academicJournals

Vol. 7(40), pp. 4754-4761, 4 October, 2013
DOI: 10.5897/AJMR2013.5399
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Ecology of pulpal and periapical flora

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Accepted 9 September, 2013

Inflammatory lesions in the periapical tissues of the teeth are a result of root canal infection following partial or complete breakdown of the pulp. Infected root canals have a complex microbial flora consisting of cocci, rods, spirochaetes, filaments and fungi which may exist as a loose collection in mist canal lumen or as dense aggregates (biofilms) adhered to dentinal walls. The biofilm environment has been found to be advantageous for bacterial living; as it helps the bacteria to survive and multiply, inducing their metabolic products that will lead in the persistence of periapical infection.

Key words: Inflammatory lesions, periapical tissues, root canal infection, bacterial.

ETIOLOGY OF PERIAPICAL DISEASE

The essential role of microorganisms in the pathogenesis of periapical lesions was demonstrated by Kakehashi et al. (1965). They made experimental pulp exposures by drilling a hole through the occlusal thickness of enamel and dentin of maxillary first molars in germ-free and conventional rats, they found that the absence or presence of a microbial flora is the major determinant of healing versus development of periapical lesions. In the conventional animals, the exposed pulps became necrotic, and periapical granulomas or abscesses occurred in all cases. However, in the germ-free rats, the exposed pulps healed with dentinal bridging while no necrotic pulps, apical granulomas or abscess developed in spite of gross food impaction.

In agreement with this was the findings Möller et al. (1981). They severed the pulps of teeth in monkeys. The amputated pulps were either immediately sealed aseptically or left open to be contaminated with indigenous oral flora for 1 week and then sealed. After 6 to 7 months, clinical, radiographic and histologic examinations of the teeth that were sealed aseptically showed no pathologic changes in their periradicular tissues. In contrast, teeth with infected root canals had inflammatory reactions in their tissues. The previous findings confirmed that bacteria are the primary etiologic factor in the development and progression of pulp and periapical diseases.

ASSOCIATION BETWEEN ROOT CANAL BACTERIA AND PERIAPICAL LESION DEVELOPMENT

There is a range of periapical responses to the root canal microbial flora that can occur. These responses may include acute periapical inflammation, chronic periapical inflammation, chronic suppurative periapical inflammation, acute periapical abscess/cellulitis, periapical osteomyelitis, periapical osteosclerosis or condensing osteitis, granulomas epithelial proliferation and cysts.

Korzen et al. (1974) studied the effects of normal oral flora and mono-infection (*Streptococcus mutans*) on the pulp and periradicular tissues of conventional and gnotobiotic rats, its results showed that the severity of pulpal and periradicular inflammation was directly related to the quantity of microorganisms. Furthermore, it showed that the degree of inflammation was less severe with monoinfection than with mixed infection. Until the early 1970s, most microbiological studies on root canal flora reported primarily the presence of facultative bacteria in this

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system. However, technologic advances in the isolation of anaerobes and increased awareness of the medical and dental progressions of the role of anaerobes in various diseases caused significant changes in medical and dental microbiology. Sundqvist et al. (1985) showed that root canal infections are multibacterial and that anaerobic organisms, namely *Bacteroides* species, play a significant role in clinical signs and symptoms of pulpal and periradicular disease. *Bacteroides* species have undergone classification changes. New genus names, *Porphyromonas* and *Provotella*, have been assigned to many of the *Bacteroides* organisms (Siqueira and Rocas, 2005).

Other investigators (Fabricius, 1982; Fabricius et al., 1982a, b) in a series of experiments, examined the importance of bacteria in the development of periradicular lesions, composition of root canal flora, and the influence of a combination of oral bacteria on oral periradicular tissues of monkeys. In one study, the researchers mechanically devitalized the pulps of monkeys, left them exposed to oral flora for 1 week, and then sealed them for 3, 6, and 35 months. Bacteriologic examinations of infected root canals at the end of these observation periods showed that 85 to 98% of the isolated bacteria were anaerobic. The most frequently found anaerobic species were *Bacteroides* and Gram positive anaerobic rods. A small percentage of facultative anaerobic bacteria were also isolated from the infected root canals. In another study, they inoculated 75 root canals of monkeys with 11 bacterial species separately, or in combinations, and sealed the access cavities for a period of 6 months. Their bacteriologic and histologic examinations showed that mixed infections have a greater capacity to cause apical lesions than do mono-infections. Furthermore, they reported that the *Bacteroides* strain did not survive in the root canals when inoculated as pure cultures, and facultative streptococci induced small periradicular lesions. To account for the possible contribution of unsampled or uncultivated bacteria in the pathogenesis of lesions, eleven isolated strains (including eight strains from one tooth, representing its total cultivable infection) from previous studies were inoculated in freshly necrotized monkey teeth, in various combinations, but always in equal proportions. The "eight-strain collection" consisted of *Bacteroides oralis, Fusobacterium, necrophorum, Fusobacterium nucleatum, Streptococcus milleri, Streptococcus feacalis (Enterococcus feacalis), Peptostreptococcus anaerobius, Actinomyces bovis* and *Propionibacterium acnes.* After six months, the "eight-strain collection" was recovered from all teeth, and interestingly, in the same proportions that it had been recovered from the original tooth (Dahlén et al., 1987). This suggested that selective pressures were at play in the root canal system that reproduced the "same infection". Other combinations did not survive effectively; some species were not recovered at all. Sundqvist et al. (1985) demonstrated a high correlation between the presence of *Prevotella melaninogenica* and clinical and radiographic signs and symptoms of periradicular pathosis. Griffee et al. (1980) also found a similar correlation between the presence of this organism and pain, sinus tract formation and foul odour. Other researchers (Yoshida et al., 1987) found that *Peptococcus magnus* and *Bacteroides* species were commonly associated with symptomatic cases. Haapasalo (1989) reported on the bacteriology of 62 infected human root canals, giving special attention to the *Bacteroides* species. His results confirmed the findings of previous investigations: almost all root canal infections are mixed, and acute symptoms are usually related to the presence of specific anaerobes, such as *Porphyromonas (Bacteroides) gingivalis, Porphyromonas (Bacteroides) endodontalis* and *Prevotella (Bacteroides) buccae*.

Brook et al. (1991) confirmed the polymicrobial nature of bacteria isolated from aspirates of periradicular abscesses in 39 patients, with anaerobic isolates being present in more than 70% of the bacteria recovered. Wasfy et al. (1992) obtained similar results in the microbiologic evaluation of periradicular infections when they found that anaerobic bacteria were the predominant flora in specimen cultures. Anearobes comprised 73% (190 of 259) of cultivable bacteria. Using molecular technique, Wang et al. (2010) investigated the occurrence of *P. gingivalis* fimA genotypes and its possible correlation with the clinical symptoms, from 158 infected root canals with apical periodontitis. Their findings showed that *P. gingivalis* was detected in 39.9% of the infected root canal samples and was found in 44.5% of *P. gingivalis* positive specimens with symptoms. Types II (69.4%) were the most frequent in the symptomatic cases followed by type IV (32.7%). The occurrence of type I (64.3%) was significantly higher than any other genotypes in the asymptomatic apical periodontitis, whereas types II and Ib were not identified. Statistical analysis revealed that the occurrences of types II, IV, and Ib fimA were associated with greater risk of clinical signs (swelling, sinus tract, or intracanal exudates) than type I.

It can be concluded from the previous findings that root canal infections are associated with great bacterial diversity and high correlation exist between the composition of the microbiota and the clinical signs and symptoms.

Nature of root canal flora

The microorganisms in nature are presented in two forms: in aqueous based environment called planktonic or found in aggregation or communities with polysaccharide matrix called biofilm. Biofilm is defined as thin layer condensations of microbes (e.g. bacteria, fungi and protozoa) that may occur on various surfaces in nature. Such films may become established on any organic or inorganic surface substrate where planktonic microorganisms prevail in water-based solution. Organisms in biofilms were shown to have stronger pathogenic potential

than those in planktonic state (Chavez de Paz et al., 2007; Siqueira et al., 2007; Svensater and Bergenholtz, 2004; Mah and O`Toole, 2001). It was proven that the biofilm bacteria are up to 1,000 times more resistant than planktonic bacteria to phagocytosis, antibodies, antibiotics, disinfectants and antimicrobials (Chai et al., 2007).

The presence of biofilm in the endodontic infections was initially reported by Nair in 1987. He reported a well condensed bacterial layer on the dentinal wall of the root canal which when visible in light microscopically, gave the palisade structure of bacterial plaques adhering to tooth surfaces. It was proven in many studies that the presence of biofilm was associated with persistent and chronic periapical periodontitis. Carr et al. (2009) examined resected root tip of failing endodontically re-treated lower molar tooth under light and electron microscope. They found complex, viable, multispecies biofilm in the entire length of the specimen. Using SEM, several studies were able to detect bacterial biofilm near the apical foramen of teeth that were resistant to endodontic treatment and with necrotic pulps or associated with chronic periapical periodontitis (Tronstad et al., 1990; Molven et al., 1991; Noiri et al., 2002; Leonardo et al., 2002).

Siqueira and Lopes (2001) examined 26 extracted teeth diagnosed as having asymptomatic periradicular lesions and were associated with extensive caries and periapical pathosis. The SEM photographs showed cocci and rods restricted to the root canal, and only one case showed dense bacterial aggregates close to the apical foramen. Clinical isolates of spore-forming gram positive aerobic rods from three patients with persistent periapical periodontitis were identified as *Bacillus subtilis* that exhibited dense meshwork-like structures in their cell surfaces. It was suggested that *B. subtilis* could form bio-films in periapical periodontitis lesions, and this might contribute to resistance to treatment in those patients (Yamane et al., 2009). Likewise, *Actinomyces* sp. oral and *Propionibacterium* were shown to be important contributors to extraradicular biofilm formation and persistent periapical infection (Wang et al., 2012)**.**

Histological examination of the root canal treated teeth with apical periodontitis was correlated with the findings of clinical observations. The results showed heavier intraradicular bacterial colonization (biofilm) associated with apical root canals of both untreated and treated teeth with apical periodontitis (Ricucci et al., 2009; Ricucci and Siqueira, 2010). In conclusion, the biofilm was proven to form in the infected root canal system as well as in the external root surface especially in cases with persistent periapical pathosis and this could be correlated to the resistance of such cases to conventional endodontic treatment and hence contributed to the treatment failure.

Flora of root canal and periradicular lesions

Current concepts suggest that the number of bacterial species in an infected root canals may vary from one to more than 12 and the number of bacterial cells from $<10^2$ to $>10^8$ per sample (Sundqvist, 1992).

Early studies (Winkler and van Amerongen, 1959) generally reported a predominance of facultative organisms over obligate anaerobic species. *Streptococcus*, Gram-negative cocci, and *lactobacilli* were most often recovered, usually found in numbers that constituted less than 50% of the total isolates reported. Through the use of improved techniques, a large variety of bacteria (genera and species) have been isolated from root canals and periradicular lesions. Other studies have found that the organisms most often found appear to be normal flora of the oral cavity; only rarely is a bacterium recovered that can be shown to originate from other parts of the body (Le Goff et al., 1997; Munson et al., 2002; Sundqvist, 1976). The composition of the microbiota from different infected root canals shows a great variability (Love, 2009; Montagner et al., 2012)

There is now a consensus of opinion that the root canal flora of non-carious teeth with necrotic pulp and diseased periapex is dominated (>90%) by obligate anaerobes (Bystrom and Sundqvist, 1981; Haapasalo, 1989; Sundqvist, 1976; Sundqvist et al., 1989) usually belonging to the genera *Fusobacterium*, *Porphyromonas*, *Eubacterium* and *Peptostreptococcus*. On the other hand, the microbial composition, even in the apical third of the root canal of periapically affected teeth with carious crowns, is less dominated (<70%) by strict anaerobes (Baumgartner and Falkler, 1991).

In general, a mixed flora of bacteria has been isolated in the various studies. Epidemiologic studies have shown that more than 200 different microbial species can be found in infected root canals, usually in combinations of 4 to 7 species per canal (Baumgartner and Falkler, 1991; Munson et al., 2002; Sundqvist, 1992; Sundqvist, 1994, Gomes et al., 2013). Theoretically, any one of these species would have the potential to be an endodontic pathogen. Studies (Haapasalo, 1989; Sundqvist et al., 1985) have suggested that several *Bacteroides* species are more likely to be involved rather than just one species. Obviously, the organisms that do eventually become involved in root canal infections have to survive a harsh selection process. Although finding several different species at once is common, it is usual for one or two of the species to dominate the mixture.

Debelian et al. (1992) recovered *Propionibacterium acnes* from root canals and blood samples taken during and after patient treatment. *Streptococci mutans* was reported to be non-cariogenic in mono-infected gnotobiotic rats by Watts and Paterson (1992) but the same organism was associated with extensive periradicular inflamemation 28 days after the creation of untreated pulpal exposures.

Sato et al. (1993) investigated the bacterial composition of necrotic pulps of human teeth by sampling the split surfaces of freshly extracted teeth and culturing for microorganisms using reliable anaerobic techniques. Of the

276 bacterial isolates, 251 (91%) were obligate anaerobes. The genera *Peptostreptococccus* (25%), *Propionibacterium* (19%), *Eubacterium* (17%) and *Fusobacterium* (13%) were most commonly recovered. *Bifidobacterium* (2%), *Lactobacillus* (1%), *Actinomyces* (1 %) and *Veillonella* (0.7%) were also recovered. The microflora of necrotic pulps of human deciduous teeth was, in the authors' conclusions, similar to that reported for the deep layers of dentinal lesions of adults.

Studies recognizing synergy or a positive correlation between species are also available. Simonson et al. (1992) reported a highly significant synergistic relationship between *Treponemadenticola* and *Porphyromonas (Bacteroides) gingivalis*, whereas Sundqvist (Sundqvist, 1992) found strong positive correlations between *Fusobacterium nucleatum* and *Peptostreptococcus micros* and *Porphyromonas (Bacteroides) endodontalis*, *Selenoomonas sputigena* and *Wolinella recta*, *E. feacalis* and *Fusobaccterium nucleatum*. Recently, Ribeiro et al. (2011) investigated the bacterial diversity in untreated asymptomatic teeth (n = 12) exhibiting periapical lesions, by 16S ribosomal-RNA (rRNA) sequence analysis. They demonstrated, that despite being highly diverse, the microbiota of primary endodontic infections is mostly represented by members of the phylum *Firmicutes* belonging to the class *Clostridia* followed by the phylum *Bacteroidetes.* Using checkerboard DNA-DNA hybridization, Sassone et al. (2012) showed differences between the composition of the microbiota in cases with exposed and unexposed pulp space. The species found in higher counts in exposed pulp space cases were *Eubacterium saburreum, Fusobacterium nucleatum* ssp. *vincentii, Tannerella forsythia, Enterococcus faecalis, Neisseria mucosa, Campylobacter gracilis* and *Prevotella nigrescens*, while in unexposed pulp space cases they were *F. nucleatum* sp. *vincentii, N. mucosa, E. faecalis, E. saburreum, C. gracilis,* and *P. gingivalis, F. nucleatum* sp. *vincentii, Campylobacter sputigena, Capnocytophagashowae, Treponemasocrenskii, Porphyromonas endodontalis, Eikenellacorrodens and Capnocytophagaochracea*.

In addition, unidentified spirochetes have been found in necrotic root canals using microbiological methods, darkfield and transmission microscopy, and molecular techniques (Brown and Rudolph, 1957; Dahle et al., 1993; Hampp, 1957; Kantz and Henry, 1974; Nair et al., 1990, 2005; Nair, 1997; Thilo et al., 1986).

Recent studies have revealed a possible role for fungi and viruses in endodontic infections (Baumgartner et al., 2000, Gomes et al., 2010). The incidence of yeasts cultured from primary endodontic infections varies from 0.5-61.5% (Akdeniz et al., 2002; Goldman and Pearson, 1969) depending on the culturing method. The most common species isolated were *Candida albicans*, and less frequently *Candida sake* and *Rodotorula mucilaginosa* (Egan et al., 2002). The occurrence of herpes viruses in periapical lesions has been investigated (Sabeti et al., 2003; Hernadi et al., 2013). Herpes simplex virus infection demonstrated no relationship with periapical disease. Periapical lesions harboring cytomegalovirus / Epstein-Barr virus dual infection tended to show elevated occurrence of anaerobic bacteria, which is symptomatic, and exhibit large size radiographic bone destruction (Sabeti and Slots, 2004).

DISTRIBUTION OF ENDODONTIC MICROORGANISMS

It is evident that bacteria can inhabit not only the main root canal, but also enter the dentin tubules, apical canal ramifications, isthmuses and other morphological irregularities of the root. A number of studies have shown that invasion of bacteria into dentin tubules occurs in 60-90% of teeth with apical periodontitis (Matsuo et al., 2003; Peters et al., 2001). There are also suggestions that bacteria found in the dentin tubules are special and unique in comparison with the microflora of the oral cavity. Existing knowledge about the ability of different species to invade dentin shows that such species as Gram-positive facultative *cocci*, *lactobacilli* and *Actinomyces* are more often found as invaders among other bacteria species. Obviously, the environment of the tubules restricts supply of nutrients to bacterial species making their life conditions less favorable (Matsuo et al., 2003; Peciuliene et al., 2008; Peters et al., 2001).

These bacteria may reach and colonize the most apical part of the root canal and thereby be in close contact with the periradicular tissues through the apical foramen and accessory foramina. Watts and Paterson (1992) found bacteria in only a minority of sections of root canals and periradicular tissues of albino rats, with and without traumatic pulpal exposures. Walton and Ardjmand (1992) found bacterial masses at the apical foramen of induced periradicular lesions in monkeys and concluded that such masses could contaminate periradicular tissues during surgery or extraction and could give a false-positive result on microbiologic sampling.

Bacteria infecting the apical region of infected root canals are predominantly anaerobic, and this dominance seems to be directly proportional to the time of infection. In a study in monkeys, Fabricius et al. (1982a, b) aimed to evaluate the distribution of different microbial species in root canal samples after different periods of time and in different parts of the root canal system. The relative proportions of anaerobic bacteria cells increased with time and facultative bacterial cells were outnumbered when the canals were infected for 90 days or more. After 90 or 180 days of infection, 85 to 98% of the bacterial cells in root-canal samples from the apical region were anaerobic. Baumgartner and Falkler (1991) cultured the apical of 5 mm of root canals of 10 teeth with carious exposures and reported that the most prevalent species were *Prevotella intermedia/nigrescens*, *Prevotella buccae*, *Peptostreptococcus anaerobius*, and *Veillonella parvula*, all of them being isolated from half of the examined cases.

Of a total of 50 bacterial isolates, 68% were strict anaerobes, demonstrating the predominance of such organisms in this site. On the other hand, Chugal et al. (2011) investigated the bacterial communities residing in the apical portion of human teeth with apical periodontitis in primary and secondary infections, using a culture-independent molecular biology approach. They demonstrated that the apical bacterial communities in primary infections were significantly more diverse than in secondary infections and sequencing findings revealed a high prevalence of *Fusobacteria, Actinomyces species* and oral *Anaeroglobus geminatus* in both types of infection. Mean time, secondary infections contained *Burkholderiales* or *Pseudomonas* species, both of which represent opportunistic environmental pathogens.

Dougherty et al. (1998) investigated the occurrence of black-pigmented anaerobic bacteria in the apical and coronal segments of infected root canals and found these bacteria in 12 of 18 cases (67%). *Prevotella nigrescens* was isolated from 9 of 12 apical segments, *Prevotella melaninogenica* from 3 of 12, *P. intermedia* from 1 of 12, and *P. gingivalis* from 1 of 12. Siqueira et al. (2004) investigated the prevalence of 11 selected putative endodontic pathogens in the apical third of infected root canals associated with periradicular lesions using a nested polymerase chain reaction (PCR) assay. Their results showed *Pseuramibacteralactolyticus* in 6 (26%), *Fusobacterium nucleatum* in 6 (26%), *Porphyromonas endodontalis* in 4 (17%), *Filifactor alocis* in 2 (9%), *Dialister pneumosintes* in 1 (4%), *P. gingivalis* in 1 (4%) and *Tannerella forsythensis* in 1 (4%). No sample yielded *P. intermedia*, *P. nigrescens*, or *Campylobacter rectus*. Of the samples examined, 17 were positive for at least 1 of the target species. Occurrence of these bacterial species in the apical third of infected root canals suggests that they can be involved in causation of periradicular lesions (Siqueira et al., 2004). Similar bacterial taxa were also demonstrated in the apical root canal system of seventeen extracted teeth with attached apical periodontitis lesions (Rocas et al., 2010). The most prevalent taxa in the apical root canal system were *Olsenellauli* (76.5%), *Prevotella baroniae* (71%), *P. endodontalis* (65%), *Fusobacterium nucleatum* (53%) and *T. forsythia* (47%). While *Streptococcus* species were more prevalent in middle/coronal samples.

Therefore, it can be concluded that bacterial profile differs according to the type of endodontic infections (primary versus secondary) and the level of the root canal system (coronal/middle/apical).

BACTERIAL SAMPLING AND COLLECTION METHODS

Precise identification of microorganisms participating in the pathogenesis of apical periodontitis is important in order to understand the disease process and to provide effective antimicrobial treatment. Traditionally, endodontic bacteria have been studied by means of cultivation-based techniques, which rely on isolation, growth and laboratory identification, by morphology and biochemical tests. For a long time, culturing and serial dilution methods were considered as standard methods used in research (Peciuliene et al., 2008). However, these methods have been demonstrated to have several limitations when it comes to microbiological diagnosis (Relman and Falkow, 1992). During the past decade, the analysis of endodontic microbiota experienced a shift from culture-based to molecular approaches. Nevertheless, it is important to differentiate between the two methods, culturing measures viable bacterial cells as colony-forming units while molecular methods measure nucleotide sequences and viable microorganisms are not required. The molecular method allows amplification of very minute quantities of DNA to detectable levels. High-throughput DNA sequencing methods have provided a deeper understanding of the oral microbiota and identified putative endodontic pathogens that were not previously found with culture-based methods. In endodontics, pyrosequencing has been used to elucidate the bacterial diversity in necrotic root canals. Li et al. (2010) demonstrated that a 600-foldincrease in the depth of coverage can be obtained with pyrosequencing when compared with traditional Sanger capillary sequencing. Siqueira et al. (2011) revealed a high degree of bacterial diversity in resected root tips of necrotic teeth by using pyrosequencing. Ozok et al. (2012) identified 606 taxa (species or higher taxon) in infected root canals, representing 25 microbial phyla or divisions. Whereas, Saber et al. (2012) identified 35,731 high-quality DNA sequences belonging to 10 bacterial phyla and 73 bacterial genera from seven symptomatic periapical lesions.

TREATMENT OF PERIAPICAL DISEASE

The elimination of endodontic infection is different from elimination and control of most other infections in the human body. Because of the special anatomic environment in the root canal and tooth, host measures that are sufficient to eliminate the infectious organisms in other sites do not suffice for complete elimination of endodontic infections. Therefore, control of an endodontic infection is a concerted effort by several host and treatment factors. Success in all aspects of this cooperation will eventually result in elimination of the infective microorganisms and healing of apical periodontitis. Studies have shown that when the root canals are properly instrumented, disinfected, and obturated, success rate of endodontic treatment was approximately 80-90% for teeth with periapical periodontitis (Sunde et al., 2002). Although, chemo mechanical cleaning and shaping is effective in spread of infection and provide symptom relief in case of apical periodontitis, systemic antimicrobial therapy might be useful for preventing the spread of pathogens to other

anatomic sites in high-risk patients. *E. faecalis* should be the target of antibiotic therapy in cases of secondary infection, whereas streptococci and strict anaerobes should be targeted in cases of primary infection (Skucaite et al., 2010).

Studies have shown that bacterial infection can be substantially reduced by standard intracanal procedures, however, it is difficult to render the canal free of bacteria as it may survive and recolonize the root canal space and this may become a focal source of persistent infection (Chivatxaranukul et al., 2008; Rocas and Siqueira, 2010, 2011). In addition, the complex anatomy of root canal system including accessory canals, grooves, and isthmuses will not allow the direct access of disinfectants during bio-mechanical preparation. Therefore, supplementary ap-proaches to reduce the load of the intra-canal microbes were suggested. These include mechanical agitation of the irrigant solution, photodynamic therapy and cold plasma therapy (Balto, 2008; Al-Madi and Balto, 2008; Ng et al., 2011; Silva et al., 2012; Pan et al., 2013).

ACKNOWLEDGEMENT

The authors express their appreciation to Dr. Sumaya Basoudan for her great efforts during the preparation of this review article.

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