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Formulation and Evaluation of a Novel Irrigant for the Disinfection of Root Canals Using a *Candida albicans* Biofilm Model

Dustin S. Reynolds
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Formulation and Evaluation of a Novel Irrigant for the Disinfection of Root Canals Using a *Candida albicans* Biofilm Model

Dustin S. Reynolds, DDS

Thesis submitted to the
School of Dentistry
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Master of Science
In
Endodontics

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Morgantown, West Virginia
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Irrigation, Penetration, Biofilm, *Candida albicans*, Clindamycin, Nystatin, Dimethyl sulfoxide

Abstract

Formulation and Evaluation of a Novel Irrigant for the Disinfection of Root Canals Using a *Candida albicans* Biofilm Model

Dustin S. Reynolds, DDS

An important goal of root canal therapy is elimination of microorganisms which are usually organized into a biofilm phenotype. Irrigants play an indispensable role in areas inaccessible to mechanical instrumentation. In this study, a novel endodontic irrigant was formulated composed of a penetrating solvent, 70% Dimethyl sulfoxide (DMSO), with a 1:1 molar ratio of an antibiotic (Clindamycin HCl) and antifungal agent (Nystatin). Nuclear Magnetic Resonance Spectroscopy (NMR) stability tests were conducted to prove the mixture was chemically stable. This study compared different concentrations of the novel irrigant to 6% sodium hypochlorite (NaOCl) using a *C. albicans* biofilm model. Furthermore, it is proposed that the novel irrigant has the ability to penetrate into dentin tubules and therefore be able to achieve better root canal disinfection.

Solutions of the irrigant were prepared and used at 100%, 50%, and 25% concentration then compared to 6% NaOCl in the following tests: ZOI, CFU, absorbance, coverage area, and penetration. Standard zones of inhibition (ZOI) trials were conducted to verify that the novel irrigant was, in fact, effective against microorganisms. *C. albicans*, a common oral fungi, and possible co-aggregate in biofilm formation, was then grown on hydroxyapatite-coated pegs (Calgary Biofilm Model) for 48 hours at 37° C and immersed in treatment solutions for one minute. Biofilm was removed from the pegs via sonication and colony forming units per ml were assayed. On additional pegs, biofilm was stained with crystal violet, washed, placed in glacial acetic acid and read at 562nm; the absorbance values reflected the amount of remaining biofilm. On other pegs the remaining biofilm was stained and analyzed with computer software to determine the coverage area. Lastly, ten extracted human teeth were prepared, sterilized, and irrigated using 6% NaOCl, Ethylenediaminetetraacetic acid (EDTA), and the novel irrigant at 100%, 50%, and 25% concentration. The teeth were then stained with Rhodamine B and viewed at 10X with a laser confocal microscope to evaluate penetration.

The ZOI studies showed that the novel irrigant possessed effective anti-microbial properties. The CFU assay positive control showed 2.9×10^6 CFUs, the irrigant concentrations from greatest to least had the following CFUs respectively: 8.7×10^3 , 2.7×10^4 , and 6.7×10^3 . No CFUs were observed with NaOCl; corresponding absorbance data revealed the following: 0.786, 0.830, 0.818 compared to a control value of 0.765. NaOCl had an absorbance of 0.843. These numbers suggest similar activity against a recalcitrant *C. albicans* biofilm. The biofilm coverage assay showed that the novel irrigant was more effective than 6% NaOCl. By utilizing the laser confocal microscope the ability of the novel irrigant to penetrate into dentin tubules was visualized.

Preliminary results using a *C. albicans* biofilm model show that all concentrations of the novel irrigant are anti-biofilm and demonstrate similar efficacy to that of 6% NaOCl. Furthermore, the novel irrigant is able to penetrate further into dentinal tubules than that of 6% NaOCl and EDTA or some combination of the two.

Dedication

To Cary: I thank you and your family for accepting me as one of your own. I could not have made this journey without you. Thank you for leaving your job, moving six hours from home, and putting up with me so that I could pursue my dreams. You are a beautiful person, an amazing woman and exceptional mother.

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To my family: Dad thank you for always providing me with every possible opportunity and for teaching me to be a friend, having never met a stranger, and always looking to help others in need. Mom, thank you for supporting me in all that I have set out to accomplish, you are my rock. Thanks for always listening and understanding, no matter what the circumstances. P.G., I hope that I have been a positive example for you. Thank you for being an amazing brother, listener, friend, and uncle. I pray that life blesses you to the fullest. Always strive for your best. I love you all and appreciate everything that you have done for me.

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Chapter 1

Introduction

Even with the evolution of modern rotary instrumentation, various new sealers, and multiple obturation techniques there is no substitute for proper chemical disinfection and physical debridement of the root canal system. Every health care professional has a disease to address, with endodontists treating pulpitis (inflammation of the pulp tissues) and apical periodontitis (inflammation and pathology associated with the apical tissues). It has long been known that microorganisms are the main cause of periapical disease (Takehashi 1965). Therefore, what is placed into the prepared root canal system for the purpose of obturation may not be as important as what is removed prior to placing a final seal. One of the goals of effective root canal therapy (RCT) is the elimination of bacteria from the root canal system and the prevention of recontamination (Bystrom & Sundqvist 1981). Endodontic therapy remains an effective means of stabilizing and preserving the natural dentition. The rate of success versus failure of endodontic therapy completed at West Virginia University from 1959 to 1979 was evaluated and determined that success had been achieved in 89.66% of 1,770 canals (Swartz 1983). Other studies report success rates of up to 96% (Sjogren 1990). Success rates of endodontic therapy are known to be better when teeth are free of bacteria after chemo-mechanical instrumentation (Bystrom 1987, Sjogren 1997). Under most clinical circumstances, endodontists are unable to create a sterile root canal environment, and the aim of treatment should be to effectively reduce the bio load in the root canal system and apical tissues by proper disinfection to a level where the body's defenses and healing mechanisms are able to prevail. Proper instrumentation is undoubtedly an important component of effective RCT and provides for the removal of a significant amount of intra-radicular infected dentin. Irrigants play an

indispensable role in areas inaccessible to instrumentation such as lateral and accessory canals, fins, and webs (Hasselgren 1988).

The properties of an ideal endodontic irrigant include possessing an anti-bacterial effect, ability to dissolve organic and inorganic tissue, have a lubricating and flushing effect, not be toxic to surrounding tissue, and not weaken tooth structure (Haapasalo 2010). Though there are currently numerous endodontic irrigants on the market, none of the presently available agents meet such requirements. In this study, a new endodontic irrigant containing 70% Dimethyl sulfoxide and an approximate 1:1 molar ratio of Clindamycin HCl and Nystatin is evaluated. This new irrigant is compared to full strength (6%) NaOCl on the ability to eliminate known endodontic pathogens, including *Candida albicans*, from a cultured biofilm grown on hydroxyapatite coated plastic pegs (Calgary Biofilm Device). The new irrigant is also compared to 6% NaOCl and 17% Ethylenediaminetetraacetic acid (EDTA) for its ability to penetrate into dentin tubules. Laser confocal microscopy was used to visualize penetration into dentin tubules of extracted human teeth.

Statement of the Problem

Will a novel endodontic irrigant composed of Clindamycin, Nystatin, and DMSO have the ability to penetrate into the dentin tubules and be anti-microbial against a *Candida albicans* biofilm phenotype as compared to full strength NaOCl?

Significance of the Problem

Although the success rate of non-surgical endodontic therapy is relatively high (92% success in cases without apical periodontitis and 74% in cases with apical periodontitis according to Phase One of the Toronto Study), it remains that if 24 million root canals are performed in the United States each year, there are 2 million failures of cases without apical periodontitis and 6 million failures of cases with apical periodontitis (Friedman 2003). With the advent of modern rotary instrumentation we are able to better clean and shape the canal space; however, it is well known that the canal anatomy and dentin tubule structure may still harbor pathogens that can contribute to endodontic failure. In a case study by Vieira et al., it was shown that a persistent intraradicular infection caused by bacteria located within dentinal tubules was the most reasonable explanation for resurgence of an apical periodontitis lesion (Vieira 2012). These areas are only accessible by chemical debridement; therefore, if the success rate of non-surgical endodontic therapy is to be increased, an endodontic irrigant capable of addressing these areas inaccessible to mechanical debridement must be developed.

Null Hypothesis

There is no significant difference in the ability of the test solution to penetrate into dentin tubules and effectively eliminate a *Candida albicans* biofilm phenotype as compared to full strength NaOCl.

Assumptions

- 1) NaOCl is the most commonly used irrigation material and thus chosen for comparison in this experiment.
- 2) Passive irrigation with a 30ga ProRinse[®] (DENTSPLY) needle results in a clinically acceptable method of irrigation.
- 3) No current irrigation materials meet the characteristics of an ideal irrigant.
- 4) Incomplete or ineffective irrigation contributes to endodontic failure and apical periodontitis.
- 5) A Calgary Biofilm Device study model is an appropriate method of evaluating irrigant effectiveness against a biofilm phenotype.
- 6) Laser Confocal Microscopy is an effective way to visualize penetration into dentin tubules.

Limitations

- 1) An in vitro experiment is simulating an in vivo situation.
- 2) There is a human element in performing all techniques.
- 3) The plastic hydroxyapatite coated pegs may not accurately simulate biofilm formation in vivo.
- 4) The root canals used will be similar, but will not have identical morphology.
- 5) Individual root canal system variability could affect the instrumentation and irrigation of any given tooth.

Delimitations

- 1) The extracted teeth were selected carefully in an attempt to minimize variability.
- 2) Only single rooted anterior teeth were selected
- 3) Teeth selected had completely formed apices
- 4) Teeth were sectioned at the mid-root level to allow uniform sampling of penetration into the canal.
- 5) All experimentation, instrumentation, and irrigation were performed by the principal investigator.

Chapter 2

Review of Literature

Sodium hypochlorite (NaOCl) in varying concentrations from 0.5% to 6% has long remained as the most commonly recommended irrigating solution utilized in RCT. It was first recommended as an antiseptic solution by Dr. Henry Dakin (Dakin 1915). In a recent survey by Dutner to members of the American Association of Endodontists (AAE), it was found that >90% of respondents used NaOCl as an irrigant (Dutner 2012). NaOCl has strong antibacterial (Bystrom 1983) and tissue dissolving effects (Rosenfeld 1978), but is toxic to periapical tissue (Gernhardt 2004) and has been suggested to degrade micromechanical characteristics of dentin (Ari 2004). In addition, NaOCl has no effect on the inorganic part of the smear layer (Baumgartner 1987). Therefore 17% EDTA is used as part of some irrigation protocols after the use of NaOCl to remove the smear layer by chelation. In 2011, Qian et al. showed that if NaOCl is used again after EDTA or citric acid as the final antibacterial rinse, it causes marked erosion of the root canal wall dentin (Qian 2011). While NaOCl remains a part of the endodontic armamentarium, there continues to be some areas of debate and concern over its use.

The subject of much discussion over the years has been which dilution of NaOCl is appropriate to use during endodontic irrigation. In a case study by Cymbler and Ardakani, it was shown that the accidental injection of sodium hypochlorite into the periapical soft tissues can elicit a severe reaction including severe pain and a burning sensation (Cymbler 1994). 5.25% NaOCl has been used as an irrigant for at least seven decades (Walker 1936). Shih's tube dilution studies showed that NaOCl had germicidal activity against *Str. faecalis* and *S. aureus*, even at a dilution of 1:1,000; however, only full strength NaOCl had an apparent immediate sterilizing effect in root canals of extracted human teeth inoculated with *Str. faecalis* or *S. aureus* (Shih 1970). A study performed by Hand et al. found that 5.25% NaOCl was significantly more

effective as a necrotic tissue solvent than 2.5% NaOCl, 1% NaOCl, 0.5% NaOCl, distilled water, normal saline solution, or 3% Hydrogen Peroxide (Hand 1978). For many years the bulk of literature had turned its attention on NaOCl's ability to dissolve necrotic tissue with no mention of its effect on vital tissue.

Rosenfeld et al. performed a study to evaluate the solvent action of 5.25% NaOCl on vital human pulp tissue that had not been disturbed by instrumentation. They found that in non-confined areas, it is apparent that NaOCl has a strong, nonspecific, surface-acting solvent action on vital, intact, young, pulp tissue (Rosenfeld 1978). Major problems have also been reported when 5.25% NaOCl is injected into human periapical tissue (Sabala 1989). Because of these types of safety concerns, Spangberg et al. recommended 0.5% NaOCl for acceptably non-cytotoxic levels (Spangberg 1973). Baumgartner and Cuenin could not detect any difference in the removal of pulpal remnants and predentin in the middle third of root canals with 5.25%, 2.5%, or 1% NaOCl delivered with either a needle or an ultrasonic device. 0.5% NaOCl left what appeared to be a few fibrils from predentin on the surface (Baumgartner 1992).

Hand et al. stated that "the surface area of tissue exposed to the test solution is important because dissolution is a function of surface contact" (Hand 1978). Koskinen et al. stressed the importance of good contact between tissue and irrigant (Koskinen 1980). Palazzi et al. concluded that the new 5.25% NaOCl solutions modified with surfactants, Hypoclean A and Hypoclean B, had surface tension values that were significantly lower than Chlor-Xtra™ and 5.25% NaOCl. It was also reported that these new irrigants have the potential to both penetrate more readily into un-instrumented areas of the root canal system and also allow a more rapid exchange with fresh solution enabling greater antimicrobial effectiveness and enhanced pulp tissue dissolution ability (Palazzi 2012). Baker et al. concluded that "the removal of micro-

organisms and debris seemed to be a function of the quantity rather than the type of irrigant” (Baker 1975). Moorer et al. found that an excess of organic matter rapidly depletes the activity of NaOCl and also lowers the pH drastically during the first moments of the reaction (Moorer 1982). Moorer concluded that “the amount of available chlorine in a hypochlorite system is important, not the mere initial strength of the hypochlorite solution. The recommended strength of antibacterial NaOCl is dependent on the amount of organic substances that are present. It can be safely stated that any hypochlorite solution that is able to dissolve tissue is, at the same time, a powerful disinfectant” (Moorer 1982). Baumgartner also concluded that “the effectiveness of low concentrations of NaOCl may be improved by using larger volumes of irrigant, by frequent exchange of irrigant, or by the presence of replenished irrigant in the canals for longer periods of time” (Baumgartner 1992). Senia et al. found that full strength NaOCl did not appear to be very effective in removing pulp tissue which remained after instrumentation. Furthermore, without direct contact with NaOCl, the deeper protected tissues were dissolved more slowly and less completely (Senia 1971). Any tissue that remains after instrumentation and in deeper areas may harbor additional bacteria that could lead to endodontic failure.

MTAD[®] (DENTSPLY) is a mixture of 3% doxycycline, 4.25% citric acid, and a detergent (Tween 80). It was introduced in 2003 by Torabinejad and Johnson at the Loma Linda University as an endodontic irrigant to address some of the shortcomings of NaOCl and other endodontic irrigants. It is considered to be a clinically effective, biocompatible endodontic irrigant (Zhang 2003). Ring et al. concluded that the cytotoxicity of NaOCl/MTAD[®] was found to be slightly less than NaOCl and NaOCl/EDTA, indicating that MTAD[®] is more biocompatible than NaOCl (Ring 2008). This finding may prove beneficial as we move toward more pulp regenerative techniques in endodontics. Doxycycline, being MTAD[®]'s antibacterial agent, is a

bacteriostatic antibiotic and does not kill bacteria; it prevents the multiplication of susceptible bacteria. It can be assumed that residual bacteria may resume growth when appropriate conditions are restored and may cause reinfection (Singla 2011). Early in-vitro studies by Torabinejad showed that MTAD[®] demonstrated superior ability in killing *E. faecalis* and exerted better anti-bacterial properties at high dilutions than did NaOCl (Torabinejad 2003). Later research however, has shown that MTAD[®] may not exhibit optimal antimicrobial properties. In a study by Kho and Baumgartner, it was shown that there was no difference in the antimicrobial efficacy for irrigation with 5.25% NaOCl and 15% EDTA versus irrigation with 1.3% NaOCl and MTAD[®] in the apical 5mm of roots infected with *E. faecalis* (Kho 2006). Dunavant found that NaOCl was significantly more efficient in elimination *E. faecalis* biofilms than MTAD[®] (Dunavant 2006). In a study performed by Johal et al., no growth of *E. faecalis* was found in root canals irrigated with 5.25% NaOCl and 15% EDTA, while 50% of canals irrigated with 1.3% NaOCl and MTAD[®] demonstrated growth of *E. faecalis* (Johal 2007). Giardino et al. showed that only 5.25% NaOCl could disaggregate and remove the *E. faecalis* biofilm each time when compared to MTAD[®] and other endodontic irrigants (Giardino 2007). In regard to anti-fungal activity, Ruff et al. showed that 6% NaOCl and 2% chlorhexidine independently were equally effective and superior to MTAD[®] and 17% EDTA independently (Ruff 2006). De-Deus et al. reported significantly faster dissolution of inorganic material by both 5% citric acid and MTAD[®] as compared with 17% EDTA (De-Deus 2007). Also, MTAD[®] has been shown to solubilize pulpal tissue (Beltz 2003). MTAD[®] has made its mark on endodontics as an adjunct to the current irrigation protocol, but some of its antibacterial efficacy has come into question, leading to the pursuit of several possibilities of alternative irrigants. In addition, as is the case

with many endodontic products, MTAD[®]'s use has been hindered by its relatively high cost and difficult handling and storage characteristics.

QMiX[™] (DENTSPLY) is an endodontic irrigant that possesses antimicrobial effects with the added ability to remove the smear layer in one ready to use solution. QMiX[™] contains EDTA, chlorhexidine (CHX) and a detergent. Mixing EDTA and CHX is known to produce a white precipitate (Rasimick 2008). There has also been concern that mixing NaOCl and CHX produces a brown/orange precipitate that could potentially be carcinogenic. Despite the CHX content, mixing QMiX[™] with NaOCl does not produce any precipitate and the solution does not change color (Stojicic 2012). The antimicrobial agent in QMiX[™], CHX, is a chemical antiseptic with activity against both gram positive and gram negative organisms. It exerts both bacteriostatic and bactericidal effects. CHX is found in many dental applications as well as some topical preparations. CHX is deactivated by anionic compounds commonly used as surfactants in detergents (Denton 2001). In a study by Stojicic et al. it was concluded that QMiX[™] and NaOCl were superior to CHX and MTAD[®] under laboratory conditions in killing *E. faecalis* and plaque bacteria in planktonic and biofilm culture (Stojicic 2012). Ma et al. concluded that QMiX[™] was equally effective in killing bacteria in dentin as 6% NaOCl (Ma 2011). In a study by Ordinola-Zapata et al. comparing 1% NaOCl, 2% CHX, 10% citric acid, 17% EDTA, and distilled water, only 1% NaOCl had a significant effect on biofilm viability and architecture (Ordinola-Zapata 2012). Mohammadi and Abbott conclude that CHX is an effective antifungal agent, but its efficacy is significantly less than NaOCl (Mohammadi 2009). The ability of QMiX[™] to remove the smear layer is comparable to EDTA (Stojicic 2012). Because CHX does not have the ability to dissolve tissue, QMiX[™] is recommended as a final rinse after NaOCl. Dai et al. concluded that the two experimental versions of QMiX[™] used in the open canal

designed experiment were as effective as 17% EDTA in removing canal wall smear layers after the use of 5.25% NaOCl as the initial rinse (Dai 2011). While QMiX™ is another addition to the endodontic irrigation protocol there are still some shortcomings of its use as a lone irrigant and inability to dissolve tissue.

Dimethyl sulfoxide (DMSO) was first synthesized by Alexander Saytzeff in 1867 by the oxidation of dimethyl sulfide (Saytzeff 1867). DMSO is basically a by-product of a process known as kraft pulping, in which wood is turned into wood pulp for many paper making applications. Lignin is the source of DMSO and wood contains approximately 25-30% lignin. In 1962, Crown Zellerbach obtained a patent for the processing of this by-product, which utilized the methyl groups of kraft lignin by adding molten sulfur to form dimethyl sulfide. Nitrogen tetroxide was then used to oxidize the dimethyl sulfide to dimethyl sulfoxide (Herschler 1965). Several peculiar chemical and therapeutic properties of DMSO were soon discovered when Dr. Stanley Jacob began seeking a relatively pure solution of DMSO for use as a cryostatic agent to protect organ transplants against freezing damage while in storage (David 1972). Perhaps the most fascinating characteristic of DMSO is its ability to penetrate living tissues without causing significant damage. This is related to its relatively compact structure and polar nature as well as its capacity to accept hydrogen bonds. Dr. Stanley Jacob at the University of Oregon Medical School then began a series of experiments to show that DMSO was an active penetrant and carrier of other substances through the skin or tissue membranes. Initial studies showed that DMSO enhanced the absorption of heparin, insulin, sodium salicylate, Evans Blue dye, sulfadiazine, aminophylline, and thio TEPA through the intact urinary bladder of the dog (Jacob 1964). DMSO is classified as a dipolar, aprotic hydroscopic solvent that appears pyramidal in shape or like a tetrahedron with the sulfur atom in the center and the two methyl groups, the

oxygen atom, and a nonbonding electron pair located at the apices (David 1972). The ability of DMSO to penetrate skin and mucous membranes may be the result of its dynamic interaction with tissue water (Szmant 1971). “The alteration in the configuration of immobile protein structures brought about by DMSO exchanging sites with bound water molecules may explain the penetration of DMSO through the skin. The transfer of DMSO across the dermal barrier is accomplished rapidly and completely without irreversible tissue damage and the alterations in protein structure are reversible” (David 1972). There have been numerous animal studies to confirm the membrane penetrability of DMSO. McDermot, Murray & Heggie investigated the percutaneous penetrability and drug carrier potentials of DMSO in guinea pigs and rabbits. They detected longer plasma duration of 1-methyl-2-hydroximinomethylpyridium methane sulfonate (P2S) when it was added to DMSO and applied topically versus intramuscular injection. This was attributed to the slow continuous passage of the compound through the skin or by DMSO interfering in some way with the metabolism or excretion of P2S (McDermot 1965). Hucker et al. found maximum serum levels of DMSO 4 to 8 hours after dermal administration; these levels then declined and 36-48 hours after application no DMSO was detected (Hucker 1967). Kligman obtained the fastest and greatest penetrability of dyes, corticosteroids, and other pharmacological agents through skin with 70-90% DMSO (Kligman 1965 Part I). Chronic toxicity studies have been made for a number of routes of administration using various concentrations of DMSO. Dermal application of 100% DMSO to backs of guinea pigs in 28 daily applications and to hairless mice given two applications a week for 30 weeks caused no skin irritation and no toxicity (Smith 1967). Kumar et al. showed that DMSO and Ethanol acted as a penetration enhancer for the transdermal delivery of Acyclovir and skin flux of the drug could be enhanced up to 107 fold compared to its aqueous solution (Kumar 2011). In addition to

being able to penetrate human membranes and potentially carry other compounds with it, some have found that DMSO exerts anti-inflammatory and pain relief ability on its own. "Pain relief from dermal administration of DMSO to affected areas seems to involve more than local analgesia, anti-inflammatory action, or vasodilation. Cutaneous application of DMSO at some distance from the affected site usually relieves pain and inflammation in the latter location, suggesting that DMSO either has been carried by the circulation to the affected area to act locally or has depressed the central nervous system pain centers or both" (David 1972). Ramirez & Luza gave intramuscular injections of 5ml of 80% DMSO two or three times a day to 42 severely disturbed psychiatric patients. They felt that DMSO had antipsychotic and antianxiety properties and that its action differed from tranquilizers in that little sedation or central depression was produced (Ramirez 1967). It was shown in a study by Formanek & Kovac that DMSO increased the infiltration of leukocytes into the traumatized area of cotton pellet implantation and suggested that the decreased formation of granulation tissue is due to an anti-inflammatory action (Formanek 1966). Kligman observed that DMSO possesses potent histamine-like properties at the site of cutaneous application, resulting in an increased blood flow to the skin (Kligman 1965 Part I). DMSO has even been shown to have antibacterial properties of its own. Jacob et al. found 20% DMSO bacteriostatic against *E. coli*, *Staph. aureus*, *Pseudomonas*, and in 5% concentration, against the tubercle bacillus (Jacob 1964). Kligman found DMSO a weak antifungal agent, which might be more effective when topical fungicides are incorporated in it (Kligman 1965 Part II). Jacob reported on studies that indicated pretreatment of tubercle bacilli resistant to 2000 micro grams of streptomycin or isoniazid with 0.5-5% DMSO rendered this organism sensitive to 10 micro grams of either drug (Jacob & Wood 1967, Jacob 1971). Kamiya et al added 5% DMSO to culture media, which restored the sensitivity of antibiotic-resistant

strains of bacteria to antibiotics and increased the sensitivity of those strains already sensitive to these agents. For example, DMSO restored the sensitivity of *Pseudomonas* to colistin. In addition, growth of *E. coli*, which is not affected by penicillin, showed growth-inhibitory effects when 5% DMSO was added to the culture (Kamiya 1966). Significant amounts of methylmethanthiosulfinate was identified in DMSO treated bacterial cultures by Tsuchiya, Iriyama, & Umezawa, which they believe contributed to the antibacterial effect of DMSO (Tsuchiya 1964). Gillchriest & Nelson believe DMSO may interfere with bacterial growth by altering the RNA conformational structure required for protein synthesis (Gillchriest 1969). In an article by Rosenbaum, continued dermal application of DMSO in patients with postoperative or posttraumatic intractable pain, post-amputation phantom pain, and tic douloureux resulted in marked pain relief in 32 of 37 patients (Rosenbaum 1965). Jacob found that over 75% of patients in a 60 patient study obtained relief of headache secondary to trigeminal neuralgia, sinusitis, cervical osteoarthritis, and temporal arteritis, when about 10-15ml of 50% DMSO were applied twice daily to the skin of the face and neck (Jacob 1965).

Clindamycin has long been used to treat odontogenic infections of endodontic origin. Clindamycin reversibly binds to 50S ribosomal subunits preventing peptide bond formation thus inhibiting bacterial protein synthesis. It is bacteriostatic or bactericidal depending on the drug concentration, infection site, and organism. Clindamycin is metabolized in the liver where clindamycin phosphate is converted to clindamycin HCl (active). Clindamycin is excreted in the urine (10%) and feces (4%) as active drug and metabolites. Clindamycin is a pregnancy risk factor category B meaning adverse events were not observed in animal reproduction studies. Clindamycin crosses the placenta throughout pregnancy and at term, but use during pregnancy has not been shown to cause adverse fetal effects. Clindamycin pharmacokinetics are not

affected by pregnancy. Small amounts of clindamycin transfer to human milk. The manufacturer does not recommend the use of clindamycin during breast-feeding. Clindamycin has not been shown to interfere with oral contraceptive activity; however, it reduces GI micro-flora, thus, oral contraceptive users should be advised to use additional methods of birth control. About 1% of clindamycin users develop pseudomembranous colitis. Prolonged use may result in fungal or bacterial super-infection, including *C. difficile*-associated diarrhea (CDAD) and pseudomembranous colitis; CDAD has been observed >2 months post-antibiotic treatment.

Nystatin binds to sterols in fungal cell membrane, changing the cell wall permeability allowing for leakage of cellular contents. Nystatin is excreted in the feces. It is a pregnancy category risk factor C. Animal reproduction studies have not been conducted. Adverse events in the fetus or newborn have not been reported following maternal use of vaginal nystatin during pregnancy. Absorption following oral use is poor. Excretion into breast milk is not known. The biological activity of polyene antibiotics (PA's), like Nystatin, sharply increases after their dissolution in DMSO and they are tenfold more efficient than their water-solved forms. DMSO is the main solvent of membranoactive polyene antibiotics such as nystatin (Ibrajimoova 2004).

The polymorphic fungus *Candida albicans* is a member of the normal human micro biome. In most individuals, *C. albicans* resides as a lifelong, harmless commensal. Under certain circumstances, however, *C. albicans* can cause infections that range from superficial infections of the skin to life-threatening systemic infections. *Candida* now ranks as the fourth most frequently encountered microbe among nosocomial bloodstream pathogens (Pfaller 1998). Several factors and activities have been identified which contribute to the pathogenic potential of this fungus. Among them are molecules which mediate adhesion to and invasion into host cells, the secretion of hydrolases, the yeast-to-hypha transition, contact sensing and thigmotropism,

biofilm formation, phenotypic switching and a range of fitness attributes (Mayer 2013). *C. albicans* may be the universal co-aggregate of endodontic biofilm formation. In a study by Baumgartner et al. the presence of *C. albicans* was detected in 21% of samples taken from root canals (Baumgartner 2000). *C. albicans*, a normal commensal of human mucosal surfaces and opportunistic pathogen in immunocompromised patients, is most frequently associated with biofilm formation. Colonization of *C. albicans* allows the development of adherent biofilm structures from which cells can then detach and cause an acute fungemia and/or disseminated infection (Ramage 2012). Microbiologists have historically studied planktonic organisms (free floating and homogeneous cells) in pure culture. However, there has been a paradigm shift as the link between sessile (surface attached and heterogeneous cells) and microbial pathogenesis and human infection is now widely accepted (Costerton 1995). Estimates suggest that up to 80% of all microorganisms in the environment exist in biofilm communities (Donlan 2002). A growing body of knowledge suggests that organisms in biofilms assume a stronger pathogenic potential than those in a planktonic state (Svensater 2004). “Biofilms are defined as highly structured communities of microorganisms that are either surface associated or attached to one another and are enclosed within a self-produced protective extracellular matrix (ECM). Biofilm formation includes protection from the environment, resistance of physical and chemical stress, metabolic cooperation, and a community-based regulation of gene expression. There has been an increased appreciation of the role that fungal biofilms play in human disease as microbes growing within biofilms exhibit unique phenotypic characteristics compared to their planktonic counterpart cells, particularly increased resistance to antimicrobial agents” (Ramage 2009). Glycoproteins and proteins supplied by saliva or gingival crevicular fluid act as the primary source of nutrients for the microbial communities of dental biofilms (Beighton 1986).

Chapter 3

Materials and Methods

Formulation of Novel Irrigant:

Chemically pure samples of Clindamycin HCl (Lot # 021M1533V) and Nystatin (Lot # 110M8715V) were purchased through Sigma Aldrich (Milwaukee, WI). DMSO was obtained through Jacob Laboratories (Portland, OR) in 70% concentration. An approximate 1:1 molar ratio of the two substances was dissolved in the DMSO (1mg Nystatin, 0.425mg Clindamycin HCL, 0.75mL of 70% aqueous DMSO [1.08mM or 0.1%wt.]). NMR spectroscopy was performed to ensure stability of the new mixture.

There was a concern that Clindamycin HCl may not be stable in the presence of Nystatin. Clindamycin is an alkyl chloride and Nystatin contains a primary amine. Thus, alkylation of the amine portion of Nystatin to form the Clindamycin-Nystatin adduct (Figure 1) is possible. While alkyl chlorides are often of low reactivity, alkylation reactions of the type shown in figure 1 can occur in DMSO and solvents containing a preponderance of DMSO. This possibility was examined using NMR.

The experiment required proton NMR data be acquired on samples of Clindamycin HCl, Nystatin, and a 1:1 mixture of the two. If no reaction occurred, the spectrum of the 1:1 mixture should be comprised of the sum of the two spectra. However, if a reaction occurred, significant changes in chemical shift of some of the protons are expected. Chemical shift is dependent upon the attached functional groups. Upon going from Clindamycin to the Clindamycin-Nystatin adduct, the chlorine is replaced by a nitrogen. Protons adjacent to a chlorine appear at below 3.44 ppm (below 3.44 means at numeric values greater than 3.44 ppm or protons adjacent to a chlorine are expected to appear downfield of 3.44 ppm and there is a shift expected due to the beta amino group to move it further downfield). If the chlorine were replaced by an amino

group, an up-field shift is expected and the same proton would be expected to appear above 3 ppm (i.e., numerically, less than 3 ppm).

Figure 1: Clindamycin-Nystatin Adduct

Clindamycin HCl, Nystatin, D₂O, and dms₂-d₆ were obtained from Aldrich (Milwaukee, WI). All materials were used as received. Samples containing Nystatin were protected from light. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Inova spectrometer operating at 600 MHz. NMR tubes were obtained from Wilmad Glass (Vineland, NJ). NMR assignments were made using a combination of TOCSY spectra, selective decoupling experiments, chemical shift and coupling information obtained from the NMR spectra. The assignments were in general agreement with literature values for clindamycin (Verdier 2000) and nystatin (Bruheim 2004) though the literature values were obtained in solvents other than D₂O/dms₂-d₆.

Stock solutions for NMR studies of Clindamycin HCl and Nystatin were prepared in a mixture of D₂O and dms₂-d₆ (3:7) at a concentration of 1 mM. Samples that only contained Clindamycin HCl or Nystatin were prepared by mixing the stock solution (0.5 mL) and then diluting with D₂O and dms₂-d₆ (3:7) (0.5 mL). The Clindamycin HCl/Nystatin sample was prepared by mixing equal amounts of the Clindamycin HCl and Nystatin stock solutions. Once prepared, the NMR samples were maintained at 25° C.

Formulation of novel irrigant bulk sample:

A sterile spatula was used to remove Clindamycin and Nystatin from their original containers which had been stored out of sunlight at 0 degrees Fahrenheit in a commercial freezer. Sterile pipets were used to remove the 70% DMSO from its stock container. In a weight boat on a digital scale, the following was measured .0212g Nystatin, .0108g Clindamycin, 15mL 70% aqueous DMSO. The powder contents were carefully emptied into a 20mL screw top vial carefully using the 70% DMSO to wash the powder residue from the weigh boat into the vial. The remaining necessary 70% DMSO was added and the mixture was vortexed until all particles were dissolved. The sample was stored in a commercial refrigerator at two degrees Celsius and the vial containing the novel irrigant was covered with aluminum foil to protect from sunlight.

Cultivation of micro-organisms:

Blood agar plates were streaked with stock samples of the following bacteria:

Escherichia coli

Pseudomonas aeruginosa

Staphylococcus aureus

Candida albicans

Lactobacillus

These five organisms were chosen to be used in preliminary screening trials. Based on the ease of growth, bi-phasic nature, and response to initial trials, *C. albicans*, was targeted for further study. The plates were placed in an incubator at 37 degrees Celsius for 24 hours.

ZOI Measurement (Biofilm and Planktonic Phenotype):

A 1 uL loop of bacteria was taken from the blood agar plates and then placed into 5 mL of Tryptic Soy Broth (TSB) in a test tube. The test tube was vortexed to ensure mixture of contents. 250 uL of bacteria-containing TSB was plated onto a 30% poloxamer (nonionic tri-block of copolymers composed of a central hydrophobic chain of polyoxpropylene flanked by two hydrophilic chains of polyoxyethylene) plate (from stock poloxamer that had been poured and allowed to reach room temperature in order to firm). An additional bacterial sample was also placed on stock Mueller-Hinton (Planktonic) agar plates. The liquid was spread with an L-spreader. Blank discs (designed to hold a specific amount of treatment solution) were allowed to soak in each solution for 10 seconds and then they were wicked on a paper towel and placed onto the inoculum of bacteria. See figure 10. The plates were placed into an incubator (at 37°C) for 24 hours. After 24 hours, the plates were removed and ZOIs were recorded for each treatment type. ZOIs were measured in mm with a digital micrometer. Plates were then placed back into the incubator and ZOIs were again recorded at 48hrs.

Figure 2: Blood Agar Plate inoculated with *C. albicans*

Figure 3: TSB culture media

Figure 4: Digital Micrometer

Figure 5: Inoculated TSB

Figure 6: L-spreaders, micropipette, disposable inoculation loops and forceps

Figure 7: *C. albicans* ZOI plates Poloxamer (Biofilm)*

Figure 8: *C. albicans* ZOI Mueller-Hinton Plates (Planktonic)*

Figure 9: *S. aureus*, *P. aeruginosa*, *E. coli* ZOI plates*

*Note that these photos differ slightly from the template that appears on the next page because two anti-biotic discs were used (ATM- Aztreonam & LVX- Levofloxacin) on either side of the NaOCl; these results were not included because the ZOIs were overtaken by NaOCl and unreliable. A blank disc was also soaked in PerioSheild™ (located between the two blank control discs); these results were also not included.

Figure 5: ZOI Diagram (Biofilm and Planktonic) 30% Poloxamer and Mueller-Hinton

100% Concentration of Novel Irrigant		50% Concentration of Novel Irrigant
25% Concentration of Novel Irrigant		70% DMSO (Solvent Alone)
6% NaOCl		Blank (No Treatment)

Creation of Bacteria Biofilm on Hydroxyapatite-Coated Pegs (Calgary Biofilm Device):

Candida albicans was chosen as the test organism. A 10 uL loop was filled with the sample of *C. albicans* and placed into 40 mL TSB in a test tube. The loop was swirled to ensure all contents had been released from the loop into the solution. The test tube was vortexed to ensure even mixing. An Innovotech microtiter tray was filled with 200 mL of *C. albicans*-containing TSB into each well (perimeter wells were excluded to eliminate the chance of evaporation during incubation period). The hydroxyapatite-coated pegs were placed into the microtiter tray of *C. albicans*-containing TSB and allowed to incubate (37°C) for 48 hours (on a rocker set to 3.5 rpm). Rocking helped to ensure even biofilm formation on each of the hydroxyapatite-coated pegs.

Treatment of Pegs with Cleansing Solutions:

Once the incubation period was complete, two more microtiter trays were filled. The first tray was filled with treatment solutions (100%, 50%, 25% concentration of Novel Irrigant as well as 70% DMSO and Full strength 6% NaOCl). See figure 13. The dilutions were made with sterile physiologic saline solution. The second tray was filled with saline solution (for a wash). The biofilm coated pegs were placed into the treatment solutions for 1 minute. The biofilm coated pegs were then placed into the saline solution to wash for 1 minute. The one minute in saline reflects the rinsing and drying of a root canal space and deactivation of the treatment solution.

Figure 6: Photo of Calgary Biofilm Device

Figure 7: Diagram of Eukaryotic Yeast Cell Membrane (Courtesy of Dr. John G. Thomas)

Figure 8: Diagram of Calgary Biofilm Device and assay template

100% CONCENTRATION OF NOVEL IRRIGANT
50% CONCENTRATION OF NOVEL IRRIGANT
25% CONCENTRATION OF NOVEL IRRIGANT
70% DMSO (SOLVENT ALONE)
6% NaOCl
NO TREATMENT (CONTROL)
EMPTY WELL

Collection of CFU Data:

A pair of sterile needle-nose pliers was used to break off pegs and place them into test tubes containing 4 mL saline. Care was used to ensure that pegs treated with each treatment solution were collected separately and the needle-nose pliers were disinfected with a 70% ethyl alcohol rinse followed by de-ionized water and allowed to dry between samples. Positive control pegs (no treatment) were also collected. The test tubes were sonicated in a water bath set at 37°C for 30 seconds. Sonicating the tooth in saline ensures that all of the biofilm bacteria are extracted from the tooth surface and come off in solution. A series of three 1 in 10 dilutions were made from each of the sonicated test tubes (sterile saline was used for dilution) and 250 uL of solution from each individual dilution was plated onto a Mueller-Hinton agar plate. Mueller-Hinton plates were placed into the incubator (at 37°C) for 24 hours. A colony-count of each plate was performed using aCOLyte software. CFU (colony forming units) were calculated based upon the dilutions that were performed in order to reflect the actual number of bacteria that were present on the peg surface after it was treated with each solution.

Figure 9: Images of CFU assay plates

Measurement of Optical Density:

Needle-nose pliers were again utilized to break off tooth pegs (cleaning the pliers with alcohol wipes in between usages, but they were allowed to dry completely in between as well to avoid removal of the biofilm). The pegs were placed into 2 mL of Crystal Violet for 15 min (tooth was completely coated in Crystal Violet to be used as a stain). Pegs were removed from Crystal Violet and placed into 2mL of de-ionized H₂O to wash for 1 min. Pegs were placed into 2 mL glacial acetic acid (at 100% strength) to remove biofilm for 15 minutes. Absorbance of solution was recorded at 562 nm.

SEM Photography:

Pegs were placed into vials of 10% neutral buffered formalin to preserve the biofilm formation. Pegs were photographed by SEM to ensure the presence of biofilm. Consistent orientation was attempted by capturing the apical 1/3 of the pegs. The samples were held down with carbon tape and a strip of copper tape was put over top of them. They were then coated with a gold palladium alloy. The thickness of the coating was between 5-10 nm. The samples were tall and round, so the copper tape makes a circuit with the gold palladium coating. The instrument used was a Hitachi S-4700 FE-SEM with an EDAX brand EDS detector in the WVU Department of Chemical Engineering, Benjamin M. Statler College of Engineering and Mineral Resources. All samples were run at 5.0 kV and a working distance of 10.5 mm. These numbers are included in the images. SE(M) means both upper and lower detectors were used (mixed), SE(L) means just the lower, and SE(U) means the upper detector was used. The lower detector is better for samples that charge or samples that have a lot of topography. The upper detector provides a clearer image in general, but doesn't show as much of the three dimensional features of the sample. Think of it as lighting a sample from above and looking down on it (upper detector) and

lighting a sample from the side and looking at it (lower detector). The mixed detector averages the two images together. Biofilm Images were identified and staged according to the following table.

Planktonic / Biofilm Staging		
Staging	Description	Characteristics
0	Free Floating	Individual Cells
I A,B	Early	Loose, open
II	Immature/Moderate	Increased biomass
III	Mature/Highly Complex	Increased biodiversity
IV A,B	Late/Apoptosis or Necrosis	Free-floating

Figure 10: Planktonic/Biofilm Staging Diagram

Figure 11: Biofilm formation (Courtesy of Dr. John G. Thomas)

Microscopy, Photography, and Biofilm Coverage Ranking Determination:

Additional pegs were stained in crystal violet for 30 seconds. Pegs were placed on microscope slides in emersion oil and viewed at 5X magnification via Zeiss microscope. Representative photographs were taken. Determination of biofilm coverage was performed by computer analysis using Image J software. Appropriate information was obtained to set the scale of measurement. An 8-bit reproduction of the original image was created. The threshold value of pixels was adjusted to gain representation of the area of biofilm coverage. The computer software then generated an outline image of the area covered including any holes in the selected area and measurement was made. An average area was obtained and statistically analyzed.

Laser Confocal Microscopy Protocol:

Extracted human single rooted anterior teeth were obtained from a local private dental practice which had been extracted as part of a normal dental treatment plan within the last 12 months. The teeth had been stored in full strength NaOCl, glycerin, and distilled H₂O. The teeth were accessed with sterile 330 and Endo-Z burs in a high speed air-driven handpiece under high speed evacuation and water spray. The teeth were then instrumented to length with EndoSequence[®] (Brassler) #40/06 rotary files at 550rpm. Sterile H₂O was used for lubrication and the procedure was performed on a bench top in the operator's hand. The prepared teeth were radiographed, placed in a plastic container of sterile water (provided by WVU SOD central dispensing and sterilization department), and were then autoclaved. A jig was created with PVS heavy body putty (3M ESPE) to be used as a mold so that blue acrylic custom tray material could be poured around the sterile instrumented teeth approximately 6mm up the root surface. See figures 18 and 19. The following irrigation protocols were conducted with a new 3mL Monoject[®] syringe equipped with a 30 gauge ProRinse[®] side-venting needle. 3mL of each solution were utilized for the time indicated. A new irrigation syringe and needle were used with each trial.

Irrigation Protocol:	6% NaOCl – 5min
	6% NaOCl – 5min
	17% EDTA – 1min
	70% DMSO – 5min
	100% Novel Irrigant – 5min
	50% Novel Irrigant – 5min
	25% Novel Irrigant – 5min
	100% Novel Irrigant – 5min
	17% EDTA – 1min
	17% EDTA – 1min
	No Treatment – only staining

The teeth were then stained with an appropriate laser fluorescent confocal microscopy stain (Rhodamine B). 0.1mM of Rhodamine B solution was prepared by adding 23.95mg of Rhodamine B dye (Aldrich Chemicals, Milwaukee, WI) to 500ml of deionized water. The dye was injected into the specimens with an additional new 3mL Monoject[®] syringe with 30 gauge ProRinse[®] side-venting needle. The teeth were stained with the 0.1mM Rhodamine B and stored in glass screw top vials for 24 hours, with no subsequent rinsing. See figure 19. Rhodamine B from the solution incorporates into demineralized tooth structure, but does not penetrate intact tooth structure. After 24 hours, the teeth were sectioned mid-root with a slow speed circular saw equipped with a diamond disc blade. A second cut was then made adjacent to the initial cut to obtain a 0.75mm circular cross section. The sections were then placed on a T-4 heated coverslip and viewed with a Zeiss laser confocal microscope using helium and neon gas at 543nm to excite the Rhodamine B. Images were obtained at 10X and keeping each specimen oriented in a similar manner.

Figure 12: Radiograph of prepared sample, cross-section of sample, slow speed diamond disc saw

Figure 13: Irrigation armamentarium, putty jig, EDTA, DMSO

Figure 14: Prepared samples ready for sectioning, blue acrylic material used to mount teeth

Chapter 4

Results

NMR: Samples of Clindamycin HCl, Nystatin, in a 1:1 mixture were examined immediately after preparation, after standing at 25°C for 24 h, after 1 week, and after 1 week plus heating to 50°C. All three samples were stable and remained unchanged by NMR. Figure 20 displays the partial proton NMR spectra of the three samples taken after 1 week. The region displayed corresponds to the proton adjacent to the chlorine of clindamycin, which is subject to replacement by the amino group in Nystatin. The set of four peaks (ratio 1:3:3:1, the quartet) is due to this proton. The single peak in the Nystatin sample that is observed in this region is due to proton on the carbon that bears the sugar residue. The spectrum obtained on the combined sample is simply the sum of the two spectra (Clindamycin HCl and Nystatin) indicating that no reaction occurred. Examination of the full spectrum of the individual and combined samples indicated that no alternative reactions occurred (i.e., the spectrum obtained for Clindamycin HCl/Nystatin was merely the sum of those for Clindamycin HCl and Nystatin, Data not shown.

Figure 15: Partial NMR spectrum of Clindamycin HCl, Nystatin, and a 1:1 mixture in the range of 4.45-4.50 ppm. The quartet in spectra of samples with clindamycin is due to the circled proton.

ZOI: The ZOI studies indicated that the novel irrigant was effective against *E. coli*, *S. aureus*, and *C. albicans* both in the planktonic form (Mueller-Hinton) and biofilm phenotype (poloxamer) in all concentrations (25, 50, and 100%). 6% NaOCl was also effective against these organisms. The novel irrigant did not appear to exhibit antimicrobial activity against the *P. aeruginosa* and *Lactobacillus*. The NaOCl did exhibit antimicrobial activity against *P. aeruginosa* and *Lactobacillus*; however, after the 48 hour reading there appeared to be colonies forming back inside the ZOI. Interesting to note was the fact that the solvent alone (70% DMSO) exhibited antibacterial activity against *E. coli* in both the planktonic and biofilm phenotype and exhibited activity against the biofilm phenotype of the *C. albicans* trial. All concentrations of the Novel Irrigant produced a “double zone” of inhibition after 48 hours in both Mueller-Hinton and poloxamer trials of *S. aureus*.

Figure 16: ZOI Results of all 5 microorganisms tested, Mueller-Hinton Media (Planktonic)

Figure 17: ZOI Results of all 5 microorganisms tested, Poloxamer Media (Biofilm)

Five additional ZOI trials were performed with *C. albicans* as the test organism and the results were averaged.

	M-H	POLX	M-H	POLX	M-H	POLX	M-H	POLX	M-H	POLX	M-H	POLX
A1	20.15	11.54	23.18	11.41	23.04	10.79	23.57	10.15	22.83	8.49	22.53	7.47
A2	22.08	11.07	23.01	10.77	24.4	11.75	25.04	11.46	22.52	9.57	21.52	8.46
B1	15.67	9.9	18.03	10.63	20.18	9.98	19.82	10.61	18.43	9.28	20.41	7.24
B2	17.03	9.74	18.51	10.66	21.58	12.03	20.79	11.08	19.39	10.27	19.59	8.23
C1	14.97	8.23	15.9	8.78	16.75	10.42	15.85	8.47	16.27	7.78	16.39	7.13
C2	15.42	9.32	15.26	9.23	17.88	9.86	17.55	9.49	16.03	8.63	16.17	8.15
D1	0	0	0	0	0	0	0	0	0	0	0	0
D2	0	0	0	0	0	0	0	0	0	0	0	0
E	0	7.76	0	7.6	0	8.43	0	8.41	0	7.53	0	7
F	76.68	63.49	73.4	75.33	61.45	78.53	62.24	82.24	66.17	77.06	68.11	81.06

Table 1: Zones of Inhibition Study, *C. albicans*, 24-hr incubation period (all measurements in mm)

A = 100% Concentration of New Product
B = 50% Concentration of New Product
C = 25% Concentration of New Product
D = 0 % Concentration of New Product (control)
E = Solvent Alone (70% DMSO)
F = 6% NaOCl

M-H = Mueller Hinton Media (Planktonic)
POLX = Poloxamer Media (Biofilm)

	(C. albicans)	
	Muller - Hinton	Poloxamer
A Avg	22.8225	10.244167
Std Dev A	1.270004474	1.4289313
B Avg	19.11916667	9.9708333
Std Dev B	5.53882335	1.2756992
C Avg	16.20333333	8.7908333
Std Dev C	0.865262265	0.9240864
D Avg	0	0
Std Dev D	0	0
E Avg	0	7.7883333
Std Dev E	0	0.5518484
F Avg	68.00833333	76.285
Std Dev F	6.065362039	6.7605821

Table 2: ZOI results of *C. albicans* trials (AVG and STD DEV)

Figure 18: ZOI Results *C. albicans* (Average of trials n=12), Mueller-Hinton Media (Planktonic)

Figure 19: ZOI Results *C. albicans* (Average of trials n=12) Poloxamer Media (Biofilm)

The 6% NaOCl was significantly more effective than the 100%, 50%, and 25% concentrations of the novel irrigant as well as the 70% DMSO alone in the ZOI study using the Mueller-Hinton agar plates (p-Value <.0001). The 100% concentration of the novel irrigant was significantly more effective than the solvent alone and the 25% concentration of the novel irrigant (p-Value <.0001) as well as 50% concentration (p-Value 0.0010). The 50% concentration of novel

irrigant was more effective than the solvent alone (p-Value <.0001) and 25% concentration (p-Value 0.0160). The 25% concentration was significantly more effective than the solvent alone (p-Value <.0001).

CFU: The CFU assay showed that there was no significant difference in the anti-microbial ability of 6% NaOCl when compared to 100% concentration of the novel irrigant.

Dilution	10^{-2}						10^{-4}						10^{-6}					
Trial	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
A	93	71	56	102	68	87	2	0	0	3	0	0	0	0	0	0	0	0
B	183	135	219	237	196	272	4	3	5	7	2	6	0	0	0	1	0	0
C	140	193	89	173	87	67	3	5	2	4	3	3	0	1	0	1	1	1
D	500	500	500	500	500	500	37	42	66	49	56	29	1	3	4	1	3	1
E	6	11	8	5	13	1	2	3	1	0	1	0	0	1	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 3: CFU Assay, *C. albicans* Results of each trial (n=6), number of colonies present at given dilutions.

A = 100% Concentration of New Product

B = 50% Concentration of New Product

C = 25% Concentration of New Product

D = 0 % Concentration of New Product

E = Solvent Alone (70% DMSO)

F = 6% NaOCl

500 signifies that the colonies were too numerous to count and was used for statistical purposes

Dilution		AVG	10^{-2}	10^{-4}	10^{-6}
A = 100% Concentration of New Product		8.33×10^3	79.5	0.833333	0
B = 50% Concentration of New Product		4.5×10^4	207	4.5	0.166667
C = 25% Concentration of New Product		3.3×10^4	124.8333	3.333333	0.666667
D = 0 % Concentration of New Product		4.65×10^5	500	46.5	2.166667
E = Solvent Alone (70% DMSO)		1.16×10^4	7.333333	1.166667	0.166667
F = 6% NaOCl		0	0	0	0

Table 4: Average of CFU Assay Results *C. albicans*

Figure 20: Graph of CFU Assay *C. albicans* Results

Optical Density Absorbance: The absorbance assay showed that the 100% concentration of the novel irrigant was more effective than 6% NaOCl and all other concentrations of the novel irrigant at biofilm removal. The solvent alone showed similar results. The positive control did not respond as expected, this could be due to the fact that untreated biofilm is easily removed from the plastic pegs by the glacial acetic acid. A lower absorbance value was indicative of greater solution anti-microbial activity.

Optical Density Absorbance Data recorded at 562nm	
	C. albicans
A = 100% Concentration of New Product	0.786
B = 50% Concentration of New Product	0.83
C = 25% Concentration of New Product	0.818
D = 0 % Concentration of New Product	0.765
E = Solvent Alone (70% DMSO)	0.787
F = 6% NaOCl	0.843

Table 5: Results of Absorbance Assay, *C. albicans*

Figure 21: Graph showing results of Optical Density Absorbance Assay, *C. albicans*

SEM Photography: The SEM assay confirmed the presence of biofilm on the pegs of our Calgary Biofilm Device. Different stages of biofilm growth were observed ranging from budding yeast cells, to pseudo-hyphae, and the more pathogenic hyphae phase. This assay clearly demonstrated the bi-phasic nature of this eukaryotic organism, *C. albicans*.

Figure 22: Bi-phasic budding yeast (Stage III), hyphae and pseudo-hyphae (Stage I)

Figure 23: Isolated single yeast cells

Figure 24: Pseudo hyphae, penetrating hyphae, example of bi-phasic eukaryote

Coverage Area Assay: The results of the biofilm coverage assay showed that 100% and 25% concentration of the novel irrigant were twice as effective at removing biofilm from the pegs of the Calgary Biofilm Device when compared to NaOCl. The 100% concentration of the novel irrigant was significantly better than NaOCl (p-Value 0.0059). 25% concentration was also significantly better than NaOCl (p-Value 0.0085). There was no significant difference in the amount of biofilm coverage remaining when comparing the three concentrations of the novel irrigant to each other as well as the solvent (70% DMSO) alone. The corresponding images on the following pages are in order from left to right, the original crystal violet stained image (as viewed under the microscope) that has been cropped for consistency in all samples, an 8-bit converted black and white image, the image with the threshold value adjusted to be inclusive of the area of biofilm coverage, and computer analysis of area measurement.

Area of *Candida albicans* Biofilm Coverage On Peg of Calgary Biofilm Device

Figure 25: Graph of Biofilm Coverage Area Assay, *C. albicans*

Figure 26: 100% Concentration of Novel Irrigant: The following represents, in order from left to right, original image obtained from the Zeiss Microscope at 5X, 8-bit Black and White image, Image adjusted with threshold value, and computer analysis of coverage area

Figure 27: 50% Concentration of Novel Irrigant

Figure 28: 25% Concentration of Novel Irrigant

Figure 29: 70% DMSO (Solvent Alone)

Figure 30: No Treatment (Positive Control)

Figure 31: 6% NaOCl

Penetration Study: The results of the penetration study showed that the mean penetration (in micrometers) of each tested combination into dentin tubules of extracted human teeth were as follows:

100% Novel	EDTA/DMSO	25% Novel	70% DMSO	50% Novel	EDTA/100% Novel	17% EDTA	EDTA/NaOCl	6% NaOCl	No Treat
601.52	362.26	311.05	308.33	276.59	257.03	140.30	129.44	77.27	17.55

Figure 32: Graph of Penetration into Dentin Tubules (micrometers)

The 100% concentration of the novel irrigant penetrated significantly further into the dentin tubules of extracted human teeth when compared to the no treatment group (p-value <0.0001), 6% NaOCl (p-value <0.0001), combination of 17% EDTA and 6% NaOCl (p-value <0.0001), and 17% EDTA alone (p-value <0.0001).

Figure 33: Comparison of Control and all concentrations of Novel Irrigant Tested

Figure 34: Confocal Laser Microscopy 10X No treatment

Figure 35: Confocal Laser Microscopy 10X 6% NaOCl Treatment

Figure 36: Confocal Laser Microscopy 17% EDTA Treatment

Figure 37: Confocal Laser Microscopy 10X 70% DMSO Treatment

Figure 38: Confocal Laser Microscopy 10X 100% Concentration of Novel Irrigant

Figure 39: Confocal Laser Microscopy 10X 50% Concentration of Novel Irrigant

Figure 40: Confocal Laser Microscopy 25% Concentration of Novel Irrigant

Figure 41: Confocal Laser Microscopy 10X Treatment with 17% EDTA and 6% NaOCl

Figure 42: Confocal Laser Microscopy 20X 100% Concentration Novel Irrigant

Discussion

As with any in vitro or bench top study, differences in results may be attributed to variations in specimens, testing procedures, instrumentation/experimentation techniques and interpretation of data. One advantage of in vitro testing is that it provides a reasonable method for technique examination and observation without the many restrictions found in a clinical setting. Conversely, it is difficult, if not impossible, for in vitro studies to reproduce clinical conditions. Conclusive evidence regarding efficacy should be drawn from many studies conducted by independent investigators looking at similar technique aspects, animal trials, and from history of clinical use.

The present study was designed to formulate and evaluate the ability of a novel irrigant in different concentrations (25, 50, and 100%) to have anti-microbial activity against a recalcitrant *C. albicans* biofilm as well as observe penetration of the irrigant into the dentin tubules of root canals prepared in an identical manner. Comparable levels of disinfection were found between the novel irrigant and full strength (6%) NaOCl. Superior penetration into dentin tubules was found with 100% concentration of the novel irrigant when compared to NaOCl and 17% EDTA or some combination of the two. All of the positive controls demonstrated microbial growth and did not show penetration into the dentin tubules supporting the experimental design.

Prepared root canals by conventional standards are chemically disinfected using some form of irrigation. The ability to chemically disinfect areas of root canals that are inaccessible to instrumentation has been considered to be essential for clinical success. Irrigation materials can reach fins, apical deltas, and lateral canals, thus improving the outcome of non-surgical root canal therapy. Unfortunately, all irrigants suffer physical short-comings such as inability to

penetrate deep into the dentin tubules and address remaining microorganisms that these areas harbor. These undesirable characteristics are incompatible with long-term endodontic success, especially when dealing with extraradicular biofilms. The objective of irrigation is to minimize the amount of bio load (microorganisms) present in the canal system while leaving the remaining dentin occupying the root canal unharmed.

Full strength NaOCl was used in this study as the control irrigant because of its known clinical success and history of use.

Recently, the materials such as MTAD[®] and QMIX[®] have attempted to replace NaOCl and the potential dangers associated with its use. At best, these new materials can be used as adjuncts with some degree of clinical success. Use of other less harmful irrigation materials can potentially decrease the chance of a sodium hypochlorite accident. However, no other endodontic irrigant seems to possess the ability to dissolve tissue like that of NaOCl.

Some studies have concluded that irrigants such as QMIX[®] when used according to manufacturer's recommendations have the ability to better disinfect root canal systems while removing the smear layer when compared to NaOCl and EDTA or some combination of the two.

The experimental groups studied containing DMSO were the only groups that demonstrated statistically significant ability to remove biofilm from pegs of the Calgary Biofilm Device. In addition, the 100% concentration of novel irrigant was statistically significant in its ability to penetrate into the dentin tubules of a prepared root canal.

After NMR analysis of the newly formulated mixture there appears to be no reaction between any of the ingredients, Clindamycin, Nystatin, and Dimethyl sulfoxide. The NMR spectroscopy study confirms the stability of the mixture. The concern always exists that this

mixture may potentially have interactions with other commonly utilized endodontic irrigants, but this question requires further testing in a laboratory setting. There is also always the possibility that certain patients may have or develop allergies to certain drugs. Clindamycin does not appear to be the allergen that other commonly used antibiotics (penicillin based drugs) seem to be; however, there is always the risk of developing Pseudomembranous colitis with the use of antibiotics. It is assumed that the dose of Clindamycin in this particular formulation is relatively low compared to an oral regimen of the drug and will not leave the root canal system. Nystatin is a commonly used antifungal agent, and hence the reason it was chosen for this formulation. It too can potentially be an allergen for some patients, though this is seen as an extremely rare occurrence. Dimethyl sulfoxide has currently only gained FDA approval in the treatment of bladder inflammation or interstitial cystitis. There is concern over whether or not DMSO could further denature collagen perhaps causing damage to the tooth structure. DMSO absorbed systemically is primarily excreted in the urine and the only listed side effect is a garlic-like odor on the breath. Again the rather miniscule amount of DMSO that the patient would be exposed to is quite small in this application and no adverse effects are anticipated. Further testing is required to verify this hypothesis. Derivatives of DMSO could possibly be substituted as a more biocompatible solvent.

The ZOI study demonstrated that all concentrations of the novel irrigant resulted in disinfection activity against *E. coli*, *S. aureus*, and *C. albicans*, as did full strength NaOCl. The novel irrigant did not show activity against *P. aeruginosa* or *Lactobacillus*. This was true for both the planktonic simulation with Mueller-Hinton agar and the biofilm phenotype simulation with Poloxamer plates. Full strength NaOCl was significantly more effective than the novel irrigant in the ZOI study. DMSO alone had activity against the biofilm phenotype of both *E. coli*

and *C. albicans*. While NaOCl did show some activity against *P. aeruginosa* and *Lactobacillus* after 24 hours, when viewed at 48 hours, it was apparent that colonies had re-grown inside the original zone of inhibition. Because *C. albicans* was the organism chosen for this study, further trials were repeated yielding similar results in both the planktonic and biofilm phenotypes. Because of these results and the unknown variable of whether or not the novel irrigant will possess the ability to dissolve tissue, it may be that the novel irrigant will not replace the use of NaOCl; rather it could be used as an adjunct to endodontic irrigation.

Pegs from the Calgary Biofilm Device were used to complete three different assays as well as obtain SEM images to verify biofilm formation. Six independent trials of performing colony forming unit dilutions were conducted using *C. albicans* as the test organism. Biofilm remaining after treatment was removed via sonication, serial dilutions were performed with physiologic saline, plated on M-H, and results were quantified using an aCOLyte colony counter. A colony forming unit is used to determine the number of viable bacterial cells in a sample per mL. A lower CFU is indicative of greater solution activity. The results suggest similar antimicrobial activity as compared to full strength NaOCl. Although the NaOCl showed no growth, this was not significantly different to the different concentrations of the novel irrigant when compared with the positive control value.

The crystal violet assay involved staining the biofilm covered plastic pegs and viewing under a Zeiss tissue microscope. Representative photographs were taken with a Nikon digital capture device. These photos were then cropped at a consistent orientation and size and converted to scale as an 8-bit gray image. Computer software was able to allow the observer to select the darker areas on the image which corresponded to remaining biofilm on the pegs. The computer software was able to map out the dark areas that were selected and obtain an average

area of coverage. The larger the dark area, corresponded with the more remaining biofilm, and therefore the less effective treatment solution. The novel irrigant did appear to have a significantly better ability to remove biofilm from the Calgary Biofilm Device compared to full strength NaOCl. The process of selecting the dark areas was a bit subjective; however, identical sample sizes were taken from each peg at the same location and consistent threshold values were used.

The absorbance assay was conducted after the remaining biofilm (having been treated with a specific treatment) was stained with crystal violet, washed with deionized water and removed from the peg into glacial acetic acid. The absorbance of the glacial acetic acid was read at a fixed wavelength of 562nm. A lower absorbance value was indicative of greater solution antimicrobial activity (Trotonda 2008). These results also suggest that the novel irrigant possesses better anti-biofilm activity compared to NaOCl.

The positive control *C. albicans* peg was viewed under SEM and representative photographs were taken to ensure biofilm formation. Quantitative analysis to determine the amount of actual biofilm coverage was unable to be computed due to variability and orientation of the pegs.

The penetration study showed that the 100% concentration of the novel irrigant was statistically more significant in its ability to penetrate the dentin tubules of extracted human teeth when compared with current irrigation protocols. This characteristic sets the novel irrigant apart from other endodontic irrigation materials and is quite unique.

Chapter 5

Conclusion

The present study indicated that a novel irrigant composed of Clindamycin, Nystatin, and 70% DMSO has equal or better ability to disinfect a recalcitrant *C. albicans* biofilm phenotype when compared to 6% NaOCl as observed through the CFU, absorbance, and biofilm coverage area assays performed using the Calgary Biofilm Device. 100% Concentration of the novel irrigant was statistically significant in its ability to penetrate into the dentin tubules of a prepared root canal in an extracted human tooth when compared to 6% NaOCl and 17% EDTA. Although the clinician should examine many studies and rely on clinically proven methods before choosing one technique or material over another, it is logical to place value in further research and evaluation of a technique or material which has been shown to provide disinfection and penetration in-vitro. Further studies are necessary to evaluate the in-vivo success of cases which have been irrigated in this manner. It must be remembered that there are numerous other factors that contribute to endodontic success. Ray and Trope concluded that the quality of the coronal seal is equally important to the successful outcome of endodontic therapy as is the quality of the apical seal (Ray 1995). Other authors agree in the importance of appropriate obturation, apical seal, and restoration in addition to an irrigation protocol that is effective.

All groups tested had similar disinfecting abilities, with the exception being 6% NaOCl in the ZOI studies which demonstrated that it may have superior ability to disinfect selected pathogens on agar plates. This in vitro study supports the need to continue research and development on a novel irrigant that not only has the ability to effectively disinfect root canal systems, but also has the ability to penetrate into the dentin tubules and address a bio load that has previously been inaccessible by modern irrigation materials and protocols.

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Appendix 1: Statistics

Rsquare	0.988755
Adj Rsquare	0.987714
Root Mean Square Error	2.110992
Mean of Response	18.42983
Observations (or Sum Wgts)	60

Table 6: One-way Analysis of Data (ANOVA, Summary of Fit) ZOI Study *C. albicans*, Mueller - Hinton (Planktonic)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treat	5	21158.760	4231.75	949.6140	<.0001*
Error	54	240.639	4.46		
C. Total	59	21399.399			

Table 7: ANOVA, ZOI *C. albicans*, Mueller-Hinton (Planktonic)

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
A	12	22.8225	0.60939	21.60	24.044
B	12	19.1192	0.60939	17.90	20.341
C	12	16.2033	0.60939	14.98	17.425
D	12	0.0000	0.60939	-1.22	1.222
E	6	0.0000	0.86181	-1.73	1.728
F	6	68.0083	0.86181	66.28	69.736

Table 8: Means of One-way ANOVA, ZOI, *C. albicans*, Mueller-Hinton (Planktonic)

A=100% Concentration of Novel Irrigant
 B=50% Concentration of Novel Irrigant
 C=25% Concentration of Novel Irrigant
 D=No Treatment (Positive Control)
 E=70% DMSO (Solvent Only)
 F=6% NaOCl
 Std Error uses a pooled estimate of error variance

Confidence Quantile	Alpha
q*	
2.95448	0.05

Table 9: Means Comparisons for all pairs using Tukey-Kramer HSD, ZOI *C. albicans*, M-H (Planktonic)

Abs(Dif)-HSD	F	A	B	C	D	E
F	-3.601	42.067	45.771	48.687	64.890	64.407
A	42.067	-2.546	1.157	4.073	20.276	19.704
B	45.771	1.157	-2.546	0.370	16.573	16.001
C	48.687	4.073	0.370	-2.546	13.657	13.085
D	64.890	20.276	16.573	13.657	-2.546	-3.118
E	64.407	19.704	16.001	13.085	-3.118	-3.601

Table 10: LSD Threshold Matrix, ZOI *C. albicans*, M-H

Positive values show pairs of means that are significantly different.

Level	Mean
F	68.008333
A	22.822500
B	19.119167
C	16.203333
D	0.000000
E	0.000000

Table 11: Connecting Letters Report, ZOI *C. albicans*, M-H

Levels not connected by same letter are significantly different.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value	
F	D	68.00833	1.055496	64.8899	71.12678	<.0001*	
F	E	68.00833	1.218782	64.4075	71.60920	<.0001*	
F	C	51.80500	1.055496	48.6866	54.92345	<.0001*	
F	B	48.88917	1.055496	45.7707	52.00761	<.0001*	
F	A	45.18583	1.055496	42.0674	48.30428	<.0001*	
A	D	22.82250	0.861809	20.2763	25.36870	<.0001*	
A	E	22.82250	1.055496	19.7041	25.94095	<.0001*	
B	D	19.11917	0.861809	16.5730	21.66537	<.0001*	
B	E	19.11917	1.055496	16.0007	22.23761	<.0001*	
C	D	16.20333	0.861809	13.6571	18.74953	<.0001*	
C	E	16.20333	1.055496	13.0849	19.32178	<.0001*	
A	C	6.61917	0.861809	4.0730	9.16537	<.0001*	
A	B	3.70333	0.861809	1.1571	6.24953	0.0010*	
B	C	2.91583	0.861809	0.3696	5.46203	0.0160*	
E	D	0.00000	1.055496	-3.1184	3.11845	1.0000	

Table 12: Ordered Differences Report, ZOI *C. albicans*, M-H

Rsquare	0.98957
Adj Rsquare	0.988604
Root Mean Square Error	2.276308
Mean of Response	14.2085
Observations (or Sum Wgts)	60

Table 13: One-way Analysis of Data (Summary of Fit) ZOI *C. albicans* Poloxamer (Biofilm)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treat	5	26547.138	5309.43	1024.674	<.0001*
Error	54	279.805	5.18		
C. Total	59	26826.944			

Table 14: Analysis of Variance, ZOI *C. albicans* (Biofilm)

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
A	12	10.2442	0.65711	8.93	11.562
B	12	9.9708	0.65711	8.65	11.288
C	12	8.7908	0.65711	7.47	10.108
D	12	0.0000	0.65711	-1.32	1.317
E	6	7.7883	0.92930	5.93	9.651
F	6	76.2850	0.92930	74.42	78.148

Table 15: Means of One-way ANOVA, ZOI *C. albicans* (Biofilm)

Std Error uses a pooled estimate of error variance

Confidence Quantile

q*
2.95448Alpha
0.05**Table 16: Means Comparisons for all pairs using Tukey-Kramer HSD, ZOI *C. albicans* (Biofilm)**

Abs(Dif)-HSD	F	A	B	C	E	D
F	-3.883	62.678	62.952	64.132	64.614	72.922
A	62.678	-2.746	-2.472	-1.292	-0.907	7.499
B	62.952	-2.472	-2.746	-1.566	-1.180	7.225
C	64.132	-1.292	-1.566	-2.746	-2.360	6.045
E	64.614	-0.907	-1.180	-2.360	-3.883	4.426
D	72.922	7.499	7.225	6.045	4.426	-2.746

Table 17: LSD Threshold Matrix, ZOI *C. albicans* (Biofilm)

Positive values show pairs of means that are significantly different.

Level	Mean
F	76.285000
A	10.244167
B	9.970833
C	8.790833
E	7.788333
D	0.000000

Table 18: Connecting Letters Report, ZOI *C. albicans* (Biofilm)

Levels not connected by same letter are significantly different.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value	
F	D	76.28500	1.138154	72.9223	79.64766	<.0001*	
F	E	68.49667	1.314227	64.6138	72.37953	<.0001*	
F	C	67.49417	1.138154	64.1315	70.85682	<.0001*	
F	B	66.31417	1.138154	62.9515	69.67682	<.0001*	
F	A	66.04083	1.138154	62.6782	69.40349	<.0001*	
A	D	10.24417	0.929299	7.4986	12.98976	<.0001*	
B	D	9.97083	0.929299	7.2252	12.71643	<.0001*	
C	D	8.79083	0.929299	6.0452	11.53643	<.0001*	
E	D	7.78833	1.138154	4.4257	11.15099	<.0001*	
A	E	2.45583	1.138154	-0.9068	5.81849	0.2744	
B	E	2.18250	1.138154	-1.1802	5.54516	0.4029	
A	C	1.45333	0.929299	-1.2923	4.19893	0.6253	
B	C	1.18000	0.929299	-1.5656	3.92560	0.7999	
C	E	1.00250	1.138154	-2.3602	4.36516	0.9495	
A	B	0.27333	0.929299	-2.4723	3.01893	0.9997	

Table 19: Ordered Differences Report, ZOI *C. albicans* (Biofilm)

Rsquare	0.641112
Adj Rsquare	0.581297
Root Mean Square Error	0.628048
Mean of Response	0.527778
Observations (or Sum Wgts)	36

Table 20: One-way ANOVA Analysis of CFU (Summary of Fit) *C. albicans*, Dilution=10⁻⁶

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Trial:	5	21.138889	4.22778	10.7183	<.0001*
Error	30	11.833333	0.39444		
C. Total	35	32.972222			

Table 21: Analysis of Variance, *C. albicans*, CFU Dilution= 10^{-6}

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
A	6	0.00000	0.25640	-0.524	0.5236
B	6	0.16667	0.25640	-0.357	0.6903
C	6	0.66667	0.25640	0.143	1.1903
D	6	2.16667	0.25640	1.643	2.6903
E	6	0.16667	0.25640	-0.357	0.6903
F	6	0.00000	0.25640	-0.524	0.5236

Table 22: Means for One-way ANOVA, *C. albicans*, CFU Dilution= 10^{-6}

Std Error uses a pooled estimate of error variance

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
A	6	75.000	111.000	12.5000	-1.816
B	6	91.500	111.000	15.2500	-0.972
C	6	141.000	111.000	23.5000	1.509
D	6	192.000	111.000	32.0000	4.118
E	6	91.500	111.000	15.2500	-0.972
F	6	75.000	111.000	12.5000	-1.816

Table 23: Wilcoxon / Kruskal-Wallis Tests (Rank Sums), *C. albicans*, CFU Dilution= 10^{-6}

ChiSquare	DF	Prob>ChiSq
23.5852	5	0.0003*

Table 24: 1-way Test, ChiSquare Approximation, *C. albicans*, CFU Dilution= 10^{-6}

Confidence Quantile

q*	Alpha
3.04160	0.05

Table 25: Means Comparisons for all pairs using Tukey-Kramer HSD, *C. albicans*, CFU Dilution= 10^{-6}

	D	C	B	E	A	F
Abs(Dif)-HSD						
D	-1.1029	0.3971	0.8971	0.8971	1.0638	1.0638
C	0.3971	-1.1029	-0.6029	-0.6029	-0.4362	-0.4362
B	0.8971	-0.6029	-1.1029	-1.1029	-0.9362	-0.9362
E	0.8971	-0.6029	-1.1029	-1.1029	-0.9362	-0.9362
A	1.0638	-0.4362	-0.9362	-0.9362	-1.1029	-1.1029
F	1.0638	-0.4362	-0.9362	-0.9362	-1.1029	-1.1029

Table 26: LSD Threshold Matrix, *C. albicans*, CFU Dilution= 10^{-6}

Positive values show pairs of means that are significantly different.

Level		Mean
D	A	2.166667
C	B	0.666667
B	B	0.166667
E	B	0.166667
A	B	0.000000
F	B	0.000000

Table 27: Connecting Letters Report, *C. albicans*, CFU Dilution=10⁻⁶

Levels not connected by same letter are significantly different.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value	
D	A	2.166667	0.3626038	1.06377	3.269561	<.0001*	
D	F	2.166667	0.3626038	1.06377	3.269561	<.0001*	
D	B	2.000000	0.3626038	0.89711	3.102895	<.0001*	
D	E	2.000000	0.3626038	0.89711	3.102895	<.0001*	
D	C	1.500000	0.3626038	0.39711	2.602895	0.0033*	
C	A	0.666667	0.3626038	-0.43623	1.769561	0.4577	
C	F	0.666667	0.3626038	-0.43623	1.769561	0.4577	
C	B	0.500000	0.3626038	-0.60289	1.602895	0.7389	
C	E	0.500000	0.3626038	-0.60289	1.602895	0.7389	
B	A	0.166667	0.3626038	-0.93623	1.269561	0.9972	
E	A	0.166667	0.3626038	-0.93623	1.269561	0.9972	
B	F	0.166667	0.3626038	-0.93623	1.269561	0.9972	
E	F	0.166667	0.3626038	-0.93623	1.269561	0.9972	
E	B	0.000000	0.3626038	-1.10289	1.102895	1.0000	
F	A	0.000000	0.3626038	-1.10289	1.102895	1.0000	

Table 28: Ordered Differences Report, *C. albicans*, CFU Dilution=10⁻⁶

Rsquare	0.91475
Adj Rsquare	0.900542
Root Mean Square Error	5.573748
Mean of Response	9.388889
Observations (or Sum Wgts)	36

Table 29: One-way ANOVA Analysis of CFU (Summary of Fit) *C. albicans*, CFU Dilution=10⁻⁴

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Trial:	5	10000.556	2000.11	64.3813	<.0001*
Error	30	932.000	31.07		
C. Total	35	10932.556			

Table 30: Analysis of Variance, *C. albicans*, CFU Dilution=10⁻⁴

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
A	6	0.8333	2.2755	-3.81	5.480
B	6	4.5000	2.2755	-0.15	9.147
C	6	3.3333	2.2755	-1.31	7.980
D	6	46.5000	2.2755	41.85	51.147
E	6	1.1667	2.2755	-3.48	5.814
F	6	0.0000	2.2755	-4.65	4.647

Table 31: Means for One-way ANOVA, *C. albicans*, CFU Dilution=10⁻⁴

Std Error uses a pooled estimate of error variance

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
A	6	64.000	111.000	10.6667	-2.018
B	6	150.000	111.000	25.0000	1.671
C	6	134.000	111.000	22.3333	0.976
D	6	201.000	111.000	33.5000	3.883
E	6	78.000	111.000	13.0000	-1.410
F	6	39.000	111.000	6.5000	-3.102

Table 32: Wilcoxon / Kruskal-Wallis Tests (Rank Sums), *C. albicans*, CFU Dilution= 10^{-4}

ChiSquare	DF	Prob>ChiSq
29.2326	5	<.0001*

Table 33: 1-way Test, ChiSquare Approximation, *C. albicans*, CFU Dilution= 10^{-4}

Level	Mean
D	46.500000
B	4.500000
C	3.333333
E	1.166667
A	0.833333
F	0.000000

Table 34: Connecting Letters Report, *C. albicans*, CFU Dilution= 10^{-4}

Levels not connected by same letter are significantly different.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value	
D	F	46.50000	3.218005	36.7121	56.28788	<.0001*	
D	A	45.66667	3.218005	35.8788	55.45454	<.0001*	
D	E	45.33333	3.218005	35.5455	55.12121	<.0001*	
D	C	43.16667	3.218005	33.3788	52.95454	<.0001*	
D	B	42.00000	3.218005	32.2121	51.78788	<.0001*	
B	F	4.50000	3.218005	-5.2879	14.28788	0.7276	
B	A	3.66667	3.218005	-6.1212	13.45454	0.8609	
C	F	3.33333	3.218005	-6.4545	13.12121	0.9020	
B	E	3.33333	3.218005	-6.4545	13.12121	0.9020	
C	A	2.50000	3.218005	-7.2879	12.28788	0.9694	
C	E	2.16667	3.218005	-7.6212	11.95454	0.9836	
E	F	1.16667	3.218005	-8.6212	10.95454	0.9991	
B	C	1.16667	3.218005	-8.6212	10.95454	0.9991	
A	F	0.83333	3.218005	-8.9545	10.62121	0.9998	
E	A	0.33333	3.218005	-9.4545	10.12121	1.0000	

Table 35: Ordered Differences Report, *C. albicans*, CFU Dilution= 10^{-4}

Rsquare	0.97572
Adj Rsquare	0.971673
Root Mean Square Error	29.44015
Mean of Response	153.1111
Observations (or Sum Wgts)	36

Table 36: One-way ANOVA Analysis of CFU (Summary of Fit) *C. albicans*, CFU Dilution= 10^{-2}

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Trial:	5	1044889.9	208978	241.1130	<.0001*
Error	30	26001.7	867		
C. Total	35	1070891.6			

Table 37: Analysis of Variance, *C. albicans*, CFU Dilution= 10^{-2}

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
A	6	79.500	12.019	55.0	104.05
B	6	207.000	12.019	182.5	231.55
C	6	124.833	12.019	100.3	149.38
D	6	500.000	12.019	475.5	524.55
E	6	7.333	12.019	-17.2	31.88
F	6	0.000	12.019	-24.5	24.55

Table 38: Means for One-way ANOVA, *C. albicans*, CFU Dilution= 10^{-2}

Std Error uses a pooled estimate of error variance

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
A	6	102.500	111.000	17.0833	-0.341
B	6	161.000	111.000	26.8333	2.111
C	6	123.500	111.000	20.5833	0.512
D	6	201.000	111.000	33.5000	3.817
E	6	57.000	111.000	9.5000	-2.281
F	6	21.000	111.000	3.5000	-3.817

Table 39: Wilcoxon / Kruskal-Wallis Tests (Rank Sums), *C. albicans*, CFU Dilution= 10^{-2}

ChiSquare	DF	Prob>ChiSq
33.1020	5	<.0001*

Table 40: 1-way Test, ChiSquare Approximation, *C. albicans*, CFU Dilution= 10^{-2}

Confidence Quantile	Alpha
q*	0.05
3.04160	

Table 41: Means Comparisons for all pairs using Tukey-Kramer HSD, *C. albicans*, CFU Dilution= 10^{-2}

Abs(Dif)-HSD	D	B	C	A	E	F
D	-51.70	241.30	323.47	368.80	440.97	448.30
B	241.30	-51.70	30.47	75.80	147.97	155.30
C	323.47	30.47	-51.70	-6.37	65.80	73.13
A	368.80	75.80	-6.37	-51.70	20.47	27.80
E	440.97	147.97	65.80	20.47	-51.70	-44.37
F	448.30	155.30	73.13	27.80	-44.37	-51.70

Table 42: LSD Threshold Matrix, *C. albicans*, CFU Dilution= 10^{-2}

Positive values show pairs of means that are significantly different.

Level	Mean
D	500.00000
B	207.00000
C	124.83333
A	79.50000
E	7.33333
F	0.00000

Table 43: Connecting Letters Report, *C. albicans*, CFU Dilution= 10^{-2}

Levels not connected by same letter are significantly different.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value	
D	F	500.0000	16.99728	448.301	551.6989	<.0001*
D	E	492.6667	16.99728	440.968	544.3655	<.0001*
D	A	420.5000	16.99728	368.801	472.1989	<.0001*
D	C	375.1667	16.99728	323.468	426.8655	<.0001*
D	B	293.0000	16.99728	241.301	344.6989	<.0001*
B	F	207.0000	16.99728	155.301	258.6989	<.0001*
B	E	199.6667	16.99728	147.968	251.3655	<.0001*
B	A	127.5000	16.99728	75.801	179.1989	<.0001*
C	F	124.8333	16.99728	73.134	176.5322	<.0001*
C	E	117.5000	16.99728	65.801	169.1989	<.0001*
B	C	82.1667	16.99728	30.468	133.8655	0.0005*
A	F	79.5000	16.99728	27.801	131.1989	0.0008*
A	E	72.1667	16.99728	20.468	123.8655	0.0024*
C	A	45.3333	16.99728	-6.366	97.0322	0.1122
E	F	7.3333	16.99728	-44.366	59.0322	0.9979

Table 44: Ordered Differences Report, *C. albicans*, CFU Dilution=10⁻²

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
RawIntDen 3	4	1.0059e+10	2.5147e+9	3.1006	0.0159*
Error	309	2.5061e+11	811029508		
C. Total	313	2.6067e+11			

Table 45: One-way Analysis of Variance of Area of Biofilm Coverage on Pegs of Calgary Biofilm Device (*C. albicans*)

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
100% Novel Irrigant	103	1686.0	2806.1	-3835	7207
25% concentration Novel	80	2200.3	3184.0	-4065	8465
50% concentration Novel	68	3744.0	3453.5	-3051	10539
DMSO	51	5504.0	3987.8	-2343	13351
NaOCl	12	31580.7	8221.1	15404	47757

Table 46: Means for One-way ANOVA, Area of Biofilm Coverage

Std Error uses a pooled estimate of error variance

Confidence Quantile	q*	Alpha
	2.74396	0.05

Table 47: Means Comparisons for all pairs using Tukey-Kramer HSD, Area of Biofilm Coverage

Level	Mean
NaOCl	31580.742
DMSO	5504.037
50% concentration Novel 5X	3744.000
25% concentration Novel 5X	2200.306
100% Novel Irrigant 5x	1686.001

Table 48: Connecting Letters Report, Area of Biofilm Coverage

Levels not connected by same letter are significantly different.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value	
NaOCl	100%	29894.74	8686.764	6058.6	53730.92	0.0059	
NaOCl	25%	29380.44	8816.102	5189.4	53571.51	0.0085*	
NaOCl	50%	27836.74	8916.989	3368.8	52304.65	0.0167*	
NaOCl	DMSO	26076.71	9137.195	1004.6	51148.85	0.0369*	
DMSO	100%	3818.04	4876.127	-9561.9	17197.96	0.9354	
DMSO	25%	3303.73	5102.980	-10698.7	17306.13	0.9670	
50%	100%	2058.00	4449.829	-10152.2	14268.17	0.9906	
DMSO	50%	1760.04	5275.362	-12715.4	16235.45	0.9973	
50%	25%	1543.69	4697.316	-11345.6	14432.96	0.9975	
25%	100%	514.30	4244.048	-11131.2	12159.82	1.0000	

Table 49: Ordered Differences Report, Area of Biofilm Coverage

*Percentages reflect % of Novel concentration

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	9	763373.08	84819.2	19.1326	<.0001*
Error	20	88664.51	4433.2		
C. Total	29	852037.59			

Table 50: One-way Analysis of Variance of Penetration into Dentin Tubules of prepared root canals (micrometers)

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
100% Novel	3	601.520	38.441	521.3	681.71
17% EDTA	3	140.304	38.441	60.1	220.49
25% Novel	3	311.053	38.441	230.9	391.24
50% Novel	3	276.588	38.441	196.4	356.78
6% NaOCl	3	77.274	38.441	-2.9	157.46
70% DMSO	3	308.330	38.441	228.1	388.52
EDTA / DMSO	3	362.259	38.441	282.1	442.45
EDTA/NaOCl	3	129.436	38.441	49.2	209.62
EDTA/Novel	3	257.027	38.441	176.8	337.21
No Treatment	3	17.547	38.441	-62.6	97.73

Table 51: Means for One-way ANOVA, Penetration Study

Std Error uses a pooled estimate of error variance

Confidence Quantile

q*	Alpha
3.54111	0.05

Table 52: Means Comparisons for all pairs using Tukey-Kramer HSD, Penetration Study

Level	Mean Penetration (micrometers)			
100% Novel	A			601.52033
EDTA / DMSO	B			362.25867
25% Novel	B	C		311.05267
70% DMSO	B	C		308.33000
50% Novel	B	C		276.58767
EDTA/Novel	B	C	D	257.02733
17% EDTA		C	D	140.30367
EDTA/NaOCl		C	D	129.43567
6% NaOCl			D	77.27400
No Treatment			E	17.54667

Table 53: Connecting Letters Report, Penetration Study

Levels not connected by same letter are significantly different.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value	
100%	N T	583.9737	54.36436	391.464	776.4838	<.0001*	
100%	6% NaOCl	524.2463	54.36436	331.736	716.7565	<.0001*	
100%	EDTA/NaOCl	472.0847	54.36436	279.575	664.5948	<.0001*	
100%	17% EDTA	461.2167	54.36436	268.707	653.7268	<.0001*	
E / D	N T	344.7120	54.36436	152.202	537.2221	0.0001*	
100%	EDTA/Novel	344.4930	54.36436	151.983	537.0031	0.0001*	
100%	50% Novel	324.9327	54.36436	132.423	517.4428	0.0003*	
25%	N T	293.5060	54.36436	100.996	486.0161	0.0009*	
100%	70% DMSO	293.1903	54.36436	100.680	485.7005	0.0009*	
70% D	N T	290.7833	54.36436	98.273	483.2935	0.0010*	
100%	25% Novel	290.4677	54.36436	97.958	482.9778	0.0010*	
E / D	6% NaOCl	284.984	54.36436	92.475	477.4948	0.0013*	
50%	N T	259.0410	54.36436	66.531	451.5511	0.0037*	
E / N	N T	239.4807	54.36436	46.971	431.9908	0.0080*	
100%	EDTA / DMSO	239.2617	54.36436	46.752	431.7718	0.0081*	
25%	6% NaOCl	233.7787	54.36436	41.269	426.2888	0.0101*	
E / D	EDTA/NaOCl	232.8230	54.36436	40.313	425.3331	0.0105*	
70% D	6% NaOCl	231.0560	54.36436	38.546	423.5661	0.0112*	
E / D	17% EDTA	221.9550	54.36436	29.445	414.4651	0.0161*	
50%	6% NaOCl	199.3137	54.36436	6.804	391.8238	0.0387*	
25%	EDTA/NaOCl	181.6170	54.36436	-10.893	374.1271	0.0746	
E / N	6% NaOCl	179.7533	54.36436	-12.757	372.2635	0.0797	
70% D	EDTA/NaOCl	178.8943	54.36436	-13.616	371.4045	0.0822	
25%	17% EDTA	170.7490	54.36436	-21.761	363.2591	0.1095	
70% D	17% EDTA	168.0263	54.36436	-24.484	360.5365	0.1202	
50%	EDTA/NaOCl	147.1520	54.36436	-45.358	339.6621	0.2354	
50%	17% EDTA	136.2840	54.36436	-56.226	328.7941	0.3216	
E / N	EDTA/NaOCl	127.5917	54.36436	-64.918	320.1018	0.4035	
17% E	N T	122.7570	54.36436	-69.753	315.2671	0.4533	
E / N	17% EDTA	116.7237	54.36436	-75.786	309.2338	0.5187	
E / 6%	N T	111.8890	54.36436	-80.621	304.3991	0.5728	
E / D	EDTA/Novel	105.2313	54.36436	-87.279	297.7415	0.6477	
E / D	50% Novel	85.6710	54.36436	-106.839	278.1811	0.8446	
EDTA	6% NaOCl	63.0297	54.36436	-129.480	255.5398	0.9708	
6%	N T	59.7273	54.36436	-132.783	252.2375	0.9792	
25%	EDTA/Novel	54.0253	54.36436	-138.485	246.5355	0.9893	
E / D	70% DMSO	53.9287	54.36436	-138.581	246.4388	0.9894	
E / 6%	6% NaOCl	52.1617	54.36436	-140.348	244.6718	0.9916	
70% D	EDTA/Novel	51.3027	54.36436	-141.207	243.8128	0.9925	
E / D	25% Novel	51.2060	54.36436	-141.304	243.7161	0.9926	
25%	50% Novel	34.4650	54.36436	-158.045	226.9751	0.9996	
70% D	50% Novel	31.7423	54.36436	-160.768	224.2525	0.9998	
50%	EDTA/Novel	19.5603	54.36436	-172.950	212.0705	1.0000	
EDTA	EDTA/NaOCl	10.8680	54.36436	-181.642	203.3781	1.0000	
25%	70% DMSO	2.7227	54.36436	-189.787	195.2328	.	

Table 54: Ordered Differences Report, Penetration Study

Percentages (100, 50, 25) reflect concentration of Novel Irrigant

NT = No Treatment (Positive Control)

E/D = 17% EDTA followed by 70% DMSO

E/N = 17% EDTA followed by 100% Novel

E/6% = 17% EDTA followed by 6% NaOCl

6%=6% NaOCl

70% D = 70% DMSO