Smear layer removal ability and antibacterial activity of endodontic irrigants

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ABSTRACT

Introduction: a variety of endodontic irrigants are available for endodontic irrigation. Irrigants must be effective in removing the smear layer created during endodontic therapy as well as eliminating bacteria.

Aim: This *in vitro* study tested various alternating sequences of sodium hypochlorite, anolyte solution (electrochemically activated water), and EDTA for their ability to do this.

Method: Forty-eight single canal teeth were randomly divided into six groups, prepared to working length, sterilised and inoculated with *Enterococcus faecalis*. Each group was assigned a different sequence of irrigants. Standard cultivation techniques were used to count the colony-forming units at each phase. Two SEM photomicrographs of each root's coronal, middle and apical thirds were taken randomly and the number of patent dentinal tubules counted. Statistical analysis was completed using One-way-ANOVA and multiple comparisons.

Results: Group 6 (n=10) protocol of 5ml anolyte followed by 3ml 18% EDTA showed the best smear layer removal results for all thirds of the canal. Chemical irrigation significantly decreased the intracanal *E. faecalis* CFUs.

Conclusion: Within the limitations of the study anolyte solution followed by EDTA showed the best smear layer removal. The various sequences of NaOCl, anolyte solution, and EDTA all had similar antibacterial results.

Keywords: Antibacterial activity; EDTA; Electrochemically activated water; Irrigants; Smear Layer; Sodium hypochlorite.

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INTRODUCTION

Endodontic treatment aims at eliminating microorganisms from the infected root canal system by mechanical and chemical methods.[1] Mechanical preparation of the canals leads to the formation of a smear layer. This is an amorphous layer of unpredictable volume, comprising remnants of pulpal tissues, micro-organisms and debris from canal preparation.[2,3] The smear layer should be removed as it may act as a substrate for remaining bacteria. Removal improves the seal of the root canal filling materials, reduces microleakage and improves the mechanical retention of the filling material to dentine.[4-8]

Bacteria remaining in the canals may penetrate the dentinal tubules as deep as 150µm in the apical two thirds of the canal and up to 400µm in the rest of the canal.[9,10] *Enterococcus faecalis* is a bacterium commonly associated with persisting endodontic disease and in secondary infections.[11] *E. faecalis* is able to survive harsh environments due to its high virulence. Within the root canal system it can bind to dentine as it possesses serine protease, gelatinases and collagen binding protein.[12]

Sodium hypochlorite is a well-established endodontic irrigant with the ability to dissolve tissues and exert an antibacterial effect.[13,14] There is some controversy over the concentration to be used in endodontic irrigation, but Clegg *et al.* (2006)[15] found that 6% sodium hypochlorite was the only concentration able to remove the biofilm and render the bacteria nonviable. Sodium hypochlorite is toxic to tissues and has been shown to reduce polymerisation of resin sealers such as Epiphany (Sybron Endo, Orange, CA, USA).[16] Furthermore, it is unable to remove the smear layer and is corrosive to endodontic instruments.[2,17-19]

These negative characteristics of sodium hypochlorite warrant the search for a replacement.[2,13]

Although EDTA has a chelating action that assists in creating smear free dentine by dissolving mineralised tissues,[20] sodium hypochlorite followed by EDTA did not produce complete smear layer removal.[21] Anolyte solution has been suggested as a replacement irrigant.[22] It does not produce a smear layer and in fact has been shown to remove any existing smear layer and exposed collagen fibrils.[23] Anolyte solution, however, is not as antibacterial as sodium hypochlorite.[18]

It would thus seem reasonable to test further combinations of these irrigants for their effectiveness in removing the smear layer. The aim of this study was, therefore, to test various alternating sequences of sodium hypochlorite, anolyte solution, and EDTA for their ability to remove the mineralised portion of the smear layer, and to eliminate bacteria.

METHODS

Ethical clearance was obtained for the use of extracted teeth as well as for the use of *E. faecalis* (clearance number M050760). Pre-operative radiographs were taken of each tooth specimen. Teeth that had root fractures, multiple canals, complicated canal forms and/or pulp stones or calcifications were excluded from the study. Forty-eight single-canal teeth were selected. They were decoronated at the level of the cemento-enamel junction and the roots cleaned of any deposits using curettes. The canals of each root were explored using a 10 K hand file (Mani, Inc., Utsunomi Ya, Tochigi, Japan). The working length was established by piercing the apex of the canal until the file was just visible at the canal apex, and 0.5 mm was subtracted from this length. A glide file path was prepared using 10 K and 15 K hand files. Thereafter the roots were prepared using ProTaper nickel titanium rotary files (Dentsply, Maillefer, Baillaigues, Switzerland) in an endodontic handpiece according to the manufacturer's instructions. The canals were prepared using S1 and S2 files, followed by a 20 K hand file, F1 rotary file, 25 K hand file and finally the F2 rotary file. Between each file, and as often as additionally necessary, the canals were rinsed with sterile distilled water. The apices of the roots were sealed with GC Fuji I (GC Corporation, Tokyo, Japan) and the orifices with EcoTemp (Ivoclar Vivadent, New York, USA) (Lot J15944) to isolate the internal environment.

The teeth were randomly divided into six groups and placed in sterile Ringer's solution for 72 hours. Four groups contained 10 roots each (test) and two groups (controls) contained four roots each. The Ringer's solutions were replaced at 24 hour intervals. The roots were sonified three times and sterilized at 121°C for 15 minutes in an autoclave. In order to maintain sterile conditions the study was conducted in a positive sterile airflow laboratory, working in a laminar flow cabinet, using sterile gloves, masks and instruments. The 48 roots were placed in sterile bottles containing Casein-peptone Soymeal-peptone Broth (CASO Broth, Merck SA (Pty) Ltd., Halfway House, South Africa) and anaerobically incubated using Anaerocult A® (Merck SA (Pty) Ltd., Halfway House, South Africa) at 37°C for three days. Sterile paper points were inserted into the canals, then placed onto CASO Agar plates and incubated anaerobically using Anaerocult A® at 37°C for 72 hours. Negative cultures confirmed that the roots were sterile and did not contain any anaerobic bacteria before the inoculation procedure.

A MacFarland Standard-I suspension (8 x 108 colony-forming units [CFU]) of *E. faecalis* (ATCC49474) was prepared. A 1% suspension was added to the Broth and incubated anaerobically using Anaerocult A® at 37°C for three days. The roots were sampled by inserting sterile paper points in the canals to soak up the inoculated broth from the canals. Each paper point was placed into a vial containing 0.9ml sterile Ringers solution. This served as a 1:10 dilution from which serial dilutions were made.

One hundred micro litres of each suspension was spread onto CASO-Agar plates in triplicate by means of the standardised glass spreading technique to quantify CFUs.[19] The roots were then irrigated for one minute for each irrigant according to the following protocols:
Group 1: (n=4) 3ml sterile distilled water; **Group 2:** (n=4) 3ml 6% sodium hypochlorite; **Group 3:** (n=10) 3ml 6% sodium hypochlorite followed by 3ml 18% EDTA; **Group 4:** (n=10) 3ml 6% sodium hypochlorite followed by 5ml anolyte solution; **Group 5:** (n=10) 0.5ml 6% sodium hypochlorite followed by 5ml anolyte solution followed by 3ml 18% EDTA; **Group 6:** (n=10) 5ml anolyte solution followed by 3ml 18% EDTA.

Thereafter the irrigants were rinsed out of the canals with 10 ml of sterile distilled water. The irrigants were all delivered using a syringe (Ultradent Inc., South Jordan, USA) and 27 gauge Endo-EZE 1" Irrigator Tip (Ultradent, Inc., USA) to within 2 mm of the canal apex. The same cultivation technique described above was used after irrigation and the CFUs of bacteria that survived the irrigation process were quantified. The percentage difference between the CFUs before and after irrigation was calculated for each group and compared using the t-test (Statistical Package and Service Solutions (SPSS) Inc, Chicago, USA). A p-value ≤ 0.05 indicated a significant statistical difference at a 95% confidence interval. The inter-group percentage differences were compared using One-way ANOVA. Multiple comparisons were made using Tukey HSD or the Tamhane test depending on the normality of the data.

The roots were prepared for Scanning Electron Microscopy according to standard methods.[24,25] Two photomicrographs were taken per third per tooth. This was done by superimposing a numbered grid over the relevant third and selecting random numbers from a statistical random number table. The selected block was magnified to 2500x. Using Image J software (U.S. National Institutes of Health, Bethesda, Maryland, USA) the open tubules in each photomicrograph were counted independently by two calibrated expert examiners. Partially open and closed tubules were not counted. Open tubules were defined as round, with no smear layer or matter overlying the tubule opening. Bacteria may be present inside the tubule such that a sealer will entomb it when penetrating the canal but may not be covering the opening. Inter-rater reliability was determined using the kappa-test. Where the examiners differed, consensus was reached after discussion. The One-way ANOVA test was used to establish intra-group and inter-group differences. A p-value ≤ 0.05 indicated a significant statistical difference at a 95% confidence interval. The Tukey HSD or Tamhane test was used for multiple comparisons.

RESULTS

Multiple comparisons showed statistically significant differences ($p<0.05$) between Group 6 and all the other groups, and between Group 5 and Group 1 for the coronal third. There were statistically significant differences ($p<0.05$) between Group 6 and Groups 1-4 for the middle third (Figure 1).

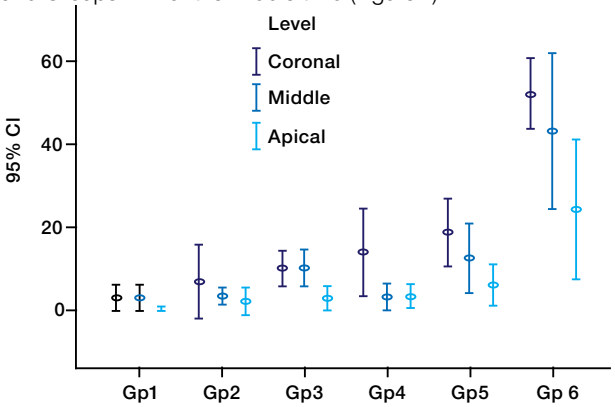

Figure 1: Graphical representation of the comparison of the mean number of patent dentinal tubules for the coronal, middle and apical thirds of all 6 groups at a 95% CI.

In the comparison of the apical thirds a statistical anomaly occurred. The one-way ANOVA showed a statistical difference but multiple comparisons failed to show where the differences were. The examiners pointed out that there was a marked visual difference observed between Group 6 and all other groups. Group 6 presented with a thinner smear layer and a larger number of patent dentinal tubules compared with other groups. The latter demonstrated thick smear layers that completely or partially covered the dentinal tubules and inter-tubular dentine.

Intra-group comparisons showed statistically significant differences in the CFUs before and after irrigation for all groups. Inter-group comparisons showed statistically significant results ($p=0.000$). Multiple comparisons revealed only a statistically significant difference ($p<0.05$) between Group 1 (sterile water) and all other groups.

Figures 2 to 4 show SEM photomicrographs (at 2500x) of the middle third of the canal. Figure 2 is of a Group 3 root after irrigation with 6% sodium hypochlorite followed by 18% EDTA. A regular distribution of open and partially open dentinal tubules can be seen (white arrow). Patches of flat smear layer are seen over a few tubules and on the intertubular dentine (black arrow).

Figure 3 is of a Group 5 root after irrigation with 6% sodium hypochlorite followed by anolyte solution followed by 18% EDTA. A low to moderate number of dentinal tubules are open (white arrow). A thick irregular smear layer can be seen covering most of the intertubular dentine and remaining dentinal tubules (black arrow).

Figure 4 is of a Group 6 root irrigated with anolyte solution followed by 18% EDTA. Regularly distributed open dentinal tubules can be seen (white arrow). A thin smear layer is loosely present over some of the inter-tubular dentine and a few dentinal tubules (black arrow).

DISCUSSION

Statistical Analysis

Previous studies have analysed the smear layer removal ability of irrigants by semi-quantitative methods. This included assessing each photomicrograph and scoring the smear layer removal on a scale.[26] In this study the actual number of open dentinal tubules was counted in each photomicrograph in order to reduce the possibility of subjective analyses. Due to the variable nature of dentine this quantitative analysis may have allowed for a larger standard deviation than that observed with semi-quantitative analyses.

Smear layer removal

Where sodium hypochlorite was the sole irrigant there was a thick irregular smear layer that was structurally different to that observed in the roots irrigated with sterile distilled water. This is in agreement with other research that has shown sodium hypochlorite cannot remove the inorganic portion of the smear layer.[2,18]

For all other irrigant sequences there was better smear layer removal in the coronal third. This may be because the irrigation solution did not reach the apical and possible middle third due to an operator error, or insufficient canal preparation. Histological differences in dentine may have also affected the smear layer removal in the apical thirds. Smear layer removal may have been improved with increased contact time, more frequent replacement or activation of the irrigants.[27-31]

Alternating the use of a tissue solvent (sodium hypochlorite) and a chelating agent (EDTA) improved smear layer removal. Alternating sodium hypochlorite with anolyte solution showed a visual trend toward improved smear layer removal compared with sodium hypochlorite alone. This was demonstrated by an increased number of patent dentinal tubules. Sodium hypochlorite produced a thick smear layer, which completely covered the dentinal tubules and inter-tubular dentine. This may indicate the role of the anolyte solution in smear layer removal.[26-29]

No one group was able to completely remove the smear layer in all thirds, but where anolyte solution was followed by 18% EDTA there was improved smear layer removal compared with other groups. Thus, anolyte solution followed by EDTA may be a promising irrigation protocol. Further research is required to establish the ideal volume, contact time and irrigation method.

Antibacterial activity

Where 3 ml of the sodium hypochlorite was used (Groups 2, 3 and 4), the CFU count after irrigation was always zero. Thus 3 ml of 6% sodium hypochlorite with surfactant molecules used for one minute was effective against *E. faecalis* under the conditions of this study.

In Group 5 (sodium hypochlorite followed by anolyte solution followed by EDTA) and Group 6 (anolyte solution followed by EDTA) the CFU count after irrigation was so close to zero that the percentage difference before and after irrigation was deemed statistically insignificant compared with the groups that had a zero CFU count (Groups 2, 3 and 4). Statistically the CFU after irrigation may be deemed insignificant but clinically the remaining microorganisms in Groups 5 and 6 cannot be discounted. Some authors

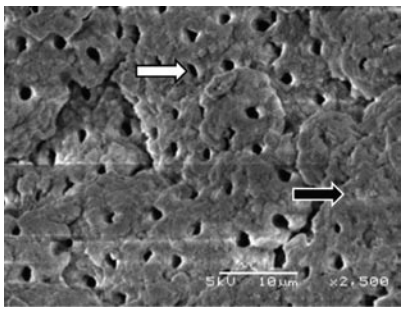

Figure 2: An SEM representative photomicrograph magnified to 2500x of the middle third of a Group 3 root after irrigation with 6% sodium hypochlorite followed by 18%EDTA. (**White arrow:** open dentinal tubule. **Black arrow:** Patches of flat smear layer over a few tubules and on the intertubular dentine.)

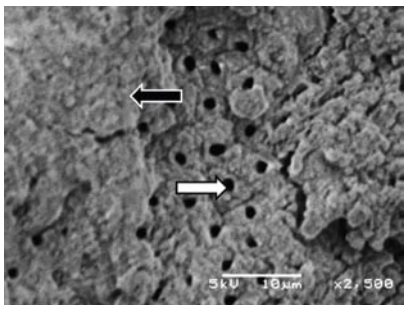

Figure 3: An SEM representative photomicrograph magnified to 2500x of the middle third of a Group 5 root after irrigation with 6% sodium hypochlorite followed by anolyte solution followed by 18% EDTA. (**White arrow:** open dentinal tubule. **Black arrow:** thick irregular smear layer covering most of the inter-tubular dentine and remaining dentinal tubules)

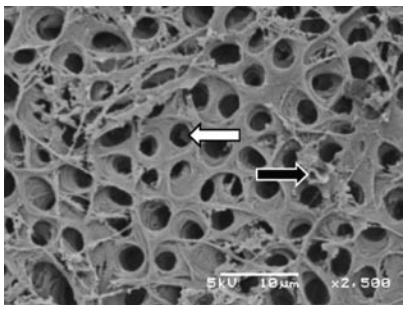

Figure 4: An SEM representative photomicrograph magnified to 2500x of the middle third of a Group 6 root irrigated with anolyte solution followed by 18% EDTA. (**White arrow:** open dentinal tubule. **Black arrow:** thin smear layer loosely present over some of the inter-tubular dentine and a few dentinal tubules)

have suggested that any remaining bacteria that are not entombed in the dentinal tubules during obturation may potentially multiply and migrate apically leading to failure of the endodontic treatment.[32,33] *E.faecalis* is particularly virulent and may survive for long periods with little or no substrate.[34-36] Group 1 (sterile distilled water) had the highest CFUs after irrigation and the percentage difference was deemed statistically significant compared with other groups. This indicates that although chemical irrigation does significantly reduce the intracanal CFU count, an antibacterial irrigant is more effective.

The limitations of this *in vitro* study include a small sample size and the use of cultivation techniques which may not be as sensitive to other remaining microbial species.

CONCLUSIONS

Within the limitations of this study, the results indicate the following:

- For any irrigant group better smear layer removal was shown in the coronal third than in the apical third.
- 5ml anolyte solution followed by 3ml 18% EDTA for one minute showed the best smear layer removal results for all thirds.
- Chemical irrigation significantly decreases the intracanal *E. faecalis* CFUs.
- Sterile distilled water is not effective in decreasing the intracanal CFUs.
- All other irrigant protocols were equally antibacterial.

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