Disinfection Efficacy of Current Regenerative Endodontic Protocols in Simulated Necrotic Immature Permanent Teeth

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Abstract

Introduction: The lack of mechanical debridement and reduced concentrations suggested for chemical debridement to maintain stem cell viability call into question the disinfection efficacy of current regenerative protocols. Current protocols vary in the concentration and type of antibiotic medicaments used. The aim of this study was to determine if simulated immature teeth infected with *Enterococcus faecalis* can be completely disinfected by following current standardized regenerative protocols and to evaluate the probable effects of residual bacteria on stem cell toxicity. **Methods:** Sixty-eight caries-free maxillary incisors were used. S1 sampling protocols were validated in both negative and positive control groups via culture, scanning electron microscopy, and confocal laser scanning microscopy. All teeth, except the negative controls, were inoculated with *E. faecalis*. The teeth were divided into the following groups: group 1, triple antibiotic paste (ciprofloxacin:metronidazole:minocycline) at concentrations of 10, 1, and 0.1 mg/mL; group 2, double antibiotic paste (ciprofloxacin:metronidazole) at concentrations of 10, 1, and 0.1 mg/mL; group 3: Ultracal XS calcium hydroxide (Ultradent, St Louis, MO); and controls, negative and positive controls. Current regenerative protocols recommended by the American Association of Endodontists were followed. S2 sampling was performed after 4 weeks and tested for bacterial presence via culturing, scanning electron microscopy, and confocal laser scanning microscopic analysis. **Results:** The data showed that calcium hydroxide and the current recommended antibiotic concentrations are not capable of completely eliminating bacteria from simulated necrotic immature permanent teeth. **Conclusions:** Overall, this study focuses on the need to re-evaluate the balance between stem cell toxicity and bacterial elimination in order to determine the appropriate concentrations and medicaments for successful regenerative endodontic procedures. *(J Endod 2016;42:1218–1225)*

Key Words

Antibiotic paste, bacteria, calcium hydroxide, regenerative endodontics

Significance

This study demonstrates the importance of adequate disinfection of the root canal system during regenerative endodontic procedures. It also outlines the antibiotic concentration that would be effective in disinfection and possibly maintain the viability of the SCAPs with minimal staining.

Treatment of young permanent necrotic teeth is challenging because of an incompletely developed root, which is difficult to fully debride, with an increased risk of a subsequent fracture because of the thin dentinal walls (1). Therefore, research studies looking into different methodologies that would regenerate the pulp-dentin complex and stimulate root closure and increase dentin thickness have been ongoing since the 1960s. During the past 30 to 50 years, Nygaard-Ostby and Schilder (2) and others have reported a series of preclinical studies and case studies on patients attempting to regenerate pulplike tissue in teeth with either vital or nonvital diagnoses. Connective tissue was shown to grow as much as several millimeters into the apical portion of the root canal system in necrotic teeth (3). The results were variable, and histologic analysis failed to reveal regeneration of a functional pulp-dentin complex. Previous reports have shown growth of the periodontium into the root canal (4). Other studies have shown cementum-, bone-, and periodontal ligament–like tissue or inflamed vital tissue formed after regenerative endodontic procedures (5–7). This lack of outcome is not surprising given the materials, instruments, medications, and knowledge base available at the time.

Trauma research has shown that immature teeth with apices larger than 1.1 mm and shorter pulp lengths have improved chances of revascularization (8). Several case reports have shown resolution of apical periodontitis and an increase in both root length and width (9), but the evidence supporting the predictability of regenerative endodontic procedures has been limited (10). Several groups have published preclinical research or case reports related to regenerative endodontic procedures to provide a better understanding of the procedure and outcomes (11).

Regenerative endodontics uses a triad of factors, namely cells like the stem cells of the apical papilla (SCAP), growth factors, and a scaffold (11). Another important factor is disinfection of the root canal space because it is well understood from the endodontic literature that elimination of bacteria from the root canal system is necessary for pulpal and periapical healing (12). Previous studies have shown that the absence of bacteria is

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critical for successful revascularization because the new tissue will stop at the level it meets bacteria in the canal space (13). However, chemical debridement of the canal system alone must be relied on because more traditional mechanical debridement is limited in immature teeth with thin dentinal walls and open apices. Previous studies have reported that instrumentation with sodium hypochlorite (NaOCl) is not sufficient to reliably create the conditions necessary for revascularization of the infected necrotic tooth (14). More recent studies have reported the use of the triple antibiotic paste (TAP) developed by Hoshino et al (15, 16) and its effectiveness in disinfection of the infected necrotic tooth, setting the conditions for subsequent revascularization. A preclinical study on dogs reported that the intracanal delivery of a 20-mg/mL solution of the TAP resulted in a greater than 99% reduction in mean colony-forming unit (CFU) levels, with approximately 75% of the root canal systems having no cultivable microorganisms present (17). Other studies have reported the use of calcium hydroxide (Ca $[OH]_2$) during these procedures (18).

The American Association of Endodontists (AAE) Regenerative Committee has established a standardized protocol for regenerative endodontic procedures. The protocol includes chemical debridement of the root canal system with 20 mL 1.5% NaOCl followed by placement of either double antibiotic paste (DAP), TAP, or $Ca(OH)_2$. However, a recent study by Ruparel et al (19) highlighted the toxicity that various concentrations of antibiotic paste have against human SCAPs. The results of the study supported using $Ca(OH)_2$ or a lower concentration of antibiotic paste (0.01–0.1 mg/mL) to maintain stem cell viability. Other studies have also shown TAP and DAP at higher concentrations can alter SCAP and periodontal ligament cell survival (20, 21). Based on these data, the current regenerative endodontic protocol uses TAP or DAP at a concentration of 0.1 mg/mL. However, it is still unclear whether these concentrations were effective in disinfecting the root canal system. Hence, this calls into question the disinfection efficacy of current regenerative protocols. Therefore, the aim of this study was 2-fold: to determine whether using $Ca(OH)_2$ or DAP or TAP (0.1, 1, and 10 mg/mL) is effective in disinfecting the canal space in simulated necrotic teeth and to determine the optimum concentration that is effective in disinfecting the canal and comparing this with known concentrations that are biocompatible for SCAPs.

Materials and Methods

Culture of the SCAPs

Human SCAPs were obtained from Dr Shi's laboratory (University of Pennsylvania, Philadelphia, PA). The cells were maintained in alpha-minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum, 1% penicillin, and 1% L-glutamine (Invitrogen). Cells were incubated at 37°C in an atmosphere of 5% CO_2. Cells were used only until passage 4 because these cells show a more differentiated phenotype after passage 4 (22).

Culture of Natural Killer Cells

Natural killer cells were isolated by our collaborators as stated previously and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin, and 1% L-glutamine (Invitrogen) (23).

Chromium-51 Release Cytotoxic Assay

The chromium-51 (Cr-51) release assay was performed by our collaborators at the University of California, Los Angeles as described previously (24). Briefly, different numbers of purified natural killer cells (effector cells) were incubated with Cr-51–labeled target cells (SCAPs) at the different effector:target (E:T) ratios. After a 4-hour incubation period, the supernatants were harvested from each sample and counted for released radioactivity in units of counts per minute (cpm) using a gamma counter. The percentage specific cytotoxicity was calculated and is shown in Figure 1:

$$\%\ \text{Cytotoxicity} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}}$$

Preparation of the Teeth

Sixty-eight single-canal maxillary incisors were used in this study. Caries-free extracted central incisors were stored in diluted 0.5% NaOCl. Teeth were sectioned 3 mm above the cementoenamel junction, accessed, and prepared using K-files and ProTaper series rotary files (Dentsply International, York, PA) until an F5 reached patency. The smear layer was removed with 2 mL 6% NaOCl, 2 mL 17% EDTA, and 2 mL 6% NaOCl in each tooth under positive pressure irrigation. The teeth were placed in sterile saline until they were sterilized. Sterilization was performed using an autoclave at 240°F at 20 psi for 40 minutes with the teeth wrapped in moist 2×2 gauze (25). Teeth were then transferred to sterile vials containing 20 mL sterile brain-heart infusion (BHI) broth medium and kept in an incubator at 37°C for 48 hours to check the efficacy of the sterilization procedure.

Cultivation of *Enterococcus faecalis* and Specimen Inoculation

Pure isolated 24-hour colonies of *Enterococcus faecalis* (ATCC 29212) grown on BHI agar plates (Sigma-Aldrich, St Louis, MO) were suspended in 20 mL sterile BHI broth medium for 8 hours. Five drops of this bacteria medium were inoculated in a new 20-mL BHI broth for 4 hours. These bacteria suspensions were adjusted to match the turbidity of 1.5×10^8 colony-forming units (CFUs)/mL (equivalent to 0.5 McFarland standard). Twenty microliters of the bacterial in

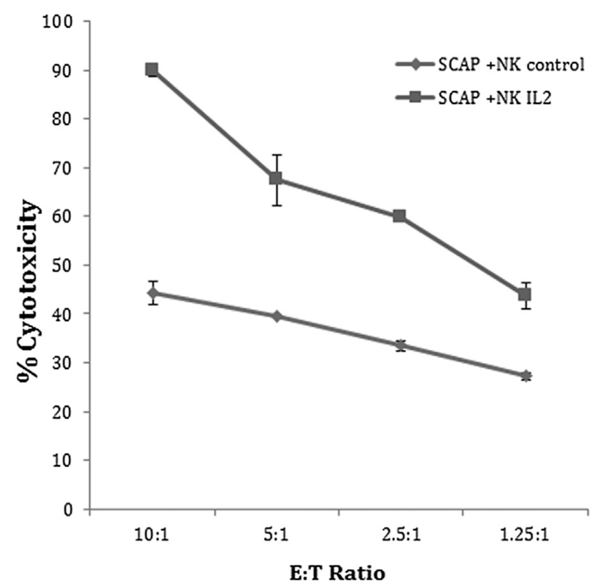

Figure 1. Increased natural killer cell cytotoxicity against the SCAPs. The Cr-51 cytotoxic assay results show that in the presence of innate immune cells SCAP cytotoxicity is increased. However, in the presence of activated innate immune cells, the cytotoxicity increases to levels as high as 90% with an E:T ratio of 10:1 and about 50% with the lowest E:T ratio of 1.25:1.

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