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# Combined Effect of Levofloxacin and N-Acetylcysteine against *Enterococcus faecalis* Biofilm for Regenerative Endodontics: An *in Vitro* Study

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**ABSTRACT**

**Introduction:** Endodontic treatment of necrotic immature teeth poses several clinical challenges. A major problem is the elimination of microorganisms from the root canal system. This study evaluates the *in vitro* antibacterial efficacy of ciprofloxacin (CIP), levofloxacin (LEV), and their combination with N-acetylcysteine (NAC) in root canals infected with *Enterococcus faecalis* (*E. faecalis*). **Methods and Materials**: A total of 120 human extracted teeth with single canals were prepared and randomly divided into six groups: Calcium hydroxide (CH), ciprofloxacin (CIP), levofloxacin (LEV), ciprofloxacin and N-acetylcysteine (CIP+NAC), levofloxacin and N-acetylcysteine (LEV+NAC), and normal saline as a positive control. According to the name of the groups, intracanal medicaments were placed into the canals and the teeth were restored with a temporary filling. After one week, intracanal medicament was removed and the final count of bacteria was measured. Antibacterial effect of medicament was assessed by measuring the percentage reduction in the colony counts (RCC) and scanning electron microscopy (SEM). The Mann-Whitney U test and the Kruskal-Wallis test were used to compare the overall antibacterial efficacy of the intracanal medicaments at significance level of 0.05. **Results**: All intracanal medicaments were significantly more effective than calcium hydroxide ($P<0.05$). The combination of LEV and NAC caused significantly higher reduction in colony count in comparison with other tested medicaments ($P=0.001$). **Conclusion**: The combination of LEV and NAC showed greater antibacterial activity compared with other tested medicaments against biofilm of *E. faecalis*. Thus, it has the potential to be used in regenerative endodontic treatments.

**Keywords**: Antibiotics; Biofilm; *Enterococcus faecalis*; Regenerative Endodontics

## Introduction

Treatment of immature permanent teeth with infected necrotic pulp is challenging [1]. Traditionally, apexification with calcium hydroxide has been used in such circumstances. Apexification induces apical barrier formation and prevents the extrusion of filling materials [2]. However, some drawbacks of this technique include possible root fracture and multiple visits for the treatment [3].

In recent years, the popularity of regeneration protocols has led to a shift towards less invasive treatment strategies in endodontics [4]. Regenerative endodontics can be defined as a biologically-based procedure which is designed to replace damaged pulpal tissues with viable ones that restore the normal function of the pulp-dentine complex [5].

Biofilms are surface-attached microbial communities encased in a self-produced slimy matrix or extracellular polymeric substance (EPS) [6]. The EPS matrix is a key feature in biofilm structure that can be considered as a major reason for the successful biofilm formation against antimicrobial agents and environmental stress compared to planktonic cells [7].

Proper root canal disinfection is a major factor that affects the success of regenerative treatments in infected immature teeth [8]. Due to thin dentinal walls of necrotic immature teeth, mechanical instrumentation is not performed thoroughly [9]. Thus, the disinfection protocol is mainly based on the use of irrigants and intracanal medicaments.

Triple antibiotic paste (TAP), containing ciprofloxacin (CIP), metronidazole, and minocycline, has proved to be effective for disinfection of the infected necrotic immature teeth [10]. It can create a favorable environment for the regeneration of vital tissues and for the successful healing of peri-radicular lesions [11]. However, this paste has shown some disadvantages such as crown discoloration and demineralization effects on root dentine that reduce its microhardness values [12]. Minocycline in this formulation is believed to be responsible for such adverse effects [13].

Calcium hydroxide (CH) is widely used as an intracanal medicament to prevent microbial re-growth [14]. It also provides additional disinfection after chemo-mechanical cleaning. In biofilms, however, the disinfecting efficacy of calcium hydroxide is limited [15]. Evidence shows that *E. faecalis* may benefit from a CH challenge and grow in mixed biofilms [16]. Also, high concentrations of CH may be toxic for stem cells [17]. Moreover, CH is less likely to be removed from the canal space with conventional filing and irrigation with sodium hypochlorite [18].

Aiming at effective intracanal medicaments for regenerative endodontics, different chemotherapeutic agents were investigated. Fluoroquinolones such as ciprofloxacin (CIP) and levofloxacin (LEV) could readily equilibrate across the biofilm [19] and therefore, they seem to be effective in stopping the growth of biofilms [20].

N-acetylcysteine (NAC) is a mucolytic agent that has been reported to inhibit biofilm formation [21]. It disrupts mature biofilm formation through decreasing bacterial adhesion on solid surfaces [22]. NAC can detach bacteria by decreasing the production of "Extracellular Polysaccharides" (EPS) [22]. Quah *et al.* [23] showed that NAC inhibits growth and eradicates *E. faecalis* biofilm. However, antibacterial character of NAC was not affected in the presence of dentine.

The development of a new disinfection regimen for regenerative endodontics may lag the grand work to achieve more predictable results. This study aimed to evaluate the antibacterial efficacy of CIP and LEV alone and combined with NAC against biofilm-producing *E. faecalis* in the root canals of extracted human teeth.

***Table 1.*** The minimum inhibitory concentration (MIC) and MBC amount of medicaments (μg/mL)

| Medicament | MIC | MBC |
|---|---|---|
| **Levofloxacin** | 0.5 | 1 |
| **Ciprofloxacin** | 1 | 2 |
| **Calcium hydroxide** | 16 | 32 |

## Materials and Methods

### *Preparation of specimens*

One-hundred and twenty recently extracted single-rooted human teeth were selected for this *in vitro* study. The study was approved by the Research and Ethics Committee of Kurdistan University of Medical Sciences. The samples were evaluated for absence of cracks, resorption, and root canal calcification. The external surfaces of teeth were cleaned with periodontal curettes. Then, they were immersed in 0.5% NaOCl solution (Golrang, Pakshoo Co. Tehran, Iran) overnight for disinfection.

Clinical crowns were cut at cementoenamel junction to obtain a standard root length of 13-15 mm. A #10 K-file (Dentsply-Maillefer, Ballaigues, Switzerland) was inserted into the root canals until its tip was visible at the apical foramen. Next, 1 mm was subtracted from this length to determine the working length. The root canals were prepared using the crown-down technique with Pro-Taper rotary files (Dentsply-Maillefer, Ballaigues, Switzerland) to F3 as the size for master apical file.

The canals were irrigated with 1 mL of freshly prepared 5.25% NaOCl solution. After instrumentation, the canals were irrigated with 17% EDTA (Aria Dent, Asia Chemi Teb, Tehran, Iran) followed by 5.25% NaOCl to remove the smear layer. A final rinse was carried out using 5 mL of sterile water. The teeth were dried with sterile paper points.

To prevent bacterial leakage, the external surfaces and apices of samples were covered with nail polish and resin, respectively. The specimens were then autoclave-sterilized in phosphate-buffered saline (PBS) for 30 min at 121°C.

### *Biofilm formation*

*E. faecalis* (ATCC 29212) was grown overnight in brain-heart infusion (BHI) broth medium (Merck, Darmstadt, Germany) to get turbidity of 0.5 McFarland standard ($1.5 \times 10^8$ cells/mL). Then, 2 mL of sterile PBS were removed and replaced with 2 mL of fresh bacterial inoculum in BHI broth. The flasks containing teeth were kept at 37°C for 30 days in an aerobic incubator. The fresh medium was introduced into the canals every 2 days to confirm the growth of bacteria. The teeth would be excluded, if any contaminants were observed.

After 30 days, the specimens were irrigated with 5 mL of sterile saline and dried with sterile gauze. The specimens were then randomly divided into 6 groups ($n$=20), according to the intracanal medicaments, as it follows: Group 1; CH, group 2; CIP, group 3; LEV, group 4; CIP+NAC, group 5; LEV+NAC, and group 6; positive control group (saline).

### *Preparation and application of the medicaments*

The minimum inhibitory concentrations (MIC) of CH, CIP and

LEV were determined to be 1 µg/mL, 0.5 µg/mL and 16 µg/mL, respectively [24]. Each medication was prepared in different concentrations of MICs: 5 times, 10 times, 50 times, 100 times, 500 times, and 1000 times (Table 1). In CH group, CH powder (Golchay, Tehran, Iran) was prepared in paste by mixing CH powder and distilled water with a powder to liquid ratio of 1:2. The paste form of CH was placed into the canal using a #40 file. To make a sticky paste, 30 µL of each concentration of the antibiotics were mixed with a sufficient amount of starch and then placed into the canal using a #40 file. The roots in group 6 were filled with saline as the positive control group.

After placing the desired material into the root canal, the coronal part of the respective tooth was sealed with Coltosol (Aria Dent, Tehran, Iran) for one week. Then, the temporary filling was removed with a fissure bur (Tizkavan, Tehran, Iran). Subsequently, the medicaments were taken out by rinsing the canals with 20 mL of sterile saline.

***Root dentine sampling and scanning electron microscopy (SEM)***

Each of the six groups of roots (Table 2) was divided into two equal subgroups: The first subgroup was used for counting "Colony Forming Units" (CFUs). Root dentine samples were taken from dentinal walls using #3, 4 and 5 sterile Gates-Glidden drills (Dentsply-Maillefer, Ballaigues, Switzerland). Each drill removed the dentine layer from the inner surface of the canal and samples were transferred to a sterile Eppendorf tube containing 100 µL of fresh sterile broth. Each sample was streaked onto BHI agar plates, and the plates were incubated at 37°C for 48 h to observe any microbial growth. The colonies on the agar plates were counted with colony counter, represented in colony-forming units (CFU) per mL.

Another subgroup was used to examine the formed biofilms and their changes after the application of the medicaments on the root canal walls. The roots were prepared for SEM analysis and observed at 25 kV with a KYKY-EM3200 electron microscope (KYKY Technology Development Ltd., Beijing, China).

***Data analysis***

The CFU data was subjected to logarithmic transformation. To compare the efficacy of different medications for the amount of reduction in CFUs/mL and log CFUs/mL, non-parametric statistical analyses were performed using the two-tailed Mann-Whitney U test and the Kruskal-Wallis test. To assess the strength and statistical significance of correlations between concentrations of antibiotics and reduction of CFUs/mL and log CFUs/mL, separate bivariate analyses were performed using the Pearson's rank correlation test. The level of significance was set at 0.05.

## Results

The antibacterial activity of each medicament was calculated from the colony counts following disinfection of infected root canals (Figure 1). No significant difference was observed between calcium hydroxide and the positive control group in the number of log CFUs/mL (two-tailed Mann-Whitney U test, $P$=0.09). The SEM image for positive control group confirmed bacterial colonization and mature biofilm formation after 30 days of incubation (Figure 2A).

For disinfection of *E. faecalis* from the roots, all intracanal medicaments were significantly more effective than calcium hydroxide ($P$<0.05). There was no significant difference in the number of log CFUs/mL among antibiotic groups (Kruskal-Wallis test, $P$=0.08) with one exception. At concentrations of 0.8 mg/mL and higher, combined treatment with LEV and NAC showed significantly higher antibacterial activity than other tested medicaments (two-tailed Mann-Whitney U test) ($P$<0.05 for log CFUs/mL).

The concentration of antibiotics had a significant inverse correlation with the number of log CFUs/mL (Pearson's rank correlation test, $r$=-0.868, $P$=0.000). Reduction in colony count (CFUs/mL) was in accordance with the destruction of the biofilm. At concentrations of 0.8 mg/mL and higher, a notable difference in biofilm structure was observed between levofloxacin with NAC and other tested medicaments (Figure 2B).

In roots with destructed biofilms, numerous particulate and some crystal-like structures were observed on the dentine surface. Such structures were larger than the bacteria and could be considered as antibiotic particles (Figure 3).

***Table 2***. Characteristics of antimicrobial agents assessed for biofilm elimination ($n$=20)

| Antimicrobial agents | Intracanal medication for seven days |
|---|---|
| **CH** | Calcium hydroxide at seven concentrations of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1 mg/mL. |
| **CIP** | Ciprofloxacin at seven concentrations of 0.0005, 0.0025, 0.005, 0.025, 0.05, 0.25, and 0.5 mg/mL. |
| **LEV** | Levofloxacin at seven concentrations of 0.016, 0.08, 0.16, 0.8, 1.6, 8, and 16 mg/mL. |
| **CIP+NAC** | Ciprofloxacin at seven different concentrations plus 8 mg/mL of N-acetylcysteine |
| **LEV+NAC** | Levofloxacin at seven different concentrations plus 8 mg/mL of N-acetylcysteine |
| **Positive control** | Saline |

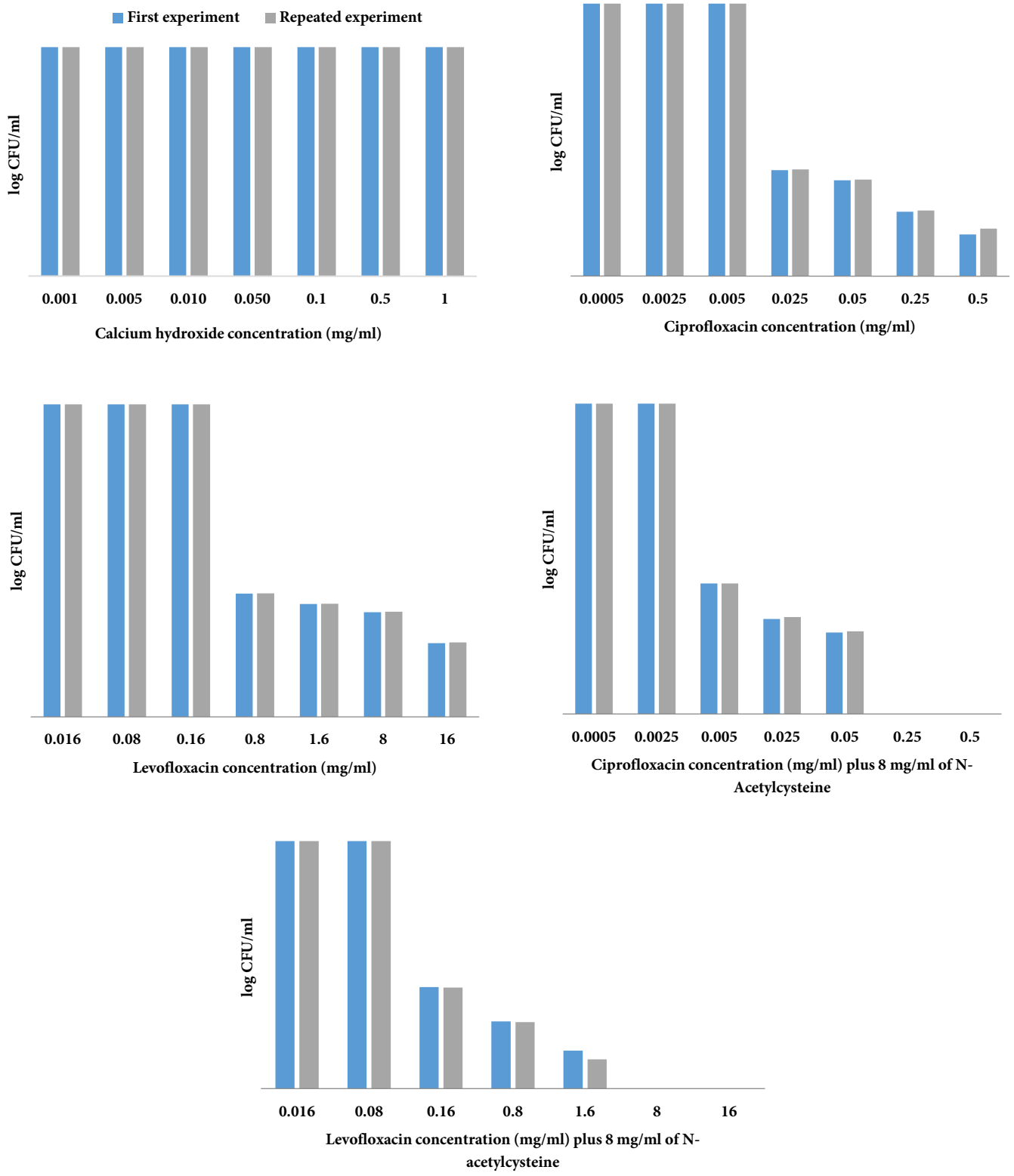


***Figure 1***. Antibacterial activity of the medicaments against *E. faecalis* biofilm in relation to different concentrations (CFU: colony-forming units)

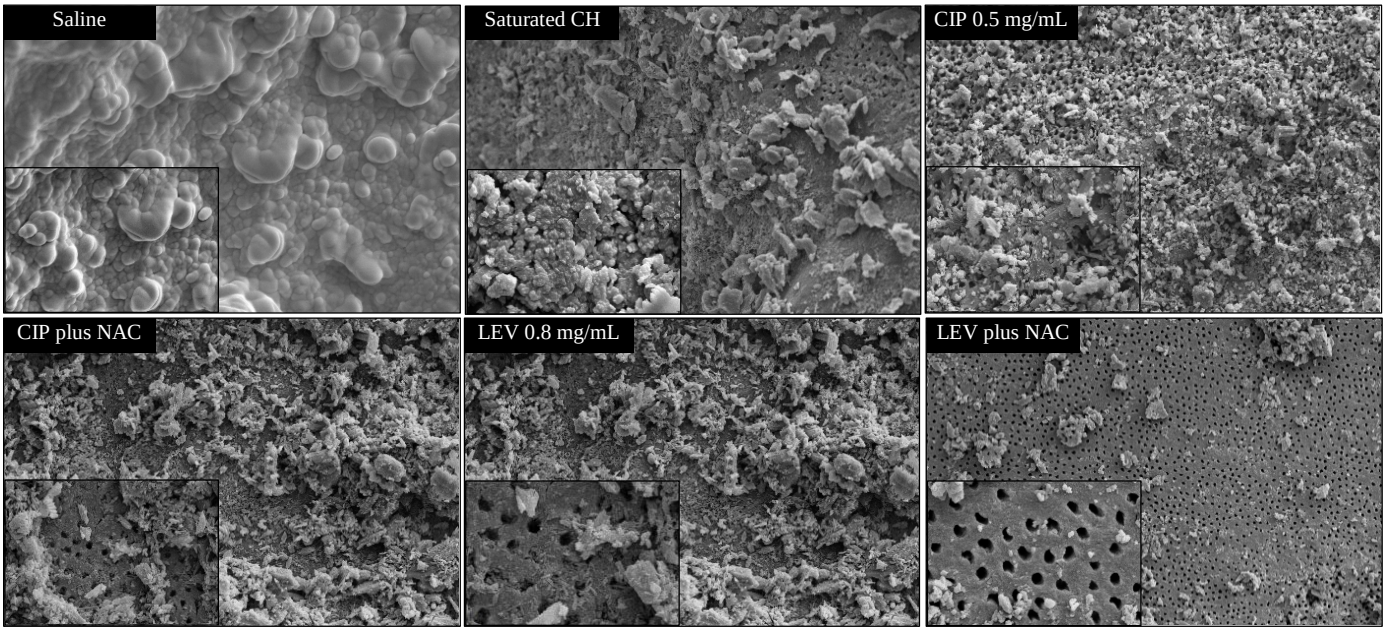


***Figure 2***: Scanning electron microscope (SEM) images of *E. faecalis* biofilms. After 30-days of incubation, the mature biofilms were treated with saline, saturated CH, CIP, ciprofloxacin plus NAC, LEV, LEV plus NAC at the indicated concentrations for 7 days and then observed using SEM at magnification of 1500× and 3000× (inset) operating at 25 kV

## Discussion

There are multiple factors that affect the results of regenerative endodontics. A thorough understanding of these factors is important to achieve a successful outcome for treatment procedures [25]. Disinfection of the canal, apex diameter, and patient age are elements to be considered for more predictable results in endodontic regenerative protocol.

Proper disinfection is the key for success in endodontic regenerative procedures [26]. Following root canal disinfection, residual viable pulp tissues or stem cells from the apical papilla may survive [27]. These tissues and cells might allow for the continuation of root development and tissue regeneration.

Bose *et al.* [28], proposed that CH and the triple antibiotic paste, when used as intra-canal medicaments, allowed for further development of pulp-dentine complex in necrotic immature teeth. Other studies also reported favorable outcomes from revascularization treatment by the use of CH and triple antibiotic paste (TAP) as intra-canal medication in necrotic immature molars [29, 30].

Antibacterial effect of TAP can provide a suitable environment for healing, as new tissue can infiltrate and grow into the radicular area [10]. Nevertheless, despite its positive effects, development of resistant bacterial strains, tooth discoloration, demineralization of the dentine and lethal effect on the stem cells of apical papilla (SCAP) are some of its pitfalls [31].

Although CH is the most frequently used intra-canal medicament [32], some concerns exist about its limited efficacy against biofilm-producing bacteria, including *E. faecalis* [15, 33]. The antimicrobial activity of CH depends on the release of hydroxyl ions into an aqueous environment [14]. Kayaoglu *et al.* [34] concluded that an increase in the pH of CH up to 8.5 could lead to an increase in collagen-binding ability of *E. faecalis*.

producing *E. faecalis* from the infected roots. Incubation of root dentine with *E. faecalis* for 21 days led to a well-developed biofilm that was highly resistant to CH dressing [35]. Similarly, in our study, it could be speculated that mature biofilms (30 days) were the reason for CH inefficacy. This might be due to the use of CH in paste form, which was shown to be incompetent in hydroxyl ion release and antimicrobial activity compared to its aqueous suspension [36].

We used fluoroquinolone family as the antibiotic of choice due to their compatibility with revascularization procedures [10] and presumed ability to penetrate into bacterial biofilms [19]. Also fluoroquinolones are believed to be highly capable of ceasing the growth of biofilms [20]. All chemotherapeutic agents showed significantly higher antibacterial activity than CH, which is a noteworthy finding.

The antibacterial activity of NAC is likely to be achieved by reducing EPS production and irreversible damage of bacterial proteins essential for growth and metabolism [37]. In our study, LEV at a concentration of 0.8 mg/mL plus 8 mg/mL NAC

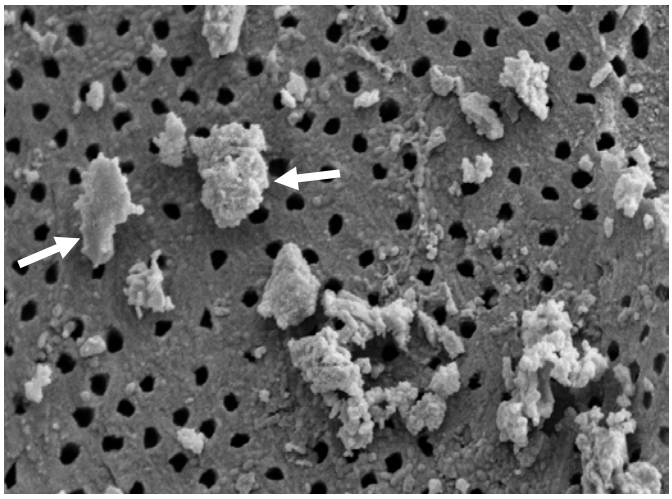

***Figure 3.*** Crystal-like structures on the dentinal wall surface

showed significantly higher antimicrobial activity than other medicaments. This finding coincides with a recent *in vitro* study reporting that NAC effectively eradicated *E. faecalis* biofilms from dentine [23]. However, similar to our observation about CIP plus NAC, combined use with NAC did not enhance the activity of alexidine against biofilm-producing *E. faecalis* [38].

Likewise, in this study, CH failed to eliminate biofilm- Recent studies have provided us with a better understanding about *E. faecalis* and mechanisms involved in persistent endodontic infections [39]. However, it should be noted that an infected root canal usually contains more than one species of pathogens [40]. Even in the existence of *E. faecalis* biofilm, ultra structural, and physiochemical properties of the root canal wall may be different in a clinical setting [41]. Nevertheless, based on our *in vitro* results, N-acetylcysteine can increase the therapeutic activity of fluoroquinolones. Thus, the combination of LEV and NAC has a higher disruptive effect on biofilms when compared to calcium hydroxide.

## Conclusion

To improve canal disinfection in the regeneration of necrotic immature teeth, using a combination of LEV and NAC, in comparison with CH, showed greater antibacterial efficacy through inducing biofilm destruction. Thus, LEV and NAC could be used as an alternative intracanal medicament for regenerative endodontics. In order to use lower concentrations of antibiotics, further studies should be conducted to compare the antibacterial effect of LEV and NAC in combination with triple antibiotic paste (TAP).

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