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The Microbial Challenge to Pulp Regeneration

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ABSTRACT

Pulp regeneration is considered in cases where the dental pulp has been destroyed because of microbial irritation. Diverse oral and food-borne micro-organisms are able to invade the pulp space, form biofilm on canal walls, and infiltrate dentinal tubules. Prior to pulp regeneration procedures, the pulp space and dentinal walls need to be sufficiently disinfected to allow for and promote regeneration. The necessary level of disinfection is likely higher than that accepted for traditional endodontic therapy, because in traditional techniques the mere lowering of bacterial loads and prevention of bacterial access to periapical tissues is conducive to healing. Moreover, several of the non-specific antimicrobials used in traditional endodontic therapy may cause significant changes in remaining dentin that interfere with its inherent potential to mediate regeneration. Non-specific antimicrobials also suppress all microbial taxa, which may allow residual virulent micro-organisms to preferentially repopulate the pulp space. Therefore, it is important for endodontic pathogens to be studied by molecular methods that allow for a broad depth of coverage. It is then essential to determine the most effective protocols to disinfect the pulp space, with minimal disruption of remaining dentin. These protocols include the topical use of effective antibiotics, including newer agents that have demonstrated efficacy against endodontic pathogens.

THE CLINICAL INDICATIONS FOR PULP REGENERATION

The dental pulp frequently succumbs to irreversible disease caused by caries, trauma, congenital abnormalities, or consequences of previous dental procedures. Endodontic therapy is generally regarded as an effective and definitive treatment for the irreversibly diseased pulp, with favorable short- and long-term outcomes. However, endodontic prognosis studies have conclusively shown that the presence of pre-operative infection, generally determined by lack of response to vitality tests and the presence of a radiolucent periapical lesion, reduces the long-term outcomes of treatment by about 15-20%, compared with cases with vital pulp (de Chevigny *et al*., 2008; Ng *et al*., 2008). While the prognosis of endodontic treatment in these cases remains favorable, the situation is more complicated in cases with young immature teeth. Root canal instrumentation, disinfection, and sealing are more technically difficult to perform in these cases. Perhaps more importantly, the tooth remains weak and is therefore susceptible to fracture due to functional stresses or minor trauma, an outcome that has been determined to range from 28-77%, depending on the degree of root maturation (Cvek, 1992). It is not known if some of the weakness in tooth structure described in this study is related to the long-term use of calcium hydroxide (Andreasen *et al*., 2002), or if it is merely related to weakness of the tooth structure, which is incompletely developed. This distinction is important, given that calcium hydroxide is rarely used long-term today, because of the availability of apexification techniques that rely on the use of an apical biodegradable matrix together with an MTA plug, with reasonable success rates reported in limited preliminary studies (Pace *et al*., 2007; Simon *et al*., 2007). It has also been shown that, in cases with an immature apex, the success rate of MTA apexification after a mean of 31 months in teeth without pre-operative lesions is 100% compared with 78% for cases with lesions (Mente *et al*., 2009), indicating that infection is still a major cause of failure. Some strengthening of the root with composite materials (Lawley *et al*., 2004) may improve the outcomes, but this remains to be evaluated.

In the initial phases of pulpal inflammation, when the immunological responses are still intact and the bacterial invasion of the tissues is minimal, several vital pulp procedures in young immature teeth have been shown to be effective, thus maintaining the radicular pulp's ability to continue tooth formation (Cvek, 1978; Witherspoon *et al*., 2006; Bogen *et al*., 2008; Bjørndal *et al*., 2010). However, following pulp necrosis, an infection spreads throughout the tissue and may extend into the periapical region, creating an environment that is not conducive to tissue regeneration without substantial disinfection procedures. It is also noteworthy to mention here that, in cases of traumatic luxation or avulsion of teeth with immature pulp, the tissue is devitalized by the acute injury. If treatment is rendered shortly after the injury, before the infection is established in the pulp space, studies have shown that the outcome of pulp revascularization with continued formation of the root apex is about

Key Words

pulp necrosis, pulp regeneration, endodontic pathogens, endodontic irrigants, topical antibiotics, microbiota.

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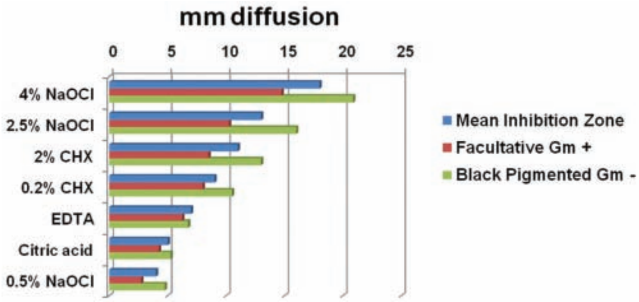

Figure 1. Comparison between common non-specific antimicrobials against 6 common endodontic pathogens: three Gram-positive and three Gram-negative bacteria. Bars show (in millimeters) inhibition zones in agar plates. Note increased efficacy among Gram-negative compared with Gram-positive bacteria. Gm: Gram. Data from Siqueira *et al.* (1998).

33% for intrusion or tooth avulsion (Andreasen *et al*., 1995a,b, 2006).

The concept of regenerating the dental pulp following pulp necrosis is not novel. Older studies have attempted to create a clean environment in the pulp space that supports the growth of healing or repair tissue from an induced blood clot with or without a collagen gel matrix (Nygaard-Østby and Hjortdal, 1971; Nevins *et al*., 1978; Nevins and Crespi, 1998). Because investigators have recognized the ineffectiveness of traditional disinfection methods in the infected dental pulp, recent case reports have proposed the use of topical antibiotics for root canal disinfection to take advantage of their efficacy and biocompatibility (Banchs and Trope, 2004; Petrino *et al*., 2010).

PATTERNS OF ROOT CANAL INVASION BY MICROBIAL PATHOGENS

It has been known for several decades that bacterial invasion of the root canal system results in the formation of a bacterial biofilm on canal walls, in complex anatomical recesses, inside the dentinal tubules, and in the immediate periapical region. Bacteria in biofilms are more resistant to disinfection procedures, because the biofilm matrix is less permeable to disinfectants or antibiotics, the bacterial cells are not in active reproduction, and the community may function as a unit in sustaining replication, metabolism, and shedding of bacterial cells to maintain the infection. Bacterial cells are smaller in diameter than dentinal tubules at the pulpal end, and therefore, bacteria penetrate more deeply into the tubules of infected teeth (Peters *et al*., 2001). In this location, they are protected from therapeutic antimicrobials in the root canal. It has also been recently shown that bacterial infiltration is deeper and affects more dentinal tubules in teeth from young, compared with old, patients (Kakoli *et al*., 2009).

Clinical and animal studies have shown that residual bacteria are key determinants of healing following endodontic treatment (Sjögren *et al*., 1997; Fabricius *et al*., 2006). Another clinical study has shown that contemporary clinical endodontic procedures are ineffective in completely eliminating bacteria that can be visualized in anatomical recesses or dentinal tubules of infected teeth (Nair *et al*., 2005).

When regenerative procedures of the dental pulp are being considered following an infection, the traditional level of root canal disinfection may not be sufficient. In most clinical endodontic cases, following chemo-mechanical disinfection of the root canal space, a well-condensed root filling is placed. Thus, if residual bacteria are left behind, their numbers are thought to be minimal, and the root filling is thought to minimize their access to sustain the periapical lesion, thus creating an environment conducive to healing in most cases. In fact, it was shown that the quality of the filling is critical only if residual bacteria are present (Fabricius *et al*., 2006). In contrast, if regeneration of the pulp is contemplated, it is likely that the disinfection procedures may need to be performed to a higher level of efficacy than is necessary in clinical endodontics. The lack of filling in the canal as the regenerative tissue is developing may be conducive to bacterial proliferation. Thus, it is necessary to maintain an aseptic environment in the pulp space following disinfection procedures for a longer period of time, to allow the new tissue sufficient time to establish itself in the root canal environment. Finally, while the emphasis in regenerative procedures is clearly on resumption of mineralization to achieve completed root formation and maturation of the tooth, it is essential that the regenerative tissue contain the elements of a robust immune system that allows it to clear any residual infection and combat any coronal, tubular, or periapical sources of new infection.

INEFFICACY AND POTENTIAL DETRIMENTAL EFFECTS OF TRADITIONAL NON-SPECIFIC DISINFECTANTS

In the treatment of endodontic infections, contemporary endodontic techniques focus on suppression of bacterial counts, through the use of mechanical shaping procedures to expose microbial niches combined with irrigation and medication with generic antimicrobial agents. In the following discussion, a review of contemporary disinfectants will be presented, with the recognition that most of these studies were performed in teeth with a mature apex, rather than on immature teeth, which are more relevant here.

The most effective agent available today appears to be sodium hypochlorite at 5.25-6% or 2.5-3% strengths (Siqueira *et al*., 1998; Fouad and Barry, 2005) (Fig. 1). However, several clinical trials have shown that this agent is effective in eliminating cultivable micro-organisms from infected root canals in only about 40-60% of cases (Byström and Sundqvist, 1985; Shuping *et al*., 2000). The small volume of hypochlorite available in the canal, its lack of permeation of anatomical recesses or dentinal tubules, or the buffering effect of dentin may contribute to this limited effectiveness of hypochlorite. When hypochlorite has been considered for regeneration procedures, concerns have been raised that it may interfere with the ability of the regenerated pulp tissue to re-attach to the dentin surface (Ring *et al*., 2008), or denature proteins of the dentin matrix which may promote tissue regeneration (Casagrande *et al*., 2010). Sodium hypochlorite may be toxic to highly proliferative cells in the apical tissues, which may play an important role in pulp regeneration, such as the stem cells of the apical papilla. Finally, sodium hypochlorite is a very unstable molecule, and does not have a lasting effect in the root canal environment, and so cannot be used to maintain an aseptic environment for regeneration to occur. For these reasons, it is not likely to be the most effective antimicrobial agent for pulp regeneration. Whether hypochlorite can be used initially because of its strong antimicrobial properties, then inactivated with 5% sodium thiosulphate, remains to be determined.

Chlorhexidine is another disinfectant commonly used in clinical endodontics. Clinical studies have shown that it is effective when used with 1% hypochlorite solution (Zamany *et al*., 2003), and *in vitro* studies have shown superiority compared with calcium hydroxide usage or controls (Cook *et al*., 2007). However, other studies have not corroborated its absolute efficacy as an irrigant or a medicament between appointments (Manzur *et al*., 2007; Paquette *et al*., 2007; Tervit *et al*., 2009). Chlorhexidine also interfered with cell attachment to dentin *in vitro*, although not to the same extent as hypochlorite (Ring *et al*., 2008).

Ethylene diamine tetraacetic acid (EDTA) is another agent used in contemporary clinical endodontics. As a chelating agent, EDTA in combination with hypochlorite is effective in removing the smear layer generated during root canal instrumentation procedures, which presumably results in better cleaning, and allows irrigants and medicaments to penetrate more deeply into dentinal tubules (Saif *et al*., 2008). EDTA has weak antimicrobial properties by itself compared with hypochlorite or chlorhexidine (Siqueira *et al*., 1998) (Fig. 1). However, perhaps more importantly in the context of pulp regeneration, is EDTA's property of releasing bioactive growth factors that may be sequestered in dentin matrix, and which may aid in the proliferation of the regenerated pulp tissue (Graham *et al*., 2006).

The topical use of antibiotics in endodontic infections is not novel; the reader is referred to Fouad (2002) for a review on the subject. However, in recent years this practice has not been popular in endodontics treatment because of the availability of less costly alternatives, and the apparent success of treatment. A series of studies identified 3 effective antibiotics against root canal bacteria that did not include beta lactams, to which many patients are allergic or could become allergic (Sato *et al*., 1993; Hoshino *et al*., 1996). The so-called triple antibiotic mix, which consists of ciprofloxacin, minocycline, and metronidazole, has been used more recently in several reports in which pulp revascularization has been documented following pulp necrosis. The purported efficacy of this mix is related to the synergistic action of antibiotics with various spectra and efficacies to suppress endodontic flora.

OUTCOME OF CURRENT CLINICAL REVASCULARIZATION TECHNIQUES

As noted before, several reports have been recently published showing revascularization or revitalization of the dental pulp, following documented evidence of pulp infection. [For a detailed report of current procedures, see Hargreaves and Law (2010).] In addition to traditional non-specific endodontic disinfectants, these reports have documented the use of the triple antibiotic mix (Banchs and Trope, 2004; Petrino *et al*., 2010), calcium hydroxide (Chueh and Huang, 2006), or formocresol (Shah *et al*., 2008). A retrospective outcome study examined the root development in thickness and in length in the recently reported cases (Bose *et al*., 2009). This study reported that the triple antibiotic mix and calcium hydroxide were effective in increasing dentin thickness and root length compared with controls, but formocresol did not achieve the same effect. The authors further concluded that calcium hydroxide was effective in extending root length only if it were placed short of the working length of the root, suggesting that as the material got closer to the apical tissues, where many of the potential progenitor cells are located, it may interfere with the revascularization process.

While these results show a favorable clinical outcome, in that the infection appeared to be controlled, and the root increased in length and thickness on post-operative radiographs, the degree to which microbial presence in the canals was actually suppressed, and the histological nature of the regenerative tissue were not evident from these studies. Clearly, the optimal outcome should be the regeneration of pulp tissue capable of forming dentin, which would add to the strength of the tooth. This new pulp tissue would also allow components of the immune system to resist future re-infection, as stated before. A series of reports from two controlled studies has been reported in which clinical revascularization procedures similar to those described before were performed after the induction of pulp necrosis and apical lesions in dogs. The efficacy of the triple antibiotic mix in eliminating cultivable bacteria from the root canals was reported to be 70-78% (Windley *et al*., 2005; Cohenca *et al*., 2010). While this may be an improvement over traditional disinfection, culturing of bacteria following antibiotic application may be problematic, since bacteria may be in the viable but uncultivable state, and the antibiotics are difficult to inactivate in the culture medium (Fig. 2) (Fouad and Barry, 2005). The histological reports from these studies reported that the mineralized tissue observed histologically at 90 days post-operatively consisted of bone or cementum, and the soft tissues were periodontal ligament or inflamed connective tissues. No new pulp tissues were demonstrated (Thibodeau *et al*., 2007; da Silva *et al*., 2010; Wang *et al*., 2010). The revascularization techniques described in these studies do not use the classic tissue-engineering triad, which consists of stem cells, scaffolds, and growth factors. They have also utilized some disinfectants that could interfere with tissue regeneration. Finally, the root canal disinfection in these experiments was likely less than optimal, and so the results appear to be a compromise that may not fulfill the objectives of true pulp regeneration.

FUTURE DIRECTIONS AND CHALLENGES IN CONTROLLING ROOT CANAL MICROBIAL INFECTION

Controlling the infection inside the pulp space is a critical initial step in pulp regeneration. The use of traditional non-specific antimicrobial agents appears to be limited in controlling the

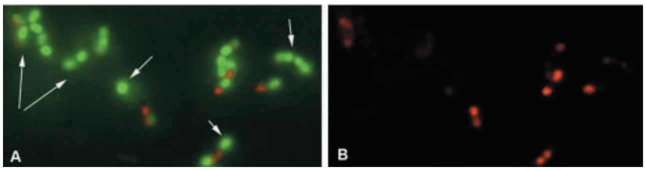

Figure 2. *Enterococcus faecalis* bacteria that have been incubated with tetracycline (25 mg/mL) for 1 hr. The bacterial cells could no longer be grown in culture, but bacterial cells remained viable, as shown in the BacLight stain. **(A)** Viable and dead cells; **(B)** dead cells. Arrows show viable cells (Fouad and Barry, 2005: figure reprinted with permission).

infection. Novel technologies that allow for better permeation and irrigation of the pulp space, such as the EndoVac system (Discus Dental, Culver City, CA, USA) have shown promising initial findings (Cohenca *et al*., 2010). Strategies that involve combining traditional antimicrobials with ultrasonic energy have also shown better permeation of complex anatomical recesses in the root canal system, and may disrupt the biofilm (Burleson *et al*., 2007; Carver *et al*., 2007). However, agents like hypochlorite and chlorhexidine may have a harmful effect on the regenerative tissue attachment to the dentin wall, or on the release of growth factors from dentin that aid in regeneration. The photodynamic effect, which involves the use of a light of specific wavelength and a dye that releases oxygen radicals at this wavelength, has shown initial promising effects in root canal infections (Fimple *et al*., 2008; Pagonis *et al*., 2010). Perhaps the ultrasonic activation of the dye may enhance the antimicrobial effects of the photodynamic effect.

The triple antimicrobial mix is clearly not effective against many root canal micro-organisms. Furthermore, because of variability of endodontic microflora, the selection of an antibiotic to use may be dictated by the patient's condition and the sensitivity profile of identifiable flora within the canal. Root canal culturing is ineffective in these circumstances, at least by itself, because almost half of the bacteria in the root canal are not cultivable, and because culturing is not a realistic technology in the dental office. Therefore, molecular identification methods, including screening for antibiotic resistance genes, would potentially allow for more appropriate selection of topical antibiotics. Finally, the use of novel antibiotics with broad spectra and enhanced bactericidal activities should be further investigated.

The research into the identity and virulence of root canal pathogens is also necessitated by the fact that non-specific antimicrobial activities may temporarily suppress but not eliminate the majority of microbial taxa, which may allow residual virulent micro-organisms to preferentially repopulate the pulp space and destroy the regenerating tissue. Hundreds of microbial taxa have been identified in endodontic infections (Fouad *et al*., 2008; Siqueira and Rôças, 2009). Therefore, efficient identification and effective targeting with antimicrobials are needed to allow for regenerative procedures. Novel methods of analysis, such as 454 pyrosequencing, may aid in this analysis (Li *et al*., 2010). These methods allow for greater depth of coverage, thus facilitating the identification of important taxa that may contribute to the virulence of the microbial community, and have important virulence determinants themselves, but are otherwise undetectable by traditional methods because of their relatively low abundance. Many of the pathogenic bacteria may constitute residual flora following therapeutic procedures that compromise the long-term success of therapy. In addition, metagenomic analyses of microflora will become necessary in the identification of key virulence determinants or expressed virulence proteins in these complex microbial communities. An example in this regard would be the use of microarrays that identify antibiotic resistance genes which may aid in rapid screening of samples for selection of optimal antibiotics.

Clearly, for pulp regeneration to be successful when an infection has developed, there should be focused efforts that combine the proper disinfection of the environment to receive the regenerative tissue with judicious, biologically sound, tissue-engineering strategies. This involves actions on multiple fronts that eventually interface to effect clinically successful outcomes.

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