

Abstract

Title of Thesis: *In Vivo* Evaluation of Contemporary Endodontic Antimicrobial Procedures

Peter Bellingham, Master of Science, 2011

Thesis Directed by: Dr. Ashraf F. Fouad, Professor and Chairman, Director of Postgraduate Endodontics, University of Maryland School of Dentistry

Introduction: Clinical assessment of the efficacy of novel root canal disinfection protocols is an important focus in endodontic research. The purpose of this randomized, masked study was to: (1) Determine and compare the incidence of bacterial DNA and growth after final rinse with MTAD or 5.25% NaOCl by conventional syringe irrigation and ultrasonic irrigation, and (2) Determine and compare colony forming units and incidence of bacterial DNA and growth before and after ProUltra PiezoFlow ultrasonic irrigation.

Methods: Canals in 20 teeth (single-rooted) were prepared using 5.25% NaOCl and 17% EDTA, then rinsed with MTAD or 5.25% in random sequence by conventional syringe and ultrasonically-activated irrigation, medicated for 7 days with Ca(OH)₂, and filled. Root canal samples were obtained before (A) preparation, after final rinse conventional syringe needle irrigation (B), and after final rinse ultrasonically-activated irrigation (C). Samples were evaluated for incidence of bacterial DNA using PCR amplification of the bacterial 16S rRNA gene. Incidence of bacterial DNA. Additionally, incidence of bacterial growth and colony counts was determined after 10 days of anaerobic incubation.

Results: Incidence of bacterial DNA and bacterial growth did not differ significantly between final rinse MTAD and 5.25% NaOCl groups, both after conventional syringe and ultrasonically-activated irrigation. Incidence of bacterial growth was significantly

less after ultrasonically-activated irrigation, and a trend was observed towards reduction in colony count.

Conclusion: The results of this randomized, masked study did not show a significant short-term antimicrobial advantage of final rinse MTAD over 5.25% NaOCl.

Ultrasonically-activated irrigation demonstrated a significant reduction in the incidence of bacterial growth and a trend towards reduction in colony counts. Bacterial presence before obturation can adversely affect the outcome of treatment. The results of this study could provide direction for new irrigation techniques to improve outcomes for the endodontic treatment of teeth.

In Vivo Evaluation of Contemporary Endodontic Antimicrobial Procedures

by
Peter Bellingham

Thesis submitted to the faculty of the Graduate School
of the University of Maryland Baltimore in partial fulfillment
of the requirements for the degree of
Master of Science
2011

Dedication

I would like to thank my family for being supportive and encouraging through out my education. This thesis is dedicated to all of you.

Acknowledgements

I am deeply indebted to my thesis supervisor and department chair, Dr. Ashraf F. Fouad. His guidance led me down a path of intellectual enlightenment through producing this work. His enthusiasm for evidence-based dentistry always kept me excited and motivated.

And a special thank you to Dr. Elaine Romberg for her invaluable help in completion of statistical analysis performed in this thesis.

Table of Contents

| Chapter | Page |
|--------------------------------------------------------------|------|
| DEDICATION..... | iii |
| ACKNOWLEDGEMENTS..... | iv |
| LIST OF TABLES..... | vi |
| LIST OF FIGURES..... | vii |
| LIST OF ABBREVIATIONS..... | viii |
| INTRODUCTION..... | 1 |
| Microbiological Goals of Endodontic Treatment..... | 1 |
| Effect of Manual Instrumentation on Root Canal Bacteria..... | 1 |
| Antimicrobial Agent: Sodium Hypochlorite..... | 2 |
| Antimicrobial Agent: MTAD..... | 3 |
| Ultrasonic Irrigation..... | 5 |
| SPECIFIC AIMS..... | 7 |
| HYPOTHESES..... | 8 |
| MATERIALS & METHODS..... | 10 |
| STATISTICAL ANALYSIS | 20 |
| RESULTS..... | 21 |
| DISCUSSION..... | 28 |
| CONCLUSION..... | 32 |
| APPENDIX I..... | 33 |
| APPENDIX II..... | 34 |
| REFERENCES..... | 35 |

List of Tables

| | |
|---------------------------------------------------------------------------------------------------------------------------------------------|------|
| Table..... | Page |
| Table 1. Incidence of bacterial growth after final rinse with MTAD or 5.25% NaOCl by conventional syringe needle irrigation (Sample B)..... | 21 |
| Table 2. Incidence of bacterial growth after final rinse with MTAD or 5.25% NaOCl by ultrasonic irrigation (Sample C)..... | 22 |
| Table 3. Incidence of bacterial DNA after final rinse with MTAD or 5.25% NaOCl by conventional syringe needle irrigation (Sample B)..... | 23 |
| Table 4. Incidence of bacterial DNA after final rinse with MTAD or 5.25% NaOCl by ultrasonic irrigation (Sample C)..... | 23 |
| Table 5. Incidence of bacterial growth before (Sample B) and after (Sample C) ultrasonic irrigation | 24 |
| Table 6. Incidence of bacterial DNA before (Sample B) and after (Sample C) ultrasonic irrigation | 25 |
| Table 7. Colony count groupings for samples B and C..... | 26 |
| Table 8. Post-hoc power analysis..... | 27 |
| Table 9. Unmasked colony count data..... | 33 |
| Table 10. Unmasked presence or absence of 16S rRNA data..... | 34 |

List of Figures

| Figure..... | Page |
|------------------------------------------------------------------------------|------|
| Fig 1. Flowchart of experimental procedures | 14 |
| Fig 2. ProUltra PiezoFlow connected to Satelec P5 piezoelectric system | 16 |
| Fig 3. Masked irrigant in ALADDIN-1000 microinfusion pump | 16 |

List of Abbreviations

NaOCl- sodium hypochlorite

CHX- chlorhexidine

EDTA- ethylenediaminetetraacetic acid

Introduction

Microbiological Goals of Endodontic Treatment

Apical periodontitis is described as an infectious disease caused by microorganisms colonizing the root canal system (1-3). Several studies have indicated that the prognosis of apical periodontitis after root canal treatment is poorer if viable microorganisms are present in the canal at the time of the root filling (4, 5). Accordingly, the ideal goal of root canal therapy is to sterilize the root canals of infected teeth. However, given the complex anatomy of the root canal system, fulfilling this goal is utopic for most cases with available instruments and techniques. The reachable goal is to reduce bacterial populations to a level below that necessary to induce or sustain disease (6).

Effect of Manual Instrumentation on Root Canal Bacteria

Mechanical instrumentation is the core method for bacterial reduction in the infected root canal. Bystrom & Sundqvist measured the reduction in bacterial loads in infected root canals after preparation with hand stainless-steel instruments and physiological saline solution (7). Their protocol caused a reduction in bacterial numbers of 100–1000-fold, but achieving bacteria-free root canals proved difficult. After five appointments, they were able to produce negative culture in only 8 of 15 (53%) infected canals. The relatively limited antibacterial efficiency of mechanical preparation was also reported by Ørstavik et al. (8).

These classic bacteriological studies were performed using stainless-steel hand instruments with a filing or reaming motion. Soon after the introduction of nickel-titanium (NiTi) endodontic hand instruments in 1988 (9), NiTi rotary instrumentation became popularized. The superelastic property of NiTi allowed safe and effective instrumentation using handpiece-driven files operated at slow speeds. Dalton *et al.* compared the ability of stainless steel K-type files and NiTi rotary instruments to remove bacteria from infected root canals using saline as the irrigating solution (10). In this study, only about one-third of the canals were rendered bacteria-free, and no significant difference was detected between canals instrumented with hand files or rotary instruments.

Antimicrobial Agents: Sodium Hypochlorite

Sodium hypochlorite is the most widely used irrigating solution. It has broad spectrum antimicrobial activity, rapidly killing vegetative and spore-forming bacteria, fungi, protozoa, and viruses (11-13). Its antibacterial effect is due to its ability to oxidize and hydrolyse cell proteins and to potentially osmotically draw fluids out of cells due to its hypertonicity (14).

Several *in vitro* studies support the strong antibacterial activity of sodium hypochlorite. Siqueira *et al.* evaluated the effectiveness of 4% NaOCl against *Enterococcus faecalis in vitro* reporting that it was significantly more effective than saline solution in disinfecting the root canal (15). In another study, Siqueira *et al.* compared the antibacterial activity of several irrigants against four black-pigmented anaerobic bacteria and four facultative bacteria using an agar diffusion test (16). Their

findings showed that the antibacterial effectiveness of 4% NaOCl and 2.5% NaOCl was significantly greater than solutions of 0.5% NaOCl, 0.2% Chlorhexidine, 2% chlorhexidine, citric acid, and EDTA. Further, Berber *et al.* assessed the efficacy of 0.5%, 2.5%, and 5.25% as intracanal irrigants associated with hand and rotary instrumentation techniques against *E. faecalis* within root canals and dentinal tubules, and found that 5.25% concentration was the most effective followed by 2.5% concentration (17).

In vivo studies have also demonstrated the excellent antibacterial activity of sodium hypochlorite. Bystrom and Sundqvist found that 0.5% NaOCl was significantly better than saline at rendering single-rooted teeth bacteria-free (18). Similarly, Shuping *et al.* found that the extent of bacterial reduction with nickel-titanium rotary instrumentation and 1.25% NaOCl irrigation was significantly greater compared to instrumentation with sterile saline (19). 61.9% of canals were rendered bacteria-free following NaOCl irrigation.

In another study, Vianna *et al.* performed chemo-mechanical preparation of human root canals containing necrotic pulp tissue with NaOCl solution or CHX gel (20). Assessment of microbial reduction with real-time quantitative-polymerase chain reaction (RTQPCR) and culturing techniques demonstrated that bacterial reduction in the NaOCl group was significantly greater than in the CHX group.

Antimicrobial Agents: MTAD

Antimicrobials such as doxycycline have been added to citric acid to increase their antimicrobial capacity (21), and some *in vitro* studies have shown promising antimicrobial efficacy. Shabahang *et al.* (22) found that MTAD is more effective in

disinfecting root canals in extracted human teeth contaminated with human saliva than 5.25% NaOCl. In another study, Shabahang and Torabinejad (23) found the combination of 1.3% NaOCl as a root canal irrigant with MTAD as a final rinse was more effective against *E. faecalis* than 5.25% NaOCl with 17% EDTA. Newberry *et al.* (24) found that the same MTAD irrigation protocol was effective in completely eliminating growth in seven of eight strains of *E. faecalis*.

In contrast, several *in vitro* studies demonstrated no benefit to MTAD. Kho *et al* (25) resected the apical 5mm roots of infected with *E. faecalis* after instrumentation and irrigation with 5.25% NaOCl/15% EDTA or 1.3% NaOCl/Biopure MTAD. Based on number of colony forming units (CFU) of *E. faecalis* per mg from the pulverized root-ends, no difference in antimicrobial efficacy between the two irrigation regimens was seen. Johal *et al.* (26) took paper point samples immediately after instrumentation and irrigation of canals incubated with *E. faecalis* for 4 weeks. Their results showed no growth of *E. faecalis* in root canals irrigated with 5.25% NaOCl/15% EDTA, while 50% of the canals irrigated with 1.3% NaOCl/Biopure MTAD exhibited growth. Dunavant *et al.* compared the efficacy of irrigants against *E. faecalis* biofilms grown on ceramic coupons (27). They found 6% and 1% NaOCl were significantly more efficient in eliminating *E. faecalis* biofilms than Smear Clear, REDTA, and Biopure MTAD.

Although *in vitro* models are useful to evaluate the potency of antimicrobial agents and their spectrum of activity, testing under *in vivo* conditions is required to establish their effectiveness. To date, there has been only one *in vivo* study evaluating the antimicrobial efficacy of MTAD. Malkhassien *et al.* prepared canals with necrotic pulps using 1.3% NaOCl, then randomly subjected the canals to a final rinse with MTAD or

saline (28). Samples taken after final rinse were enumerated by epifluorescence microscopy and by colony-forming-unit (CFU) counts after 14 days of incubation. Bacterial densities were not significantly different between final rinse saline and MTAD groups. Prepared canals were further medicated interappointment with a 2% chlorhexidine gel. Bacterial counts determined from samples taken after this interappointment medication also did not differ significantly between the MTAD and control groups.

Ultrasonic Irrigation

In 2005, Gutarts *et al.* published research on a novel ultrasonically activated irrigating needle (29). The 25-gauge needle was connected to a piezoelectric ultrasonic unit, and allowed irrigating solution to be delivered during ultrasonic activation. The mesial roots of lower molars with vital pulps were prepared with hand and rotary instruments with 6.0% NaOCl irrigation after every third file. Groups which received supplemental irrigation of 6.0% NaOCl through the ultrasonically activated needle exhibited significantly higher mean percentage canal and isthmus cleanliness on histological examination. A similar study by the same group found significantly higher cleanliness values with respect to biofilm/necrotic debris removal in mandibular molars with necrotic pulps (30). Carver *et al.* took microbiological samples before and after ultrasonically activated irrigation, which were cultured anaerobically and enumerated. Supplemental ultrasonic irrigation group produced a significant reduction in CFU counts in infected necrotic human molars (31). In addition, a significantly higher percentage of the canals cultured had no bacteria following the addition of ultrasonic irrigation (80%)

than following hand/rotary instrumentation alone (27%). The ultrasonically activated needle tested in these studies was the prototype for the commercially available ProUltra PiezoFlow.

Specific Aims

Aim 1

Determine and compare the incidence of bacterial DNA and growth after final rinse with MTAD or 5.25% NaOCl by conventional syringe irrigation and ultrasonic irrigation.

Aim 2

Determine and compare colony forming units and incidence of bacterial DNA and growth before and after ProUltra PiezoFlow ultrasonic irrigation.

Hypotheses

Hypothesis 1

H₀: There is no significant difference in the incidence of bacterial growth between conventional syringe needle final rinse MTAD and 5.25% NaOCl groups.

H_A: The conventional syringe needle final rinse MTAD group has a lower incidence of bacteria growth than the conventional syringe needle final rinse 5.25% NaOCl group.

Hypothesis 2

H₀: There is no significant difference in the incidence of bacterial growth between the ProUltra Piezoflow ultrasonic final rinse MTAD and 5.25% NaOCl groups.

H_A: The ProUltra PiezoFlow ultrasonic final rinse MTAD group has a lower incidence of bacterial growth than the ProUltra PiezoFlow ultrasonic final rinse 5.25% NaOCl group.

Hypothesis 3

H₀: There is no significant difference in incidence of bacterial DNA between conventional syringe needle final rinse MTAD and 5.25% NaOCl groups.

H_A: The conventional syringe needle final rinse MTAD group has a lower incidence of bacterial DNA than the conventional syringe needle final rinse 5.25% NaOCl group .

Hypothesis 4

H₀: There is no significant difference in incidence of bacterial DNA between ProUltra PiezoFlow ultrasonic final rinse MTAD and 5.25% NaOCl groups.

H_A: The ProUltra PiezoFlow ultrasonic final rinse MTAD group has a lower incidence of bacterial DNA than the ProUltra PiezoFlow ultrasonic final rinse 5.25% NaOCl group.

Hypothesis 5

H₀: There is no significant difference in the incidence of bacterial growth before and after ProUltra PiezoFlow ultrasonic irrigation.

H_A: The incidence of bacterial growth before ProUltra PiezoFlow ultrasonic irrigation is greater than after ProUltra PiezoFlow ultrasonic irrigation.

Hypothesis 6

H₀: There is no significant difference in the incidence of bacterial DNA before and after ProUltra PiezoFlow ultrasonic irrigation.

H_A: The incidence of bacterial DNA before ProUltra PiezoFlow ultrasonic irrigation is greater than after ProUltra PiezoFlow ultrasonic irrigation.

Hypothesis 7

H₀: There is no significant difference in the CFU count before and after ProUltra PiezoFlow ultrasonic irrigation.

H_A: CFU count is greater before ProUltra PiezoFlow ultrasonic irrigation than after ProUltra PiezoFlow ultrasonic irrigation.

Materials and Methods

Research Design

This study was conducted in the postgraduate endodontic clinic of the University of Maryland Baltimore Dental School. The project is a prospective randomized double-blind clinical trial of final rinse MTAD vs. 5.25% NaOCl administered by conventional syringe irrigation and supplemental ultrasonic irrigation in teeth with necrotic pulps and periapical lesions. All subjects were randomly assigned to one of two treatment irrigation solution groups.

Subject Recruitment and Selection

Subjects presenting for non-surgical endodontic treatment for primary endodontic infections were identified from the regular pool of patients presenting to the postgraduate endodontic clinic of the dental school for routine and emergency treatment.

Screening

Subjects were screened for study participation following the usual clinical recommendation for non-surgical endodontic treatment. The patients' medical histories were reviewed and an oral examination performed, including periapical radiographs as necessary, to confirm the need for non-surgical endodontic treatment. The non-surgical endodontic and experimental procedures were explained verbally and in writing. Informed consent was obtained prior to study enrollment.

To the best of our knowledge, there is no available data to suggest any differences in the potential covariate effects of agents used in the study based on gender, ethnicity, or race. Therefore, participation in this study is not limited by these variables. Exclusion criteria are for the purpose of ascertaining that subjects do not have medical conditions or concomitant medications that may confound the study. An inclusion and exclusion checklist was cross-referenced with the participants during screening to ensure their qualification and well-being. Criteria for study participation are described next.

Inclusion Criteria

- The patient were 14 years of age or older at the time of enrollment
- The tooth being instrumented was diagnosed to have pulp necrosis and a periradicular lesion at least 3 mm in diameter
- No history of previously completed endodontic treatment on the tooth
- The tooth has a mature apex
- The tooth has only one root

Exclusion Criteria

- Any systemic debilitating disease such as:
 - Diabetes mellitus
 - Liver disease or liver failure
 - Rheumatoid arthritis
 - Neoplastic disease or its treatment
 - Chronic corticosteroid therapy

- Chronic hepatitis B or C infection
- History of hepatitis A infection in the past year
- Chronic alcoholism
- Any systemic disease that compromises the immune system
- Allergy to tetracycline or doxycycline, or history of side effects such as intolerance to sunlight with administration of tetracycline or doxycycline
- Women who stated that they are pregnant at the time of initial treatment
- Anyone who had taken antibiotics in the preceding month or required prophylactic antibiotic before dental treatment
- Current smokers, or patients who stopped smoking in the last 4 years

Randomization and Allocation

Subjects were randomly assigned to receive final rinse irrigation with either BioPure MTAD or 5.25% NaOCl. Masking tape concealed the contents of the solution from the clinical provider.

Root Canal Access and Isolation

Following isolation of the experimental tooth with a rubber dam, the field was disinfected with 30% H₂O₂ and then 5% tincture of iodine, then 5.25% NaOCl, followed by 5% sodium thiosulphate to inactivate the halogens. Caries and/or existing restorations, if present, were removed, and then the disinfection/inactivation sequence were repeated,

making sure that the fluids do not seep into the chamber. The pulp chamber was then be accessed with a new sterile bur.

Root Canal Preparation and Sampling

A flowchart of experimental procedures is outlined in Figure 1. The working length was established with a Root ZX (J. Morita Mfg. Corp., Irvine, CA) and radiographically to within 1 mm of the radiographic apex for each canal. Sample A will be taken after working length determination. A sterile #10-20 K-type hand files (Dentsply Maillefer, Tulsa, OK) will be placed to within 1 mm of estimated working length and pumped 5 times with minimal reaming motion to accumulate dentin shavings and intracanal debris. The file and two paper points also taken to length were then placed in collection vials: one molecular and one culturing. Molecular samples were collected in sterile, DNA- and RNA-free vials containing 1.5 ml of filter sterilized 10 mM Tris-HCl. Culturing samples were collected in 1mL Liquid Dental Transport Medium (Anaerobe Systems, Morgan Hill, CA).

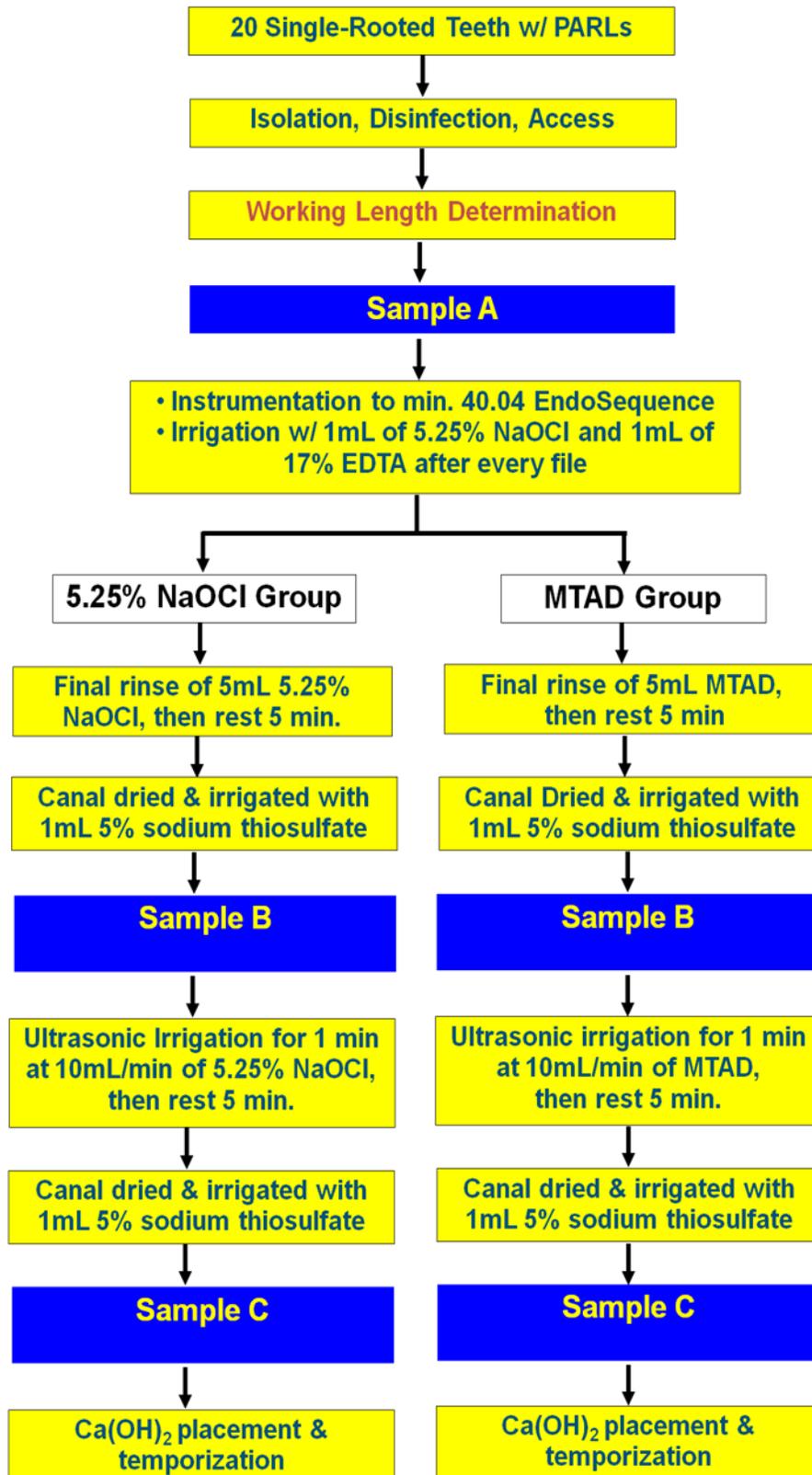


Figure 1. Flowchart of experimental procedures.

The canal orifice was enlarged with size #2-4 Gates Glidden bur, and the canal initially cleaned and shaped to a #20 K-type hand file (Dentsply Maillefer, Tulsa, OK). A crown down technique was then be used to enlarge the coronal portion, then the mid root, and finally the apical third of the canal will be enlarged to a minimum 0.04 size 40 EndoSequence rotary file (Brassler). After each file, 1ml of 2.6% NaOCl and 1ml of 17% EDTA was delivered through a 27-guage ProRinse needle (Dentsply Tulsa Dental) placed 1 to 2mm short of the working length. At this point the root canals were randomly assigned to the MTAD or 5.25% NaOCl irrigation group. A final post-instrumentation irrigation was performed with 5ml of masked 5.25% NaOCl or 5mL of MTAD solution. The solution was left to rest in the canal for 5 minutes, as recommended in the manufacturer's instructions for BioPure MTAD. The canal was then dried with sterile paper points, 5% sodium thiosulfate was used to neutralize sodium hypochlorite, and the root canal was dried again with sterile paper points. At this point the second bacterial sampling (Sample B) occurred in the same manner previously described.

Upon completion of the second sampling (Sample B), the ultrasonic irrigation protocol was performed. The ProUltra PiezoFlow was connected to the Satelec P5 piezoelectric ultrasonic system (Dentsply Tulsa Dental) (Figure 2), and the rear-aperture was connected by Luer-lock to the masked irrigant which is placed in an ALADDIN-1000 microinfusion pulp (World Precision Instruments, Sarasota, FL) (Figure 3). The needle was placed at 75% of the working length established for instrumentation ultrasonic irrigation was performed for 1 minute at 10mL/minute. At the conclusion of 1 minute, the solution was allowed to rest for another 4 minutes in the canal. High-speed suction was maintained at all times on the distal aspect of the experimental tooth. The

canal was then dried with sterile paper points, 5% sodium thiosulfate was used to neutralize sodium hypochlorite, and the root canal was dried again with sterile paper points. Then, the third bacterial sampling (Sample C) occurred in the manner previously described.

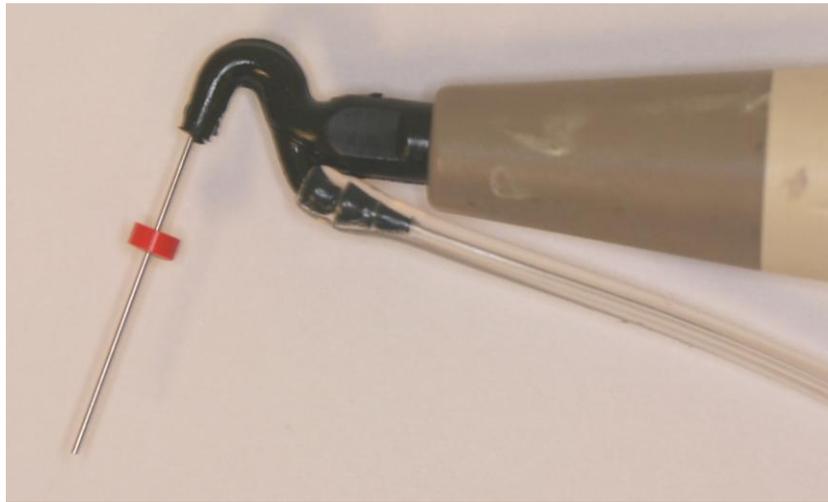


Figure 2. ProUltra PiezoFlow connected to the Satelec P5 piezoelectric ultrasonic system.



Figure 3. Masked irrigant in ALADDIN-1000 microinfusion pump.

Completion of Root Canal Therapy

Calcium hydroxide (Ca(OH)₂) was then placed as an intracanal medicament. The teeth were restored temporarily with Cavit and Fuji IX. The second appointment was at least 7 days after the first appointment, and involved further irrigation and obturation of the canals according to standard techniques.

16S rRNA Gene-Based PCR Analysis

An aliquot (~500ul) of each sample was taken for DNA extraction. DNA was extracted according to the protocol described for the QIAamp DNA mini kit (Qiagen, Valencia, Calif). The pellet was suspended in 180 µl of enzyme solution (20 mg of lysozyme per ml, 20 mM Tris HCl [pH 8.0], 2 mM EDTA, 1.2% Triton) and incubated for 30 min at 37°C. Proteinase K (20 µl) and RNaseA (4 µl at 100 mg/ml) were added, and the specimen incubated for 2 min at room temperature. Buffer AL (200 µl) was added, and the specimen was vortexed and incubated at 56°C for 30 min and then for 15 min at 95°C. Ethanol (200µl at 96 to 100%) was added, followed by vortexing and brief centrifugation. The mixture was then added to a QIAamp spin column and centrifuged at 8,000 rpm for 1 min. The column was then placed in a clean 2-ml collection tube, 500 µl of buffer AW1 was added, and the mixture was centrifuged at 8,000 rpm for 1 min. The column was again placed in a clean 2-ml collection tube, and 500 µl of buffer AW2 was added, followed by centrifugation at 14,000 rpm for 3 min. Then, buffer AE (200 µl) was added, followed by centrifugation at 8,000 rpm for 1 min. The elutions were combined for a total yield of 400 µl, which was aliquoted in sterile, DNA- and RNA-free conical tubes and frozen at -20°C until use. PCR amplification of 16S rDNA was performed with

the universal 16S rRNA gene-specific primer pair previously published by Fouad *et al.* (32): AGA GTT TGA TCC TGG CTC AG. PCR amplification was performed in a thermal cycler (PE9700 or PE2400; Perkin-Elmer Applied Biosystems, Foster City, Calif.) in a volume of 50 μ l containing 10 μ l of extracted sample DNA or 5 μ l of extracted control stock bacterial DNA (see below), 5 μ l of 10X PCR buffer, 0.25 μ l of 5 U of *Taq* DNA polymerase (Eppendorf, Cologne, Germany) per μ l or 0.5 μ l of HotStar *Taq* (Qiagen), 1.5 mM MgCl₂, 0.2 mM concentrations of each of the four deoxynucleoside triphosphates (Takara, Otsu, Shiga, Japan), and a 0.5 μ M concentration (500 ng) of each (sense and antisense) primer; the balance consists of sterile ultrapure water. The PCR conditions used were generally as follows: the initial denaturation was at 94°C for 2 min for Eppendorf *Taq* or 15 min for HotStar *Taq*. This was followed by 30 cycles of denaturation at 94°C for 15 s, annealing at a temperature that depended on the primer (Table 1) for 15 s, and extension at 72°C for 45 s. The final extension was at 72°C for 5 min, and then the products were cooled to 4°C until they were removed. The amplification products were analyzed by 2% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 2 mM EDTA [pH 8.3]). The Power Pac 1000 apparatus (Bio-Rad, Hercules, Calif.) is set at 110 mA for 2 h or 95 V for 1 h. The gels were stained with 0.5 μ g of ethidium bromide per ml for 30 min and destained with water for 20 min. The PCR products were visualized under UV light with an Alpha Imager (Alpha Innotech Corp., San Leandro, Calif.).

Cell Culturing

For culturing, samples were collected in in 1mL Liquid Dental Transport Medium (Anaerobe Systems, Morgan Hill, CA) vials. The dental transport medium was serially

diluted (10- and 100-fold) and plated on CDC anaerobic agar plates (BD sciences, Franklin Lakes, NJ) and incubated under anaerobic conditions (miniMACS Aneerobic work station – Microbiology International, Fredrick, MD) at 37°C for 10 days. Morphologically distinct colonies were counted.

Statistical Analysis

Hypothesis 1

Chi-square was used, unless, when computing chi-square the expected value was less than 5 in more than 20% of cells. In that case, Fisher's Exact Test was used.

Hypothesis 2

Fisher's Exact Test was used.

Hypothesis 3

Fisher's Exact Test was used.

Hypothesis 4

Fisher's Exact Test was used.

Hypothesis 5

McNemar Test was used.

Hypothesis 6

McNemar Test was used.

Hypothesis 7

Fisher's Exact Test was used.

Results

All Sample A specimens tested positive for bacterial growth and bacterial DNA. Molecular sample 9A was not analyzed due to contamination.

Hypothesis 1

Table 1 shows incidence of bacterial growth after conventional syringe needle irrigation with test irrigants. The incidence of bacterial growth after conventional syringe needle irrigation with MTAD was not significantly different from the incidence of bacterial growth after conventional syringe needle irrigation with 5.25% NaOCl (Fisher's Exact Test; $p=0.50$).

Table 1. Incidence of bacterial growth after final rinse with MTAD or 5.25% NaOCl by conventional syringe needle irrigation (Sample B).

| Irrigant | Bacterial Growth | No Bacterial Growth |
|-------------|------------------|---------------------|
| MTAD | 5 (50%) | 5 (50%) |
| 5.25% NaOCl | 4 (40%) | 6 (60%) |

Hypothesis 2

Table 2 shows incidence of bacterial growth after ultrasonic irrigation with test irrigants. The incidence of bacterial growth after ProUltra PiezoFlow ultrasonic irrigation with MTAD was not significantly different from the incidence of bacterial growth after ProUltra PiezoFlow ultrasonic irrigation with 5.25% NaOCl (Fisher's Exact Test; $p=0.42$).

Table 2. Incidence of bacterial growth after final rinse with MTAD or 5.25% NaOCl by ultrasonic irrigation (Sample C).

| Irrigant | Bacterial Growth | No Bacterial Growth |
|-------------|------------------|---------------------|
| MTAD | 2 (20%) | 8 (80%) |
| 5.25% NaOCl | 2 (20%) | 8 (80%) |

Hypothesis 3

Table 3 shows incidence of bacterial DNA after conventional syringe needle irrigation with test irrigants. The incidence of bacterial DNA after conventional syringe needle irrigation with MTAD was not significantly different from the incidence of bacterial DNA after conventional syringe needle irrigation with 5.25% NaOCl (Fisher's Exact Test; $p=0.50$).

Table 3. Incidence of bacterial DNA after final rinse with MTAD or 5.25% NaOCl by conventional syringe needle irrigation (Sample B).

| Irrigant | Bacterial DNA | No Bacterial DNA |
|-------------|---------------|------------------|
| MTAD | 10 (100%) | 0 (0%) |
| 5.25% NaOCl | 9 (90%) | 1 (10%) |

Hypothesis 4

Table 4 shows incidence of bacterial DNA after ultrasonic irrigation with test irrigants. The incidence of bacterial DNA after ultrasonic irrigation with MTAD was not significantly different from the incidence of bacterial DNA after ProUltra PiezoFlow ultrasonic irrigation with 5.25% NaOCl (Fisher's Exact Test; $p=0.53$).

Table 4. Incidence of bacterial DNA after final rinse with MTAD or 5.25% NaOCl by ultrasonic irrigation (Sample C).

| Irrigant | Bacterial DNA | No Bacterial DNA |
|-------------|---------------|------------------|
| MTAD | 9 (90%) | 1 (10%) |
| 5.25% NaOCl | 9 (90%) | 1 (10%) |

Hypothesis 5

Table 5 shows matched incidence of bacterial growth before and after ultrasonic irrigation. The incidence of bacterial growth after ProUltra PiezoFlow ultrasonic irrigation was significantly less than the incidence of bacterial growth before ProUltra PiezoFlow ultrasonic irrigation (McNemar's Test; $p=0.03$).

Table 5. Incidence of bacterial growth before (Sample B) and after (Sample C) ultrasonic irrigation.*

| Matched Samples | Number |
|-----------------------------------------|--------|
| No presence of bacteria before or after | 11 |
| Bacteria before, but none after | 5 |
| Bacteria before, lower bacteria after | 3 |
| Bacteria before, more bacteria after | 1** |

*See Table 9 in Appendix I for unmasked colony count data.

**Difference was so small it was probably due to sampling bias.

Hypothesis 6

Table 6 shows matched incidence of bacterial DNA before and after ultrasonic irrigation. The incidence of bacterial DNA after ProUltra PiezoFlow ultrasonic irrigation was not significantly less than the incidence of bacterial DNA before ProUltra PiezoFlow ultrasonic irrigation (McNemar's Test; $p=0.50$).

Table 6. Incidence of bacterial DNA before (Sample B) and after (Sample C) ultrasonic irrigation.*

| Matched Samples | Number |
|----------------------------------------------|--------|
| No presence of bacterial DNA before or after | 1 |
| Bacterial DNA before, but none after | 1 |
| Bacterial DNA before, bacterial DNA after | 18 |

*See Table 10 in Appendix II for unmasked presence or absence of 16S rRNA data.

Hypothesis 7

The bacterial growth obtained from the results were graded as follows:

1. no growth: 0 microorganisms
2. light growth: 1-100 microorganisms
3. moderate growth: >100 microorganisms

Table 7 shows colony count groupings before (Sample B) and after (Sample C) ultrasonic irrigation. The colony count after ProUltra PiezoFlow ultrasonic irrigation was not significantly less than colony count before ProUltra PiezoFlow ultrasonically activated irrigation (Fisher's Exact Test; $p=0.17$).

Table 7. Colony count groupings for samples B and C.

| Colony Count Groupings | Before Ultrasonic Irrigation | After Ultrasonic Irrigation |
|------------------------|------------------------------|-----------------------------|
| No Growth: 0 | 11 (55%) | 16 (80%) |
| Light Growth: 1-100 | 8 (40%) | 4 (20%) |
| Moderate Growth: >100 | 1 (5%) | 0 (0%) |

A post-hoc power analysis was performed using the proportion of no growth for samples taken before ProUltra PiezoFlow ultrasonic irrigation (55%) and after ProUltra PiezoFlow ultrasonic irrigation (80%) (Table 8). Power was equal to 0.52 with an n of 20 in each group, a one-tailed test, a $p \leq 0.05$, and an effect size of 0.25 (medium effect size) (33). With the planned $n = 32$ at the beginning of the study, power would be equal to 0.69. A power of 0.80 would require a sample of 42 subjects (34).

Table 8. Post-hoc power analysis.

| N | Power |
|----|-------|
| 20 | 0.51 |
| 32 | 0.69 |
| 42 | 0.80 |

Discussion

This study was designed as a randomized, double-blinded clinical trial primarily to test the antibacterial efficacy of final rinse MTAD in comparison to final rinse 5.25% NaOCl. In addition, the antimicrobial effectiveness of the ProUltra PiezoFlow ultrasonic irrigating needle was evaluated. The *in vivo* nature of this study makes the steps in root canal therapy more clinically relevant than *in vitro* studies. *In vitro* studies can control for poor access, determine working lengths accurately by visualizing files at the apical foramen, and instrument teeth without regard to difficult access or clinical time constraints. Therefore, this *in vivo* study may be more clinically relevant.

Results from the study demonstrated that 1 minute of ultrasonically activated irrigation was significantly effective in producing bacteria-free canals in single-rooted teeth with pulp necrosis. Cleaning and shaping protocol with conventional syringe needle final rinse of 5.25% NaOCl or MTAD resulted in 45% of cases exhibiting bacterial growth. After 1 minute of supplemental ultrasonic irrigation with MTAD or 5.25% NaOCl, the percentage of cases exhibiting bacterial growth was significantly reduced to 20%. These results are consistent with those of Carver *et al.* who found that a supplemental 1 minute of ultrasonic irrigation in the mesial roots of lower molars significantly reduced the percentage of canals exhibiting bacterial growth from 73% to 20% (31). With respect to CFU reduction, our findings showed that the CFU count after ProUltra PiezoFlow ultrasonic irrigation was not significantly less than CFU count before the ultrasonically activated irrigation. However, a post-hoc power analysis indicated that we would likely find a significant difference if the sample size were 42.

Acoustic streaming and cavitation have been proposed as mechanisms responsible for the antimicrobial efficacy of ultrasonically activated irrigation. Ahmad (35) showed that ultrasonically activated files produce rapid movement of fluid in streaming patterns close to the file, continuously moving irrigants around, thereby producing shear stress which can remove debris and bacteria from the wall, recesses, and isthmuses (36). Others have suggested that cavitation is the primary mechanism responsible for the destruction of bacteria after exposure to ultrasonics. Cavitation is the radical oscillation and subsequent violent collapse of gas bubbles in the acoustic field, which results in the generation of shock waves, high temperatures, and free radicals. Yumita et al (37) showed that transient cavitation could damage cell walls and cell membranes. Joyce et al (38) showed that low-frequency ultrasound caused cavitation-induced de-agglomeration of bacterial biofilms. However, Ahmad found that cavitation is minimal and typically restricted to the tip of the ultrasonically activated endodontic file (39). The most likely reason for the trend toward greater reduction of intracanal bacteria in this study was deaggregation of biofilm bacteria by cavitation and/or acoustic microstreaming, in combination with the intense flushing activity of a constantly replenished supply of irrigant.

In our model, a final rinse of MTAD was not significantly better than a final rinse of 5.25% NaOCl in rendering root canals free of bacterial growth or DNA. These findings were not unexpected. According to Torabinejad et al. (40), the action against bacteria of MTAD is caused by the doxycycline present in the mixture. However, tetracyclines have a bacteriostatic effect through inhibition of protein synthesis, not a bacteriocidal effect. Additionally, it has been shown that the growth phase of specific

endodontic pathogens has a major effect on its susceptibility to endodontic disinfectants. Starved *E. faecalis* cells have been found to be 10,000 times more resistant to endodontic medicaments than cells in logarithmic growth phase (41).

Our findings are different from two early *in vitro* MTAD studies. Shabahang *et al.* found that root canals contaminated with whole saliva exhibited significantly less bacterial growth after irrigation with MTAD compared with 5.25% NaOCl (22). Another study found that 1.3% NaOCl irrigation with MTAD final treatment was the only irrigation protocol to eliminate all *E. faecalis* from human tooth cementum and dentin samples (23). The better bacteriocidal effect of MTAD in these two experiments may have been because of a carryover effect of the doxycycline in the MTAD preparation. In both studies, the root canal and external surface of the tooth was soaked in irrigant. Bacterial growth was determined by turbidity after placement of the entire tooth in tubes containing BHI broth. Doxycycline on the enormous external surface of the study teeth may have been carried over to the BHI broth. Portenier *et al.* (42) demonstrated that 100% MTAD prevented bacterial growth on culturing plates down to the dilution of 1:1000, although the bacteria were not killed.

All initial sample A specimens tested positive for bacterial DNA. However, only one sample B specimen and two sample C specimens tested negative for bacterial DNA. The particular value of this molecular approach was that uncultivable bacterial species are included in the analysis, allowing higher sensitivity in determining failure in elimination of bacteria from the root canal environment. However, the molecular technique can also detect free-floating DNA from ruptured bacterial cells. Aside from non-cultivable bacteria, this may have contributed to the high number of specimens

testing positive for bacterial DNA. Vianna *et al.* (20) performed real-time quantitative-polymerase chain reaction on root canals after receiving irrigation of NaOCl and CHX, also using primers for the highly-conserved 16S rRNA regions. None of their 32 samples achieved 0 rRNA copy numbers after the irrigation protocol, which supports our findings. Future studies should utilize real-time PCR technology in order to quantify bacterial load reduction, instead of investigating absolute absence of bacterial DNA.

A significant limitation of this study is that it does not take into consideration the possibility of residual doxycycline activity. The substantive properties of doxycycline may have an antimicrobial effect beyond the immediate period of application. Future studies should include a second sampling 7 to 10 days after irrigation regimens. Sequencing of the residual bacteria should also be considered, as MTAD may or may not exert antimicrobial activity against species known to be involved in persistent endodontic infections. A higher level of evidence study, however, would assess long-term healing of periapical disease with and without application of MTAD.

Conclusion

It is generally accepted that the success of endodontic treatment relies on thorough elimination of bacteria from the root canal system before obturation. To that end, the results of this study could provide direction for new irrigation techniques to improve outcomes for the endodontic treatment of teeth. This is the first *in vivo* study that uses molecular techniques to evaluate the antimicrobial effectiveness of the ProUltra PiezoFlow ultrasonic irrigating needle, and to compare the antimicrobial effectiveness of a final rinse of BioPure MTAD to a final rinse of 5.25% NaOCl. 3 of 40 molecular samples exhibited no bacterial DNA presence. Incidence of bacterial DNA and bacterial growth did not differ significantly between final rinse MTAD and 5.25% NaOCl groups, indicating no added benefit of an MTAD final rinse. Importantly, the incidence of bacterial growth was significantly less after ultrasonic irrigation, and a trend was observed towards reduction in colony count. These results add to previous research indicating this new irrigant agitation and delivery device imparts a significant antimicrobial advantage.

Appendix I

Table 9. Unmasked colony count data.

| Patient | Sample B Colony Counts | Sample C Colony Counts |
|-------------------|------------------------|------------------------|
| B01 (5.25% NaOCl) | 0 | 0 |
| B03 (5.25% NaOCl) | 96 | 0 |
| B06 (5.25% NaOCl) | 0 | 0 |
| B07 (5.25% NaOCl) | 48 | 0 |
| B08 (5.25% NaOCl) | 32 | 48 |
| B12 (5.25% NaOCl) | 0 | 0 |
| B13 (5.25% NaOCl) | 0 | 0 |
| B14 (5.25% NaOCl) | 0 | 0 |
| B17 (5.25% NaOCl) | 16 | 4 |
| B18 (5.25% NaOCl) | 448 | 0 |
| B02 (MTAD) | 0 | 0 |
| B04 (MTAD) | 48 | 0 |
| B05 (MTAD) | 0 | 0 |
| B09 (MTAD) | 0 | 0 |
| B10 (MTAD) | 32 | 16 |
| B11 (MTAD) | 0 | 0 |
| B15 (MTAD) | 64 | 8 |
| B16 (MTAD) | 0 | 0 |
| B19 (MTAD) | 28 | 0 |
| B20 (MTAD) | 0 | 0 |

Appendix II

Table 10. Unmasked presence or absence of 16S rRNA data.

| Patient | Sample B | Sample C |
|-------------------|----------|----------|
| B01 (5.25% NaOCl) | presence | presence |
| B03 (5.25% NaOCl) | presence | presence |
| B06 (5.25% NaOCl) | presence | negative |
| B07 (5.25% NaOCl) | presence | presence |
| B08 (5.25% NaOCl) | negative | negative |
| B12 (5.25% NaOCl) | presence | presence |
| B13 (5.25% NaOCl) | presence | presence |
| B14 (5.25% NaOCl) | presence | presence |
| B17 (5.25% NaOCl) | presence | presence |
| B18 (5.25% NaOCl) | presence | presence |
| B02 (MTAD) | presence | presence |
| B04 (MTAD) | presence | presence |
| B05 (MTAD) | presence | presence |
| B09 (MTAD) | presence | presence |
| B10 (MTAD) | presence | presence |
| B11 (MTAD) | presence | presence |
| B15 (MTAD) | presence | presence |
| B16 (MTAD) | presence | presence |
| B19 (MTAD) | presence | presence |
| B20 (MTAD) | presence | presence |

References

1. Kakehashi S, Stanley HR, Fitzgerald RJ. The Effects of Surgical Exposures of Dental Pulps in Germ-Free and Conventional Laboratory Rats. *Oral Surg Oral Med Oral Pathol* 1965;**20**:340-349.
2. Sundqvist G. Bacteriological studies of necrotic dental pulps. Odontological Dissertation No. 7. Umea, Sweden: Umea University; 1976.
3. Moller AJ, Fabricius L, Dahlen G, Ohman AE, Heyden G. Influence on periapical tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys. *Scand J Dent Res* 1981;**89**(6):475-484.
4. Sjogren U, Figdor D, Persson S, Sundqvist G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. *Int Endod J* 1997;**30**(5):297-306.
5. Katebzadeh N, Sigurdsson A, Trope M. Radiographic evaluation of periapical healing after obturation of infected root canals: an in vivo study. *Int Endod J* 2000;**33**(1):60-66.
6. Siqueira JF, Jr., Rocas IN. Clinical implications and microbiology of bacterial persistence after treatment procedures. *J Endod* 2008;**34**(11):1291-1301 e1293.
7. Bystrom A, Sundqvist G. Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. *Scand J Dent Res* 1981;**89**(4):321-328.
8. Orstavik D, Kerekes K, Molven O. Effects of extensive apical reaming and calcium hydroxide dressing on bacterial infection during treatment of apical periodontitis: a pilot study. *Int Endod J* 1991;**24**(1):1-7.
9. Walia HM, Brantley WA, Gerstein H. An initial investigation of the bending and torsional properties of Nitinol root canal files. *J Endod* 1988;**14**(7):346-351.
10. Dalton BC, Orstavik D, Phillips C, Pettiette M, Trope M. Bacterial reduction with nickel-titanium rotary instrumentation. *J Endod* 1998;**24**(11):763-767.
11. Siqueira JF, Jr., Magalhaes KM, Rocas IN. Bacterial reduction in infected root canals treated with 2.5% NaOCl as an irrigant and calcium hydroxide/camphorated paramonochlorophenol paste as an intracanal dressing. *J Endod* 2007;**33**(6):667-672.
12. Sen BH, Safavi KE, Spangberg LS. Antifungal effects of sodium hypochlorite and chlorhexidine in root canals. *J Endod* 1999;**25**(4):235-238.
13. Waltimo TM, Orstavik D, Siren EK, Haapasalo MP. In vitro susceptibility of *Candida albicans* to four disinfectants and their combinations. *Int Endod J* 1999;**32**(6):421-429.
14. Pashley EL, Birdsong NL, Bowman K, Pashley DH. Cytotoxic effects of NaOCl on vital tissue. *J Endod* 1985;**11**(12):525-528.

15. Siqueira JF, Jr., Machado AG, Silveira RM, Lopes HP, de Uzeda M. Evaluation of the effectiveness of sodium hypochlorite used with three irrigation methods in the elimination of *Enterococcus faecalis* from the root canal, in vitro. *Int Endod J* 1997;**30**(4):279-282.
16. Siqueira JF, Jr., Batista MM, Fraga RC, de Uzeda M. Antibacterial effects of endodontic irrigants on black-pigmented gram-negative anaerobes and facultative bacteria. *J Endod* 1998;**24**(6):414-416.
17. Berber VB, Gomes BP, Sena NT, Vianna ME, Ferraz CC, Zaia AA, et al. Efficacy of various concentrations of NaOCl and instrumentation techniques in reducing *Enterococcus faecalis* within root canals and dentinal tubules. *Int Endod J* 2006;**39**(1):10-17.
18. Bystrom A, Sundqvist G. Bacteriologic evaluation of the effect of 0.5 percent sodium hypochlorite in endodontic therapy. *Oral Surg Oral Med Oral Pathol* 1983;**55**(3):307-312.
19. Shuping GB, Orstavik D, Sigurdsson A, Trope M. Reduction of intracanal bacteria using nickel-titanium rotary instrumentation and various medications. *J Endod* 2000;**26**(12):751-755.
20. Vianna ME, Horz HP, Gomes BP, Conrads G. In vivo evaluation of microbial reduction after chemo-mechanical preparation of human root canals containing necrotic pulp tissue. *Int Endod J* 2006;**39**(6):484-492.
21. Torabinejad M, Khademi AA, Babagoli J, Cho Y, Johnson WB, Bozhilov K, et al. A new solution for the removal of the smear layer. *J Endod* 2003;**29**(3):170-175.
22. Shabahang S, Pouresmail M, Torabinejad M. In vitro antimicrobial efficacy of MTAD and sodium hypochlorite. *J Endod* 2003;**29**(7):450-452.
23. Shabahang S, Torabinejad M. Effect of MTAD on *Enterococcus faecalis*-contaminated root canals of extracted human teeth. *J Endod* 2003;**29**(9):576-579.
24. Newberry BM, Shabahang S, Johnson N, Aprecio RM, Torabinejad M. The antimicrobial effect of biopure MTAD on eight strains of *Enterococcus faecalis*: an in vitro investigation. *J Endod* 2007;**33**(11):1352-1354.
25. Kho P, Baumgartner JC. A comparison of the antimicrobial efficacy of NaOCl/Biopure MTAD versus NaOCl/EDTA against *Enterococcus faecalis*. *J Endod* 2006;**32**(7):652-655.
26. Johal S, Baumgartner JC, Marshall JG. Comparison of the antimicrobial efficacy of 1.3% NaOCl/BioPure MTAD to 5.25% NaOCl/15% EDTA for root canal irrigation. *J Endod* 2007;**33**(1):48-51.

27. Dunavant TR, Regan JD, Glickman GN, Solomon ES, Honeyman AL. Comparative evaluation of endodontic irrigants against *Enterococcus faecalis* biofilms. *J Endod* 2006;**32**(6):527-531.
28. Malkhassian G, Manzur AJ, Legner M, Fillery ED, Manek S, Basrani BR, et al. Antibacterial efficacy of MTAD final rinse and two percent chlorhexidine gel medication in teeth with apical periodontitis: a randomized double-blinded clinical trial. *J Endod* 2009;**35**(11):1483-1490.
29. Gutarts R, Nusstein J, Reader A, Beck M. In vivo debridement efficacy of ultrasonic irrigation following hand-rotary instrumentation in human mandibular molars. *J Endod* 2005;**31**(3):166-170.
30. Burleson A, Nusstein J, Reader A, Beck M. The in vivo evaluation of hand/rotary/ultrasound instrumentation in necrotic, human mandibular molars. *J Endod* 2007;**33**(7):782-787.
31. Carver K, Nusstein J, Reader A, Beck M. In vivo antibacterial efficacy of ultrasound after hand and rotary instrumentation in human mandibular molars. *J Endod* 2007;**33**(9):1038-1043.
32. Fouad AF, Barry J, Caimano M, Clawson M, Zhu Q, Carver R, et al. PCR-based identification of bacteria associated with endodontic infections. *J Clin Microbiol* 2002;**40**(9):3223-3231.
33. Cohen J. *Statistical Power Analysis for the Behavioral Sciences*, Second Edition. Hillsdale, NJ: Lawrence Erlbaum Associates, Inc., 1988.
34. Borenstein M, Rothstein H, Cohen J, Schoenfeld D, Berlin J. *Power and Precision*. Release 2.00 ed. Englewood, NJ: Biostat, Inc., 2000.
35. Ahmad M, Pitt Ford TJ, Crum LA. Ultrasonic debridement of root canals: acoustic streaming and its possible role. *J Endod* 1987;**13**(10):490-499.
36. Ahmad M. Effect of ultrasonic instrumentation on *Bacteroides intermedius*. *Endod Dent Traumatol* 1989;**5**(2):83-86.
37. Yumita N, Nishigaki R, Umemura K, Morse PD, Swartz HM, Cain CA, et al. Sonochemical activation of hematoporphyrin: an ESR study. *Radiat Res* 1994;**138**(2):171-176.
38. Joyce E, Phull SS, Lorimer JP, Mason TJ. The development and evaluation of ultrasound for the treatment of bacterial suspensions. A study of frequency, power and sonication time on cultured *Bacillus* species. *Ultrason Sonochem* 2003;**10**(6):315-318.
39. Ahmad M, Pitt Ford TR, Crum LA, Walton AJ. Ultrasonic debridement of root canals: acoustic cavitation and its relevance. *J Endod* 1988;**14**(10):486-493.

40. Torabinejad M, Shabahang S, Apécio RM, Kettering JD. The antimicrobial effect of MTAD: an in vitro investigation. *J Endod* 2003;**29**(6):400-403.
41. Portenier I, Waltimo T, Orstavik D, Haapasalo M. The susceptibility of starved, stationary phase, and growing cells of *Enterococcus faecalis* to endodontic medicaments. *J Endod* 2005;**31**(5):380-386.
42. Portenier I, Waltimo T, Orstavik D, Haapasalo M. Killing of *Enterococcus faecalis* by MTAD and chlorhexidine digluconate with or without cetrimide in the presence or absence of dentine powder or BSA. *J Endod* 2006;**32**(2):138-141.