Etiologic role of root canal infection in apical periodontitis and its relationship with clinical symptomatology

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Abstract: Evidence shows the polymicrobial etiology of endodontic infections, in which bacteria and their products are the main agents for the development, progression, and dissemination of apical periodontitis. Microbial factors in necrotic root canals (e.g., endotoxin) may spread into apical tissue, evoking and supporting a chronic inflammatory load. Thus, apical periodontitis is the result of the complex interplay between microbial factors and host defense against invasion of periradicular tissues. This review of the literature aims to discuss the complex network between endodontic infectious content and host immune response in apical periodontitis. A better understanding of the relationship of microbial factors with clinical symptomatology is important to establish appropriate therapeutic procedures for a more predictable outcome of endodontic treatment.

Keywords: Periapical Periodontitis; Periapical Diseases; Lipopolysaccharides; Cytokines; Matrix Metalloproteinases.

Introduction

Apical periodontitis is mainly a consequence of root canal infection, characterized by inflammation and destruction of periradicular tissues resulting from the interaction between microbial factors and host immune response.[1] Evidence has reinforced the microbial role in apical periodontitis;[2] however, given the diversity of the endodontic microbiota and its different virulence factors, the exact pathogenic roles of microbial species have been under investigation to determine whether any particular group of bacteria is associated with specific endodontic symptoms and clinical signs.

Gram-negative bacteria predominate in root canals of teeth with pulp necrosis and periapical lesions.[3,4,5,6,7,8,9,10,11,12] Among the virulence factors of gram-negative bacteria, lipopolysaccharides (LPS/endotoxins) are especially important in endodontic infection because of their biological effects, which lead to a complex interplay with host factors, resulting in clinical symptomatology, inflammatory reaction, and resorption of mineralized tissues.[4,11,12,13,14,15,16,17,18,19,20] On the other hand, lipoteichoic acid (LTA), present in gram-positive bacteria, shares its pathogenic properties with lipopolysaccharides (LPS),[21,22] resulting in well-known injuries to the dental pulp and periapical tissues. In general, both LPS and LTA are

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able to potently activate monocytes/macrophages, causing rapid release of cytokines at periradicular sites related to tissue destruction.[21,22]

Destruction of the periodontal ligament is triggered by degradation of the extracellular matrix by metalloproteinases (MMPs),[23] involving periradicular inflammation and bone destruction mediated by proinflammatory cytokines.[24]

LPS are the most potent stimuli for immune cells regarding the release of several inflammatory mediators (e.g., IL-1α, IL-1β, TNF-α, IL-6, PGE_2, IL-10, and MMPs)[11,12,17,25,26,27,28,29,30] and, consequently, they are associated with clinical symptomatology.[11,12,13,15,16,17,18,19,20,31]

This review of the literature aims to discuss the complex network between endodontic infectious content [(bacteria and virulence factors (endotoxins and LTA)] and host immune response in apical periodontitis. A better understanding of the relationship of microbial factors with clinical symptomatology is important to establish appropriate therapeutic procedures for a more predictable outcome of endodontic treatment.

Etiologic role of bacteria in root canals

The oral cavity has one of the highest rates of microorganisms. Although viruses, fungi, yeasts, and protozoa can be found in this ecosystem, bacteria account for a larger number,[10,32] about 10,[10] distributed in 700 species or phylotypes. Approximately 40 to 60% of these bacteria have not yet been cultured.[10,32,33]

The enclosed anatomy of the dental pulp provides an effective primary barrier against its microbial colonization, once the teeth are part of the oral cavity. Actually, as long as the enamel layer is intact, bacteria will not reach the pulp through the crown. Furthermore, root walls are similarly naturally impermeable. Nevertheless, it is clinically apparent that the dental pulp does become infected.[34]

Pathways of infection

Under appropriate conditions, the normal oral microbiota may give rise to opportunistic pathogens if access to dental pulp tissues occurs. Openings in the physical barriers of dentin (enamel and cementum) by means of caries, cracks, or traumatic injuries create pathways for bacteria into the root canal system.[2,32,35] Other routes are exposed dentinal tubules; direct pulpal exposure; restorative procedures; lateral canals of teeth with periodontal involvement; and entry into the systemic circulation, known as anachoresis.[2,36] The most common route of contamination is dental caries, inducing successive inflammatory responses in the pulp tissue, ending with pulp necrosis if appropriate therapeutic measures are not adopted.[34] Having overcome either or both physical and/or biological barriers, establishment of infection will depend upon the survival of microorganisms within the pulp space.[34]

Pulpal response to injuries

The pulp tissue normally reacts to a biological, physical, or chemical injury, provoking tertiary (reactionary or reparative) dentinal deposition, accompanied by moderate inflammatory cell infiltration.[37] Fibrosis and premature aging of the pulp may accompany resolution.[37]

Once bacteria invade and colonize the dentin, their removal is very difficult. Although pulpal infection produces an immune response in the dental pulp, the immune response is not enough to eradicate the pathogens. This occurs because immune cells and molecules cannot reach into the dentin effectively, as the dental pulp tissues are entrapped by the dentin and also as a result of restricted vascularization.[38] One should recall that the pulp consists of a highly vascularized and highly innervated connective tissue, encased into rigid walls, in communication with the periodontium and with the rest of the body only through the apical foramen, apical deltas, and accessory canals. Thus, from a practical clinical point of view, the pulp is an end organ without collateral circulation.[34]

If the pulpal vascular system becomes dysfunctional, pulpal infection typically progresses and causes pulpal disease (ranging from reversible to irreversible pulpitis), which will eventually lead to total necrosis, depending on how host defense mechanisms can cope with the increased numbers of (virulent) bacteria and their products. If left untreated, pulpal disease will spread beyond the apex of the tooth leading to periapical disease. Initially, only the periodontal ligament will be involved in the periapical reaction. However, resorption of cementum

(and dentin) and breakdown of the alveolar bone ensues and all periodontal tissues end up affected.[39] It is a cascade reaction which starts with dental caries and then progresses to pulpal disease, pulp necrosis, and periapical disease. The latter may have systemic presentation with clinical signs such as high temperature, malaise, and leukocytosis; in susceptible patients, for whom subacute bacterial endocarditis is a potential risk, there could be life-threatening implications.[34,40,41]

Bacteria colonizing the root canal damage periradicular tissues; and periradicular inflammation can be observed even before the entire root canal becomes necrotic.[42,43] As the infection progresses, the cellular infiltrate intensifies and tissue destruction continues with the formation of small abscesses and necrotic foci in the pulp, which eventually leads to total pulp necrosis.[44]

After pulp necrosis, usually as a sequelae of dental caries, the root canal environment provides a selective habitat for the establishment of a mixed microbiota with predominance of anaerobic bacteria.[45] To exert its pathogenic effects, the root canal microbiota must either invade periradicular tissues or evoke (by their products and/or structural components) a defense response in the host for establishment of apical periodontitis.[46]

Microbial challenge emanating from the root canal system elicits an inflammatory response in periradicular tissues, in an attempt to prevent the spread of the infectious process into bone tissue and beyond. Periradicular diseases can give rise to a multitude of clinical and radiographic presentations and can be regarded as infectious disorders caused by endodontic infections.[38,45,46,47]

Classification of bacteria based on Gram staining

Bacteria are traditionally classified as gram-positive and gram-negative after Gram staining. Gram-positive bacteria are those whose cell walls have a single thick layer of peptidoglycans. By using Gram staining with crystal violet, the bacteria are stained purple or blue, as they retain the dye even when exposed to alcohol. Gram-negative bacteria are those with a thinner cell wall and a second lipid membrane outside the cell wall, which is exclusively found in these bacteria. In the staining process, the lipid in this outermost membrane is dissolved by alcohol and releases the first dye, crystal violet. At the end of the staining process, the cells are visualized by the second dye, safranin, which gives them a red color.[48]

Figure 1 shows bacterial penetration into dentinal tubules and examples of Gram staining morphology.

Table 1 shows the bacterial genera most commonly found in endodontic infections according to their Gram staining characteristics and gaseous requirements. Some Gram-positive species detected in root canals are strict anaerobes, including *Eubacterium, Filifactor, Parvimonas, Peptostreptococcus, Pseudoramibacter* and

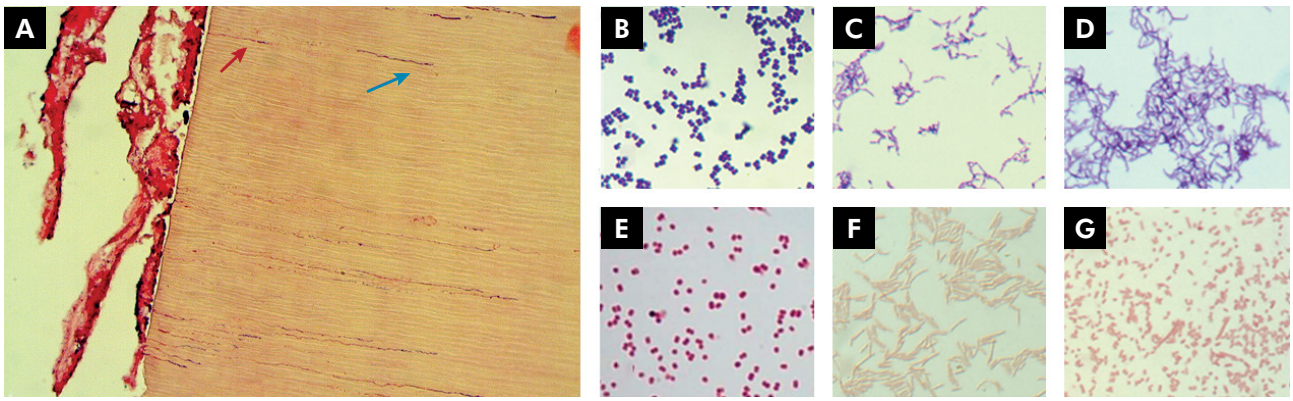

Figure 1. Bacterial penetration into dentinal tubules and examples of Gram staining morphology. A- Histological section of an instrumented root canal (Taylor modification of Brown & Brenn stain; original magnification x20) showing the root canal wall covered by biofilm and some dentinal tubules clogged with gram-negative bacteria (red arrow) and gram-positive bacteria (purple arrow); B- Gram-positive cocci; C-D- Gram-positive rods; E- Gram-negative cocci; and F-G- Gram-negative rods (Courtesy Dr Jefferson J.C. Marion).

Table 1. Bacterial genera commonly occurring in endodontic infections according to the Gram-staining characteristics and gaseous requirements.

Bacteria morphology	Obligate anaerobes	Facultative anaerobes
Gram-positive cocci	*Finegoldia*	*Enterococcus*
	Parvimonas	*Gemella*
	Peptoniphilus	*Staphylococcus*
	Peptostreptococcus	*Streptococcus**
Gram-negative cocci	*Veillonella*	*Neisseria*
Gram-positive rods	*Actinomyces*	
	Eggertella	
	Eubacterium	*Actinomyces**
	Filifactor	*Corynebacterium*
	Lactobacillus	*Lactobacillus**
	Olsenella	*Propionibacterium**
	Propionibacterium	
	Pseudoramibacter	
Gram-negative rods	*Alloprevotella*	
	Bacteroides	
	Camphylobacter	
	Dialister	*Capnocytophaga*
	Fretibacterium	*Eikenella*
	Fusobacterium	*Haemophilus*
	Porphyromonas	
	Prevotella	
	Tannerella	
	Treponema	

*Some species can also be strict anaerobes.

Propionibacterium. Other Gram-positives are facultative anaerobes, including *Actinomyces**, *Lactobacillus**, *Streptococcus** (*some species can be strict anaerobes), *Enterococcus, Gemella* and *Staphylococcus*.[9,10,32]

Some members of the gram-negative community are strict anaerobes, including *Fusobacterium, Porphyromonas, Prevotella, Tannerella* and *Treponema*. Others are facultative anaerobes such as *Capnocytophaga, Eikenella, Neisseria*, among others.[9,10,32]

Escherichia coli is a gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium found in the gastrointestinal tracts of humans and other vertebrates, and rarely isolated from root canals. However, it is widely used in laboratories because it is easy and inexpensive to grow, not particularly virulent, and it is an excellent host for producing several proteins. It was one of the first organisms to have its genome sequenced, in 1997. More than 90% of endotoxin studies were conducted on *E. coli* LPS.[49]

Bacterial virulence factors

Bacterial virulence factors include the structural components and products of bacterial metabolism. The latter are responsible for direct damage to pulp tissue, whereas structural components of the bacterial cell, such as lipopolysaccharides (LPS) and lipoteichoic acid (LTA), can injure tissues indirectly by activation of an immune response.[50]

LPS, or endotoxin, is a major constituent of the cell wall of gram-negative bacteria and is secreted in vesicles by growing organisms or released during the disintegration of bacteria after their death. Endotoxin is one of the most important virulence factors involved in the development of periapical inflammation and bone destruction, activating immunocompetent cells and leading to the release of a variety of proinflammatory mediators. Lipid A is the bioactive component of LPS responsible for most of the host's immune response.[51,52,53,54]

LTAs are present on the surface of gram-positive cells, such as *Enterococcus faecalis*, and have adhesive properties, as they adsorb to hydroxyapatite, binding through its lipid portion. They stimulate leukocytes, monocytes, and macrophages to release inflammatory mediators.[55] Thus, LTA may be associated with resistance to medications used during endodontic treatment,[56,57] also playing an important role in biofilm formation, providing bacterial resistance to antibiotics or disinfectants. It contributes to the virulence of *E. faecalis* by facilitating aggregation substance formation and plasmid transfer.[56]

LPS and LTA activate the immune system by similar mechanisms. Both bind to CD14, activating Toll-like receptor signaling and inducing the production of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-8 (IL-8), interleukin-12 (IL-12), and anti-inflammatory cytokines such as interleukin-10 (IL-10).[58,59] At low concentrations, LPS and LTA stimulate the innate response of the host defense system. At higher levels, they have been related to pain of pulp origin and to periradicular inflammation.[16,60]

Based on the facts and considerations above, it is clear that infection of the root canal system is the primary cause of apical periodontitis. In addition, virulence factors help the bacteria invade the host, cause disease, and evade host defenses, thereby worsening periradicular diseases.

The endodontic microbiota

Since Miller[61] demonstrated the presence of bacteria in necrotic pulp tissue, the role of oral microorganisms in the pathogenesis of pulpal and apical periodontitis has become increasingly evident. However, knowledge about the nature of the endodontic microbiota depends upon the recognition of the microorganisms present in the pulp space, which relies on contemporary knowledge and technology.[34]

Methods for microbial detection in root canals

Currently, there are several methods for microbial identification, including culture- and non-culture-based techniques. Traditionally, microorganisms in endodontic samples have been identified by various cultivation procedures, which rely on isolation, growth, and laboratory identification, using morphology and biochemical tests. However, the prevalence of some oral pathogens could have been underestimated by culture-based techniques as such approaches may fail to grow certain bacteria, especially fastidious anaerobic microorganisms such as spirochetes.[4,9,10,62,63]

Culture is a widely used method for evaluating the antimicrobial efficacy of root canal procedures against viable bacteria in root canal infection.[2] Correlations between absence of cultivable bacteria and a favorable treatment outcome have been reported.[64] In order to recover microorganisms from the necrotic pulp and from diseased periapical tissues and study their properties, stringent anaerobic sampling and cultivation techniques are necessary.[2] Improvements in anaerobic techniques have permitted a more detailed knowledge of the microbiota within the infected root canals and its association with periapical lesions.[4,63]

Advances in molecular techniques have improved the identification of several novel and as-yet-uncultivated bacterial species. Molecular techniques include polymerase chain reaction (PCR) and its variations (nested PCR, real-time quantitative PCR), cloning and sequencing, and next generation sequencing (NGS). NGS is a high throughput sequencing technology that has enabled the parallel sequencing of several microbiological samples by PCR amplification of a phylogenetic marker, the 16S rRNA. It also provides the most accurate detection of abundant and rare members of the microbial community. Furthermore, an advantage of this method is that it does not require laborious cloning to obtain microbial sequences.[65] All of the above-mentioned techniques are based on bacterial identification through the 16S rRNA gene, a region of bacterial DNA present in all microorganisms that is well preserved and very specific to each species.[63]

Unlike the techniques based on the 16S rRNA gene, the checkerboard DNA-DNA hybridization or DNA-DNA hybridization was introduced for hybridizing large numbers of DNA samples against large numbers of DNA probes on a single support membrane.[66] This method is fast, has adequate sensitivity, and it is relatively inexpensive, overcoming several limitations of microbial culture, allowing the study of the communities present in clinical samples.[66,67]

Molecular methods overcome the limitations of culture-dependent methods; however, despite the inherent limitations of each method, the combined results of both cultural and molecular studies are necessary to improve the understanding of the endodontic microbiota.[68] For example, the sensitivity of microbial culture is approximately 10^4 to 10^5 cells for target species using nonselective media and 10^3 using selective media, while for PCR it ranges from 10 to 10^2 cells, depending on the technique used. Nested PCR brings the detection limit down to about 10 cells.[69] The detection limits of the checkerboard DNA-DNA hybridization method ranges from 10^3 to 10^4.[63,66,70]

Factors related to the composition of the root canal microbiota

The major ecological factors that define the composition of the root canal microbiota are the availability of nutrients, redox potential variability, and bacterial interactions.[5,6,32,34,71] Bacterial interactions might be the key element to the growth and survival of organisms in their habitat. On the other hand,

bacterial interaction can also have an effect on the microbial population itself, inhibiting the growth of particular species and perhaps increasing the chances of the host to cope with the infective agent and to restore health. Other factors involved are pH level, temperature, and host defense mechanisms.[5,6,32,34,71]

It has been reported that there is a difference between the microbiota of a root canal subjected to any source of oral contamination for some time and the microbiota of closed canals. In closed canals, the microbiota is predominantly anaerobic, while in open canals the recovered/detected microorganisms are mostly facultative anaerobes.[34,45,47,72] Moreover, anaerobes are also more frequently detected in root canal samples from clinically symptomatic teeth,[4,7,9,10,32,45,47,67,72-78] which have a bacterial community profile that is significantly different from asymptomatic (chronic) lesions. This has also been observed for teeth with primary versus post-treatment apical periodontitis.[7,9,10,32,77,79-81]

In addition, the bacterial community profile exhibits a high interindividual variability and there are significant geographic differences in the composition of the endodontic microbiota, which may have implications in terms of efficacy of antimicrobial protocols used in different countries.[10,32]

Overall microbial composition of the root canal

Similarly to the oral cavity, over 500 different bacterial species or phylotypes have already been detected in infected root canals by culture or by molecular methods such as 16S rRNA gene sequencing or metagenomic sequencing. Bacteria are the most commonly found microorganisms in root canals, belonging to 20 phyla according to present knowledge.[32]

Table 2 shows some of the microorganisms isolated/detected from root canals according to their phyla. Of the major reported phyla, Firmicutes, Fusobacteria, and Bacteroidetes were the most abundant in acute infections, while Firmicutes, Bacteroidetes, and Actinobacteria were the most abundant in chronic infections.[76,77,81] In addition, fungi, yeasts,[10,80,82-86] viruses,[87-90] and Archaea[71,91] were also detected in root canals.

These microorganisms can be found suspended in the lumen of the root canal (planktonic form) or adhered to the walls of the root canal, forming a biofilm (sessile form). They can penetrate into dentinal tubules and into lateral, secondary, and accessory canals. They can be found between the gutta-percha and the root canal walls, and they can also form biofilms in the extraradicular region. The colonization of these sites is directly related to the time of infection and to the composition of the microbiota.[32]

Classification of endodontic infections

Endodontic infections can be classified according to their anatomical location (intraradicular or extraradicular infection) and to how long it took microorganisms to reach the root canal (primary, secondary, or persistent infection). Usually, primary and secondary/persistent endodontic infections are located intraradicularly, and may originate in extraradicular infections if left untreated or inadequately treated.[9,10,32]

Primary infected root canals are untreated canals where microorganisms were able to access and colonize the pulpal tissue and impair its function. Their microbial profile consists of 10–30 species per canal,[9,10,32,92] and the species present in the apical region may have a major role in the pathogenesis of apical periodontitis.

Fusobacterium, Porphyromonas, Prevotella, Parvimonas, Tannerella, Treponema, Dialister, Filifactor, Actinomyces, Olsenella, and *Pseudoramibacter* predominated in the canals. Some facultative or microaerophilic streptococci are also commonly found in primary infections.[9,10,32]

Persistent/secondary infected root canals are usually associated with post-treatment apical periodontitis, which indicates that there was a failure in endodontic treatment. In this case, microorganisms may have tolerated the chemomechanical procedures (persistent infection) or invaded the canal via coronal leakage of the root filling (secondary infection).[7,9,10,32,85]

The microbiota found in cases of endodontic failure is composed of a more restricted group of species when compared to primary infections. Canals apparently well treated have been shown to harbor fewer than five species. On the other hand, teeth with unsatisfactory root filling may harbor 10 to 30 species, a number similar to that of primary infections.[9,32]

Facultative anaerobic and gram-positive bacteria predominated in canals with endodontic treatment failure, which may be due to increased resistance

Table 2. Microorganisms most commonly isolated/ detected from root canals distributed according to the phyla.

Firmicutes	Actinobacteria	Fusobacteria	Proteobacteria	Spirochaetes	Bacteroidetes	Synergistetes
Dialister pneumosintes	*Actinomyces gerencseriae*	*Fusobacterium necrophorum*	*Campylobacter gracilis*	*Treponema amylovorum*	*Alloprevotella tannerae*	
Dialister invisus	*Actinomyces israelii*	*Fusobacterium nucleatum* ssp *nucleatum*	*Campylobacter rectus*	*Treponema. denticola*	*Porphyromonas endodontalis*	*Pyramidobacter piscolens*
Eggerthella lenta	*Actinomyces naeslundii*	*Fusobacterium nucleatum* ssp *polymorphum*	*Eikenella corrodens*	*Treponema lecithinolyticum*	*Porphyromonas gingivalis*	
Enterococcus. faecalis	*Actinomyces odontolyticus*		*Haemophilus aphrophilus*	*Treponema maltophilum*	*Prevotella denticola*	
Filifactor alocis	*Actinomyces viscosus*			*Treponema medium*	*Prevotella intermedia*	
Finegoldia magna	*Propionibacterium acnes*			*Treponema pectinovorum*	*Prevotella melaninogenica*	
Parvimonas micra	*Propionibacterium propionicum*			*Treponema socranskii*	*Prevotella nigrescens*	
Peptoniphilus asaccharolyticus	*Slackia exigua*			*Treponema vincentii*	*Tannerella forsythia*	
Peptostreptococcus anaerobius						
Pseudoramibacter alactolyticus						
Selenomonas sputigena						
Streptococcus anginosus						
Streptococcus constellatus						
Streptococcus intermedius						
Streptococcus mitis						
Streptococcus sanguis						
Veillonella parvulla						

to instrumentation and to antiseptic agents.[5,6,82,85] According to Molander et al.,[82] facultative anaerobes, especially gram-positive ones, can survive in a quiescent phase with low metabolic activity for some time, and factors such as coronal leakage during or after root canal treatment can change the nutritional conditions and contribute to bacterial growth.

E. faecalis, a facultative gram-positive bacterium, is capable of surviving in an environment with scant availability of nutrients and minimal commensality with other bacteria.[83,93] It presents

different virulence and resistance mechanisms, which hinder its eradication from root canals.[22] An important collagen-binding protein of *E. faecalis* microbial surface components is Ace (adhesion of collagen from *E. faecalis*),[93] which is related to its ability to invade dentinal tubules and adhere to collagen in the presence of human serum.[94,95]

Streptococcal species are also frequently found in root canals of teeth with post-treatment disease. Other bacterial species, including anaerobic bacteria such as *Parvimonas micra*, *Propionibacterium* species and *Pseudoramibacter alactolyticus* are commonly reported in persistent intraradicular infections. *Candida* species are more frequently found in root canals of teeth with endodontic failure than in those with primary infections .[9,10,32,79,85]

Extraradicular infections are mostly caused by intraradicular infections. They are characterized by apical abscesses and extraradicular biofilms. However, there are also independent extraradicular infections, such as apical actinomycoses, caused by *Actinomyces* spp. and *Propionibacterium* spp., requiring periapical surgery for their resolution.[32,36,96]

Apical periodontitis may be acute or chronic, depending on several factors. Acute infection is usually caused by a community of highly virulent bacteria, probably due to the presence of species with greater virulence or to the synergism between species and is characterized by a high concentration of bacteria and tissue invasion, together with decreased host resistance.[46] Chronic apical periodontitis, by contrast, is usually associated with low virulence of the bacterial community involved. Bacterial persistence in the root canal system may be related to its organization in biofilms, not allowing for host defense due to the anatomical location of the infection.[46]

Acute apical abscess is the most common form of extraradicular infection. Microbial communities present in acute apical abscesses are complex, with a predominance of strict anaerobic microorganisms (approximately 90% of the isolates), mainly gram-negative bacilli, including *Fusobacterium, Prevotella, Porphyromonas, Dialister*, and *Treponema,* and gram-positive cocci. They are polymicrobial infections where the occurrence of strict anaerobes is 3 to 4 times greater than that of facultative anaerobes.[76,78,97,98,99] However, although the concentration of strict anaerobes is higher in periapical abscesses, their diversity is lower than that found in the root canals of periapical abscesses.[98,99]

Chronic apical abscess is characterized as an inflammation in the periapical region with formation and discharge of pus slowly through a fistula, recognized as a pathognomonic sign of this lesion. Radiographically, there are radiolucent signs of apical bone destruction, however without significant discomfort for the patient.[100] The endodontic microbiota of this pathology has been scarcely characterized in the literature, probably due to its low prevalence (between 9.7% and 18.1%).[101] Rôças et al.[75] used checkerboard DNA-DNA hybridization to identify microorganisms in the root canals of nine patients diagnosed with chronic apical abscess. *Porphyromonas endodontalis* (9/9 - 100%), *Dialister invisus* (8/9 - 89%), *Parvimonas micra* (7/9 - 78%), and *Solobacterium moorei* (7/9 - 78%) were the most prevalent species in these cases. Tennert et al.,[102] after sequencing the 16S rRNA genes of isolated microorganisms, reported the presence of *Anaerococcus prevotii*, *Atopobium rimae*, *Dialistes invisus*, and *Fusobacterium nucleatum*.

Bacterial biofilm is a community of microcolonies of bacterial cells involved in an extracellular matrix of polysaccharides, adhered to a solid substrate wet or liquid medium, from which bacteria can obtain their nutrients. Biofilm is a form of protection against bacterial development in hostile environments. It can be formed by one or multiple species.[96,103,104,105,106,107]

Periradicular dental biofilm is characterized by microorganisms adhered to cementum and/or dentin in the apical portion of the root, surrounded by an external polysaccharide layer known as glycocalyx, which forms an intermicrobial matrix. The structure of the polysaccharide matrix that surrounds the biofilm limits the access of defense molecules (antibodies and complement) and of phagocytic cells (macrophages and neutrophils).[108] The prevalence of intraradicular bacterial biofilms is high, whereas extraradicular biofilms are considered rare and are normally dependent on intraradicular infections.[106]

Bacteria in biofilm differ phenotypically from their planktonic form, since a number of genes are regulated to optimize their phenotypic properties in

a given environment.[107] Clinically, one of the main characteristics of bacterial biofilms is their greater resistance to antimicrobial agents.[109]

Figure 2 illustrates the radiographic aspects and microbiological findings observed in each type of infection. There is a great diversity of microbial colonies in primary intraradicular infections (Figure 2, A-B) and a lower diversity in secondary ones (Figure 2, C-D). A large diversity of microbial colonies with a predominance of black-pigmented bacteria can be found in acute periapical abscesses (extraradicular infections) (Figure 2, E-F), whereas endodontic treatment failures may be due to the presence of extraradicular apical biofilms, on which microorganisms such as *Actinomyces israelii* may be present (Figure 2, GH).

In summary, although the infectious nature of endodontic pathosis has been established for many decades, with the development of modern microbiological diagnostic techniques, the composition of these infections has been improved and redefined.

The key role of endotoxin

All gram-negative bacteria, and only these microorganisms, have a differentiated external cell membrane with hydrophobic constituents composed of polysaccharides (sugar polymers), lipids (complexes containing fatty acids), and proteins. This structure is called LPS or endotoxin, highlighting the main components and biological effects of the molecule.[3]

Endotoxins are secreted into vesicles during the bacterial growth phase or released during cell death. They are also released when the cell is chemically treated to remove LPS. LPS represents the major antigenic surface of gram-negative bacteria, presenting microbiological and immunological significance.[51,52]

Approximately 75% of the cell surface of gram-negative bacteria is composed of this molecule, which is essential for cell growth, decrease in membrane permeability, and structural integrity and stability, as well as for protection against external damage.[52,110]

Endotoxins are heat-stable and, therefore, usual sterilization processes are ineffective for

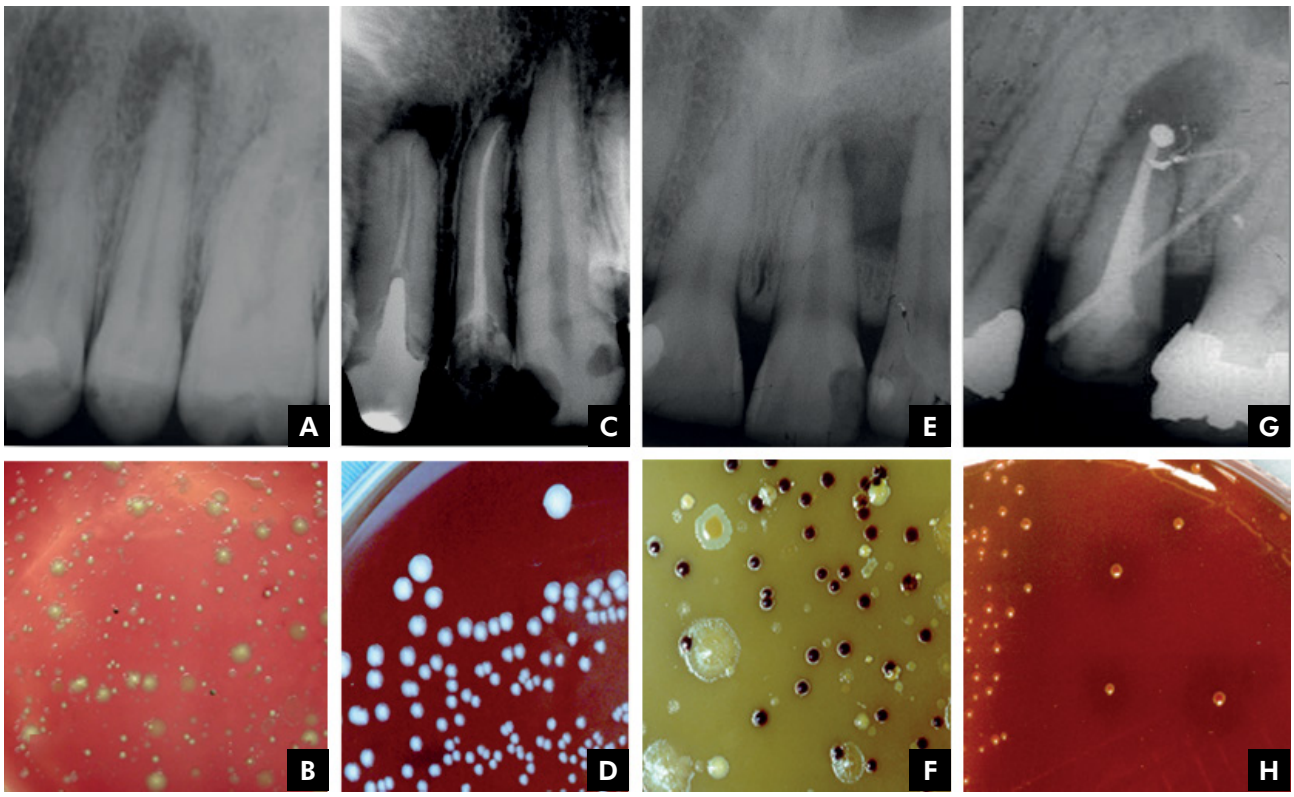

Figure 2. Radiographic aspects and microbial culture plates of different types of endodontic infections. A-B - Primary infection; C-D - Secondary infection; E-F- Periapical abscess; and G-H- Apical Biofilm

their destruction.[111] To degrade them chemically, strong acids or bases or pyrolysis must be used, and different heating protocols are known: 180°C for 3 hours,[111,112] 200°C for 4 hours,[113] or 250°C for 3 hours according to the Cambrex/Lonza manual. Other processes that lead to the inactivation of endotoxins are ionizing radiation and treatment with polymyxin B. In endodontic therapy, hydroxyl ions present in the calcium hydroxide paste can hydrolyze LPS, degrading lipid A and neutralizing its residual effect after cell lysis.[54,114]

LPS is known to be the most toxic constituent of bacterial endotoxin, having no structural homologue among multicellular organisms.[115]

In general, bacterial LPS are composed of three structural domains: lipid A, core, and repeating O-antigen.[116,117,118] The lipid A moiety exerts most of the endotoxic activities, being regarded as the endotoxic principle of LPS.[116] The chemical structure of lipid A is composed of fatty acids with 15 to 17 carbon atoms, linked to two amino-sugar molecules (glycosamines), to which two phosphate radicals are bound, and a protein residue bound to the phosphate radicals. The positions of the phosphate radicals, as well as the number, type, and site of bonds seem to determine the inflammatory potential of different LPS.[92]

Polysaccharide moiety is a potent antigen that can stimulate antibody formation even at submicrogram concentrations.[119] The recognition of LPS occurs through lipid A, which in turn activates different intracellular signaling pathways through its binding to proper receptors, according to the structure of the acyl chain.[120] The major receptors are located in the cell membrane of monocytes, called Toll-like receptors 4 (TLR4).[121,122]

Studies have shown that lipid A can vary among different bacterial species, depending on the number of phosphate groups and on the amount and position of fatty acids in the molecule.[53,120,123] These variations are closely related to the change in TLR4 signaling and, consequently, to their immunostimulatory effects.[118,120,124] TRL-4 mutations are likely to influence susceptibility to gram-negative infection, or the course of infection once it is established, as they block LPS signaling, whereas overexpression greatly increases LPS signaling.[115] Moreover, changes in the microenvironment, such as hemin concentration and temperature, can structurally alter this bioactive portion.[125]

LPS structure is remarkably heterogeneous among bacterial species, thus evoking different patterns of inflammatory response. The LPS molecule can also vary among different strains of single species and, consequently, exhibit different inflammatory potentials.[53,18,126]

More than 90% of endotoxin studies have been conducted on enterobacterial LPS,[49] of which *Escherichia coli* LPS is the best known. LPS from gram-negative bacteria such as *Prevotella and Porphyromonas* isolated from the oral cavity are able to produce classic manifestations that are less damaging to host tissues than those from *E. coli*.[127] On the other hand, Fusobacterium spp. LPS presents a similar structure to that of gram-negative enteric bacilli,[128] contributing to the high virulence of this microorganism.[127] According to Martinho et al.,[126] *Fusobacterium nucleatum* induces a greater expression of IL-1β and TNF-α cytokines compared to *Porphyromonas gingivalis*. These two bacteria have different patterns of macrophage activation, which may contribute to the immunopathogenesis of apical periodontitis.

LPS is essential for bacterial survival because it protects the bacterium from host defense cell stimuli.[129] It has many biological activities including fever induction, adjuvant activity, Schwartzman reaction, cytotoxicity, blood clotting, and fibrinolysis, among others. LPS can also stimulate production of bradykinin, which is a potent pain mediator.[130]

Siqueira Junior and Rôças[46] mention several biological effects of LPS, as follows:

a) Activation of macrophages/monocytes with consequent synthesis and release of proinflammatory cytokines (IL-1β, IL-6, CXCL8 or IL-8, TNF-α), prostaglandins, nitric oxide, and oxygen-derived free radicals. These substances are chemical mediators of inflammation and most of them can stimulate bone resorption;

b) Activation of the complement system. Some products of complement activation are chemotactic to inflammatory cells (C5a), act as opsonins (C3b), and can increase vascular permeability (C3a and C5a).

c) Activation of the Hageman factor, the first step of the intrinsic pathway of coagulation, triggering the

coagulation cascade or the production of bradykinin, an important chemical mediator of inflammation;

d) Induction of the expression of leukocyte adhesion molecules in endothelial cells, which are important in the early stages of inflammation;

e) Stimulation of osteoclast differentiation and bone resorption, particularly via interactions with TLR4 in osteoblast lineage cells. LPS induces RANKL expression in osteoblasts and stimulates these cells to secrete interleukin (IL)-1, IL-6, prostaglandin E2 (PGE2), and TNF-α, each of which is known to induce osteoclast activity and differentiation.

f) LPS may be mitogenic to B lymphocytes and epithelial cells.

g) LPS can stimulate naive B cells in the absence of T helper cells. At low concentrations, LPS stimulates specific antibody production. At high concentrations, this molecule can cause nonspecific polyclonal activation of B cells.

h) It has been recently demonstrated that trigeminal afferent neurons express the TLR4 and CD14 receptor complex and that LPS activation of TLR4/CD14 may trigger intracellular signaling cascades, leading to peripheral release of neuropeptides and central nociceptive neurotransmission. Hence, it is assumed that one of the pain mechanisms associated with bacterial infectious processes could result from direct effects of LPS on sensory fibers via interaction and direct activation of the TLR4/CD14 complex.

LPS may evoke pain through activation of the Hageman factor or through neurotoxic properties when acting on presynaptic nerve terminals, direct sensitization of nociceptors, sensitization and up-regulation of the transient receptor potential cation channel, subfamily V, member 1 (TRPV1).[18]

LPS concentrations found in infected root canals seem to promote a direct sensitization of receptors that activate the pain mechanism associated with bacterial infections, also sensitizing trigeminal sensory neurons.[131,132]

The presence of endotoxin has been reported in samples taken from vital pulp,[13] irreversible pulpitis,[13] necrotic pulp,[11,12,13,15,16,17,20,25,28,29,30,113,131,133,134,135,136,137,138,139] root canals of teeth with endodontic failure,[19,20,26,132] and

Table 3. Total endotoxin levels in initial samples from root canal reported in previous studies.

Author	Pulpal / Periodontal tissue status	LAL Method	Endotoxin concentration
Schein and Schilder (1975)[13]	Vital pulp / healthy periodontium	Gel clot	0.007 μg/mL
	Irreversible pulpitis / healthy periodontium	Gel clot	0.075 μg/mL
Schein and Schilder (1975)[13]	Pulp necrosis / apical periodontitis	Gel clot	0.192 μg/mL
Jacinto et al. (2005)[15]		QCL	18016.50 EU/mL
Vianna et al. (2007)[133]		QCL	151.61 EU/mL
Martinho and Gomes (2008)[16]		QCL	323.27 EU/mL
Gomes et al. 2009[113]		QCL	212.23 EU/mL
Martinho et al. (2010a)[11]		TKA	9.19 EU/mL
Martinho et al. (2010b),[134] (2012),[17] (2014)[25]		TKA	7490.00 pg/mL
Martinho et al. (2011b)[141]		QCL	34.20 EU/mL
Martinho et al. (2011b)[141]		KQCL	7.49 EU/mL
Martinho et al. (2011b)[141]		TKA	9.19 EU/mL
Oliveira et al. (2011)[135]		KQCL	192.37 EU/mL
Gomes et al. (2012)[20]		TKA	7.49 EU/mL
Xavier et al. (2013)[136]		KQCL	153.13 EU/mL
Marinho et al. (2014)[137]		TKA	18.70 EU/mL
Marinho et al. (2015)[29]		TKA	32.43 EU/mL
Herrera et al. (2015)[30]		TKA	21.83 EU/mL
Cardoso et al. (2015)[131]		KQCL	10.92 EU/mL
Herrera et al. (2017)[139]		TKA	27.72 EU/mL
Endo et al. (2012),[19] Gomes et al. (2012)[20]	Previous root canal treatment / apical periodontitis	TKA	3.96 EU/mL
Duque et al. (2018)[140]	Vital pulp / chronic periodontal disease	TKA	0.10 EU/mL

QCL: Chromogenic endpoint assay (Quantitative chromogenic LAL); TKA: Turbidimetric kinetic assay; KQCL: Chromogenic kinetic assay.

from root canals of teeth associated with periodontal disease.[140] Table 3 shows some of the endotoxin levels reported in initial samples collected from the root canals.

LPS have been detected in 100% of root canals with necrotic pulp, with significantly higher levels in symptomatic teeth.[12,15,20,141] Moreover, even though LPS levels were higher in primary endodontic infections than in secondary/persistent infections, endotoxins were detected in all samples.[20] This shows that endotoxins are extremely strong stimulators of inflammatory reactions, even at low concentrations.

There is a correlation between higher levels of endotoxins and a greater area of bone destruction in periapical tissues,[142] as well as with the presence of specific clinical features found in primary endodontic infections.[20,46] Increased endotoxin levels in infected root canals may be associated with the severity of periapical disease, as well as with the development of clinical symptoms.[20]

Clinical endodontic researchers have investigated not only bacterial LPS in infected root canals, but also correlated higher endotoxin levels with clinical signs, symptoms, and radiographic findings.

Interplay of infectious/endotoxic contents with inflammatory mediators and clinical symptomatology

The role of cytokines

Periapical immune response is a second line of defense that seeks to localize the infection of the root canal system by confining it and preventing its dissemination and systemic involvement.[2] This immune response is initially comparable to the pulpal response to microbial infection, characterized by a cell infiltrate of polymorphonuclear neutrophils (PMNs) and monocytes, with the subsequent additional feature that the periradicular bone is destroyed. The intensity of bacterial invasion of periradicular tissues depends on the number of pathogenic bacteria and on their degree of virulence.[2] Once bacteria and their virulence factors come into contact with periradicular tissues, they stimulate the synthesis and expression of different mediators that will attract inflammatory cells to the area.[17] These factors, depending on host resistance, may stimulate the development of an acute inflammatory response (acute apical periodontitis or acute periradicular abscess), or a chronic response (chronic apical periodontitis or chronic periradicular abscess).[46] The destruction of periapical tissues seems to be mainly indirect via host-derived stimuli rather than by the direct effects of bacteria on the bone.

Periapical immune response is predominantly a reaction to bacterial infection present in necrotic root canals.[46,143,144] Immunocompetent cells settle in periapical areas in an attempt to prevent the spread of the infectious microbiota.[17] Among these cells, macrophages are the defense cells responsible for the rapid recognition of pathogens and rapid presentation of lymphocytes and other cells of the immune system.[17] Macrophages are stimulated predominantly by the bacterial endotoxin.[12,132] Lymphocytes express different sets of inflammatory cells, proinflammatory and immunoregulatory cytokines and chemokines, which are considered important mediators in periapical immune response to infection.[132,143]

Among the various host degradative pathways, considerable interest has been focused on the study of cytokines, not only because of their role in regulating the humoral immune system and in cellular responses against invasive bacteria, but also as mediators of periapical tissue destruction.[145]

Inflammatory cytokines produced from host cells (e.g., monocytes/ macrophages) reflect root canal conditions and determine the local immune process within the periapical environment.[29] After the macrophage and PMN activation by bacterial components, a cascade of proinflammatory cytokines, including IL-1α, IL-1-β, and TNFα, is triggered.[144]

Cytokines are small signaling molecules that mediate host responses to infection, inflammation, and trauma. Proinflammatory cytokines initiate or enhance systemic inflammation while anti-inflammatory cytokines reduce inflammation and promote healing.[144] Examples of proinflammatory cytokines include IL-1 and TNF-α, whereas IL-10 is an important anti-inflammatory cytokine. Some cytokines can have both proinflammatory and anti-inflammatory properties, such as IL-6, as it can inhibit TNF-α and IL-1 and activate IL-10 at the same time. Cytokines work with each other in a homeostatic network regulation to prevent the constant state of inflammation.[144]

Interleukin-1β (IL-1β) and interleukin-6 (IL-6) act as proinflammatory cytokines during apical periodontitis, initiating or intensifying systemic inflammation.[144,146] In addition, they also stimulate osteoclast differentiation and bone resorption in chronic apical periodontitis, inducing the secretion of chemokines during the destruction of periodontal tissues.[146]

On the other hand, the reactions provoked in the host organism by proinflammatory cytokines can be prevented by suppressing the activity of these cytokines through the activity of anti-inflammatory cytokines, such as interleukin 4 (IL-4), interleukin 10 (IL-10), IL-13, and the transforming growth factor beta (TGF-β).[146]

Proinflammatory cytokines, such as IL-1β, IL-2, IL-8, IL-17, IFN-γ, and TNF-α, are detected in the interstitial fluid of periapical lesions, where the high concentration of bacteria found in root canal infection was correlated with a higher rate of detection of proinflammatory cytokines.[147,148]

Gram-negative bacterial species tend to induce a greater proportion of TNF- α, but other cytokines, for example IL-1β, IL-6, IL-8, and IL-10, increase their levels during the course of endodontic infection.[144]

TNF-α, IL-1β, and IL-6 are examples of important cytokines in the acute phase of inflammation.[144,148] On the other hand, LTA, present in gram-positive bacteria, has pathogenic properties similar to those of LPS,[21,22] resulting in well-known injuries to the dental pulp and periapical tissues. Overall, both LPS and LTA are able to potently activate monocytes/macrophages, causing rapid release of cytokines at periradicular sites related to tissue destruction.[21,22] However, by comparing the cytokines released by LTA in immune cells with the cytokine released by LPS of gram-negative bacteria, LTA has a power that is 100 to 1,000 times lower than that of LPS.[144] While LPS is a potent inducer of proinflammatory cytokines and IL-10, LTA exhibits less induction of proinflammatory cytokines and does not induce IL-12 and the subsequent formation of IFN-γ,[149,150] which can be explained by the use of different TRLs.[149] TRL2 appears to be the primary mediator of the innate immune response to the LTA of several gram-positive bacteria[151] and is highly induced in inflamed dental pulps.[152,153]

TNF-α is the cytokine most abundantly detected in endodontic infection.[29,144] TNF-α is a potent immune mediator of acute and chronic inflammatory responses, with the potential to increase bone resorption.[154] It is considered the main mediator of the acute inflammatory response induced by gram-negative bacteria and other infectious microorganisms. LPS is the most important stimulus to activate the production of TNF by macrophages, although activated T cells, NK, and mast cells can also secrete this cytokine. IFN-γ produced by T and NK cells increases the synthesis of TNF by LPS-stimulated macrophages.[155]

TNF-α stimulates the production of collagenase and prostaglandin E_2 (PGE_2), factors related to the induction of chemokines, cytokines, cell adhesion molecules, and bone resorption.[17] PGE_2 can induce or inhibit IL-6, another proinflammatory cytokine that stimulates osteoclast differentiation and bone resorption in chronic apical periodontitis.[17] IL-6 can also induce or inhibit PGE_2, depending on the duration of the lesion, bacterial load, stimulated signaling pathways, and cytokines.[17] PGE_2 was directly and indirectly related to most of the inflammatory and destructive alterations in apical lesions, such as vasodilation, increased vascular permeability, and collagen degradation.[17] In the same study, PGE_2 was also positively related to TNF-α and IL-1β.[17]

IL-1 has the main function of mediating the host's inflammatory response to infections and to other stimuli, similarly to the effects of TNF. It acts on endothelial cells by inducing the expression of surface molecules that mediate leukocyte adhesion. Macrophages are the main source of IL-1 production, with neutrophils and endothelial cells involved in their production. There are two forms of IL-1, alpha (α) and beta (β), the latter of which is the most commonly found in the human circulatory system.[155] IL-1α plays a critical role in protecting the body from external invaders such as bacteria and viruses, and it is also involved in bone resorption.[156] IL-1β has been correlated with clinical signs/symptoms and with greater bone resorption.[157]

In addition to mediating the production of potent inflammatory molecules, such as prostaglandins, leukotrienes, platelet-activating factor, and cytokines, and positively regulating endothelial cell adhesion molecules, IL-1 affects several processes of the innate immune response. It has been implicated as a central mediator during bone and tissue destruction processes.[158,159]

IL-6 is a cytokine that mediates the host's response to infection and has been observed in exudates (EX) associated with endodontic apical lesions.[160] In addition, IL-6 was positively correlated with the area of the radiographic lesion in the study by Martinho et al.,[17] thus confirming its role in bone resorption in chronic inflammatory periodontitis. However, the same study demonstrated that PGE_2 and IL-6 together were negatively correlated with the size of the radiographic lesion, confirming that the relationship between these cytokines is complex and difficult to establish in many respects.[17] IL-6 has potent proinflammatory effects at local and systemic levels, including the acute-phase inflammatory response, in which C-reactive protein (CRP) can be detected in response to IL-6 action.[160] Although IL-6 is detected in all analyzed samples of root canals, whether healthy or with apical periodontitis, in the latter cases its expression is significantly increased.[160] IL-6 expression is induced by IL-1β and TNF-α during the early stages of inflammation, and all act synergistically, promoting the recruitment of PMNs and monocytes, shifting from acute to chronic inflammation, which induces activation of MMPs and stimulation of osteoclastogenesis and bone resorption.[160]

IL-8 is mainly produced by monocytes/macrophages and in smaller amounts by fibroblasts, endothelial cells, keratinocytes, melanocytes, hepatocytes, and chondrocytes. Its stimuli are usually IL-1, TNF-α, and IFN-g. IL-8 can be inhibited by corticosteroids and cyclosporine A. It is a chemokine, thus increasing chemokinesis and acting as a chemiotactic factor. The term 'chemokine' comes from the contraction of 'chemotactic cytokines'. Other chemokines include MIP-1a, MIP-1b, MCP, eotaxin, and RANTES. IL-8 provides potent migratory stimulus for the cells of the immune system, mainly neutrophils, also determining an increase in the expression of adhesion molecules by endothelial cells. It also activates PMNs, increasing oxidative metabolism. It antagonizes the production of IgE stimulated by IL-4, but it does not affect the production of other immunoglobulins.[150]

IL-10 is produced by monocytes, macrophages, and lymphocytes,[161,162] originally identified for their ability to antagonize cellular immunity.[163] Among its characteristics, immunosuppression is noteworthy, as it depresses the activation of mononuclear cells and prevents the production of mediators of inflammation.[163,164] IL-10 inhibits antigen presentation in monocytes by negative regulation of MHC class II[163] and the *in vitro* expression of costimulatory molecules.[165] Its biological effects would be a consequence of its ability to inhibit many of the functions of activated macrophages, such as the production of IL-12 and TNF-α.[155]

Although many of the cytokines involved in the immunoregulatory network were identified in apical periodontitis, the longitudinal expression of this network and its effects on proinflammatory cytokines still need to be further investigated.

The role of matrix metalloproteinases

Matrix metalloproteinases are members of an enzyme family that require a zinc ion in their active site for catalytic activity. MMPs are critical for maintaining tissue allostasis. MMPs are active at neutral pH and can therefore catalyze the normal turnover of extracellular matrix (ECM) macromolecules such as interstitial and basement membrane collagens, proteoglycans such as aggrecan, decorin, biglycan, fibromodulin, and versican, as well as accessory ECM proteins such as fibronectin.[166]

The degradation of the ECM by MMPs seems to be an important trigger for the progression of the inflammatory process.[148,167]

Proinflammatory cytokines, such as IL-1β and TNF-α, are able to stimulate, either directly or indirectly, the release of MMPs in the periapical/periodontal region, maintaining a persistent inflammatory process.[30,168] MMPs are deeply involved in the pathogenesis of pulp, periodontal, and periapical tissue destruction.[168]

MMPs are commonly organized in groups, based partly on historical assessment of the substrate specificity of the MMP and partly on the cellular localization of the MMP. These groups are the collagenases [MMP 1 (interstitial collagenase), MMP 8 (neutrophil collagenase), MMP 13 (collagenase 3), MMP18 (collagenase 4)], the gelatinases [MMP 2 (gelatinase A), 9 (gelatinase B)], the stromelysins (MMP 3, 10, 11), membrane-type MMPs (MT-MMPs 14, 15, 16, 17, 23A, 23B, 24, 25), and other MMPs.[169]

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are of particular interest in endodontics, because there is

evidence that these MMPs play an important role in the pathogenesis of pulp, periodontal, and periapical tissue destruction.[170,171] They are responsible for the degradation of gelatin (denatured collagen) and type IV collagen, the major component of basement membranes.[172]

MMP-2 can also degrade collagens V, VII, and X, decorin, elastin, and fibronectin.[172,173] Its collagenolytic activity is shown by its action on fibroblasts, the main constituent cells of connective tissue of the periodontal ligament, responsible for collagen production.[30] Fibroblasts secrete MMP-2 when induced by endodontic content in primary endodontic infections, and this process contributes to the progression of periapical inflammation and tissue destruction.[30]

It has been reported that the expression of MMP-9 in inflamed pulps has shown higher levels than those recorded in clinically healthy pulps.[167] MMP-9 was detected in endothelial cells, osteoblasts, fibroblasts, and inflammatory cells, and could be released during the inflammatory reaction induced directly by bacteria or indirectly by proinflammatory cytokines, evidencing the role of MMPs in the pathogenesis of inflammation,[27] where its synthesis is controlled by proinflammatory cytokines.[167]

Ahmed et al.[167] showed a correlation between gram-negative bacteria and MMP-9 hyperactivity in symptomatic periapical lesions. MMPs also act in the processes of bone resorption and destruction of periapical tissues, promoting direct degradation of the ECM, exhibiting a correlation between the concentration of gram-negative bacteria and MMP-9 expression in symptomatic periapical lesions.[167]

The destruction of the periodontal ligament is initiated by the degradation of extracellular membrane and serine proteases.[174] Connective tissue destruction is essentially controlled by MMPs, which contributes to the destruction of gingival tissue and alveolar bone surrounding the teeth.[23] MMP activity requires a balance with the intrinsic inhibitors known as tissue inhibitors of MMPs (TIMPs),[27,175] because an excessive production of MMPs leads to accelerated matrix degradation and tissue destruction, which is associated with pathological conditions such as periodontitis and apical periodontitis.[168,176]

Specific Quantikine ELISA kits (R&D Systems, Minneapolis, MD, USA) have been used for measurement of both cytokines (IL-1α, IL-1β, TNF-α, and PGE_2) and MMPs (MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13). Cytokines and MMPs are measured indirectly after stimulation of host cells (macrophages and fibroblasts) with infectious contents,[17,29,30] or directly from samples of the periapical region.[27,140]

Regardless of the method used, the data obtained in the studies mentioned above reveal that MMPs are involved in apical periodontitis because they interact with complex networks, which include cytokines, in the development of clinical features and severity of bone destruction.

Association of microorganisms with endodontic clinical features

The microbiota of infected root canals consists of a complex polymicrobial population. In such heterogeneous community, interactions among several microbial species may play a significant role in the balance between individual microorganisms. Combinations of bacteria are more potent at inducing pathological state in the host (*e.g.*, apical periodontitis) than are single strains. Moreover, complex interactions of species result in characteristic clinical pictures that cannot be achieved by individual species alone.[6]

Gram-negative species, such as *Fusobacterium, Prevotella,* and *Porphyromonas,* are likely to have some clinical significance due to the presence of endotoxin. LPS has many biological activities, including fever induction, adjuvant activity, Schwartzman reaction, cytotoxicity, blood clotting, fibrinolysis, and production of bradykinin, which is a potent pain mediator.[4-7,130] However, the cell walls of gram-positive bacteria such as *Peptostreptococcus and Eubacterium* spp. can also produce inflammatory reactions due to the presence of peptidoglycans and LTA. They enhance the pathogenicity of "black-pigmented *Bacteroides*" and are also related to acute symptoms and destruction of periapical tissues. The combination of *P. micros* and *Prevotella* spp. was associated with clinical features such as pain and swelling.[4-7] One explanation for the synergy between these species is the known enhancement of the endotoxin effect by gram-positive superantigens.[7] Superantigens interact with antigen-presenting cells (APCs), such as macrophages and T cells, inducing cell proliferation

and massive cytokine production, which leads to clinical symptomatology.[177]

A long-held desire in endodontic microbiology has been to find a single or at least a group of bacterial species that is responsible for acute symptoms.[32,78] However, while several bacterial species seem to be more prevalent when associated with pain, the very same species have also been encountered in asymptomatic cases. The possibility exists that some of these species really play a role in making the bacterial mixed community more virulent. Several other factors can be regarded as influential to the development of symptoms, including differences in virulence among clonal types of the same species, bacterial interactions in the multispecies community, resulting in collective pathogenicity, total and specific bacterial counts, and host-related factors.[32]

Endodontic symptomatology includes history of pain, spontaneous pain, pain on palpation (POP), and tenderness to percussion (TTP). Clinical signs mean presence of sinus swelling in periodontal tissues, presence of apical periodontitis, status of the root canal such as dry canal, and presence of clear, hemorrhagic, or purulent exudate.

Several works using the strict anaerobic culture technique reported an association between specific bacteria and endodontic signs and symptoms.[4-7,72-74,85,178-181]

Nowadays, with the advance of molecular microbiology, thanks to which more than 500 species or phylotypes have been detected in root canals, it seems even harder to associate a species or group of species with clinical symptoms and signs. Nevertheless, the bacterial community profiles associated with teeth with symptomatic apical periodontitis are significantly different from asymptomatic lesions. The same has been observed for teeth with primary versus post-treatment apical periodontitis.[32]

Association of LPS with clinical features

Anaerobic gram-negative bacteria have been frequently isolated from root canals of endodontically involved teeth; consequently, their endotoxins may affect the periapical tissues and exert a role in the pathogenesis of inflammatory lesions of pulpal origin.[15]

Since the work by Schein and Schilder,[13] the relationship between endotoxin levels and presence of endodontic clinical signs and symptoms has been investigated. These authors found a significant correlation between endotoxin levels and presence of exudate and radiolucent areas.[13]

Horiba et al.[31] showed that teeth with clinical symptoms contained higher levels of endotoxin than those that were asymptomatic. Jacinto et al.[15] reported a positive association between endotoxin and symptomatic cases (e.g., spontaneous pain, TTP, POP, swelling, and purulent exudate), which exhibited higher levels of endotoxin than asymptomatic cases. A negative association was reported between the endotoxin present in the root canals and asymptomatic teeth.[15]

Martinho and Gomes[16] found a positive correlation between LPS and TTP in primary infected root canals. Higher levels of endotoxin were found in teeth with clinical symptomatology.

Martinho et al.[11] reported that larger areas of bone destruction, identified by the size of the radiolucent area, were related to higher levels of endotoxin. Additionally, a correlation was found between levels of endotoxins and the number of gram-negative bacterial species. Moreover, higher levels of endotoxin were detected in teeth with exudate.[12]

Endo et al.,[19] after investigating endotoxin levels in teeth with post-treatment apical periodontitis, reported that higher levels of endotoxin are related to a larger radiolucent area (> 5 mm).

Gomes et al.[20] compared root canal samples collected from primary and secondary infections with median levels of endotoxins found in primary and secondary endodontic infections with apical periodontitis by correlating LPS contents with clinical/radiographic findings. Endotoxins were detected in 100% of the values of 7.49 EU/mL and 3.96 EU/mL, respectively ($p < .05$). The median value of endotoxins found in the presence of clinical symptoms was significantly higher than in asymptomatic teeth with primary infections ($p < .05$). A positive correlation was found between endotoxin contents and a larger radiolucent area (> 3 mm) ($p < .05$).[20]

Martinho et al.,[18] in their systematic review and meta-analysis, evaluated the relationship between endotoxin levels and presence of clinical signs/

symptoms and radiographic features in patients with endodontic infection. Among the 385 articles identified in their initial search, 30 were included for full-text appraisal and only eight studies[11-13,15,16,19,20,31] met the inclusion criteria for the systematic review.

The meta-analysis revealed that individuals with teeth with TTP ($p = 0.04$; I^2 57%)[11-13,15,16,19,20] and previous episode of pain (PEP, $p = 0.001$; I^2 81%)[15,16,31] had higher levels of endotoxin than their counterparts. These correlations are consistent with the hypothesis that LPS in clinical infections is related to the production of pain and mechanical allodynia.[18]

Size of radiographic lesion > 2 mm (SRL, $p = 0.02$; I^2 68%) was also associated with higher levels of endotoxin.[11,13,19,20,31] Previous studies have also demonstrated this association, where the endotoxin content of teeth with radiolucent areas is five times as great as that of teeth without them.[183]

Presence of root canal exudation ($p = 0.0007$; I^2 0%) was associated with higher levels of endotoxins,[11,12,31] indicating acute inflammation in a periapical lesion. Overall, the meta-analysis provided strong evidence that endotoxin is related to the presence of clinical signs/symptoms and radiographic features in patients with endodontic infection.[18]

It is important to highlight that not only the levels of endotoxin are implicated in the presence/development of symptoms and severity of bone destruction, but also the bacterial community involved in the infection, its interplay (synergism/antagonism), and consequently, the type of bacterial LPS and its lipid A structure.[18]

Since a more complex gram-negative bacterial community is associated with a primary endodontic infection, it is clear that higher levels of endotoxins will be present in these teeth compared with teeth with secondary infections.[20] However, both situations have been associated with the relation between endotoxin levels and bone destruction in periapical tissues as well as with the development of clinical features. Thus, it is important to correlate endotoxin levels with immune stimuli by the expression of inflammatory mediators.

Association of LPS with cytokines

LPS is considered the major etiologic component responsible for pathophysiology of inflammation and post-infectious sequelae. It is able to potently activate monocytes/macrophages, causing rapid release of cytokines in periradicular sites related to tissue destruction.[21,22]

Gram-negative species tend to induce a higher ratio of cytokines, particularly TNF-α. However, interleukins such as IL-1β, IL-6, IL-8, and IL-10 are also increased during the course of endodontic infection. Chemokines such as CXCL2 and CXCL10 are also detected in endodontic infections. Chemokines activate inflammatory cells and also influence angiogenesis.[144]

Positive correlations have been established between the number of gram-negative bacteria, endotoxin contents, and the levels of TNF-α, IL-1β, and PGE_2 found in primarily infected root canals.[11,12,144]

Although LPS is known to induce cytokine production, the amount of cytokines can be lowered to control inflammation by providing immune cells with 'LPS tolerance.' This can be done by exposing immune cells to low doses of LPS. The ability to develop tolerance is also better in young than in older adults.[144]

Cytokines/MMPs and clinical symptomatology

The virulence factors present in gram-negative- (LPS) and in gram-positive bacteria (LTA) are responsible for stimulating host cells to express various cytokines, most of which are harmful to the host organism. Specific functions are assigned to each cytokine and the effects of their interactions are implicated in the development of clinical signs and symptoms, as well as in the mechanisms of bone resorption exhibited by apical periodontitis.

The study of Martinho et al.[26] showed several correlations between clinical symptomatology and cytokine expression. The presence of POP has been positively associated with TNF-α and IFN-γ, whereas higher levels of IL-4 and IL-13 decreased the chances of POP. The chance of having TTP is increased in the presence of higher levels of TNF-α and IFN-γ, whereas elevated levels of IL-4, IL-5, and IL-13 were considered a protective factor. Larger size of bone destruction (>3 mm) was positively associated with TNF-α and negatively associated with higher levels of IL-4.

Due to the large number of studies that have linked the role of proinflammatory cytokines to the pathogenesis and progression of periapical/

periodontal disease, the possibility of interventions with the manipulation of anti-inflammatory cytokines as adjuvant therapies in patients with these diseases indicates a new era in endodontic regeneration.

An important interplay has been established between cytokines and other inflammatory mediators, including MMPs. It has been reported that proinflammatory cytokines such as IL-1α, IL-1-β, IL-6, and TNFα can enhance MMP expression and production in human periodontal ligament cells.[184] MMPs are involved in the breakdown of the ECM and in the processing of a variety of biological molecules.[185] They play an important role in many physiological and pathological processes.[154,185-187] Herrera et al.[30] showed that root canal contents from primary endodontic infections have gelatinolytic activity for MMP-2. The authors also reported that endotoxin levels achieved after chemomechanical preparation (CMP) do not have antigenicity against fibroblasts for MMP-2 and MMP-9 expression.[30]

Table 4 shows the main cytokines and MMPs reported in previous studies and their involvement in the immune response/clinical symptomatology of apical periodontitis.

Tissue breakdown in apical periodontitis is the result of the imbalance in the interplay between the local host immune response and different inflammatory mediators stimulated by infection of the root canal system. Clinicians should be guided by endodontic protocols that allow the reduction of higher levels of endodontic infectious contents and, consequently, the reduction of complex interplay mechanisms. This, in turn, will lead to the restoration of the balance between host immune response and inflammatory mediators in the periapical region.

Treatment of the endodontic infection

The objective of endodontic treatment is the removal of pain of pulp and periapical origin and the prevention and treatment of pulpal and periapical diseases. To achieve that, principles of infection control are of great importance. These principles should be adopted before the beginning of the endodontic treatment, with antisepsis of the operative field and the use of instruments and sterile substances, followed by crown decontamination and CMP, where the mechanical action of the instruments throughout the root canal, combined with the use of auxiliary chemical substances and efficient irrigation, will reduce the levels of microorganisms and their virulence factors in the root canal system.[32]

Technological advances in instrumentation have brought significant improvements in the ability to shape the root canals; however, recent micro-CT studies have revealed that around 10 to 50% of the root canal area remains intact after the action

Table 4. Main cytokines and MMPs reported in previous studies and their involvement in the immune response / clinical symptomatology of apical periodontitis.

Author	Biomarker	Primary function	Significant association with clinical features			
			POP	TTP	EX	> SRL
Martinho et al. (2010a),[11] (2014),[25] Cardoso et al. (2016)[132]	IL-1β	Expression of adhesion factors, diapedesis, fever	+	+	+	+
Martinho et al. (2012)[17]	IL-6	Acute phase response, fever				+
Martinho et al. (2010a),[11] (2012),[17] (2014),[25] Cardoso et al. (2016)[132]	TNF-α	Cardinal signs of inflammation, fever	+	+	+	+
Martinho et al. (2011)[12]	PGE2	Vasodilatation, Collagen degradation		+	+	
Shin et al. (2002),[170] Martinho et al. (2016a)[27]	MMP-1	Collagen degradation				+
Martinho et al. (2016a)[27]	MMP-2	Collagen degradation		+		+
Martinho et al. (2016a)[27]	MMP-9	Collagen degradation	+			+

POP: Pain on palpation; TTP: Tenderness to percussion; EX: Exudation; > SRL: Higher size of radiographic lesion.

of the instruments.[188-190] These areas may harbor bacterial biofilm that remains unchanged after CMP, compromising treatment outcome. Moreover, with the advent of the rotary instrumentation system, instrumentation time has been decreased as well as the contact time of the auxiliary chemical with the substrates present in the root canals. This emphasizes the need for efficient irrigation with effective antimicrobial substances.

Several substances have been recommended for use during CMP, including mainly sodium hypochlorite (NaOCl), chlorhexidine (CHX), and 17% ethylenediaminetetraacetic acid (EDTA).[191] NaOCl is the main endodontic irrigant used, because of its antibacterial properties and ability to dissolve organic tissues.[192] CHX, usually at 2% and in a gel-based format, has been proposed as an alternative to NaOCl because of its antimicrobial activity against gram-negative and gram-positive bacteria,[191] substantivity,[193,194] and lower cytotoxicity.[195] EDTA is widely used in clinical practice since it removes the contaminated smear layer and debris formed during CMP, opening the dentinal tubules to receive an intracanal medication (ICM) or an endodontic sealer.[196] Despite the consecrated properties of each of these irrigants, none of them is sufficiently capable of thoroughly disinfecting the root canal system. Thus, CMP should not be focused only on the mechanical approach and on passive irrigation.

Different systems of mechanical activation of irrigants to improve endodontic disinfection have been reported in the literature,[197,198] including manual dynamic activation with gutta-percha cones,[199] endodontic instruments or special brushes,[197] vibrating systems activated by sonic energy,[199,200] use of ultrasonic[201] or laser energy[202] to activate the irrigants, and apical negative-pressure irrigation systems.[203] Protocols designed to improve intracanal decontamination by a specific chemical action include ozone[204] and direct laser/light-activated disinfection.[205-207]

From a semantic point of view, and given that chemical substances used as irrigants are already 'active', the term 'activation' of the irrigants does not describe the process properly. Similarly, 'passive' ultrasonic irrigation (PUI) is in fact active; however, when it was first introduced, the term 'passive' was related to the 'noncutting' action of the ultrasonically activated file.[209]

PUI has been used to enhance the chemical action of endodontic irrigants, whether antimicrobial activity (NaOCl / CHX), tissue-dissolving activity (NaOCl), or chelating activity (EDTA). After CMP, the enlarged canal allows not only a better flow and backflow of the irrigants, but also the free vibration of the ultrasonic tip,[139] particularly in the apical third, where it is almost impossible to achieve a complete renewal of the irrigant with conventional irrigation.

Ultrasonic activation is reported to be more effective than needle irrigation in removing bacteria from root canals.[139,209] However, in relation to the reduction of endotoxin levels, its action is controversial, as Herrera et al,[139] reported reduction, while Nakamura et al.[209] did not. Nevertheless, the use of ultrasound allows a more intense flow of EDTA against root canal walls and into areas that are not reachable by root canal instrumentation and conventional irrigation, acting on remaining endotoxins.[139] Additionally, this protocol could improve the outcome of endodontic treatment because EDTA has the capacity to rise the rate of LPS-lipoprotein binding by increasing the disaggregation of the endotoxin.[210] Lipoprotein-bound LPS does not stimulate host immune cells and is less active than free LPS to induce proinflammatory cytokine expression.[211]

In order to complement the action of CMP, the use of an efficient ICM for an adequate time period is indicated, particularly in those cases with persistent exudate, TTP, and or POP. Other indications not related to infection are the presence of teeth with an open apex, insufficient time to conclude treatment, fatigue of the patient or operator, among others.[32]

Calcium hydroxide-based ICMs have been widely used as an antimicrobial strategy in the management of infected root canal systems. Nevertheless, there is no evidence to suggest that completion of root canal treatment in a single visit or over two or more visits, with or without ICM, makes any difference in term of effectiveness or complications.[212] The antimicrobial effects of calcium hydroxide are probably due to protein denaturation and damage to DNA and cytoplasmic membranes,[213] while its detoxifying activity occurs by hydrolysis of lipid A in fatty sugars and nontoxic amino acids.[54,114.]

Even after ICM, microorganisms and their by-products may still remain in the root canal. However, their presence does not necessarily means treatment failure, unless there is a pathway of communication linking the root canal to the periapical/periodontal tissues. The threshold levels of endotoxin present in the root canal to induce damage to periapical tissues still have to be determined; however, evidence shows that minimal amounts of endotoxins can induce or maintain an inflammatory process.

The treatment of teeth with suspected apical biofilm after conventional endodontic treatment has been attempted consists of the surgical removal of the apical portion of the tooth, followed by retrograde instrumentation and obturation. At present, there is no auxiliary chemical or ICM capable of destroying the apical biofilm or disorganizing its structure.

It is evident that the integration of new technologies and materials into endodontic therapy can improve the clinical outcome. Nonetheless, after root canal filling, a well-adapted definitive coronal restoration should be placed to prevent coronal microleakage, root canal reinfection, and the risk of root fracture.[32]

Final remarks

Although the polymicrobial etiology of apical periodontitis has been described, the role of individual microbial species in the development, progression, and dissemination of periradicular diseases has not yet been well established. The prevalence of several microbial species has been highlighted in endodontic infections; however, the pathogenic process of apical periodontitis is also related to the number and virulence of the microorganisms and to host's resistance. The virulence of the microorganisms may be affected by the synergistic relationships between the bacteria found in the root canal system.

The association between certain anaerobic species and endodontic signs and symptoms, such as the presence of spontaneous pain, TTP, POP, swelling, and purulent exudate, is directly related to the cytotoxicity of each species. Because of the high toxicity of gram-negative bacterial species and their main virulence factor (endotoxin), stimulating immune cells to release different inflammatory mediators, endodontic therapy should not only rely on reducing/eliminating planktonic and sessile microorganisms and substrates,[214] but also on reducing endotoxins to levels that are compatible with periradicular tissue healing.[32,64,136]

The residual endotoxins found after CMP, if access to periradicular tissues is allowed, would modulate host immune responses by the expression of different cytokines and MMPs.[30,54] Moreover, it has been shown that an elevated inflammatory state caused by metabolic disorders can impact the clinical outcome of periapical lesions and interfere with wound healing after endodontic treatment. Immunoregulatory cytokines produced by various cell types, including immune cells and adipose tissue, play an important role in this interrelationship.[38]

In conclusion, research is necessary to better understand microbial communities and the complex networks activated by the infectious/inflammatory content of an infected root canal, which will act in the development of clinical features and influence the severity of bone destruction. Moreover, improvement in endodontic instruments, instrumentation systems, irrigants, and ICM, along with the development of novel therapeutic approaches, are crucial for more effective procedures for treating infected root canals.

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