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THESIS APPROVAL PAGE FOR MASTER OF SCIENCE IN ORAL BIOLOGY

Title of Thesis: DEVELOPMENT AND EVALUATION OF A TWO-CHAMBER MODEL FOR THE IN VITRO ANALYSIS OF BACTERIAL LEAKAGE FOLLOWING ENDODONTIC THERAPY

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Master of Science Degree 09-Jun-2023

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DEVELOPMENT AND EVALUATION OF A TWO-CHAMBER MODEL FOR THE *IN VITRO* ANALYSIS OF BACTERIAL LEAKAGE FOLLOWING ENDODONTIC THERAPY

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THESIS SUBMITTED TO UNIFORMED SERVICES UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF ORAL BIOLOGY
IN
ENDODONTICS

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Abstract

Introduction: This *In vitro* study evaluated a two-chamber model for determining the frequency of bacterial leakage in root canal systems. This study evaluated root canals obturated with BC bioceramic sealer and AH Plus resin epoxy sealer in conjunction with gutta percha, when challenged with human saliva. The aim of this study was to determine if the current laboratory-based methods for evaluating bacterial leakage are sufficient. **Materials and Methods**: A model consisting of two chambers, one containing human saliva and one containing brain heart infused broth was evaluated. Two separate evaluation studies were conducted with a total of 35 extracted, single rooted teeth, each containing a single canal system. The teeth were then monitored for bacterial leakage by the formation of turbidity in the BHI broth. Each incident of bacterial leakage as demonstrated by turbidity was recorded. **Results**: 100% of experimental teeth leaked. No statistical analysis was performed due to lack of data. **Conclusion**: Future research should investigate the effectiveness of cold plasma sterilization to bacterial leakage models and strive to improve the physical design of the bacterial leakage apparatus.

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Introduction / Literature Review:

The aim of root canal therapy is the prevention and treatment of apical periodontitis. Successful root canal therapy can only be accomplished if the decontamination of the canal is followed by obturation with an acceptable material that effectively seals and prevents leakage. According to the American Association of Endodontists (AAE) Glossary of Terms, obturation is defined as: "To fill the shaped and debrided canal space with a temporary or permanent filling material (AAE Glossary of Terms)." The goals of obturation in endodontics are not only to fill the root canal space, but to fill it with an appropriate material (1). In order to deliver the most effective and reliable results to our patients, we must decide which obturation material to use based on evidence. This decision is best made when we define our desired requirements of an obturation material (1). It has been demonstrated repeatedly that endodontic chemo-mechanical irrigation procedures cannot produce completely sterile canals (2). In addition, the human mouth can host up to 700 species of bacteria, some of which may be pathogenic and harmful if they enter the canal space (3). Because of this, our obturation material choices should set our treatment up for success via dentinal bonding, dentinal tubule penetrance, antimicrobial activity, and fluid tight sealing, resulting in reduced microbial penetrance and eventual leakage (1). The most commonly used obturation material in endodontics is Gutta Percha (GP). GP will not create an acceptable seal with radicular dentin on its own (4). Therefore, a sealer is necessary to prevent leakage. A

sealer prevents contamination of the root canal and obturation material, leading to apical periodontitis (4).

According to the AAAE Guide to Clinical Endodontics, “Root canal sealers are used in conjunction with a biologically acceptable semi-solid or solid obturating material to establish an adequate seal of the root canal system (AAE Guide to Clinical Endodontics).” Sealers plays a critical role in the success of endodontic treatment, and therefore need to perform in an ideal manner. The ideal properties of sealers have been described by Grossman: tackiness allowing good adhesion to the radicular wall, air and fluid tight sealing, radiographic visibility, ease of use, setting properties with minimal shrinkage or favorable expansion, esthetic compatibility, antibacterial action, low or no solubility, biocompatibility, and ease of removal via common medicaments (5).

The clinical performance of endodontic sealers is best explained by their physiochemical and biological properties (6). These properties will dictate the ability of the sealer to create a bacteria tight seal and determine its biocompatibility (6). Two commonly used endodontic sealers are resin epoxy and bioceramic sealers. For the purposes of this study, the properties of resin epoxy sealers, specifically AH Plus (AHP) (DENTSPLY DeTrey), and Bioceramic Sealers, specifically EndoSequence BC Sealer (BCS) (BC Sealer, Brassler, Georgetown, GA) will be briefly discussed.

Resin epoxy sealers:

In the past, resin epoxy sealers were considered the gold standard for endodontic obturation because of their long history of successful use. According to the manufacturer: AHP has many desirable characteristics such as high radiopacity, little shrinkage upon setting, biocompatibility, and low solubility (AH Plus IFU). These characteristics are important, as the quality of the root canal filing is affected by the shrinkage upon setting and solubility of the materials used (7). According to Marin-bauza et al, AHP has shrinkage at a level that is acceptable by ANSI/ADA standards (8). Although within acceptable limits, this shrinkage results in gap formation between the sealer and dentinal wall (9). The gap formation leaves the canal vulnerable to bacterial contamination, resulting in microbial leakage (1). Regarding biocompatibility, there is conflicting reports of cell cytotoxicity for AHP (6,10). According to Leonardo et al, AHP has good biocompatibility and tissue tolerance when used as an endodontic sealer (10). However, a recent article published by Mann states that AHP displayed sealer toxicity during fibroblast cell viability testing (6). Although there is a long history of success with resin epoxy sealers, and more specifically AHP, there is room for improvement in the physiochemical and biological properties. In pursuit of improvement, bioceramic sealers were developed to address the issues that plague resin epoxy sealers.

Bioceramic Sealers

Bioceramic materials developed for use in medicine and dentistry are tolerated by human tissues due to their inert nature, and ability to incorporate into the tissues (1). The properties of bioceramics mirror the desired properties of endodontic sealers through their antibacterial action and biological compatibility (6). According to Trope, gap formation caused by shrinkage in resin epoxy sealers may be avoided if using bioceramic sealer and bioceramic coated or impregnated gutta percha through bond formation with the radicular dentin (1). The exact mechanism of bioceramic based sealer bonding to root dentin is unknown (7). The leaching of calcium ions from bioceramic sealer provides one possible mechanism by which bioceramic sealers bond with the dentin wall, forming an apatite like connection (11). Candeiro et. al describes a possible explanation for the release of these ions through the mechanism of action of calcium silicate cements during settting (11). Sarkar et. al confirmed the bioactivity of Mineral Trioxide Aggregate (MTA), a calcium silicate bioceramic, and experimentally demonstrated its ability to chemically bond to dentin (12).

The bioceramic sealer that will be investigated in this experimental protocol is EndoSequence BC Sealer (BSC). BCS is a premixed bioceramic composed of zirconium oxide, calcium silicates, calcium phosphate monobasic, calcium hydroxide, filler and thickening agents. (13). BCS demonstrates radiopacity, capacity to penetrate dentin tubules via flowability, antibacterial action via alkaline pH and release of calcium ions, and biocompatibility (11). Unlike other bioceramic materials, BCS is premixed, and therefore

requires hydration from the dentinal tubules in order to become dimensionally stable (13). Therefore, BCS is not overly sensitive to moisture and blood contamination when confronted with these conditions in the canal orifices (1). In fact, BCS has been shown to expand upon setting (14). This is in sharp contrast to the shrinkage that occurs during the setting reaction of resin epoxy sealers. This expansion should in theory provide a fluid tight seal between the obturation material and the dentinal wall. This claim is refuted by Mann, who found that BC Sealer had a higher solubility than is acceptable by ISO standards (6). Because of BC Sealers biological and physiochemical properties, the idea of using it as the solo obturation material is appealing. It may even be possible to forgo the use of GP as an obturation vehicle altogether. As a solo obturation material, BCS could be utilized as a primary monoblock (15). A primary monoblock with a modulus of elasticity approximating that of dentin would strengthen the root canal following obturation (15). The modulus of elasticity of dentin is reported by Osiri et al, as 8.60 $\pm$ 0.86 Gigapascal (GPa)(16). When BCS was used to obturate teeth *in vitro* in conjunction with BC Points gutta percha (BCP), it's modulus of elasticity was reported to be 2.54 $\pm$ 0.13 GPa (16). Unfortunately, these values are not cohesive with the modulus of elasticity of dentin, and therefore do not support the theory of BC Sealer being used as a primary monoblock (15,16). However, Osiri et al, did report that BCS could reinforce prepared roots because of its ability to bond to root dentin regardless of its low modulus of elasticity (16).

In order to test the efficacy of sealers, different leakage tests are employed. Some of these tests include protein leakage, dye leakage, and bacterial penetration. For our study, we chose a bacterial leakage model. Many studies have utilized a monoculture in their leakage models, focusing on tenacious bacterial sources such as *E. faecalis* (17). For our experimental protocol, we wanted to simulate an *in vitro* model that more closely represents the polymicrobial nature of the oral flora. Utilizing commercially available pooled saliva (Lee Biosolutions, Item #991-05-P, Maryland Heights, MO), we will be able to challenge our leakage model with a proliferative bacterial assault.

The aim of this study is to determine if the two chamber bacterial leakage apparatus can be used to determine if there is a significant difference in bacterial leakage as measured by turbidity in root canals obturated with BCS, or BCS and TGP when compared to root canals obturated with AHP and TGP. We hypothesize that the apparatus would be successful and that canals obturated with BCS alone or BCS and TGP will have less bacterial leakage than canals obturated with BCS and TGP, or AHP and TGP. The null hypothesis was that all GP and sealer combinations will demonstrate equal bacterial leakage.

Many issues were identified in the experimental protocol including physical flaws, and possible issues with cold plasma sterilization. Due to these issues, the focus of the experiment shifted to improving the experimental protocol and investigating cold plasma

sterilization for evaluation of the seal of endodontic materials when challenged with oral flora.

Bacterial Viability Study

The bacterial viability study was conducted using a test tube model in order to determine how long the bacteria will remain viable in the growth medium. This viability study determined the interval at which the saliva-BHI solution was replenished in the experimental model in order to ensure continual contamination of the coronal portion of the tooth at an appropriate interval in experimental conditions. Ninety mL of BHI broth was inoculated with 30 mL of saliva. A test tube was then filled from the inoculated BHI broth and placed in a UV spectrophotometer. The initial spectrophotometer reading was .01 nanometers (nM). Saliva was set to be replenished when bacterial levels dropped to below 25% of .01 nM (See Figure 1.1). Based on the readings of the spectrophotometer, the interval to replenish the saliva-BHI solution was set to three days for the experimental protocol.

Evaluation of Pooled Human Saliva

To determine the diversity of the salivary sample, the saliva-BHI solution was plated on chocolate and blood agar plates. The sample yielded both alpha and beta hemolytic streptococci. Additional analysis by light microscopy demonstrated that mostly gram-positive cocci were present in gram staining (See Figure 1.2, Figure 1.3). Based on

these findings, it was determined that the alpha and gamma hemolytic streptococci were able to survive the freezing and thawing process necessary to procure the saliva from a commercial source, and therefore would be challenging our endodontic obturation materials in the experimental model.

Next, the bacteria were tested for aerotolerance with thioglocylate broth (See Figure 1.4). After 24 hours, the broth showed turbidity, indicating the presence of aerotolerant anaerobes.

These bacterial viability studies provided valuable information about the salivary sample that was used to contaminate our bacterial leakage apparatus. The results of the studies proved that the commercially procured saliva could challenge our endodontic obturation materials with a metabolically diverse and polymicrobial flora.

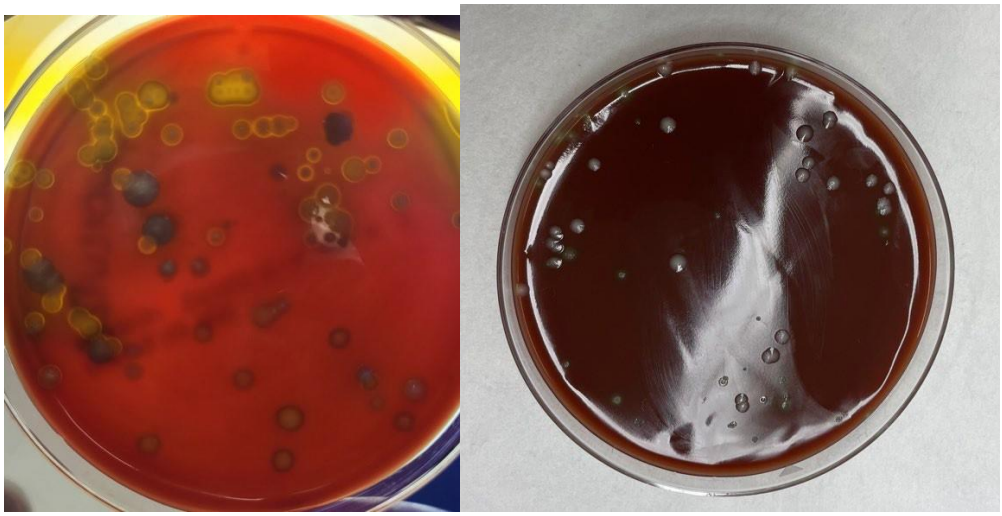


Figure 1.2, 1.3: Saliva cultured on blood agar, (left), and chocolate plates, (right), yielding alpha and beta hemolytic streptococci.

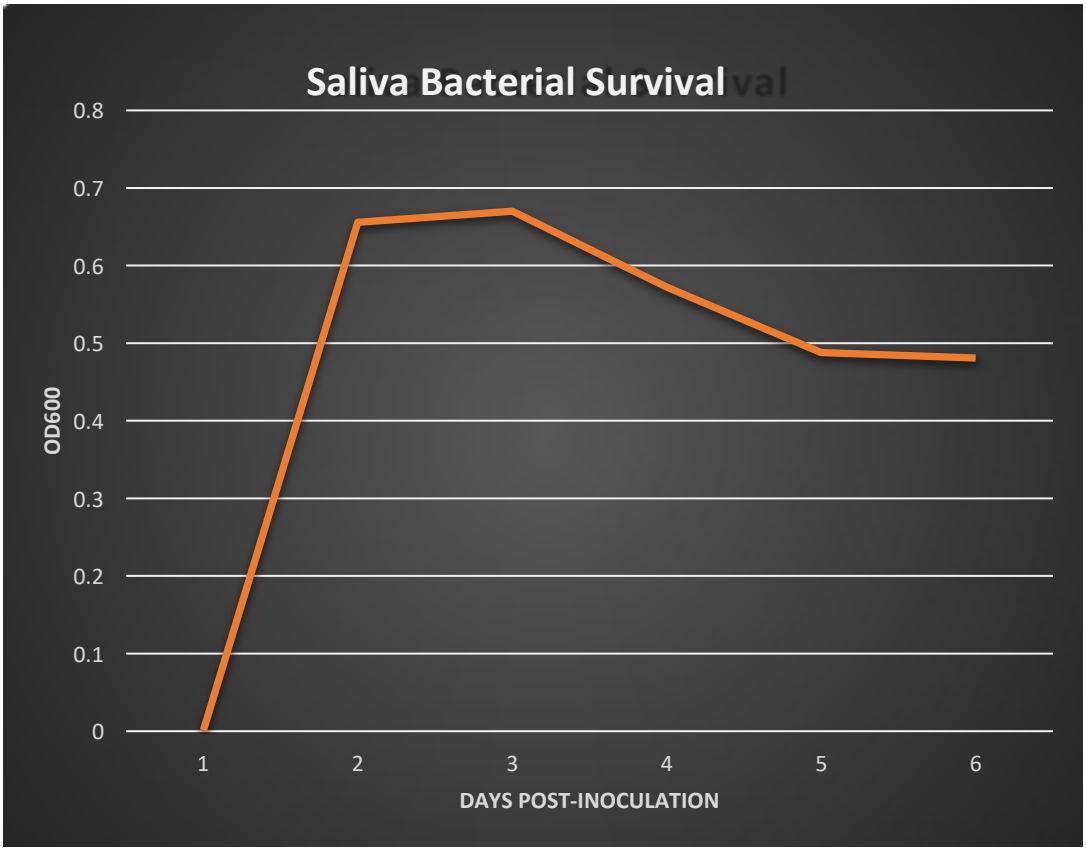


Figure 1.1: OD600 of bacteria throughout 7 days of growth. Optical Density of the saliva-BHI broth sample at a wavelength of 600 nM.

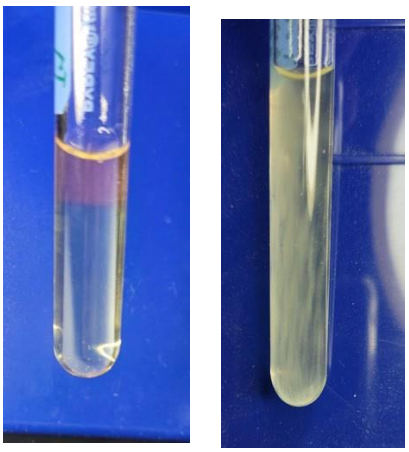


Figure 1.4: Un-inoculated thioglycolate broth showing presence of oxygen in upper third of tube (left). Thioglycolate broth 24 hours after inoculation showing turbidity throughout tube indicating the presence of aerotolerant anaerobes (right)**.**

Methods and Materials

Two Chamber Model Evaluation 1.0

Our experimental model was determined to be exempt from the Institutional Review Board (IRB).

Tooth Selection:

Approximately 250 teeth were collected from private dental offices in accordance with the Fort Gordon Collection of Extracted Teeth Standard Operating Procedures (SOP). Teeth were screened clinically and radiographically to qualify for inclusion in the experiment. Clinically, teeth needed to be mandibular or maxillary incisors or premolars with the appearance of a single canal system and an intact root system from CEJ to apical foramen. Radiographically, images were taken in the bucco-lingual and mesial-distal direction to confirm the presence of a single canal, and lack of aberrant anatomy such as lateral canals, isthmi, accessory and secondary canals. Overall, 192 teeth were eligible for experimental purposes.

Experimental Design:

Nineteen single canal teeth were selected for the evaluation. 3 experimental groups of 5 teeth each were formed, with one group of 2 positive control teeth, and one group of 2 negative control teeth. Group 1: Obturation with BCS. Group 2: Obturation with TGP and BCS. Group 3: Obturation with TGP and AH Plus. The positive control teeth were instrumented, but not obturated. The negative control teeth were un-accessed, un-instrumented, and coated completely with nail varnish. Each group was designated using randomization by Microsoft Excel. All teeth were decoronated using sterile diamond burs (NeoDiamond Football 150 micrometer coarse grit, Patterson Dental, St Paul, MN) at the level of the CEJ. All teeth were prepared by the same operator using a standardized instrumentation, irrigation, and obturation technique. Patency was determined by the passage of a standard size 10 stainless steel K file (Henry Schein, Melville, NY) through the apical foramen before and after completion of instrumentation. Working Length was determined by subtracting 1 mm from the length determined by the passage of a stainless-steel K file through the anatomic apical foramen. Teeth were instrumented utilizing a crown down protocol. Hand instrumentation was utilized to a size 10 and 15 stainless steel K file to working length, followed by rotary instrumentation with vortex blue (Dentsply Sirona, Charlotte, NC) nickel titanium files and profile (Dentsply Sirona, Charlotte, NC) nickel titanium files to one size larger than the first file to bind at working length, or maximum ISO 80.04.

The canals were irrigated with 1 mL of 8% NaOCl (Clorox, Oakland, CA) between each hand file and rotary file. The final rinse consisted of 3 mL of 8% NaOCl followed by 3 mL of 17% Ethylenediaminetetracetic Acid (EDTA) (Benco Dental, Pittson, PA), followed by 3 mL of 8% NaOCl. All teeth were obturated by the same operator. Group 1 was obturated using the syringe supplied with the BCS packaging. All teeth in Group 1 obturated with BCS were confirmed radiographically to be complete and free of voids. All other experimental groups were obturated using warm vertical compaction. All sealers were used according to manufacturer's instructions. A Kerr Elements (Kerr Endodontics, Brea, CA) heating tip was used to sear the gutta percha master cone below the orifice level. Standard gutta percha condensers were used to condense the gutta percha apically. A Kerr Elements gutta percha delivery handpiece was used to incrementally backfill gutta percha to the orifice level. In order to ensure occlusion of all lateral canal systems, the outside of the middle third of each tooth was coated in nail varnish (LA Color Last, Ontario, CA) leaving the coronal and apical 2 mm uncovered. Obturated teeth were stored for three weeks at 68 degrees F in Dulbecco's Phosphate Buffered Saline (DPBS) (Atlanta Biologicals, Atlanta, GA) to ensure setting of the sealer.

Microbial Leakage Apparatus

The two-chamber microbial leakage apparatus used as our experimental model was modified from an experimental protocol described in a previous study (16). A 10 mL

Wheaton Tubular Serum Glass vial (DWK Life Sciences, Millville, NJ) was used with a closure modified by punching a hole in the rubber stopper. A 1.5 mL plastic Eppendorf centrifuge tube (Fisher Scientific, Hampton, NH) was modified to fit the tooth within the modified tube, so that the apical portion of the tooth extended through a portal of exit at the bottom of the tube, with the coronal portion of the tooth held within the lumen of the tube (See Figure 2.1, Figure 2.2). The tooth/Eppendorf centrifuge tube/rubber stopper interface was sealed on both sides of the stopper with industrial grade silicone (Henkel Corporation, Rocky Hill, CT) to prevent leakage from the coronal chamber of the dual chamber system to the apical chamber. The testing apparatus was sterilized via cold plasma sterilization to ensure sterility before commencement of the experimental protocol. The glass assay vial was filled with 11 mL of BHI broth so that the apical 2 mm of the root was immersed in the broth (Figure 2.3). The bacterial leakage apparatus was sealed to the glass vial with industrial grade silicone. The coronal portion of the Eppendorf centrifuge tube was filled with 1.5 mL of a 1:3 (v/v) ratio of a solution composed of commercially obtained human saliva (30 mL) mixed in BHI broth (90 mL). This 1.5 mL increment was removed and replaced every three days, as determined by the bacterial growth viability study. The system was incubated at 37 C° in a CO_2 incubator and checked daily for the appearance of turbidity in the apical BHI broth over 14 days. This study was originally planned for a 45-day duration, however due to contamination of the study samples, the duration of the study was cut short, and was terminated on Experimental Day 14.

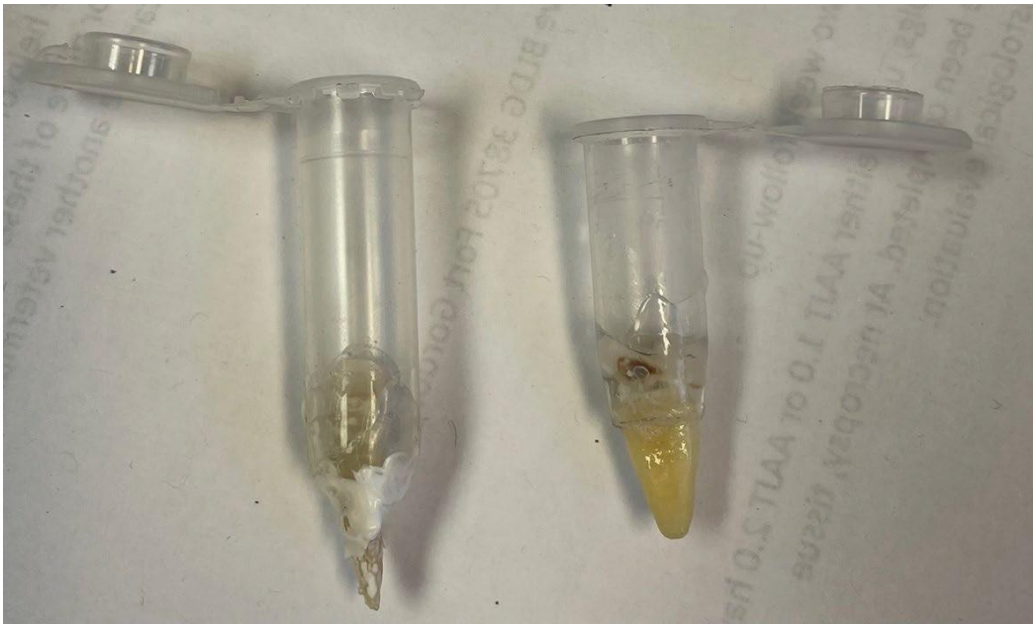


Figure 2.1: Plastic Eppendorf centrifuge tube modified and sealed with industrial silicone (left) and cyanoacrylate (right).

Figure 2.2: Bacterial leakage apparatus without experimental tooth.

Figure 2.3: Bacterial leakage apparatus sealed with silicone.

Results:

The two-chamber model evaluation yielded immediate contamination of the negative control samples. This resulted in negation of the experimental model. Experimental samples were fed on Experimental Day 0, 3, 6, 9, and 12. The experiment was concluded on Day 14, following the confirmation of contamination of the negative control samples via culture, plating and gram staining. Even though the negative samples were immediately contaminated, the experiment was conducted through Day 14, and results are given in Table 1.0.

	Experimental Days													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
GROUP 1: BCS		**1,2**						**5**					**3**	**4**
GROUP 2: TGP + BCS		**2**				**5,4,1,3**								
GROUP 3: TGP + AH PLUS		**5**	**3**			**1**							**4,2**	
POSITIVE CONTROL		**2**					**1**							
NEGATIVE CONTROL	**1,2**													

Table 1.0: Experimental Group vs. Experimental Day of Observed Turbidity. Negative Controls showed white sediment later identified as bacteria via culturing and gram staining, verifying contamination.

Statistical Analysis

No statistical analysis was completed, due to insufficient data.

Discussion:

The results of the first two-chamber model highlighted flaws in the construction of the bacterial leakage apparatus. One flaw that was immediately identified, was the lack of a window in which sterilization of the bacterial leakage apparatus was confirmed prior to the commencement of the experiment. In other bacterial leakage models, the

sterilization of the bacterial apparatus was confirmed prior to commencement of the experiment by incubation of the apparatus with BHI Broth growth medium for a predetermined time and set temperature (18). In our study, all portions of our experimental model were sterilized separately by cold plasma sterilization before the construction of the leakage apparatus. The teeth were placed inside Eppendorf centrifuge tubes and sealed with industrial grade silicone. The teeth / tube apparatus, rubber stoppers and glass vials were all sterilized separately prior to construction. Immediately upon assembly of the bacterial leakage apparatus, issues of potential contamination and lack of aseptic technique were identified. Even though the bacterial leakage apparatus was constructed under a laminal flow biosafety cabinet, sterile gloves and sterile instruments were not used during construction and manipulation of components of the apparatus. Additionally, prior to assembly, the teeth were not adjusted to a uniform length, and therefore a predetermined level of BHI broth was not calculated to ensure the required submersion of the apical 2 mm of the experimental teeth. As a result, during the construction of the apparatus, when placing the Eppendorf centrifuge tube through the rubber stopper, the height had to be adjusted many times to ensure that the apical 2 mm of the tooth was submerged appropriately in growth medium. This repeated manipulation may have resulted in contamination of the bacterial leakage apparatus. The cold plasma mechanism of sterilization and continued sterility of the leakage apparatus following construction was not challenged prior to beginning the

experiment due to the lack of incubation of the apparatus while submerged in BHI broth for a predetermined time interval. Success of our sterilization technique was assumed, and due to immediate turbidity observed in many samples, it was impossible to determine if the bacterial growth was due to failure of the cold plasma sterilization, contamination during assembly, or failure of the sealing material.

The experimental design was based on the leakage apparatus described by DeDeus in his 2007 article investigating sealing ability and obturation techniques in oval shaped canals (18). Although trying to mimic a successful experimental model, our experimental model was faced with many challenges. First, the Eppendorf centrifuge tube was sealed by a hinged, locking lid. This was intended to be a mechanism by which easy access could be achieved while refreshing the salivary challenge, but instead proved to be problematic. In order to open and close the lid, cotton plier forceps had to be employed to stabilize the Eppendorf centrifuge tube. If cotton plier forceps were not used to stabilize the tube, the torque created when opening and closing the lid disrupted the silicone seal between the Rubber stopper and the glass vial. Even though all re-applications of saliva were completed under sterile conditions, the disruption to the seal may have been a potential source of contamination.

Although an Eppendorf centrifuge tube was used successfully in the DeDeus study, the use of an Eppendorf centrifuge tube proved to be problematic in our experimental

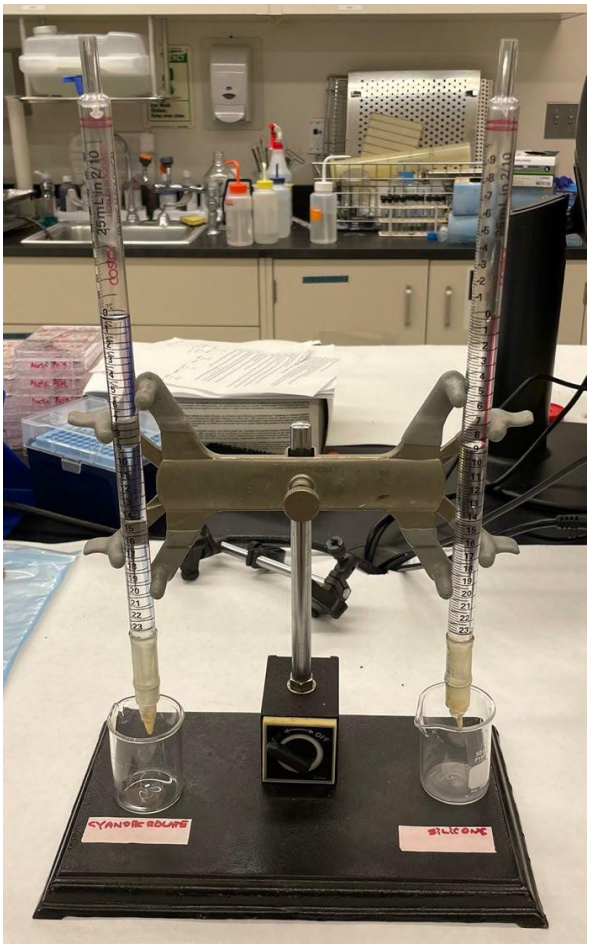
model for many reasons (18). The chemical composition of the Eppendorf centrifuge tube is polypropylene (19). Because of polypropylenes inability to bond with cyanoacrylate, we needed to consider an alternative sealing material. In other experimental models, the bacterial leakage apparatus was sealed successfully with various other sealants including industrial grade silicone and resin(20)(21)(22)(23). As a result, silicone was selected to seal our experimental model in lieu of cyanoacrylate.

Many issues with the experimental model, construction of the leakage apparatus, and sealing materials were identified in the first study. To address these issues, and to confirm the validity of our experiments, a second model evaluation study was undertaken.

Two Chamber Evaluation 1.0 (a)

As previously mentioned, the silicone seal at the rubber stopper and glass vial interface was disrupted during opening and closing of the Eppendorf centrifuge tube lid while replenishing saliva over the duration of the experiment. Additionally, since there was immediate turbidity of select samples, the integrity of the silicone / tooth / Eppendorf Centrifuge tube seal was in question. Due to these issues, Super Glue Corp (Rancho Cucamanga, CA) resin adhesive was tested against silicone in an additional study to determine if a better seal could be achieved. Two teeth were fitted into Eppendorf centrifuge tubes and sealed with either Super Glue or industrial grade silicone. The

locking lid of the Eppendorf centrifuge tube was removed, and the teeth were mounted and connected to a 25 mL pipette. Twenty-five mL of water was placed inside the pipette, placing a gravitational pressure on the seal (See Figure 3.1). The silicone assembly immediately started to leak, but the Super Glue assembly did not demonstrate any leakage. This small study was the basis for the decision to use Super Glue as the sealant of choice for the second evaluation, replacing the silicone.



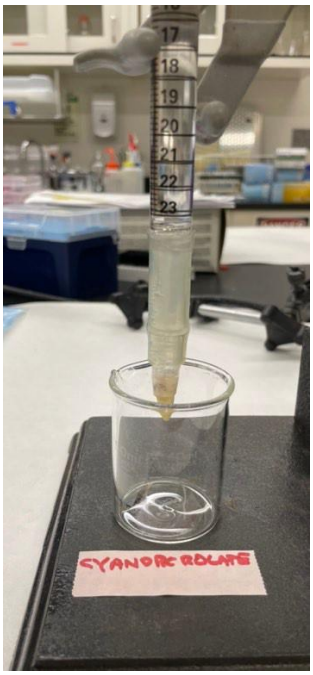


Fig 3.0, 3.1: Super Glue versus Silicone leakage study. No leakage detected with Super Glue, Silicone had immediate leakage.

Two Chamber Model Evaluation 2.0

For the second evaluation, it was important to address the issues identified by the first evaluation. In order to address the materials issues, Super Glue (cyanoacrylate) was used as a sealer instead of industrial grade silicone. To address lack of aseptic technique, a strict sterile technique was implemented to include assembly of the leakage apparatus under a bio-safety cabinet, as well as use of sterile gloves and forceps for manipulation. To improve issues with sterilization and apparatus construction, once teeth were mounted into the Eppendorf centrifuge tube, teeth were sealed with Super Glue and

examined underneath a microscope to ensure the integrity of the tooth/Eppendorf centrifuge tube interface. The teeth were then pre-fitted to the rubber stopper and sealed before sterilization. The height of BHI to be allocated in each glass vial was pre-determined by fitting each individual tooth/rubber stopper/Eppendorf centrifuge tube apparatus to the vials and measuring the height that would allow the apical 2 mm of the experimental teeth to be submerged. These changes contributed to decreasing manipulation during assembly of the bacterial leakage apparatus following sterilization. With these changes, our hope was to improve the model and provide proof of concept, allowing us to develop a model that can be effectively used for bacterial leakage investigations.

Materials and Methods

Sixteen single rooted teeth were selected for the second study. Three experimental groups of 3 teeth were formed, each with one group of 2 positive control teeth, and one group of 2 negative control teeth. The experimental teeth were de-coronated, instrumented and obturated in the same fashion as the teeth in the two-chamber evaluation 1.0.

Results

Experimental Results

Unfortunately, immediately after inoculation of our experimental teeth with saliva-BHI Broth our negative control teeth exhibited turbidity. Because our negative controls were eliminated, the results of our study were effectively negated. All samples demonstrated turbidity within a 14-day period, further demonstrating a failure of the model (Table 2.0).

	Experimental Days													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
GROUP 1: BCS						**2**		**3**						**1**
GROUP 2: TGP + BCS					**2**		**3**	**1**						
GROUP 3: TGP + AH PLUS			**2**				**1**							**3**
POSITIVE CONTROL					**1**		**2**							
NEGATIVE CONTROL	**1,2**													

Table 2.0: All samples demonstrated turbidity by experimental day 14.

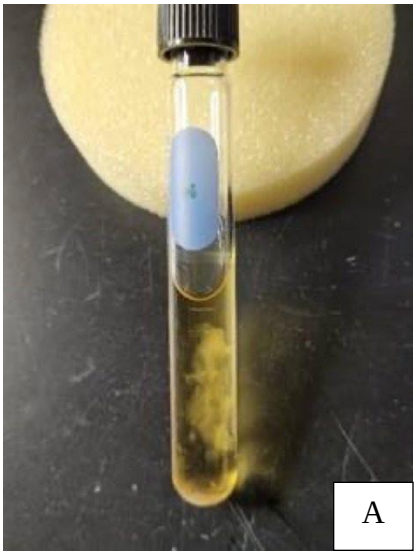
The results of the experiment were insignificant and were unsuccessful in verifying our experimental model.

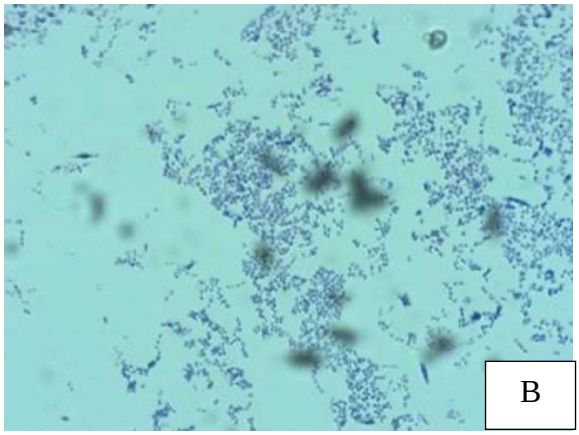
Statistical Analysis

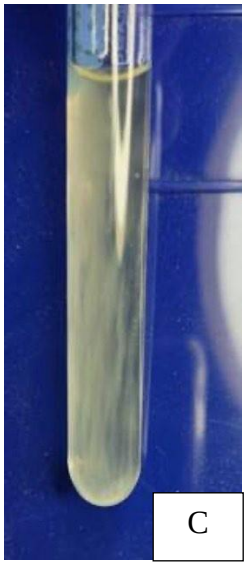
No statistical analysis was completed, due to insufficient data.

Bacterial Analysis

Following the failure of our samples in the second evaluation, the turbid broth was sampled and plated, which allowed us to confirm the types of bacteria present in the salivary sample. Because we had determined the composition of our salivary sample, we were able to compare the results of bacteria present in the turbid broth to the bacteria from the saliva that was analyzed directly before the first study. We were able to confirm that the bacteria present in the turbid broth was in fact, the same as the bacteria from our salivary sample. This bacterium contained great microbial diversity including gram positive and negative microbes (Figure 2.B), hemolytic and non-hemolytic microbes (2.D), as well as the presence of aerobic and anaerobic microbes (Figure 2.A, 2.D).







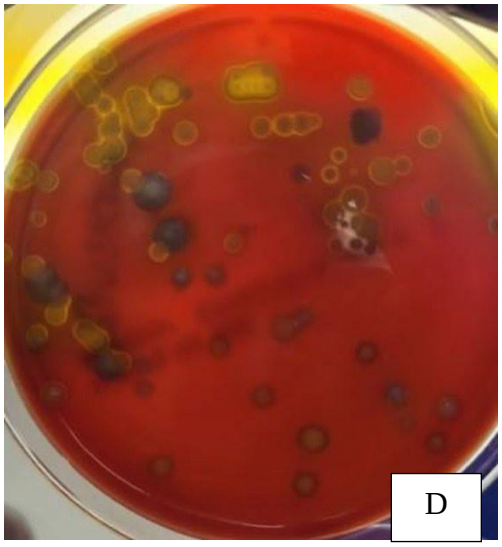


Figure 2A: Flocculent growth just below the surface of the media indicating possible aerobic and aerotolerant microbes.

Figure 2B: Gram positive and gram variable organisms in the turbid BHI broth.

Figure 2C: Presence of turbidity in a thioglycollate medium demonstrating the presence of anaerobes.

Figure 2D: A blood agar plate of the saliva after 24 hours growth demonstrating the diversity of hemolytic and non-hemolytic colony types.

Discussion

In the Two Chamber Model Evaluation 2.0, we implemented many changes in hopes of improving our experimental model. One of the first changes was an attempt to verify the cold plasma sterilization mechanism before adding our first application of saliva and commencing our experiment. Additionally, our aseptic technique was enhanced during construction of the leakage apparatus by using sterile gloves and forceps underneath the laminar flow bio-safety cabinet. To verify sterility following construction of the apparatus, the leakage apparatus was incubated for 7 days at 37° C and 100% humidity in a CO_2 incubator. After the incubation period, no samples had demonstrated turbidity which indicated sterility in our model. However, after commencement of our experiment, all our samples demonstrated turbidity by 14 days. It is possible that had we extended our incubation period to 14 days, that turbidity may have been demonstrated before the addition of the saliva. Therefore, it is impossible to rule out contamination of the teeth due to lack of effectiveness of plasma sterilization. It is possible that the only source of contamination in our experimental model occurred during construction, or due to an insufficient seal, but because of the short duration before turbid growth in the BHI Broth, all possible contamination mechanisms must be considered. In many bacterial leakage studies, the method of sterilization reported is the overnight application of ethylene oxide or dioxide gas (18) (24) (21) (23). Due to the lack of access to this

mechanism of sterilization, our option was cold plasma sterilization.

Plasma is a state of being of matter, which is neither solid, liquid or gas (25). Plasma sterilization creates plasma from various gasses at either low or high temperatures (25). The state of plasma contains reactive species that lends it capable of disinfection and sterilization (25). There are different types of plasma disinfection and sterilization depending on the temperature of the particles within the plasma, the power source utilized to charge the plasma, and the type of gas utilized (25). Non-thermal or cold plasma sterilization with oxygen, argon or various other gasses has been shown to be effective for surface disinfection of medical and dental devices (25). Non- thermal plasma, or cold plasma, can be used to disinfect medical devices through various mechanisms including direct contact of plasma to device, or plasma activated mediums that are then bathed on the medical devices (25). Plasma has been shown to inactivate bacteria, fungi and viruses, as well as effectively inactivate toxins (25). There is question however, as to whether it is effective against certain viral species such as virus's encased in envelopes, and bacteria encased in biofilms (25). The inactivation mechanisms by which plasma works are variable and depend on the type of gas used, but generally cause the production of reactive oxygen species and electrical charge which ultimately disrupts bacterial, fungal and viral activity and inactivates toxins (25). When plasma is discussed in literature, it is discussed as a disinfector/sterilizer. The Centers for Disease Control and Prevention (CDC) defines disinfection as "a process that eliminates many or all pathogenic

microorganisms, except bacterial spores, on inanimate objects". The CDC defines sterilization as "a process that destroys or eliminates all forms of microbial life". There is a significant difference between these two definitions, and to use them together to describe cold plasma sterilization is confusing. It is unclear if all forms of cold plasma sterilization provide complete sterility, or if it instead only disinfects objects that it encounters.

There are some examples of cold plasma sterilization being utilized in dental literature, but most have tested the efficacy of a single microbe such as *E. faecalis* instead of a polymicrobial model which is consistent with our evaluation as well as *in vivo* oral conditions (26). For example, in an *in vivo* model low pressure cold plasma oxygen emission was able to effectively sterilize *E. faecalis* on glass slabs (26). Another study demonstrated the use of argon gas to effectively sterilize titanium implant surfaces that had been previously infected (27). Although promising, a glass slab and titanium implant are not comparable to a permeable tooth containing dentinal tubules and variable porous anatomy such as lateral and accessory canals. Another more applicable study looked at the effectiveness of cold plasma sterilization with oxygen and argon gas in extracted teeth that had been cleaned, shaped, and autoclaved (28). The experimental teeth were then incubated with *E. faecalis* to form biofilms and exposed to cold plasma sterilization (28). This experiment found that cold plasma sterilization could possibly fully eradicate *E. faecalis* biofilms (28). Although this model was closer to our experimental model, the

teeth were unobturated, allowing the plasma to interact directly with the surfaces that were contaminated. Despite the differences in the experimental models in the literature and our experiment, the literature did support plasma sterilization as an effective mechanism for the disinfection/sterilization of our experimental teeth (27) (28) (29) (25). As mentioned above, a significant difference in both the experimental examples and our evaluation lies in the ability of the plasma to contact all surfaces of our experimental model directly. In the articles referenced, the cold plasma was in direct contact with the surface in which sterilization was desired. For our pilot study, our teeth were cleaned, shaped and obturated, closing off the internal portion of the tooth to sterilization. Because of the configuration of our bacterial leakage apparatus, it was impossible for the plasma to come into contact with all surfaces of our experimental model. It is possible that if we had used another mechanism of sterilization such as ethylene oxide or dioxide gas that this issue may have been addressed. It is well known that endodontic chemo mechanical preparation does not fully eradicate microbes (2). As such, it is possible that the chemo mechanical preparation of the endodontic teeth was ineffective at eradicating all the microbes from the canal space, and the dentinal tubules which were potentially infected with bacteria. There may have also been biofilms present in the experimental teeth that were infectively penetrated by the NaOCl storage medium or irrigation procedures followed in the experimental protocol. Therefore, it was essential to the

experimental model that the mechanisms of sterilization following the preparation of the experimental teeth was effective in order to fully examine the effectiveness of the endodontic sealer against microbial penetration when exposed to oral flora via salivary contamination.

Not only was the mechanism of sterilization in question following the failure of evaluation study 2.0, but also the materials chosen for the experimental model were now in question. Due to the issues encountered with using industrial grade silicone in the first evaluation, Super Glue was substituted as the sealant material for evaluation 2.0. Cyanoacrylate has demonstrated successful use in many experimental models in various literature references (18). Unfortunately, the use of cyanoacrylate in our experimental model was fraught with issues. First, due to the size of the lumen of our Eppendorf centrifuge tube and the ovoid form of our experimental teeth, there were large gaps between the tooth and the Eppendorf centrifuge tube that required many applications over time of cyanoacrylate to occlude the gap. Although inspection of the junction between the tooth and the Eppendorf centrifuge tube was examined under a microscope, it is possible that the seal may have contained air bubbles or incorporated bacteria that was unsuccessfully eliminated via the plasma sterilization process. One variable that was not considered, is the inherent lack of sterility of the Super Glue itself. Although it is reasonable to assume that there would be little if any bacteria inherently present in the Super Glue, it is not a sterile product, which may have contributed to contamination

during the construction of the bacterial leakage apparatus. Like in the first evaluation, the junction between the tooth and Eppendorf centrifuge tube was sealed, as well as the junction between the rubber stopper and the glass vial. During construction of the bacterial leakage apparatus, it was observed that excess cyanoacrylate was expressed into the BHI broth in some of the experimental samples. This accidental expression of cyanoacrylate may have contributed to contamination of the BHI broth. Additionally, the cyanoacrylate formed a film on the outside of the glass vial creating a fog that was later removed with acetone. This fog was necessary to remove, as its presence interfered with our ability to identify turbidity in our bacterial samples. Due to the necessity of removing the fog, excessive manipulation of the apparatus may have been a source of bacterial contamination.

It was theorized that the cyanoacrylate would have a more tenacious bond between the rubber stopper and glass vial than the industrial grade silicone had demonstrated. However, the bond was still insufficient to withstand the forces of the opening and closing of the Eppendorf centrifuge tube when replenishing the saliva over the course of the experiment. The seal between the rubber stopper and the glass vial was disrupted in select samples despite reinforcing the Eppendorf centrifuge tube with cotton plier forceps to reduce torquing forces while opening the Eppendorf centrifuge tube.

Overall, the inability of cyanoacrylate to seal the junction between the tooth and

the Eppendorf centrifuge tube in one application, the lack of inherent sterility, the incompatibility with the glass vial and the lack of a tight seal between the rubber stopper and glass vial proved to be problematic to our experimental model and provided several sources of potential bacterial contamination. Because of these failures, we are currently pursuing a method by which we can confirm a seal between the tooth and the Eppendorf centrifuge tube before and after assembly, and before sterilization.

Conclusions

There are inherent flaws in bacterial leakage experimental models that have been demonstrated throughout multiple literature references. In fact, the Journal of Endodontics, which is the official journal of the American Association of Endodontists quit accepting bacterial leakage studies for publication. It may be questioned as to why we chose this method to evaluate the sealing efficacy of bioceramic sealers as a solo obturation material.

Had our model proven successful and capable of delivering statistically significant results, our findings could have been viewed as a foundation which could be extrapolated to an *in vivo* model utilizing live animals in the future, and depending on the results of those studies, into a human model as well. The development of a bacterial leakage study was viewed as a necessary first step to obtaining validity for to the use of bioceramic sealer as a solo obturation material before implementation of this obturation method in

a publishable experiment. Although our experimental model ultimately failed, much was gained from the failures and can be translated into the success of projects taken on by future residents choosing to continue this research. Recommendations for future directions include changes in the model design that eliminate the snapping lid of the Eppendorf centrifuge tube, utilizing a mechanism that allows for the removal and replenishment of the experimental saliva-BHI Broth without torquing forces. The reduction in torquing forces would reduce torque on the seal, and theoretically reduce the chances of contamination through manipulation of the leakage apparatus over the duration of the experiment. Additionally, implementation of a strict sterile technique in all interactions with the experimental model should be enforced. In order to eliminate the variable of cold plasma sterilization, utilization of ethylene oxide sterilization or another proven mechanism of sterilization is recommended.

In conclusion, the use of a two-chamber bacterial leakage model is an exciting and viable research topic that may be pursued through various research channels with appropriate implementation and modification of the publicly available protocols.

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