequential occasions using luminometry and bioluminescence imaging: (i) background, (ii) after inoculation, (iii) after irrigating the inoculated teeth with 3 mL of a nonantimicrobial irrigant delivered either 1 mm (group 1, $n = 15$) or 5 mm (group 2, $n = 15$) from working length (WL) using a 28G safety-ended irrigating needle, (iv) after an additional 3 mL irrigation (total 6 mL). Intragroup and intergroup comparisons were made using Wilcoxon matched pairs and Mann–Whitney tests, respectively.

Results In group 1, there was a mean $\log_{10}$ decrease in bacteria of 0.68 ± 0.26 after 3 mL of irrigant compared with 1.19 ± 0.48 after 6 mL ($P < 0.001$); in group 2 the mean $\log_{10}$ decrease was 0.58 ± 0.28 after 3 mL of irrigant compared with 0.69 ± 0.35 after 6 mL ($P < 0.02$) (Wilcoxon matched pairs). Using 3 mL of irrigant, needle depth did not have a significant effect on reduction of intracanal bacteria ($P = 0.407$), but the effect became significant when 6 mL of irrigant was used ($P < 0.002$) (Mann–Whitney tests).

Conclusions The mechanical efficacy of 6 mL of irrigant in reducing intracanal bacteria was significantly greater when delivered 1 mm compared with 5 mm from WL.

Keywords: bacteria, bioluminescence, irrigation, irrigant needle depth, root canal.

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Introduction

A goal of root canal chemomechanical debridement is the removal of bacteria, which have been shown to be essential for the development of periapical disease (Kakehashi *et al.* 1965, Möller *et al.* 1981, Fabricius *et al.* 1982a,b). However, scanning electron microscopy (SEM) has revealed that portions of the root canal are routinely left untouched following root canal preparation (Mayer *et al.* 2002, Hulsmann *et al.* 2003). These findings are supported by data collected from clinical paper point samples showing that endodontic chemomechanical preparation methods do not reliably render canals bacteria-free, regardless of irri-

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gation and instrumentation regimens (Byström & Sundqvist 1981, 1983, 1985, Lin *et al.* 1991, Shuping *et al.* 2000, Card *et al.* 2002).

Early *in vitro* studies indicated that the proximity of the irrigating needle to the apex played an important role in the removal of dentine (Brown & Doran 1975) and gel particles (Chow 1983) from simulated canals and the removal of radiopaque dentinal shavings from extracted teeth (Abou-Rass & Piccinino 1982). Recent studies on endodontic irrigation have concentrated on irrigant antibacterial or chelating activity (Siqueira *et al.* 1996, Shabahang *et al.* 2003, Torabinejad *et al.* 2003) or irrigating devices (Briseno *et al.* 1992, Walters *et al.* 2002). In contrast, there are few data available on the mechanical ability of irrigation to remove bacteria from root canals. This may be partly due to the difficulties in predictably recovering the entire canal sample for quantification and statistical analysis. Although paper point sampling is clinically convenient, it is limited in that paper points only hold what is displaced from the canal into the paper and do not provide information on what remains in the root canal. The latter is arguably of greater importance if the objective is to assess the effects of chemomechanical debridement to render the root canals free of bacteria. Furthermore, data from sequential treatment procedures are not feasible with some detection methods that use radiolabelled bacteria (Rollison *et al.* 2002) or histological methods (Walters *et al.* 2002).

Recently a nondestructive *in vitro* method to study the efficacy of sequential chemomechanical instrumentation procedures has been developed (Sedgley *et al.* 2004). This method uses real-time optical biophotonic imaging and luminometry to visualize and quantify photon emission from the bioluminescent reporter strain *Pseudomonas fluorescens* 5RL after it has been inoculated into root canals. Bioluminescent bacteria have been successfully applied for real-time monitoring of bacterial physiology (Heitzer *et al.* 1998) and biofilms and infections in mice (Contag *et al.* 1995, Francis *et al.* 2001, Kadurugamuwa *et al.* 2003). Bioluminescence emissions from the inoculum can be correlated with cell counts obtained using conventional culturing methods. The primary advantage of this approach over alternative methods is that it provides real-time quantitative assessment of bacteria in the root canal as opposed to qualitative assessment of bacteria displaced into paper points in the root canal or visualized by SEM. Moreover, intracanal bioluminescent bacteria can be quantified over sequential endodontic procedures without destroying the tooth, thereby allowing multiple readings using the same tooth sample which provides its own baseline reading. This method has been used in a previous study which showed that a minimum of 100 bioluminescent bacterial cells in the root canal were required for detection and that the volume of irrigant used significantly influenced the efficacy of irrigation in reducing numbers of bacteria remaining in the canal (Sedgley *et al.* 2004). However, in that study, the irrigant was delivered to a depth of 1 mm from working length (WL), which may not always be clinically practical. Therefore, the aim of the present study was to test the hypothesis that the mechanical effectiveness of irrigation in reducing bacterial counts in root canals is dependent on the depth of placement of the irrigation needle into the root canal.

Materials and methods

Teeth

Thirty permanent cuspids that were 25 mm long with straight single roots, clinically intact crowns, no restorations, no cracks and no caries were selected from the collection of extracted teeth stored in 50% glycerine/50% ethyl alcohol at the University of Michigan School of Dentistry, Ann Arbor, MI, USA. The teeth were autoclaved at 121 °C for 15 min at 26 psi and stored at 25 °C in 100% humidity until use. Teeth subsequently identified as having abnormal anatomy or calcified root canal systems visible on radiographs were excluded.

Access openings were prepared using tungsten carbide burs in a high-speed handpiece with constant water spray. Root canal WL was set at 24 mm. Canals were instrumented based on the protocol used by predoctoral students at the University of Michigan. This technique uses a crown-down approach with nickel-titanium rotary and hand instruments (Dentsply Tulsa Dental Products, Tulsa, OK, USA). Orifice openers and ProFile .06 Series 29 Rotary Ni-Ti files were used to prepare the coronal two-thirds of the root canal and Profile .04 Ni-Ti hand files were used to prepare the apical one-third to a minimum apical size 60.

Copious irrigation with 5.25% sodium hypochlorite was used between instruments. After instrumentation, the smear layer was removed from canal walls by ultrasonic treatment for 4 min each in 17% EDTA and 5.25% NaOCl (Haapasalo & Ørstavik 1987). Canals were irrigated with distilled water and dried with paper points. Root apices were coated with nonbiolumines-

cent nail varnish to seal the apical foramen. Teeth were autoclaved at 121 °C for 15 min at 26 psi and stored in 100% humidity at 25 °C until use. Canals were dried with sterile paper points (Kerr USA, Romulus, MI, USA) immediately before experiments.

Bacteria

All investigations used *Pseudomonas fluorescens* 5RL, a salicylate-inducible bioluminescent reporter strain harbouring the plasmid pUTK21, a *nah sal-lux* reporter plasmid (approximate size 120 kb) which contains a salicylate-inducible *luxCDABE* gene cassette from *Vibrio fischeri* (Shaw *et al.* 1988, King *et al.* 1990).

Bacterial cultures were prepared from −80 °C frozen stocks in 300 mL Erlenmeyer flasks containing 100 mL LB broth (Difco; Becton, Dickinson and Company, Sparks, MD, USA) with sodium salicylate (50 μg mL^{-1}) (LBS) in aerobic conditions at 25 °C with shaking. After 24 h, a subculture was prepared and grown to OD_{600} 0.190, corresponding to a final concentration of approximately 1×10^9 microorganisms mL^{-1}. Ten-fold serial dilutions were prepared in sterile distilled nuclease-free water supplemented with sodium salicylate (50 μg mL^{-1}) (H_2O/Sal). A dilution corresponding to approximately 1×10^6 cells mL^{-1} was selected for experiments based on data on initial bacterial cell counts *in vivo* (Byström & Sundqvist 1981). Viable counts were obtained by plating serial dilutions in triplicate on LBS agar, and counting colony forming units after 48 h aerobic incubation at 25 °C.

Bacterial suspensions in H_2O/Sal (15 μL) were pipetted into the root canal to WL using sterile fine-pointed gel-loading pipette tips (Fisherbrand; Fisher Scientific, Pittsburgh, PA, USA) previously confirmed to fit loosely at the WL in root canals prepared to apical size 60.

Irrigation procedures

Canals were irrigated with 3 mL H_2O/Sal delivered via a sterile 28G safety-ended (side delivery) endodontic irrigating needle (Max-I-Probe; Dentsply Limited, Weybridge, Surrey, UK) attached to a 3 mL sterile plastic syringe (BD Syringe; Becton Dickinson and Company, Franklin Lakes, NJ, USA). The needle tip was placed either 1 mm (group 1, 15 teeth) or 5 mm (group 2, 15 teeth) short of the WL. Following irrigation, the canal contents were immediately aspirated into the delivery needle and the canals were dried using sterile absorbent paper points placed to WL and replaced until the canals were dry. Teeth were irrigated on two occasions with 3 mL (designated T3 and T6), and the rate of irrigant delivery was approximately 3 mL $(15\ s)^{-1}$. A new sterile needle and syringe combination was used for each procedure for each tooth. Contact between the root surface and any extruding irrigant was prevented by holding the tooth inverted with sterile forceps during the irrigation and drying stages.

Measurement of bioluminescence

Bioluminescent imaging (BLI) was undertaken at the Michigan Small Animal Imaging Resource (Department of Radiology, University of Michigan). Imaging was conducted on a cryogenically cooled IVIS™ system (Xenogen Corp., Alameda, CA, USA) coupled to a data-acquisition PC running LivingImage™ software (Xenogen Corp.). The imaging system includes a highly sensitive charge-coupled device (CCD) camera, a dark imaging chamber to minimize incident light, and software to quantify the results. The CCD camera can detect small numbers of photons as well as operating as a traditional camera to create real-time images with extremely low noise. Teeth were positioned in the dark imaging chamber at 25 °C. A grey scale tooth surface image was collected in the chamber under dim illumination, followed by acquisition and overlay of the pseudocolour image representing the spatial distribution of detected photons emerging from bioluminescent bacteria within the tooth. An integration time of 1 min was used for acquisition of the luminescent image (photons s^{-1} cm^{-2} sr^{-1}). Signal intensity was quantified as the sum of all detectable photons (photons s^{-1}) within a region of interest prescribed over the entire tooth.

Bioluminescence measurements were also obtained using a single tube luminometer (Vega-FB14; Zylux Corporation, Oak Ridge, TN, USA) (ZL) with each tooth placed apex first into a glass vial positioned in the luminometer with the apical 16 mm available for bioluminescence measurement (photons s^{-1}). These data were correlated with those obtained using BLI to provide independent confirmation of data.

To assess the mechanical efficacy of irrigation in removing bacteria from the root canal, bioluminescence was quantified after irrigating the inoculated root canals with a nonantimicrobial irrigant delivered either 1 mm (group 1, $n = 15$) or 5 mm (group 2, $n = 15$) from WL using a 28G safety-ended irrigating needle. For each tooth, bioluminescence was measured: (i) before bacterial inoculation (background), (ii) immediately after inoculation (T1), (iii) after 3 mL irrigation

(T3), and (iv) after 6 mL irrigation (T6). All inoculation and irrigation procedures were performed in sequence.

Data analysis

Photon emission from bioluminescent bacteria in each tooth at T1, T3 and T6 were calculated by subtracting background photons from each measurement. A $\log_{10}$ transformation of bacterial photons was performed due to the extensive range of photon readings. Since all experimental groups did not have normally distributed data, nonparametric tests were used for statistical analysis. Nonparametric repeated-measures analysis of variance tests (Friedman tests) were used to detect differences between T1, T3 and T6 within each group. Intragroup comparisons were made using Wilcoxon matched pairs tests. Intergroup comparisons were made using Mann–Whitney tests. Significance was set at $P < 0.05$. Statistical analysis was performed using Prism 4 for Macintosh software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Viable counts obtained by culturing showed that approximately 2.24×10^4 bacteria were inoculated into the canals. One tooth from group 2 was eliminated during irrigation procedures after BLI imaging showed an intense spot of luminescence on the crown (a tooth coloured restoration not previously noted) which might have interfered with measurement of bioluminescence from the bacteria.

Correlation between all measurements obtained from the two independent detection devices was high ($R^2 = 0.83$) (Fig. 1). Statistical analyses were performed on BLI $\log_{10}$ transformed data. Table 1 also shows the percentage of bacteria and cell numbers remaining after irrigation.

Friedman tests showed a highly significant reduction in bacterial numbers for group 1 and group 2 following irrigation (both $P < 0.0001$). Wilcoxon matched pairs tests showed a significant reduction in bacteria following 6 mL of irrigant compared with 3 mL for group 1 ($P < 0.001$) and group 2 ($P < 0.02$). Mann–Whitney tests showed that while 3 mL of irrigant delivered 1 mm from WL resulted in a mean $\log_{10}$ decrease of 0.68 ± 0.28 compared with 0.58 ± 0.28 when the same volume was delivered 5 mm short of WL, this difference was not significant ($P = 0.407$). However, 6 mL of irrigant delivered 1 mm from WL resulted in a mean $\log_{10}$ decrease of 1.19 ± 0.48 compared with 0.69 ± 0.35 when the same volume was delivered 5 mm short of WL ($P < 0.002$). Representative BLI images are shown in Fig. 2.

Discussion

The purpose of these investigations was to evaluate the effects of sequential irrigation procedures delivered at two different depths using bioluminescent reporter bacteria and real-time imaging analysis. Because the method is noninvasive, evaluation of more than one irrigation procedure was possible. Sequential readings

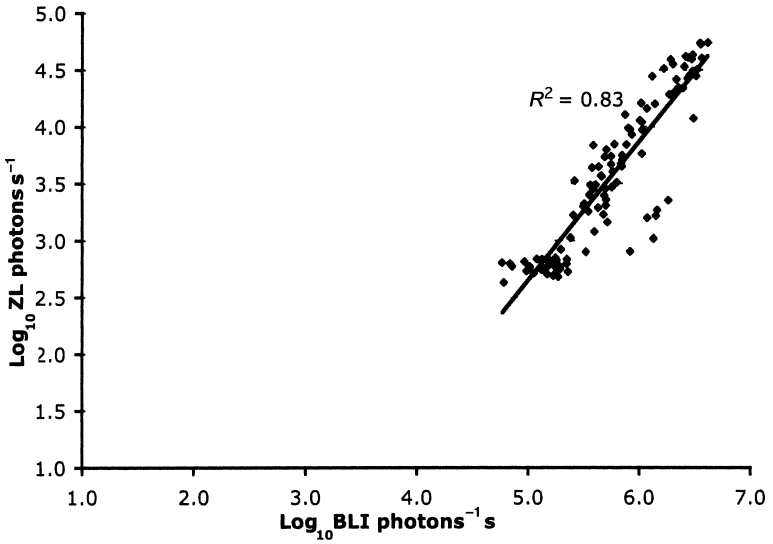

Figure 1 Correlation between bioluminescence detection devices in irrigation experiments.

Table 1 Influence of needle depth placement and volume on bacterial counts

	Group 1 (1 mm from WL) ($n = 15$)			Group 2 (5 mm from WL) ($n = 14$)		
	T1	T3	T6	T1	T3	T6
Bioluminescence detected in bacteria inoculated in root canals (photons s^{-1})						
Log_{10}						
Mean	6.37	5.69	5.18	6.37	5.79	5.68
SD	0.26	0.26	0.48	0.21	0.28	0.35
Data analysis						
T1, T3, T6			$P < 0.0001$[a]			$P < 0.0001$[a]
Group 1 (T6)	–	$P < 0.001$[b]	–	–	–	–
Group 2 (T3)	–	$P = 0.407$[c]	$P < 0.001$[c]	–	–	–
Group 2 (T6)	–	$P = 0.861$[c]	$P < 0.002$[c]	–	$P < 0.02$[b]	–
Percentage of bacteria remaining in root canal						
Mean	100	25.24	9.05	100	32.87	26.40
SD	–	14.85	5.99	–	21.80	20.22
Absolute numbers of bacteria remaining in root canal						
Mean	2.24×10^4	5.65×10^3	2.03×10^3	2.24×10^4	7.36×10^3	5.91×10^3
SD	–	3.33×10^3	1.34×10^3	–	4.88×10^3	4.53×10^3

T1, inoculation; T3; after 3 mL irrigation; T6; after 6 mL irrigation.
[a]Friedman test.
[b]Wilcoxon matched pairs test.
[c]Mann–Whitney test.

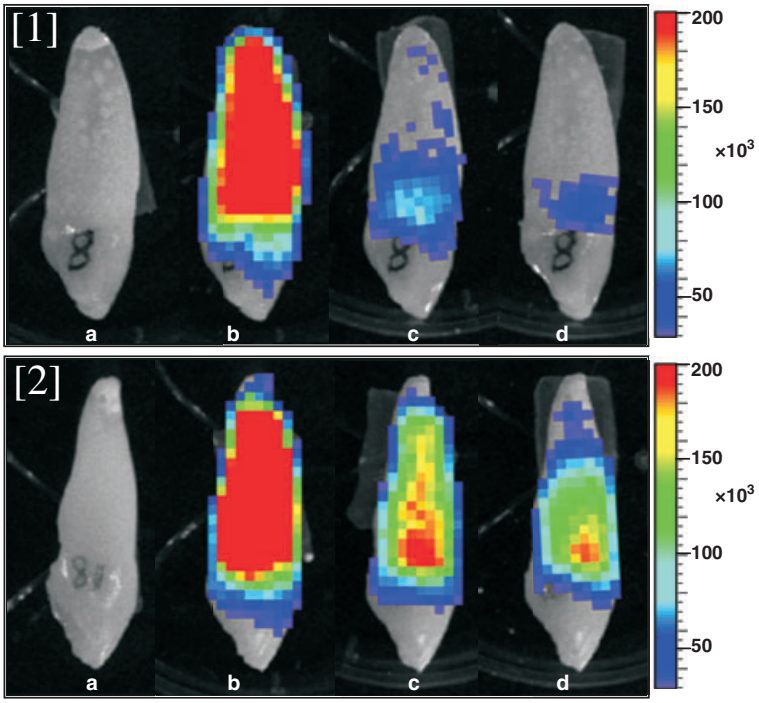

Figure 2 Images of representative teeth from (1) group 1 and (2) group 2. (a) Background, no bacteria, (b) 2.24×10^4 *Pseudomonas fluorescens* 5RL in root canal. Bacterial cell count was determined by culturing the same volume of inoculum. (c) After 3 mL irrigation using 28-gauge Max-I-Probe needle positioned 1 mm from WL (group 1) or 5 mm from WL (group 2). (d) After 6 mL irrigation. Colour bar on right gives luminescence image units (photons s^{-1} cm^{-2} sr^{-1}).

and images could be obtained for each tooth, including baseline counts for each experimental tooth, compared with a method utilizing radiolabelled bacteria which allows only one reading (Rollison *et al.* 2002). Two independent methods of luminescence quantification were used, with high correlation between devices ($R^2 = 0.83$). BLI data have been shown to provide the most sensitive screening method at both lower and

upper limits of detection, as well as real-time measurements and images (Sedgley *et al.* 2004) and therefore these data were analysed in the present investigation. The method provides an alternative to traditional *in vitro* methods using paper point samples and further supports previous reports about the difficulties of completely removing bacteria from root canals (Byström & Sundqvist 1981, 1983, 1985, Lin *et al.* 1991, Shuping *et al.* 2000).

Pseudomonas fluorescens 5RL, a Gram-negative rod 2–3 μm in length, was selected for these experiments based on strong bioluminescent activity associated with its reporter plasmid pUTK21, enhanced upon exposure to salicylate. The images in Fig. 2 result from photons emitted from bacteria in root canals imaged by an extremely sensitive CCD camera. The photons are emitted in all directions from their source and therefore a clear outline of the root canal would not be expected. It is important to note that no attempt was made to 'grow' *P. fluorescens* 5RL in the root canals. The bioluminescent bacteria were introduced into the root canals after instrumentation and sterilization, and therefore cannot be directly compared with a root canal flora present as a biofilm attached to the root dentine present, for example, in teeth prior to treatment. It is reasonable to speculate that irrigation would be mechanically less efficient in the removal of bacteria in an attached biofilm compared with bacteria in a planktonic state in a root canal.

Early *in vitro* studies reported that the proximity of the needle to the apex plays an important role in the removal of debris (Brown & Doran 1975, Abou-Rass & Piccinino 1982, Chow 1983), but the influence of needle depth on removal of bacteria from root canals has not been previously evaluated. In the present study, the influence of needle depth became apparent using 6 mL of the irrigant (Table 1); 6 mL of irrigant delivered 1 mm from WL resulted in a mean $\log_{10}$ decrease of 1.19 ± 0.48 compared with 0.69 ± 0.35 when the same volume was delivered 5 mm short of WL ($P = 0.002$). These findings support previous observations that little fluid exchange and displacement of particles occurs beyond the tip of the needle in simulated canals (Chow 1983).

An antimicrobial irrigant was intentionally not used in these investigations as the objective was not to kill bacteria, but to assess the mechanical efficacy of irrigants when delivered at different distances from the apex in the removal of bacteria. However, antimicrobial irrigants, such as sodium hypochlorite (NaOCl), would be expected to reduce the numbers of viable bacteria in root canals (Byström & Sundqvist 1983, Siqueira *et al.* 2002). Byström & Sundqvist (1981) showed that the combination of mechanical instrumentation and 6 mL of saline irrigation (using a 23G 'end delivery' needle) *in vivo* reduced the number of bacterial cells in paper points samples from initial counts of 10^4–10^6 to 10^2–10^3 by the end of the appointment. When 0.5% NaOCl was used as the irrigant *in vivo*, fewer species were cultured from samples over the course of treatment, although data on absolute cell numbers were not provided (Byström & Sundqvist 1983).

Increasing the volume of irrigant significantly reduced bacterial load regardless of needle depth (Table 1). Mean $\log_{10}$ values obtained after 6 mL of irrigant delivered 1 mm from WL (1.19 ± 0.48) were comparable with those previously reported using the same method (mean $\log_{10}$ decrease of 1.14 ± 0.25, $n = 5$ teeth) (Sedgley *et al.* 2004). The addition of mechanical instrumentation to the irrigation regime would be expected to further reduce bacterial numbers (Byström & Sundqvist 1981, Shuping *et al.* 2000, Card *et al.* 2002) and will be investigated in future studies.

Approximately 9% of the bacteria remained after 6 mL of irrigant was delivered 1 mm from WL compared with 26% when the irrigant was delivered 5 mm from WL in cuspids prepared uniformly to an apical preparation size of 60 (Table 1). Larger sized apical preparations have been associated with a greater reduction in numbers of bacteria following chemomechanical preparation (Card *et al.* 2002, Rollison *et al.* 2002), possibly due to the ability to place the needle tip more apically, based on the present findings. The size of the needle will partly determine the depth of placement as a 21-gauge needle can reach the apex of a size 80 canals, a 23-gauge needle to size 50, a 25-gauge needle to size 45, and a 30-gauge needle to size 20 (Chow 1983). The 28-gauge needle used in this study was placed loosely to WL and it could be hypothesized that irrigation would be most effective in large canals using narrow gauge needles, as better currents could be produced. In addition, irrigation needle tip design could influence the efficacy of irrigation; an *in vitro* study showed that, of the several needles and irrigation devices investigated, the side delivery Max-I-Probes were the most effective in removing dye introduced into clear artificial canals (Kahn *et al.* 1995). The present method could be applied to investigate the influence, if any, of needle-tip design on the efficacy of irrigation.

Apical extrusion of irrigant was avoided in these investigations by sealing the apical foramina with nail varnish. However, from a clinical perspective it is important to note that care is required when irrigating close to WL, particularly in open apex cases and when using an irrigant with the tissue solvent capacity and toxicity of NaOCl (Moorer & Wesselink 1982).

Conclusions

Depth of needle placement was a significant factor in the reduction of bacterial counts when irrigating root canals; 6 mL of irrigant was significantly less effective in removing bacteria when delivered 5 mm from the WL compared with 1 mm from the WL ($P < 0.002$).

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