Efficacy of a Novel Microelectronic Device Against an Endodontic Biofilm in a Tooth Model

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THESIS

Submitted as partial fulfillment of the requirements
for the degree of Master of Science in Oral Sciences in the
Graduate College of the University of Illinois at Chicago, 2019

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ACKNOWLEDGEMENTS

I would like to thank my thesis committee. Thank you, Dr. Xie, for advising me throughout this process and for being such a great mentor. Thank you, Dr. Mathew, for planting the seed for this research project, for helping me to gain an understanding of the relevant scientific principles, and for your constant enthusiasm. And thank you, Dr. Johnson, for your always steady presence and for your help in focusing this project and reviewing the thesis drafts.

I would also like to thank Dr. Wu, for your help in structuring and focusing the experiment, and for allowing us to use your lab. Thank you, Dr. Jin, for being so helpful in all stages of carrying out the experiment, from the beginning to the end. And thank you, Dr. Li, for also being so helpful in all stages of the experiment and for your help in advising the experiment and analyzing the results.

Finally, thank you to my beautiful wife, Catherine, for putting up with me these past two years through the long days and long hours, and for supporting me in my goal of becoming an endodontist and attaining my masters. You are truly amazing and I could not have done this without you.

EHS

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ANOVA	Analysis of Variance
CE	Counter electrode
CFU	Colony forming units
EAL	Electronic apex locator
EC	Electrochemical disinfection
EPT	Electronic pulp testing
NSRCT	Nonsurgical root canal treatment
OCP	Open circuit potential
PBS	Phosphate-buffered saline
PPI	Positive pressure irrigation
PUI	Passive ultrasonic irrigation
RE	Reference electrode
WE	Working electrode
WL	Working length

SUMMARY

This study was a follow-up on a previous experiment which showed a statistically significant reduction in bacterial viability when using electrochemical disinfection on a single-species bacterial biofilm cultured in a 96-well plate. The purpose of this study was to investigate the effectiveness of electrochemical disinfection when using a tooth model cultured with a mixed-species bacterial biofilm.

A total of 60 single-canal permanent teeth were cut to 15mm in length and cultured with a mixed-species biofilm containing *Enterococcus faecalis, Fusobacterium nucleatum*, and *Porphyromonas gingivalis*. They were then divided into five groups. Group 1 tooth samples were irrigated with phosphate-buffered saline (PBS), group 2 tooth samples were irrigated with PBS in combination with electrochemical disinfection (EC), group 3 tooth samples were irrigated with 1.5% NaOCl, group 4 tooth samples were irrigated with 1.5% NaOCl in combination with EC, and group 5 tooth samples were irrigated with 6% NaOCl.

There was a statistically significant 80.7% reduction in bacterial viability in group 2 compared to group 1. There were no statistically significant differences with respect to bacterial viability between groups 2 - 5.

EC was found to be effective in reducing bacterial viability in a tooth model when used with PBS; however, no statistically significant bacterial viability reduction was identified when EC was combined with NaOCl compared with EC alone or NaOCL alone.

I. INTRODUCTION

1. The Role of Microbiota and Bacterial Biofilms in Endodontic Infections

In the field of endodontics, it has been established that the presence of microorganisms in root canals is the primary cause of pulpal disease and apical periodontitis. Bacteria have long been thought to be a primary culprit for disease (Kakehashi *et al*., 1965; Möller *et al*., 1981), but more recently other microorganisms, such as yeasts in the form of Candida and Saccharomyces (Lana *et al*., 2001) and viruses such as the Epstein-Barr virus (EBV) and human cytolomegavirus (HCMV), have been found to play major roles as well (Sabeti *et al*., 2003). The bacterial inhabitants of infected root canal systems consist primarily of gram-negative obligate anaerobes, such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum,* and gram-positive facultative anaerobes such as *Enterococcus faecalis* (Sundqvist 1994). Resolution of apical periodontitis is dependent on the removal of bacteria in root canals (Basmadjian *et al*., 2002). Bacteria are primarily organized in biofilms on the walls of root canals (Svensater and Bergenholtz 2004). A biofilm is defined as a sessile community of cells attached to a surface and intertwined with a polysaccharide matrix. When organized in a biofilm community, bacteria are able to efficiently communicate with each other through quorum sensing and undergo advantageous metabolic and phenotypic changes, all of which fortify these bacteria against easy removal compared to their planktonic counterparts (Donlan 2002). Instrumentation of canal walls with both hand instruments and powered-files have been found to significantly reduce bacterial viability in canals (Dalton *et al*., 1998), but instrumentation alone leaves 35% or more of the canal walls untouched due to isthmuses, ovoid canals, and inaccessible accessory and lateral canals (Peters *et al*., 2001).

Moreover, bacteria are known to inhabit the dentinal tubules, out of the reach of traditional instrumentation techniques (Stuart *et al.,* 2006).

2. Current Methods of Canal Disinfection

Given these hurdles thwarting disinfection by strictly mechanical means, a chemomechanical approach has been the accepted method of root canal disinfection (Shuping *et al.,* 2000), with high strength sodium hypochlorite (NaOCl) being found to be the most effective irrigant due to its properties of tissue dissolution (Hand *et al.,* 1978), broad-spectrum antibacterial effects (Zehnder 2006), and ability to disrupt bacterial biofilm (Spratt *et al.,* 2001).

However, the same properties that make NaOCl a highly effective irrigant also make it particularly cytotoxic (Gernhardt *et al*., 2004). With a predominance of anaerobic bacteria being found in the apical 5mm of a root canal (Baumgartner and Falkler 1991), efficient exchange of irrigation in the apical third is imperative (Chow 1983), which requires enlargement of a root canal apex to at least a size 35 file (Salzgeber and Brilliant 1977). Excessive irrigation pressure and increased proximity to the apical foramen can lead to extrusion (Mitchell and Yang 2010), leading to post-operative pain and, on rare occasions, a hypochlorite accident (Hülsman and Hahn 2000). Moreover, bacteria can inhabit up to 650 μm deep into dentinal tubules (Zou *et al*., 2010), out of the reach of NaOCl expressed through a syringe (Wong and Cheung 2014).

Adjunctive tools and methods have been developed over the years to increase the efficacy and safety of NaOCl. Increasing the temperature of NaOCl has been found to result in enhanced tissue dissolving ability due to improved reaction kinetics (Cunningham and Balekjian 1980). Sonic activation, most commonly associated with the EndoActivator™ (Advanced Endodontics, Santa Barbara, CA, USA), facilitates penetration of irrigant into isthmuses and deeper into dentinal

tubules (Townsend *et al*., 2009) through the placement of a plastic tip vibrating at up to 166 Hz near working length (WL), resulting in less extrusion compared to positive pressure irrigation (PPI). Some studies have shown, however, that passive ultrasonic activation (PUI), which involves the vibration of a file at a much higher frequency (up to 30 kHz), cleans canals significantly better than sonic activation (Wiseman *et al*., 2011). The vibration of a file at such a high frequency causes acoustic microstreaming, which creates shear stresses on the canal walls, helping to remove debris and microbiota in its path (Van der Sluis *et al*., 2007). Ultrasonically-activated NaOCl has been found to result in significantly cleaner canals and isthmuses compared to PPI (Goodman 1985).

The EndoVac™ (Kerr Dental, Orange, CA, USA) is another adjunctive tool for root canal disinfection. Rather than projecting irrigant from a needle placed near WL, the EndoVac instead uses negative pressure, drawing down irrigant from the pulp chamber to the apical third of a canal through a micro-cannula. Compared to PPI, it has been found to result in superior cleanliness of the apical third (Siu and Baumgartner 2011), less extrusion (Mitchell and Yang 2011), and less post-operative pain (Gondim *et al*., 2010). As with PPI, it requires enlargement of a canal to at least a size 35 file in order for the micro-cannula tip to be able to reach WL.

A recent development in the field of endodontics is the GentleWave® System (Sonendo, Inc, Laguna Hills, CA), which utilizes "Multisonic Ultracleaning Technology" to comprehensively clean entire root canal systems, including previously inaccessible isthmuses and accessory canals. A high-volume of degassed 3% NaOCl, 8% ethylenediaminetetraacetic acid (EDTA), and saline solution is projected into the pulp chamber of a tooth, creating a cavitation cloud that results in an "implosion of thousands of microbubbles [which] creates an acoustic field of broadband frequencies that travels through the procedure fluid into the entire root canal system" (Sigurdsson

et al., 2017). The powerful shear force that is created enables efficient debridement of the canal walls and results in significantly faster tissue dissolution compared to PPI (Haapasalo *et al*., 2014). Furthermore, the slightly negative-pressure system ensures minimal extrusion of irrigant (Haapasalo *et al*., 2016). Drawbacks of the GentleWave System include the high initial and ongoing operating costs in addition to the extra time and effort required to fabricate a customized "platform" and run the GentleWave Procedure. While the theory behind the GentleWave System and the results of the initial *in vitro* studies are encouraging, long-term studies are not yet available to justify universally the high costs and extended treatment times.

Despite the many advances in root canal disinfection protocol, it is understood that, while non-surgical root canal treatment (NSRCT) overall has a very high success rate, the success rate has not significantly improved for many decades (Friedman 2002). In particular, the success of NSRCT on teeth with preoperative lesions have a success rate of only 82% which is low compared to the 93% success rate in teeth without (de Chevigny *et al*., 2008). Given the current state of affairs, there is value in finding a safer and more effective disinfection protocol.

3. Electrochemical Disinfection

Electrochemistry is the branch of science that deals with current flow caused by chemical changes in the environment. It is largely used to study the corrosion processes in metals and hazardous environments. Recently, however, electrochemistry has been harnessed for clinical applications, such as microbial disinfection, drug delivery and sterilization. Low amperage current has been found to effectively disinfect drinking water (Patermarakis and Fountoukidis 1990), medical catheters (Davis *et al*., 1982), and even dental implants *in vitro* (Mohn *et al*., 2011). Indeed, electrochemistry has a long history in the field of dentistry. In the early 1900s, it was used

for the purposes of disinfecting root canals and was first investigated on a scientific basis in 1931 by Grossman and Appleton in their study, "Experimental and Applied Studies in Electro-sterilization" published in the *Dental Cosmos* (Grossman and Appleton, 1931). Its bactericidal action primarily arises from its forming various bactericidal agents on-site, including HOCl, Cl_2, OCL-, ClO_2. These are highly reactive oxidizing agents. A previous *in vitro* study (Segu *et al*., 2018) demonstrated that a potential difference of -9 volts given for 5 minutes resulted in an 80-95% reduction of *in vitro E. faecalis* biofilm grown on a 96-well plate. This study also demonstrated the superior biocompatibility of electrochemistry compared to NaOCl.

4. Objective

The purpose of this study was to follow-up on a previous study (Segu *et al*., 2018) by evaluating the efficacy of electrochemical disinfection (EC) in a more clinically relevant tooth model inoculated with a mixed-species bacterial biofilm. EC was combined with phosphate-buffered saline (PBS) and with low-strength NaOCL. The control groups which did not undergo EC consisted of PBS, low-strength NaOCl, and high-strength NaOCl. The null-hypothesis was that there would be no difference in post-intervention bacterial viability between any of the groups.

II. MATERIALS AND METHODS

1. Tooth Model Sample Preparation

Sixty permanent, single-canal anterior and premolar teeth were collected from dental offices located in the Chicago suburban area and from within the dental school and stored in 10% formalin. The exclusion criteria for the teeth were as follows: fractures, multiple canals, open apices, root dilacerations, and resorptive defects. The teeth were decoronated and standardized to a length of 15mm and the external surfaces of the roots were debrided and cleansed. Patency in the canals was attained using size 10 C-files (Roydent Dental Products, Johnson City, TN), and the tooth samples were instrumented up to a size 30 with a .04 taper with powered instrumentation using Dentsply ProFile files (Dentsply Sirona, York, PA) up to 1mm short of the apical foramen. Super-glue was used to seal-off the apical foramen and nail varnish was used to seal the external surface of the teeth and prevent contamination.

The tooth samples were placed in a 500ml wide-mouth jar covered with deionized water and then steam autoclaved for 20 minutes at 121ºC. After sterilization, the water in the jar was replaced with 200ml of freshly prepared and sterilized Schaedler broth. All jars were incubated at 37°C for 3 days to ensure sterility. Turbidity of Schaedler broth was considered a sign of contamination.

2. Biofilm Formation

An infected tooth model for the mixed-culture biofilm was adapted from a previous study (Xie *et al*., 2012) with minor modifications. *E. faecalis*, *P.gingivalis* and *F.nucleatum* were anaerobically grown in Schaedler broth supplemented with Hemin (0.001%, MPbio, 194025) and

Vitamin K1 (0.0001%, Sigma, V3501-1G) for 48 hours at 37ºC, then washed with PBS (0.05M, pH 6.8). The bacteria were inoculated into jars with the tooth samples containing 200ml of Schaedler broth supplemented with Hemin and Vitamin K1, with the final inoculum containing *E. faecalis* ($1x10^4$ CFU/ml), *P. gingivalis* ($1x10^6$ CFU/ml) and *F. nucleatum* ($1x10^6$ CFU/ml). All jars were placed in an anaerobic jar (BD 260610) with Gaspak (BD260001) and incubated anaerobically at 37°C for 21 days to allow for biofilm formation and maturation within the canals of each tooth sample. During incubation, 100ml of culture medium was replaced every 2 days and aliquots of cultures from each group were checked by the gram-staining method under light microscopy to ensure the growth of bacteria and to rule out contamination. After 21 days, the 60 tooth samples were removed from the jars and randomly divided into five groups of 12 teeth using an aseptic technique.

3. Electrochemical Setup

The electrochemical setup involved a Gamry Interface 1000 potentiostat (Gamry Instruments, Warminster, PA), which is a tool that allows an operator to customize the voltage potential difference between a working electrode and a reference electrode (Figure 1). The potentiostat was connected to a laptop computer (Acer Inc., New Taipei City, Taiwan). The Gamry software installed on the laptop computer was used to customize the following electrochemical sequence:

Electrochemical Sequence

Step 1: 50 seconds of open circuit potential (OCP)
Step 2: 5 minutes of the potentiostatic scan administering -9V
Step 3: 50 seconds of OCP

The purpose of the OCP preceding and following the potentiostatic scan was to passively measure the free potential and ensure the electrochemical stability of the system. Only during the 5-minute potentiostatic scan phase was a voltage applied to allow current to flow through the electrodes into the root canals.

On the other end, the potentiostat was connected to a working electrode (WE), reference electrode (RE), and a counter electrode (CE). A potential difference is created between the WE and the RE. The purpose of the CE is to aid in current flow based on the potential difference between the WE and RE without compromising the stability of the system. In the electrochemical setup, the WE was connected to a hand-file placed inside of the tooth sample, and the reference electrode was connected to a 2-inch long platinum wire, which was used due to platinum's excellent corrosion resistance properties. The CE was also connected to the platinum wire, distal to the RE with respect to the tooth sample (Figure 2).

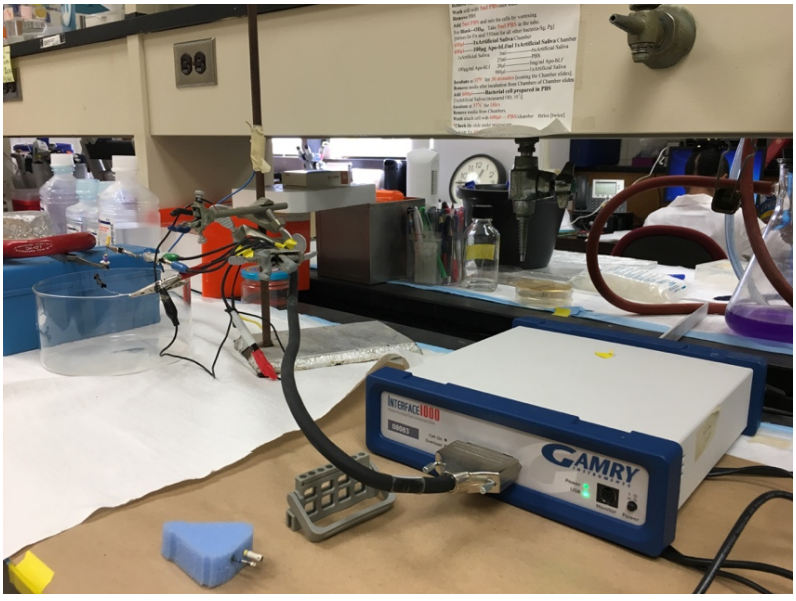

Figure 1. The electrochemical setup, including the Gamry Interface 1000 potentiostat

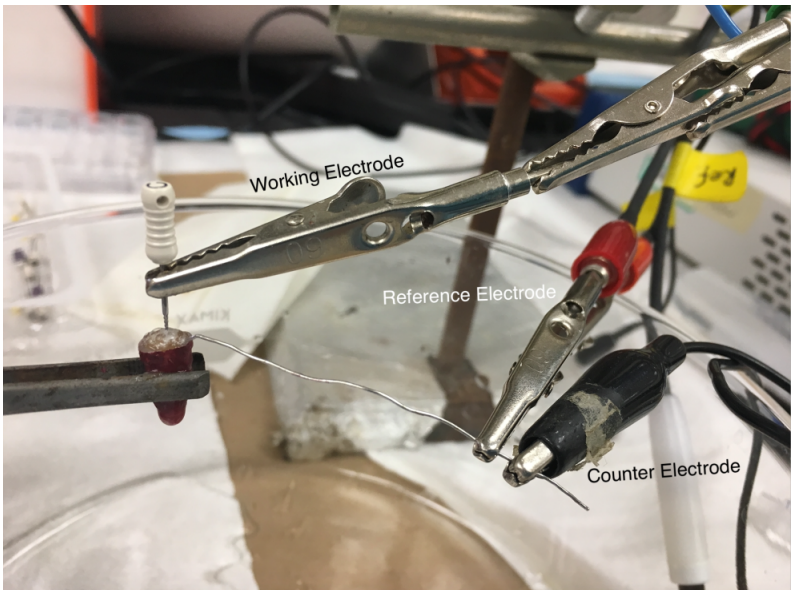

Figure 2. The working electrode, reference electrode, and counter electrode connected to a hand file and platinum wire

4. Treatment Groups

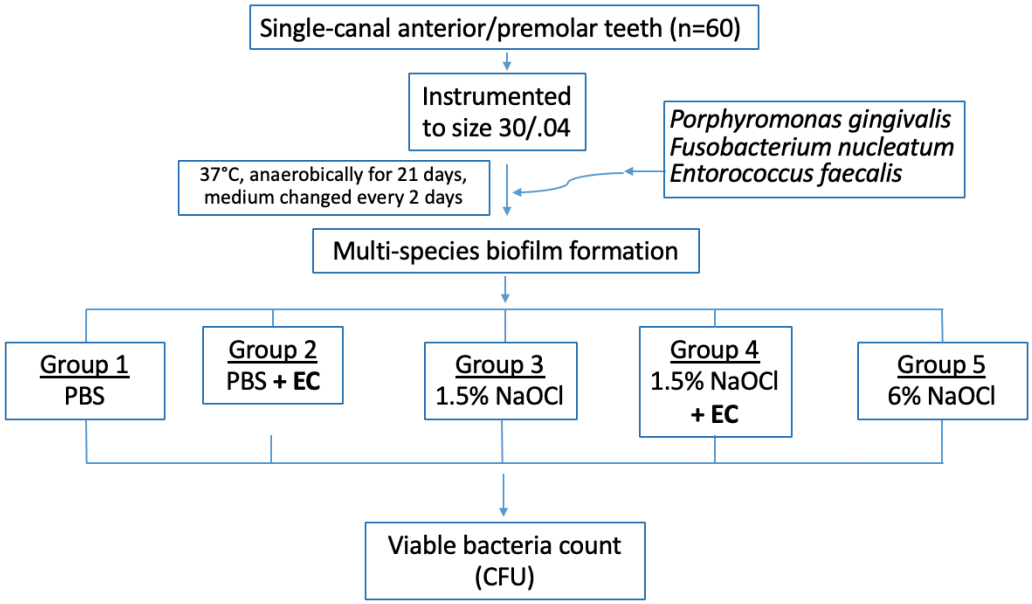

Figure 3. Experimental design flowchart

The groups were as follows (Figure 3):

Group 1: Phosphate-buffered saline (PBS)
Group 2: PBS with electrochemical disinfection (EC)
Group 3: 1.5% NaOCl
Group 4: 1.5% NaOCl with EC
Group 5: 6% NaOCl

In group 1, each tooth was handled with sterile gauze and irrigated with 3ml of PBS using a .27 gauge side-vented needle (Henry Schein, Melville, NY) placed just short of binding in the canal with a constant up-and-down motion. A size 15 C-file was then placed to WL and the tooth sample was left untampered with for 7 minutes, then rinsed with 3ml of PBS. In group 2, each tooth was rinsed with 3ml of PBS and a size 15 C-file was placed to WL. The WE was then connected to the file, and the platinum-wire connected to the RE and CE was placed onto the occlusal surface of the tooth in contact with the PBS and in solution with the working electrode. The electrochemical sequence was then completed, and the tooth was then rinsed with 3ml of PBS. Micro-bubble formation around the file during the electrochemical sequence was noted (Figure 4).

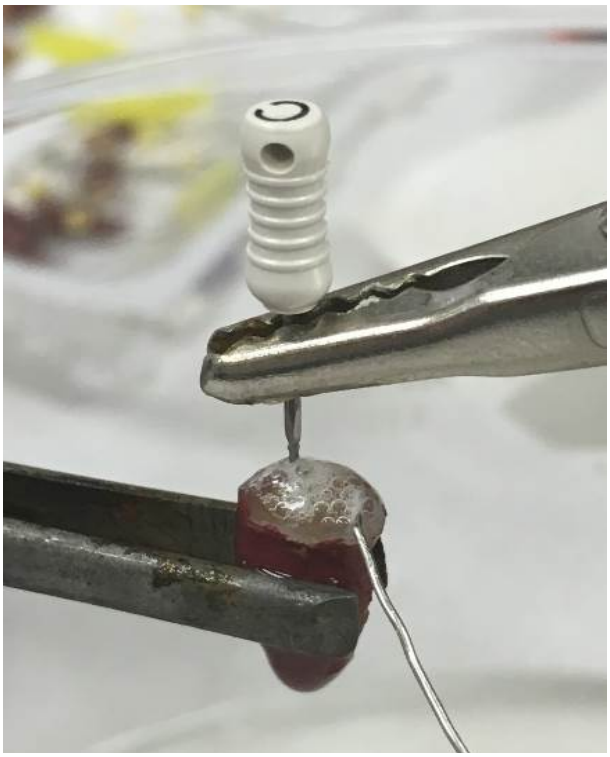

Figure 4. Bubble formation during electrochemical treatment

In group 3, each tooth was irrigated with 3ml of 1.5% NaOCl, immediately rinsed and deactivated with 1ml of 5% $Na_2S_2O_3$, followed by 1ml of PBS. A size 15 C-file was then placed to WL, and the tooth was left untampered with for 7 minutes, then rinsed with 1ml of PBS. In group 4, 3ml of 1.5**%** NaOCl was used to irrigate each tooth, followed by 1ml of 5% $Na_2S_2O_3$, which was followed by 1ml of PBS. A size 15 C-file was placed to WL, and the working electrode was attached to the hand-file while the tip of the platinum wire was placed on the occlusal surface of the tooth in contact with the PBS and in solution with the working electrode. The electrochemical sequence was completed, and the tooth was rinsed with 1ml of PBS.

In group 5, 3ml of 6% NaOCl was used to irrigate each tooth, followed by 1ml of 5% $Na_2S_2O_3$, which was followed by 1ml of PBS. A size 15 C-file was placed to WL, and the tooth was left untampered with for 7 minutes, followed by irrigation with 1ml of PBS.

5. Bacteria sampling/counting

After irrigation with PBS, each tooth sample was then instrumented to WL with a size 30/.06 Dentsply ProFile rotary file, recapitulating five times. The canal of each tooth sample was then dried with five sterile size 25/.04 taper paper points (Brasseler USA, Savannah, GA). Each rotary file and all paper points were placed into a 1.5ml Eppendorf tube containing 900 µl of PBS. For bacterial viability testing, the samples in the 1.5ml Eppendorf tubes were then 10-fold serial diluted and plated on a CDC anaerobic blood agar plate (Fisher, 221734). The plates were incubated anaerobically at 37°C for 48 hours and 4 weeks. The viable colonies were then enumerated under light microscopic examination.

6. Data Analysis and Statistics

The data were analyzed using a one-way ANOVA ($P < 0.05$) and a Tukey post hoc test using Minitab 19 statistical software (Minitab, LLC, State College, PA).

III. RESULTS

Figure 5. Group 1 (PBS) bacterial viability on a blood agar plate

Figure 6. Group 2 (PBS + EC) bacterial viability on a blood agar plate

Means

Factor	N	Mean	StDev	95% CI
PBS	12	35389	22996	(29283, 41495)
PBS + EC	12	6829	4753	(723, 12935)
1.5% NaOCl	12	737	1057	(-5370, 6843)
1.5% NaOCl + EC	12	1272	2123	(-4834, 7378)
6% NaOCl	12	55.5	69.1	(-6050.7, 6161.7)

Pooled StDev = 10555.0

TABLE I. MEANS, STANDARD DEVIATIONS, AND 95% CONFIDENCE INTERVALS

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
PBS	12	35389	A
PBS + EC	12	6829	B
1.5% NaOCl + EC	12	1272	B
1.5% NaOCl	12	737	B
6% NaOCl	12	55.5	B

Means that do not share a letter are significantly different.

TABLE II. MEANS AND TUKEY POST HOC TEST RESULTS

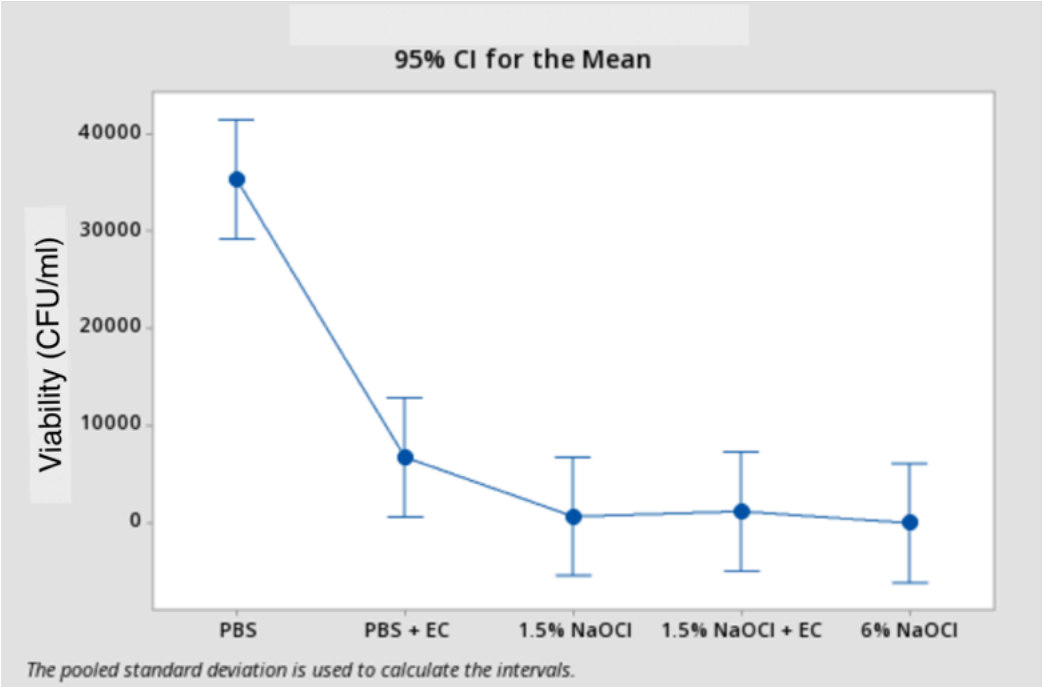

Figure 7. Means and 95% confidence intervals of the post-interventional bacterial viability

After 48 hours, the colony forming units (CFU) from the growth of *E. faecalis* were counted (Figures 5 and 6). The results of the statistical analysis can be found in TABLE I, showing the respective means, standard errors, and 95% confidence intervals of each group. Figure 7 illustrates the data using a line graph with whiskers denoting the 95% confidence intervals. Analysis with one-way ANOVA found a significant difference between the five groups ($P = 1.14 \times 10\text{-}11$). Post hoc analysis using the Tukey post hoc test found a significant difference in bacterial viability between group 1 and groups 2, 3, 4, and 5 ($P<.005$). There was not a significant difference in bacterial viability detected between groups 2, 3, 4, or 5 (TABLE II). Examples of potentiostatic scans showing the change in current as a function of time can be seen in Figures 8 and 9. An example of open circuit potential before and after the potentiostatic scan can be seen in Figure 10, with voltage changing as a function of time.

After 4 weeks, no viable colonies of the obligate anaerobic bacteria *F. nucleatum* or *P. gingivalis* could be identified on any plates, most likely due to excessive oxygen being introduced into the system during handling.

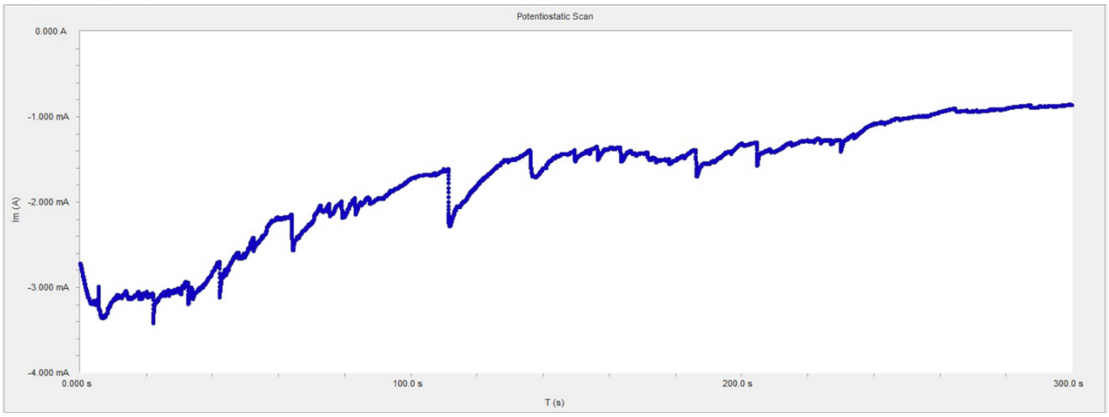

Figure 8. Potentiostatic scan of a sample from group 2, PBS + EC

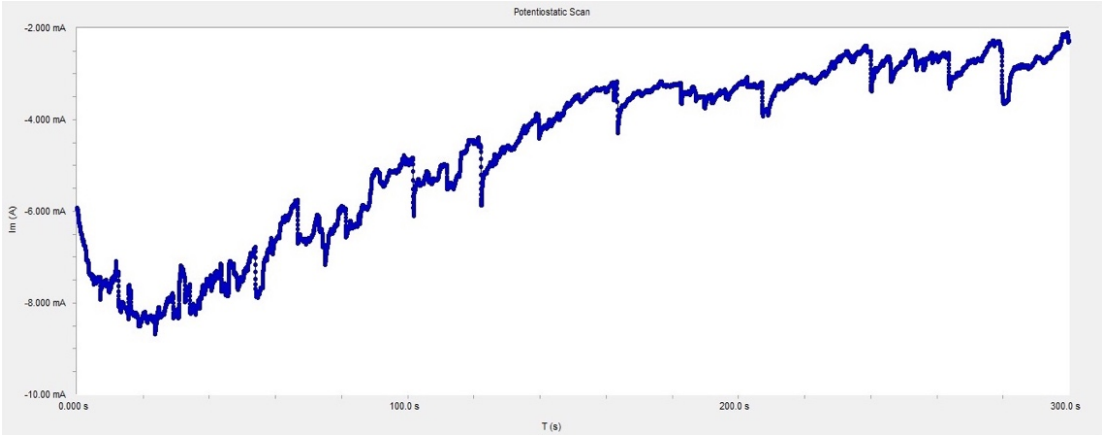

Figure 9. Potentiostatic scan of a sample from group 4, 1.5% NaOCL + EC

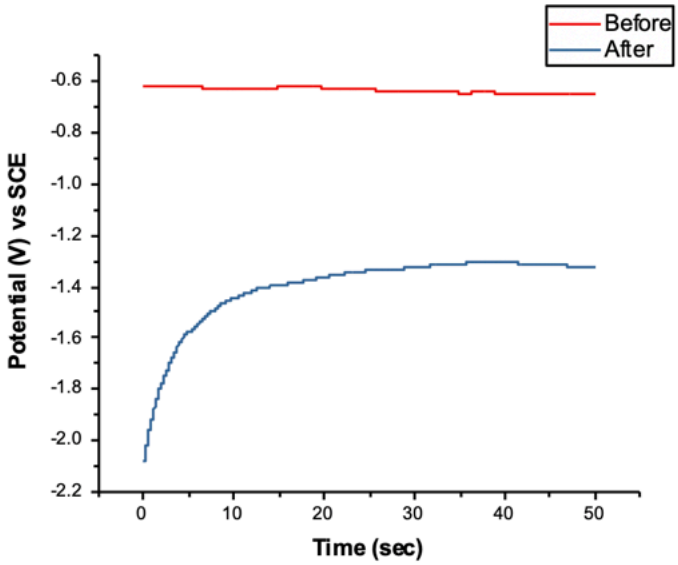

Figure 10. An example of open circuit potential before and after the potentiostatic scan

IV. DISCUSSION

1. Problems with Current Disinfection Techniques

NaOCl has been found to be the most effective irrigant for root canal disinfection, owing to its broad-spectrum antibacterial activity, ability to remove the organic portion of the smear layer, and tissue dissolution properties. There are, however, drawbacks to the use of NaOCl. The same properties that make NaOCl an effective endodontic irrigant also make it cytotoxic to human cells if accidentally forced into the periapical tissues, causing rapid hemolysis, ulceration, and destruction of fibroblasts and endothelial cells (Pashley *et al*., 1985). Even a small amount of extruded NaOCl can lead to a disproportionately fulminant and acute reaction, commonly referred to as a sodium hypochlorite accident. The symptoms include sudden pain, heavy bleeding, swelling, and neurological deficits that may take weeks or months to resolve (Guivarc'h *et al*., 2017). While rare, a survey of diplomates of the American Board of Endodontists found that 42% of respondents reported at least one hypochlorite accident in their careers (Kleier *et al*., 2008). Some authors have posited that lower concentrations of NaOCl are less cytotoxic (Spangberg *et al*., 1973), though this assumption has been contested (Yesilsoy *et al*., 1985). Besides the potentially destructive sequelae to the periapical tissues, 5.25% NaOCl has also been found to potentially reduce the flexural strength and elastic modulus of dentin compared to 0.5% NaOCl and saline, which could lead to premature fracturing of a tooth (Sim *et al*., 2001).

Irrigation with NaOCl is traditionally performed with an open-ended or side-vented needle using a positive-pressure irrigation (PPI) technique, which must be extended to within 1-2mm of WL in order to fully address the apical extent of a canal system (Boutsioukis *et al*., 2010). This often requires significant enlargement of a canal and increases the risk of inadvertent extrusion of irrigant past the apex. Additionally, PPI by itself cannot fully address the vastly complex root

anatomy, including isthmuses and accessory canals (Haapasolo, *et al*. 2005**).** NaOCl is thus, a highly effective but imperfect irrigant, and there is value to continued research to evaluate new methods that can help to maximize its effectiveness, minimize its ill-effects, or perhaps replace it altogether.

2. Mechanism of Action of Electrochemical Disinfection

Adjunctive disinfection techniques have been developed to aid in increasing efficacy, safety, and conservation of tooth structure. They include passive ultrasonic irrigation, sonic irrigation, the EndoVac, and the Sonendo GentleWave, among others. Another possible and novel tool for root canal disinfection comes from the field of electrochemistry. Electrochemistry is the study of the movement of electrons caused by reduction-oxidation ("redox") reactions, where reduction is the addition of electrons and oxidation is the loss of electrons. In an electrolytic cell, the reduction reactions occur at the cathode and the oxidation reactions at the anode (Figure 11). A potentiostat, such as the one used in this study, is a tool that makes it possible to control the potential difference between a working electrode (anode) and a reference electrode (cathode), and thus the flow of electrons (current). In this study, the anode was the file placed into the canal, and the cathode was the platinum wire placed on the occlusal surface of the tooth. In water, electrochemical disinfection at the anode occurs through the production of highly active oxidizing agents, such as Cl_2, OCl-, HOCl, and ClO_2 (Figure 12). In an *in vitro* pilot study investigating the electrochemical disinfection of dental implants, Mohn found that a current of 7.5 mA applied for 15 minutes led to complete disinfection at the anodic implant (Mohn *et al*., 2011).

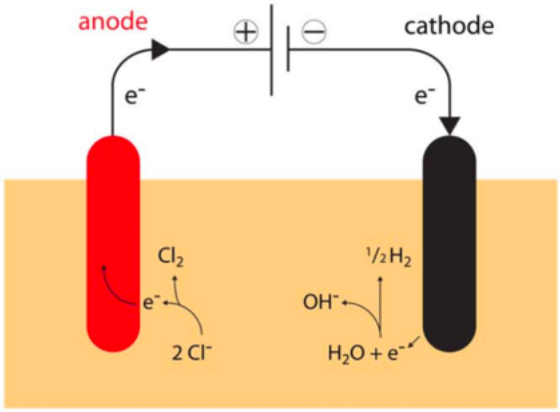

Figure 11. An electrolytic cell showing redox reactions occurring at the anode and cathode (Mohn *et al.,* 2011)

anode	cathode
$2H_2O \rightarrow O_2 + 4H^+ + 4e^-$	$H_2O + e^- \rightarrow 1/2H_2 + OH^-$
$2Cl^- \rightarrow 2Cl^- + 2e^-$	$O_2 + e^- \rightarrow O_2^-$
$Cl_{2(aq)} + H_2O \rightarrow HOCl + H^+ + Cl^-$	$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$
$2Cl^- \rightarrow 2Cl^- + 2e^-$	
$2Cl^- \rightarrow Cl_2$	
$Cl^- + H_2O \rightarrow Cl^- + HO^- + H^+$	
$H_2O \rightarrow HO^- + H^+ + e^-$	
$2HO^- \rightarrow H_2O_2$	
$Cl_2 + 2OH^- \rightarrow H_2O + OCl^- + Cl^-$	
$Cl_2 + 4H_2O \rightarrow 2ClO_2 + 8H^+ + 8e^-$	

Figure 12. Putative reactions occurring at the anode and cathode of an electrolytic cell in water (Mohn *et al.,* 2011)

3. Previous Studies on Electrochemical Disinfection

Previous studies involving electrochemistry in the field of endodontics have found that electrochemical activation of NaOCl led to significantly enhanced tissue dissolving properties (Ertugrul *et al*., 2015). In an *in vitro* study involving a single-species biofilm of *E. faecalis* grown in a tooth sample, the antibacterial effects of electrochemical activation of NaOCl were found to be comparable to passive ultrasonic activation and significantly more effective than the control (Maden *et al*., 2017). A study by Segu *et al.* investigated the efficacy of electrochemical disinfection on single-species biofilms of *E. faecalis* grown on 96-well plates as a proof-of-

concept. The authors found that the antibacterial properties of EC was time and voltage-dependent, with a potential difference of -9V over 5 minutes exhibiting superior effectiveness compared to lower voltages and shorter times. There was a statistically significant 80-95% reduction in bacterial viability compared to the control when -9V was applied for 5 minutes.

4. Safety of Electrochemical Disinfection

With respect to safety, previous studies investigating the effects of the electronic pulp tester (EPT) and electronic apex locator (EAL) on pacemaker function are instructive and informative. An *in vitro* experiment (Garofalo *et al*., 2002) studied pacemaker interference caused by five different EALs. Four out of the five EALs exhibited no pacemaker interference when directly connected to the pacemaker leads. While one EAL did cause interference, the authors ultimately concluded that EAL use in patients with pacemakers is safe because: 1) modern pacemakers are encased in a metal shield for the purpose of blocking out electromagnetic interference: 2) an EAL is never directly connected to a patient's pacemaker in clinical practice, as they were in this study: 3) in practice, the circuit produced by EALs is limited to the head region and does not come into contact with the chest, and: 4) EALs operate on a low voltage, 7-9V battery, leading to low-level signals. Similar to the EAL, EC of a tooth would create a circuit that does not cross that chest, with the circuit being contained within an individual tooth. Also, the voltage used in the EC method is only -9V, similarly creating only low-level signals.

Another study (Wilson *et al*., 2006) evaluated the effects of the EAL and EPT on pacemaker function in an *in vivo* setting. With a sample size of 27 patients, no electrical stimulation or interference on pacemaker functioning could be detected. This is particularly encouraging because the EPT uses anywhere from 15-350V and completes a circuit that courses

from the tooth being tested and through the arm to the fingers touching the EPT tester. This is in contrast to EC, which uses only -9V and has a circuit that is only contained to within a tooth.

Regarding heat build-up caused by the application of an electrical current, a previous study (Segu *et al*., 2018) detected no heat build-up when different solutions were subjected to -9V. This seems to help with ruling out tissue-damaging heat build-up as a potential concern with EC.

5. *In Vitro* Tooth Model

The current study was a follow-up to a previous study (Segu *et al*., 2018) with the intention of translating the same concept to a tooth model. Two pairs of variable and control were used: group 1 was the PBS control, while group 2 added the variable of EC. Similarly, group 3 was the 1.5% NaOCl control, while group 4 added the variable of EC. Group 5, 6% NaOCl, was included in this study to stand as the "gold standard" representing the concentration of NaOCl most commonly used during root canal treatment. Of particular interest was whether 1.5% NaOCl with EC was as effective as high strength 6% NaOCl, and whether PBS plus EC was as effective as 1.5% NaOCl. In order to most closely simulate *in vivo* conditions, a mixed-species biofilm consisting of *E. faecalis, P. gingivalis, and F. nucleatum* was grown in an anaerobic environment. *E. faecalis* is a gram-positive facultative anaerobic cocci and has been implicated as the most-commonly isolated species of bacteria (Sundqvist 1994) in secondary infections of root canals, due to its various virulence factors including lipotechoic acid (Baik *et al*., 2011), its ability to invade deep into the dentinal tubules (Love 1996), its proton pump which resists the action of $CaOH_2$ (Byström *et al*., 1985), and its ability to survive in nutrient-poor conditions for extended periods of time (Stuart *et al*., 2006). It should be noted, however, that recent research using pyrosequencing techniques have found secondary infections to be similarly as diverse in

composition as primary infections, with *E. faecalis* representing just one of many species (Hong *et al*., 2013). *P. gingivalis* (a gram-negative anaerobic rod) and *F. nucleatum* (a gram-negative spindle-shaped rod) are also some of the most commonly identified pathogens in both primary and secondary endodontic infections.

6. Analysis of Results

The results of the current study showed that EC is effective in reducing intracanal bacterial viability in an *in vitro* tooth model. The null-hypothesis was rejected. When comparing groups 1 (PBS) and 2 (PBS with ED), there was a statistically significant 80.7% drop in bacterial viability in the electrochemical group (Figures 5 and 6). There was not, however, a significant difference in bacterial viability between groups 3 and 4, where 1.5% NaOCl was used as the irrigating solution. It is notable that there was a statistically significant difference between groups 1 and 2 but not groups 3 and 4, and there are a couple of possible explanations. One is that the small amount of bactericidal chemicals that are produced through the EC method is dwarfed by the amount of bactericidal chemicals contained in even a diluted 1.5% NaOCl solution; in other words, the effects of EC are essentially overshadowed by the effects of NaOCl. Relatedly, it is also possible that, given the high level of effectiveness of 1.5% NaOCl, the sample size of this study was simply insufficient for identifying a statistically significant *increase* in effectiveness.

Continuing on with the analysis of the results, another comparison of interest was between groups 2 (PBS with EC) and 3 (1.5% NaOCl). It was found that, while the difference was not statistically significant, 1.5% NaOCl appeared to be more effective than PBS with ED. Finally, while not statistically significant, high-strength 6% NaOCl appeared to most effectively removed bacteria, resulting in the lowest bacterial viability score among all groups.

Notable in the graphs of the potentiostatic scans for groups 2 and 4 (Figures 8 and 9), one notices the progressive but lurching decreasing of current as a function of time. Most of the time, the current would start at approximately -2 to -6 mA, and steadily decrease towards -1 mA by the 4 minute mark. This was thought to be due to the rapid formation of bubbles due to the reactions catalyzed by the current (Figure 4), resulting in a continuous loss of fluid. Once the current went below -1 mA, it was found that the system was at high risk of cutting out due to the platinum wire no longer being in solution with the hand file. To avoid this, PBS was occasionally replenished on the occlusal surface of the tooth sample. These replenishments can be discerned on the potentiostatic scan graphs by the large vertical increases in current.

Bookending the active potentiostatic scan phase of electrochemical disinfection with two 50 second-long segments of open circuit potential (Figure 10) was important for being able to observe and measure the surrounding system's voltage potential in order to ensure the stability of the system before and after the potentiostatic scan.

7. Analysis of Study Design

An effort was made in the study design to minimize as much as possible the influence of confounding variables. A uniform volume of 6ml of total irrigant was maintained across all groups. As much as possible, a consistent and uniform amount of time that irrigant was allowed to sit inside of the canal of a tooth was also maintained across all groups. Groups that did not undergo EC nonetheless were left to sit untampered with for 7 minutes with a size 15 C-file in the canal in order to closely align with the amount of time necessary for the EC sequence to run. All groups utilizing NaOCl were flushed immediately with $Na_2S_2O_3$, which inhibits the effects of NaOCl. This was done because previous efforts running an EC sequence with NaOCl as the

solution were unsuccessful. The more consistent performance was attained when NaOCl was deactivated and replaced with PBS.

8. Limitations of Study

A significant limitation to our study was the occasional cutting off of the circuit when running an electrochemical cycle. The cutting off would tend to occur when the solution on the occlusal surface of the tooth would evaporate, causing the reference electrode to no longer be in solution with the working electrode. As explained previously, this was addressed by occasionally replenishing the occlusal surface solution with drops of PBS in order to maintain the circuit. While this was not an ideal solution to this problem, it was necessary in order to allow the electrochemical cycle to complete. Work is currently being done to improve the performance and reliability of a microelectronic device, and it is hoped that this is no longer an issue in future studies.

Additionally, the loss of all CFUs at 4 weeks made it impossible to evaluate the effects of each intervention on *P. gingivalis* and *F. nucleatum*. These are slow-growing, obligate anaerobic bacteria and their losses are likely due to handling errors introducing oxygen. Future studies should limit handling errors in order to make it possible to evaluate the viability of these bacteria.

Another limitation to our study was not taking a pre-intervention sample of each group in order to help establish a baseline of bacterial presence for each tooth. Instead, only a post-intervention sampling was taken. The possible lack of uniformity in pre-interventional bacterial levels combined with the small sample size of 12 per group could be seen as weakening the results and conclusions of our study. The colony-forming unit (CFU) counts varied quite considerably between samples in every group. Overall, however, the contours of the final results and comparisons between group appeared to be consistent and reliable.

9. Future Studies

Future studies should evaluate using higher voltages and shortening treatment times in an effort to improve the effectiveness and clinical practicality of EC. The development of a more stable apparatus with microelectronic interface is also an important future step toward potentially turning EC into a clinically viable adjunctive endodontic device. A follow-up study may replicate this current study using a multiple-canal tooth model in order to evaluate the efficacy of EC with more complicated tooth anatomy and to evaluate its practicality when used to cleanse multiple canals simultaneously. Comparisons to current adjunctive tools, such as PUI and the GentleWave System, in terms of canal cleanliness and bacterial viability, should be carried out in future studies.

V. CONCLUSIONS

The findings of this study evaluating the effectiveness of EC indicate that, in an *in vitro* environment using single-canal tooth samples populated with a mixed-culture bacterial biofilm, EC is effective in reducing bacterial viability. Conversely, EC in combination with 1.5% NaOCl did not result in additive bactericidal activity compared to 1.5% NaOCl by itself.

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