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ABSTRACT

The Effects on the Antimicrobial Properties of Hoshino's Triple Antibiotic Paste when Chlorhexidine Gluconate (0.12%) is Substituted for the Propylene Glycol and Macrogol Ointment Mixture

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The purpose of this *in vitro* agar-diffusion study was to evaluate the effect on the antimicrobial efficacy of Hoshino's triple antibiotic paste when 0.12% chlorhexidine gluconate is substituted for the traditional propylene glycol and macrogol ointment carrier solution. Ciprofloxacin (200 mg), metronidazole (500 mg), and minocycline (100 mg), were mixed with either chlorhexidine or a 1:1 combination of propylene glycol and macrogol ointment. Seven microorganisms, including: *E. coli, P. aeruginosa, S. aureus, E. faecalis, S. sanguis, F. nucleatum, and C. albicans*, were incubated and plated onto triplicate blood agar plates. Two blank paper discs were positioned onto each agar plate, one saturated with chlorhexidine and the three antibiotics, and the other containing propylene glycol/macrogol ointment and the antibiotics. After appropriate incubation, a blinded, independent observer measured the zones of inhibition around each disc. Mixtures containing chlorhexidine showed significantly larger zones of inhibition in every trial ($p<.004$, ANOVA test). Control plates with chlorhexidine only, or propylene glycol and macrogol ointment only (no antibiotics added), further exhibited the antimicrobial properties of chlorhexidine, and the lack thereof for propylene glycol/macrogol ointment. In conclusion, substituting 0.12% chlorhexidine gluconate for propylene glycol and macrogol ointment as the carrier in Hoshino's triple antibiotic paste increased the antimicrobial efficacy of the mixture.

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CHAPTER I

INTRODUCTION

Regeneration of necrotic teeth has been the desire of dental practitioners since Nygaard-Ostby and Hjortdal attempted the procedure in the 1960's (1). Many others have made efforts to revascularize immature teeth with apical periodontitis, but their lack of success was likely due to their lack of technology and understanding of the necessary scientific principles. According to Trope, we now have this understanding, and the applicable technology. Practitioners can disinfect a pulp canal, place a scaffold in said canal, and then seal off this same tooth sufficiently to allow the body to revitalize the previously necrotic space (2). The most accepted protocol utilizes a triple antibiotic paste as the disinfectant, and mineral trioxide aggregate (MTA) as the sealant. This revascularization process occurs most predictably in teeth with open apices (3).

Much research has been completed in an attempt to find the perfect mixture of antibiotics to sufficiently disinfect the canal. Most regeneration experts currently hold Hoshino's triple antibiotic paste as the decontaminator of choice. Hoshino completed both *in vitro* and *in situ* studies using the paste for sterilization in the 1990's (4,5,6), and since that time there is a trend in the research towards using this combination of ciprofloxacin, metronidazole, and minocycline (3Mix). Numerous clinical studies, and *in vivo* research, have also shown that this specific combination of antibiotics is the most successful (7,8,9,10); but no study to date has confirmed that the carrier ointment utilized to place and maintain the antibiotics is the most efficient one available.

The carrier plays a critical role in the triple antibiotic paste, because the antibiotics cannot disinfect the canal if they are not maintained consistently within the

tooth. Hoshino's protocol calls for a mixture of macrogol ointment and propylene glycol (MP) to be used as the carrier for the triple antibiotic paste. This MP ointment has been accepted as the carrier of choice by those who can easily acquire the two materials needed. However, many practitioners and pharmacists do not readily have access to macrogol ointment and propylene glycol. This has resulted in some using sterile saline, root canal sealer (9), or a myriad of other transporters without any scientific studies to prove their effectiveness. Other dentists may not attempt the regeneration procedure at all, because of the inconvenience, or lack of an acceptable alternative to the MP mixture.

The concern is that the lack of consistency among carriers will damage the validity of clinical studies in the field of regeneration. Worse yet, the revascularization attempts may be unsuccessful if practitioners resort to an untested carrier that actually impedes the efficacy of the triple antibiotic mixture (3Mix). There is also a concern that the propylene glycol in the MP ointment is potentially toxic at high concentrations (11). The ideal carrier would have a high level of safety, would have substantivity, and would have disinfectant properties of its own that would not hinder the effectiveness of the antibiotics that it is transporting.

Chlorhexidine gluconate has been used as a dental disinfectant for many years due to its broad antibacterial action (12). It has been shown to be safe (13), it is well-known for its substantivity, and it is readily available among dental practitioners. Using chlorhexidine gluconate as the carrier for Hoshino's triple antibiotic paste (3Mix) has not been previously reported. By using this *in vitro*, agar-diffusion study suggested by Torabinejad and Stowe (14,15), the author will attempt to determine if the substitution of

chlorhexidine gluconate for the macrogol ointment and propylene glycol mixture will alter the antimicrobial efficacy of the triple antibiotic paste utilized for regeneration.

STATEMENT OF THE PROBLEM

Is there a difference in the antimicrobial efficacy of the triple antibiotic paste utilized for regeneration when chlorhexidine (CHX) is substituted as the carrier solution in lieu of the traditional macrogol ointment and propylene glycol mixture (MP)?

SIGNIFICANCE OF THE PROBLEM

The current protocol for disinfection in regeneration cases uses Hoshino's triple antibiotic paste with macrogol ointment and propylene glycol (MP) as the vehicle of choice. These are ingredients that are commonly found in Japan, and in some research institutions in the United States, but they are not readily available to most practitioners. Because of their unfamiliarity with these ingredients, many practitioners have decided not to attempt the regeneration procedure – leading to fewer numbers of case studies. Others have used carriers that have not been researched – leading to potentially faulty case studies.

If chlorhexidine (CHX) does not negatively alter the antimicrobial efficacy of the three antibiotics in the protocol, it could readily be used as a replacement for the macrogol ointment and propylene glycol mixture (MP) called for in Hoshino's studies. This would allow many more practioners to attempt a regeneration case, resulting in more revitalized teeth, and many more case studies to review. Chlorhexidine (CHX) has been used in dentistry for a variety of disinfection techniques because of its broad antibacterial

action, substantivity, and degree of safety. Chlorhexidine is also readily available at most pharmacies and dental offices.

HYPOTHESIS

There is no significant difference between using a macrogol ointment and propylene glycol mixture versus chlorhexidine as the carrier for Hoshino's triple antibiotic paste.

DEFINITION OF TERMS

3Mix – the triple antibiotic part of Hoshino's triple antibiotic paste consisting of ciprofloxacin (200mg), metronidazole (500mg), and minocycline (100mg).

Aerobic – an organism that requires the presence of air or free oxygen for life.

Anaerobic – an organism that lives in the absence of air or free oxygen.

Antibiotic Efficacy – the power that a given antibiotic has to prevent, inhibit, or destroy the life of its intended organism.

Apical Periodontitis – inflammation of the periodontal ligament surrounding the root apex of a tooth, usually a consequence of pulpal inflammation or necrosis.

Carrier – the means by which the disinfectant reaches, and is maintained within, the necrotic root canal space.

Cementoenamel Junction – the surface at which the enamel of a crown and the cememtum of the root of a tooth are joined.

Chlorhexidine Gluconate – imidodicarbonimidic diamide, a chemical antiseptic utilizing membrane disruption as the mechanism of action, it is effective against gram +/- organisms that is commonly used in dentistry as an oral rinse.

Disinfection – cleaning a surface of some or all of the pathogenic organisms which may cause infection.

Foramen – a small opening, or orifice, at the end of a fully developed human tooth by which the tooth is innervated and supplied with blood vessels.

Immature Apex – undeveloped bottom of the human tooth, still very open and not yet formed the completed foramen.

In situ – in the natural or normal place, with no invasion of neighboring tissues.

In vitro – in an artificial environment, such as within a test tube.

In vivo – in the living body, in the normal environment.

Macrogol Ointment – polyethylene glycol, often used as an excipient in pharmaceutical products to serve as an ointment base, tablet binding, film coating, or lubricant, and used in Hoshino's triple antibiotic paste as part of the carrier.

Microbial Growth – the development of a microorganism.

MP – the carrier part of Hoshino's triple antibiotic paste consisting of macrogol ointment and propylene glycol.

Necrotic – the sum of morphological changes indicative of cell death, the pulpal diagnosis of a tooth that no longer retains living tissue within its pulp.

Propylene Glycol – propane-1,2-diol, an organic compound that is faintly sweet, colorless, clear, viscous liquid that is hydroscopic and used in Hoshino's triple antibiotic paste as part of the carrier.

Regeneration – the restoration or new growth by an organism of tissues that have been lost, removed, or injured.

Revascularization – the restoration of the blood circulation to an organ or area that previously lost said vascularization.

Scaffold – a temporary structure for holding materials during repair, specifically for holding, or positioning, the necessary cellular components needed during tooth revascularization.

Sterilization – the literal destruction of all living organisms from a certain area.

Substantivity – the ability of a substance to exist for an unusually long, or extended, period of time within its own right.

Zones of Inhibition – the area of no bacterial growth around an antimicrobial agent in the disk-diffusion test.

ASSUMPTIONS

(1) Practitioners are having a difficult time obtaining propylene glycol and macrogol ointment. (2) Revascularization is possible in teeth with open apices. (3) Hoshino's triple antibiotic paste is effective for canal sterilization. (4) Chlorhexidine is readily available to most practitioners. (5) Chlorhexidine is safe when used in a manner consistent with the regeneration protocol. (6) The bacteria used in this experiment can be found in necrotic teeth.

LIMITATIONS

(1) Antibiotic paste efficiency across a bacterial lawn on an agar plate is simulating the efficiency across irregular dentin in a root canal. (2) The effect of the triple antibiotic paste on the bacteria tested is representing the effect of the paste on other species of bacteria found in necrotic root canals. (3) An *in vitro* experiment is simulating an *in vivo* situation. (4) The 3Mix is being mixed with each respective carrier (MP or CHX) by a human, and the mixtures may not be precisely the same. (5) There is a human element in observing zones of inhibition.

DELIMITATIONS

(1) Only the three antibiotics in Hoshino's 3Mix are being tested – no other antibiotic combinations. (2) Only eight bacteria will be cultured. (3) The experiment is limited to agar plates where bacteria have been inoculated and then incubated for a set period of time. (4) The same amount of 3Mix and carrier (either MP or CHX) will be used each time. (5) Factory new bottles of each product will be used that have been

tested at the factory for quality control. (6) The observer will be blinded and independent. (7) Each plate in each group will be incubated under the same standard conditions (time, temperature, and chamber used will all be the same - and often the experiments will occur simultaneously).

CHAPTER II

REVIEW OF LITERATURE

Revascularization of necrotic teeth has been attempted by numerous authors over several decades. Early dentists' attempts were often without much success, but these early trials were absolutely necessary so that when the technology became available, regeneration of a previously non-vital tooth could become a reality. Nygaard-Ostby and Hjortdal completed a series of experimental investigations in the 1960's and 70's, in which they removed the pulp from several canals and endeavored to see new tissue appear within these empty canals (1). In 1971 they used a protocol not unlike what practitioners are trying today. They removed the contents of the canal, induced bleeding from the periapical area, and sometimes partially filled the canal as a scaffolding. The authors reported deposition of connective tissue and cellular cementum in some of their teeth, and in others they noted "formations" of varying degrees. They even concluded that in the teeth in which they had found cellular components they were "completely successful," and that the major contributing factor seemed to be the presence or absence of a blood clot. One of today's prominent figures in the field of regeneration, Dr. Trope, has acknowledged that although their ideas were well before their time, their experiments were "mostly unsuccessful" due to their lack of technology and understanding of the necessary scientific principles (2).

Johnson, Goodrich, and James concluded that revitalization can occur in teeth with immature root development. Their studies on replantation showed that teeth with apical closure were likely to become necrotic after avulsion, but those teeth with incomplete root development often regained their vitality (16). In a study out of

Belgium, it was shown that revascularization after cryopreservation had a very high success rate in teeth with open apices. Some of the teeth with open apices in this experiment were that way due to their level of immaturity, and others were prepared via an apicoectomy (17). Hargreaves believes the open apices to be one of four key precepts in obtaining revascularization. Other important factors include proper disinfection of the necrotic canal, a resorabable matrix to enhance tissue in-growth, and a coronal seal following treatment. He also noted that instrumentation and sodium hypochlorite irrigation are not adequate steps for disinfecting the canal space prior to regeneration (3). In his prospective, he points to the use of Hoshino's "3Mix-MP" triple antibiotic paste as the disinfectant most likely to create the necessary conditions. If used properly, Hargreaves states that "biologically based endodontic therapies can result in continued root development, increased dentinal wall thickness, and apical closure when treating cases of necrotic immature permanent teeth" (3).

Hargreaves also mentions that a scaffold is needed to permit the ingrowth of tissue. Trope gives the step-by-step protocol for regenerative cases in an article from 2008 (2), and confirms that a blood clot to the level of the cementoenamel juction is a proper scaffold following sterilization of the canal's lumen (18). He also points to Hoshino's triantibiotic paste as the correct antibiotic mixture for attempting this type of endodontic technique, and gives the specific mixing directions for the 3Mix-MP paste (2). This includes the use of ciprofloxacin, metronidazole, and minocycline as the three antibiotics, and macrogol ointment and propylene glycol as the carriers. After the canal has been disinfected and a scaffold has been placed, the final step in the protocol is placement of an MTA barrier as the coronal sealant.

Several authors in the dental traumatology literature have also found success with regeneration using various mixtures of antibiotics. One case study showed thickening of the canal wall followed by apical closure that was confirmed thirty months after treatment (8). A different case study showed an immature second lower right premolar with a draining sinus tract that became revascularized via Trope's protocol, and its resulting apex was very similar to the adjacent and contralateral teeth (7). In yet another article out of Dental Traumatology from 2004, the authors had a 91% success rate of revascularization of replanted immature dog teeth (19).

Hoshino's triple antibiotic paste has become the gold standard in the field of regeneration due to the numerous articles proving its success. In 1993, Sato, Hoshino, Uematsu, and Noda authored a paper where they sought to find the perfect drug combination to combat bacteria obtained from carious and endodontic lesions. Each of their attempts included ciprofloxacin, metronidazole, and a third variable antibiotic. They recovered no bacteria following any of the treatments, but ample amounts on the control plates that received no treatment (5). Researchers quickly realized that they now had achieved a way in which to sterilize a root canal. Hoshino did not stop there, however, because he still wanted to find the perfect trifecta of antibiotics for this purpose. His articles show that he was leaning towards the tetracycline derivative, minocycline, but was concerned about the drugs propensity to cause tooth stain (5).

Hoshino et al completed a study in 1996, in which they confirmed that none of the individual drugs in their combination was sufficient to eradicate all of the bacterial biofilm from a canal. They were attempting to clarify the antibacterial effect of the ciprofloxacin, metronidazole, minocycline mixture via *in vitro* research, and they

concluded that it truly was sufficiently potent to kill the microorganisms found in the infected dentine of root canals (4). A similar group completed an *in situ* study the same year, in which they tested the same antibiotic mixture in root canals of extracted teeth. They found that bacterial recoveries decreased quickly over time, and after forty-eight hours of treatment there was no bacteria recovered from the canals (6). It was concluded that this triple antibiotic paste could penetrate through dentin *in situ*, but it is of interest to note that the authors ultrasonically irrigated all of the canals with EDTA prior to the treatments.

The next step in Hoshino's quest was determining the proper carrier to transport and maintain the antibiotics within the canals. He co-authored a paper in the *International Journal of Endodontics*, in which he used either root canal sealer or a combination of macrogol ointment and propylene glycol (MP). The group found that the placement of either paste into the canals of necrotic primary teeth resulted in success both radiographically and symptomatically (9). They coined this "Lesion Sterilization and Tissue Repair" (LSTR) therapy, and they concluded that "3Mix-MP should be used rather than 3Mix-sealer" (9). At this point, Hoshino and fellow researchers had developed what they believed to be the ideal sterilization paste, which is commonly referred to as "3Mix-MP". An article in the *Journal of Endodontics* in 2005 tested this combination in immature dog teeth with apical periodontitis. They found that the samples cultured after irrigation with sodium hypochlorite still had a high bacterial load, but those sampled after treatment with the antibiotic paste were nearly free of microorganisms (10). They again summarized the necessary steps to achieve

revascularization, including: disinfection of the canal, a resorbable matrix to encourage the in-growth of new tissues, and a coronal seal.

Apart from Hoshino's article that concluded macrogol ointment and propylene glycol should be used instead of root canal sealer, very little has been said in the literature about the carrier in the paste. Macrogol ointment is an international proprietary name for polyethylene glycol (PEG). PEG is a polyether that is used in a host of products, including: laxatives, skin creams, sexual lubricants, paintballs, hydraulic fluids, and toothpastes. It is a chemical that is believed to have a low degree of toxicity, but there is little in the research about its effect with long-term, low-dosage application to the human body. Propylene glycol is produced by Dow Chemicals, and it is an organic, colorless, odorless liquid. This chemical is used in various medicines, tobacco products, perfumes, coolants, and tattoo ink. Propylene glycol is generally regarded as safe, and in a study from 1972 it was determined to have no carcinogenic potential with dietary levels equivalent to 2-5 g/kg/day in rats (20).

There has been some research over the years warning of the possible toxicity of propylene glycol. It is interesting to note that the FDA does allow it to be used as a direct food additive, however, they state that it alters the acid/base balance in the human body. In addition, it is not approved for use in cat food due to safety concerns. In 1970, Martin and Finberg published a case report of an individual who experienced "toxic symptomotology" after ingesting large doses of vitamin C that had been suspended in propylene glycol (11). They concluded that this carrier may be dangerous to small children, and that further research should be initiated to investigate this potential. In a subsequent article in 1972, Ruddick concluded that propylene glycol possesses a low

toxicity, and that it could be employed within limits as a solvent (21). In a more recent article out of the *American Academy of Dermatology*, it was surmised that propylene glycol is well-suited for topical preparations, but it does have the potential to cause irritant skin reactions and allergic sensitization (22).

One article was located in the *Journal of Endodontics* with the keyword search of "propylene glycol." This particular article was in regards to calcium hydroxide, and its use as an antimicrobial agent within root canals. Calcium hydroxide dissolves in water to produce hydroxide ions that are effective in eradicating bacteria. However, according to the aforementioned research, the use of nonaqueous mixing vehicles with calcium hydroxide is not clearly understood. Following the experiment, the authors concluded that propylene glycol rendered the conductivitiy of the calcium hydroxide paste essentially zero. Furthermore, the nonaqueous mixture actually impeded the effectiveness of the calcium hydroxide (23). It is a common finding that the carrier products in Hoshino's paste are safe, however, there is some concern as to their toxicity over a long period of time, and possibly to their effectiveness. Futhermore, these products are difficult to obtain in our country, and there is a general lack of research regarding their use.

The current study is attempting to find if there a difference in the antimicrobial efficacy of the triple antibiotic paste utilized for regeneration when chlorohexidine is substituted as the carrier solution in lieu of the traditional macrogol ointment and propylene glycol mixture. Chlorhexidine gluconate is a solution that is very well known in the dental community, and first came on the scene as a general disinfectant. Chlorhexidine, or imidodicarbonimidic diamide, is a bactericidal agent and chemical

antiseptic that disrupts the membranes of gram +/- organisms. In a review of the chlorhexidine literature prior to 1972, Hirst referred to chlorhexidine as one of the most promising antimicrobial substances found in the dental literature (12). He went on to say that it was the solution's local antibacterial action, and the fact that it was absorbed into the hydroxyapatite of tooth surfaces, that made it safe for use long-term. In 1961, Birch and Melville completed an experiment in which chlorhexidine was first introduced as an endodontic irrigant (24). Later that decade, in 1964, another study used chlorhexidine as a potential irrigant, and commented on its ability to increase the permeability of dentin (25).

Using chlorhexidine as an antimicrobial during root canal therapy is a common thread in the literature since those 1960's articles. Today, it is not only offered in different concentrations as an acceptable irrigant, but it is also available in a form that is impregnated into gutta-percha points. The Activ Point, recommended as an interappointment filling material out of Langenau, Germany, is designed to aid in the sterilization of canals. In a recent article in the *Journal of Endodontics*, the authors reviewed the use of gutta-percha impregnated with chlorhexidine and other medicaments. Although they concluded that no interappointment medicine can replace proper instrumentation and irrigation during treatment, their studies did show that these modified gutta-percha cones had sufficient antimicrobial activity to eliminate what is left of the endodontal pathogens (26).

Chlorhexidine was introduced at 0.12% in a mouthrinse in the United States in 1986. Since that time, it has been proven that the solution is very effective at reducing oral flora, both supra- and sub- gingivally, and that it has a very high safety margin. In

addition, there has never been a reported case of bacterial resistance to the drug, making it a nearly ideal antimicrobial agent (13). The safety of chlorhexidine was again confirmed in vivo in dogs in a *JOE* study from 2002, in which it was shown that the solution was very well tolerated. In addition, the authors stated that in cases in which a tooth was immediately obturated following endodontic treatment, chlorhexidine resulted in better periapical repair than the more traditional sodium hypochlorite (27).

Chlorhexidine was shown in one study to be very cytotoxic to human fibroblasts in vitro. This was found in a paper in which the authors were attempting to determine the solution's safety for use in wound healing. The authors stated that numerous reports showed it was safe as an oral rinse, but following their study, they showed concern with chlorhexidine's use in direct exposure to connective tissue (28). Long-term exposure of the human body to chlorhexidine is proven to be safe in numerous studies. Some of the most impressive are those involving its use as a drug in local delivery agents to treat periodontal disease. One such research project used a split-mouth design to confirm chlorhexidine's safety and efficacy as a subgingivally placed drug delivery system. It also showed the solution to have the sought after substantivity property, which means chlorhexidine remains active for much longer than most medicaments (29). Jeffcoat et al reiterated these same results in a very similar study, and stated that the only adverse side-effects they had found were some tooth discoloration or sensitivity to very high doses of the drug (30).

In a 1999 research paper from the IEJ, the authors used a method much like the one in the current study, to test chlorhexidine's effectiveness against *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus salivarius*, *S. pyogenes*, *Escherichia coli*, and

Candida albicans. They found that 2% chlorhexidine was effective as an endodontic irrigant against the aforementioned microbes (31). They stated that sodium hypochlorite (5.25%) was the superior irrigant, but that chlorhexidine was strongly antimicrobial and "not toxic if extruded to the periapical tissues through the apex." Siqueira et al confirmed that chlorhexidine was effective in vitro against *E. faecalis* in 2002 (32). In addition, chlorhexidine was shown to be effective against all of the microorganisms used in the current experiment in a *JOE* article from 1999. In this study, the authors tested three different medicaments against several bacterial strains, including: *Actinomyces odontolyticus, Candida albicans, Enterococcus faecalis, Escherichia coli, Fusobacterium nucleatum, Psuedomonas aeruginosa, Staphylococcus aureus,* and *Streptococcus sanguis*. They discerned that chlorhexidine had a bactericidal effect on all of these microbes at all concentrations tested, and regardless of short time periods of contact (33).

In an attempt to study chlorhexidine's potential as a carrier agent of the triple antibiotic mixture in the regeneration protocol, the current study is using the previously mentioned research methodology found in the *International Journal of Endodontics* in 1999. The method involves plating several strands of bacteria, and then determining if there is a difference in antimicrobial action between the currently used 3Mix-MP, and the proposed 3Mix-CHX. A zone of inhibition is recorded for each mixture, and then the results are analyzed statistically (31). This same methodology was employed by Torabinejad et al in 1995 to determine the antibacterial efficacy of root end filling materials. The authors tested four materials against nine facultative bacteria. They first grew the bacteria on solid media, and then placed each filling material onto the surface of the agar plates. The diameter of the antibacterial effects were measured in millimeters

(15). In 2005 a very similar methodology was used for a study published in the *Journal of Endodontics*. The researchers inoculated agar plates with a total of eight bacterial strands, and then cut two 5mm wells into the surface of each plate. They were testing the effect of two mixing agents on the antibacterial efficiency of ProRoot Mineral Trioxide Aggregate. They placed one of each of the mixtures into the wells in the agar plates, and then following an incubation period, measured the zones of inhibition surrounding each well (14). This approach has been chosen as the method for the current research paper, and is being closely simulated.

In an attempt to study the antimicrobial effects of endodontic products, it is critical to choose the correct bacteria to represent the total population of microorganisms found within a necrotic root canal. One group of authors studying endodontic irrigants chose *Staphylococcus aureus, Enterococcus faecalis, Streptococcus salivarius, Streptococcus pyogenes, Escherichia coli,* and *Candida albicans* in a famous study from 1999 (31). In another research project comparing irrigants, the scientists thought is prudent to include both facultative aerobes-anaerobes and obligate anaerobes. In their study they chose four of the previously mentioned bacteria, as well as *Actinomyces odontolyticus, Fusobacerium nucleatum,* and *Streptococcus sanguis* among others (33). In the previously mentioned article out of the JOE in 2005, the authors used a total of eight bacteria that appear to be the best representation of the total population in a necrotic canal (14). Like the methodology, the idea for the microorganisms used in the current paper were obtained from that article.

Sundqvist et al discovered that *Enterococcus faecalis* plays a major role in cases of failed root canal therapy. They discovered that this gram-positive organism was the most

commonly discovered species in incomplete or defective root fillings, as well as in untreated root canals within an otherwise fully obturated tooth (34). *Actinomyces* are also often present in many endodontic infections, especially those associated with abscesses or cellulites. Like some *Enterococcus* species, they are often coupled with endodontic treatment that failed to heal (35). *Streptococcus sanguis* is another bacteria that was chosen for this study, because it grows readily under anaerobic conditions. It is commonly found in the root canals of diseased teeth, and it has the ability to adhere very well to hard tissue (36). *Staphylococcus aureus* is also a robust species, as shown by clinical research from the *JOE* in 2006. The study found that gram-positive facultative anaerobic cocci were more prevalent than a host of other bacterial strands following interappointment treatment with various medicaments (37).

The gram-negative *Fusobacterium nucleatum* has been found to be the prevailing bacterial species in diseased root canals (38). It has also been positively associated with a host of other microorganisms being used in the present study. According to Baumgartner, *Candida albicans* is found in pulpal disease about 20% of the time. This is a fungi that is often associated with infected root canals, but seldom detected in periradicular aspirates (39). Many other studies link the eight bacterial strains chosen for this experiment with pulpal disease. Each microorganism was chosen for its prevalence, or its hearty nature that makes it difficult to eradicate. The author of the current paper believes that if the carrier solutions used have antimicrobial properties, the bacteria chosen will be sufficient to bring this fact to light.

CHAPTER III

MATERIALS & METHODS

Escherichia coli, *Enterococcus faecalis*, *Psuedomonas aeruginosa*, and *Candida albicans* were each inoculated into 5 mL samples of LB broth (Fisher Scientific, Hampton, NH) and incubated aerobically for 24 hours at 37 degrees Celsius. *Staphylococcus aureus, Streptococcus sanguis,* and *Fusobacterium nucleatum* were each grown in chopped-meat glucose broth (Fisher Scientific) and incubated anaerobically (Figure 1) for 24 to 48 hours at 37 degrees Celsius. All bacteria were obtained from clinical stock strains in the West Virginia University Department of Pathology Laboratory (Morgantown, WV). One-hundred microliters of each suspension, with the turbidity standardized to McFarland 4.0, were spread on agar plates with an L-spreader for complete inoculation of the agar surface (Figure 2). There were four different platings for each of the seven bacterium, resulting in twenty-eight total agar plates.

A 6 mm blank paper disc (Becton, Dickinson, and Company, Sparks, MD) was fully saturated with a 20 microliter mixture of Hoshino's paste (3Mix + MP) (Figure 11) and positioned onto each agar plate. This combination was created by combining ciprofloxacin (200mg), metronidazole (500mg), and minocycline (100mg) (Figure 3 & 4) with a 1:1 mixture of propylene glycol (Paddock Laboratories, Minneapolis, MN) (Figure 5) and macrogol ointment (MP) (Gallipot Incorporated, St. Paul, MN) (Figure 6). A mixture of the three antibiotics, in the same amounts, and 0.12% chlorhexidine gluconate (CHX) (Colgate-Palmolive, New York City, NY) (Figure 7) was saturated onto a second blank paper disc (Figure 10) and also placed onto each agar plate (3Mix + CHX). A control agar plate for each of the individual microorganisms previously mentioned was

also made with the same 6 mm paper discs. Only the MP or CHX was placed on each disc on the control plates (Figure 12 & 13), without any addition of antibiotics (3Mix).

All plates were incubated at 37 degrees Celsius for 24 to 48 hours (Table 1) as required for an even lawn of bacterial growth. A blinded, independent observer then measured the zones of inhibition around each mixture. The zones were assessed in millimeters by taking an average of three measurements (north to south, east to west, and north-east to south-west). An analysis of variance (ANOVA) test was used to determine if significant differences in zones of inhibition occurred between experimental groups (3Mix + MP) and (3Mix + CHX) with a confidence level of $p<.05$.

Table 1. Incubation Times – All plates were incubated at 37 degrees Celsius for 24 to 48 hours, with exact times for each blood agar plate's incubation shown below.

BACTERIA/YEAST	**Time of Incubation**		**Time of Reading**	
Escherichia Coli (EC)	1/26/10, 12:17	1/26/10, 12:18	1/27/10, 12:30	1/27/10, 12:31
	1/26/10, 12:19	1/26/10, 12:20	1/27/10, 12:32	1/27/10, 12:34
Pseudomonas aeruginosa (PA)	1/26/10, 12:27	1/26/10, 12:28	1/27/10, 12:36	1/27/10, 12:37
	1/26/10, 12:29	1/26/10, 12:30	1/27/10, 12:38	1/27/10, 12:40
Staphylococcus aureus (SA)	1/26/10, 12:47	1/26/10, 12:49	1/28/10, 10:45	1/28/10, 10:46
	1/26/10, 12:50	1/26/10, 12:52	1/28/10, 10:47	1/28/10, 10:49
Enterococcus faecalis (EF)	1/26/10, 12:33	1/26/10, 12:35	1/27/10, 12:42	1/27/10, 12:43
	1/26/10, 12:36	1/26/10, 12:37	1/27/10, 12:44	1/27/10, 12:45
Streptococcus sanguis (SS)	1/26/10, 12:57	1/26/10, 12:58	1/28/10, 10:37	1/28/10, 10:38
	1/26/10, 12:59	1/26/10, 13:00	1/28/10, 10:40	1/28/10, 10:41
Fusobacterium nucleatum (FN)	1/26/10, 13:03	1/26/10, 13:04	1/28/10, 10:31	1/28/10, 10:32
	1/26/10, 13:05	1/26/10, 13:06	1/28/10, 10:33	1/28/10, 10:34
Candida albicans (CA)	1/26/10, 12:42	1/26/10, 12:43	1/27/10, 12:50	1/27/10, 12:51
	1/26/10, 12:44	1/26/10, 12:45	1/27/10, 12:52	1/27/10, 12:54

CODE	
Plate 1	**Plate 2**
Plate 3	**Control Plate**

Figure 1. Anaerobic Chamber – Anaerobic microorganisms were incubated in this Bactron 1.5 Anaerobic Environmental Chamber (Sheldon Labs, Crystal Springs, MS).

Figure 2. Inoculation of Agar Plate – An "L-spreader" was used to disperse bacteria onto blood agar plates prior to the placement of the antibiotic-soaked paper discs.

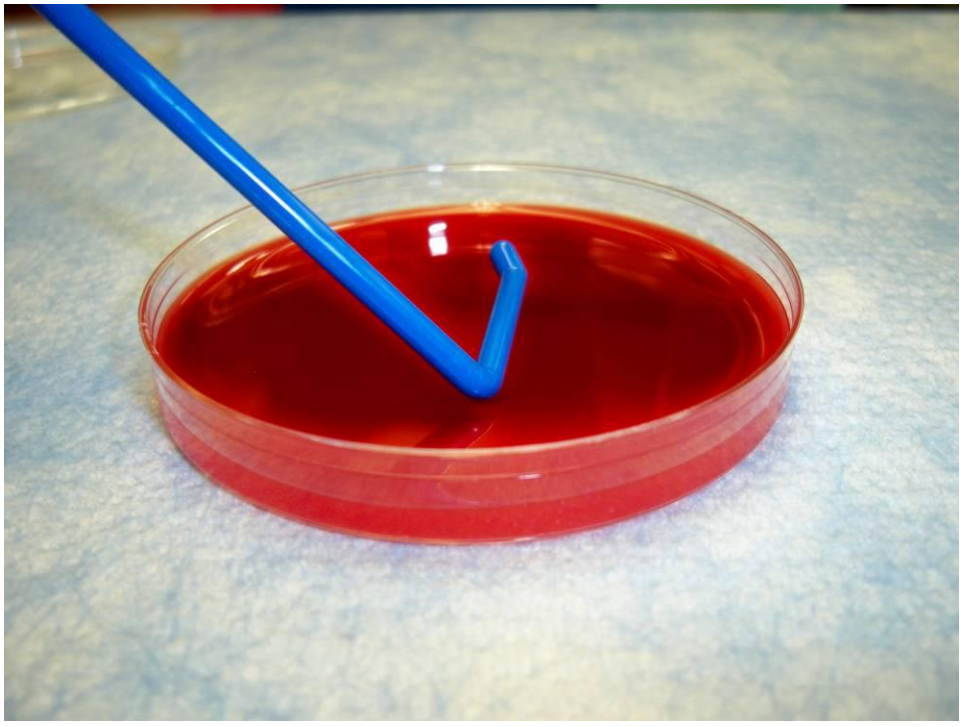


Figure 3. Triple Antibiotic Mixing with Mortar and Pestle – Tablets of Ciprofloxacin (200 mg), Metronidazole (500 mg), and Minocycline (100 mg) were crushed into a powder mixture with a mortar and pestle.

Figure 4. Mix of Ciprofloxacin, Metronidazole, & Minocycline – A yellow hue was noted in the antibiotic mixture as a result of minocycline's distinct yellow color.

Figure 5. Propylene Glycol – Solvent was drawn from the one pint bottle of propylene glycol USP (Paddock Labs, Minneapolis, MN) to create the MP mixture.

Figure 6. Macrogol Ointment – This is the one pint bottle of polyethylene glycol 400 NF (Gallipot Inc, St. Paul, MN) from which solvent was drawn to create the MP mixture.

Figure 7. Chlorhexidine Gluconate – Chlorhexidine gluconate (0.12%) was drawn from this 16 fluid ounce bottle of PerioGard (Colgate-Palmolive, New York City, NY).

Figure 8. Mixes of MP and CHX – The triple antibiotic powder (center) was mixed with PerioGard (Right) to create 3Mix+CHX, and with propylene glycol/macrogol ointment (Left) to create 3Mix+MP.

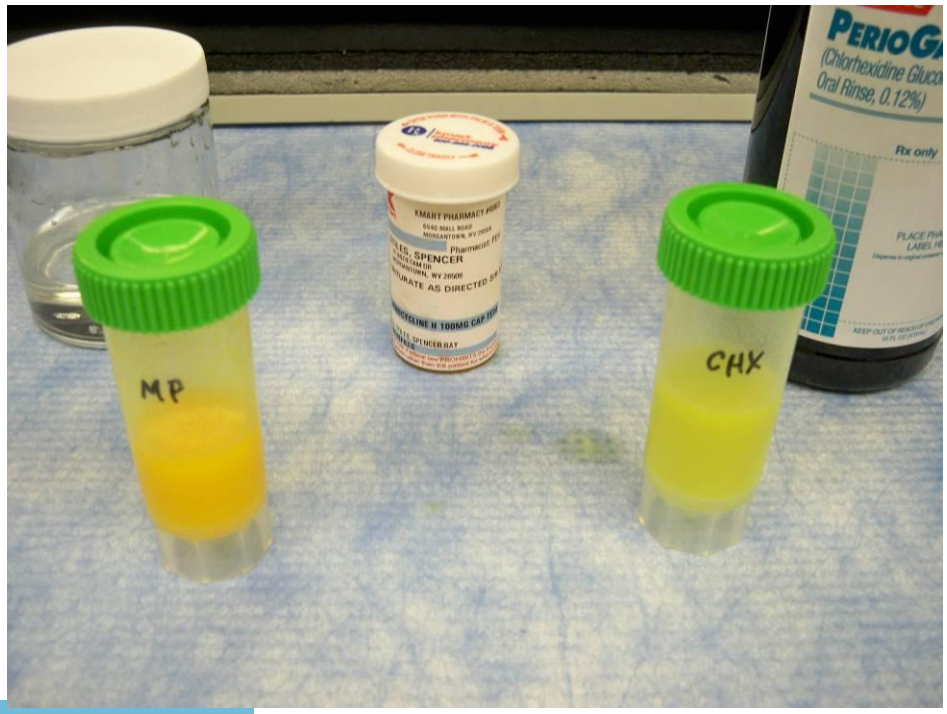


Figure 9. Prepared 6mm Discs – Blank paper discs were saturated with (from Left to Right) 3Mix+CHX, 3Mix+MP, CHX only, or MP only.

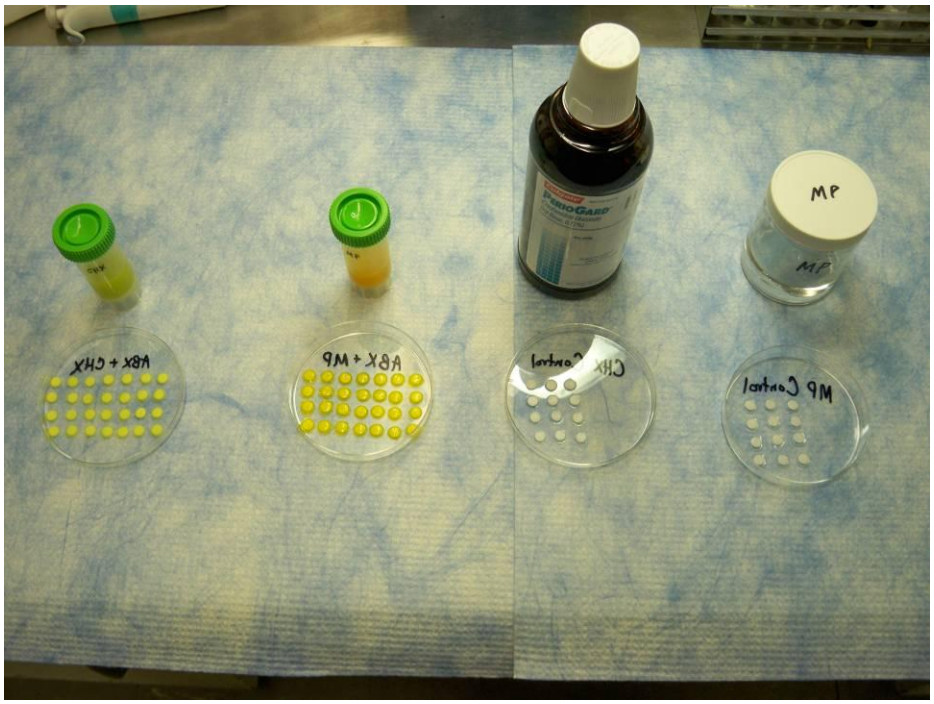


Figure 10. Discs Saturated with 3Mix + CHX

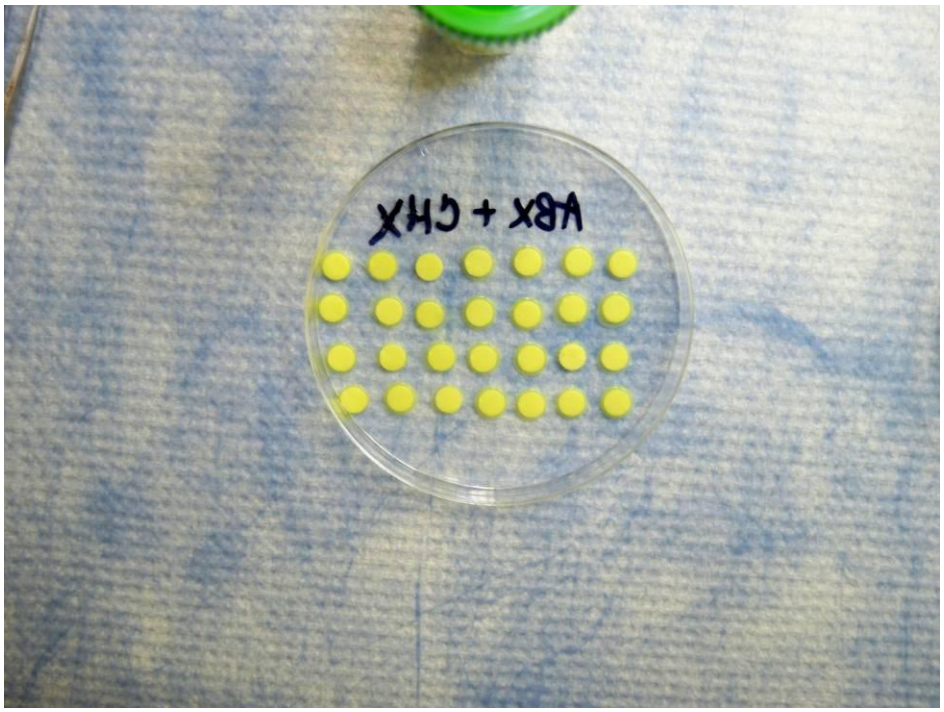


Figure 11. Discs Saturated with 3Mix + MP

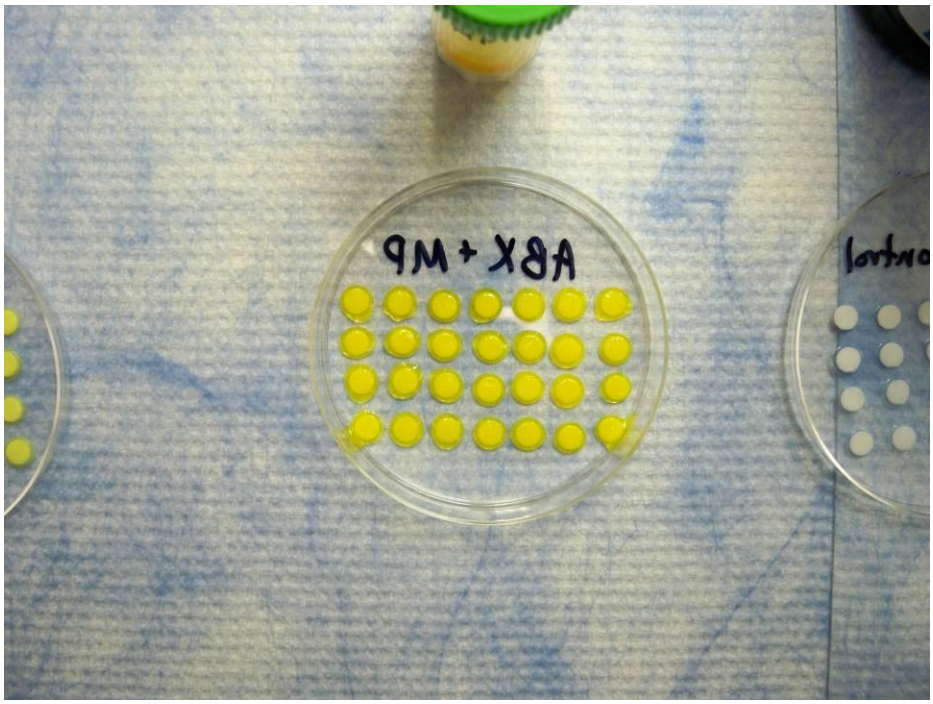


Figure 12. Discs Saturated with MP Only

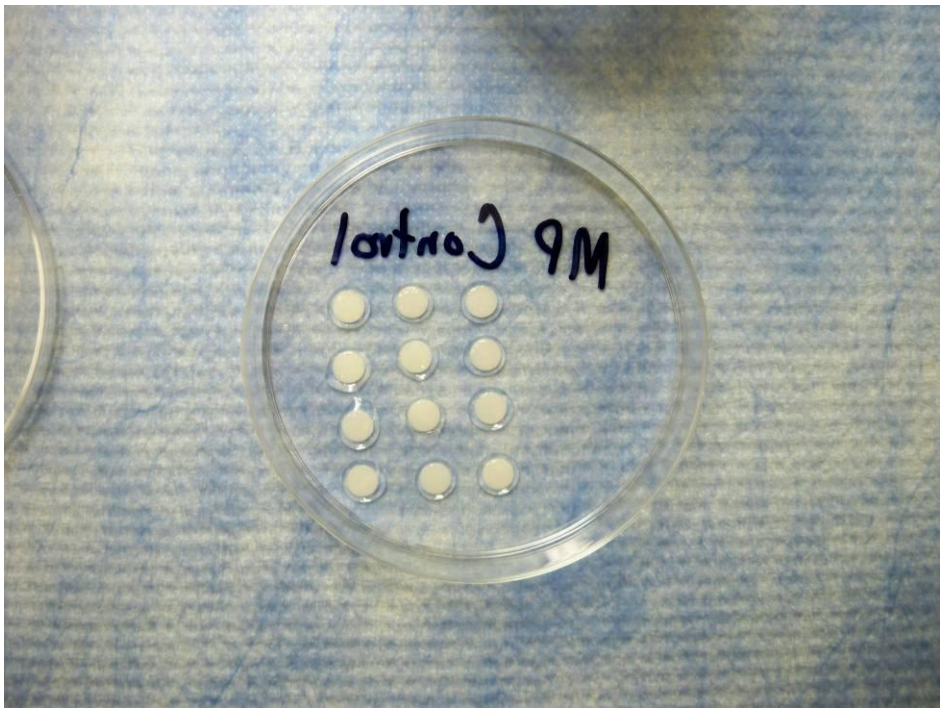


Figure 13. Discs Saturated with CHX Only

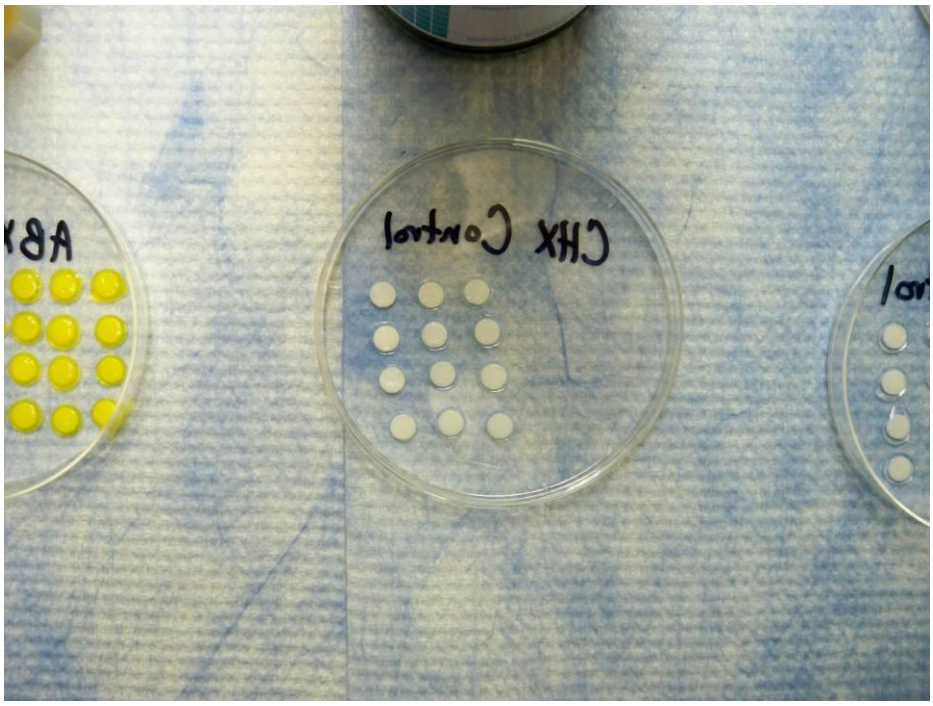
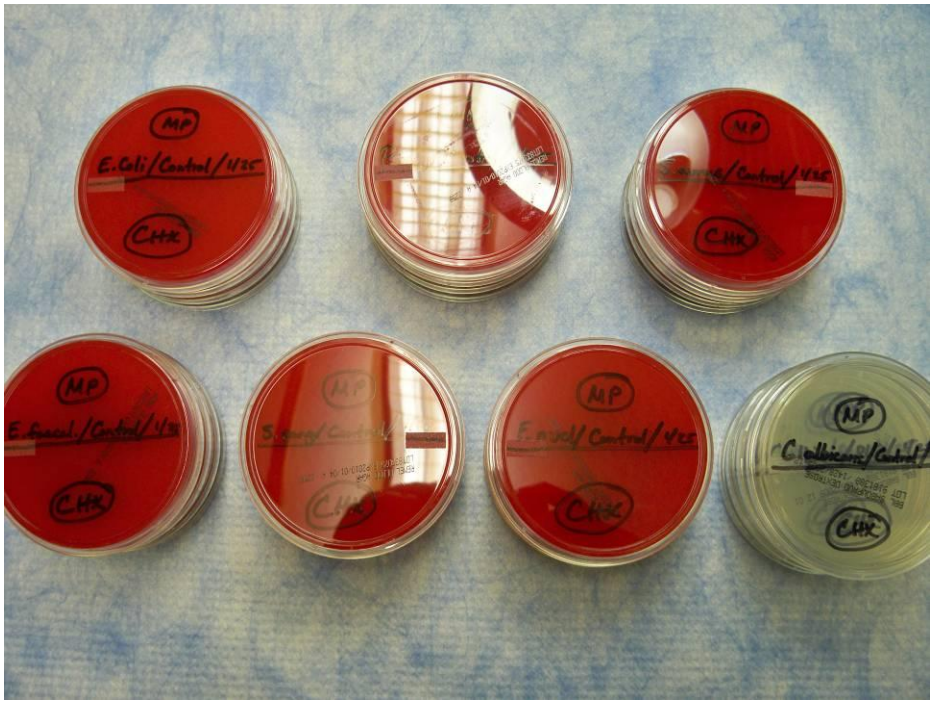


Figure 14. Example of Agar Plate Correctly Labeled – All blood agar plates were divided with an indelible marker into two sections (MP and CHX), and labeled with the name of the bacteria, plate number, and date.

Figure 15. All Agar Plates Labeled (7x4=28 Total Plates) – One stack of four plates was made for each microorganism, and the stacks were then placed into the incubator for 24 to 48 hours. The stack in the lower right corner are sabouraud dextrose plates (not blood agar like all of the others), upon which *C. albicans* was grown.



CHAPTER IV

RESULTS

Table 2 and Figures 16 and 17 display data collected while measuring the zones of inhibition around each saturated paper disc. Mixtures containing chlorhexidine gluconate (CHX) showed greater antimicrobial activity in every test than those containing the propylene glycol and macrogol ointment (MP) mixture ($p<.004$). This statement is inclusive of the triplicate plates, as well as the control plates, where CHX always revealed a zone of inhibition while MP never displayed said zone.

An ANOVA statistical analysis (Table 3-8) reveals that the mean zone of inhibition for CHX was 23, while the mean zone for mixes that incorporated MP was 20. *Fusobacterium nucleatum* was the most resistant bacteria to the various triple antibiotic mixtures, with *Escherichia Coli* showing the largest zones of inhibition. The bacteria that exhibited the most intra-plate difference was *Enterococcus faecalis* where the mean zone size for CHX was 21, while the mean size for MP was 14. Visual results are also included by way of several pictures of the experimental agar plates.

Table 2. Zones of Inhibition (mm) – These zones were measured in millimeters, and each measurement was made by a blinded, independent observer. The final measurement was achieved by taking an average of three separate observations for each zone.

BACTERIA/YEAST	MP Plate 1	CHX Plate 1	MP Plate 2	CHX Plate 2	MP Plate 3	CHX Plate 3	MP Control	CHX Control
Escherichia Coli (EC)	32.0	35.0	33.0	36.0	33.0	38.0	0.0	7.0
Pseudomonas aeruginosa (PA)	30.0	31.0	30.0	32.0	28.0	31.0	0.0	7.0
Staphylococcus aureus (SA)	20.0	22.0	22.0	26.0	22.0	24.0	0.0	14.0
Enterococcus faecalis (EF)	14.0	21.0	16.0	22.0	14.0	21.0	0.0	8.0
Streptococcus sanguis (SS)	22.0	25.0	22.0	25.0	21.0	22.0	0.0	13.0
Fusobacterium nucleatum (FN)	7.0	8.0	7.0	8.0	6.0	7.0	0.0	19.0
Candida albicans (CA)	15.0	18.0	16.0	18.0	17.0	19.0	0.0	8.0

Figure 16. Zones of Inhibition per Microorganism (Stacked Column) – This graph illustrates the spectrum of bacterial resistance to the various triple antibiotic pastes from the most susceptible (*E. coli*) to the least (*F. nucleatum*).

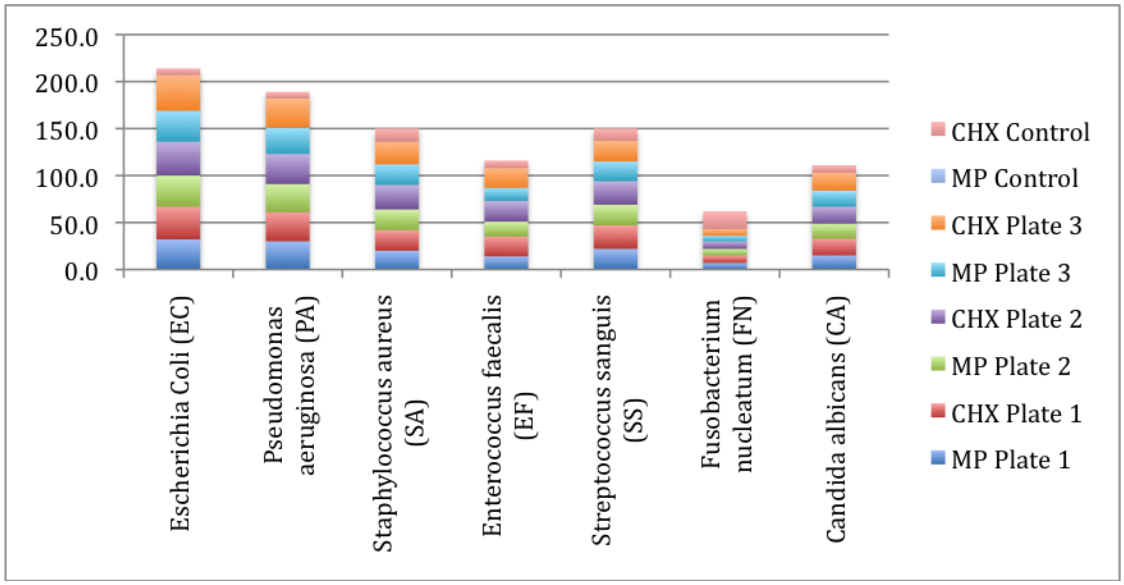


Figure 17. Zones of Inhibition per Agar Plate (Stacked Column) – This graph illustrates that the total antimicrobial action of CHX was greater than that of MP per agar plate. In addition, it displays the significant contrast between the zones of inhibition measurements between the two control groups.

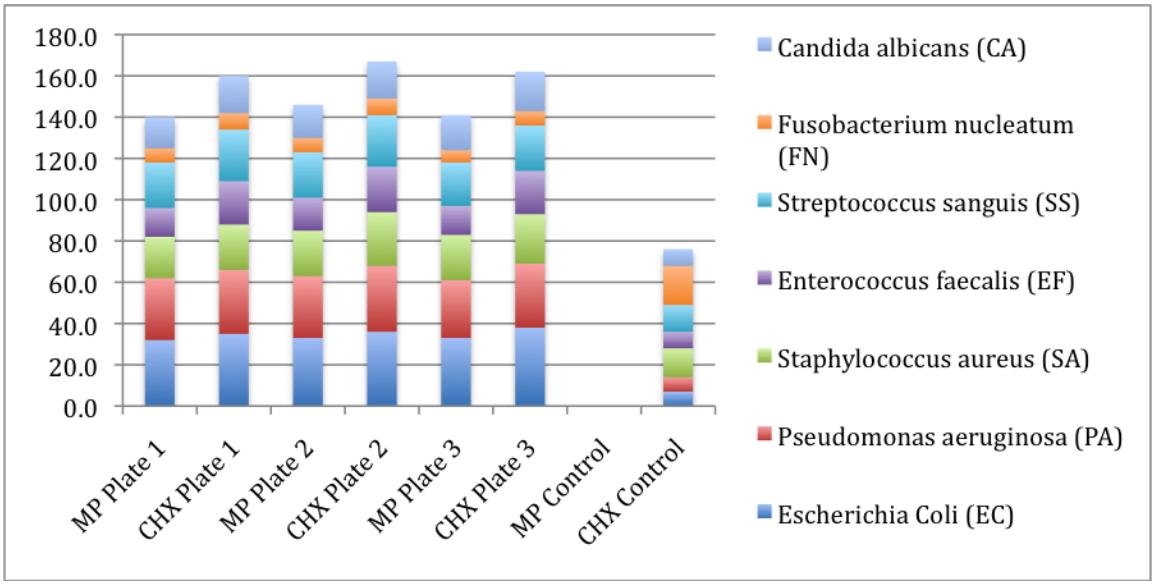


Table 3. Summary of Fit – This is a multiple regression table displaying summary statistics.

RSquare	0.989144
RSquare Adj	0.984104
Root Mean Square Error	1.091089
Mean of Response	21.80952
Observations (or Sum Wgts)	42

Table 4. Analysis of Variance (ANOVA) – This table illustrates the statistically significant difference between the antimicrobial properties of 3Mix+CHX versus 3Mix+MP. The substitution of CHX into the paste was significant to p<.004.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	13	3037.1429	233.626	196.2462
Error	28	33.3333	1.190	**Prob > F**
C. Total	41	3070.4762		<.0001*

Table 5. Effect Tests – This table, and the following three, display the least squares significance levels for the effects of organism, liquid, and organism combined with liquid.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Organism	6	6	2915.8095	408.2133	<.0001*
Liquid	1	1	91.5238	76.8800	<.0001*
Organism*Liquid	6	6	29.8095	4.1733	0.0040*

Table 6. Least Squares Means Table (Organism)

Level	Least Sq Mean	Std Error	Mean
Candida albicans (CA)	17.166667	0.44543540	17.1667
Enterococcus faecalis (EF)	18.000000	0.44543540	18.0000
Escherichia Coli (EC)	34.500000	0.44543540	34.5000
Fusobacterium nucleatum (FN)	7.166667	0.44543540	7.1667
Pseudomonas aeruginosa (PA)	30.333333	0.44543540	30.3333
Staphylococcus aureus (SA)	22.666667	0.44543540	22.6667
Streptococcus sanguis (SS)	22.833333	0.44543540	22.8333

Table 7. Least Squares Means Table (Liquid)

Level	Least Sq Mean	Std Error	Mean
CHX	23.285714	0.23809524	23.2857
MP	20.333333	0.23809524	20.3333

Table 8. Least Squares Means Table (Organism * Liquid)

Level	Least Sq Mean	Std Error
Candida albicans (CA),CHX	18.333333	0.62994079
Candida albicans (CA),MP	16.000000	0.62994079
Enterococcus faecalis (EF),CHX	21.333333	0.62994079
Enterococcus faecalis (EF),MP	14.666667	0.62994079
Escherichia Coli (EC),CHX	36.333333	0.62994079
Escherichia Coli (EC),MP	32.666667	0.62994079
Fusobacterium nucleatum(FN),CHX	7.666667	0.62994079
Fusobacterium nucleatum (FN),MP	6.666667	0.62994079
Pseudomonas aeruginosa (PA),CHX	31.333333	0.62994079
Pseudomonas aeruginosa (PA),MP	29.333333	0.62994079
Staphylococcus aureus (SA),CHX	24.000000	0.62994079
Staphylococcus aureus (SA),MP	21.333333	0.62994079
Streptococcus sanguis (SS),CHX	24.000000	0.62994079
Streptococcus sanguis (SS),MP	21.666667	0.62994079

Figure 18. Least Squares Means Plot – This graph visualizes the greater zones of inhibition for CHX over MP on each of the bacteria. It also displays the profound difference in this effect on *E. faecalis.*

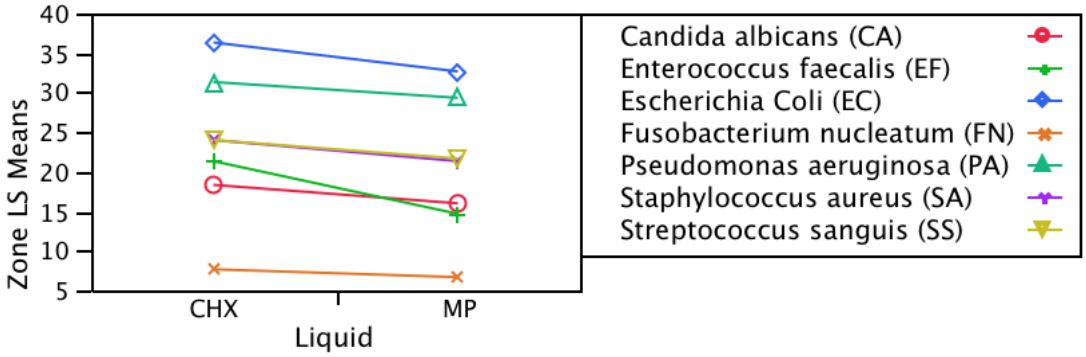


Figure 19. Example of *E. coli* Experimental Plate – This shows an agar plate following incubation with E. coli and the two antibiotic-soaked discs (top with MP liquid, bottom with CHX). Notice the relatively large zones of inhibition around each disc on the *E. coli* blood agar plating.

Figure 20. Example of *E. coli* Experimental Control Plate – This plate contains the same microorganism (E. coli) as above, except without 3Mix added onto either disc. MP is on the top (NO zone of inhibition) and CHX is one the bottom (zone of inhibition present).

Figure 21. Example of *P. aeruginosa* Experimental Plate – This shows an agar plate following incubation with *P. aeruginosa* and the two antibiotic-soaked discs (top with MP liquid, bottom with CHX). Notice the relatively large zones of inhibition around each disc on the *P. aeruginosa* blood agar plating.

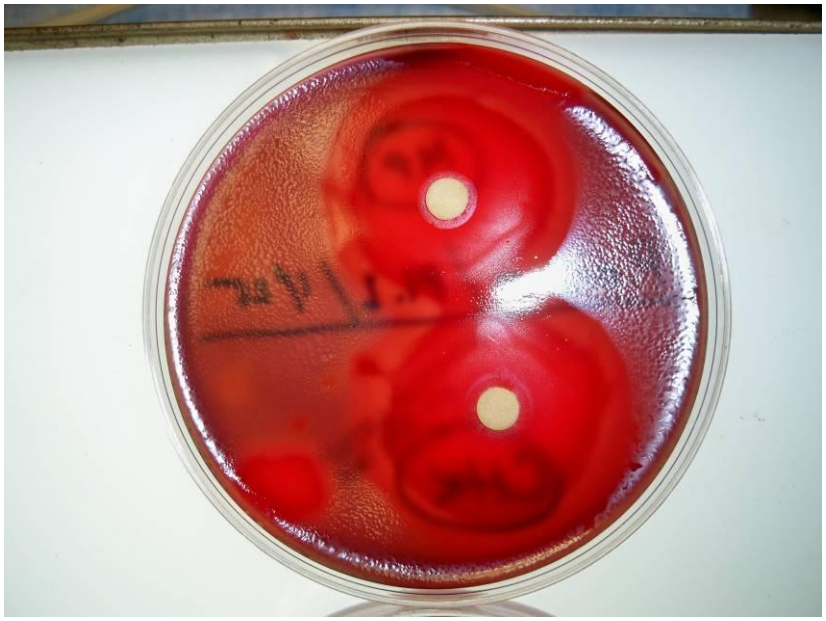


Figure 22. Example of *P. aeruginosa* Experimental Control Plate – This plate contains the same microorganism (*P. aeruginosa*) as above, except without 3Mix added onto either disc. MP is on the top (NO zone of inhibition) and CHX is one the bottom (zone of inhibition present).

Figure 23. Example of *C. albicans* Experimental Plate -- This shows an agar plate following incubation with *C. albicans* and the two antibiotic-soaked discs (top with MP liquid, bottom with CHX). Notice the irregular borders around the zones of inhibition on the *C. albicans* sabouraud dextrose plating.

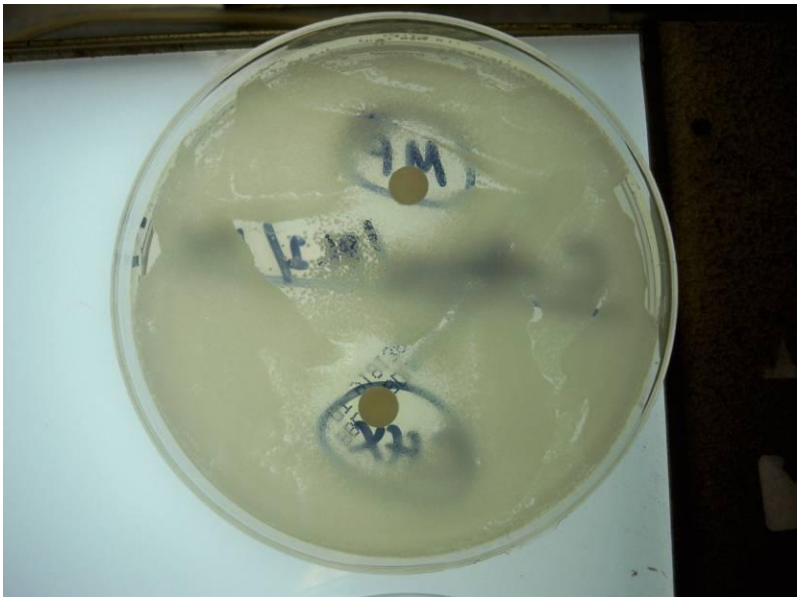
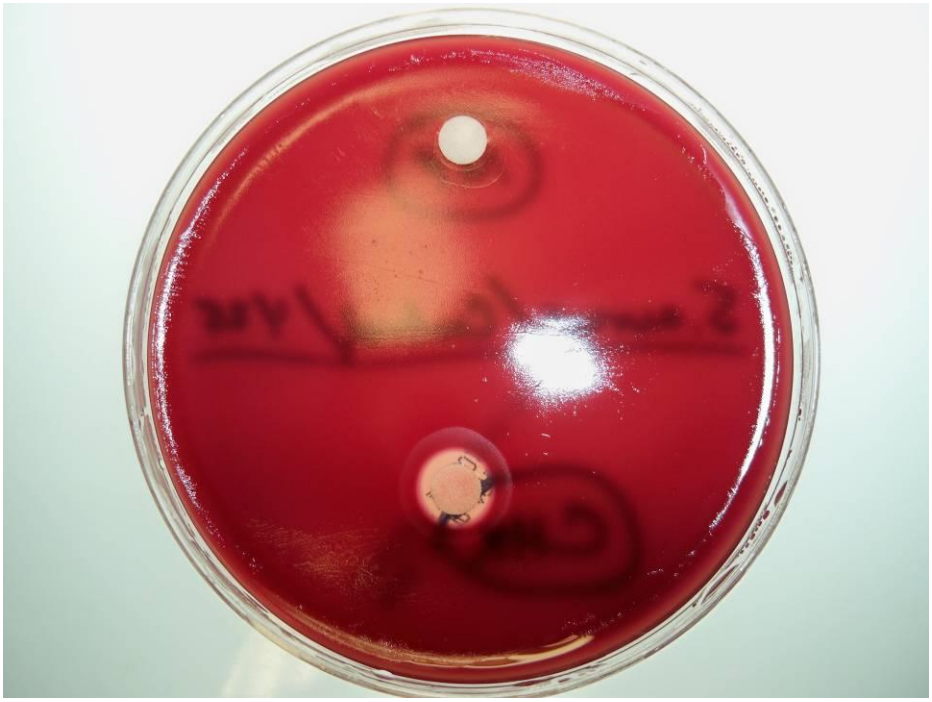


Figure 24. Example of *S. aureus* Experimental Plate – This shows an agar plate following incubation with *S. aureus* and the two antibiotic-soaked discs (top with MP liquid, bottom with CHX). Notice the different appearance of the zones of inhibition on a plate containing a microorganism that was plated anaerobically.

Figure 25. Example of *S. aureus* Experimental Control Plate – This plate contains the same microorganism (*S. aureus*) as above, except without 3Mix added onto either disc. MP is on the top (NO zone of inhibition) and CHX is one the bottom (zone of inhibition present).



DISCUSSION

The purpose of this study was to determine if there is a difference in the antimicrobial efficacy of the triple antibiotic paste utilized for regeneration when 0.12% chlorhexidine gluconate (CHX) is substituted as the carrier solution in lieu of the traditional macrogol ointment and propylene glycol mixture (MP). It was assumed that the average practioner's unfamiliarity with the MP used in the current protocol has led many to use untested carriers, or completely forego regeneration cases. It was also assumed that CHX is readily available, and is safe when used in a manner consistent with the regeneration protocol. It was determined that if sound research could show that CHX did not impede the antimicrobial efficacy of the three antibiotics in the protocol, it could be used as a replacement in future regeneration techniques.

This research showed that chlorhexidine gluconate (CHX) had a synergistic effect on the antimicrobial efficacy of the triple antibiotic paste (3Mix) tested in this study. Because the antibiotics mixed with chlorhexidine (3Mix + CHX) showed larger zones of inhibition in every assay than the antibiotics mixed with propylene glycol and macrogol ointment (3Mix + MP); one can conclude that CHX was positively assisting the antibiotics in their function, or MP was negatively affecting the mixture. Since the control plates showed CHX with zones of inhibition in every trial, and MP with no zone of inhibition in any trial, it is evident that CHX is indeed effective against the chosen microorganisms, while MP is not. This is not a novel idea, as CHX was chosen for this experiment because of its known broad antibacterial action (12), its substantivity, and its availability.

This experiment allows the practitioner to now use chlorhexidine gluconate (CHX) as the liquid carrier in their triple antibiotic paste with the newfound confidence that their mixture is more antimicrobial than 3Mix + MP mixtures *in vitro*. It can be stated that CHX mixtures showed significantly more antimicrobial efficacy than propylene glycol and macrogol ointment mixtures against each bacterium chosen for this research ($p<.004$). Furthermore, because of past research we know that CHX is a safe alternative for use intraorally (13), and we have established the usefulness of the data collected in similar *in vitro* agar-diffusion studies (14,15).

The bacteria and yeast chosen for this study were meant to represent the total population of microorganisms found within a necrotic tooth. Analogous groups of microbes have been chosen for past similar studies (14,31), and it is assumed that the effect of the various mixtures in this experiment on the microorganisms selected is representative of the effect of the mixtures on other species of bacteria found in necrotic root canals. It was determined that 3Mix + CHX was significantly more effective against each of the seven microorganisms selected than was 3Mix + MP. This experiment included gram-positive, gram-negative, aerobic, and anaerobic bacteria; as well as C. albicans, a yeast that has been shown to be found very often in pulpal disease (39). There was no significant difference between the effect of the antibiotic paste on aerobes versus its zone size of anaerobes.

E. faecalis plays a major role in failed root canal therapy (34), and is considered one of the most difficult bacteria to eradicate from a root canal system. It is interesting to note that this was the very microorganism to which the CHX had its most profound effect. The roughly parallel lines in Figure 18 exhibit the fact that CHX showed

approximately the same amount of increased effectiveness as compared to MP. However, the line representing this difference in efficacy against *E. faecalis* is noticeably of increased steepness. This anomaly is worth further research to determine if CHX is indeed "super effective" against this known offender.

During this research notes were made regarding the physical properties of the two different liquid carriers. The 1:1 mixture of macrogol ointment and propylene glycol (MP) was much more turbid and thick than the CHX mixture. These ointment, or lubricant-like, properties of MP may allow it to better maintain the 3Mix within the root canal system when compared to a less dense liquid. However, this oil-based nature may also cause it to block infected dentin tubules, or remain intact in the canals for a lengthy duration resulting in an increased opportunity of allergic sensitization (22). Further research is indicated to confirm or refute these hypotheses. There is also some recent concern regarding chlorhexidine's potential cytotoxic effect on human fibroblasts and connective tissue (26,28), a topic of importance that requires further clinical trials. Future studies will make the use of CHX in regeneration more practical, but for now we can reject the null hypothesis that there is no significant difference in the antimicrobial properties between using MP versus CHX as the carrier for Hoshino's triple antibiotic paste.

CHAPTER V

SUMMARY

Regeneration of necrotic teeth is the ultimate form of root canal therapy. Attempts have been made to revitalize teeth for many years with varying success. Numerous antibiotics and antimicrobials have been offered as the panacea of disinfectants for this purpose. With each mixture that has been utilized for this regeneration procedure, some type of carrier ointment or liquid has been called upon to transport the antimicrobial into the canal. Although many studies, and clinical trials, have assessed the success rates of the various antibiotics, no known study has directly observed the effect of the carrier ointment.

The current study was an attempt by the author to shed new light on the carriers used in regeneration, and to offer a novel carrier as a choice to practitioners. Hoshino's triple antibiotic paste is the most widely used mixture to disinfect root canals for regeneration, utilizing propylene glycol and macrogol ointment as the carriers. These ingredients are not readily available, and have not been reviewed in the dental literature. The current study sought to replace these carriers with 0.12% chlorhexidine gluconate, a carrier that has antimicrobial properties of its own, is readily available to practitioners, and is thoroughly researched.

It was hypothesized that there would be no difference in the antimicrobial efficacy of Hoshino's triple antibiotic paste if chlorhexidine were substituted for propylene glycol and macrogol ointment. An *in vitro* agar-diffusion model was used, and the null hypothesis was rejected because it was determined that mixtures including chlorhexidine showed significantly more antimicrobial action than those with propylene glycol and

macrogol ointment. The chlorhexidine did not impede the antibiotics in the mixture, and in fact, worked synergistically against every microorganism tested to create larger zones of inhibition. Randomized clinical trials are needed to demonstrate the effectiveness of chlorhexidine in regneration more clearly, but this *in vitro* study offers practitioners a new option in carriers for the triple antibiotic paste.

CONCLUSION

Chlorhexidine gluconate increased the antimicrobial efficacy of Hoshino's triple antibiotic paste. When mixed with the three antibiotics, it showed a statistically significant increase in antibacterial action over that of propylene glycol and macrogol ointment. Chlorhexidine alone also exhibited antimicrobial results, while propylene glycol and macrogol ointment had no inherent antimicrobial activity.

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