

# Antibacterial effects of different low-level doses of double antibiotic paste on multispecies biofilms in regenerative endodontics: A confocal laser scanning microscopy study

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## Abstract

**Aim:** This research aimed to assess the comparative efficacy of various concentrations of double antibiotic paste used on multispecies biofilms in regenerative endodontic procedures.

**Methodology:** Seventy caries-free human teeth with a single root that were extracted for orthodontic purposes were gathered. A total of 900 µL of bacterial suspension was prepared, comprising three bacterial species: *E. faecalis* (ATCC 43146), *A. naeslundii* (ATCC 10556), and *S. sanguinis* (ATCC 29212). Six experimental groups (n=10/group) were randomly created with the infected dentin slices: 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 5 mg/mL, 10 mg/mL of double antibiotic paste and a control group. Confocal laser scanning microscopy (CLSM) analysis was conducted to evaluate the antimicrobial activity. Two samples from each group were analyzed with a scanning electron microscope. The live/dead bacteria percentages were analyzed via the Kruskal-Wallis test. The tests were conducted with a significance level of 5%.

**Results:** Greater live/dead bacteria ratios were observed in the control group, and the differences compared to other groups were statistically significant. Additionally, the numbers of live/dead bacteria observed after the 0.5 mg/mL and 1 mg/mL DAP doses were applied were significantly higher compared to the 10 mg/mL DAP concentration. All DAP doses exhibited some degree of antibiofilm activity; however, 5 mg/mL and 10 mg/mL were the most effective.

**Conclusion:** In regenerative endodontics, the 5 mg/mL and 10 mg/mL doses of DAP demonstrated more effective activity against multispecies biofilms than lower doses.

**Keywords:** Antibacterial agents, biofilm, regenerative endodontics, regenerative medicine, confocal laser scanning microscopy

## Introduction

The regeneration of pulp tissue offers the potential for extended utilization of natural teeth by ensuring reliable preservation of the dental root structure. Regenerative endodontic procedures (REPs) have been coming to the fore in recent years because of their success and excellent survival rates in both mature and immature permanent teeth (1, 2). However, to date, only one study has reported the formation of pulp-like tissue after induced bleeding, which is the sole approach approved in the United States for managing pulpal necrosis in immature permanent teeth. This may be due to the high level of cytotoxicity associated with the antibiotic pastes used to eliminate infections as well as the differences in cell populations and growth factors among patients. At present, there is no treatment that combines biocompatible disinfection with the ability to regenerate the dentin-pulp complex in a predictable manner. This underscores the significant oral health challenge regarding the therapeutic care of permanent teeth with necrotic pulp and immature apices, indicating the need for research into reliable regenerative treatment methods (3).

The most crucial stage for effective endodontic regeneration treatment is the efficient eradication of preoperative infection (4).

Also, root canal irrigation may expose dentin tubules and collagen fibrils, enhancing their suitability for stem cell attachment (1). Minimal mechanical instrumentation preserves the integrity of the bacterial biofilm on the walls of the root canal, and remaining pathogens may give rise to new bacterial biofilm (5). In this context, the importance of disinfection protocols emerges.

Contemporary antibacterial strategies are implemented in two stages: the utilization of double or triple antibiotic pastes (DAP/TAP) intracanal to disinfect after pulp extirpation, followed by the application of Regenerative Endodontic Procedure (REP) strategies to eradicate remaining bacteria. In regenerative endodontics, disinfection regimens advocate for the use of several substances, including triple antibiotic paste (TAP) (containing minocycline, metronidazole, and ciprofloxacin) and double antibiotic paste (DAP) (containing metronidazole and ciprofloxacin) (0.1-1.0 mg/ml doses), or calcium hydroxide (6). It has been suggested that both low and high concentrations of antibiotic combinations may provide longer lasting antibiofilm effects compared to the  $\text{Ca}(\text{OH})_2$  formula currently employed in clinical practice (7). In contrast to calcium hydroxide, which shows little disinfection efficacy even when used with several antimicrobial agents, combinations of double and triple antibiotic pastes demonstrate excellent antibiofilm activity. The results of such studies prompted us to analyze the effects of different doses of antibiotics on bacterial biofilm (8, 9).

In the studies conducted, it was observed that low concentrations of antibiotic pastes have antibacterial activity comparable to high doses of antibiotics (10).

Furthermore, dentin that underwent treatment with all doses of DAP exhibited a notably extended residual antibacterial action in comparison to dentin treated with TAP at equivalent concentrations (11).

The most extensively studied bacterial strain among the examined strains was *E. faecalis* (ATCC 19433) (gram-positive facultative anaerobe); however, the prevalence of primary endodontic infections ranges from 10% to 45% to 80% in patients that had been previously treated (12, 13). This trend may present a limitation for endodontic regeneration studies. According to conducted research, the majority of strains found in infected root canals are anaerobic gram-negative bacteria, whereas a smaller portion consist of gram-positive anaerobic rods. In summary, there is significant variability and diversity in terms of strains within the endodontic microbiota (14). Limited publications have investigated the multispecies biofilm on human dentin. When evaluating the efficiency of antibacterial drugs, it is crucial to assess their ability to suppress biofilm formation. Numerous studies have reported antibacterial effects on *E. faecalis* (11, 15, 16), but the formation of multispecies biofilm on human dentin has received relatively little attention in research (17-19). Examining the antibacterial properties of antibiotics on multispecies biofilm is crucial.

Our work is the first study in the literature examined the efficacy of various dosages of DAP used in regenerative endodontic procedures against multispecies bacterial biofilm formation. Therefore, the objective of this study was to evaluate and contrast the efficacy of various dosages of DAP used in regenerative endodontic procedures and determine the effective dose against three bacterial species: *E. faecalis* (*E. f*), *A. naeslundii* (*A. n*), and *S. sanguinis* (*S. s*).

## Materials and Methods

This study protocol was approved by the Hamidiye Scientific Ethics Committee of the Health Sciences University (Nu: 4.7.18/124).

### Preparation of dentin samples

Seventy caries-free single-rooted human teeth extracted for orthodontic reasons were collected following a meticulous cleaning process. The crown was cut with a water-cooled low-speed diamond blade (Isomet, Buehler, Lake Bluff, IL, USA). After that, the roots were then cut horizontally, 3 mm above the cemento-enamel junction, resulting in 2 mm thick dentin discs. An average of 4-5 dentin slices were obtained from each tooth. Afterwards, the dentin slices were polished using silicon carbide abrasive sandpaper with a maximum grit size of 1200 in order to achieve a consistent thickness. An ultrasonic bath was used to remove the smear from the samples. The dentin samples underwent a 10-minute rinsing process in a saline solution and were subsequently autoclaved at a temperature of 121°C.

## Multispecies biofilm model

*E. faecalis* (ATCC 43146) was incubated in an aerobic environment at 37°C for 24 hours, *S. sanguinis* (ATCC 29212) in 5% CO<sub>2</sub> at 37°C for 48 hours, and *A. naeslundii* (ATCC 10556) in an anaerobic environment at 37°C for 48 hours. An average of 50 µL (10<sup>6</sup> CFU/ml) of each microorganism species was added to each dentin sample. The infected dentin samples were placed in 24-well plates containing sterilized brain heart infusion (BHI) with 1% sucrose (BHIS). The plates were placed in an environment with sufficient oxygen (37°C and 5% CO<sub>2</sub>) for a period of 14 days to allow biofilm formation. The broth was changed every other day to maintain bacterial survival. After a period of 14 days, the dentin samples were meticulously washed with sterile PBS (PBS, Sigma-Aldrich, Buenos Aires, Argentina) in order to remove any germs that were not securely adhered. The infected dentin slices, with a total of 10 slices in each group, were randomly divided into six different experimental groups: 0.125, 0.25, 0.5, 1, 5, 10 mg/ml DAP, and a control group (Table 1).

**Table 1.** Group names of the study

| Group Name      |
|-----------------|
| 0.125 DAP group |
| 0.25 DAP group  |
| 0.5 DAP group   |
| 1 DAP group     |
| 5 DAP group     |
| 10 DAP group    |
| Control group   |

## Treatment of samples with medicaments

Ciprofloxacin and Metronidazole were crushed and mixed in a sterile mortar to obtain DAP powder. To prepare diluted DAP medications, 500, 250, 50, 25, 12.5, and 6.25 mg of DAP powder were separately mixed with 50 mL of sterile water until completely dissolved. Afterwards, 4 grams of methylcellulose powder (Methocel 60 HG, Sigma-Aldrich) were slowly added to each diluted DAP solution while vigorously stirring at room temperature for a period of 120 minutes.

This process resulted in the formation of uniform pastes of 0.125 to 10 mg/mL concentrations of DAP. DAP was administered to the pulp side of the dentin pieces. The medications continued for a duration of 7 days. In order to preserve a moist atmosphere and avoid the dehydration of the DAP.

## Confocal laser scanning microscopy and scanning electron microscopy

For the purpose of evaluating effectiveness against microorganisms, a total of eight samples from each group were produced specifically for analysis using confocal laser scanning microscopy (CLSM) (Fig. 1).

A LIVE/DEAD™ BacLight™ viability kit L7012 (Molecular Probes Inc., Eugene, OR, USA), which includes SYTO 9 and propidium iodide (PI) was used for the staining of the dentin samples (20).

The dentin slices were evaluated using a mosaic technique of 3D reconstruction. Two random regions were selected for analysis in each slice. Each area consisted of nine sub-areas measuring 300 × 300 µm. The total number of locations that were examined for each sample was 18. The areas that were selected for CLSM imaging (Leica SP2 CL5Mt, Leica Microsystems Inc., Heidelberg, Germany). This imaging is a 40× lens that was selected from the root canal area towards the cementum side.

The Z-stacks were obtained by collecting a sequence of segments across the tissue at appropriate step size settings of 0.35 µm. The resulting images had a resolution of 512 × 512 pixels. The evaluation and quantification of the data were performed using the specialized software, Imaris 7.2 (Bitplane Inc., St. Paul, MN, USA).

An approximate 480/500 nm was the excitation emission peak for SYTO 9, whereas an approximate 490/635 nm was the peak for PI. Both dyes were excited by light.

Two samples of each group were prepared for scanning electron microscopy (SEM) analysis (at magnifications of 1,000× and 5,000×) (Fig. 2).

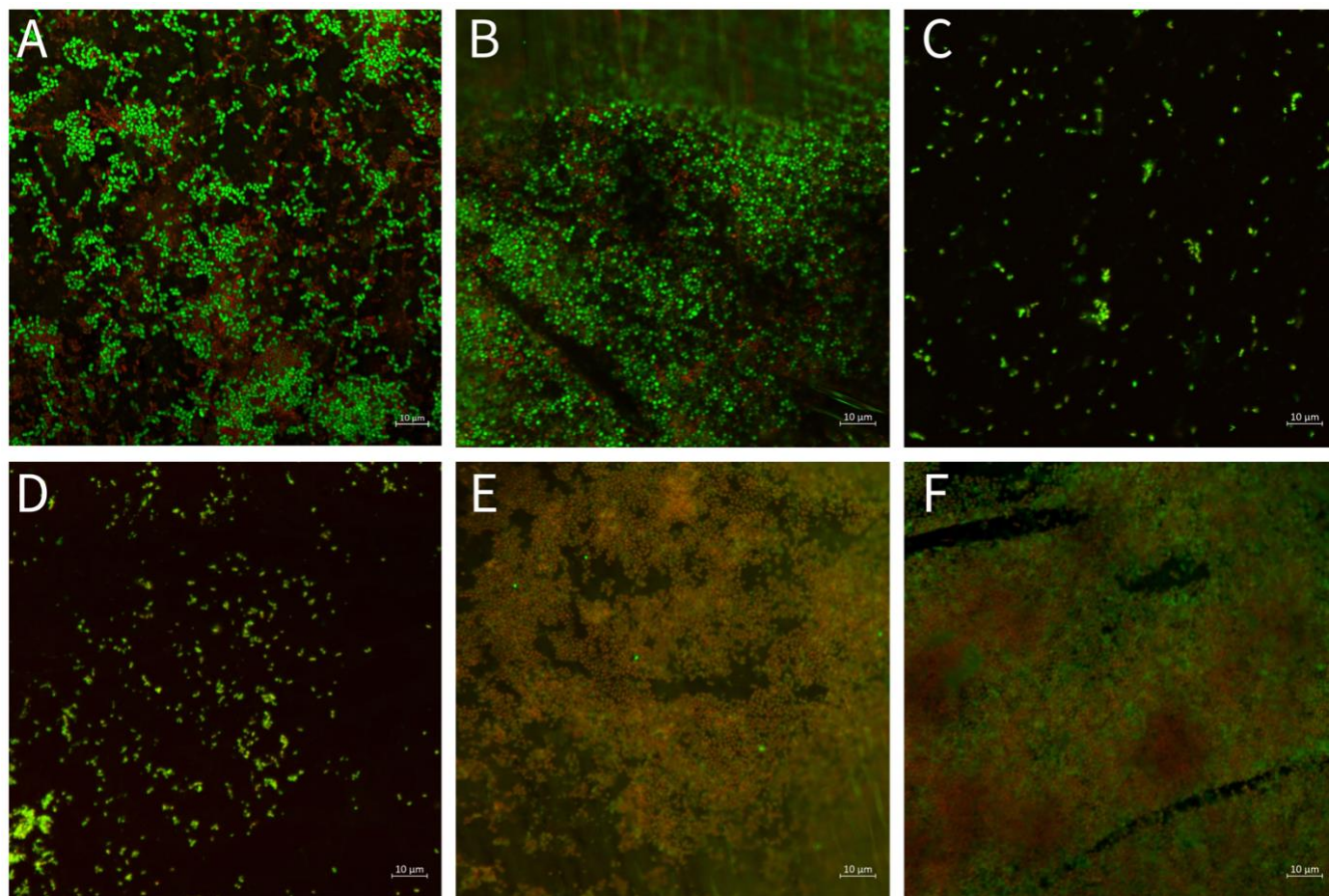
The sample was coated with palladium using a sputter coating technique (BAL-TEC SCD005, Bal-Tec AG, Balzers, Liechtenstein). It was then dehydrated with ethanol and dried to the critical point using a critical-point drier (BAL-TEC CPD030; Bal-Tec AG).

Live and dead cell numbers in the images were analyzed using ZEN Lite 2.5 software (Carl Zeiss Microscopy GmbH, Jena, Germany).

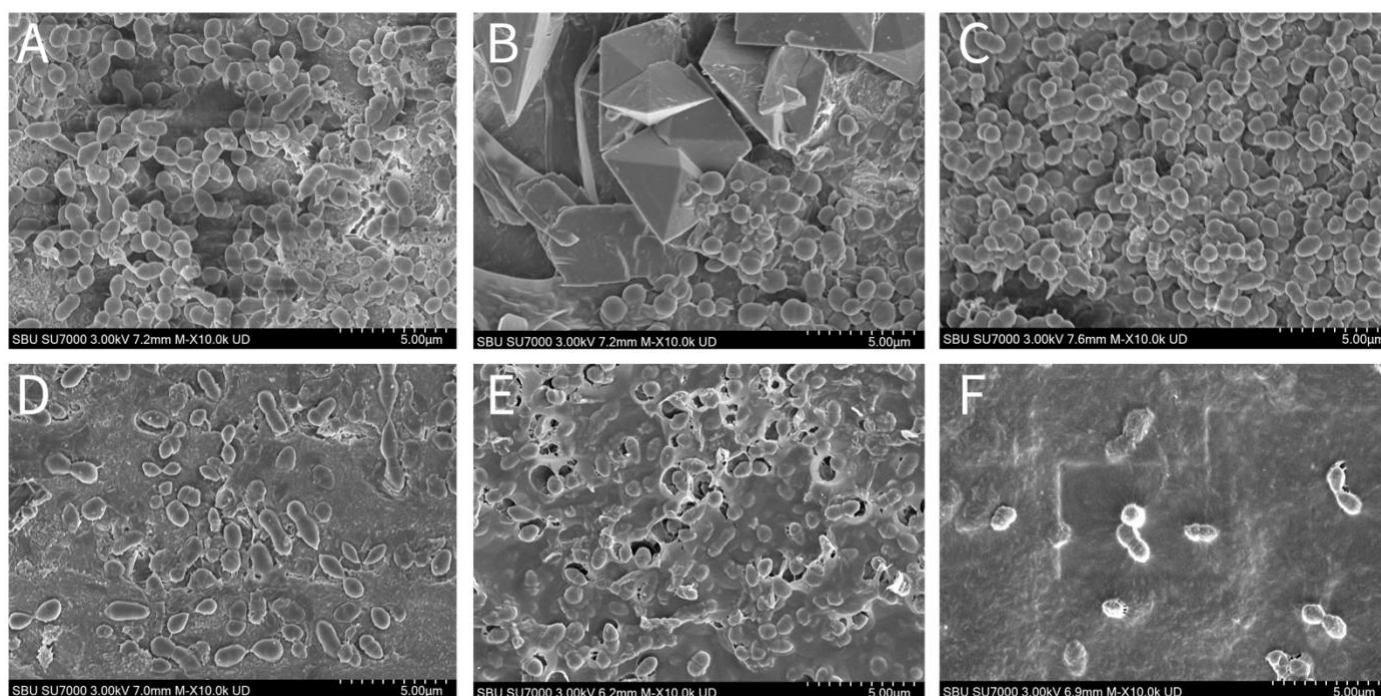
## Statistical analysis

Analyses were performed by using SPSS software (IBM SPSS Statistics version 20, IBM Inc., Armonk, NY, USA).

The data were not normally distributed. A Kruskal-Wallis test was conducted to compare the proportions of live and dead bacteria and ascertain variations in the quantity of deceased cells. The tests were conducted at a significance level of 5%.



**Figure 1.** Confocal laser scanning microscopy images representing the mean percentage of bacterial viability of different groups: (A) control, (B) 0.125 mg/ml double antibiotic paste (DAP), (C) 0.25 mg/ml DAP, (D) 1 mg/ml DAP, (E) 5 mg/ml DAP, (F) 10 mg/ml DAP (1,000× zoom) (green point shows live cells, red point shows dead cells).



**Figure 2.** Scanning electron microscopy images of multispecies biofilms of different groups: (A) control, (B) 0.125 mg/ml double antibiotic paste (DAP), (C) 0.25 mg/ml DAP, (D) 1 mg/ml DAP, (E) 5 mg/ml DAP, (F) 10 mg/ml DAP (1000× zoom).

## Results

The number of live/dead organisms in the control group was statistically higher than in all other groups. The group treated with 0.125 mg/dL of DAP had a statistically significant higher number of live/dead organisms compared with the groups with doses of 0.25, 0.5, 1, 5, and 10 mg/mL.

The number of live/dead organisms in the group treated with 0.25 mg/mL of DAP was higher than those that received higher doses of 1, 5, and 10 mg/mL.

Additionally, the numbers of live/dead bacteria in the groups treated with DAP doses of 0.5 and 1 mg/mL were significantly higher than in the group treated with 10 mg/mL of DAP.

There was no statistically significant difference between the groups treated with 5 and 10 mg/mL doses (Table 2).

All DAP doses exhibited some degree of antibiofilm activity; however, 5 and 10 mg/mL were the most effective against biofilm formation.

**Table 2.** Mean live/dead bacteria cells of all groups.

|             | N  | Mean | SD   | Min | Max | F      | p     |
|-------------|----|------|------|-----|-----|--------|-------|
| Control     | 5  | 2.54 | 0.58 | 1.9 | 3.2 | 42.839 | 0.000 |
| 0.125 mg/ml | 5  | 1.63 | 0.19 | 1.4 | 1.9 |        |       |
| 0.25 mg/ml  | 6  | 1.29 | 0.10 | 1.1 | 1.4 |        |       |
| 0.5 mg/ml   | 6  | 1.10 | 0.04 | 1   | 1.1 |        |       |
| 1 mg/ml     | 6  | 0.95 | 0.06 | 0.8 | 1.1 |        |       |
| 5 mg/ml     | 6  | 0.85 | 0.03 | 0.7 | 0.8 |        |       |
| 10 mg/ml    | 6  | 0.67 | 0.09 | 0.5 | 0.7 |        |       |
| Total       | 40 | 1.25 | 0.61 |     |     |        |       |

## Discussion

In recent years, the number of articles investigating peptides, nanoparticles, or natural products in root canal disinfection has significantly increased (21). However, the European Endodontic Society advises against the use of antibiotic pastes in canal treatment, and the World Health Organization encourages the reduced use of antibiotics. Despite these recommendations, the American Association of Endodontists' regenerative endodontic guidelines mention the use of antibiotic pastes, and practitioners commonly employ these drugs for disinfection purposes (6). Furthermore, recent studies have demonstrated the superior antibacterial effects of double antibiotic pastes over calcium hydroxide (8, 9). Therefore, using the proper dosage is crucial. In regenerative endodontic procedures (REPs), achieving antibacterial efficacy is key to clinical success. The aim of this study was to evaluate the antibacterial effectiveness of different doses of a dual antibiotic paste used to ensure antibacterial efficacy.

Both DAP and TAP are recommended because they have residual antibiofilm effects. However, compared to TAP, DAP has longer-lasting residual antibiofilm effects (11) and minimum tooth discoloration potential (10, 22). One study proposed that 1 mg/mL of DAP-loaded methylcellulose system was efficient in eliminating 3-week-old *E. faecalis* biofilm. Other studies have shown

that 1 mg has a direct antibiofilm effect on *E. faecalis* (15, 23). In the present study, various DAP concentrations (0.125, 0.25, 0.5, 1.5, and 10 mg/mL) were tested on multispecies biofilms, and DAP doses of 5 mg/mL and 10 mg/mL were found to have significantly higher antibiofilm effects compared to 1 mg/mL.

Different carriers can be used for diluted antibiotic pastes. For example, various studies have employed sterile water (7, 11) and methylcellulose powder (22). In this study, diluted antibiotic patches were obtained using methylcellulose (22).

In this study, 10 mg/ml of DAP successfully eliminated the entire biofilm formation over a period of 7 days. Every other concentration of antibiotic paste diminished the existing biofilm, but none of them completely eradicated it. The antibiotic concentration necessary for complete biofilm eradication in this investigation (10 mg/mL) was comparable to the concentrations documented in prior studies (10 mg/mL) (16). In that study, a two-species biofilm was used to evaluate the effect of diluted antibiotic medicaments. To our understanding, that examination of the antibiofilm effects of different antibiotic doses on a well-established multispecies biofilm is a more accurate representation of real clinical conditions (7).

In a clinical study, microbiota was examined in samples collected at various stages using paper points from 15 cases during regenerative endodontic treatment (RET). After performing a root canal treatment (RET), a

molecular analysis identified the presence of 10 specific pathogens commonly found in endodontic infections: *Actinomyces naeslundii*, *Porphyromonas endodontalis*, *Parvimonas micra*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Filifactor alocis*, and *Treponema denticola* (20).

In the root canal system, there are mostly facultative and strict anaerobic bacteria colonies (24). The biofilm model in this study used *E. faecalis* because it has been widely isolated from persistent endodontic infections (25). *S. sanguinis* was used in addition to *E. faecalis* because it is frequently seen in mature, permanent teeth (26). A third bacteria, *A. naeslundii*, which is most common in immature, necrotic, permanent teeth, is also used in this bacterial biofilm (27).

Many studies have examined the formation of biofilms consisting of a single species, particularly *E. faecalis*, on dentin (7, 11, 15, 16). However, few studies have investigated the formation of biofilms that include many species in human dentin (28).

In addition to standardizing various tests and techniques for the strains under investigation, it will be beneficial to enhance the repeatability, comparability, and predictability of clinical success. In this study, as in many other studies, confocal laser scanning microscopy (CLSM) was utilized.

Ensuring a proper equilibrium between the antibacterial properties and the potential harm to cells is critical when using any suggested intracanal medicament (11). DAP at a dose of 0.125 mg/ml was reportedly not toxic to DPSCs (Reference). In one study, this concentration was neither toxic nor effective against an *E. faecalis* biofilm (11, 29). In this study, similar findings were obtained; this concentration was ineffective against a multispecies biofilm. The American Association of Endodontists (AAE) recommended using 0.01-0.1 mg/ml concentrations of antibiotic medicaments in regenerative endodontics (11). This dose might be ineffective against bacterial biofilms in root canal systems. Providing the essential antibacterial efficacy, which is crucial for RET success in clinical settings, can only be achieved by using the correct antibacterial agents in appropriate doses. There is a need to develop products and medicaments that can be utilized to disinfect root canal systems during RETs, that do not negatively affect DPSCs, and that do not cause adverse effects, such as antibiotic resistance (20).

This study's limitations include the inability to fully replicate a clinical environment, the influence of culture medium conditions on antibacterial activity, being conducted on tooth sections rather than whole teeth, and the inability to rinse with EDTA.

## Conclusion

In regenerative endodontics, the 5 mg/ml and 10 mg/ml doses of DAP demonstrated more effective antibiofilm activity against a multispecies biofilm than lower doses.

## Disclosures

**Ethical Approval:** Ethics committee approval was received for this study from the Hamidiye Scientific Ethics Committee of the Health Sciences University, in accordance the World Medical Association Declaration of Helsinki, with the approval number: 2022-007

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