Archives of Oral Biology 70 (2016) 88–93

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/aob

Residual antibiofilm effects of various concentrations of double antibiotic paste used during regenerative endodontics after different application times

Daniel B. Jenks[a], Ygal Ehrlich[a], Kenneth Spolnik[a], Richard L. Gregory[b], Ghaeth H. Yassen[b,*]

[a] Department of Endodontics, Indiana University School of Dentistry, Indianapolis, IN, USA
[b] Department of Biomedical and Applied Sciences, Indiana University School of Dentistry, Indianapolis, IN, USA

ARTICLE INFO

Article history:
Received 1 March 2016
Received in revised form 9 May 2016
Accepted 6 June 2016

Keywords:
Double antibiotic paste
Calcium hydroxide
Antibacterial effect
Enterococcus faecalis
Bacterial biofilm

ABSTRACT

Objective: We investigated the residual antibiofilm effects of different concentrations of double antibiotic paste (DAP) applied on radicular dentin for 1 or 4 weeks.
Design: Dentin samples were prepared (n = 120), sterilized and pretreated for 1 or 4 weeks with the clinically used concentration of DAP (500 mg/mL), low concentrations of DAP (1, 5 or 50 mg/mL) loaded into a methylcellulose system, calcium hydroxide ($Ca(OH)_2$), or placebo paste. After the assigned treatment time, treatment pastes were rinsed off and the samples were kept independently in phosphate buffered saline for 3 weeks. Pretreated dentin samples were then inoculated with *Enterococcus faecalis* and bacterial biofilms were allowed to grow for an additional 3 weeks. Biofilms were then retrieved from dentin using biofilm disruption assays, diluted, spiral plated, and quantified. Fisher's Exact and Wilcoxon rank sum tests were used for statistical comparisons (α=0.05).
Results: Dentin pretreatment for 4 weeks with 5, 50 or 500 mg/mL of DAP demonstrated significantly higher residual antibiofilm effects and complete eradication of *E. faecalis* biofilms in comparison to a 1 week pretreatment with similar concentrations. However, dentin pretreated with 1 mg/mL of DAP or $Ca(OH)_2$ did not provide a substantial residual antibiofilm effect regardless of the application time.
Conclusions: Dentin pretreatment with 5 mg/mL of DAP or higher for 4 weeks induced significantly higher residual antibiofilm effects in comparison to a 1 week pretreatment with the same concentrations.

1. Introduction

Root canal disinfection is an indispensable step during endodontic regeneration procedures. The disinfection is usually achieved using root canal irrigation solutions such as sodium hypochlorite (NaOCl) as well as intracanal medicaments such as calcium hydroxide ($Ca(OH)_2$) or various antibiotic pastes. However, a plethora of recent in vitro evidence recommended the use of low concentrations of NaOCl (Martin et al., 2014) and antibiotic pastes (Althumairy, Teixeira, & Diogenes, 2014; Kim et al., 2015) in an attempt to create a balanced disinfection protocol that can eliminate root canal pathogens without damaging stem cells and dentin endogenous proteins within the root canal system (Labban, Yassen, Windsor, & Platt, 2014). On the other hand, recent studies suggested that $Ca(OH)_2$ is a stem cell friendly medicament (Althumairy et al., 2014; Ruparel, Teixeira, Ferraz, & Diogenes, 2012) and significantly improved the attachment of apical papillae cells to dentin (Kitikuson & Srisuwan, 2016). Both double (DAP) and triple (TAP) antibiotic pastes have been clinically used in endodontic regeneration (Nagata et al., 2014; Nevins & Cymerman, 2015) and were found to have comparable antibacterial properties against different endodontic pathogens (Sabrah, Yassen, & Gregory, 2013; Sabrah et al., 2015a). A recent study suggested that a 1 week treatment with a low concentration of DAP (1 mg/mL) loaded into a methylcellulose system as well as a clinically used concentration of $Ca(OH)_2$ were efficient in eliminating 3-week old *Enterococcus faecalis* biofilm (Tagelsir, Yassen, Gomez, & Gregory, 2016).

In endodontic regeneration, the absence of obturating material in the canal during the development of newly formed tissue may enable the residual or new pathogens to multiple and initiate a new biofilm. Therefore, it is advised to provide a higher level of disinfection in comparison to traditional endodontic treatment (Fouad & Nosrat, 2013). Additionally, the developing new tissues, regardless of their type, require adequate time to establish their

* Corresponding author at: Department of Biomedical and Applied Sciences, Indiana University School of Dentistry, 1121 W. Michigan St Indianapolis, IN, 46202, USA.
E-mail address: gyassen@iupui.edu (G.H. Yassen).

http://dx.doi.org/10.1016/j.archoralbio.2016.06.006
0003-9969/

structure within the root canal. Consequently, the bacteria-free environment should be maintained for an extended period of time in comparison to regular root canal therapy (Fouad & Verma, 2014). Therefore, disinfection during regenerative endodontics may require antimicrobial agents with considerable levels of substantivity (Fouad, 2011). Indeed, both TAP and DAP were suggested to have an extended residual antibiofilm effect after their removal (Sabrah et al., 2015b). Additionally, DAP was proposed to have longer residual antibiofilm properties in comparison to similar concentrations of TAP (Sabrah et al., 2015b).

No clear consensus is available regarding the interappointment application time of intracanal medicaments during endodontic regeneration procedures. While the minimum application time clinically reported in the literature is 1 week (Ding et al., 2009; Paryani & Kim, 2013), other studies have applied these medicaments for up to 11 weeks (Shimizu et al., 2013; Thibodeau, 2009). The American Association of Endodontists (AAE) recommended an application time of 1–4 weeks with consideration of additional treatment time in cases with persistent infection (American Association of Endodontists, 2015). The aim of this study was to investigate the residual antibiofilm effects of various concentration of DAP loaded into an aqueous methylcellulose system and applied for 1 or 4 weeks. We hypothesized that DAP exerts similar residual antibiofilm effects regardless of the concentration used or application time.

2. Materials and methods

2.1. Dentin sample preparation

Sound human permanent teeth (n = 120) were collected after obtaining Institutional Review Board approval (IRB, 1408897632). The teeth were kept in 0.1% thymol solution at 4 °C and used within 6 months. The crowns were removed using a water-cooled diamond saw and the roots were used to obtain 120 standardized dentin slabs with the dimensions of $4 \times 4 \times 1.5\ mm^3$. A Rotoforce 4 polishing unit (Struers, Cleveland, OH) was used to polish the pulpal side of each dentin specimen with abrasive papers (1200–4000 grit; Struers) under running water. Dentin slabs were then sonicated with 1.5% NaOCl (Value Bleach; Kroger, Cincinnati, OH) and 17% EDTA (VISTA, Racine, WI) for 4 min. Each sample was then wrapped with a cotton pellet saturated with sterile water, placed in Whirl-pak bags (Sigma-Aldrich, St Louis, MO), sterilized with ethylene oxide gas, and kept at 4 °C until used.

2.2. Preparation of medicaments used in the study

A total of 5 antimicrobials were investigated in the current study including clinically used concentrations of DAP (500 mg/mL), three low concentrations of DAP (1, 5 and 50 mg/mL) and $Ca(OH)_2$ (UltraCal XS, Ultradent, South Jordan, UT). The clinically used DAP was prepared by mixing 500 mg of equal portions of metronidazole and ciprofloxacin (Champs Pharmacy, San Antonio, TX) with 1 mL of sterile water (Kim et al., 2015). The low concentrations of DAP (1, 5, and 50 mg/mL) were loaded into a methylcellulose system as described in previous studies (Tagelsir et al., 2016; Yassen, Sabrah, Eckert, & Platt, 2015) to create a clinically applicable antibiotic medicament that can be injected into a root canal system (Algarni, Yassen, & Gregory, 2015). In summary, 2500, 250 and 50 mg of DAP were dissolved independently in 50 mL of sterile water. Then, 4 gm of methyl cellulose powder (Methocel 60 HG, Sigma-Aldrich, St. Louis, MO) was slowly added to each DAP solution over 120 min under maximum stirring to obtain pastes with 1, 5, and 50 mg/mL of DAP. A DAP free placebo paste were also prepared and used as a control group. No untreated positive control group was used in the current study as our earlier pilot study has shown no difference between the untreated positive control samples and dentin samples treated with the placebo paste. A recent study has also found no difference between infected dentin treated with aqueous methylcellulose based paste and that treated with normal saline (Tagelsir et al., 2016).

2.3. Treatment of dentin samples

In order to be able to precisely quantify the residual indirect antibiofilm effects of medicaments, sterilized rather than infected dentin samples were pretreated with various medicaments in the current study as described in a recent report (Sabrah et al., 2015b). Sterilized dentin slabs were placed individually in separate wells of sterile 96 well microtiter plates (Fisherbrand, Fischer Scientific) with the pulpal side (treatment side) facing upward. Samples were then randomly divided into 5 treatment groups and 1 control group (n = 20 per group). The pulpal side of each dentin slab received 200 μL of one of the treatment pastes (1, 5, 50, 500 mg/mL of DAP or $Ca(OH)_2$) or the control placebo paste. All treated samples were then incubated for 1 or 4 weeks (n = 10 per group at each time point) at 37 °C and 100% humidity. The two treatment times were selected based on the clinical endodontic regeneration procedure recommended by AAE (American Association of Endodontists, 2015). After the assigned treatment time, the treatment paste was rinsed off from each sample using 5 mL of sterile saline followed by 5 min of irrigation with 5 mL of 17% EDTA. Dentin slabs were then immersed in 200 μL phosphate buffered saline (PBS) and incubated at 37 °C for 3 weeks before growing the bacterial biofilm.

2.4. Bacterial strain and media

E. faecalis (29212; ATCC, Manassas, VA) was grown initially on anaerobic blood agar plates (Bio-Merieux, Durham, NC). A sterile broth of brain heart infusion (BHI) supplemented with 5 g of yeast extract/L (BHI-YE) was inoculated with colonies of *E. faecalis* and incubated at 37 °C with 5% CO_2 for 24 h.

2.5. Biofilm growth on treated dentin samples

After 3 weeks of immersion in PBS, dentin slabs were transferred independently into wells of sterile 96-well microtiter plates with the treated surface facing upward. Then, 190 μL of fresh BHI-YE and 10 μL of an overnight *E. faecalis* culture (10^6 CFU/mL) were added to each well. The slabs were incubated anaerobically for 3 weeks at 37 °C and the culture media was replenished 2 times a week. After incubation, each dentin slab was gently washed with sterile saline to remove unattached bacteria. One randomly selected sample from each experimental group at each time point was processed for confocal laser scanning microscopy (CLSM) and the remaining 9 samples in each group were utilized for biofilm disruption assays. A negative untreated control group was also used in the current study to confirm the absence of any bacterial contamination from outside sources within the experimental setting of this study. Briefly, untreated sterilized dentin slabs (n = 3) were individually placed in 200 μL of bacteria-free BHI-YE media and incubated for 3 weeks under the same anaerobic conditions described earlier with regular replacement of BHI-YE. After 3 weeks, biofilm disruption assays were performed to confirm the lack of any bacterial biofilm in the uninfected dentin slabs.

2.6. Biofilm disruption assay

The biofilm disruption assays were conducted as described in recent studies (Sabrah et al., 2015b; Tagelsir et al., 2016). Briefly, dentin slabs were placed into sterile test tubes containing 2 mL of sterile saline. To detach the bacterial biofilm, samples were