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Degradation and Removal of Porphyromonas gingivalis Lipid 654 by Common Endodontic Intracanal Irrigants and Medicaments

Michelle A. Hack
mhack@uchc.edu

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Degradation and Removal of *Porphyromonas gingivalis*
Lipid 654 by Common Endodontic Intracanal Irrigants
and Medicaments

Michelle Anne Hack

B.A., The Johns Hopkins University, 2005
D.D.S., University of Maryland, Baltimore College of Dental Surgery, 2010

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Degradation and Removal of *Porphyromonas gingivalis* Lipid 654 by
Common Endodontic Intracanal Irrigants and Medicaments

Presented by

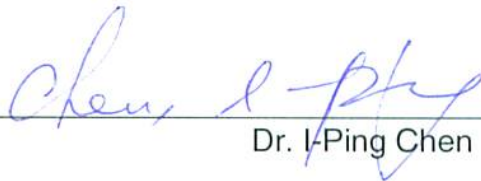
Michelle Anne Hack, DDS

Major
Advisor



Dr. Frank Nichols

Associate
Advisor



Dr. I-Ping Chen

Associate
Advisor



Dr. Blythe Kaufman

Associate
Advisor



Dr. Kamran Safavi

University of Connecticut

2015

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Abstract

Porphyromonas endodontalis is a black pigmented Gram-negative organism that has been identified as a common pathogen of the necrotic root space and within endodontic periapical lesions. *P. endodontalis* synthesizes several classes of complex biologically active lipids, which may promote osteoclastogenesis and osteoclast activation. Specifically, in teeth with necrotic pulps *P. endodontalis* Lipid 654 of the serine lipid class has been shown to activate pathways that could lead to apical bone destruction. Phylogenetically related *Porphyromonas gingivalis* synthesizes structurally identical Lipid 654. Objectives: The first purpose of this study is to isolate *P. gingivalis* Lipid 654 and examine the capacity of common intracanal irrigants and medicaments to remove this lipid class from mineralized discs. The second purpose of this study is to evaluate the capacity of common endodontic intracanal irrigants and medicaments to degrade Lipid 654 to breakdown product Lipid 430. Methods: *P. gingivalis* (ATCC #33277, type strain) was grown under anaerobic conditions and centrifuged. *P. gingivalis* total lipids were recovered using the Bligh and Dyer phospholipid extraction procedure and were fractionated by semipreparative normal phase HPLC. A known amount of isolated *P. gingivalis* Lipid 654 was applied to bovine dentin discs and exposed to 0.5% NaOCl, 5.25% NaOCl, calcium hydroxide in saturated solution, and sterile water for 1 minute, 15 minutes, or 1 week. Removal of Lipid 654 and degradation of Lipid 654 to Lipid 430 was assessed by

mass spectrometric analysis. Results: Lipid extraction and MS analysis revealed that 1 and 15 minute exposures of NaOCl at 0.5% and 5.25% and 1 week calcium hydroxide treatment can remove Lipid 654 from bovine dentin discs. One week calcium hydroxide treatment can hydrolyze Lipid 654 into the Lipid 430 form. Conclusions: Our results show that *P. gingivalis* Lipid 654 can be removed from bovine dentin discs and hydrolyzed into the Lipid 430 form by common endodontic irrigants and medicaments.

Introduction:

Apical periodontitis manifests as bone destruction caused by host response to microorganisms and microbial byproducts in the necrotic root canal system [1]. *Porphyromonas endodontalis* is a Gram negative pathogen commonly found in necrotic root canals and apical lesions related to primary and secondary endodontic infections [2-4]. The involvement of *P. endodontalis* in the progression of apical periodontitis is believed to be related to release of destructive byproducts, which activate innate host cell response pathways [5]. Serine lipids of *P. endodontalis*, specifically the Lipid 654 and Lipid 430 classes are biologically active complex lipids that are identical in structure to those lipid classes produced by *Porphyromonas gingivalis* [6]. Our unpublished data suggest the activity of these lipids may play a role in both the bone and soft tissue destruction that is associated with apical periodontitis in humans.

The goal of endodontic therapy is to eradicate microbial irritants and proinflammatory substances from the root canal system and to obturate the cleaned and shaped root canal space to prevent future recontamination. This is accomplished through a combination of mechanical instrumentation of canal walls and irrigation with antimicrobial substances to remove and destroy irritants within the canal [7]. Sodium hypochlorite (NaOCl) is perhaps the most effective irrigant due to its bactericidal effect on endodontic pathogens including vegetative bacteria, spore-forming bacteria, fungi, protozoa, and viruses [8], its tissue dissolution capacity, and its ability to remove organic components of the

smear layer [9]. NaOCl acts by disrupting oxidative phosphorylation and DNA synthesis in bacterial cells and has been shown to be effective in root canal disinfection at concentrations of 0.5% to full strength at 5.25% [10]. Calcium hydroxide is an intracanal medicament used for root canal disinfection by creating an alkaline environment through release of calcium and hydroxyl ions over time [11]. Calcium hydroxide is slow acting and has been shown to be significantly more effective when exposed for a duration of 7 days versus 10 minutes [12]. It has been previously shown to have tissue dissolving capacity and to specifically suppress apical bone destruction by attenuating the virulence of *P. endodontalis* LPS [13].

Examination of *P. endodontalis* Lipid 654 or *Porphyromonas gingivalis* Lipid 654 removal or degradation by these common endodontic antimicrobial agents has not occurred. We hypothesize that sodium hypochlorite at two concentrations and calcium hydroxide will remove *P. gingivalis* Lipid 654 from bovine dentin discs and degrade *P. gingivalis* Lipid 654 to the Lipid 430 form.

Specific Aims:

The specific aims of this study are:

1. Isolate *P. gingivalis* Lipid 654
2. Determine the capacity of common endodontic irrigants and medicaments to remove Lipid 654 from mineralized surfaces of bovine dentin discs. A predetermined amount of Lipid 654 will be adsorbed to a mineralized dentin disc surface. The surface will be exposed to the indicated intracanal irrigants and medicaments, then the remaining bacterial lipid on the discs will be quantified. All experimental groups will be exposed for short time intervals of 1 and 15 minutes and calcium hydroxide will also be exposed for 1 week at physiological temperature. The mineralized bovine dentin discs will be acidified with 1% acetic acid and residual lipids will be extracted into chloroform and quantified by mass spectrometry. This experiment will demonstrate the capacity for endodontic intracanal irrigants and medicaments to remove adsorbed bacterial Lipid 654 from mineralized surfaces.
3. Determine the capability of common endodontic irrigants and medicaments to degrade *P. gingivalis* Lipid 654 to the Lipid 430 class. Samples will be treated as stated above. The rate of degradation is determined by measuring the presence of Lipid 654 breakdown product,

Lipid 430 and the ratio between the amount of recovered lipid from each of the two lipid classes.

Background Information:

The role of bacteria and bacterial byproducts in the progression of pulpal disease and apical periodontitis has been demonstrated in classic studies and is well accepted [14, 15]. Microorganisms and their byproducts have the ability to mediate changes in the dentin and pulp, which initiate pulpal inflammation and can lead to pulp necrosis and periapical lesion development [16]. Bone is in a continuous process of remodeling through osteoclast resorption and deposition by osteoblasts. As pulpal necrosis progresses to periapical disease, this homeostasis is disturbed resulting in periapical bone destruction through activation of osteoclast mediated bone resorption and inhibition of new bone formation [17].

The human immune response is divided into innate immunity and adaptive immunity. Innate immunity consists of nonspecific defense mechanisms that react immediately to foreign antigens. These defense mechanisms include physical epithelial barriers, phagocytic leukocytes, dendritic cells, natural killer cells, and circulating plasma proteins. Adaptive immunity is an antigen-specific immune response developed over a longer period of time and is less involved in initial pulpal defense against pathogens. It becomes activated when the innate immune system is not able to eliminate an infection. Humoral immunity is adaptive immunity mediated by antibodies produced by B lymphocytes and cell-mediated immunity is adaptive immunity mediated by T lymphocytes [18].

The microorganisms found in primary root canal infections are typically dominated by Gram negative and obligatory anaerobic bacteria [2]. Specific

byproducts of Gram negative bacteria within the necrotic root canal system have been identified as promoters of inflammation and periapical tissue pathogenesis. Lipopolysaccharide (LPS) is the primary component of the outer membrane of all Gram negative bacteria and plays an important role in stimulating secretion of pro-inflammatory cytokines. It is composed of polysaccharides, fatty acids, and phosphate and is highly recognized as a virulence factor in the destruction of periapical tissues [19]. Lipid A is the moiety of LPS responsible for its toxicity. It interacts with macrophages and monocytes to elicit a strong host immune response. However, LPS is not inherently destructive. It stimulates competent cells like macrophages, neutrophils, and fibroblasts to release damaging chemical mediators such as tumor necrosis factor, interleukin (IL)-1, IL-5, IL-8, alpha-interferon, and prostaglandins that in turn initiate the host response [20]. LPS maintains its virulence after cell death [21]. LPS freed from microorganisms, due to multiplication or cell death, binds to mineralized tissues and inhibits osteoblast differentiation and mineralization of bone [13].

At the onset of microbial insult and pulpal inflammation, the number of antigen presenting cells of the innate immune system rapidly increases at the site of tissue injury [22]. Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs) that are involved in pathogen detection. TLRs are transmembrane proteins found on antigen presenting cells as well as many other cell types, and play an important role in the recognition of microbial products. TLRs recognize pathogen-associated molecular patterns (PAMPs) on invading organisms, which are molecular pattern receptors that are evolutionarily

conserved from insects to humans [23]. Activation of TLRs initiates complex intracellular signaling cascades. As TLRs engage with their respective PAMPs, co-stimulatory molecules are expressed and secretion of pro-inflammatory cytokines is induced [24]. Ten human and twelve mouse TLRs have been identified to date. Toll-like Receptor 2 (TLR2) has been found in both human and mouse cellular plasma membrane and reacts with multiple microbial ligands including lipoteichoic acid, lipoproteins, and peptidoglycan, and human and mouse Toll-like Receptor 4 (TLR4) is known to respond to LPS [25]. Previous studies have associated TLR2 and TLR4 activity with periodontal inflammation and the Gram negative periodontal pathogen, *P. gingivalis* has been shown to signal via TLR2 and TLR4 [26-28]. Pro-inflammatory cytokines produced via TLR2 activation cause damage and bone loss in associated alveolar bone, as shown in studies using TLR2 knockout mice [29].

Several classes of biologically active complex lipids, distinct from Lipid A, are produced by *P. gingivalis* and *P. endodontalis*. These lipids can act as virulence factors. Specifically, the serine lipids have been shown to engage human and mouse TLR2. This activates the innate immune system and likely plays a role in mediating inflammatory responses. Clark et al. identified two serine lipid classes from *P. gingivalis* that act as TLR2 ligands [30]. In a mass spectrometry assessment, 654, 640, and 626 *m/z* negative ions make up the Lipid 654 class, referred to collectively as Lipid 654 (Figure 1) and the 430, 416, and 402 *m/z* negative ions make up the Lipid 430 class, referred to collectively as Lipid 430 (Figure 2).

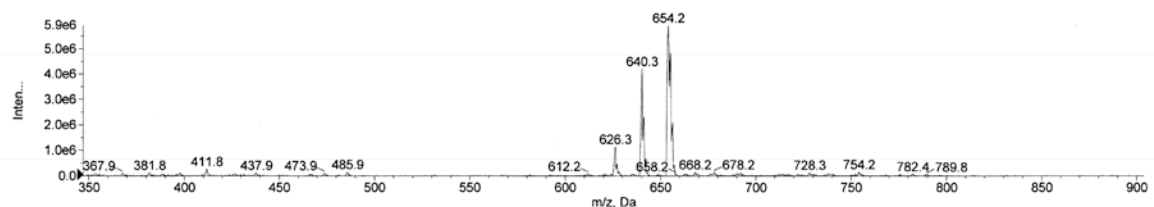


Figure 1. Mass spectrometry of Lipid 654 with peaks representing m/z 654, 640, and 626 negative ions.

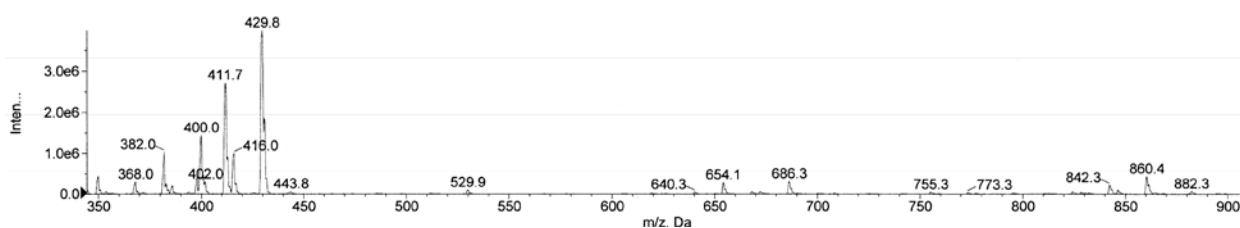


Figure 2. Mass spectrometry of Lipid 430 with peaks representing m/z 430, 416, and 402 negative ions.

The Lipid 430 class is the deesterified or nonesterified form of the Lipid 654 class. It is thought to be a breakdown product of Lipid 654 and is isolated in far less abundance from *P. gingivalis* than Lipid 654. Both lipid classes are made up of identical amino acid base structures, however Lipid 654 contains an ester-linked fatty acid where Lipid 430 does not [30]. Due to this physical difference, Lipid 430 is soluble in neutral or basic aqueous environments and Lipid 654 is not water soluble unless sonicated [30]. Another important distinction between the Lipid 654 and Lipid 430 involves their biologic activity. Lipid 430 has been shown to inhibit osteoblast differentiation at doses that are 4 to 10 times lower than Lipid 654. However, in comparison to Lipid 654, Lipid 430 is a weak

activator of TLR2 [Wang et al., submitted]. These lipid classes are structurally distinct from LPS Lipid A when assessed via mass spectrometric/nuclear magnetic resonance spectroscopy.

The serine lipids produced by *P. gingivalis* have been shown to be identical in structure and have consistent MS/MS spectra to those produced by phylogenetically related *P. endodontalis* [6]. *Porphyromonas endodontalis* is a Gram negative pathogen commonly found in root canals and apical lesions related to primary and secondary endodontic infections [2-4, 16]. The structure of *P. endodontalis* Lipid 654 is shown in Figure 3. It contains two fatty acids: 3-hydroxy iso C17:0 is amide linked to glycine and iso C15:0 is held in ester linkage by a β carbon of 3-hydroxy iso C17:0. The dipeptide head group is composed of glycine and a terminal serine. The similar structure of Lipid 430 is shown below in Figure 4.

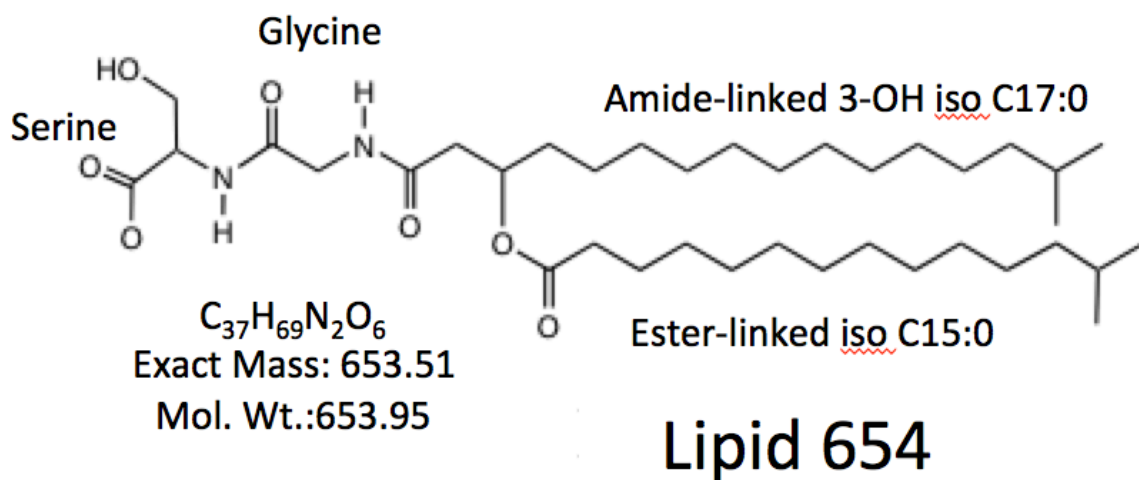


Figure 3. Structure of Lipid 654

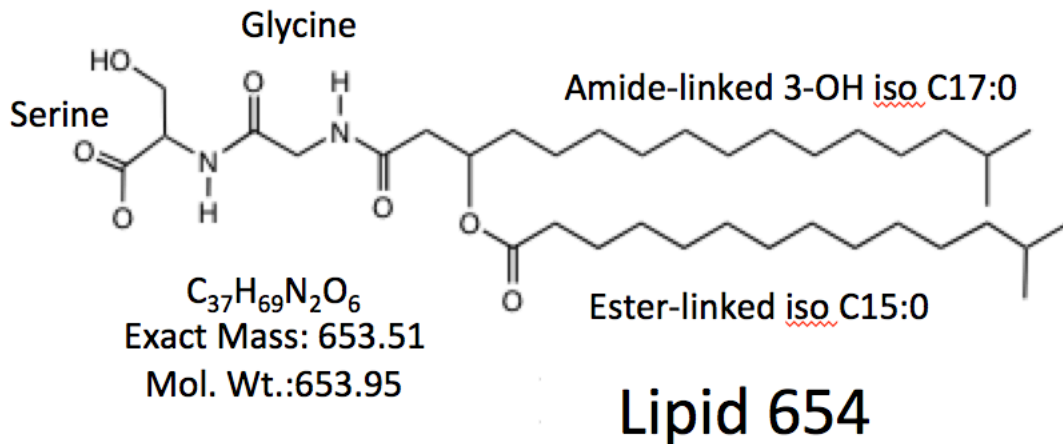


Figure 4. Structure of Lipid 430

Bacterial lipids from cell walls of Bacteroidetes species like *P. gingivalis* and *P. endodontalis* are present in all teeth with pulpal necrosis and apical periodontal lesions [31].

The goal of endodontic therapy is to eradicate microbial irritants and proinflammatory substances from the root canal system and to obturate the cleaned and shaped root canal space to prevent future recontamination. Complete cleaning and disinfection is not possible through mechanical means alone due to irregularly shaped and branching root canal systems with unique isthmuses and ramifications [32]. These mechanically inaccessible areas may house bacteria and bacterial byproducts, allowing for the possibility of resurgence of infection. The persistence of microorganisms in the root canal space after mechanical debridement is the main cause of endodontic treatment failure [33]. Because bacterial lipids found in the necrotic pulp space trigger inflammation independently of bacterial cell viability, removing and degrading

these residual lipids in addition to killing bacteria, may facilitate healing. Chemical means are used to further decontaminate these mechanically inaccessible areas of the irregular root canal system. Torabinejad et al. described the properties of the ideal root canal irrigant or medicament as one that completely removes the smear layer, penetrates into dentinal tubules, disinfects dentinal tubules, has sustained antibacterial action after use, has no negative impact on physical properties of dentin, does not interfere with sealing ability of restorative materials, does not discolor the tooth, is inexpensive, is convenient to use, and is nonantigenic, nontoxic, and noncarcinogenic to local tissue [34]. Haapasalo later added that an ideal irrigant should have a washing action, reduce friction, improve cutting of dentin by instruments, control temperature, dissolve organic and inorganic matter, penetrate deep within the root canal system, kill both planktonic and biofilm microbes, and detach biofilms [35]. The irrigant should also have low surface tension for flow into areas that are difficult or impossible to access mechanically, dissolve soft tissue for ease of removal in those areas instruments cannot reach, and act as a lubricant during mechanical instrumentation [36].

Many different irrigants and medicaments have been tried over the years in an effort to destroy and remove bacteria and bacterial byproducts from the necrotic root canal system. Everything from inert saline to highly toxic substances like formaldehyde have been used historically in endodontics. Several of these substances have fallen out of favor due to toxicity, limited effectiveness, and other qualities that make them less than ideal compared to the irrigants and

medicaments commonly used today. For example, Grossman advocated the use of antibiotic root canal dressing in the 1940s and early 1950s, but this practice was no longer advised by the late 1950s due to concerns over allergy and antibiotic resistance [37, 38]. Here, we will review some of the key historic endodontic irrigants and medicaments.

Chloramines are produced by reaction of hypochlorous acid (HOCl) and an amine, amide, or imide and slowly release chlorine. There are several chloramines, but chloramine T has been historically used as an endodontic irrigant. Chloramine T is a chlorine-releasing compound of sodium *p*-toluene sulfonchloramide that is formed by combining sodium 4-toluene sulfonamide and HOCl. It is water soluble up to 12% and hydrolyzes to form HOCl [39]. It is effective against algae, bacteria, viruses, fungi, and mycobacteria. The method of action involves interference with bacterial metabolism and destruction of DNA by oxidation. Its use was first reported in 1916 by Dakin et al. as a disinfectant to treat necrotic tissue wounds during World War I [40]. Austin and Taylor published multiple reports on the disinfection properties of chloramine T in 1918. They described chloramine T as a non-irritating antiseptic with “detoxicating action”, but found it did not have the capacity to dissolve necrotic tissue [41-44]. Later studies described the slow bactericidal action of chloramine T dependent on pH, temperature, and concentration. The antibacterial properties decrease as pH increases, making its practical use best in acidic environments with long exposure time [39, 45]. Chloramine T is no longer recommended for use in endodontics because its effectiveness and ease of use do not surpass the more

modern irrigants typically used today.

Quaternary ammonium compounds are bactericidal cationic surface-active agents that alter the permeability of the bacterial cell membrane. These surfactants interfere with membrane lipid bilayer integrity, interrupt transduction, disrupt membrane barrier function, impede action of membrane proteins, and ultimately interfere with growth and viability of microorganisms [46]. They have been considered suitable nonspecific root canal irrigants due to their activity against Gram positive and Gram negative organisms and fungi [47]. In the 1950s Strindberg was the first to recommend the use of quaternary ammonium compounds in endodontics after favorably comparing brand name Biosept with the more toxic and corrosive sulfuric acid [48]. Salvizol and Solvidont are two brand names of quaternary ammonium compounds with the active ingredient *bis-dequalinium* acetate that have been used as root canal disinfecting irrigants more recently. Their desirable properties include low tissue toxicity, low surface tension for good flow, and the ability to remove the smear layer as well as or better than ethylenediaminetetraacetic acid (EDTA) [47, 49, 50]. The bactericidal action of quaternary ammonium compounds has been demonstrated in numerous studies [51-53]. However, many studies also exist questioning clinical safety and ability of these irrigants to remove debris [51, 54-56]. Haikel et al. showed Salvizol only desorbed 2% of protein albumin from apatite surfaces in comparison to 30-70% for NaOCl at concentrations of 0.5% to 6% [56] and Engstrom and Spangberg found 1% Biosept was toxic to HeLa cells and recommended against its use clinically in 1969 [51]. Cetrimide is a bactericidal cationic surfactant containing a

mixture of quaternary ammonium salts that has been shown to decrease mechanical stability within bacterial biofilms and eradicate *Enterococcus faecalis* *in vitro*. It is utilized most commonly as an adjunct to other irrigants such as chlorhexidine, EDTA, citric acid, MTAD, and tetraclean to enhance their desirable properties [57-59]. Cetrimide is not commonly used in standard endodontic irrigation protocols, but research from the last five years shows favorable results. An *in vitro* study by Kaushik et al. found exposure to 0.2% cetrimide alone caused maximum reduction in levels of *E. faecalis* compared to 5.25% NaOCl, 3% hydrogen peroxide, 10% citric acid, and 17% EDTA [60]. However, quaternary ammonium compounds in general should be used with caution clinically due to high allergenic potential [61].

Hydrogen peroxide (H_2O_2) was discovered in 1818 by Louis Jacques Thenard and first produced in a pure form through vacuum distillation in 1894 by Richard Wolffenstein [62]. In 1915 Dakin described H_2O_2 as a mildly effective antiseptic [63]. It is an oxidizing agent active against bacteria, yeast, fungi, spores, and viruses through production of hydroxyl radicals which cause cell death by interfering with macromolecules including proteins, membrane lipids, and DNA [41, 64]. Hydrogen peroxide has been used as a bactericidal endodontic irrigant in concentrations of 1% to 30%, but primarily at a concentration of 3%. It has also been shown to physically dislodge necrotic tissue and dentin debris [65], but does not have the capacity to dissolve tissue. In the past, H_2O_2 has been used as an adjunct in repeated alternation with sodium hypochlorite to achieve both tissue removal and dissolution, but studies have

shown the alternating use of 2% or 5% sodium hypochlorite with 3% H₂O₂ was less effective at dissolving necrotic tissue than NaOCl alone [66]. It has also been shown that the combination of H₂O₂ with sodium hypochlorite was not significantly better at destroying *E. faecalis* than irrigation with sodium hypochlorite alone [67]. The use of H₂O₂ as an endodontic irrigant is not currently recommended because it has not been shown to dissolve tissue, increase removal of intracanal debris, or reduce bacterial load within the root canal as effectively as more widely used alternatives such as varying concentrations of NaOCl [64].

Sodium hypochlorite is the most important endodontic irrigant used today. In water it ionizes into sodium and hypochlorite ions to form equilibrium with HOCl. NaOCl is most effective against microbiota in environments with low to neutral pH, where most of the chlorine exists as HOCl [35]. It acts by disrupting oxidative phosphorylation and DNA synthesis in bacterial cells. The antibacterial efficacy of NaOCl is concentration dependent [68], but it has been shown to be effective in root canal disinfection from 0.5% to full strength at 5.25% [10]. It has a wide-spectrum and is nonspecific, killing bacteria, spores, and viruses [9]. NaOCl is inexpensive, easy to store, and widely available. Sodium hydroxide is added to commercially available bleach solutions to increase the pH and ensure stability of the solution. Household bleach usually contains 0.01-0.05% sodium hydroxide to slow decomposition of sodium hypochlorite into sodium chloride and sodium chlorate. NaOCl was first recommended as a bleaching agent in the late 1700s and Labarraque recommended its use to prevent infectious diseases at

the turn of the nineteenth century [9]. Its bactericidal action has been reported since the early 1900s. First, by Dakin as an antimicrobial wash to treat necrotic tissue in soldiers' open wounds in World War I, then in multiple studies by Austin and Taylor [41-44, 63, 69]. Crane was the first to suggest NaOCl for use as an endodontic irrigant in 1920 [70]. NaOCl dissolves essentially all exposed organic matter within the root canal in a concentration dependent manner [71, 72]. Clinically, it has a very low allergenic potential [73]. This may be partially due to the fact NaOCl dissolves necrotic tissue more effectively than vital tissue [41]. Like all endodontic irrigants, NaOCl use should be confined within the root canal system to avoid the rare complication of a sodium hypochlorite accident. This occurs when NaOCl is extruded outside of the tooth into surrounding tissues. The resulting host response can manifest as local edema or mild ecchymosis only to severe extensive mediastinal ecchymosis. While unpleasant, sodium hypochlorite accidents are rare and almost never life threatening and the benefits of using NaOCl as an endodontic irrigant far outweigh this risk [74]. While precautions must be considered in the use of NaOCl, it fulfills more of the desired characteristics proposed for an ideal irrigant than current alternatives and is recommended as the main irrigant in endodontics. However, Buttler et al. reported that 0.58% to 5.20% NaOCl does not detoxify *E. coli* LPS [75]. Lipids that contain saturated fatty acids will not react with NaOCl, leaving the possibility for continued virulence if this is the only irrigant used.

Calcium hydroxide is a slow acting intracanal medicament that is widely used in endodontics for its antimicrobial activity, tissue dissolving capacity,

inhibition of tooth resorption, and induction of hard tissue repair [11, 33]. It is a mildly water-soluble white powder with highly alkaline pH of 12.5. Calcium hydroxide slowly dissociates into calcium and hydroxyl ions when in an aqueous environment [76]. The hydroxyl ions are extremely active ions that are thought to damage bacterial membranes, denature proteins, and damage bacterial DNA when in direct contact [11]. Hermann first introduced calcium hydroxide for use in dentistry in 1920 [77]. Sjögren showed calcium hydroxide is ineffective as an intracanal disinfectant with an application time of only 10 minutes, but an application time of 7 days effectively removed cultivable bacteria that had survived mechanical instrumentation of the canal [12]. Due to its slow acting nature, calcium hydroxide is most commonly used in endodontics as an interappointment canal medicament. Previous studies have reported the toxic LPS Lipid A molecule is hydrolyzed into innocuous fatty acids and amino sugars by calcium hydroxide, discontinuing LPS activity *in vitro*. It is the only endodontic irrigant or medicament shown to inactivate the negative effects of LPS [13, 31, 78, 79]. Calcium hydroxide has a very important role in endodontic therapy, but compared to NaOCl it has a limited antimicrobial spectrum and is not active against all endodontic microbiota. It also cannot reliably eliminate bacteria from the inside of dentinal tubules due to its physical properties and lack of flow. Calcium hydroxide should always be used clinically in combination with other irrigants and medicaments [11].

Chlorhexidine is a synthetic cationic bisbiguanide with two symmetric 4-chlorophenyl rings and two biguanide groups connected by a central

hexamethylene chain [80]. It is available in varying concentrations in solution and gel forms. This antimicrobial agent is lipophilic and hydrophobic and interacts with negatively charged phosphate groups on microbial cell walls to increase permeability and to allow for penetration of the chlorhexidine molecule into the cell [81]. In very low concentrations it is bacteriostatic and will cause leakage of cellular contents like phosphorus and potassium. At high concentrations it has a bactericidal effect by coagulating the cellular cytoplasm, causing cell death. It is effective against Gram negative and Gram positive bacteria, yeast, fungi, and select viruses including herpes, cytomegalovirus, and HIV [82]. The use of chlorhexidine was first introduced in medicine in 1954 for debriding skin, mucous membranes, and wounds. It was reported for oral use in 1959 to control dental bacterial plaque and became more commonly used in dentistry in the 1970s [82]. When used as an antimicrobial intracanal medicament, chlorhexidine has been shown to be as effective or more effective than calcium hydroxide [83, 84]. Liquid concentrations of 0.2% to 2% have been shown to have similar antimicrobial activity as 5.25% NaOCl [85, 86]. However, unlike NaOCl, chlorhexidine is unable to dissolve vital or necrotic tissue [87] and its effect on microbial biofilms is significantly lesser than NaOCl [81]. In the root canal, positively charged chlorhexidine adsorbs onto dentin and maintains continued antimicrobial action on the dentin surface beyond the time the medication is applied in a feature called substantivity. White et al. showed the substantivity of 2% chlorhexidine solution lasted for 72 hours after endodontic irrigation [88] and Rosenthal et al. showed the substantivity lasted for up to 12 weeks [89]. Allergy to chlorhexidine

is very rare. Other side effects such as contact dermatitis and dysgeusia are uncommon and seem to be dose dependent. Chlorhexidine is highly biocompatible [81]. Previous bacterial lipid studies have shown chlorhexidine has little to no effect on inactivating the Lipid A portion of LPS and does not adequately reduce remaining LPS in the root canal system after chemomechanical preparation [82].

The removal and degradation of *P. endodontalis* Lipid 654 with intracanal irrigants and medicaments may hinder the progression of apical periodontitis in endodontic infections. Lipid 654 produced from *P. endodontalis* and *P. gingivalis* is structurally identical [6]. The effect of NaOCl at different concentrations and calcium hydroxide on Lipid 654 is not fully understood. We examined the capacity of these substances to remove Lipid 654 produced from *P. gingivalis* from mineralized surfaces and the capacity for chemical degradation of Lipid 654 to the Lipid 430 form.

Materials and Methods:

For this investigation, Lipid 654 was prepared from *P. gingivalis* using HPLC as previously described [90]. Briefly, *P. gingivalis* was grown in pure culture followed by extraction of lipids and purification of the Lipid 654 preparation as described below.

Bacterial growth- Bacteria were grown in broth culture as previously described. *P. gingivalis* (ATCC #33277, type strain) was inoculated into basal

(peptone, trypticase and yeast extract) medium supplemented with hemin and menadione (Sigma-Aldrich) and brain heart infusion [90]. Culture purity was verified by Gram stain, lack of growth in aerobic culture and formation of uniform colonies when inoculated on brain heart infusion agar plates and grown under anaerobic conditions. The suspension cultures were incubated for four days in an anaerobic chamber flushed with N₂ (80%), CO₂ (10%) and H₂ (10%) at 37°C and the bacteria were harvested by centrifugation (2000 x g for 2 hour).

Lipid extraction, fractionation and characterization- Lipids were extracted from lyophilized bacterial pellets. Generally 2 to 4g of bacterial pellet was extracted for each semipreparative fractionation. The bacterial samples were weighed and dissolved in chloroform:methanol:water (1.33:2.67,1, v/v/v, 2g of bacterial pellet in a total of 20 ml of solvent) as previously described [90]. The mixture was vortexed at 15 min intervals for 2 hours and the mixture was supplemented with 6 ml of chloroform and 6 ml of (2N KCl + 0.5N K₂HPO₄). The mixture was vortexed and centrifuged (2000 x g) at 20°C for 4 h. The lower organic phase was removed and dried under nitrogen. The dried extract was reconstituted in HPLC solvent (hexane:isopropanol:water, 6:8:0.75, v/v/v, 24 ml) and vortexed [90]. The sample was centrifuged at 2500 x g for 10 minutes and the supernatant removed for HPLC analysis. Semipreparative HPLC fractionation was accomplished using a Shimadzu HPLC system equipped with dual pumps (LC-10ADvp), automated controller (SCL-10Avp) and in line UV detector (SPD-10Avp). Lipids were fractionated using normal phase isocratic separation (Ascentis[®]Si, 25 cm x 10

mm, 5 μ m, Supelco Analytical) with a solvent flow of 1.8 ml/min and 1 min fractions. HPLC solvent was composed of hexane:isopropanol:water (6:8:0.75, v/v/v). The effluent was monitored at 205 nm. Replicate fractionations were pooled and dried under nitrogen. The dried samples were reconstituted in HPLC solvent for mass spectrometric analysis as described below. Fractions containing Lipid 654 were pooled and dried before additional HPLC fractionation.

Acidic HPLC fractionation of Lipid 654 and Lipid 430- Lipid 654 fractions were further purified by elution over the same HPLC column at 1.8 ml/min but using HPLC solvent supplemented with 0.1% acetic acid. Purity of Lipid 654 was verified by electrospray-mass spectrometry (ESI-MS or MS/MS) as described below. Lipid 430 mass spectral characteristics were verified using ESI-MS and MS/MS on a quadrupole time of flight (QTOF) (Sciex QStar instrument).

Application of Lipid 654 to bovine dentin discs- Lipid 654 was dissolved in chloroform to achieve a concentration of 0.02 μ g/ μ l and 5 μ l of this solution was applied to bovine dentin discs (Immunodiagnostic Systems) and air-dried. Discs were 5mm in diameter and 0.3mm thick. Three discs were allocated into individual glass tubes for the indicated test solution treatment groups. Test solutions were irrigants and medicaments common to endodontic practice exposed for 1 and 15 minute time periods. The calcium hydroxide group was also exposed for the duration of 1 week at physiologic temperature. The experimental solutions included 0.5% NaOCl, 5.25% NaOCl, calcium hydroxide aqueous

suspension, and a sterile water control irrigant. Once the discs were processed, the supernatant was removed and 1 ml of 1% acetic acid was added to each tube. The discs dissolved rapidly and once this was completed, the aqueous samples were extracted three times with 1 ml of chloroform. The organic extracts were pooled and dried. Prior to mass spectrometric analysis, the samples were dissolved in 100 μ l of HPLC solvent (hexane:isopropanol:water, 6:8:0.75, v/v/v) and 2 μ l of each solution was analyzed by mass spectrometric analysis as described below.

Mass spectrometry- Dentin extracts were injected over a normal phase column (Ascentis[®] Si, 3 cm x 2.1 mm, 5 μ m, Supelco Analytical) interfaced with an API Qtrap 4000 instrument from Sciex. Neutral HPLC solvent was delivered under isocratic conditions with a Shimadzu LC-10ADvp pump at a flow rate of 100-120 μ l/min. Collision energies for Lipid 654 and Lipid 430 negative ion products were typically between -30 and -55 volts depending on the precursor ion under investigation. Negative ion ESI was carried out at -4,500 V, with a declustering potential of -90 V, focusing potential of -350 V, and entrance potential of -10 V. Multiple reaction monitoring (MRM) negative ion transitions for Lipid 654 were 653.5/131.1, 653.5/306.2, 653.5/349.3 and 653.5/381.4 m/z and for transitions for Lipid 430 were 430.2/140.9, 430.3/173.1 and 430.3/382.3 m/z . Lipid 430/Lipid 654 ratios were calculated using only the 430.3/382.3 and 653.5/381.4 m/z transitions. Lipid peaks were integrated electronically and

these were used to calculate the recovery of Lipid 654 on the discs as well as the conversion of Lipid 654 to Lipid 430 on the discs.

Statistical Analysis- Since the results typically were not normally distributed, data are expressed using Box-Whisker plots. Statistical testing included Analysis of Variance (ANOVA) with pairwise comparisons using the Scheffe test. For data sets that did not satisfy a normal distribution test, the results were depicted as Box Whisker plots and either Kruskal-Wallace or Mann Whitney U rank order tests were applied. A p value of ≤ 0.05 was considered to be significant

Results:

Analysis of *P. gingivalis* Lipid 654 extracts treated with sterile water, calcium hydroxide in a saturated sterile water solution, 0.5% NaOCl, and 5.25% NaOCl for 1 minute, 15 minutes, or 1-week intervals revealed these medicaments do impact the amount of Lipid 654 and Lipid 430 recovered after treatment. Lipid 654 recovery from the bovine dentin discs after treatment is illustrated in Figure 5.

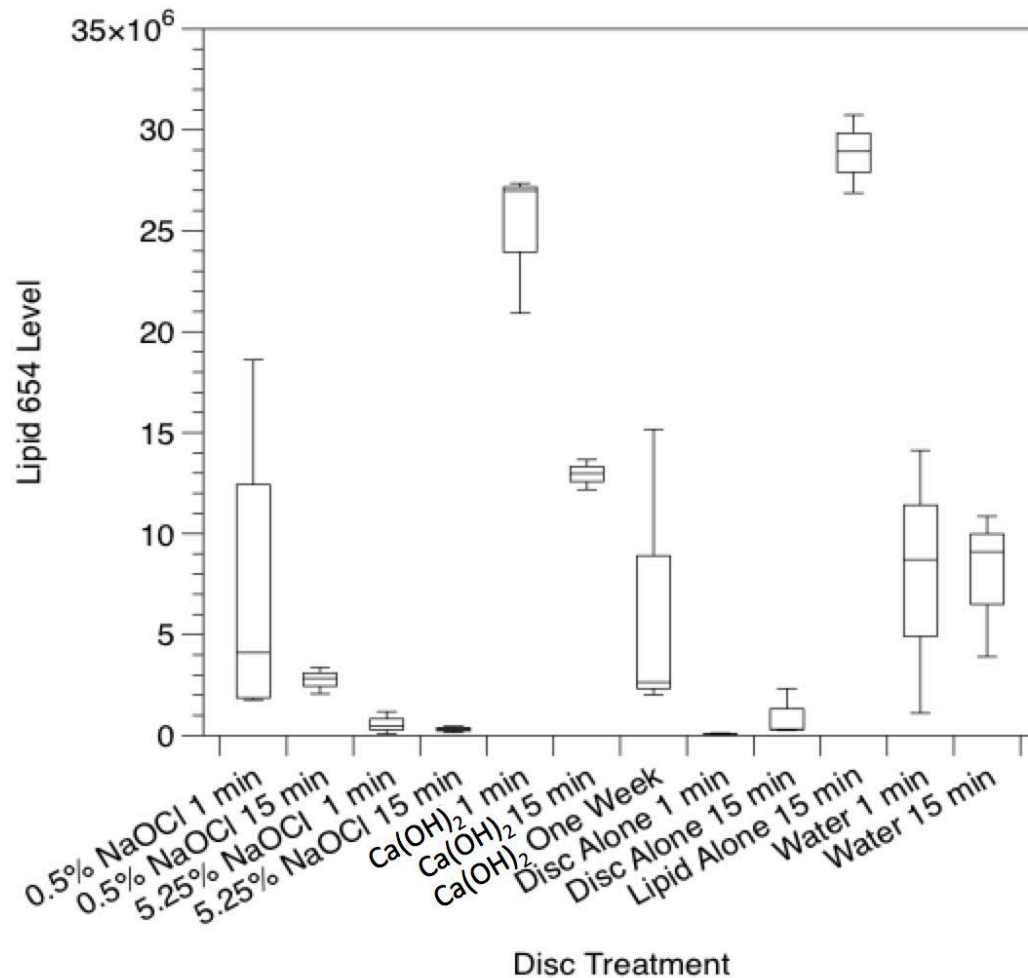


Figure 5. Lipid 654 recovered from bovine dentin discs after exposure to sterile water, calcium hydroxide ($\text{Ca}(\text{OH})_2$) in a saturated sterile water solution, 0.5% NaOCl, and 5.25% NaOCl for 1 minute, 15 minute, or one week intervals.

More Lipid 654 was recovered from the bovine dentin discs in the positive control of lipid alone versus any other treatment group. This finding was highly significant ($p < 0.01$) in all cases except for the comparison between lipid alone and calcium hydroxide treatment for 1 minute. Less Lipid 654 was recovered from bovine dentin discs of the negative control samples in the disc alone group when compared to all other treatment groups except for 5.25% NaOCl at both 1 and 15 minute exposures. Less Lipid 654 was recovered from the 5.25% NaOCl

1 and 15 minute exposure groups than the disc alone negative control, but these differences were not significant.

There was no difference in recovery of Lipid 654 or Lipid 430 by 0.5% NaOCl compared to 5.25% NaOCl for any time of exposure. The 0.5% and 5.25% NaOCl treatment groups at both 1 and 15 minutes had significantly less remaining Lipid 654 than calcium hydroxide treatment for corresponding 1 or 15 minutes exposure periods ($p < 0.05$). However, all NaOCl groups acted similarly to calcium hydroxide exposure for 1 week for Lipid 654 recovery. There was no significant difference in any of the NaOCl treatment groups for Lipid 654 recovery when compared to 1 week of calcium hydroxide treatment.

The difference in Lipid 654 and Lipid 430 recovery between 1 minute and 15 minute exposure times was not significant for the control groups of lipid alone or bovine dentin disc alone. Exposure time of 1 minute compared to 15 minutes had no impact on amount of Lipid 645 or Lipid 430 recovered from the bovine dentin discs after treatment with 0.5% NaOCl, 5.25% NaOCl, or sterile water. No significant difference was found in the recovery of Lipid 654 for calcium hydroxide treatment for 15 minutes versus 1 week, but samples for both fifteen minute and 1 week exposure times had significantly less lipid recovery than exposure for 1 minute ($p < 0.05$).

Lipid 430 is a breakdown product of Lipid 654 after hydrolysis of the ester-linked fatty acid. Unlike Lipid 654, it is soluble in neutral or basic aqueous environments [30]. Figure 6 shows the recovered Lipid 430 for each treatment group. More Lipid 430 was recovered from bovine dentin discs treated with

calcium hydroxide for 1 week than all other treatment groups and this difference was significant for all comparisons except for calcium hydroxide exposure for 1 week versus treatment with calcium hydroxide for 1 minute or lipid alone for 15 minutes ($p < 0.05$). Time of exposure did have an impact on treatment with calcium hydroxide. In the recovery of Lipid 430, no significant difference was found between calcium hydroxide treatment for 1 minute versus 15 minutes or 1 week, but significantly more lipid was recovered with calcium hydroxide treatment for 1 week compared to 15 minutes ($p < 0.05$).

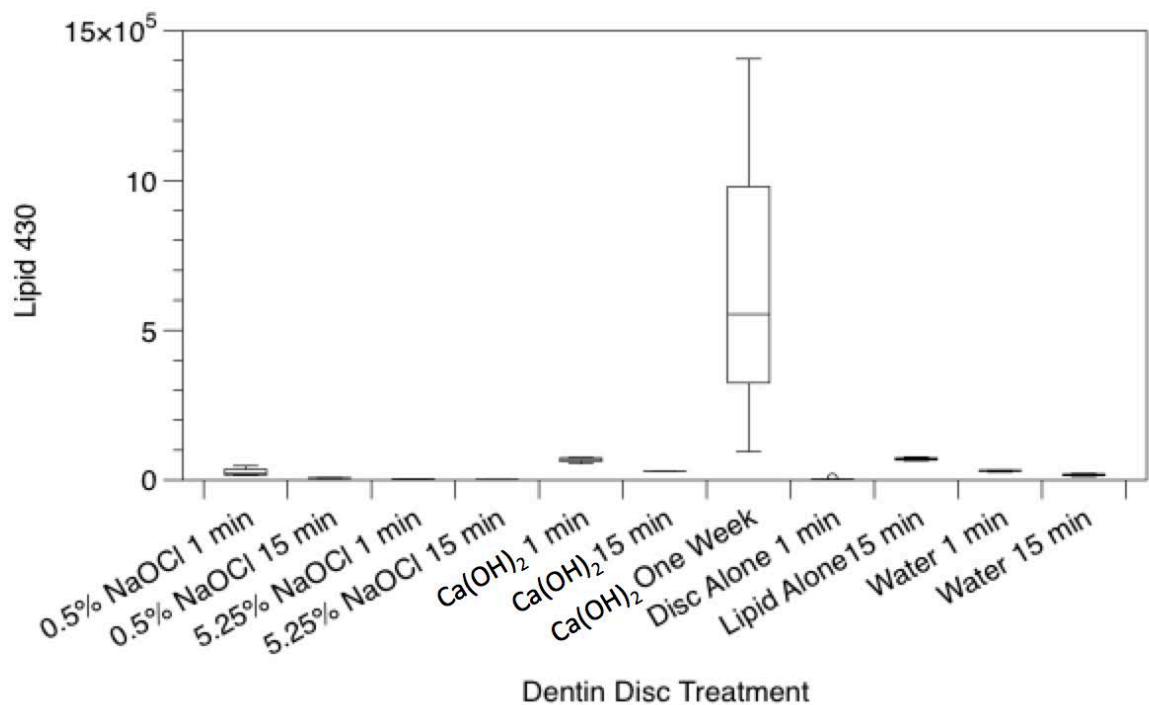


Figure 6. Lipid 430 recovered from bovine dentin discs after exposure to sterile water, calcium hydroxide ($\text{Ca}(\text{OH})_2$) in a saturated sterile water solution, 0.5% NaOCl, and 5.25% NaOCl for 1 minute, 15 minute, or 1 week intervals.

Figure 7 shows the ratio of recovered Lipid 430 versus Lipid 654 for each experimental group as determined using mass spectrometry. The highest ratio was observed with the Lipid 654 applied to bovine dentin discs exposed to calcium hydroxide for 1 week. These results suggest an increased conversion of Lipid 654 to Lipid 430 in this group compared to the other groups.

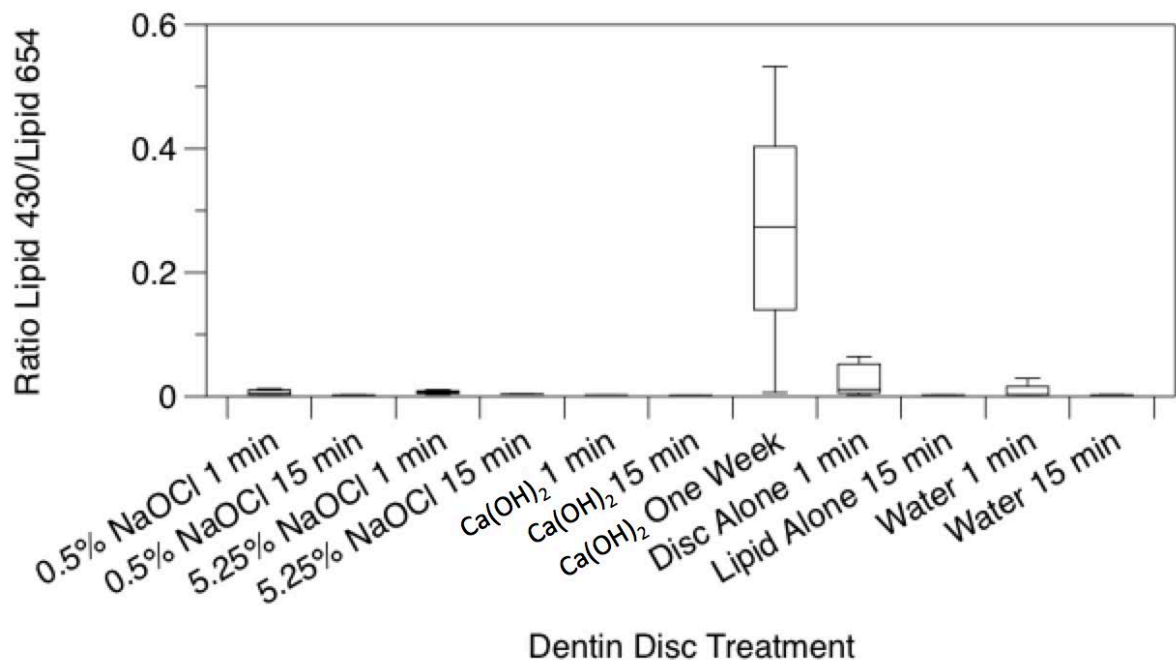


Figure 7. Ratio of Lipid 430 to Lipid 654 recovered from bovine dentin discs after exposure to sterile water, calcium hydroxide ($\text{Ca}(\text{OH})_2$) in a saturated sterile water solution, 0.5% NaOCl, and 5.25% NaOCl for 1 minute, 15 minute, or 1 week intervals.

Discussion:

Our study demonstrated that Lipid 654 from *P. gingivalis* can be reliably removed from bovine dentin discs with common endodontic irrigants and medicaments and that Lipid 654 is hydrolyzed into the Lipid 430 form when

exposed to calcium hydroxide for the duration of 1 week. Because the structure of Lipid 654 and Lipid 430 produced from *P. gingivalis* and *P. endodontalis* is identical, we believe that these lipid classes isolated from *P. endodontalis* will respond in a similar manner to such treatment.

In a clinical study, Endo et al. reported higher levels of LPS in infected root canals were related to larger size of associated radiolucent lesions in the periapical bone[91]. Gomes et al. described similar findings when they found positive correlations between higher LPS levels in primary and secondary endodontic infections and size of the associated radiolucent lesion and higher LPS levels in primary infections and increased clinical symptoms[92]. They called for establishment of clinical protocols focused on the neutralization and elimination of not only Gram negative bacteria, but also their harmful byproducts, in endodontic therapy.

Similarly, Lipid 654 has been shown to promote inflammatory and bone cell activation processes that may lead to apical periodontitis [6]. This underscores the importance of removal of Lipid 654 in endodontics clinically. Members of the *Bacteroidetes* phylum are the major producers of Lipid 654. *Bacteroidetes* make up almost half of the gastrointestinal microorganisms and are also common in periodontal disease. Serine lipids specifically have been recovered in diseased periodontal tissues and serum [30].

Our study demonstrates that Lipid 654 is effectively removed from bovine dentin discs by 0.5% NaOCl and 5.25% NaOCl after as little as 1 minute exposure time and by calcium hydroxide after 1 week of exposure. The

concentration of NaOCl from diluted 0.5% to full strength 5.25% did not significantly impact lipid removal capability. NaOCl is significantly more effective at removing Lipid 654 from bovine dentin discs when compared to calcium hydroxide at identical 1 or 15 minute exposure times, but acts similarly to calcium hydroxide exposure of 1 week.

The bovine dentin discs used in this study contained a baseline amount of detectable lipid, as can be seen in the analysis of the disc alone treatment groups. Less Lipid 654 was recovered from negative control samples of the bovine disc alone when compared to all other treatment groups except for 5.25% NaOCl at both 1 and 15 minute exposures. However, this finding was not statistically significant. This suggests the 5.25% NaOCl not only removed the Lipid 654 that was applied experimentally, but possibly also baseline lipid that already existed in these bovine dentin discs.

We found Lipid 654 is hydrolyzed to the Lipid 430 form when treated with calcium hydroxide for 1 week. In a study of the ability of common endodontic irrigants and calcium hydroxide to break down bacterial LPS, Buck et al. demonstrated that alkaline solutions degrade LPS more effectively than acidic and that exposure of LPS to calcium hydroxide for two and five days caused more degradation than exposure for only 1 day [21]. Our results regarding Lipid 654 correspond with these findings. The breakdown product Lipid 430 class has been shown to be significantly biologically active with the ability to inhibit osteoblast differentiation gene expression in cells from wild type mice, but shows only slight activity in cells from TLR2 knock out mice. This suggests Lipid 430

affects osteoblast differentiation with cell activation events, and is not fully dependent on engagement with TLR2 [Nemati et al., submitted]. Lipid 430 is recovered at a much lower rate than Lipid 654 in lipid extracts from *P. gingivalis* [30], but is recovered in higher quantities from *P. gingivalis* lipid extracts of chronically inflamed tissue from diseased periodontium. Additionally, Lipid 430 has been found to be biologically active at levels four to tenfold lower than those of Lipid 654 [Wang et al., submitted]. This accentuates the importance of removing both Lipid 654 and Lipid 430 clinically, not simply hydrolyzing the former to the latter. Lipid 430 is soluble in neutral or basic aqueous environments while Lipid 654 is not water soluble unless sonicated [30]. Therefore, Lipid 430 may be more easily removed by endodontic solutions clinically than Lipid 654. The removal of Lipid 654 versus Lipid 430 by endodontic irrigants and medicaments requires further study. In unpublished studies we evaluated the ability of calcium hydroxide to alter the biologic activity of serine bacterial lipids. We found calcium hydroxide treatment de-esterified Lipid 654 and significantly reduced its effect on the release of the inflammation mediator tumor necrosis factor alpha.

The exact amount of time needed for calcium hydroxide to kill bacteria clinically is not known. In an *in vivo* study, Sjögren showed exposing chemomechanically debrided necrotic pulps to calcium hydroxide for only 10 minutes resulted in elimination of cultivable bacteria in half of the experimental cases while a 7 day exposure resulted in elimination of cultivable bacteria from all experimental samples[12]. Therefore, long-term application of calcium

hydroxide has been suggested as an interappointment dressing to destroy bacteria that may survive initial chemomechanical preparation of the canal and is an argument for two-visit treatment of necrotic pulps. There is ample evidence that a bacteria-free status cannot be achieved in a chemomechanically prepared necrotic pulp space after 1 endodontic treatment visit [93, 94]. However, multiple studies have not found a significant difference in outcome between 1 visit treatment compared to 2 visit treatment with calcium hydroxide interappointment dressing in teeth with necrotic pulps [95-97]. Because bacterial lipids have been shown to be active well after cell death [21], it is possible that removal and degradation of these lipids are important considerations in addition to achieving bacteria-free status of the prepared canal. Our study supports the use of calcium hydroxide as a long-term intracanal medicament for the purpose of removing Lipid 654 from the canal and also degrading it to the Lipid 430 form.

In summary, the results of our study demonstrate the ability of NaOCl at 0.5% and 5.25% concentrations and long-term 1 week calcium hydroxide treatment to remove *P. gingivalis* Lipid 654 from bovine dentin discs. We also demonstrate that long-term 1 week calcium hydroxide treatment can hydrolyze *P. gingivalis* Lipid 654 into the Lipid 430 form.

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