



# The antimicrobial properties, cytotoxicity, and differentiation potential of double antibiotic intracanal medicaments loaded into hydrogel system

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Received: 1 February 2018 / Accepted: 20 June 2018 / Published online: 25 June 2018  
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## Abstract

**Objective** This study evaluated the antimicrobial properties, cytotoxicity, and mineralization potential of methylcellulose hydrogels loaded with low concentrations of double antibiotic pastes (DAP).

**Materials and methods** The direct and residual antibacterial effects of 1, 5, and 10 mg/mL of DAP loaded into hydrogels as well as calcium hydroxide (Ca(OH)<sub>2</sub>) were tested against single-species biofilms of *Enterococcus faecalis* and dual-species biofilms (*Enterococcus faecalis* and *Prevotella intermedia*). The effects of DAP hydrogels on proliferation and mineralization of dental pulp stem cells (DPSC) were tested using MTT assays, alkaline phosphate activity (ALP), and alizarin red staining. Fisher's exact tests, Wilcoxon rank sum tests, and one-way ANOVA were used for statistical analyses ( $\alpha = 0.05$ ).

**Results** All tested concentrations of DAP hydrogels as well as Ca(OH)<sub>2</sub> demonstrated significant direct antibacterial effects against single- and dual-species biofilms. However, only 5 and 10 mg/mL of DAP hydrogels exhibited significant residual antibacterial effects against both types of tested biofilms. Only 1 mg/mL of DAP hydrogels did not have significant negative effects on DPSC viability, ALP activity, and mineralization nodule formation. However, 5 and 10 mg/mL of DAP hydrogels caused significant negative effects on cytotoxicity and mineralization nodule formation of DPSC.

**Conclusions** Hydrogels containing 1 mg/mL DAP offered significant direct antibacterial effects against single- and dual-species biofilms without causing significant negative effects on viability, ALP activity, and mineralization nodule formation of DPSC.

**Clinical relevance** The methylcellulose-based hydrogel proposed in this study can be used clinically as a biocompatible system to deliver controlled low concentrations of DAP.

**Keywords** Antimicrobials · Cytotoxicity · Double antibiotic paste · Hydrogels · Mineralization

## Introduction

Endodontic regeneration is currently the treatment of choice to manage immature permanent teeth with necrotic pulps. Recent

clinical trials suggested that this procedure can improve healing of the periapical area as well as increase the length and width of radicular dentin [1, 2]. Root canal disinfection utilizing irrigation solutions and intracanal medicaments is a fundamental step during endodontic regeneration [3]. Numerous in vitro and in vivo studies have been proposed to optimize the disinfection procedure during endodontic regeneration [4]. The optimization techniques have been focused on maintaining the survivability and function of the stem cells of apical papillae (SCAP) in the periapical area [5, 6].

Calcium hydroxide (Ca(OH)<sub>2</sub>) or various antibiotic mixtures were proposed as intracanal medicaments during endodontic regeneration [7]. Ca(OH)<sub>2</sub> was suggested to maintain the biocompatible environment [8] and offer direct antibacterial effects [9]. However, there are multiple concerns regarding the ability of Ca(OH)<sub>2</sub> to offer an elevated and extended

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level of disinfection required for some cases of endodontic regeneration [10, 11]. On the other hand, various antibiotic mixtures such as triple (TAP) or double (DAP) antibiotic paste were proposed to offer an extended and aggressive level of disinfection [11–13]. However, these antibiotic mixtures were found to be cytotoxic to SCAP [6, 8] and dental pulp stem cells (DPSC) [14, 15]. Therefore, recent studies proposed to use a low concentration of these antibiotic mixtures in an attempt to provide antibacterial effects without compromising the biological environment within the root canal system [6, 15, 16].

The delivery of controlled low concentration of antibiotic into the root canal spaces offers a clinical challenge, and multiple methods have been suggested in the literature to overcome this issue. One of the suggested approaches is to encapsulate low concentrations of antimicrobials into an injectable, self-assembled, biomimetic nanomatrix gel [17]. Another proposed approach is to load these low concentration antibiotics into an electrospun polymer fibers that can be temporarily inserted into the root canal [18, 19]. A third suggested approach is to incorporate these low concentrations within an injectable methylcellulose hydrogel with controlled molecular weight [9, 20]. As reported by others, these approaches examined the direct antimicrobial properties as well as the cytotoxicity of these delivery systems [9, 15, 18, 19]. However, none of the previous studies have intensively evaluated the residual antimicrobial properties and the effect on the stem cell differentiation potential of these delivery systems. Thus, the aims of the current study were two-fold. First, to investigate the direct and residual antimicrobial properties of low concentrations of DAP loaded into methylcellulose hydrogel against single- and dual-species biofilms. Second, to evaluate the effects of these DAP hydrogels on the cytotoxicity and differentiation of DPSC.

## Materials and methods

### Preparation of antimicrobials used in the study

In the current study, low concentrations of DAP (1, 5, and 10 mg/mL) were prepared in methylcellulose hydrogels as detailed in recent studies [15, 20]. In brief, 5, 25, and 50 mg of equal parts of ciprofloxacin and metronidazole (Champs Pharmacy, San Antonio, TX, USA) were slowly dissolved in 5 mL of sterile water under continuous stirring in a sterilized environment to form 1-, 5-, and 10-mg/mL DAP solutions, respectively. Then, 0.4 g of methylcellulose powder (Methocel 60 HG, Sigma-Aldrich, St. Louis, MO, USA) was gradually incorporated into each DAP solution under controlled vigorous stirring to obtain a creamy injectable consistency of 1, 5, and 10 mg/mL of DAP. A DAP-free hydrogel and a Ca(OH)<sub>2</sub> paste (UltraCal XS, Ultradent, South Jordan, UT, USA) were also used as controls in this study.

### Dentin sample preparation

De-identified human teeth were used after obtaining local IRB approval (IRB# 1408889870, 2016) to prepare a total of 196 radicular dentin samples (4 × 4 × 1.5 mm) using a diamond saw (IsoMet; Buehler, Lake Bluff, IL, USA) and automatic polishing unit (Rotoforce 4, Struers, Cleveland, OH, USA) as described in previous studies [20, 21]. The samples were then sonicated using 1.5% NaOCl (Value Bleach; Kroger, Cincinnati, OH, USA), 17% EDTA (VISTA, Racine, WI, USA), and sterile water to remove the smear layers. Each sample was independently sterilized with ethylene oxide, stored at 4 °C, and used within 1–3 weeks for antibacterial testing.

### Bacterial strain and media

*Enterococcus faecalis* (ATCC strain 29212, American Type Culture Collection, Manassas, VA, USA) and *Prevotella intermedia* (ATCC strain 25611) were independently grown anaerobically on blood agar plates (Bio-Merieux, Durham, NC, USA). Colonies of *E. faecalis* and *P. intermedia* were then inoculated in a sterile broth of brain heart infusion (BHI) supplemented with 5 g of yeast extract/L (BHI-YE) and incubated anaerobically for 24 h at 37 °C and 5% CO<sub>2</sub> atmosphere.

### Direct antibacterial testing

Sterile dentin samples ( $n = 84$ ) were individually inserted into wells of sterile 96-well microtiter plates (FisherBrand, Fischer Scientific) with the root canal sides oriented outward. Half of the dentin samples ( $n = 42$ ) received a mixture of 190  $\mu\text{L}$  of fresh BHI-YE and 10  $\mu\text{L}$  of overnight *E. faecalis* culture ( $10^6$  CFU/mL). The other half of the dentin samples received a mixture of 190  $\mu\text{L}$  of fresh BHI-YE, 5  $\mu\text{L}$  of overnight *E. faecalis* culture (approximately  $1 \times 10^6$  CFU/mL), and 5  $\mu\text{L}$  of overnight *P. intermedia* culture ( $1 \times 10^6$  CFU/mL). All infected dentin slabs were incubated anaerobically at 37 °C for 3 weeks and the growth media was replaced every 7 days. Furthermore, uninfected sterile dentin samples (negative control) were used in this experiment for each type of culture to verify the lack of bacterial contamination though the course of the experiment ( $n = 14$ ). Each negative control dentin sample received 200  $\mu\text{L}$  BHI-YE and was incubated anaerobically for 3 weeks with weekly replacement of BHI-YE. After the incubation period, dentin samples infected with single- and dual-species cultures were transferred to new wells of sterile 96-well microtiter plates containing 50  $\mu\text{L}$  of fresh BHI-YE and each type of infected dentin was randomized into seven experimental groups ( $n = 7$  per group). Aliquots of cultures from each dual-species group were taken out at the end of 3-week incubation, stained by Gram method, and inspected under

light microscope to confirm the growth of the two species of bacteria. The infected slabs were treated with 100  $\mu\text{L}$  of 1, 5, or 10 mg/mL DAP, placebo paste,  $\text{Ca}(\text{OH})_2$ , or received sterile water (positive control). The sterile dentin slabs in the negative control group received no treatment. All slabs were then incubated for 1 week at 37 °C and 100% humidity. After that, each sample was gently rinsed off with 1 mL of sterile water for 1 min to remove the treatment pastes. Biofilm disruption assays were performed for all dentin samples [9, 21]. Briefly, dentin samples were individually placed into sterile test tubes containing 2 mL of sterile saline, sonicated, and vortexed to displace the biofilms. The dislodged biofilms were diluted, spiral plated onto blood agar plates, incubated anaerobically for 24 h, and quantified using an automated colony counter (Synbiosis, Inc., Frederick, MD, USA).

### Residual antibacterial effects

Sterile dentin samples ( $n = 84$ ) were inserted in 96-well microtiter plates as described earlier and pretreated for 1 week with the same antibacterial treatment groups described earlier ( $n = 14$ ). After treatment, the antimicrobial pastes were washed off from dentin samples using sterile saline followed by 17% EDTA (5 mL each). Dentin samples were then immersed independently in 200 mL of sterile phosphate-buffered saline (PBS) and stored at 37 °C for 1 week before growing the bacterial biofilm. After that, half of dentin samples from each treatment group were infected with an overnight *E. faecalis* culture. The remaining half of dentin samples ( $n = 42$ ) from each treatment group were infected with an overnight dual-species culture as described earlier in the direct antibacterial experiment. Biofilms from both cultures were allowed to grow anaerobically for 3 weeks with weekly replenishment of growth media. Non-infected sterile dentin samples were also used in this experiment as described earlier ( $n = 14$ ). After 3 weeks of incubation, biofilm disruption assays were conducted for each dentin sample as detailed earlier.

### Cell and culture conditions

Human dental pulp stem cells (DPSC) (AllCells, Alameda, CA, USA) were cultured in 24-well plates (Cat: TP9024, Alkali Scientific Inc.) at concentrations of  $3 \times 10^4$  cells/well in  $\alpha$ -MEM (HyClone Laboratories Inc., South Logan, UT, USA) with 10% heat-inactivated FBS (Biowest, Kansas city, MO, USA) and 1% penicillin/streptomycin (Lonza, Allendale, NJ, USA).  $\beta$ -Glycerolphosphate of 10 mM and 50 mg/ml ascorbic acid were added to the  $\alpha$ -MEM for the ALP and mineralization groups to create an osteogenic environment and induce differentiation of the DPSC to mineral-forming cells. Transwell chamber inserts (1.0  $\mu\text{m}$  pore size, Corning, NY, USA) were used to separate the DPSC below the membrane from the treatments that were added on top of

the chamber membrane. Cells were cultured for 3 days for the proliferation assays or 7 days for ALP and mineralization assays. One day after cell plating, 100  $\mu\text{L}$  of each of the experimental groups (1, 5, and 10 mg/mL DAP, DAP-free placebo,  $\text{Ca}(\text{OH})_2$ , and untreated control) was added to the top chamber, followed by 750  $\mu\text{L}$  of culture media. The bottom chamber contained 500- $\mu\text{L}$  media, for a total volume of 1.35 mL in each well. Culture media was changed in the ALP and mineralization assays after 3 days in culture in osteogenic media.

### Proliferation assay

Cell metabolic activity, a measure of cell proliferation, was determined using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (MTS) (Promega Cat: G5421). The MTS assay was performed following the manufacturer's specifications after incubation of the DPSCs with the treatment groups for 3 days. Duplicate aliquots from each well were measured at 0, 1, 2, and 3 h using a spectrophotometer set at 490 nm.

### ALP assay

An alkaline phosphatase (ALP) assay was performed using the Sigma-Aldrich Brown Box for ALP kit. Cells were lysed with 100  $\mu\text{L}$  of mRIPA lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% Igepal-CA 630, 0.25% sodium deoxycholate) supplemented with 10  $\mu\text{g}/\text{mL}$  leupeptin hydrochloride, 10  $\mu\text{g}/\text{mL}$  aprotinin, 10  $\mu\text{g}/\text{mL}$  pepstatin). The lysates were collected and sonicated for 5 min, centrifuged for 5 min, and the supernatant collected. Then, 5  $\mu\text{L}$  of each cell lysate was assayed in triplicate with 100  $\mu\text{L}$  of ALP substrate solution (40 mg of p-nitrophenyl phosphate (p-NPP), 10 mL of alkaline buffer, and 10 mL of  $\text{dH}_2\text{O}$ ), incubated at 37 °C for 1 h, and 95  $\mu\text{L}$  NaOH added to stop reaction. A standard curve of 4-nitrophenol and NaOH was also created. Optical absorbance was measured at 405 nm. A protein assay was performed using the Pierce BCA Assay kit from Thermo Scientific #23225 following the manufacturer's protocol.

### Mineralization assay

DPSC were cultured with the treatments in 24-well transwell chambers and differentiated with ascorbic acid and  $\beta$ -glycerolphosphate. After 7 days, the cells were stained for calcium deposits using Alizarin Red-S. First, the cells were fixed in 3.7% formaldehyde in PBS for 10 min (PBS, HyClone Laboratories, Inc.). A standard curve was made with 1% ( $w/v$ ) cetylpyridinium chloride (CPC) (Sigma #C0732), and 40 mM Alizarin Red-S Solution pH 4.2 (Sigma-Aldrich) was created for comparison. Then, 500  $\mu\text{L}$  of Alizarin Red-S was added to each well and the plate left on the shaker for

30 min to fully stain the samples. Wells were subsequently washed 5 times with tap water followed by a 15-min PBS wash. The PBS was suctioned off, and 500  $\mu\text{L}$  of 1% (w/v) CPC was added to each well for 15 min to extract the Alizarin Red-S. Absorbance of eluted Alizarin Red-S was measured in triplicate at 562 nm in a 96-well plate. The calcium concentration in each well was determined from the standard curve and was based on the pre-determined and known ability of Alizarin in binding 2 mol of calcium per mol of dye.

## Statistical analyses

For the antibacterial experiments, some experimental groups did not exhibit any bacterial growth. Therefore, Fisher's exact tests were utilized to evaluate the significant differences in the presence or absence of any bacterial growth. Additionally, Wilcoxon rank sum tests were used to compare the antibiofilm effects of various treatment groups that demonstrated bacterial growth. For DPSC experiments, one-way ANOVA followed by pair-wise tests were used for statistical comparison. The significance level was set at 0.05. Three independent experiments were conducted for all biological experiments, and each individual experiment was performed in triplicate.

## Results

### Direct antibacterial effects

*E. faecalis*-infected dentin samples treated with 1, 5, and 10 mg/mL DAP, as well as  $\text{Ca}(\text{OH})_2$  demonstrated significant antibiofilm effects in comparison to the positive control groups or groups treated with placebo paste, regardless of the type of biofilm used ( $P < .0001$ ) (Table 1). No significant differences were found between *E. faecalis*-infected dentin treated with 1, 5, and 10 mg/mL DAP and  $\text{Ca}(\text{OH})_2$ . However, dual-species-infected dentin treated with 5 mg/mL DAP, 10 mg/mL DAP, and  $\text{Ca}(\text{OH})_2$  demonstrated

significantly higher antibiofilm effects in comparison to dual-species-infected dentin treated with 1 mg/mL DAP ( $P < .0001$ ). The differences between single- and dual-species-infected dentin were not significant in any treatments except 1 mg/mL DAP.

### Residual antibacterial effects

Dentin pretreated with 5 and 10 mg/mL DAP exhibited significantly higher residual antibacterial effect in comparison to 1 mg/mL DAP,  $\text{Ca}(\text{OH})_2$ , placebo paste, or positive control, regardless of whether a single-species or dual-species biofilm was used ( $P < .0001$ ) (Table 2). No significant differences were found between dentin treated with 1 mg/mL DAP,  $\text{Ca}(\text{OH})_2$ , placebo paste, and positive control regardless of the type of biofilm.

### Effect of medicaments on the proliferation of DPSC

$\text{Ca}(\text{OH})_2$ , 1 mg/mL of DAP, and DAP-free hydrogel did not cause significant decreases in the proliferation of DPSC in comparison to the untreated control DPSC (Fig. 1), suggesting that the treatments were not cytotoxic to the cells. Both 5 and 10 mg/mL DAP induced significant decreases in DPSC proliferation in comparison to the untreated negative control DPSCs.

### Effects of medicaments on ALP activity of DPSC

The DAP-free methylcellulose hydrogel caused a significant increase in the ALP activity of DPSC ( $P < .001$ ) in comparison to the untreated control DPSCs and all other treatment groups (Fig. 2).  $\text{Ca}(\text{OH})_2$ , as well as 5 and 10 mg/mL of DAP, led to significant decreases in ALP activity in comparison to the 1 mg/mL group ( $P < .01$ ). However, no significant difference in ALP activity was found between 1 mg/mL of DAP and the untreated control. Furthermore, no significant differences in ALP activity were found between the untreated

**Table 1** The direct antibiofilm effects of the different antimicrobials against single- and dual-species biofilms represented as the mean (SE) of the log CFU/mL

Type of treatment	Single-species biofilms Mean log <sub>10</sub> (SE)	Number of positive samples	Dual-species biofilms Mean log <sub>10</sub> (SE)	Number of positive samples
10 mg/mL DAP	0 (0.0) <sup>Aa</sup>	0/7	0 (0.0) <sup>Aa</sup>	0/7
5 mg/mL DAP	0 (0.0) <sup>Aa</sup>	0/7	0 (0.0) <sup>Aa</sup>	0/7
1 mg/mL DAP	0 (0.0) <sup>Aa</sup>	0/7	1.6 (0.57) <sup>Bb</sup>	4/7
Calcium hydroxide	0 (0.0) <sup>Aa</sup>	0/7	0 (0.0) <sup>Aa</sup>	0/7
DAP-free hydrogel	5.9 (0.02) <sup>Ba</sup>	7/7	6.75 (0.03) <sup>Ca</sup>	0/7
Positive control	5.9 (0.01) <sup>Ba</sup>	7/7	6.69 (0.05) <sup>Ca</sup>	7/7
Negative control	0.00 (0) <sup>Aa</sup>	0	0.00 (0) <sup>Aa</sup>	0

Different uppercase letters indicate statistical significance between treatment groups within a similar biofilm group. Different lowercase letters indicate statistical significance between the single-species and dual-species biofilms for each type of treatment

**Table 2** The residual antibiofilm effects of different antimicrobials against single- and dual-species biofilms represented as the mean (SE) of the log CFU/mL

Type of treatment	Single-species biofilms Mean log <sub>10</sub> (SE)	Number of positive samples	Dual-species biofilms Mean log <sub>10</sub> (SE)	Number of positive samples
10 mg/mL DAP	0 (0.0) <sup>Aa</sup>	0/7	0 (0.0) <sup>Aa</sup>	0/7
5 mg/mL DAP	0 (0.0) <sup>Aa</sup>	0/7	0 (0.0) <sup>Aa</sup>	0/7
1 mg/mL DAP	4.99 (0.1) <sup>Ba</sup>	7/7	4.8 (0.13) <sup>Ba</sup>	7/7
Calcium hydroxide	4.98 (0.05) <sup>Ba</sup>	7/7	5.1 (0.07) <sup>Ba</sup>	7/7
DAP-free hydrogel	4.98 (0.06) <sup>Ba</sup>	7/7	4.8 (0.08) <sup>Ba</sup>	7/7
Positive control	4.9 (0.09) <sup>Ba</sup>	7/7	5.2 (0.12) <sup>Ba</sup>	7/7
Negative control	0.00 (0) <sup>Aa</sup>	0/10	0.00 (0) <sup>Aa</sup>	0/10

Different uppercase letters indicate statistical significance between treatment groups within a similar biofilm group. Different lowercase letters indicate statistical significance between the single-species and dual-species biofilms for each type of treatment

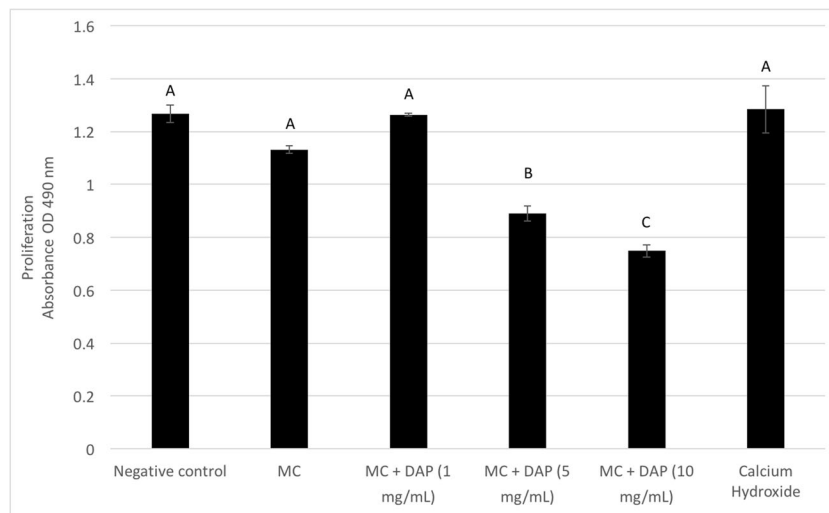
negative control and those treated with Ca(OH)<sub>2</sub>, 5 mg/mL DAP or 10 mg/mL DAP.

**Effects of medicaments on the mineralization capacity of DPSC**

The DAP-free hydrogel and Ca(OH)<sub>2</sub> induced significant increases in mineralized nodule formation (Fig. 3) in comparison to the untreated control DPSCs and all tested concentrations of hydrogels containing DAP (1, 5, and 10 mg/mL) (*P* < .0001). No significant differences in mineralized nodule formation were observed between DPSC treated with 1 mg/mL DAP and untreated control DPSC. However, 5 and 10 mg/mL DAP evoked significant reductions in mineralized nodule formation in comparison to the untreated control DPSC (*P* < .001) and those treated with 1 mg/mL DAP (*P* < .001).

**Discussion**

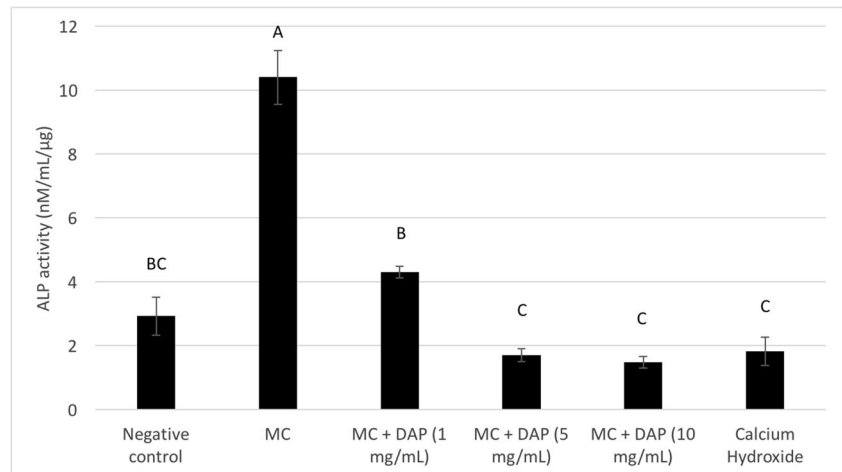
DAP has emerged in multiple clinical reports as a substitute for commonly used antimicrobials during regenerative endodontics [22, 23]. Recent in vitro studies proposed that DAP has superior antimicrobial properties than Ca(OH)<sub>2</sub> [10, 11]. Furthermore, DAP was found to cause minimum or no tooth discoloration in comparison to minocycline containing TAP [24]. However, just like other antibiotic mixtures, delivering an efficient and controlled concentration of DAP into the root canal during endodontic regeneration is a topic of great interest. In the current study, the antimicrobial properties of methylcellulose hydrogel containing relatively low concentrations of DAP were tested using standardized samples of human radicular dentin rather than a complete root model. This translational model is increasingly used in the literature due to the



**Fig. 1** Proliferation assays performed on DPSC treated with various concentrations of the DAP hydrogels, Ca(OH)<sub>2</sub>, placebo gel (MC), and the untreated control. The proliferation assay was stopped after 3 h and absorbance at 490 nM measured. Compared to the negative control, the percentage (%) DPSC survival for each group was 89, 100, 70, 59, and

101% for the MC, 1 mg/mL DAP, 5 mg/mL DAP, 10 mg/mL DAP, and Ca(OH)<sub>2</sub> groups, respectively. Different letters indicate statistical significance. Three independent experiments were conducted with similar results. The mean ± SE is shown for a representative experiment

**Fig. 2** ALP activity assays of DPSC treated with various concentrations of the DAP hydrogels, Ca(OH)<sub>2</sub>, placebo gel (MC), and the untreated control. Different letters indicate statistical significance. Three independent experiments were conducted with similar results. The mean ± SE is shown for a representative experiment



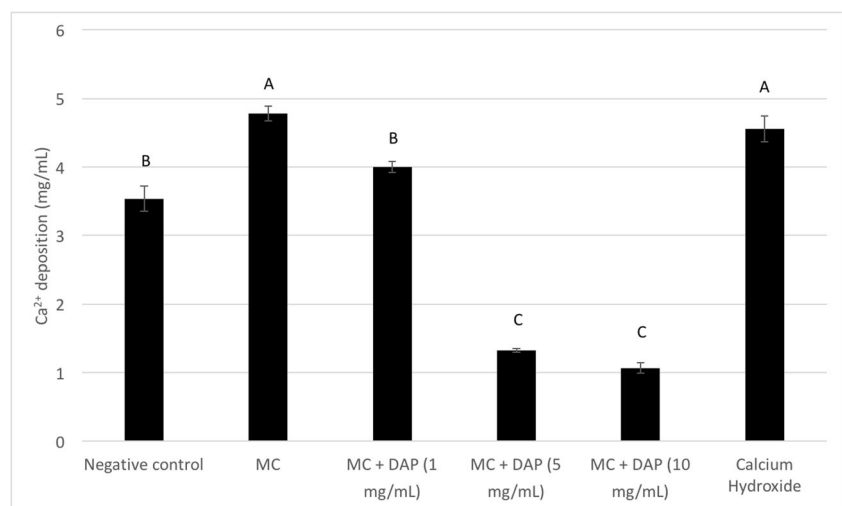
standardized and reproducible measurements obtained from this model [21], which was reflected by the low variability obtained in the current study, as well as previous studies that used a similar model [9, 11, 13]. Additionally, this model was found to allow the continuous growth of biofilm on the surface of dentin samples, as well as the penetration of microorganism within the dentinal tubules [13].

The current study demonstrated that all tested concentrations of DAP (1, 5, and 10 mg/mL) as well as Ca(OH)<sub>2</sub> exhibited significant and substantial direct antibacterial effects against single- and dual-species biofilms, which generally agrees with recent studies using single [9] or multispecies biofilms [20]. However, the dual-species biofilm was significantly more resistant to 1 mg/mL DAP in comparison to single-species biofilm. This indicates that more complicated multi-species biofilms that are clinically present in infected root canals may be more resistant to lower concentrations of DAP. It is worth noting that 1 mg/mL of DAP is the concentration that is currently recommended by American

Association of Endodontists (AAE) during endodontic regeneration procedures [7].

The empty canal space during the regeneration process may promote the repopulation of residual bacteria inside the root canal system [25]. Indeed, a recent *in vivo* study demonstrated that residual bacteria have a negative effect on the outcomes of endodontic regeneration [26]. Therefore, the ability of various concentrations of DAP to exert a residual antibacterial effect was also tested in the current study. Both 5 and 10 mg/mL of DAP demonstrated significant and substantial residual antibacterial effects in comparison to 1 mg/mL DAP and Ca(OH)<sub>2</sub>. The residual antibacterial properties of DAP have been also demonstrated in recent publications [11, 20]. However, the exact mechanism for this residual effect is not fully understood. Due to the limited dissolution of DAP in water, it is logical to assume that the hydrogels containing DAP tested in this study have both soluble and insoluble portions of DAP. The ability of the soluble DAP portion to bind to dentin as well as the ability of the insoluble DAP portion to

**Fig. 3** Mineral deposition by DPSC treated with various concentrations of the DAP hydrogels, Ca(OH)<sub>2</sub>, placebo gel (MC), and the untreated control. Different letters indicate significant differences. Different letters indicate statistical significance. Three independent experiments were conducted with similar results. The mean ± SE is shown for a representative experiment



adsorb to dentin and to penetrate the dentinal tubules might explain the residual antibacterial properties of DAP at the higher concentrations reported in this study. However, further investigations are needed to confirm this hypothesis.

In the current study, two bacterial species were used to form the in vitro dual-species biofilms on the radicular dentin samples, namely *E. faecalis* and *P. intermedia*. These bacteria were selected due to their ability to co-exist together and form a well-established mixed biofilm in vitro [27]. Nevertheless, the presence of these specific bacteria in the infected root canal of an immature tooth is not clear. Recent study concluded that the microbial profile of infected immature teeth is similar to that of primarily infected permanent teeth [28]. However, the same study found that the most detected bacteria in the infected root canal of immature teeth were *Actinomyces naeslundii* and *Porphyromonas endodontalis* [28].

In the current study, DPSC rather than SCAP were used for the biological assays. Current studies demonstrated similar osteo/odontogenic differentiation potential of DPSC and SCAP [29, 30]. However, other study proposed that SCAP are a subset of DPSC with potent dental stem/progenitor cells [31]. One of the interesting findings of this study is that the DAP-free methylcellulose hydrogel system did not elicit any significant negative effects on the proliferation of DPSC, which indicates that the system is biocompatible. Furthermore, 1 mg/mL of DAP hydrogel did not exhibit any significant cytotoxic effect. Previous studies using a relatively similar direct cytotoxic model have shown that 1 mg/mL DAP solutions resulted in survival of approximately 50% [8] of SCAP and 0% of dental pulp fibroblasts [14]. Our study also demonstrated that 5 and 10 mg/mL DAP contributed to concentration-dependent cytotoxic effects in comparison to 1 mg/mL DAP. However, in the 5 and 10 mg/mL DAP groups, 70 and 59% survival of DPSC, respectively, was observed. Previous studies have shown that 5–10 mg/mL of DAP solution exhibited 0–15% viability of pulp fibroblasts and SCAP [8, 14]. Collectively, these proliferation results indicate that the methylcellulose hydrogel system used in our study may improve the survival of DPSC at higher concentrations of DAP.

Our study also demonstrated that the DAP-free methylcellulose hydrogel system caused a significant increase in ALP activity and mineralization of DPSC. These findings provide additional support that the delivery system proposed in this study is biocompatible. The biocompatible nature of the hydrogels used in the current study could be explained by the ability of methylcellulose at specific concentrations to improve cellular metabolic activity [32]. Our study also found a concentration-dependent negative effect of DAP on the ALP activity and mineralization of DPSC. However, 1 mg/mL DAP demonstrated no significant negative effects on ALP activity and mineralization of DPSC in comparison to the untreated control cells. On the other hand, a previous study

demonstrated that concentrations as low as 0.39 µg/mL of TAP caused significant reductions in the mineralization capacity of SCAP and pulp fibroblasts [33]. Collectively, this may indicate that the methylcellulose hydrogel system used in our study was able to improve the cell differentiation capacity of 1 mg/mL DAP. However, the hydrogel system used in the present study was not able to completely prevent the negative effects of higher concentrations of DAP on cell proliferation and differentiation.

The non-cytotoxic nature of Ca(OH)<sub>2</sub> and its ability to significantly improve mineralization found in this study is consistent with other studies [8, 34]. Higher mineralization activity is expected in any materials that have the ability to release calcium ions [35]. However, the well-documented ability of Ca(OH)<sub>2</sub> to improve mineralization and hard tissue formation should be viewed with caution within the context of endodontic regeneration. Excessive calcification within the root canal can lead to partial or complete obliteration of the root canal system, negatively affecting the ability to regenerate the tissues, and to complicate future endodontic treatments [36, 37]. A recent clinical study found that the use of Ca(OH)<sub>2</sub> as an intracanal medicament during endodontic regeneration was associated with a higher frequency of intracanal calcification (77%) in comparison to the use of TAP and DAP intracanal medicaments (46%) [38]. It is worth noting that the European Society of Endodontology is in favor of using Ca(OH)<sub>2</sub> as an intracanal medicament during endodontic regeneration [39]. The use of Ca(OH)<sub>2</sub> or low concentrations of antibiotic mixtures during endodontic regeneration is still an open question that warrant further investigations. However, it is important to provide multiple disinfection options during regenerative endodontics that enable the endodontist to make a clinical decision on case by case basis taking into consideration the ability to overcome root canal infection, maintaining the vitality of stem cells as well as avoiding the adverse effects of intracanal medicaments such as tooth discoloration and formation of intracanal calcification.

In the current study, all the biological experiments were performed within 3–7 days of DPSC culture. This can be justified by the temporary nature of these intracanal medicaments when applied clinically. Furthermore, the design of this study was intended to give a comprehensive understanding of the antibacterial and biological aspects of the tested medicaments within a time frame (1 week) that is relevant for clinical applications. Indeed, a 1-week application time is the recommended minimum application time of intracanal medicaments during endodontic regeneration procedures [7]. Further investigations are warranted to examine the mineralization of DPSC exposed to methylcellulose hydrogels containing DAP at multiple time points. In addition, future studies will examine the angiogenic and adipogenic potential of DPSC exposed to methylcellulose hydrogels containing different

concentrations of DAP. In vivo studies are also needed to confirm the results found in this in vitro study before any clinical extrapolation.

## Conclusion

This study indicates that Ca(OH)<sub>2</sub> as well as methylcellulose hydrogels containing as low as 1 mg/mL of DAP can provide a significant direct antibacterial effect against single- and dual-species biofilms. However, a concentration of 5 mg/mL of DAP or higher may be needed to provide an extended residual antibacterial effect. Our findings also indicate that the methylcellulose hydrogel containing 1 mg/mL of DAP was not cytotoxic and did not have a significant adverse effect on the proliferation and differentiation of DPSC. However, increasing the concentration of DAP within the suggested hydrogel system can lead to significant negative effects on proliferation and mineralization of DPSC. Thus, the methylcellulose hydrogel can be used as a biocompatible and injectable system for the delivery of controlled low concentration antibiotic intracanal medicaments.

**Funding** This study was supported by Indiana University School of Dentistry

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All procedures performed in studies involving human teeth were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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