

7-1-2016

Serine Dipeptide Lipid and Prostaglandin E2 Recovery in Pulps and Tooth Apices: Relationship to Pulpal Status

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Recommended Citation

Tobin, Aniuska, "Serine Dipeptide Lipid and Prostaglandin E2 Recovery in Pulps and Tooth Apices: Relationship to Pulpal Status" (2016). *Master's Theses*. 981.

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Serine Dipeptide Lipid and Prostaglandin E2 Recovery in Pulp and Tooth

Apices: Relationship to Pulpal Status

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DDS, University "Gran Mariscal de Ayacucho", 2003

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Dental Science

At the

University of Connecticut

2016

APPROVAL PAGE

Master of Dental Science Thesis

**Serine Dipeptide Lipid and Prostaglandin E2 Recovery in Pulp and
Tooth Apices: Relationship to Pulpal Status**

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Acknowledgements

I wish to express my most sincere gratitude to all the people that made this project possible. I want to thank my principal advisor Dr. Nichols for helping me to develop and complete this project, for your patience, time, effort and knowledge, without your guidance and continuous help this thesis would not have been possible.

To Dr. Safavi, there are no words to express my gratitude; you make UConn Endodontology program great. Thank you for believing in me, for giving me the opportunity to be part of this great family, and for your patience and infinite knowledge; I could not have ask for a better mentor.

I will like to thank my other committee members, Dr. Chen and Dr. Kaufman. Dr. Chen thank you so much for all the help with my protocol, reviewing the thesis and presentation, you have extraordinary knowledge in research; as well for all your help in the clinic, I really appreciate your guidance and teaching. To Dr. Kaufman thank you for all your help with this project, for all your support, for taking charge of our literature reviews and for continuously preparing us for our boards, you do make a difference in our preparation.

Dr. Zhu, thank you for all your help in the clinic, for your knowledge and to always have such a positive and happy attitude. It is always very fulfilling to see you working with the students, you are a great example to follow.

To my co-residents, Yousef, Luis, Jeremiah, Saleh, Jonathan, Homan and Fatima, I wish you all the best in your future careers, thank you for your friendship and for making this residency so special. To the rest of the DC2 family, Colleen, Aimee,

Purvi and Debbie, thank you for making DC2 great and for all your patience and help with us.

A special thanks to Debbie Osborne, for all her help through out these three years, you will always have a special place in our lives. And to Erica Roman, our newest family member, thank you for coming with great attitude and eager to learn, you are doing a great job.

To my friends from other departments, thank you for welcoming my family and I from the first day and making us feel that Connecticut is our home. A special thanks to DC5 and DC8 for helping me collect the samples for this project.

To my family, I am truly blessed to have the family that I have, you have supported me every single step of the way even through the distance, and specially to my mom Katuska, my brother Jesus, my auntie Muriel for coming here and helping me through this last semester, I love you with all my heart.

And my final thanks goes to my co-resident, partner and best friend, my husband Alejandro, you are my pillar; I could not have done this with out your support, I love you. And to our daughter Isabella, for being such a great little girl and always understanding when mom had to study, all this is for you I love you.

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Abstract

Introduction: Apical periodontitis is characterized by inflammation and destruction of the periapical tissues that is primarily caused by polymicrobial infection of the pulp, which is dominated by Gram-negative species. Gram-negative bacteria have different virulence factors, e.g. cell wall endotoxin and serine lipids, which are toxic to the periapical tissues leading to activation of the host immune response and inflammatory reaction. *Porphyromonas endodontalis* (*P.endodontalis*), a Gram-negative bacterium, has been shown to populate necrotic root canal systems and may contribute to apical bone loss. Moreover, it is shown to synthesize novel serine dipeptide lipid classes (Lipid 654 and Lipid 430). Lipid 654 is significantly increased in necrotic pulps whereas Lipid 430 remains at comparable levels between healthy and necrotic pulps of human teeth. This finding suggests that the Lipid 430 is likely not heavily involved in the inflammatory reaction of necrotic pulps. However, to date, the levels of lipid 654 and 430 in periapical tissues of teeth with apical periodontitis are still unknown. We hypothesize that the conversion of the lipids in the periapical tissue could be increased from that which is found in the necrotic tissue inside of the root canal space. We also evaluated the levels of prostaglandin E₂ (PGE₂) in these samples and the possible correlation with the levels of the serine dipeptide lipids.

Materials and methods: The lipid extracts from 12 extracted teeth (molars and incisors) with necrotic pulps with apical periodontitis were obtained from the necrotic apical pulpal tissues as well as from the periapical tissues of the same teeth. Third molars with vital pulps and intact premolars (n=12) were collected and processed as

negative controls. PGE₂, Lipid 654 and Lipid 430 were identified by liquid chromatography-multiple reaction monitoring mass spectrometry (LC-MRM MS).

Results: The Lipid 654 levels were significantly elevated in the teeth with pulpal necrosis versus healthy pulp contents, but no significant difference was seen in Lipid 654 levels between the canal and periapical samples for teeth with necrotic pulps. PGE₂ levels detected in pulps and from periapical tissues of necrotic teeth with apical periodontitis increased significantly compared to the levels measured in the pulpal tissues from the same teeth. The ion abundances for Lipid 430 transitions were not detectable in most of the pulp and periapical samples.

Conclusions: Lipid 654 ions were elevated in teeth with necrotic pulps and apical periodontitis in the intracanal and periapical portions of the teeth, which supports its participation in the activation of the innate immune system. This elevation of Lipid 654 can also indirectly cause bone destruction associated with apical periodontitis. It is unclear at this time if the lipid species of Lipid 430 represent precursor or breakdown products (or both) for the constituent lipid species of Lipid 654. Elevated PGE₂ levels in the apical samples of the teeth with apical periodontitis are in concordance with other studies showing the association of the PGE₂ with bone destruction and inflammation.

Introduction

Description of Apical Periodontitis

Apical periodontitis is characterized by inflammation and destruction of the periapical tissues; it involves changes in the alveolar bone, periodontal ligament and cementum and is primarily caused by microbial infection of the pulp (1). Apical periodontitis can be caused by a combination of exogenous and endogenous factors. The exogenous factors include the bacteria and their toxins (e.g. lipopolysaccharide (LPS), lipoteichoic acid and others) and metabolic byproducts, mechanical irritants, chemical agents, foreign bodies and trauma. Endogenous factors include the host's metabolic products, cytokines (e.g. Interleukin 1 (IL-1), tumor necrosis factor (TNF)) and other inflammatory mediators (e.g. Prostaglandin E₂) (2). These irritants will activate the host immune and inflammatory response (3).

Relationship of Bacteria and Apical Periodontitis

The relationship of the presence of bacteria with apical periodontitis has been well documented (4,5,6,7). Miller first demonstrated the presence of different types of bacteria in the necrotic pulp over a century ago in 1890 (4). The role of the microorganism in the etiology of apical periodontitis remained uncertain for many years until Kakehashi *et al.* in 1965 evaluated germ free rats with pulpal exposures and showed that the pulps remained vital and had hard-tissue formation whereas conventional rats with pulp exposures had pulpal necrosis (5). Similar results were

observed in humans in Sundqvist's study in 1976, which further confirmed the role of bacteria in the development of apical periodontitis (6). With an advanced anaerobic culturing technique he observed that bacteria were recovered only in intact teeth that were necrotic after trauma with radiographic evidence of apical periodontitis and not in those that were necrotic and had no evidence of apical periodontitis. He therefore demonstrated that necrotic pulps in the absence of infection could not induce and perpetuate periapical lesions (6). In 1981 Moller *et al.* in an animal study using *Macaca fascicularis* monkeys also provided strong evidence about microbial causation of apical periodontitis. When intact vital pulps were intentionally devitalized under aseptic conditions, he showed absence of pathological changes in periapical region and only the devitalized infected pulps showed apical lesions (7). Collectively there is considerable evidence that bacteria constitute a major etiological factor in the development of apical periodontitis.

Host Immune Response to Apical Periodontitis

As previously discussed, the bacteria and noxious bacterial by-products activate the host immune response with the subsequent inflammatory reaction that will produce the pathologic changes in the periapical tissues. The host immune response includes both innate and adaptive immune responses.

The Innate immunity includes the recognition of structures shared by groups of microbes (pathogen-associated molecular patterns (PAMPs)) by patterns recognition receptors (PRRs) such as Toll like receptors (TLRs) which can be expressed on the cell

surface of immune cells including macrophages, dendritic cells, mast cells, natural killer cells, neutrophils and eosinophils which become activated during inflammation (8). Examples of PAMPs are bacterial lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid (LTA), bacterial DNA, double-stranded RNA, and glucans. As TLRs engage with their respective PAMPs, there can be expression of co-stimulatory molecules and stimulation of secretion of pro-inflammatory cytokines (9). To date, ten human and twelve mouse TLRs have been identified. 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 (10). Engagement of human and mouse Toll-like Receptor 4 (TLR4) by LPS also triggers the biosynthesis of diverse mediators of inflammation, such as TNF- α and IL-1 β , and activates the production of co-stimulatory molecules (10,11).

The expression of these inflammatory cytokines, chemokines and co-stimulatory molecules during the innate immunity response is essential for the activation of the adaptive immune response (12). The adaptive immune response is an antigen-specific response that develops over time that involves B and T lymphocytes and specific receptors in each cell that recognize and binds to foreign or self-antigens. The naive T cells circulate between the lymphatic system and blood circulation until they encounter foreign antigens presented by antigen-presenting cells. There are several T-cell subpopulations, categorized by their functions: (1) T helper cells (T_H), (2) T regulatory cells (T_{reg}), (3) T suppressor cells (T_S), and (4) T cytotoxic (cytolytic) (T_C) cells (**Error! Reference source not found.**). The B cells mainly participate in the production of

antibodies that constitute the host humoral immune response, but are also capable of antigen presentation and other functions attributed to macrophages (8).

Inflammatory Mediators

Several different types of biochemical mediators are released as part of the innate inflammatory response to apical periodontitis. Primarily derived from plasma and cells, the main function of these inflammation mediators is to produce vasodilatation, increase vascular permeability and to recruit inflammatory cells, mainly neutrophilic leukocytes and macrophages, from blood circulation to the site of tissue injury. Some inflammatory mediators can also cause tissue injury (**Error! Reference source not found.**).

Examples of inflammatory mediators that cause vasodilatation are histamine, prostaglandins, nitric oxide, and calcitonin gene-related peptide (CGRP). Bradykinin, leukotrienes, and substance P increase vascular permeability; Chemokines and Tumor necrosis factor (TNF) participate in leukocyte activation and chemotaxis (**Error! Reference source not found.,Error! Reference source not found.**). All these inflammatory mediators have been shown to be present in apical periodontitis (**Error! Reference source not found.**).

One of these inflammation mediators that is of our interest is prostaglandins (PG), they are bioactive lipids that have been implicated in inflammation and in the induction of bone resorption (16). PG has been established to have a role in inflamed dental pulps, and to participate in bone resorption and cyst formation (17).

Prostaglandin E₂ (PGE₂) has been quantified in teeth with exudation and in periapical tissue, being directly and indirectly implicated with most of the inflammatory and destructive changes in apical lesions and increasing vascular permeability and collagen degradation (18).

Bacteria in Primary Root Canal Infection

The primary root canal infection is polymicrobial with approximately equal proportions of Gram-positive and Gram-negative strict anaerobes bacteria, predominantly dominated by Gram-negative species (19,20,21). Black-pigmented anaerobic Gram-negative rods are part of the normal microbiota at various sites of the human body and are often isolated from mixed infections at these sites. They belong to the family *Bacteroidaceae* and are included in the genera *Prevotella* and *Porphyromonas* (21). *Porphyromonas endodontalis* is associated with endodontic infections. It has been isolated from infected dental root canals and periapical abscesses of endodontic origin (21,22,23). *P. endodontalis* is thought to contribute to the destruction of periapical tissues through the release of virulence factors including lipopolysaccharide (LPS) (24,25,26,27). Siqueira et al. showed Black-pigmented species were detected in 80% of abscessed teeth. *Porphyromonas endodontalis* was found in 70% of the pus samples, *Porphyromonas gingivalis* in 40%, and *Porphyromonas intermedia* in 10%. *P. gingivalis* was always found associated with *P. endodontalis* in the abscessed teeth (28).

Gram-negative bacteria have different virulence factors that are toxic to the periapical tissues; as well they contain endotoxin in their cell wall. Endotoxin is composed of complex polysaccharides, lipid A and associated proteins. It has been named lipopolysaccharide (LPS), emphasizing its chemical structure, and is released during multiplication or bacterial death, causing a series of biological effects that lead to an inflammatory reaction and resorption of mineralized tissues. Lipid A is the region of the endotoxin molecule responsible for its toxic effects (29).

LPS and other complex bacterial serine lipids

LPS is one of the most studied microbial initiators of inflammation, including periodontal and endodontic pathogenic processes (**Error! Reference source not found.**). LPS adheres irreversibly to mineralized tissues thereby promoting the synthesis and release of cytokines that activate osteoclasts, such as Interleukin 1 (IL-1) and tumor necrosis factor (TNF) and stimulates the release of prostaglandin E₂ that also influences osteoclasts. (29). As previously described Prostaglandin E₂ is known to promote vasodilation, inflammatory responses and to stimulate osteoclast mediated bone resorption (**Error! Reference source not found.**).

LPS is detected by the Toll-like receptor 4 (TLR4) of the innate immune system present on macrophages and endothelial cells. Gram-negative bacteria are generally recognized by TLR4, however there is controversy regarding recognition of the Gram-negative bacteria because studies have shown that *P. gingivalis* LPS can signal via TLR2, TLR4, or both (30). Concern exists that TLR2 effects attributed to LPS or lipid A

of *P. gingivalis* may actually be accounted for by contaminating TLR2 ligands, such as phosphorylated dihydroceramide lipids (31).

Besides Lipid A that is contained in the LPS of Gram-negative bacteria, other classes of biologically active complex lipids have been shown to be produced by *P. gingivalis*, *P. endodontalis* and other oral *Bacteroidetes* species. These complex lipids called dihydroceramides possess the capacity to stimulate the secretion of inflammatory cytokines, inhibit osteoblast differentiation, and promote osteoclastogenesis (32,33).

Several novel complex lipids produced by *P. gingivalis*, termed phosphorylated dihydroceramides have been identified (34,35). They potentiate interleukin-1b (IL-1b) mediated secretion of inflammatory mediators from fibroblasts, including prostaglandin E₂, and alter gingival fibroblast morphology and adherence (9). Clark *et al.* identified two serine lipid classes from *P. gingivalis* that act as TLR2 ligands (36), the Lipid 654 class and the Lipid 430 class; both promote the release pro-inflammatory cytokines. Lipid 430 may also be a precursor to Lipid 654 and is isolated in far lower abundance in *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. Due to this physical difference, Lipid 430 is soluble in neutral or basic aqueous environments and Lipid 654 is not water soluble unless sonicated (36).

Lipid 654 produced by *P. gingivalis* and other oral *Bacteroidetes* species (*Prevotella intermedia*, *Tannerella forsythia*, *Capnocytophaga ochracea*, *C. gingivalis* and *C. sputigena*) may play a critical role in the development of destructive periodontal disease by promoting bone loss and inhibition of bone formation as it has been

previously reported (35,37,38). *P. endodontalis* is an organism that is phylogenetically related to *P. gingivalis* and is usually present in association with *P. gingivalis* in apical abscesses (24). *P. endodontalis* produces analogous phosphorylated ceramide lipids as *P. gingivalis*. Like *P. gingivalis* lipids, *P. endodontalis* lipids inhibit osteoblast differentiation through engagement of TLR2 (39).

In a recent investigation the lipid fractions of *P. endodontalis* were identified using liquid chromatography-mass spectrometry and it was seen that *P. endodontalis* inherently produced analogous complex serine lipids when compared to the lipids produced by *P. gingivalis*, with Lipid 654 as the most abundant species within this lipid class (40). The structure of *P. endodontalis* Lipid 654 is shown in Figure 1. 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. It was also seen that Lipid 654 of *P. endodontalis* acts as a ligand of human and mice TLR2 (40).

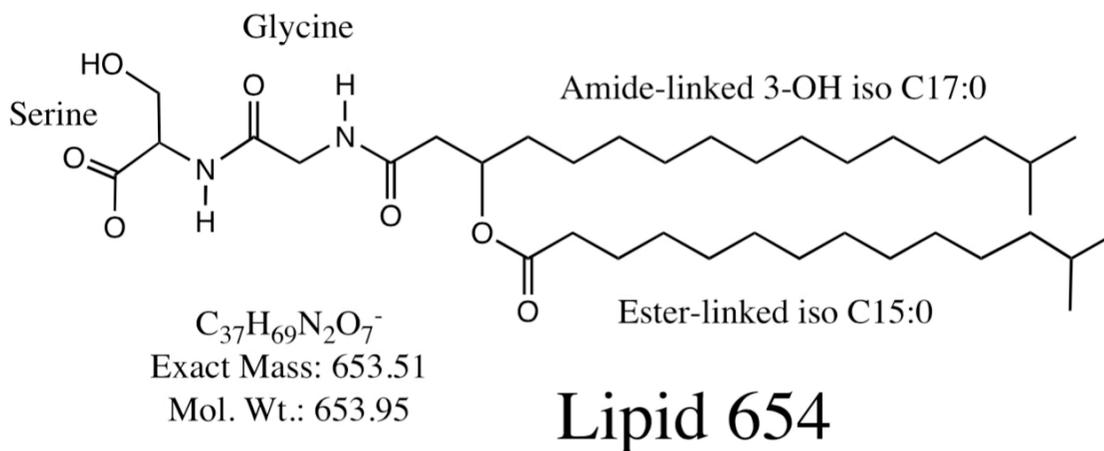


Figure 1. Structure of Lipid 654

Lipid extracts of human impacted third molars have been evaluated using liquid chromatography multiple reaction monitoring mass spectrometry (LC-MRM MS), specifically quantifying the Lipid 654 class. It was found that lipid extracts from impacted teeth contain minimal amounts of Lipid 654. These findings suggest that in human tissues not directly exposed to bacteria, these lipids are not recovered in appreciable levels (40). Recent studies reveal that lipid extracts from diseased human gingival tissue, carotid atheroma and serum, demonstrate Lipid 654 in levels far exceeding the phosphorylated dihydroceramide lipids of *P. gingivalis* (41).

In the same study that identified the lipid fractions of *P. endodontalis* was also found that there was an increase of Lipid 654 in necrotic pulps in comparison to vital pulp controls, however there was not a significant increase in the Lipid 430 versus Lipid 654 in the necrotic pulp inside of the canal. It was concluded the Lipid 430 was not more abundant in teeth with necrotic pulps and likely was not heavily involved in the inflammatory reaction (40). It remained uncertain if the Lipid 430 represents a precursor or breakdown product of the Lipid 654. It was also noticed that the two lipid classes differ in their solubility characteristics. Other studies have evaluated the Lipid 654 and Lipid 430 levels in other chronically inflamed tissues and have observed elevation of both lipid levels (41).

The bacterial lipids stimulate inflammatory mediator secretion, such as prostaglandin E₂, IL-6, and TNF- α , inhibit osteoblast differentiation and function, and induce osteoclast formation (**Error! Reference source not found.**). We postulate that Lipid 654 whether produced by *P. endodontalis* or *P. gingivalis*, has a critical role in the development of periapical bone destruction in teeth with necrotic pulps.

Objectives and Specific Aims

The purpose of this investigation was to isolate lipids from healthy or diseased pulps and examine the serine lipid levels in extracts from pulpal tissue and periapical tissues of extracted teeth with necrotic pulps and apical periodontitis, to determine if Lipid 654 and Lipid 430 are of any importance in pulpal disease and periapical disease and if there is a difference between the levels of the lipids inside the canal versus the apical portion of the root. We also evaluated the levels of prostaglandin E₂ in these samples and if there is a correlation with the levels of the serine dipeptide lipids.

The specific aims are:

- To quantify bacterial Lipid 654 and Lipid 430 levels in lipid extracts from pulpal tissue and the periapical tissue of extracted teeth, and to compare the levels between vital and necrotic tissue samples.
- To quantify levels of PGE₂ in the same samples using liquid chromatography multiple reaction monitoring mass spectrometry (LC-MRM MS)

Materials and Methods

Sample Collection and Processing

Extracted teeth included two basic categories of clinic samples. One group included teeth extracted that were confirmed by electric pulp testing to have necrotic pulpal contents. The teeth in this group also had a periapical radiolucency confirmed by radiograph (disease group) and no periodontal involvement (no probing over 5 mm or to the apex), included molars and anterior teeth. The control group included teeth that were extracted for reasons other than pulpal problems. They included intact bicuspid extracted to allow for orthodontic treatment or fully impacted third molars that were surgically removed. The teeth were collected in compliance with the IRB protocol approved by IRB Institutional Review Board at the University of Connecticut Health.

A total of 12 teeth with necrotic pulps and apical periodontitis and 12 teeth with vital pulps (control) were collected. The extracted teeth samples were immediately stored frozen until processing samples in the laboratory. The apical 2-3 mm of each root was removed using a high speed hand piece and sterile fissure bur and placed in an individual glass tube, the exposed pulp contents of the remaining root were reamed with Hedstrom hand files. Each Hedstrom file was cut at the shank and placed in an individual glass tube. Tooth samples were labeled in pairs to facilitate comparison of both the apical portion and the canal contents for each sample.

Recovery of lipid 654, Lipid 430 and PGE₂

Lipids were extracted from the paired samples using the chloroform/methanol lipid phospholipid extraction procedure described by Bligh and Dyer (42) as modified by Garbus (43). Lipid extracts were fractionated by high-performance liquid chromatography (HPLC) as previously described (35). Replicate HPLC fractionations were pooled and evaluated using electrospray-mass spectrometry (ESI-MS). ESI-MS analysis of lipid fractions was accomplished using a Sciex QTrap 4000 mass spectrometer system. Multiple reaction monitoring was used to quantify Lipid 654, Lipid 430 and PGE₂ using previously optimized ion transitions for each of these products. Although several ion transitions were acquired for each lipid product, only one transition was selected for the data analysis as shown below. This mass spectrometric method quantifies the specific bacterial lipids that are thought to contribute to the development of pulpal disease and bone loss.

The primary negative ion indicating the dominant form of Lipid 654 ion is the *m/z* ion 653.5. Two additional transitions were monitored for Lipid 654 but these were used primarily to confirm the presence of Lipid 654. The MRM transition used for quantifying Lipid 654 was *m/z* 653.5 to *m/z* 381.4. The ion abundances reflecting Lipid 430 and prostaglandin E₂ (PGE₂) levels were additionally examined. Lipid 430 was monitored using the *m/z* 432.3 to *m/z* 382.3 and PGE₂ was monitored by MRM MS as the *m/z*

351.6 to m/z 333.3 transition. We have previously identified this transition as the dominant transition representing authentic PGE₂ in lipid extracts of tissues and tooth samples.

Each lipid sample was injected manually into a Shimadzu (10ADVP) HPLC system interfaced with a QTrap 4000 mass spectrometer (ABSciex). Samples were eluted over a normal phase silica gel column (2.1 mm x 3 cm, 5 μ m, Ascentis, Supelco) using isocratic separation with HPLC solvent (hexane: isopropanol: water, 6:8:0.75, v/v/v). Flow rate of HPLC separation was 0.15 ml/min, the optimal ion transitions, unique to Lipid 654, Lipid 430 and PGE₂ were chosen from previously acquired tandem mass spectra using product ion scan mode.

The Multiple Reaction Monitoring (MRM) collision energy (CE) and declustering energy (DP) were optimized for the specified ion transitions using ramp scanning of the potentials while directly infusing the highly enriched Lipid 654. The optimal CE and DP potentials for Lipid 654 were -52 and -90 volts, respectively. Both entrance and collision cell exit potentials were set to -10 volts. Lipid ion transition peaks were integrated using the Analyst software feature, and the percentage abundance of each lipid class was calculated from the integrated ion transitions.

Statistical Analysis

The recovery of bacterial lipids and prostaglandin E₂ was not normally distributed. Thus the results are depicted using a Whisker box plot format and a Kruskal

Wallace comparison. This was followed by a Mann-Whitney U test to evaluate significant differences between sample categories. A p-value of less than 0.05 was considered significant for statistical comparisons.

Results

Liquid Chromatography-Multiple Reaction Monitoring Mass Spectrometry (LC-MRM MS) revealed the presence of Lipid 654 in all samples. The ion abundances of Lipid 654 were minimal in lipid extracts from teeth with vital pulps, in the canal portion as well as in the periapical samples (see Figure 2). In contrast, the ion abundances of Lipid 654 for the teeth with necrotic pulps and apical periodontitis were significantly higher than the vital teeth. Yet, no statistically significant difference was seen between the canal portion and the periapical samples of teeth with apical periodontitis (see Figure 2).

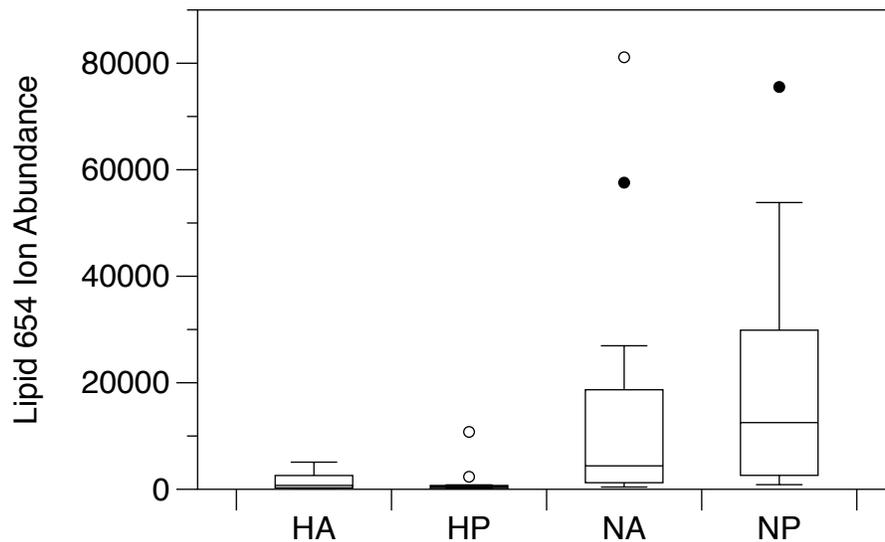


Figure 2: Comparison of Lipid 654 ion levels in lipid extracts from control teeth with vital pulps and teeth with necrotic pulps and apical periodontitis using LC-MRM MS. The dots indicate outliers. Lipid 654 ion was recovered in necrotic pulp lipid extracts in the canal and periapical samples. The lipid samples represent healthy teeth apices (HA), healthy teeth pulp canal tissues (HP), necrotic pulp teeth apices (NA), necrotic pulp canal tissues (NP)

In our study, Lipid 430 ion transitions were not detectable in most of the samples of teeth with vital pulps as well from the teeth with necrotic pulps and apical periodontitis root tip samples.

Additionally prostaglandin E₂ (PGE₂) levels were compared in all samples. Using MRM-MS we found that the PGE₂ levels in teeth with necrotic pulps with apical periodontitis were significantly higher ($p < 0.01$) in the periapical root samples in comparison with the intracanal portion and in comparison to the teeth with vital pulps as well (see Figure 3).

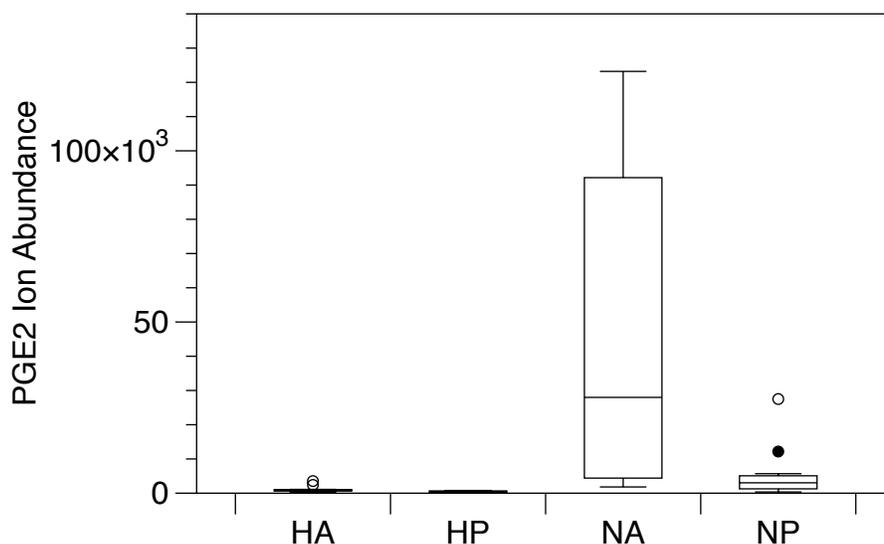


Figure 3: Comparison of prostaglandin (PGE₂) levels from control teeth with vital pulps and teeth with necrotic pulps and apical periodontitis using LC-MRM MS. PGE₂ was recovered in significantly higher levels ($p < 0.01$) in the apical portion of the teeth with apical periodontitis samples in comparison with controls and with the intracanal sample of the teeth with apical periodontitis. Healthy teeth apices (HA), healthy teeth pulp canal tissues (HP), necrotic pulp teeth apices (NA), necrotic pulp canal tissues (NP)

The results in Figure 3 suggest that a shift in the ratio of PGE₂ to Lipid 654 occurs with the necrotic teeth apical tissues. The ratios of PGE₂/Lipid 654 were calculated and summarized using Box Whisker plots as shown in Figure 4.

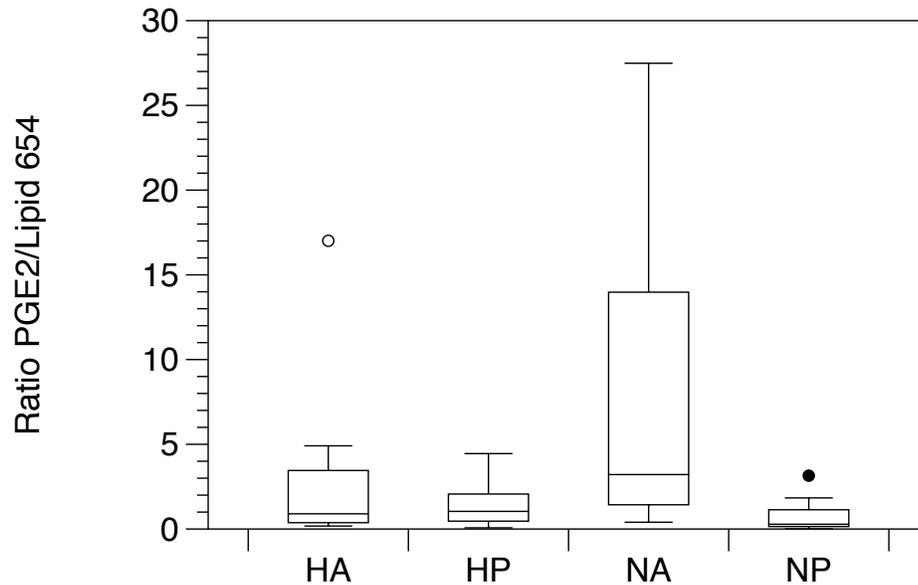


Figure 4. Comparison of PGE₂/Lipid 654 ratios in the teeth samples. The ratios for individual determinations for each type of tooth samples were depicted in Box Whisker plots and analyzed by Mann-Whitney U test. The ratios for necrotic teeth were significantly different ($p=0.0008$) whereas the ratios for healthy teeth were no significantly different ($p=0.9081$). Healthy teeth apices (HA), healthy teeth pulp canal tissues (HP), necrotic pulp teeth apices (NA), necrotic pulp canal tissues (NP)

These results suggest that the PGE₂ levels in and on the apical portions of necrotic teeth increase dramatically over the pulpal tissues from the same teeth, indicating that the necrotic pulp tissues are not capable of generating significant amounts of PGE₂ and therefore the elevated PGE₂ at the root apices is produced from

the tissues at the apex of the tooth. Though it appears that the necrotic pulp tissues contain less PGE₂ than the healthy pulp tissues, this difference is not significant.

Outliers were observed in both the teeth with vital pulps and teeth with necrotic pulps and apical periodontitis in the canal and periapical area of both samples. The recovery of bacterial lipids and prostaglandin E₂ was not normally distributed, thus the results are depicted using a Whisker box plot format and a Kruskal Wallace comparison followed by a Mann-Whitney U test were used to evaluate for significant differences between sample categories.

Discussion

Apical periodontitis is caused by a combination of exogenous and endogenous factors that include bacteria and their toxins and metabolic byproducts, mechanical irritants, chemical agents, foreign bodies and trauma, host's metabolic products, cytokines and other inflammatory mediators (2). The response to these various types of irritants is manifested as an inflammatory reaction regulated by the innate and adaptive immune mechanisms of the host (3).

Black-pigmented anaerobic Gram-negative rods are part of the normal microbiota at various sites of the human body and are often isolated from mixed infections at these sites. *P. gingivalis* and *P. endodontalis* belong to the phylum *Bacteroidaceae* and are included in the genera *Porphyromonas* (21). *Porphyromonas endodontalis* is associated with endodontic infections. It has been isolated from infected dental root canals and periapical abscesses of endodontic origin (21,22,23).

A recent study identified a new serine lipid class of *P. endodontalis*, Lipid 654 and Lipid 430 that act as ligands for human and mouse TLR2. It was also reported that Lipid 654 is present in canals of teeth with necrotic pulps, but no significant detectable levels of Lipid 430 were observed (40).

The present study investigated the levels of Lipid 654 and Lipid 430 as well as the levels of PGE₂ in teeth with apical periodontitis. Two different comparisons were made. Levels of prostaglandin E₂ measured in the canal portion were compared to the

levels measured in the apical portion of the same teeth. These were also compared to control teeth with vital pulps and the disease group with apical periodontitis.

In our results we observed significantly elevated levels of Lipid 654 in the canals of teeth with apical periodontitis in comparison to the control teeth with vital pulps. This was consistent with the findings of a recent study by Blondin *et al.* (40). There were some differences in study design between our study and that of Blondin *et al.* In our study we compare the ion levels of Lipid 654 and Lipid 430 between the canal portion and the periapical portion of the same teeth while Blondin looked at the intra canal portion from the coronal aspect of the root. We found that teeth with apical periodontitis had significantly higher levels of Lipid 654 in comparison with the teeth with vital pulps, but no significant difference between the canal and the periapical samples of necrotic teeth were found.

In other studies Lipid 430 has been recovered at a much lower levels than Lipid 654 in lipid extracts from *P. gingivalis* (36). Additionally, Lipid 430 has been found to be biologically active at lower levels than those of Lipid 654 (32). Previously the Lipid 430 was detected in the canal portion of teeth with necrotic pulps (40), however we hypothesized that the ion levels of Lipid 430 could be different in the apical portion of the samples, as this could be the place where the hydrolysis of the Lipids occurs. Interesting, there was not sufficient ion abundance to detect Lipid 430 in most of the samples. This finding was the same in teeth with vital pulps as well for the teeth with necrotic pulps and apical periodontitis. One reason for this finding could be the fact that in our study the samples were taken from the apical third of the root, which could

represent less tissue and by as a result less lipid was recovered. It remains unclear at this time if Lipid 430 represents a precursor or breakdown product (or both) for the constituent lipid species of Lipid 654.

Additionally we evaluated the levels of prostaglandins E_2 in all samples. Bacterial lipids stimulate inflammatory mediator secretion, such as IL-6, TNF- α and PGE_2 (9). Many investigators have demonstrated the role of prostaglandins in the gingival tissues and their relationship to disease status, inflamed and uninfamed dental pulps, and studied their roles in bone resorption and cyst formation (44,45). PGE_2 has been quantified in teeth with exudation and in periapical tissue, being directly and indirectly implicated with most of the inflammatory and destructive changes in apical lesions and increasing vascular permeability and collagen degradation (46).

Cohen *et al.* has quantified the levels of PGE_2 and $PGF_{2\alpha}$ in uninfamed, chronically inflamed, and acutely inflamed human dental pulp tissues. The values of PGE_2 in their inflamed tissue were 28 times higher than the value in the uninfamed pulp tissue (47). An animal study demonstrated that prostaglandins (PGE_2 and PGI_2) were involved in acute periapical lesions induced in rats, and that the main cellular source of the PG production was macrophages. PGE_2 and PGI_2 also increased osteoclast-mediated bone resorption (48).

Another study investigated the correlation between different clinical signs/symptoms and radiographic features with the levels of different cytokines (IL-1 β , IL-6, IL-10, PGE_2 and TNF- α) in teeth with primary endodontic infection and apical periodontitis. Raw 264.7 macrophages were stimulated with bacterial contents during 24

hours and then measure the cytokines production. They found that primary endodontic contents can potentially stimulate macrophages to produce a wide variety of cytokines. Specifically they found over production of IL-6 and PGE₂ and they positively correlated to each other in pain on percussion (45).

In the present study, we found significantly higher levels of PGE₂ on the apical portions of teeth with apical periodontitis, especially in the periapical samples, with very low levels in the canal samples of the teeth with apical periodontitis and even less in the control healthy samples. These findings are in accordance with other studies evaluating prostaglandins and apical periodontitis.

As previously described, bacterial lipids stimulate the secretion of inflammatory mediators, such as prostaglandin E₂ (9). Our results suggest that the PGE₂ levels in and on the apical portions of necrotic teeth increase dramatically over the pulpal tissues from the same teeth, indicating that the necrotic pulp tissues are not capable of generating significant amounts of PGE₂ which is to be expected since bacteria and bacteria by-products (LPS, serine lipids) will stimulate only live macrophages to release IL-1 β , TNF- α , IL-6, PGE₂ (46). Though it appears that the necrotic pulp tissues contain less PGE₂ than the healthy pulp tissues, this difference is not significant.

In summary, apical periodontitis is a complex disease that has multiple interactions of several factors that ultimately leads to the bone destruction of the periapical tissues. It has been seen that not just LPS from the bacteria will activate TLRs but the serine lipids specifically activate TLR2 as well, which inhibits osteoblast differentiation and these lipids induce monocyte TNF- α secretion. We observed

significant elevation of Lipid 654 levels in teeth with apical periodontitis as well as the increased levels of PGE₂ in the apical portion of these samples. In our investigation, we did not identify the presence or absence of clinical symptoms, or the size of the periapical lesion in the apical periodontitis samples. Further investigations to observe correlation between these variables and the levels of Lipid 654 and PGE₂ could be beneficial. We also found that there was not enough recovery of ion abundance of Lipid 430 in most of the samples to be quantified, further investigation will be required to assess if the lipid species of Lipid 430 represent precursor or breakdown products (or both) for the constituent lipid species of Lipid 654. Of high importance are further studies in therapy to inactivate these lipids and bacteria endotoxins.

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