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Alteration of Biological Properties of Bacterial Lipids by Calcium Hydroxide Treatment

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Alteration of Biological Properties of Bacterial Lipids by Calcium Hydroxide Treatment

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2.Abstract

The importance of the role played by bacteria in the pathogenesis of pulpal and apical disease has been established. One of the characteristics of apical periodontitis is apical bone resorption, which is due to apical immune response to bacterial infection. Recently, novel bacterial complex lipids have been reported to be inflammatory activators. These novel bacterial lipids stimulate prostaglandin E2, IL-6, and TNF-α secretion, inhibit osteoblast differentiation and function, and induce osteoclast formation. Also the bacterial lipids were observed in infected or necrotic root canals. A recently discovered bacterial lipid that contains serine was observed to produce the effects described above but also has been shown to mediated these effects through engagement of Toll-like receptor 2. These new findings imply that bacterial lipids could be important virulent factors that cause apical bone resorption. The purpose of this study was to investigate whether treatment of the serine bacterial lipid with calcium hydroxide alters its biological action as measured by the mouse macrophage cell secretion of TNF-α. Calcium hydroxide-mediated alteration of biological properties of the bacterial lipids may be another important reason for the beneficial effects obtained with calcium hydroxide use in clinical endodontics. In the present investigation, mouse macrophages were stimulated with bacterial lipids with or without calcium hydroxide treatment. The cell culture supernatants were analyzed for TNF-α1 by ELISA. Untreated cells cultured without lipids served as negative controls. Cells cultured with LPS served as positive controls. We found that anti-mouse TLR2 antibody significantly reduced the effect of total lipid. Also, total lipid

significantly promoted the release of TNF-alpha from macrophages. Calcium hydroxide treatment significantly reduced the effect of the serum lipid effect on the release of TNF-alpha. This study demonstrated that serine bacterial lipid of *P. gingivalis* could participate in apical bone resorption associated with pulpal disease. It also demonstrates the effect of calcium hydroxide on this lipid. Based on these findings, the rationale for use of calcium hydroxide in root canal therapy can be expanded to include the hydrolysis of bacterial lipids that are known to promote immune cell activation and bone resorption. These results also provide an additional argument to perform two-visit non-surgical root canal treatment using calcium hydroxide as an intracanal medicament for better disinfection of the necrotic root canals.

3.Review of Literature

3.1.Physiology of the pulp

The dental pulp is an organ enclosed in the lumen of the tooth, surrounded by dentin. Its health represents the vitality of the tooth. The pulpal tissue is mainly composed of connective tissue that contains blood vessels, ground substances, interstitial fluid, odontoblasts, fibroblasts and other components. The pulpal tissue is a unique organ because the soft tissues are enclosed inside hard dentin and enamel leading to a low compliance environment. In addition, the pulp also has an exceptionally responsive sensory system due to its rich supply of sensory fibers [1]. Furthermore, the pulp contains cells involved in producing inflammatory responses to noxious stimuli including but not limited to dendritic antigen presenting cells [2-5]. Inflammatory reactions usually result in vasodilation, edema and increased volume of the inflamed tissue, the limited pulp space leads to an increased tissue pressure [6, 7]. This rise in the tissue pressure, together with the exposure to inflammatory mediators leads to cell injury and finally pulp necrosis [8].

Teeth are subjected to mechanical and thermal stimulation every day. These stimuli may cause trauma to the pulp. Though the pulp has a low compliance environment, it also has an inherent plasticity and is capable of repairing and regenerating areas of tissue damage [9-12]. In addition to the formation of reparative dentin for protection of the pulp from noxious stimuli, the resident stem cells present

in the pulp can facilitate regeneration of damaged pulpal tissue. Dental caries is the second most common infectious disease of mankind, second only to the common cold [13]. It represents the main cause for pulp necrosis. If caries when not identified and eliminated in its earliest stage can cause damage to the pulpal tissue due to invasion of bacteria into the pulp chamber. Invasion of the dentinal tubules is another pathway into the pulp that can be facilitated by trauma, caries, restorative procedures, tooth wear and cracks. [14, 15]. Invasion of bacteria and its products in the dentinal tubules may cause a decrease in dentin permeability, formation of tertiary dentin or immune mediated inflammation [16-19]. The process of pulp necrosis takes place early in part because of detection of foreign antigens by pattern recognition receptors (PRRs) available on the odontoplastic processes. Engagement of the innate immune response will decrease dentin permeability and stimulate the formation of tertiary dentin. Moreover, the detection of foreign antigens activates the resident dendritic cells and the inflammatory pathways (NF-κB) [20, 21]. If dental caries is allowed to progress, the microbial insult to the pulp will persist leading to an increase in the inflammatory infiltrate in the area, vasodilation and edema of the tissues. During this process, the chronic inflammatory response will increase including both cell-mediated and humeral inflammatory infiltrates [22]. The prolonged increase in tissue pressure can lead to irreversible damage leading to pulpal necrosis. Once pulpal necrosis is initiated in the tissue, it can propagate to include the whole pulpal tissue. As the pulp lacks collateral circulation, pulpal necrosis will spread to the apex of the root leading to apical periodontitis [23].

3.2. Pulpal and periapical infections

Pulpal infection has has many interesting features. Miller was a pioneer in creating the bacteriological basis for apical infections [24]. His paper titled "Microorganisms of the human mouth" was the first paper to suggest the presence of bacteria in dental caries and its effect in pulpal and apical destruction [25]. Miller was able to identify several species of bacteria in the root canals in necrotic teeth. Then, Onderdonk 1901 proved that bacteria are the main etiological factor in pulpal pathosis. He was the first to recommend culturing every root canal before obturation [26].

Next came the focal infection theory (1909-1937) in which Rosenow suggested that bacteria from the mouth can spread through the blood stream to distant organs in the human body causing the spread of infection to these organs [27], Hunter in 1911 supported this theory, leading to a consensus that all pulpally infected teeth should be extracted, even if they could otherwise be saved by treatment [28, 29].

Kakehashi in 1965 [30] demonstrated the importance of the presence of infection for the development of the periapical lesions. In his studies, pulp infection caused by exposure to the oral environment in conventional rats resulted in the development of periapical lesions, whereas, in germ free rats with pulps exposed to the oral cavity, no periapical lesions formed. In addition, in germ free rats, dentin bridges were formed, which demonstrated the potential for hard tissue regeneration in the absence of infection. Sundqvist in 1976 [31] also demonstrated the causal relationship between periapical lesions and infection. He used the VPI method to

study the bacteriology of 32 teeth with intact crowns but necrotic pulps due to trauma. Of 32 samples, he was able to isolate bacteria only from those teeth with periapical radiolucency. Based on culture results, the prevalence of anaerobic bacteria in necrotic canals was 90%. Lesions in patients who experienced symptoms such as pain or swelling were found to harbor a greater number of different bacterial strains.

3.3. Pathogenesis of periapical lesions

Nair in 2006 defined apical periodontitis as an inflammatory disorder of the periradicular tissues caused by persistent microbial infection within the root canal system of the affected tooth. The pathogenesis of periapical lesions is described by many authors [32-34]. From a histopathological perspective, the lesions are classified according to the distribution of inflammatory cells within the lesion, whether epithelial cells are present in the lesion, if the lesion was transferred into a cyst and the relationship between cystic cavity and the root canal apex [32].

Normal pulp usually contains a few inflammatory cells [2]. When microorganisms infect the apical tissues through the apical portion of the root canal, acute apical periodontitis is initiated. It is initiated by the neuro-vascular response of inflammation, which causes hyperemia, vascular congestion and periodontal ligament edema, and leads to extravasation of neutrophils [33]. Upon the multiplication and death of Gram-negative bacteria at the apex, organisms can release lipopolysaccharides (LPS) [35]. LPS in turn, attracts polymorphonuclear leukocytes (PMNLs) to the site of infection –the newly initiated apical periodontitis- by

chemotaxis [2]. At this stage, no changes can be observed radiographically as the integrity of the periodontium has not been affected yet [33]. When PMNLs are attracted to the site of injury, they phagocytose the microorganisms and release both leukotrienes, prostaglandins, hydrolytic enzymes and reactive oxygen metabolites. Leukotrienes attract more neutrophils and macrophages while macrophages potentiate the activation of osteoclasts. Bone resorption will take place in few days. And the periapical radiolucency will be noticeable in the radiographs at this point [36]. LPS also activates macrophages to produce chemical mediators such as pro-inflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and chemotactic interleukin-8 (IL-8) that can contribute to the pathogenesis of apical periodontitis. The local effects of IL-1 are boosting the adhesion of leukocytes to the endothelial walls, stimulation of lymphocytes, potentiation of neutrophil activation, increased production of prostaglandins and proteolytic enzymes, enhancement of bone resorption and inhibition of bone formation [37]. IL-6 is synthesized by both lymphoid and non-lymphoid cells and its synthesis is enhanced by IL-1, TNF-α and interferon-γ (IFN-γ) [38]. IL-6 participates in the regulation of the production and control of some IL-1 effects. The presence of TNF-α in human apical periodontitis lesions and root canal exudates of the teeth with apical periodontitis is reported in several studies [39-41]. IL-8 is a member of the chemotactic cytokine family [42]. It is produced by macrophages and fibroblasts under the influence of IL-1β and TNF-α. It plays an important role in the acute phase of apical periodontitis during massive infiltration of neutrophils. The outcomes of

acute primary apical periodontitis could be spontaneous healing –if no infection is involved-, expansion of the lesion into bone potentially causing alveolar abscess formation or drainage to the oral cavity through sinus tract or transforming to chronic apical periodontitis.

If the microbial irritation to the periapical tissues persists for extended periods, the periapical lesion that is dominated by PMNLs will be occupied by a larger number of macrophages, lymphocytes and plasma cells. These cells will be encapsulated by collagenous connective tissue. In this stage, the pro-inflammatory cytokines derived from macrophages (IL-1, IL-6 and TNFα) act as lymphocyte stimulators [33]. T-cells also play a dominant role in down-regulating the lesion in this stage. The population of T-cells exceeds B-cells in chronic lesions [43-46], which may indicate specific antibacterial reactivity. Two types of T-lymphocytes are present in equal amounts are found in this stage. These are T-suppressor cells (Ts) and T-helper cells (Th) [47-49]. Studies have shown that Th cells aid in the development of the periapical lesion while Ts cells play a role in the cessation of the growth of the lesion [34, 48]. Th cells play an important role in promoting bone resorption as they can stimulate B-cells to produce antibodies. When Th cells are activated, they elaborate the cytokine lymphotoxin [50]. Th cells are also capable of producing macrophage migration-inhibitory factor (MIF) and gamma interferon [51], they cause the activation of macrophages. Macrophages also elaborate the bone resorptive monokines IL-1, TNFα and PGE_2[34]. Macrophage derived TNFα [52] and T-lymphocyte derived TNF-β [53] both possess numerous systemic and local effects

similar to IL-1. Chronic lesions can remain latent and asymptomatic. During this time, radiographic examination will not show any changes in the size of the periapical radiolucent area. However, disturbance to this equilibrium by any factor can cause microorganisms to proceed into the periapical area leading to a flare-up and recurrence of symptoms. Hence, the microorganisms now can be found extra-radicularly. Rapid enlargement of the periapicial area in the radiographs during this stage can also be seen. The term periapical granuloma is used to designate chronic apical periodontitis. Periapical granulomas usually enlarge in an irregular manner due to the proliferation of the infiltrate cells in all directions. Upon extracting a tooth with periapical granuloma, the lesion can be removed in toto during the extraction. This is due to the attachment of the fibrous connective tissue capsule, that is formed of collagenous fibers, to the root surface [33].

If a periapical granuloma remains untreated, periapical cysts may form as a sequel to chronic apical periodontitis. Although all cysts develop from chronic granulomas, not all chronic granulomas are transformed to cysts. Two types of cysts can be present: a true periapical cyst in which the cystic cavity is completely enclosed in epithelial lining, and the periapical pocket cyst in which the cystic cavity is connected to the root canal. The latter was also described as a Bay cyst [54, 55].

3.3.1. Periapical true cyst

True cyst genesis takes develops in three phases [56]. The first phase is represented by the proliferation of the epithelial cell rests. The second phase involves

the formation of an epithelium-lined cavity (the cystic cavity). Two theories have been proposed to explain development of the cystic cavity: (a) The nutritional deficiency theory assumes that the cystic cavity is formed due to necrosis and degeneration of the central cells of the epithelial strands from limited sources of nutrition. The products of cell degeneration in the necrotic area will attract neutrophilic granulocytes. Different micro-activities take place in the necrotic area such as epithelial cell degeneration; leukocytic infiltration and tissue exudates combine to form the cystic cavity lined with stratified squamous epithelium. The second theory for cystic cavity formation is the abscess theory. This theory hypothesizes that epithelial cells enclose the exposed connective tissue surfaces and the epithelium proliferates to surround the abscess formed by cell lysis. The third phase of true cyst genesis is represented by growth of the cystic cavity; however, the mechanism of growth is still not completely defined. Various theories relate the growth of cystic cavity to osmotic pressure [57, 58]. However, the fact that the lumen of pocket cyst can also enlarge when it is open to the root canal caused the rejection of the osmotic pressure theory [32, 54]. After the formation of a cystic lumen, degenerated neutrophils release prostaglandins [59], which in turn, diffuse into the surrounding tissues through the porous epithelial wall [56]. The area surrounding the epithelium contains numerous T-lymphocytes [60] and macrophages that produce inflammatory cytokines. Prostaglandins and inflammatory cytokines cause the activation of osteoclasts and lead to bone resorption [33].

3.3.2. Periapical pocket cyst

The bacteria present in the apical portion of the root canal are assumed to attract neutrophils to the apical foramen [32, 54]. Here, a mirco-abscess forms and is in contact with the root apex forming an epithelial collar with the epithelial attachment [61]. More neutrophils are attracted to the area by chemotaxis due to the existence of microbes and their products in the root canal apex. But these attracted neutrophils end up in the cystic lumen, which acts as a death trap for the transmigrating neutrophils. The sac-like cystic cavity enlarges due to accumulation of the necrotic cells and it extends into the periapical area [32, 54]. Enlargement of the pocket cyst and associated bone resorption mechanisms are believed to develop similar to the periapical true cyst [33].

3.4.The role of anaerobes in periapical infections

The oral cavity represents a pool of large number of microorganisms. Although microorganisms could be bacteria, viruses or fungi, in the oral cavity bacteria predominate over other microorganisms. [62]. Likewise, necrotic root canals provide an ideal environment for the habitat of oral bacteria. This is facilitated by the lack of a host defense since a functional circulation in the canal is lost but may also be influenced due to the presence of the porous dentinal tubules in the walls of the root canal. The dentinal tubules allow the passage of microorganisms into the dental pulp and their colonization [63, 64]. In spite of the presence of hundreds of species of

bacteria in the oral cavity, a smaller number of these species could be found in the root canals (about 20-30 species only) [65, 66]. The factors affecting the habitat of bacteria in the root canals are the temperature, pH, bacterial receptors, type of available nutrients, and oxygen tension [67].

Facultative bacteria are the usual initial colonizers of the root canals. In time, oxygen supply decreases as well as the blood supply. Hence, an anaerobic environment evolves. Therefore, anaerobic bacteria eventually predominate. Anaerobes survive in harsh environments because they are able to produce their nutrients from the necrotic pulpal tissue, inflammatory exudates, salivary components or the metabolic products of bacteria [68, 69].

As we know, anaerobic bacteria colonize the root canal in the form of "biofilms". Biofilms represent clusters of multiple species of bacteria that "co-aggregate" to form a complex integrated microbial community. Ricucci in 2010 found biofilm arrangements in 77% of the inraradicular root canals studied [70]. The presence of bacterial species in biofilms increases their growth and resistance to antimicrobials. It leads to a broader range of growth, more metabolic diversity, less competition with other microorganisms, genetic exchange, enhanced pathogenicity and symbiotic nutrition [71]. Infected dentinal tubules are present in 70-80% of all apical periodontitis lesions [72, 73].

The relation between anaerobic microorganisms and inflammation and pain attracted considerable attention of scientists. Anaerobes in bacterial biofilms in the

root canals may cause neutrophil chemotaxis inhibition, therefore, inhibit phagocytosis. They may also be responsible for the increased resistance to antibiotics [74, 75] and the production of enzymes and endotoxins [76, 77], which may lead to persisting painful periapical lesions [78, 79].

Anaerobic infections cause necrosis of the pulpal tissue and abscess formation. The clinical manifestations to the anaerobic infections are swelling, pain and fever, associated with purulent discharge of foul odor related to the bacterial metabolites such as ammonia, indoles, urea and amino acids, all of which will stimulate the pulp neurons [80]. The significant role of anaerobes in the pathogenesis of periapical infections has been confirmed experimentally. In 1981, Moller found several bacterial species on pulpal tissues of monkey teeth mechanically exposed for 7 days. These teeth were sealed afterwards and examined after 6 months. The bacterial species found were α-hemolytic streptococci, enterococci, coliforms and anaerobic bacteria such as *Bacteroides* and eubacteria [81]. Radiographic periapical lesions were found in 90% of the teeth. In 1982, Fabricius demonstrated that the ratio between anaerobes/aerobes increases with time after sealing the exposed pulp tissue. As the ratio was 3.9 at 90 days, it has increased to 11.3 at 1,060 days [82]. In 1986, a study by Tronstad used six patients with asymptomatic lesions. Tronstad was able to gain access to the periapical lesion surgically, and revealed the presence of aerobes and anaerobes. *Porphyromonas gingivalis* as well as *P. endodontalis* were isolated [83].

Most prevalent bacterial species found in the root canals belong to the following phyla: *Firmicutes* (*Enterococcus, Eubacterium, Lactobacillius*, etc), *Bacteroidetes* (*Porphyromonas, Pervotella, Tannerella*, etc), *Actinobacteria* (*propionibacterium*, etc), *Fusobacteria* (*Fusobacterium*), *Spirochaetes* (*Terponema*, etc) [31, 66, 82, 84].

3.5. *Porphyromonas gingivalis* and its virulence factors

Black pigmented Gram-negative anaerobic bacteria are frequently found in the infected root canals and endodontic abscesses, such as *Porphyromonas gingivalis*, *P. endodontalis, Prevotella intermedia* and *Prevotella nigrescenes* [78, 85]. Traditional culture and molecular genomic methods were used to confirm the presence of those species in the root canals or abscesses [86] [87]. *P. gingivalis* and *P. endodontalis* in root canals directly correlated with acute symptoms of endodontic infections [65, 88]. In addition, *P. gingivalis* and *Fusobacterium nucleatum* were found to be co-colonizers in biofilms of apical lesions [89]. Recent studies found a synergistic relationship between *F. nucleatum* and *P. gingivalis*. As the biofilm formed by *F. nucleatum* is enhanced by *P. gingivalis*, the attachment of *P. gingivalis* to the host cells is enhanced by *F. nucleatum* [90, 91].

The species of the genus *Porphyromonas* usually colonize human and/or animal tissues. These species are associated with the oral cavities of humans, dogs, cats and non-human primates. Members of the genus *Porphyromonas* can also be

found in warm-blooded animals. The members of genus *Porphyromonas* are 0.5-0.8 by 1.0-3.5 μm in diameter. They are obligate anaerobes, non-spore forming, asaccharolytic non-motile rods. They are characterized by the production of a large number of enzymes, proteins and end products of their metabolism. All of which are active against a broad spectrum of host proteins. They help in the evasion of host defenses by hydrolyzing specific host defense compounds include proteinase inhibitors, immunoglobulin, iron-containing proteins, bactericidal proteins, extracellular matrix proteins and proteins involved in phagocytic functions [92].

P. gingivalis produces multiple virulence factors that are capable of promoting bone and tissue destruction [93-95]. *P. gingivalis* may also be critical to the transition from gingivitis to periodontitis. *P. gingivalis* also interacts with other host microbiota to produce destructive compounds leading to the progression of the disease [96].

Virulence factors are defined as "molecules that result in the establishment and maintenance of a species associated with or within the confines of a host." [96]. For the bacteria to be pathogenic, colonization of the host is the first step which can be achieved by adherence. Bacterial invasion of the host should transgress the host's external protective tissue barriers and overcome the opposing movement of tissue fluids by the host. Finally, the bacteria must find a suitable habitat for its survival. Virulence factors may be essential for the process of colonization. They could be fimbriae, lipopolysaccharides, exopolysaccharides, outer membrane proteins and

outer membrane vesicles. As bacterial species colonize the host tissues in the form of biofilms, they produce other factors in the form of antibacterial molecules. These molecules help to protect specific bacteria from competing organisms found within the same biofilm [96].

3.5.1. The Bacterial Capsule

Many investigators found the capsule of *P. gingivalis* to be a very important virulence factor. Its main role is antiphagocytic. Several *P. gingivalis* strains were studied by electron microscopy using Ruthenium red staining and routine lead acetate staining. This approach showed the presence of an electron dense layer covering the outer membrane. This layer is the polysaccharide capsule [97-101]. Encapsulation of *P. gingivalis* is strongly linked to their ability to exhibit decreased autoagglutination and become more hydrophilic if compared with the non-capsulated strains [102, 103]. The *P. gingivalis* capsule also increases the resistance to phagocytosis, serum resistance and decreased induction of polymorphonuclear leukocyte chemiluminescence [102-106].

3.5.2. Outer membrane proteins

Gram negative bacteria possess a complex double layered cell wall that is due to its multilayered cell envelope. The cell envelope is composed of an inner cytoplasmic membrane and a thin peptidoglycan layer in the periplasmic space, which is surrounded by an asymmetrical outer membrane. The components of the outer membrane are: the complex lipopolysaccharide, lipoproteins and peripheral and

transport proteins. Transport proteins are the components that connect the outer membrane to the peptidoglycans giving the cell envelope its structural integrity. The outer cell membrane of Gram negative bacteria has numerous short fimbriae or if motile, long thick flagella. Furthermore, the outer cell membrane contains lipopolysaccharides and hemagglutinins. The outer cell membrane contains protein complexes. *P. gingivalis* has an outer membrane that contains at least 20 major proteins ranging in size from 20 to 90 kDa [96, 107].

Mihara and Holt [108-110] found that a 24-kDa protein of *P. gingivalis* that stimulates thymidine incorporation in human gingival fibroblasts. The purified 24-kDa protein was named ”fibroblast activating factor” due to its ability to stimulate fibroblasts. The same protein was found to function as a cell proliferation factor for eukaryotic cells and promotes bone resorption. Watanabe et al. found that the same protein promotes polyclonal C cell activation. He also found that the purified 24-kDa protein can promote interleukin-1 secretion from mouse peritoneal macrophages [111].

3.5.3. Bacterial fimbriae

Most Gram negative bacteria have numerous, thin hair like structures on their surface. These structures were first named pili and were believed to be important in red blood cell agglutination. Today, these pili are referred to as fimbriae due to their thin hair like structure. Fimbriae are composed of two classes: either those involved in the adhesion to other bacteria, cells and soft and hard tissues (type specific

fimbriae) or are involved in bacterial conjugation (sex pili). The later type is characterized by being longer and more flexible compared with the type specific fimbriae. The fimbriae usually range in size from 3-25nm in diameter and 3-25μm in length. Their distribution may vary as some bacterial species were found to have 10 fimbriae per cell, other species possess as many as 1000. Except for one or two strains, all *P. gingivalis* strains are found to express fimbriae. They are arranged in a peritrichous fashion on the cell surface [97]. The fimbriae of *P. gingivalis* promote binding to the host tissues and saliva coated hydroxyapatite [96].

3.5.4. Lipopolysaccharide (LPS)

Lipopolysaccharide is "a major virulence factor" as it exerts a plethora of biological effects which result in the amplification of inflammatory reactions" [79]. From an immunological antibody response, LPS is a non-specific weak antigen and antibodies produced against LPS are typically poor in neutralized its effects. As we mentioned before, LPS is located in the outer membrane of the bacterial cell wall. LPS is composed of O-specific polysaccharide, the common core and a lipid component referred to as Lipid-A [112]. The most important biological activitiy of the bacterial LPS is attributed to Lipid-A [113]. Any slight modification to lipid-A leads to a change in the biological effects of LPS such as toxicity, pyrogenecity, macrophage activation and complement activation [113, 114]. Lipid-A is composed of di-glucosamine, phosphate and fatty acids [115]. Lipid-A structure treatment with weak base leads to the release of hydroxy fatty acids from Lipid-A [114, 116]. LPS

are large molecules found to have variable molecular weights. The smallest LPS reported was found to be larger than 1000 Daltons [117, 118].

Generally, LPS administration causes mast cell degranulation and release of histamine and heparin [119]. In addition, LPS causes the release of collagenase from host cells [120]. Furthermore, LPS causes the release of tumor necrosis factor from macrophages [121]. LPS is capable of inducing fever even if administered in small amounts. Fever is due to the release of interleukin-1 from macrophages. IL-1 in turn acts on the hypothalamus, the thermoregulator of the body [122]. LPS promotes the adherence of endothelial cells and instigates the release of oxygen radicals, which enhances neutrophil mediated injury [123]. In addition, LPS is a non-specific stimulator of B-lymphocytes [124, 125].

In chronic periapical lesions associated with microorganism contamination, small amounts of LPS can induce a periapical inflammatory response [76, 82]. The presence of LPS in necrotic pulps was confirmed experimentally. Both studies of Schein and Schilder [126] and Dahlén and Bergenholtz [77] used the limulus lysate test to measure LPS. Schein and Schilder found higher levels of LPS in pulpless root canals associated with radiographic periapical lesions, more than vital pulp teeth that had no radiographic periapical lesions. Dahlén and Bergenholtz revealed a direct relationship between the limulus titer and the quantity of Gram negative bacteria isolated from infected pulps [79].

LPS, being a causative factor in inducing perapical inflammatory lesions and causing more destruction, is a topic explored in several studies. Dwyer and Torabinejad [127] were able to initiate the formation of large apical radiolucent areas in cat canines when LPS was inoculated into the pulp chamber. However, no periapical radiolucencies were found in the teeth pulp chambers inoculated with control vehicle (saline of detoxified LPS). Neutrophils dominated in the inflammatory exudate. Fewer numbers of lymphocytes and macrophages not typically observed. Osteoclasts were observed in areas of bone resorption. Dahlén et al. [128] observed periapical bone destruction in 6 of 27 teeth when LPS or oral Fusobacteria was sealed for three months in the root canals of monkey molars and premolars. Upon histological examination, chronic periapical inflammation together with bone resorption was found. Also, there was evidence of elevated enzyme activity by bone cells. Warfvinge et al. tested the time intervals required for LPS to produce inflammatory lesions in monkeys [129]. Class V cavities were prepared with a dentin barrier of less than 1 mm thickness covering the pulp. A paste of lyophilized LPS from oral organisms was mixed with saline and placed on the floor of the prepared class V cavities and sealed. The teeth were histologically examined after 8 and 72 hours. Bovine serum albumin was used as a control. While the control samples resulted in mild neutrophilic infiltration, the experimental groups showed increased leukocytic infiltration from 8 to 72 hours.

LPS may have a dominant role in the pathogenesis of peripical bone resorption. LPS stimulates the secretion of bone resorbing mediators such as

prostaglandin-E2 through Lipid-A moiety [113, 130]. LPS is known to be a stable macromolecule that can be shed from the cell wall of some bacteria during their life cycles as a result of cell lysis [131]. Hence, LPS is capable of persisting in the root canals even after the death of bacteria. Although they (what are you referring to? The bacteria or the LPS persist in small concentrations, they are capable of producing inflammatory reactions [113, 132]. LPS is also capable of stimulating osteoclast activity and may stimulate other bone cells to secrete pro-inflammatory cytokines such as TNF-α and IL-6. These cytokines can also promote bone resorption activity [54].

3.6. Novel Lipids of *P. gingivalis*

Recent studies found that *P. gingivalis* synthesizes novel complex lipids termed phosphorylated dihydroceramide lipids [133, 134]. These phosphorylated dihydroceramide lipids include three classes: free non-phosphorylated dihydroceramides (DHC), phosphoethanolamine dihydroceramides (PE DHC), and phosphoglycerol dihydroceramides (GH DHC). These lipids were found to stimulate IL-1β mediated secretion of inflammatory mediators from fibroblasts, such as PGE_2. They also affect the morphology and adherence of gingival fibroblasts [133, 135]. *P. gingivalis* lipids inhibit osteoblast differentiation by engagement of TLR2 [136]. They induce the secretion of monocyte TNF-α. The bacterial lipids have several effects such as secretion of inflammatory mediators including PGE_2, IL-6 and TNF-α.

They also inhibit differentiation of osteoblasts and interfere with their capacity to form mineral deposits. These bacterial lipids also stimulate osteoclastogenesis. They were identified from infected root canals which implies that they could be a causative factor for apical periodontitis [136].

More recent studies [135, 137] attributed TLR2 activation to phosphoethanolamine dihydroceramides. These studies found that the total lipid extract of *P. gingivalis* stimulates mouse dendritic cell activation and suppresses bone deposition, which is mediated by osteoblasts via TLR2. A very recent study with unpublished data by Clark et al. [138] was able to clarify using advanced HPLC separations, that phosphoethanolamine dihydroceramide lipids are not capable of activating TLR2 from human cells. Therefore, this study concluded that phosophoethanolamine dihydroceramide lipids do not engage TLR2 in mouse dendritic cells and the results reported in previous studies could be related to the presence of another lipid contamination.

In the same study, Clark et al. [138] identified two new serine lipid classes of *P. gingivalis*, Lipid 654 and Lipid 430 classes. These lipids act as ligands for human and mouse TLR2. As Lipid 654 contains an ester linked fatty acid whereas Lipid 430 which is related structurally to Lipid 654 contains only one fatty acid that is amide linked to a glycine-serine head group. In addition, Lipid 430 is soluble in aqueous solvents where the pH is basic or neutral. However, Lipid 654 class is insoluble, except if it is sonicated to form liposome preparations. Based on this study, a new

hypothesis can be posited, that is Lipid 654 and Lipid 430 classes of *P. gingivalis* play a dominant role in the pathogenesis of periapical lesions in which bone destruction is enhanced and bone formation is inhibited.

3.7. Calcium Hydroxide intracanal medication

In root canal treatment, total bacterial elimination by instrumentation is almost impossible [84, 139]. Therefore, intracanal medication with calcium hydroxide was proposed. Hermann first introduced calcium hydroxide as an intracanal medicament in 1920. Nowadays, it is the most widely used intracanal medication in the world. Calcium hydroxide has multiple beneficial effects in the field of endodontics. The main reason for its use is its antibacterial property [116, 132]. In addition, calcium hydroxide is capable of dissolving tissues [140]. Furthermore, its ability to prevent root resorption and promote hard tissue formation has encouraged endodontists to use it routinely [141, 142]. The antibacterial activity of calcium hydroxide is attributed to its high pH resulting from the release of a hydroxyl ions [143]. Calcium hydroxide when dissolved in aqueous solvent causes an alkaline pH (about 12.5) [144]. Bacteria are usually not capable of surviving such a high pH. Some bacterial strains can survive in pH levels ranging from 6 to 9 such as *Escherichia coli*, *Proteus vulgaris, Enterobacter aerogenes* and *Pseudomonas aeroginosa*. Other bacterial species can tolerate higher pH levels from 9 to11, such as enterococci [145]. On the other hand,

Prevotella intermedia, Fusobacterium nucleatum and *Porphyromonas gingivalis* can survive in PH ranging from 8 to 8.3 [143].

Apparently, the high pH level of calcium hydroxide causes denaturation of bacterial proteins [143]. The ionic bonds maintaining the tertiary structure of the proteins break by alkalinization, leading to changes that cause the loss of biological activity of these enzymes. On the other hand, the hydroxyl ions released from calcium hydroxide are capable of causing damage to the bacterial DNA by splitting of the strands causing the destruction of genes [146]. Replication of DNA will be prevented accordingly. One of the main strengths of calcium hydroxide is its ability to cause damage to the bacterial cytoplasmic membrane by inducing LPS degradation. The hydroxyl group reacts with the ester-linked hydroxyl fatty acid, leading to an alteration of Lipid-A structure. This alteration in Lipid-A renders the LPS inactive [114, 132].

As calcium hydroxide has all of these harmful effects to bacteria, it could be thought as being harmful to human tissues as well. However, calcium hydroxide has limited solubility and diffusibility, which confines its effects to the root canal environment where it is applied, preventing its spread to the periapical tissues –if it is not physically extruded beyond the apical foramen [143].

Calcium hydroxide is a slow acting medication; it should be applied for several days to gain optimal benefit from its usage. Sjögren et al. compared the application of calcium hydroxide for 10 minuts versus one week. He found that 10

minutes was not sufficient to eliminate bacteria, while placement of calcium hydroxide into the root canal for one week resulted in sufficient bacterial elimination [147]. However, the exact time needed for calcium hydroxide to gain the best results is still not known.

4. Hypothesis

Calcium hydroxide alters the biological activities of bacterial lipids.

5. Aim

The purpose of this study was to investigate whether treatment of bacterial lipid with calcium hydroxide alters its biological action as measured by the mouse macrophage cell secretion of TNF-α1.

6. Study Design

In this bench top study, mouse macrophages were stimulated with bacterial lipids or calcium hydroxide-treated bacterial lipids. The cell culture supernatants were analyzed for TNF-α1by ELISA. Cells cultured without tested material served as negative controls. Cells cultured with LPS served as positive controls.

6. Materials and Methods

Bacterial growth, lipid extraction, fractionation and characterization done as described in a pervious study. [138]The mouse macrophage cell line RAW 264.7 was obtained from American TypeCulture Collection (ATCC, Manassas, Va). Cells were grown in Eagle's minimum essential medium (ATCC) supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc, Logan Utah) and 1% antibiotic/antimycotic cocktail (300 units/mL penicillin, 300 μg/mL streptomycin, 5 μg/mL amphotericin B [Gibco BRL, Gaithersburg, Md]) under standard cell culture conditions (37°C, 100% humidity, 95% air, and 5% CO2).

Bacterial lipids were purified from *P. gingivalis* (ATCC 33277, type strain). For calcium hydroxide treatment, calcium hydroxide was placed into 50 μg purified lipids for 7 days in the incubator. After incubation the lipids were extracted and purified again for cell treatment.

For cytokine assay, mouse macrophages were seeded into the wells (10^5 cells/1 mL medium per well) of 24-well flat-bottom plates (Becton Dickinson, Oxnard, Calif).The plates were incubated for 24 hours. Then 5 μg of bacterial lipids or calcium hydroxide-treated bacterial lipids were added into each well. After 24-hour incubation, culture media were collected and analyzed for TNF-α1 by ELISA (R&D Systems, Inc, Minneapolis, Minn). Cells cultured without tested material served as negative controls. Cells cultured with LPS served as positive controls. LPS has been shown to stimulate mouse macrophages to produce TNF-α1. Therefore, cells treated

with 1 μg/mL LPS (Sigma, Saint Louis, Mo) were used as positive controls to make sure that the macrophages could be activated and that the ELISA detection method worked properly.

The TNF-α1 difference between treatment and control groups was analyzed using one-way analysis of variance (ANOVA). A p value less than 0.05 is considered significant. Post hoc tests were done with Scheffe's test. The experiments were repeated with two replicates.

7. Results

7.1. The effect of Anti-TLR2 antibody on the stimulation of TNF-alpha by total lipid and lipid 654 from macrophages

Compared to the control group, total lipid significantly stimulates the production of TNF-alpha ($P < 0.01$) (Fig. 1). Anti-TLR2 antibody significantly reduced the effect of total lipid ($P < 0.01$) (Fig. 1). Lipid 654 shows the same effect as total lipid to stimulate the production of TNF-alpha ($P < 0.01$) (Fig. 1). Anti-TLR2 antibody also significantly reduced the effect of lipid 654 ($P < 0.01$) (Fig. 1).

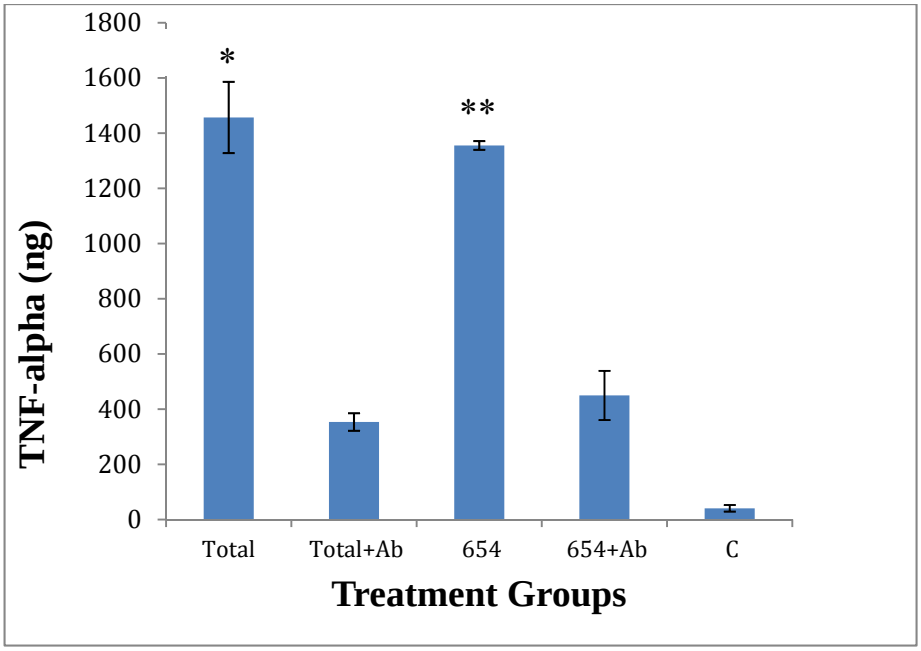

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Figure. 1. TNF-alpha (ng/ml) production by monocytes when exposed to total lipid or lipid 654 extracted from *P. gingivalis*. Anti-TLR2 antibody significantly reduced the effect of total lipid and lipid 654 for the production of TNF-alpha. C, Control. *p < 0.01 versus Control and Total+Ab groups. **p < 0.01 versus control and 654+Ab groups.

7.2. The effect of calcium hydroxide treatment on total lipid and lipid 654 for their stimulation of TNF-alpha production from macrophages

Total lipid and lipid 654 significantly promote the release of TNF-alpha from macrophages ($P < 0.01$) (Fig. 2). Calcium hydroxide treatment significantly reduced the effect of total lipid on the release of TNF-alpha ($p < 0.01$) (Fig. 2). The same result was observed when cells were exposed to calcium hydroxide treated lipid 654 ($p < 0.01$) (Fig. 2).

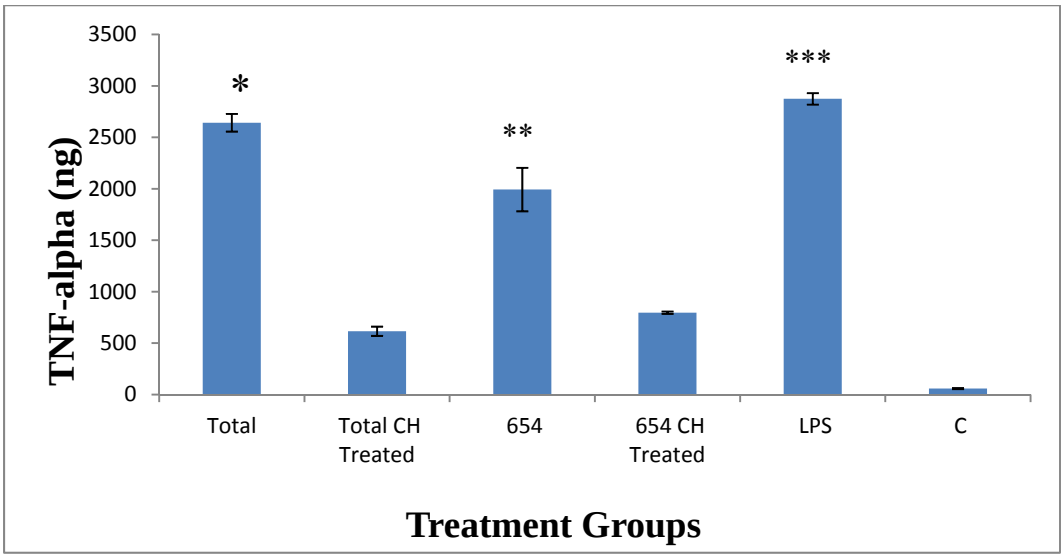

Figure. 2. TNF-alpha (ng/ml) production by monocytes when exposed to total lipid, lipid 654, calcium hydroxide treated total lipid and lipid 654. LPS treated cells served as a positive control. Cells without treatment served as a negative control. *$p < 0.01$ versus Control and Total CH Treated groups. **$p < 0.01$ versus control and 654 CH Treated groups. ***$p < 0.01$ versus control group.

8. Discussion

Our study demonstrated that phosphorylated dihydroceramide lipids of *P. gingivalis* stimulate the release of TNF-α from mouse macrophages via TLR-2.

Toll-like receptors (TLRs) are "a group of pathogen-associated pattern recognition receptors, which have been identified as a key participants in the innate recognition of pathogens" [148]. They are considered as trans-membrane receptors. TLRs are usually located on the surface of immune cells. Therefore, activation of immune cells results in promotion of inflammatory reactions that are stimulated by bacterial by-products [149-151]. Bacterial lipoproteins cabable of engaging TLR2 include lipoproteins, peptidoglycans and *Staphylococcus aureas* lipoteichoic acid [152-154]. On the other hand, TLR4 is the target receptor for LPS [155], which is believed to be one of the causative factors of periapical bone resorption. A study by Kikuchi et al [151] demonstrated the ability of LPS to induce osteoblast formation by stimulating RANKL release from osteoblasts. Both TLR2 and TLR4 are expressed from monocytes and macrophages [156].

Engagement of TLR receptors causes the release of proinflammatory cytokines from cells such as IL-1β, IL-6 and TNF-α [153, 157, 158]. Previous histological studies were able to link periapical lesions in patients with severe periodontal disease with the expression of both TLR2 and TLR4 [159]. Recent studies were able to demonstrate that TLR2 is as essential receptor for initiation of osteoclastogenesis for *P. gingivalis*. On the other hand, TLR4 is not necessary for the

induction of the same process [148, 160]. They found that TLR2 deficient mice were incapable of the production of periapical lesions after their inoculation with *P. gingivalis.*

Pathological bone loss resulting from bacterial infections usually takes place through the engagement of receptors for pathogen-associated molecular pattern (PAMP) molecules. Engagement of TLR2 is also involved with stimulation of osteoclasts and/or inhibition of osteoblasts [137]. Bone deposition and secretion of bone matrix proteins is mediated by osteoblasts. These proteins include type 1 collagen, osteocalcin (OC), osteopontin (OPN), bone sialoprotien (BSP) and proteoglycans [161]. Osteoblasts are also important in the regulation of hydroxyapatite crystal deposition.

Osteoblasts are regulated by bone morphogenetic proteins that stimulate the expression of bone matrix proteins [161]. On the other hand, osteoclasts (large multinucleated specialized giant cells) are responsible for bone resorption [136]. They differentiate from hemopoietic stem cell lineage of monocytes and macrophages [162]. The role of osteoclasts in the process of bone resorption is very significant. A member of the tumor necrosis factor (TNF) ligand superfamily, RANKL (Receptor activator of nuclear factor κB Ligand) is involved in the production of osteoclasts [148]. RANKL exists in two forms, a soluble form and a transmembrane form. They both stimulate osteoclast generation if the cells are cultured with macrophage colony-stimulating factor (M-CSF) [163]. Although some studies found that TNF-α

dependent osteoclastogenesis take place in the presence of RANKL [164]. Other studies found that osteoclast differentiation stimulated by phosphorylated dihydroceramide lipids from *P. gingivalis* and *P. endodontalis* takes place independently from the classic stimulated RANKL [148, 165]. Also unpublished data of a study by Mirucki et al. [165] suggested that phosphorylated dihydroceramide lipids of *P. endodontalis* not only stimulate the release of TNF-α from monocytes via TLR2 but also have an inhibitory effect on osteoblast differentiation and affect their ability to cause deposition of mineral nodules. The study was able to isolate successfully phosphorylated dihydroceramide lipids from *P. endodontalis*. Although this study found analogous phosphorylated ceramide lipids of *P. gingivalis, P. endodontalis* do not possess phosphoglycerol dihydroceramide lipids as does *P. gingivalis*.

The structure of Lipid 654 (Figure 3) contains two fatty acids: 3-hydroxy iso $C_{17:0}$ is amide linked to glycine and the iso $C_{15:0}$ is held in ester linkage by a β carbon of 3-hydroxy iso $C_{17:0}$. the dipeptide head group is composed of glycine and a terminal serine. *P. gingivalis* also synthesizes another lipid, called Lipid 430, that does not contain the esterified iso $C_{15:0}$ fatty acid. Lipid 430 also engages TLR2 as described below. Calcium hydroxide treatment of Lipid 654 was demonstrated to produce Lipid 654 using mass spectrometric analysis of the calcium hydroxide-treated Lipid 654.

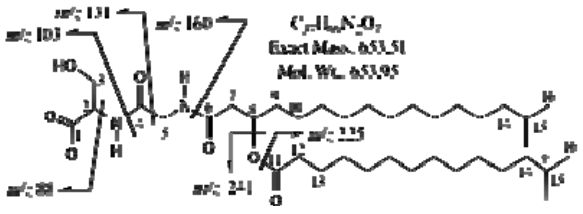

Figure 3: The proposed structure of Lipid 654.

Mass spectrometry of Lipid 654 (Figure 4) shows that 654, 640 and 626 negative ions represent the three constitutive species of the Lipid 654 class. Also, there are no negative ions recovered between 1300 and 1450 indicating that lipid A is not present in this lipid preparation. The 690 negative ion is an artifact due to recombination of 654 with another chemical moiety.

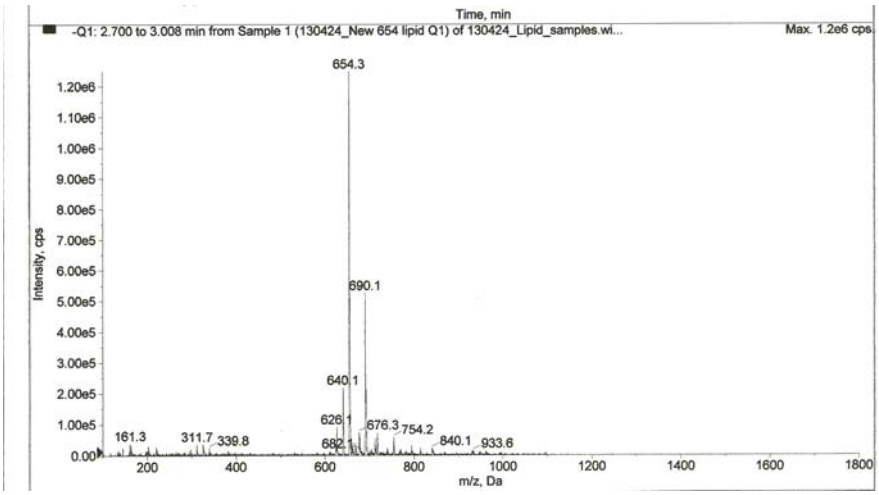

Figure 4: Mass spectrometry of Lipid 654. Lipid 654 was dissolved in HPLC solvent and infused into an ABSciex QTrap 4000 instrument at a flow rate of 100 ul/ml and used in the negative ion mode.

Although Lipid 654 shares all the structural characteristics with the previously described lipid class termed "Flavolipin", there are significant differences between Lipid 654 and Flavolipin in the biological activity. Flavolipin was named after the organisms of the *Flavobacterium* genus from which it was isolated. Although Flavolipin was found to act as a TLR4 ligand, Lipid 654, as well as Lipid 430, do not activate via TRL4 but they function as ligands for TLR2 [138].

Sodium hypochloride is the most popular irrigant used in the field of endodontics. It reacts with unsaturated fatty acids by hydrolysis. It binds to the chlorine ion carbon and hydroxyle group, leading to an inactivated, non-virulent bacterial by-product. However, phosphorylated dihydroceramide lipids of *P. gingivalis* contain saturated fatty acids that will not react with sodium hypochlorite. The absence of the double bonds will not allow the hypochlorous acid molecule to react with the lipid molecule. Therefore, using sodium hypochlorite as the only irrigant without any adjuvant medication is not sufficient as it may leave some amounts of these lipids in the instrumented, irrigated root canals. Consequently, healing will not occur [165]. In this study, we evaluated in this study the efficacy of calcium hydroxide on these serine lipids.

The main reason for performing two-visit non-surgical root canal treatment is calcium hydroxide application in the root canals. Although the time needed for calcium hydroxide to achieve a proper antibacterial effect is not known. Sjögren et al. [147] found that application of calcium hydroxide in the root canals for 10 minutes yielded 50% bacteria free samples while its application for 7 days yielded 100% bacteria free samples. When calcium hydroxide was left in the canal for one week the bacteria was not able to recover even after five weeks. Although many studies found no significant difference in the outcome of non-surgical root canal treatment when performed in one or two visits [166-168]. Other studies proved that one visit is not capable of achieving bacteria free canals [169, 170]. Therefore, medication with calcium hydroxide is recommended. Calcium hydroxide is the most commonly used root canal dressing due to its antimicrobial effect [116, 132]. Its antimicrobial activity is related to its high pH (12.5), and the release of a hydroxyl group from its molecule. Safavi et al. [132] found that the hydroxyl group reacts with the easter-linked hydroxyl –fatty acid molecule of LPS, leading to alteration of Lipid-A. In this study, we found that calcium hydroxide is capable of significant reduction on the release of TLR2-dependent TNF-α release from the macrophages by either total lipids and Lipid 654 of *P. gingivalis*. These results are probably explained by the calcium hydroxide hydrolysis the ester-linked fatty acid of the Lipid 654 lipid. The hydroxyl group of calcium hydroxide acts on the carbonyl group of the lipid producing a stable molecule that is unable to be engaged in further reactions.

This study demonstrates the role of serine lipids of *P. gingivalis* in promoting macrophage activation. It also demonstrates the effect of calcium hydroxide on this Lipid 654 when exposed to the calcium hydroxide for several days. Based on these findings, we now believe that calcium hydroxide use is important as a pulpal medicament for disinfection of necrotic pulpal tissues and further support the view that endodontic therapy should include two visits of non-surgical root canal treatment.

9. Summary and conclusion:

1. Total lipids of *P. gingivalis* stimulate the release of TNF-α from mouse macrophages via TLR-2, as well as Lipid 654.

2. Anti-TLR-2 antibody significantly reduced the effect of total lipids and Lipid 654 of *P. gingivalis*.

3. Treatment of total lipids and Lipid 654 of *P. gingivalis* with calcium hydroxide significantly reduced their effect on the release of TNF-α.

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