2018

Antibacterial Activity of Current Endodontic Repair Materials Against *Enterococcus faecalis*.

Michael A. Tran

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Antibacterial Activity of Current Endodontic Repair Materials Against *Enterococcus faecalis*

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Thesis submitted to the School of Dentistry
At West Virginia University
In partial fulfillment of the requirements
For the degree of

Master of Science
In
Endodontics

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Morgantown, West Virginia
2018

Keywords: Endodontics, Bioceramic, *Enterococcus faecalis*, Antibacterial Activity, Direct Contact Test, Perforation Repair, ProRoot MTA, BC Fast Set Putty, Biodentine, NeoMTA Plus

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Abstract

Antibacterial Activity of Current Endodontic Repair Materials Against *Enterococcus faecalis*

Michael A. Tran, D.D.S.

Introduction: Root canal perforations caused by pathologic or iatrogenic means have been shown to cause serious complications leading to overall tooth loss. Bacterial infection of the affected site results in failure of the root canal as well as an inflammatory response leading to the destruction of supporting tissues. Although a multitude of endodontic repair materials have been tested over the past several decades, recent introduction of bioceramic materials have shown favorable outcomes to repairs. Bioceramics not only exhibit excellent biocompatibility, but also may possess antibacterial properties due to their strong alkaline pH. The aim of this *in vitro* study was to compare the antibacterial properties of the following endodontic repair materials: grey ProRoot MTA, Biodentine, EndoSequence BC Root Repair Material Fast Set Putty and NeoMTA Plus against *Enterococcus faecalis*.

Materials and Methods: A modified direct contact test was utilized to expose grey ProRoot MTA, Biodentine, EndoSequence BC Root Repair Material Fast Set Putty and NeoMTA Plus to a bacterial suspension of *Enterococcus faecalis* strain ATCC 29212. Each material (n =4 per time period) was exposed to the bacterial suspension 10 minutes after allocation to its designated well and antibacterial activity was measured at 1H, 12H, 24H and 48H. Negative controls (n =4 per time period) consisted of each material exposed to LB broth (growth media) while positive controls (n =4 per time period) were the bacterial suspension without any of the tested materials. Enumeration of viable bacteria was conducted using Invitrogen's Bacteria Counting Kit in combination with flow cytometry analysis. A two-way ANOVA with repeated measures on time was conducted. Significance was set at $P \leq .05$.

Results: At 48H, mean viable bacteria count for ProRoot MTA ($2.76125 \pm .452167$) $\times 10^6$ /mL, Biodentine was ($3.01050 \pm .410705$) $\times 10^6$ /mL, BC Fast Set Putty was ($2.90525 \pm .372005$) $\times 10^6$ /mL, and NeoMTA was ($2.96900 \pm .294599$) $\times 10^6$ /mL were significantly lower than the positive controls (29.68550 ± 1.849175) $\times 10^6$ /mL ($P < .001$). There were no statistically significant differences between the materials.

Conclusion: Grey ProRoot MTA, Biodentine, BC Fast Set Putty, and NeoMTA Plus exhibited similar antimicrobial properties when subjected to a modified direct contact test against *Enterococcus faecalis* strain ATC 29212. Flow cytometry analysis can be used as an alternative method for assessment of viable bacteria load.

Dedication

To my parents, Khanh and Angie: Thank you for always being there for me. Your tough love, words of wisdom, encouragement and support have shaped me to become who I am today. I am forever appreciative of everything that the two of you have done to raise me. I love you both.

Acknowledgements

AAE Foundation/Dentsply - Thank you for your generosity in awarding the West Virginia University Endodontic Department an Innovation in Research Grant (Fund# 2R388) which supported this research project.

Dr. Marvin Speer - Thank you for your mentorship and guidance over the past 2 years. Your tips from private practice experience have been tremendously helpful.

Dr. Larry Hildebrand - Thank you for giving me the opportunity to pursue a career in endodontics by accepting me into the program. It is inspirational to see the dedication you have towards WVU Endodontics and how you are always looking for ways to improve our program.

Dr. Mark Byron - Thank you for your guidance both clinically and with writing my thesis. Your transition to Graduate Endodontics Director has been seamless and the wealth of knowledge you have to share has been priceless.

Dr. Tom Borgia - Thank you for accepting me into the program and sharing all of your advice/insight from your many years of endodontic experience.

Dr. Elizabeth Kao - Thank you for serving as chair of my committee. Your expertise in dental research was crucial in guiding me through this process.

Dr. Bryan Weaver - Thank you for sharing your vast knowledge of Medicine and Oral Surgery. Your class was very informative regarding the management of medically compromised patients and you have always been accommodating to residents that are not part of your OMFS program.

Mr. Chris Waters - This project would not have taken off without your help or support. Thank you for showing me around the lab, ordering materials, and helping me with experiment design. You are much appreciated and I can't thank you enough!

Dr. Kathleen Brundage - Thank you for staying patient with me and explaining the details of how flow cytometry works! I would not have been able to complete this project if I had not found your department and skills.

Dr. Constance Weiner - Thank you for showing me how to run SPSS and helping me understand the statistics of my project.

Dr. Travis Moore - What can I say, we finally made it thru! I admire your hard work and punctuality. Congrats on your baby boy and I wish you the best of luck with your practice in North Carolina!

Dr. Cade Brawley - I'm going to miss all of our jokes in the resident room. I'm excited for you to start working with Maupin. I can't wait for a few years from now where you'll be teaching us all of the latest advancements in Endodontics.

Dr. Mehran Malakpour - It is very motivating to see an individual as dedicated as you to the art of Endodontics. Coming straight out of dental school, you are an extremely talented clinician and you'll definitely be one of the best once you graduate.

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List of Symbols, Abbreviations or Nomenclature

American Association of Endodontists - AAE

Beckton Dickinson - BD

Blood Agar Plate - BAP

Colony Forming Unit - CFU

EndoSequence Root Repair Material Fast Set Putty - ERRM or BC Fast Set Putty

Ethoxy Benzoic Acid - EBA

Fluorescein Isothiocyanate - FITC

Forward Scatter - FSC

Hank's Balanced Salt Solution - HBSS

Hour - H

Luria-Bertani Broth - LB Broth

Mineral Trioxide Aggregate - MTA

Photo Multiplying Tube - PMT

Side Scatter - SC

Chapter 1

Introduction

A root canal perforation is defined as a communication between the root canal system and the external tooth surface caused by mechanical, iatrogenic, or pathologic means (1,2). The occurrence of iatrogenic perforations as a result of endodontic treatment has been reported in the literature between 2% to 12% (3). Coronal perforations may result from crown-root angulations, calcifications of the pulp chamber and orifices, anatomic variations, and excessive removal of coronal dentin (4). Excessive flaring and aggressive instrumentation of curved roots may contribute to midroot perforations (5). Inappropriate cleaning and shaping techniques leading to blocked or transported canal systems may lead to apical perforations (5). Kvinnsland et al. reported 73% of iatrogenic perforations occur in the maxillary arch compared to the remaining 23% in the mandibular arch (3). A possible influence to the increased percentage of perforations in the maxilla may be the operator's underestimation of the palatal inclination of roots in the upper jaw.

Serious complications have been documented from root perforations and therefore it is important to repair these areas as soon as possible (4–10). Bacterial infection originating from the root canal, periodontal tissues, or both, prevents healing and leads to inflammatory sequelae to the exposed supporting tissues (4–10). Down-growth of gingival epithelium to the perforation site can emerge, particularly in the crestal area by lateral perforation or perforation in the furcations of multi-rooted teeth (10). The apical migration of epithelium creates a periodontal pocket, leading to chronic inflammation of the perforation site that is maintained most likely by continuous ingress of irritants from the pocket (6,10–12). Consequently, painful

conditions, suppurations resulting in tender teeth, abscesses, and fistulae including bone resorptive processes may arise (6,10).

According to Tsesis and Fuss, successful treatment of a root canal perforation is dependent on whether or not the bacterial infection of the perforation site can either be prevented or eliminated (10). In addition to sealing the perforation, materials with antibacterial properties are believed to improve prognosis of the repair.

Repair materials such as amalgam, gutta percha, Cavit, calcium hydroxide, glass ionomer cement, intermediate restorative material, composite resin and Super EBA have been tested throughout the years with varying degrees of success (4,13–16). With the development of bioceramic materials, success rates of these repairs have steadily improved (4,5,16).

The aim of this *in vitro* study was to compare the antibacterial properties of the following endodontic repair materials: ProRoot MTA, Biodentine, EndoSequence BC Root Repair Material Fast Set Putty, and NeoMTA Plus against *Enterococcus faecalis*.

Statement of the Problem

Out of the current endodontic repair materials available on the market, which product has the greatest antibacterial activity to *Enterococcus faecalis*? The most commonly used technique for enumerating bacteria involves time consuming methods of serial dilution of samples followed by plating onto agar media (17). Can flow cytometry analysis serve as an alternative method to accurately determine bacteria load?

Significance of the Problem

Root perforations can dramatically decrease the prognosis of a tooth. Successful treatment depends on a variety of factors, one being that bacterial infection of the affected site be prevented or eliminated. This study will provide information on the antibacterial efficacy of current endodontic repair materials, thus giving clinicians a guideline on which products are most viable for treatment. The successful utilization of flow cytometry can improve upon the tedious and error-prone process of enumerating bacteria in a sample.

Null Hypothesis

There is no significant difference in antibacterial efficacy over time for the four endodontic repair materials tested: grey ProRoot MTA (Dentsply Tulsa Dental Specialties, Johnson City, TN), EndoSequence Root Repair Material (BC) Fast Set Putty (Brasseler USA, Savannah, GA), Biodentine (Septodont, Lancaster, PA), and NeoMTA Plus (Avalon Biomed Inc., Houston, TX). Furthermore, there is no significant difference in the accuracy of bacterial enumeration when comparing the serial dilution/plating method to flow cytometry analysis.

Assumptions

1. The direct contact test is a quantitative and reproducible method for simulating the contact between bacteria and repair material in a root canal.
2. The operator of the flow cytometer was knowledgeable and well versed in properly handling the instrument.

Limitations

1. This was an *in vitro* experiment and the results may or may not replicate the clinical environment.
2. Operator error may have occurred during the experimentation process.
3. The sample size was small for each group.
4. Endodontic infections comprise of biofilms which are difficult to eradicate in the root canal system. The responses of different bacteria to dental materials may vary depending on the environment and time they are exposed to.

Delimitations

1. Bacteria enumeration was conducted via flow cytometry analysis.
2. All samples were prepared by the principal investigator.
3. The 4 most current bioceramics available on the market were selected for experimentation.
4. The following experiment tested only one species of bacteria in its planktonic form. *Enterococcus faecalis* was the bacteria chosen due to its ability to survive the harsh conditions of the root canal system.
5. Antibacterial efficacy was measured over a 48 hour time period.

Chapter 2

Review of Literature

The four materials tested in this investigation are considered bioceramics. Bioceramics are ceramic materials specifically designed for medical and dental use. They are inorganic materials that include alumina and zirconia, bioactive glass, coatings and composites, hydroxyapatite and resorbable calcium phosphates, and radiotherapy glasses (18). Bioceramics are known to have both biocompatible and bioactive (defined as a material that forms a layer of hydroxyapatite when immersed in a simulated body fluid or a solution containing inorganic phosphate) properties (19). During setting, a hydration reaction takes place, forming calcium hydroxide which then dissociates into calcium and hydroxyl ions (18). The calcium hydroxide release has the potential to interact with phosphates in the tissue fluid to form hydroxyapatite (18). Bioceramics in endodontics are classified as powder/liquid systems or premixed systems (18). Powder/liquid systems allow customization of the material's consistency, but produce considerable waste. Premixed systems have the advantage of uniform consistency and lack of waste, but require moisture from the surrounding tissue to set.

MTA is mainly composed of tricalcium silicate, tricalcium aluminate, tricalcium oxide, silicate oxide, mineral oxide and bismuth oxide (4). When mixed with water, calcium hydroxide forms releasing calcium ions necessary for cell attachment and proliferation. Furthermore, this continuous release of calcium hydroxide allows MTA to maintain a $\text{pH} \geq 11.9$ therefore creating an antibacterial environment (20). In a literature review, several studies have demonstrated MTA's capacity to promote hard tissue formation allowing it to form hydroxyapatite on its surface and providing a biologic seal (21). One of the major disadvantages of MTA is the

approximate set time of 3 to 4 hours allowing the opportunity for potential wash out of the material (4). The degree of MTA solubility is a matter of debate among investigators, but from the available literature it is documented that with an increase in water-to-powder ratio, the release of calcium from MTA increases, thereby accelerating its solubility (20,22,23). MTA placement has resulted in discoloration of teeth limiting its ability to be used in esthetic cases (24,25). Bismuth oxide is responsible for the staining because it dissociates into dark color crystals of metallic bismuth and oxygen when exposed to visible and ultraviolet light. Over-oxidation of bismuth oxide, which can occur when in contact with sodium hypochlorite solution during root canal cleansing, can also lead to discoloration (24).

There have been several studies on the antibacterial and antifungal properties of MTA with conflicting results. One investigation noted that MTA has an antibacterial effect on some facultative bacteria and no effect on any species of strict anaerobes (26). In another antimicrobial study, MTA and Portland cement exhibited diffusion in agar without inhibition of growth against *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 6633), *Candida albicans* (ICB/USP-562), a wild fungus, and a mixture of both the bacterial and fungal species (27). Al-Hazaimi et al. stated that grey ProRoot MTA requires lower concentrations than white ProRoot MTA to exert the same antibacterial effect against *E. faecalis* and *S. sanguis* (28). Additional studies have also evaluated the replacement of distilled water with Chlorhexidine to mix with MTA powder (29,30). Holt et al. noted that the zones of inhibition against *Enterococcus faecalis* were significantly larger for MTA samples mixed with 2% chlorhexidine compared to sterile water (29). However, it is important to note that adding various liquids can adversely affect

compressive strength, setting time, and other properties of MTA (29). Possible reasons for disagreement among the antibacterial and antifungal properties of MTA in the literature may be due to the various tested species of microorganisms, the manufacturer of the preparing material, as well as the concentration and the type of MTA used in these studies (22).

Biodentine (Septodont, Lancaster PA), commercially available in 2009, is considered a second generation bioceramic material that is packaged as a powder/liquid system (18). The material is based off previous MTA cement technology and is composed mainly of tricalcium silicates with dicalcium silicate as a second core material (31). The liquid provided with Biodentine contains calcium chloride which acts as an accelerator and a hydrosoluble polymer that serves as a water reducing agent (31). When compared to MTA, Biodentine is easier to handle, stronger mechanically, and has a shorter setting time (approximately 12 minutes) (32). Grech et al. demonstrated negative solubility values for Biodentine which was attributed to the deposition of substances such as hydroxyapatite on the material surface when in contact with synthetic tissue fluids (33). Additionally, discoloration of the tooth crown has been reported to be minimal to none due to the replacement of bismuth oxide with zirconium oxide as a radio-opacifier (24,32). Of the few studies conducted regarding Biodentine's antibacterial and antifungal properties, one investigation reported that both Biodentine and ProRoot MTA had similar efficacy when paired against *Candida albicans* and *Enterococcus faecalis* (34).

EndoSequence Root Repair Material (ERRM) (Brasseler USA, Savannah, GA), available in North America since 2008, is a pre-mixed bioceramic delivered in a moldable putty form. The chemical composition includes calcium silicates, zirconium oxide, tantalum oxide, calcium

phosphate monobasic, and fillers (18). According to the manufacturer, ERRM Fast Set Putty otherwise known as BC Fast Set Putty, has superior handling characteristics, high biocompatibility, possesses anti-bacterial properties due to its pH of 12 or above, and has a shortened set time of 20 minutes. Several *in vitro* studies have shown that ERRM displays similar cell viability to MTA in both fresh and set conditions (35–37). In one particular study, ERRM demonstrated higher osteoblast differentiation compared to MTA in a three-dimensional culture system (38). ERRM has significantly less coronal tooth discoloration when compared to the MTA line of materials (24). At the current time, there appears to be only one study which has analyzed the antibacterial properties of the moldable putty. Using a modified direct contact test, Lovato and Sedgley reported similar antibacterial efficacy between ERRM regular set putty, ERRM syringeable paste, and ProRoot MTA against several clinical strains of *Enterococcus faecalis* (39).

NeoMTA Plus (Avalon Biomed Inc., Houston, TX) is marketed as an economic MTA formulation designed to improve on the physiochemical properties while maintaining the biocompatibility of the original MTA. Recently released in 2015, the number of studies on the material is limited. The composition of NeoMTA Plus is a finely ground powder consisting of tricalcium silicate, dicalcium silicate, and tantalum oxide as a radiopacifying agent (40). The powder is mixed with a water-based gel that improves the handling and placement (41). Siboni et al. reported that values of NeoMTA Plus for porosity and solubility were high when the material was immersed in Hank's Balanced Salt Solution (HBSS) after setting for a period of 50% longer than the final setting time according to ISO 6876:2012 (41). The major limitation with this study is that when MTA-based materials are exposed to bodily fluids, the calcium and

hydroxyl ions from the materials combine with the phosphate of the surrounding fluids causing the precipitation of a superficial layer of calcium phosphate to fill the open voids (42). Due to the use of tantalum oxide as a radiopacifier, NeoMTA Plus does not result in tooth discoloration when exposed to sodium hypochlorite (43).

As facultative anaerobes, *Enterococci* have the ability to grow in the presence or absence of oxygen (44). *Enterococcus faecalis* has been associated with both primary and secondary root canal infections (44,45). Due to its survivability and virulence factors, *E. faecalis* has proven to be difficult to eradicate from the root canal system. These factors include the ability to invade dentinal tubules (44,46), bind to collagen (46), endure prolonged periods of starvation (44), enter and recover from the viable but nonculturable (VBNC) state (47), inhibit the cytokine-producing functions of Th-1 and Th-2 cells (48,49) and form biofilms (44). Furthermore, studies have shown that calcium hydroxide, a commonly used intracanal medicament, has been ineffective at killing *E. faecalis* despite the high pH produced (44,50). Proposed reasons include: a) Although *E. faecalis* is unable to survive in environments having a pH of 11.5 or greater, the buffering capacity of dentin makes it highly unlikely that the high pH of calcium hydroxide (>11.5) is attained within the dentin tubules. b) *E. faecalis* maintains passive pH homeostasis via cell membrane permeability to ions and the cytoplasm's buffering capacity. c) *E. faecalis* possesses a proton pump where cations/protons are pumped into the cell to lower the internal pH (44,50).

Flow cytometry is a technology that is used to analyze the physical and chemical characteristics of particles, usually cells, in a fluid as it passes through at least one laser (51).

Flow cytometers are designed for various applications which include but are not limited to the study of eukaryotic cells, prokaryotic cells, cell organelles, sub-micron particles and isolated chromosomes (52). There are 3 main components to a flow cytometer: fluidics, optics and electronics (53). When a fluid sample containing a cell suspension is introduced to the instrument, sheath fluid directs the sample to the sensing/interrogation region where they intersect one or several laser beam paths (**Fig. 1**). By controlling the force and speed of the sheath fluid, a phenomenon called hydrodynamic focusing, the sample cells are arranged into a single file so that the laser light encounters each cell separately (52). As the cells pass through the laser beam, light scatter is measured by multiple detectors in the form of forward scatter (FSC) and side scatter (SS) (**Fig. 2**). Forward-scatter light is proportional to a cell-surface area or size whereas side-scattered light is proportional to cell granularity or internal complexity (53). Furthermore, cell samples can be stained and emitted fluorescence is measured with diode detectors or photomultiplier tubes (PMTs). A voltage pulse, otherwise known as an event, is created when a particle enters the laser beam and starts to scatter or fluoresce (53). This measured voltage is then processed and analyzed by the computer to create a data plot.

Figure 1. Overview of flow cytometer. (54)

Figure 2. Light scattering properties of a cell. (53)

With the aid of a flow cytometer, accurate enumeration of bacteria can be performed without the use of traditional plating techniques. Using one of the commercially available bacteria counting kits, the sample population is diluted, stained, mixed with microsphere particles and applied to a flow cytometer for analysis (55). For example, the Invitrogen Bacteria Counting Kit supplies a stain (SYTO) which is a high-affinity nucleic acid stain that easily penetrates both gram-positive and gram-negative bacteria and results in a bright green fluorescent signal (55). When bound to nucleic acids, SYTO green-fluorescent nucleic acid stains have excitation and emission spectra similar to those of fluorescein (FITC) and can be visualized using optical filters appropriate for fluorescein (55). The stained bacteria are then compared to a fix concentration of microsphere particles. As a result, the density of bacteria in a sample can be determined from the ratio of bacterial signals to microsphere signals in the cytogram (55).

Chapter 3

Materials and Methods

Bacteria

Enterococcus faecalis ATCC 29212 (ATCC, Manassas, VA) was the bacterial strain used in this experiment. In Sedgley's study on the "Antibacterial Activity of EndoSequence Root Repair Material and ProRoot MTA against Clinical Isolates of *Enterococcus faecalis*", an initial bacterial suspension of 3×10^7 CFU/mL was used (39). It was decided to use a similar concentration of 3×10^7 CFU/mL in the present investigation. The inoculum was prepared as follows:

Enterococcus faecalis strain ATCC 29212 was taken from -80°C stock and plated on blood agar plates (BAP) (Thermo Fisher Scientific, Waltham, MA) and incubated aerobically for 24 hours at 37°C (**Fig. 3**). A stock solution was created using the freshly grown bacteria from one of the BAPs. 15mL micro-centrifuge tubes were labeled with the organism ID and date. The stock solution was prepared as follows:

Tube #1 (Initial bacteria stock solution)

- 10mL of Luria-Bertani (LB) broth was placed in tube #1.
- A 10µL lambda loop was used to take a sample swab of bacteria from the BAP (**Fig. 4**).
- The lambda loop with bacteria was placed into tube #1 and agitated to disperse the bacteria into the media.
- Solution was vortexed.
- Stock solution was incubated aerobically for 24 hours at 37°C.

- Solution was vortexed again before McFarland test reading (McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range to standardize microbial testing (56)).
- Test tube was placed in DEN-1 Densitometer (BioSan, Warren, MI) to obtain McFarland test reading = 5.5 = 1.65×10^9 CFU/mL (**Fig. 5**)

Figure 3. *Enterococcus faecalis* ATCC 29212 plated on blood agar plate (BAP).

Figure 4. 10µL lambda loop was used to swab bacteria and placed in tube #1.

Figure 5. McFarland test reading of tube #1 in DEN-1 densitometer.

Tube #2 (Diluted bacteria stock solution)

- In order to obtain a concentration of 3×10^7 CFU/mL, tube #1 was diluted further using LB broth according to the following calculation:
 - $C_1V_1=C_2V_2$, where C_1 = concentration of stock solution, V_1 = volume of stock solution needed to make the new solution, C_2 = final concentration of new solution, V_2 = final volume of new solution
 - $(1.65 \times 10^9)(x) = (3 \times 10^7)(10\text{mL})$
 - $X = 0.1818\text{mL}$
 - $10\text{mL} - 0.1818\text{mL} = 9.818\text{mL}$
 - 9.818mL of LB broth was placed into micro-centrifuge tube #2
 - 0.1818mL of stock solution from tube #1 was placed into tube #2 to achieve a total volume of 10ml with a concentration of 3×10^7 CFU/mL.
 - Solution was vortexed.

Growth was verified by using a sterile pipette and removing 0.5mL (500 μ L) of stock solution out of tube #2 and placing it on the center of a BAP. Using an L-spreader, the stock solution was evenly distributed in a circular pattern on the BAP. The sample was incubated aerobically at 37°C and checked at 24 hours to ensure that adequate growth was present.

Preparation of Materials

Grey ProRoot MTA (Dentsply Tulsa Dental Specialties, Johnson City, TN), EndoSequence Root Repair Material BC Fast Set Putty (Brasseler USA, Savannah, GA), Biodentine (Septodont, Lancaster, PA), and NeoMTA Plus (Avalon Biomed Inc., Houston, TX) were the endodontic repair materials compared for antibacterial efficacy using a modified direct contact test based on

Orstavik's and Sedgley's study (39,57) (**Fig. 6-9**). The test involves placing a fixed amount of material in a designated well of the 96-well plate and then exposing the material to the diluted bacteria stock solution.

Figure 6. Packaging of Grey ProRoot MTA.

Figure 7. Packaging of EndoSequence Root Repair Material BC Fast Set Putty.

Figure 8. Packaging of Biodentine.

Figure 9. Packaging of NeoMTA Plus.

The antibacterial efficacies of the materials were measured at four different time points: 1 hour, 12 hours, 24 hours, and 48 hours. Thus, four 96-well plates were used to correspond with each time point. On a single 96-well plate, 24 out of the 96 wells were used (4 rows + 6 columns) (**Fig. 10**). Each material had a dedicated row for a negative control (LB broth growth media + material), positive control (bacteria only), and four experimental replicates (material + bacteria) (**Fig. 10**). In total, including both controls and experimental replicates, there were 96 samples analyzed (24 samples at each time point) over the course of the experiment.

ProRoot MTA, Biodentine, and NeoMTA Plus was mixed and handled according to the manufacturer's directions. BC Fast Set Putty did not require preparation as it comes ready to use in a pre-mixed syringe. According to the manufacturers, the setting time is ~ 2 hours and 45 minutes for ProRoot MTA, ~12 minutes for Biodentine, ~15 minutes for NeoMTA, and ~20 minutes for BC Fast Set Putty.

In order to standardize the volume of material, the 1.5mm diameter end of an amalgam carrier was used to allocate a 3mm in length plug of each material to the bottom of its designated well (**Fig. 10**). Once each material was mixed or dispensed, it was added immediately to its corresponding well of the 96-well plate.

Figure 10. Experimental setup on 96-well plate.

Antimicrobial Assays

A 250 μ L of bacterial suspension from tube #2 was added to the positive control and experimental replicate wells. A 250 μ L of LB growth media was added to the negative control wells. The 250 μ L aliquots were distributed 10 minutes after each material was added to its corresponding well. The lid cover was placed over the 96-well plate and secured with Parafilm to reduce evaporation of either the growth media or bacterial suspension (**Fig. 11**). All samples were incubated aerobically at 37°C based on its designated time point: 1H, 12H, 24H and 48H (**Fig. 13**).

Figure 11. Parafilm was used to secure lid on 96-well plate.

Figure 12. 96-well plates were incubated aerobically at 37°C.

Flow Cytometry Preparation and Analysis

At the end of each time point, a sterile pipette was used to gently mix the suspension in each well before taking a 150µL sample and placing it in a 10mL micro-centrifuge tube. In order to prepare for flow cytometry analysis, Invitrogen's Molecular Probes Bacteria Counting Kit (Invitrogen, Carlsbad, CA) was used. As part of the instructions, the kit required the bacteria culture to have a 1mL sample with a final density of approximately 10^6 CFU/mL. Therefore, 850µL of LB broth growth media was added to the 150µL well sample in order to create 1mL solutions which were subjected to Mcfarland standards. Once the Mcfarland readings were obtained for each 1mL solution, the samples were diluted accordingly to achieve a final density of approximately 10^6 CFU/mL. The positive controls at 1H and the experimental replicates at the 1H, 12H, 24H, and 48H time points produced a Mcfarland reading of $0.25 = 10^7$ CFU/mL. As

a result, the 1mL solution was diluted by a factor of 10. Positive controls at 12H, 24H, and 48H gave a Mcfarland reading of $1 = 10^8$ CFU/mL, resulting in a dilution factor of 100. The negative controls at all time points did not require further dilution. The following dilution factor equation was used for both the 1:10 and 1:100 dilutions:

- Example of 1:10 dilution

- $DF = VF/VI$, where DF = dilution factor, VF = final volume, VI = initial volume
 - $10 = 1000\mu\text{L} / X$
 - $X = 100\mu\text{L}$
- 100 μL was taken out of the initial 1mL sample and placed in a new 10mL micro-centrifuge tube
- 900 μL of LB broth was then added to create a new 1mL sample that's 1:10 diluted from the original 1mL sample.
- Solution was vortexed

- Example of 1:100 dilution

- $DF = VF/VI$, where DF = dilution factor, VF = final volume, VI = initial volume
 - $100 = 1000\mu\text{L} / X$
 - $X = 10\mu\text{L}$
- 10 μL was taken out of the initial 1mL sample and placed in a new 10mL micro-centrifuge tube
- 990 μL of LB broth was then added to create a new 1mL sample that's 1:100 diluted from the original 1mL sample.
- Solution was vortexed

1 μ L of SYTO BC bacteria stain (Invitrogen Bacteria Counting Kit Component A) was added to the 1mL sample (density of $\sim 10^6$ CFU/mL), vortexed, and incubated aerobically at room temperature for 5 minutes (**Fig. 13**). Next, the microsphere standard suspension (Invitrogen Bacteria Counting Kit Component B) was resuspended by waterbath sonication for 5 minutes (**Fig. 14, 15**). 10 μ L of the microsphere suspension was then added to the stained cell preparation and vortexed.

Figure 13. 1 μ L of SYTO BC Stain was used for each sample.

Figure 14. 10 μ L of the microsphere standard suspension was used for each sample.

Figure 15. Microsphere standard suspension resuspended in waterbath sonication for 5 minutes.

Prepared samples were brought to WVU's Flow Cytometry and Single Cell Core Facility (HSC North Room 2160) for analysis on the Becton Dickinson (BD) LSRFortessa cell analyzer (Becton Dickinson, Franklin Lakes, NJ) using the FSC photomultiplier tube (PMT) (**Fig. 16**). Approximate fluorescence and emission maxima was 480/500 nm for SYTO BC stain bound to DNA. All samples were run at low speed for 60 seconds. Data was compiled in FACSDiva 8.0 software from BD Biosciences. Each sample produced a data plot of FSC (forward scatter = size/volume of cell) versus FITC (fluorescence of cell) (**Fig. 17**). Both FSC and FITC are recorded photons of light and therefore have no unit of measurement. Overall this graph shows that the bacteria are smaller (less FSC) and stain brighter (greater FITC) when compared to the microspheres. Each dot on the data plot represents either a particle or cell that has passed through the lasers of the flow cytometer. **Table 1** shows the # of events computed from the data plot in **Fig. 17**. "All Events" represents the number of particles or cells which was analyzed by the flow cytometer. "Syto (+)" represents the number of live bacteria (light blue) detected within the boxed region. "Beads" represent the number of microspheres (red) detected within the boxed region. The boxed regions are drawn around the densest areas of cells or particles. The beads box is smaller because microspheres are more uniform in size and shape compared to biological cells like bacteria. The bacteria will be of different sizes depending on whether they are in the process of replicating, dying or quiescent. It is important when drawing the boxed region that it encompasses all the single cells but excludes doublets or clumps.

Figure 16. Becton Dickinson (BD) LSRFortessa cell analyzer.

Using the plot of forward scatter versus fluorescence, the number of viable bacteria was calculated according to the following equation (**Fig. 17 & Table 1**):

$$- \quad (\text{Syto (+) events} / \text{Beads events}) \times \text{dilution factor} = \# \text{ of bacteria} \times 10^6/\text{mL}$$

Figure 17. An example of the data plot of forward scatter (x-axis) versus fluorescence (y-axis) for ProRoot MTA experimental replicate at time 1H.

Tube: Pro 5

Population	#Events
All Events	9,397
Beads	5,515
syto(+)	2,328

Table 1. The # of events computed from previous data plot (**Fig. 18**) for ProRoot MTA experimental replicate at time 1H.

Statistical Analysis

IBM Statistical Package for Social Sciences version 25 (SPSS; Chicago, IL) was used for the tabulation and statistical analysis of the results obtained. A two-way ANOVA with repeated measures on time was conducted. Wilk's Lambda was performed to elucidate multiple comparisons of antibacterial activity between the materials and controls. Significance was set at $P \leq .05$.

Chapter 4

Results

At each time period, four experimental replicates were conducted for each of the 4 products along with four positive and four negative controls. Combining data at each time point, the mean for each group ($\pm$ standard deviation) are shown below (**Table 2-5**). At 48H, the mean bacterial count for the negative control was $(.25150 \pm .066003) \times 10^6/\text{mL}$ and for the positive control was $(29.68550 \pm 1.849175) \times 10^6/\text{mL}$.

At 48H, the mean bacterial load for ProRoot MTA was $(2.76125 \pm .452167) \times 10^6/\text{mL}$, Biodentine was $(3.01050 \pm .410705) \times 10^6/\text{mL}$, BC Fast Set Putty was $(2.90525 \pm .372005) \times 10^6/\text{mL}$, and NeoMTA was $(2.96900 \pm .294599) \times 10^6/\text{mL}$.

The Wilk's Lambda for Multivariate Tests had a significant main effect for time (meaning there was decreased bacterial growth for all materials from 1H to 48H) with $P < .001$; ($F = 43.472$) (**Fig. 18 & Table 6**). However, the interactions of time * material failed to reach significance (no difference between materials) with $P = 0.548$ (**Fig. 19, Table 6 and Table 11**).

1 H Time Point; # of Bacteria x 10 ⁶ /mL			
	Mean	Std. Deviation	N
Negative Control	0.043700	0.0046311	4
Positive Control	10.776000	0.6636862	4
ProRoot MTA	4.305250	0.6636356	4
BC Fast Set Putty	4.300500	1.0781248	4
Bondentine	4.786500	0.7793474	4
NeoMTA Plus	4.584250	0.5552845	4
Total			24
Experimental Group Average	4.49412	.740385	

Table 2. Mean and standard deviation of viable bacteria count for all materials at 1H.

12 H Time Point; # of Bacteria x 10 ⁶ /mL			
	Mean	Std. Deviation	N
Negative Control	.053075	.0075398	4
Positive Control	15.493000	1.0952808	4
ProRoot MTA	4.112250	.7236212	4
BC Fast Set Putty	3.785750	.7132159	4
Bondentine	4.381500	.5513864	4
NeoMTA Plus	4.387500	.7420478	4
Total			24
Experimental Group Average	4.16675	.664972	

Table 3. Mean and standard deviation of viable bacteria count for all materials at 12H.

24 H Time Point; # of Bacteria x 10 ⁶ /mL			
	Mean	Std. Deviation	N
Negative Control	.19300	.038927	4
Positive Control	23.83225	2.373396	4
ProRoot MTA	3.52000	.818982	4
BC Fast Set Putty	3.22225	.520073	4
Bondentine	3.62050	.402630	4
NeoMTA Plus	3.67400	.475189	4
Total			24
Experimental Group Average	3.50919	.546222	

Table 4. Mean and standard deviation of viable bacteria count for all materials at 24H.

48 H Time Point; # of Bacteria x 10 ⁶ /mL			
	Mean	Std. Deviation	N
Negative Control	.25150	.066003	4
Positive Control	29.68550	1.849175	4
ProRoot MTA	2.76125	.452167	4
BC Fast Set Putty	2.90525	.372005	4
Bondentine	3.01050	.410705	4
NeoMTA Plus	2.96900	.294599	4
Total			24
Experimental Group Average	2.91150	.359427	

Table 5. Mean and standard deviation of viable bacteria count for all materials at 48H.

Multivariate Tests of ProRoot MTA vs. Controls		F	Sig.
Time	Wilk's Lambda	128.844	.000
Time * Materials	Wilk's Lambda	25.714	.000

Table 6. Wilk's Lambda for Multivariate Tests computed for time only and interactions of time * material for ProRoot MTA vs. Controls.

Multivariate Tests of BC Fast Set Putty vs. Controls		F	Sig.
Time	Wilk's Lambda	92.208	.000
Time * Materials	Wilk's Lambda	21.443	.000

Table 7. Wilk's Lambda for Multivariate Tests computed for time only and interactions of time * material for BC Fast Set Putty vs. Controls.

Multivariate Tests of Biodentine vs. Controls		F	Sig.
Time	Wilk's Lambda	114.738	.000
Time * Materials	Wilk's Lambda	24.257	.000

Table 8. Wilk's Lambda for Multivariate Tests computed for time only and interactions of time * material for Biodentine vs. Controls.

Multivariate Tests of NeoMTA Plus vs. Controls		F	Sig.
Time	Wilk's Lambda	139.743	.000
Time * Materials	Wilk's Lambda	26.908	.000

Table 9. Wilk's Lambda for Multivariate Tests computed for time only and interactions of time * material for NeoMTA Plus vs. Controls.

Multivariate Tests of Experimental Materials without Controls		F	Sig.
Time	Wilk's Lambda	43.472	.000
Time * Materials	Wilk's Lambda	.890	.548

Table 10. Wilk's Lambda for Multivariate Tests computed for time only and interactions of time * material for Experimental Materials without Controls.

Tests of Between-Subjects Effects for Experimental Materials without Controls	
Time	Sig.
1H	.788
12H	.577
24H	.696
48H	.812

Table 11. Tests of Between-Subjects Effects for Experimental Materials without Controls

Figure 18. Antibacterial activity of all materials + controls vs. time.

Figure 19. Antibacterial activity of all materials only vs. time.

Discussion

When it comes to selecting endodontic repair materials, clinicians base their decision on several factors which include sealing capability, biocompatibility, antibacterial activity, cost, and handling characteristics (10). The purpose of this study was to determine the antibacterial activity of four current endodontic repair materials on the market: grey ProRoot MTA, Biodentine, BC Fast Set Putty, and NeoMTA Plus against *Enterococcus faecalis* strain ATC 29212.

This *in vitro* investigation was based on Sedgley's study showing that EndoSequence Root Repair premixed putty and syringeable paste, as well as white ProRoot MTA, possess similar antibacterial properties against exposure to clinical strains of *E. faecalis* for 1-hour (39). One of the major differences between the studies was the enumeration of bacteria. In the present study, flow cytometry was incorporated whereas in Sedgley's study serial dilutions were prepared, plated, and CFU/mL were calculated after aerobic incubation for 24-48 hours. There are several disadvantages to the serial dilution method which include being time consuming, labor intensive, error prone (operator experience), costly and relatively imprecise (17). Since agar plates are limited to a range of 10-400 colonies, multiple dilution steps are needed when cell concentrations are high (17). As a result, these dilution steps are sensitive to pipetting and handling errors by the operator. Furthermore, when presented with an unknown concentration, several dilution steps are required to ensure that at least one hit a good range or "dilution window". In theory by plating in triplicate, three agar plates should yield countable and statistically valid numbers of CFU (17). However, multiple plates will be needed in order to achieve this "dilution window" increasing overall material costs. As described earlier, flow cytometry involves the measurement of the chemical and physical characteristics of a cell or

particle in a fluid stream as it passes through a measuring device. Advantages of flow cytometry pertaining to this experiment include high speed analysis of a large number of cells and a reduction in operator errors due to minimum preparation of the sample. The disadvantages of this technique are that the instrument is extremely expensive, complex, requires management by a highly trained specialist and on-going maintenance by service engineers.

In Sedgley's study, the experimental materials were preincubated at 37°C in >95% humidity for 30 minutes and 24 hours before 1-hour exposure to *E. faecalis* strains. The rationale for measuring four different time points (1H, 12H, 24H, 48H) in the current investigation was to determine if the four materials possessed extended antibacterial activity past the 1-hour mark. Furthermore, unlike Sedgley's study, bacteria were introduced 10 minutes after each material was added to its corresponding well in order to simulate clinical conditions. Although Sedgley's study was also looking at the antibacterial activity of the setting reaction of the materials tested, it is rather unrealistic to apply these materials to a patient's tooth and wait 30 minutes much less 24 hours for the material to set before continuing with treatment.

The pilot studies consisted of two experimental replicates for each of the four materials along with positive and negative controls. Samples were subjected to flow cytometry analysis at four time points (1H, 12H, 24H and 48H). During these initial tests, the bacterial suspension in the designated wells had completely evaporated by the 24H time point. As a result, Parafilm was used in this study to wrap the lids of the 96-well plates in order to prevent evaporation.

The results of this investigation showed that ProRoot MTA, Biodentine, BC Fast Set Putty and NeoMTA Plus possessed antibacterial properties against *Enterococcus faecalis* ATCC 29212 when the materials were mixed and handled according to the manufacturer's directions. However, the differences between materials failed to reach significance and therefore the null hypothesis tested was accepted. At 1 hour, all experimental materials exhibited approximately a two-fold decrease in bacterial load as compared to the positive control ($4.49 \times 10^6/\text{mL}$ vs. $10.78 \times 10^6/\text{mL}$). At 48 hours, all experimental materials resulted in a tenfold decrease in bacterial load as compared to the positive control ($2.91 \times 10^6/\text{mL}$ vs. $29.69 \times 10^6/\text{mL}$).

MTA was introduced in 1993 by Dr. Mahmoud Torabinejad as a root-end filling material for surgical endodontic procedures. Since then, its clinical applications have expanded to include perforation repair, pulp capping, pulpotomy and apexification (4). The 1H antibacterial effects of grey ProRoot MTA from this investigation (mean reduction of 60%) were similar to the results that Sedgley found in her study; mean $\log_{10}$ viable counts for white ProRoot MTA (4.12 ± 1.26) were significantly lower than for nonexposed controls (7.40 ± 0.3) ($P < .0001$) resulting in a mean reduction of 44%. Several other studies have reported MTA having antimicrobial action against facultative bacteria such as *Enterococcus faecalis* and *Streptococcus sanguis* (26,28,29,58). Although MTA has been shown to have antibacterial effects, the clinician will also have to take into account that the material needs to be mixed to a desired consistency, has difficult handling properties, long setting time and a potential for tooth staining.

Biodentine was launched in the dental market as a 'dentin substitute'. It was formulated using MTA-based cement technology and claims improvements of some of the properties such as physical qualities and handling (59). Compared to MTA, there are very few studies regarding Biodentine's antibacterial attributes. Bhavana found that Biodentine had the largest zone of inhibition against *Streptococcus mutans*, *Enterococcus faecalis*, *Escherichia coli* and *Candida albicans* when compared to commercial glass ionomer cements and MTA (60). In the current study, Biodentine demonstrated antibacterial properties and performed equally compared to the other materials tested.

BC Fast Set Putty is a ready to use, premixed bioceramic material recommended for perforation repair, apical surgery, apical plug, and pulp capping (37). Due to its premixed nature, the material has excellent handling characteristics and is fairly easy to manipulate. Since the fast set putty is fairly new to the market, there is minimal research regarding its antibacterial activity. In the current study, the 1H antibacterial effects of BC Fast Set Putty (mean reduction of 60%) were similar to Sedgley's findings; mean $\log_{10}$ viable counts for BC Regular Set Putty ($4.55 \pm .85$) were significantly lower than for nonexposed controls (7.40 ± 0.3) ($P < .0001$) resulting in a mean reduction of 38%. Although the manufacturer claims a set time of 20 minutes for the fast set putty, the results of this investigation demonstrate that material's antibacterial properties are not negatively affected by early exposure prior to manufacturer's recommended set time.

Out of the group of materials, NeoMTA Plus is the newest bioceramic. Compared to traditional MTA, the tricalcium silicate material is more finely grounded. The handling

challenges that accompany the powder-water mixture of previous generations of MTA are circumvented by mixing NeoMTA Plus with a water-based gel. This allows the clinician to vary the consistency from a puttylike mixture to a thinner viscosity (61). The manufacturer states the indications include the application of this material for vital pulp therapies (pulp capping, pulpotomy or cavity liner/baase), root apexification, root repair (resorption or perforation), root-end filling and sealing of root canals (41). To date, the author is not aware of any published studies in the literature regarding the antibacterial activity of NeoMTA Plus. In the current investigation, NeoMTA Plus exhibited similar antibacterial properties compared to ProRoot MTA, Biodentine, and BC Fast Set Putty. As with the other materials, the antibacterial effect of NeoMTA Plus can most likely be attributed to its high pH (>11.0) (41).

A major limitation of the current *in vitro* investigation was the use of *Enterococcus faecalis* in its planktonic form. Endodontic infections are polymicrobial in nature, with obligate anaerobic bacteria conspicuously dominating the microbiota (62). Due to the depleted nutritional levels and harsh environment within a tooth, the environment favors biofilm formation which can be difficult to eradicate. Furthermore, the buffering capacity of dentin may negatively affect the sustaining high pH produced by the current endodontic repair materials. Future studies using polymicrobial biofilms in an extracted tooth model are needed in order to evaluate the true antibacterial effectiveness of current endodontic repair materials.

Chapter 5

Conclusion

In conclusion, within the limitations of the present study, ProRoot MTA, Biodentine, BC Fast Set Putty, and NeoMTA Plus exhibited similar antimicrobial properties when subjected to a modified direct contact test against *Enterococcus faecalis* strain ATC 29212. All materials demonstrated a significant reduction in bacterial growth compared to nonexposed controls after 48 hours. Flow cytometry analysis provides a convenient alternative method for accurate enumeration of bacteria albeit being cost prohibitive due to the expensive equipment cost and therefore is usually limited to individuals in an academic setting.

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Appendix

Group	Baseline	12_hours	24_hours	48_hours
Pro_Root_MTA	4.221	4.033	3.998	2.919
Pro_Root_MTA	4.599	4.007	3.915	2.437
Pro_Root_MTA	3.424	3.328	2.294	2.360
Pro_Root_MTA	4.977	5.081	3.873	3.329
BC_Fast_Set_Putty	3.604	3.508	2.914	2.626
BC_Fast_Set_Putty	5.572	4.427	3.637	3.376
BC_Fast_Set_Putty	4.794	4.301	3.688	3.030
BC_Fast_Set_Putty	3.232	2.907	2.650	2.589
Biodentine	5.465	4.921	4.042	3.599
Biodentine	5.455	4.788	3.845	2.924
Biodentine	4.175	3.973	3.455	2.874
Biodentine	4.051	3.844	3.140	2.645
Neo_MTA	3.775	3.402	3.238	2.649
Neo_MTA	4.693	4.311	3.879	3.046
Neo_MTA	5.015	5.154	4.247	3.338
Neo_MTA	4.854	4.683	3.332	2.843
Negative_Control	0.046	0.042	0.170	0.270
Negative_Control	0.048	0.059	0.180	0.329
Negative_Control	0.044	0.056	0.251	0.236
Negative_Control	0.037	0.055	0.171	0.171
Positive_Control	10.399	14.931	20.556	27.332
Positive_Control	11.372	16.975	23.762	30.183
Positive_Control	10.034	14.457	24.976	31.786
Positive_Control	11.299	15.609	26.035	29.441

Data collection for statistical analysis.

BD FACSDiva 8.0

Tube: BC 5

Population	#Events
All Events	8,397
Beads	4,894
syto(+)	1,764

Data plot of forward scatter (x-axis) versus fluorescence (y-axis) and computed # of events for BC Fast Set Putty experimental replicate at time 1H.

BD FACSDiva 8.0

Tube: Bio 5

Population	#Events
All Events	8,713
Beads	4,538
syto(+)	2,480

Data plot of forward scatter (x-axis) versus fluorescence (y-axis) and computed # of events for Biodentine experimental replicate at time 1H.

BD FACSDiva 8.0

Tube: Neo 5

Population	#Events
All Events	9,500
Beads	5,635
syto(+)	2,127

Data plot of forward scatter (x-axis) versus fluorescence (y-axis) and computed # of events for NeoMTA Plus experimental replicate at time 1H.

BD FACSDiva 8.0

071618 Time 48h-Pro 5

Tube: Pro 5

Population	#Events
All Events	7,949
Beads	5,868
syto(+)	1,430

Data plot of forward scatter (x-axis) versus fluorescence (y-axis) and computed # of events for ProRoot MTA experimental replicate at time 48H.

BD FACSDiva 8.0

071618 Time 48h-BC 5

Tube: BC 5

Population	#Events
All Events	8,162
Beads	5,527
syto(+)	1,674

Data plot of forward scatter (x-axis) versus fluorescence (y-axis) and computed # of events for BC Fast Set Putty experimental replicate at time 48H.

BD FACSDiva 8.0

071618 Time 48h-Bio 5

Tube: Bio 5

Population	#Events
All Events	8,159
Beads	5,686
syto(+)	1,662

Data plot of forward scatter (x-axis) versus fluorecence (y-axis) and computed # of events for Biodentine experimental replicate at time 48H.

BD FACSDiva 8.0

071618 Time 48h-Neo 5

Tube: Neo 5

Population	#Events
All Events	8,517
Beads	5,184
syto(+)	1,579

Data plot of forward scatter (x-axis) versus fluorescence (y-axis) and computed # of events for NeoMTA Plus experimental replicate at time 48H.