

An *In Vitro* Investigation on Antibacterial Effectiveness of Ciprofloxacin and Levofloxacin, Alone or in Combination with N-acetylcysteine as Intracanal Medicaments Against Biofilm-producing *Enterococcus faecalis*

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Introduction

- The principal cause of root canal treatment failure is thought to be persisting infections subsequent to endodontic therapy caused by polymicrobial communities which are capable to adapt to the new environmental conditions set by the treatment (1). The frequent detection of *Enterococcus faecalis* from root canals of teeth with failed endodontic treatment has led to an intense research interest in this bacterium (2, 3). In fact, *E. faecalis* is **nine times** more likely to be found in failed root canal treatment cases than primary endodontic infections (4).

- Although *E. faecalis* possesses several virulence factors (5), its capability to secrete serine protease and the collagen-binding protein for adhesion to the root canal (6) and survive prolonged periods of starvation until recovery upon addition of human serum (7), seems to play an important role in the establishment of persistent endodontic infections. Moreover, *E. faecalis* is able to penetrate dental tubules (8) and form biofilms in medicated root canals (9) which offer many benefits to cells living in that are not observed for planktonic cells, including much more resistance to host immune responses and antimicrobial agents (10).

- Since *E. faecalis* biofilm formation in medicated root canals can lead to endodontic failures (5, 9), it is necessary to eradicate these biofilms by use of different strategies, including mechanical instrumentation, chemical irrigation, and intracanal medication (11). The latter approach is the focus of this study which has apparently been shown to contribute to further reduction of the root canals microbial flora (12).
- The most widely used intracanal medicament is calcium hydroxide ($\text{Ca}[\text{OH}]_2$) which has antibacterial activity due to its high pH (13). However, given the limited effectiveness of $\text{Ca}(\text{OH})_2$ in preventing regrowth of endodontic bacteria (14) as well as its inadequacy in eradication of *E. faecalis* from the root canal (9, 15, 16), more effective medicaments are needed in order to combat these bacteria.

- To this purpose, local application of chemotherapeutic agents within the root canal system can be considered as an effective strategy, but only after selection of the appropriate agents (17).
- Among different chemotherapeutic agents, ciprofloxacin (CIP) and levofloxacin (LEV) were reported to efficiently diffuse into the bacterial biofilms (18, 19). Several previous studies also reported antimicrobial effectiveness of CIP against intracanal and periodontal *E. faecalis* (17, 20), and LEV was found to be even more active than CIP against biofilm bacteria (19, 21).

- Another promising chemical for intracanal medication is N-acetylcysteine (NAC), a mucolytic agent which was reported to inhibit biofilm formation by decreasing bacterial adhesion on surfaces and even detaching them, as well as reducing the production of extracellular polysaccharides (22, 23). It is speculated that combination of NAC with other antimicrobial substances can result in enhanced efficacy against bacteria in biofilms, including *E. faecalis* (24). Thus, the aim of this study was to evaluate the antibacterial efficacy of CIP and LEV alone and combined with NAC against biofilm-producing *E. faecalis* in root canals of extracted human teeth.



Materials *and* methods

Preparation of the Roots

- One hundred and twenty-three recently extracted single-rooted human teeth with straight roots, checked by radiographs, were prepared as described elsewhere (25).

Biofilm Formation

- Eighty two out of 123 roots were randomly divided into **eight groups** (Table 1). **For groups 1 to 7**, *E. faecalis* (American Type Culture Collection 29212) was grown on brain-heart infusion (BHI) agar (Merck, Germany) for 24 h at 37°C and 1.5 ml of its fresh inoculum in BHI broth (Merck, Germany) adjusted spectrophotometrically to match the turbidity of a McFarland 1 scale (3×10^8 cells/ml) (Biowave, S2100, UK) was placed in the plastic tubes containing each specimen, and every 3 days new inoculum with the same turbidity was introduced into the tubes. The same method was done for **group 8 using the** same fresh, sterile BHI broth but without bacterial inoculum. A sample was taken every the third day and was cultured on agar plates to confirm the purity of *E. faecalis* in the tubes of groups 1 to 7 and the sterility of the procedure for the test tubes of group 8. These methods were carried out for **30 days**.
- During this period, the test tubes were incubated at 37°C in an aerobic incubator.
- After the 30-days period, the roots were washed with phosphate-buffered saline (PBS). Root canals were then irrigated with 5 ml of saline and dried with sterile paper points.

Preparation and Application of Medicaments

- For groups 1 to 5, the minimum inhibitory concentration (MIC) of $\text{Ca}(\text{OH})_2$, CIP and LEV was measured by using broth microdilution method (26, 27), and each of the medicaments (Table 1) was prepared at seven different concentrations of MIC, 5 MIC, 10 MIC, 50 MIC, 100 MIC, 500 MIC and 1000 MIC, by dissolving their powders in distilled water. Then 30 μl of each of the concentrations was mixed with sufficient and equal amount of dental powder to make a sticky paste. Under aseptic conditions, the canals of groups 1 to 5 were filled with respective medicaments using a nonsurgical manual MTA carrier (Dentsply, Tulsa Dental). The roots in group 6 were filled with water of standardized hardness (WSH) as controls. Coronal and apical orifices were sealed with wax and each of the specimens was wrapped with aluminium foil to prevent dehydration, then placed in a sterile plastic tube and incubated at 37°C for seven days.
- At the end of the one week-period, the medicaments and WSH were removed by rinsing the canals with 20 ml of sterile saline and the roots were air-dried.

Root Dentin Sampling and SEM Analysis

- Each of the eight groups of roots (Table 1) was divided into two equal subgroups;
- **Subgroup 1** was used for colony forming unit (CFU) counting; Root dentin samples were taken using a previously described method of collection (25), followed by preparation of 10-fold dilutions in saline, from which 0.1 ml were spread plated onto BHI agar plates, incubated at 37°C for 48 hours, and CFUs per 1 ml were counted.
- **Subgroup 2 was** used to examine the structure of the biofilms formed on the root canal wall and their changes after the medications;
- The roots were prepared for SEM analysis as described previously (9), then were observed at 25 kV with a KYKYEM3200 instrument (KYKY Technology Development Ltd., Beijing, China).
- The CFU counting experiments were repeated at 4 weeks interval; The remaining 41 roots out of 123 were divided similar to table 1 as to have seven roots in each of the five medication groups (groups 1 to 5) and two specimens in each of the control groups (groups 6 to 8). The steps of biofilm formation, preparation and application of medicaments, root dentin sampling and CFU counting were repeated as mentioned above.

Statistical analysis

- The data for CFU/ml were subjected to logarithmic transformation. To compare the effectiveness of the different medicaments on the reduction of CFU/ml and log CFU/ml, non-parametric statistical analyses were performed using the two-tailed Mann-Whitney U test and the Kruskal-Wallis test, both allowing for continuous variables and non-normal distribution. To assess the strength and statistical significance of correlations between the concentration of the antibiotics and the reduction of CFU/ml and log CFU/ml, separate bivariate analyses were performed by use of the Pearson rank correlation test.

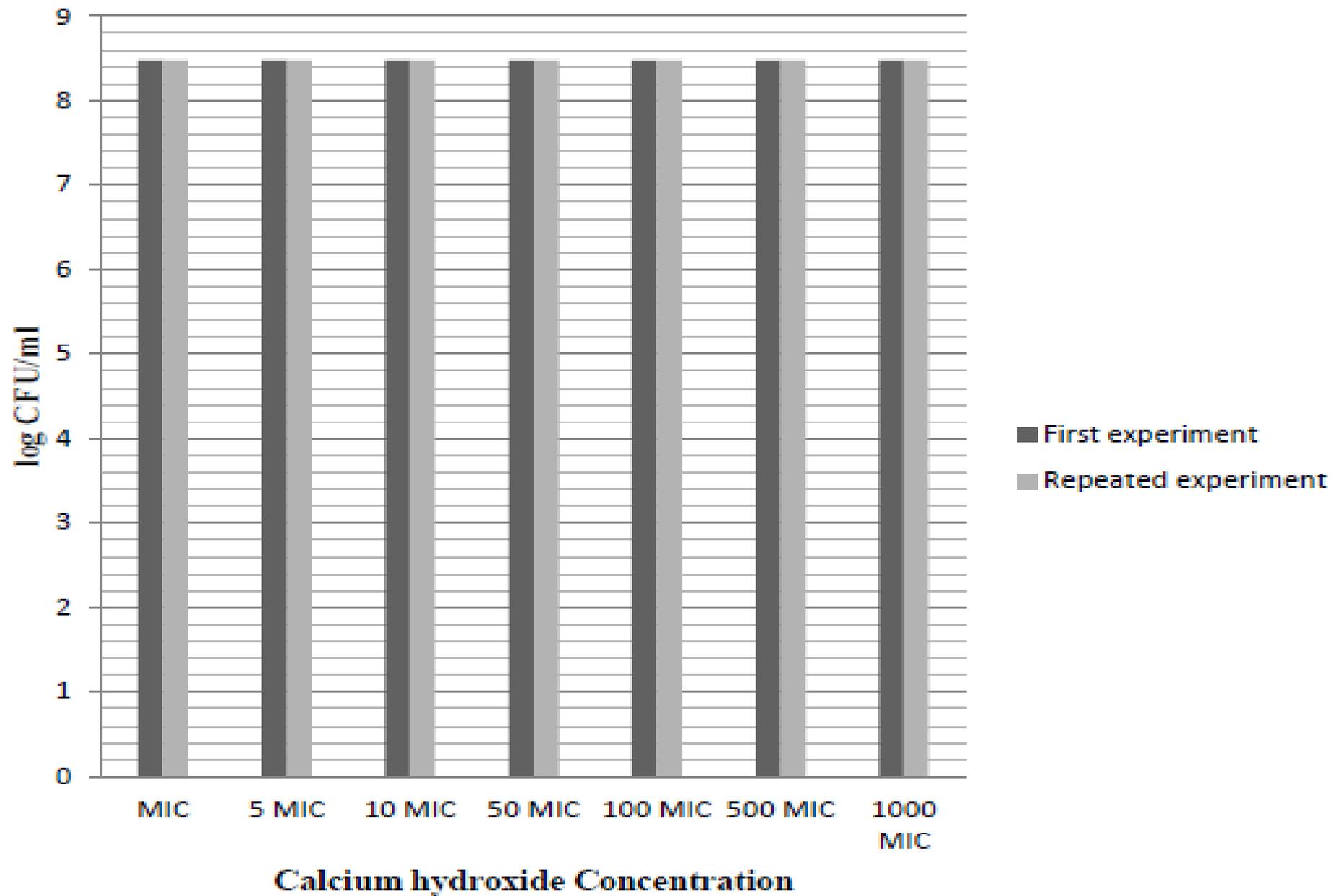


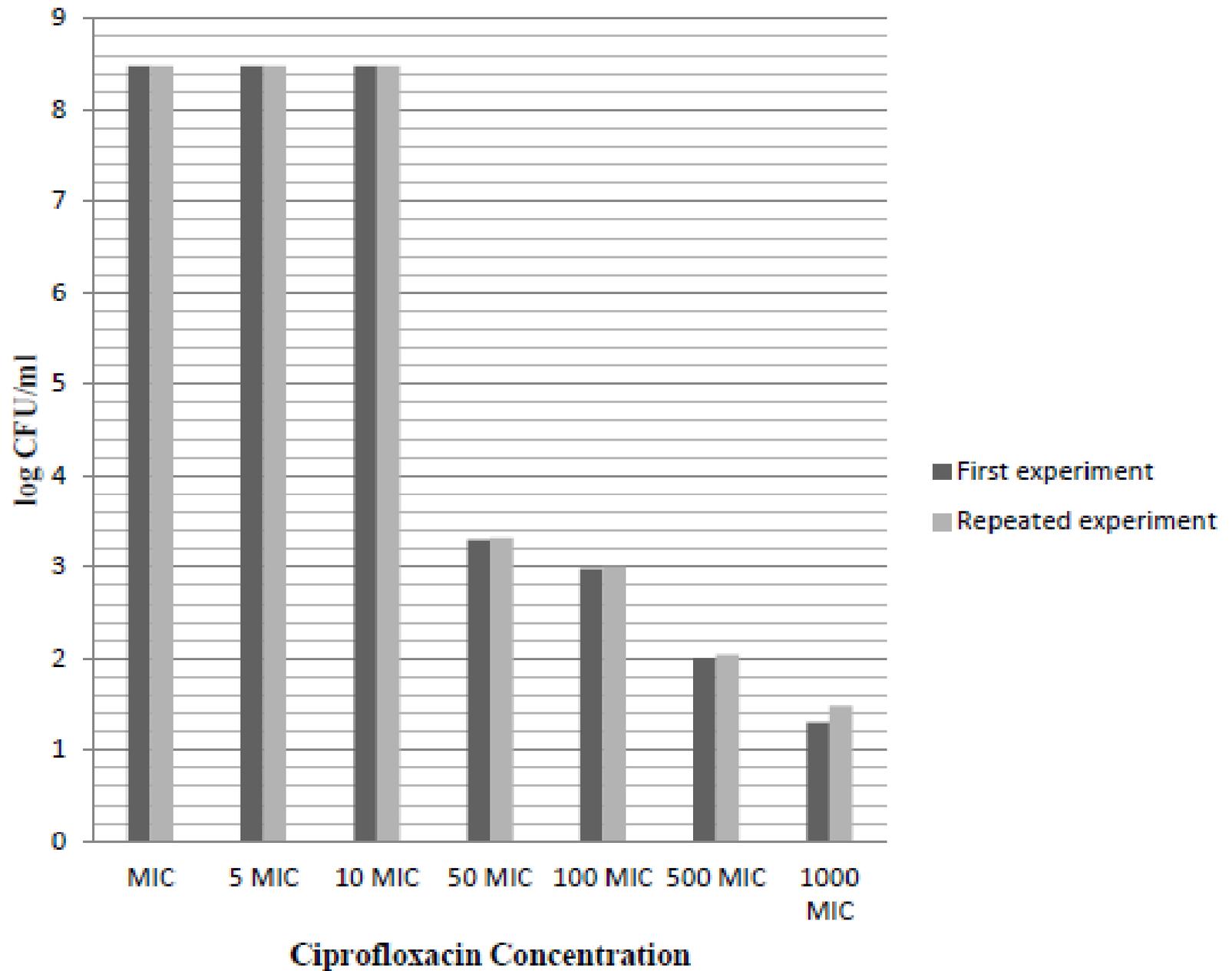
Results

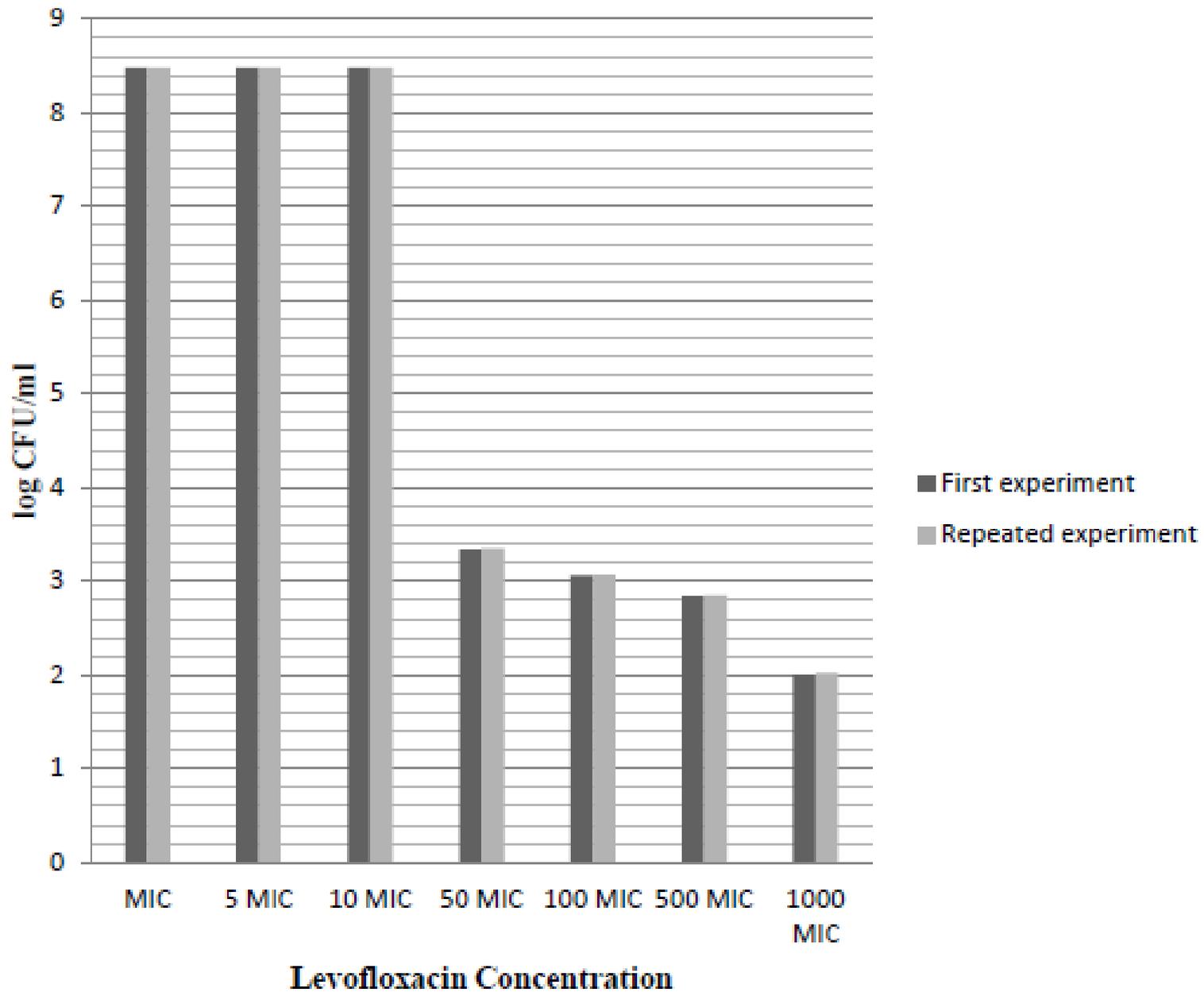
- The MICs of CIP, LEV and Ca(OH)₂ were 1 µg/ml, 0.5 µg/ml and 16 µg/ml, respectively. The antimicrobial activity of each of the medicaments at different concentrations was evaluated repeatedly by counting the CFU/ml and calculating the log CFU/ml (Fig. 1).

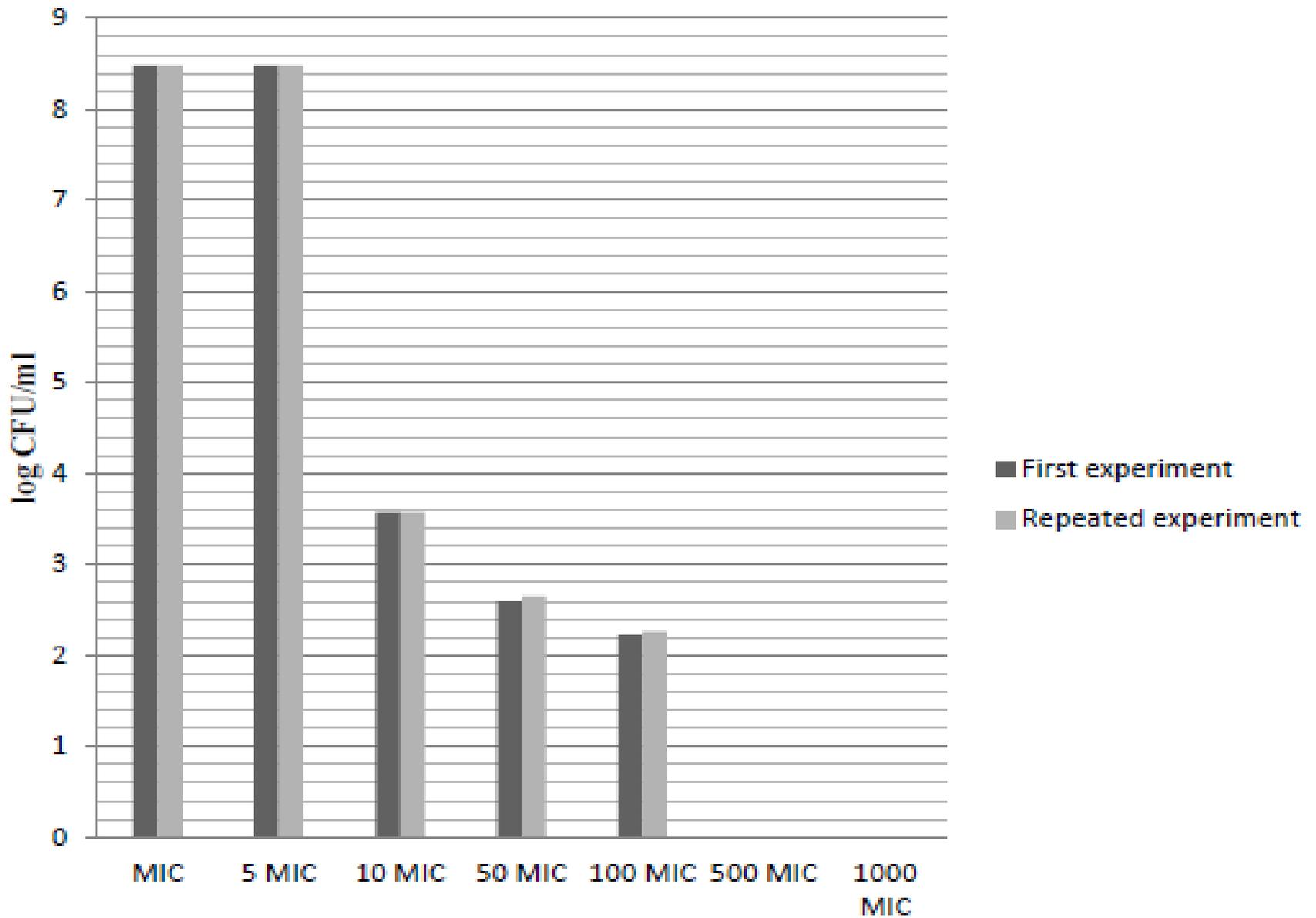
- Figure 1. The antimicrobial activity of the five different medicaments against biofilm producing *E. faecalis* in root canals. 30 days-old *E. faecalis* biofilms were treated for 7 days with the five intracanal medicaments at seven different concentrations and log CFU/ml were calculated.
- (A) calcium hydroxide; group 1.
- (B) ciprofloxacin; group 2.
- (C) levofloxacin; group 3.
- (D) ciprofloxacin plus 8 mg/ml of N-acetylcysteine; group 4.
- (E) levofloxacin plus 8 mg/ml of N-acetylcysteine; group 5.

CFU, colony-forming units.

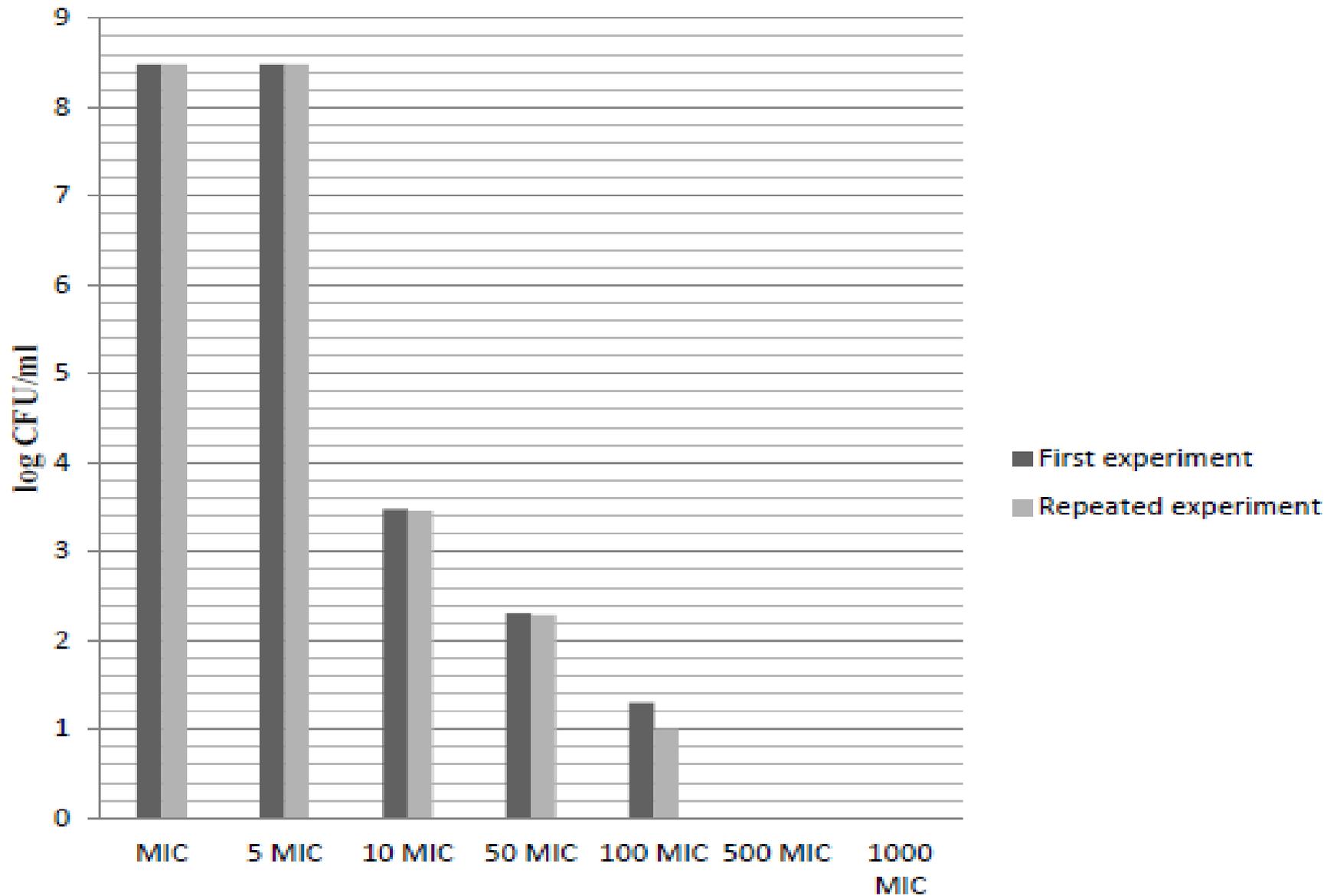








Ciprofloxacin Concentration plus 8 mg/ml of N-acetylcysteine



Levofloxacin Concentration plus 8 mg/ml of N-acetylcysteine

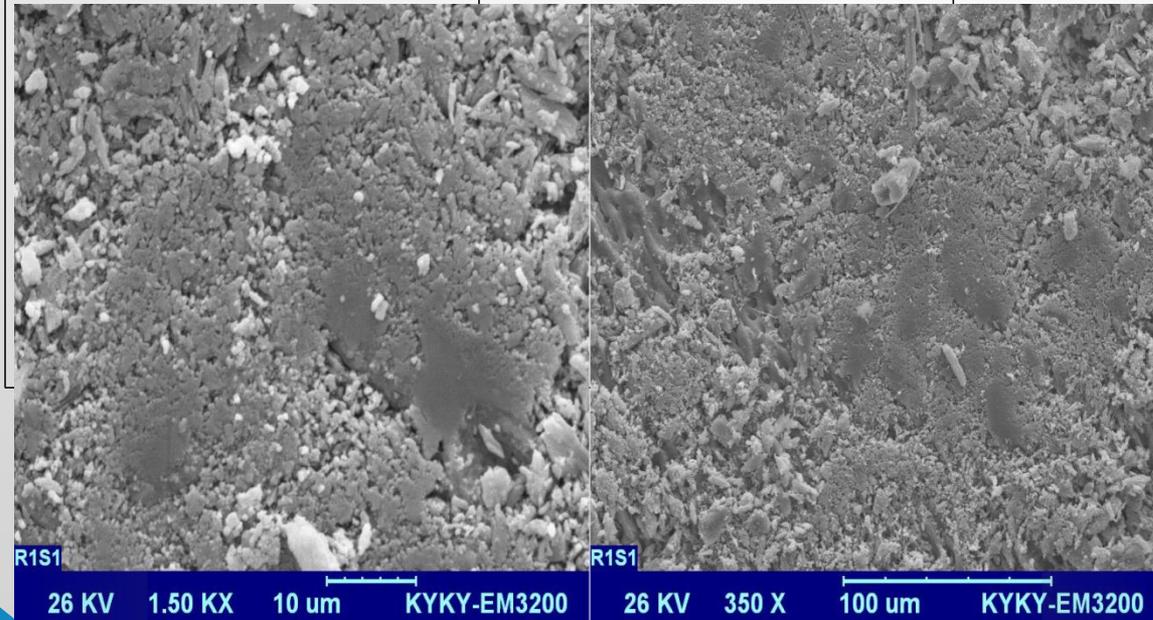
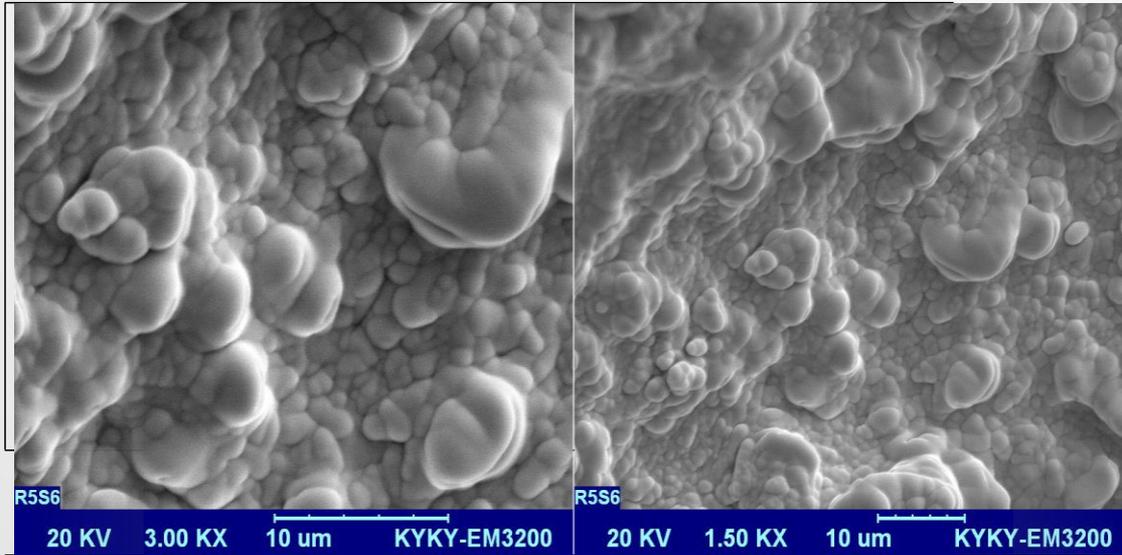
- After one-week medication, no significant difference was observed between group 1 and group 6 in the number of CFU/ml and log CFU/ml (two-tailed Mann-Whitney U test, $P > 0.05$) while every of the four antibiotic groups showed significantly better antibacterial activity than groups 1 and 6 (two-tailed Mann-Whitney U test, $P < 0.01$ for CIP and LEV in CFU/ml and log CFU/ml, $P = 0.001$ for CIP + NAC and LEV + NAC in CFU/ml, and $P < 0.05$ for CIP + NAC and LEV + NAC in log CFU/ml). There was no significant difference in the number of CFU/ml and log CFU/ml among groups 2 to 5 (Kruskal-Wallis test, $P > 0.05$) with one exception; at concentrations of 50 MIC and above, **combined treatment with LEV and NAC showed significantly higher antibacterial activity than LEV alone** (two-tailed Mann-Whitney U test, $P < 0.01$ for CFU/ml and $P < 0.05$ for log CFU/ml).

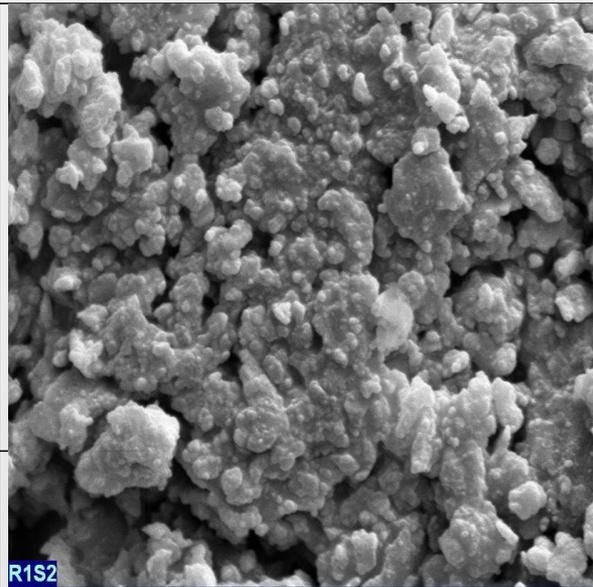
- The antibiotics concentration was significantly negatively correlated with the number of CFU/ml (Pearson rank correlation test, $r = -0.820$, $P = 0.000$), as well as the number of log CFU/ml ($r = -0.868$, $P = 0.000$).
- According to SEM results, group 8 showed the root canals with no bacterial colonization after the 30 days of incubation while mature biofilms were formed in group 7 . Similar to group 6, group 1 (Fig. 2A) showed colonization of all the specimens, regardless of its concentration.

Figure 2. Scanning electron microscopic micrographs of *E. faecalis* colonizing root canals at 30 days. (A) roots medicated for 7 days with calcium hydroxide at concentration of 500 MIC. Magnifications are 3000 × for the left column, and 1500 × for the right column. (B) roots medicated with ciprofloxacin at the seven increasing concentrations plus 8 mg/ml of N-cetylcysteine. S stands for concentration; S1= MIC, S2 = 5 MIC, S3 = 10 MIC, S4 = 50 MIC, S5 = 100 MIC, S6 = 500 MIC and S7 = 1000 MIC. Magnifications are 1500× for the left column, and 350× for the right column. The cells were coated with gold palladium and SEM was done at 25 kV.

Figure2

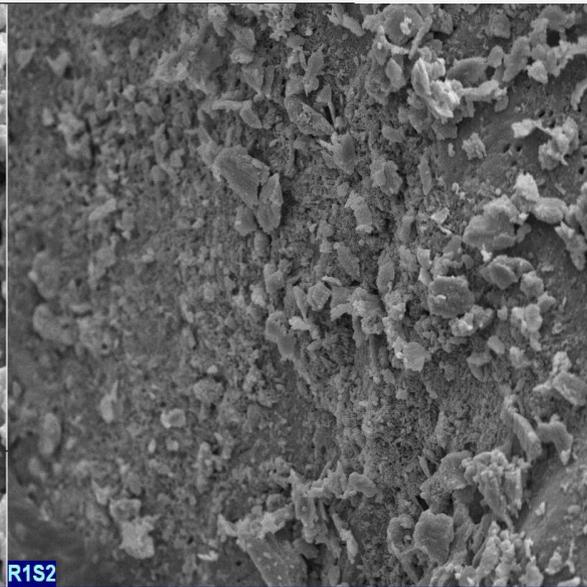
1 A





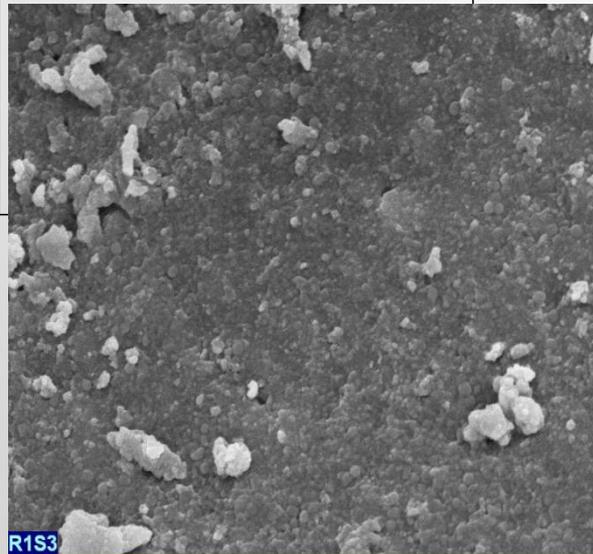
R1S2

26 KV 1.50 KX 10 um KYKY-EM3200



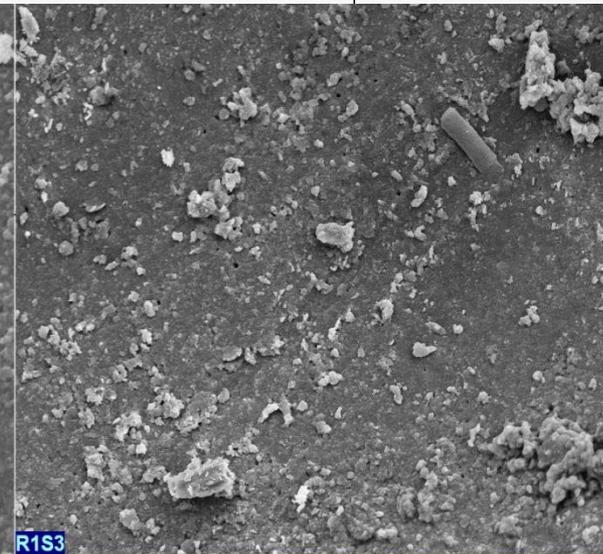
R1S2

26 KV 350 X 100 um KYKY-EM3200



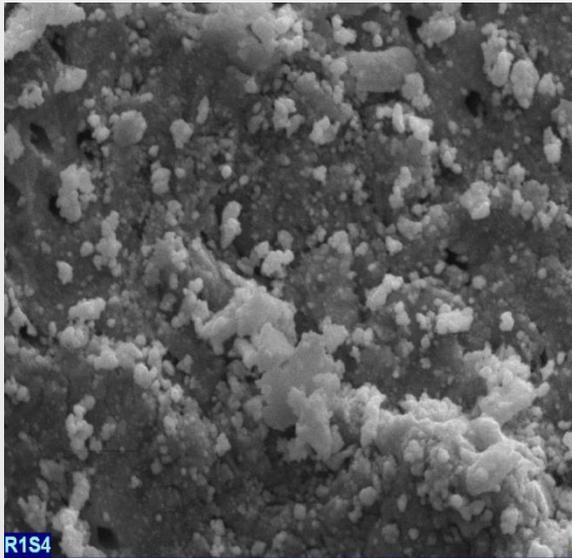
R1S3

26 KV 1.50 KX 10 um KYKY-EM3200



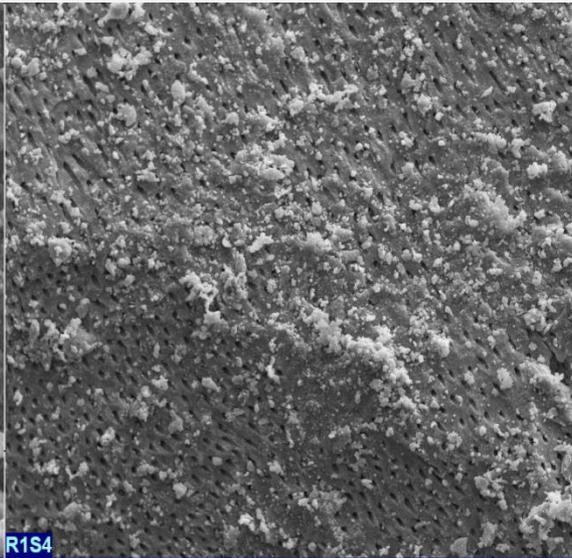
R1S3

26 KV 350 X 100 um KYKY-EM3200



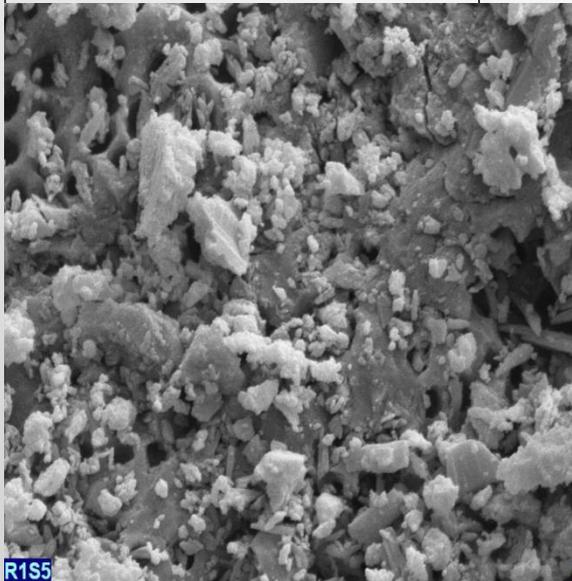
R1S4

26 KV 1.50 KX 10 um KYKY-EM3200



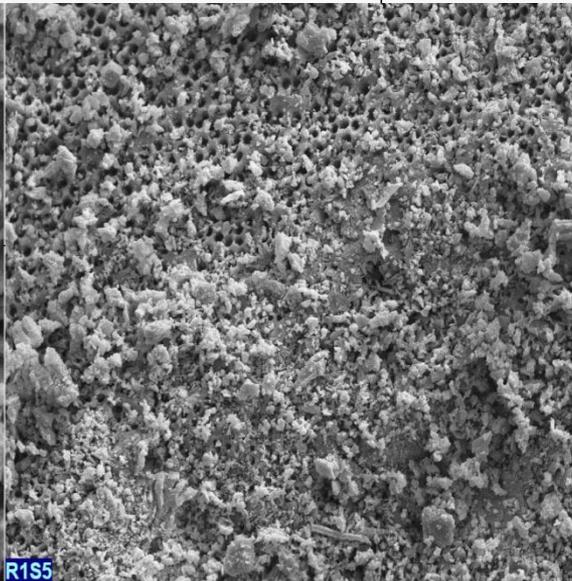
R1S4

26 KV 350 X 100 um KYKY-EM3200



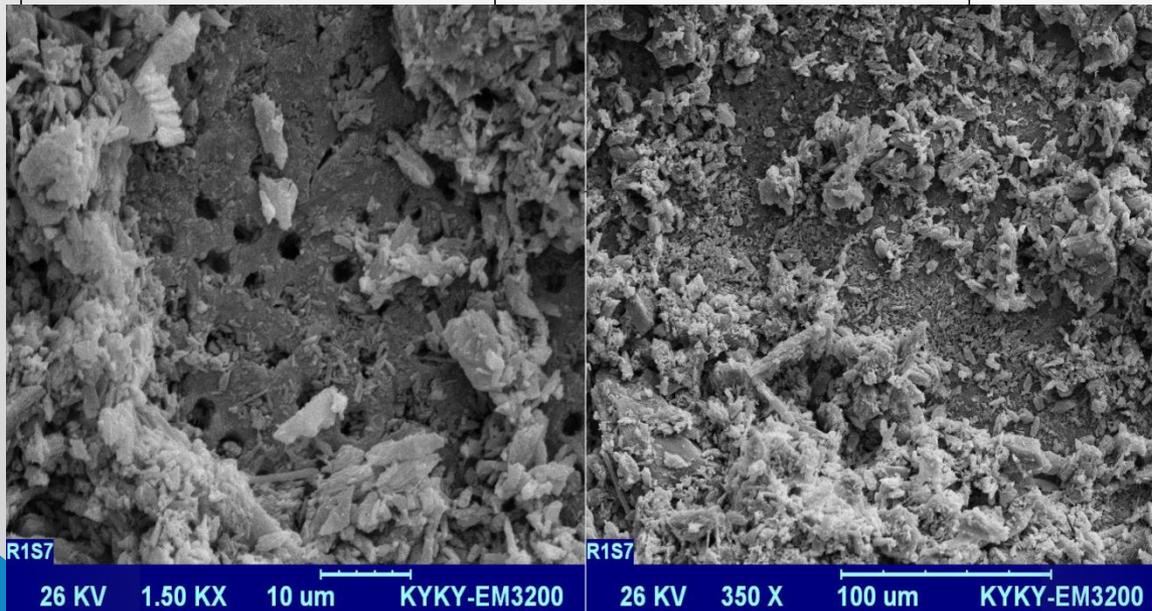
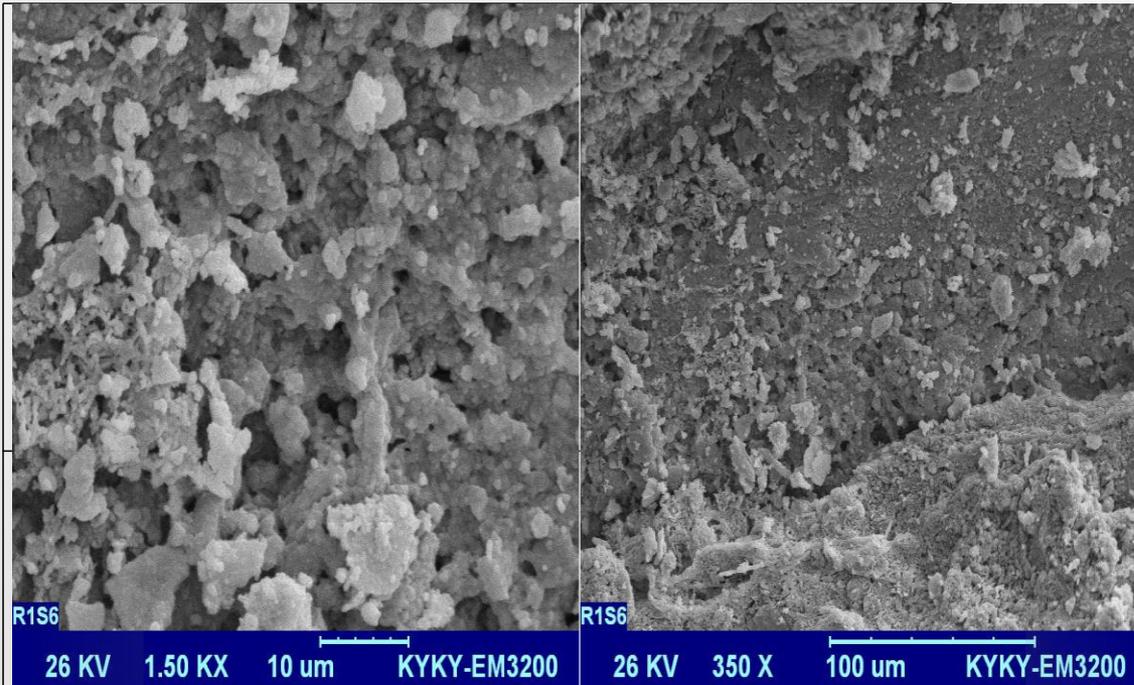
R1S5

26 KV 1.50 KX 10 um KYKY-EM3200



R1S5

26 KV 350 X 100 um KYKY-EM3200

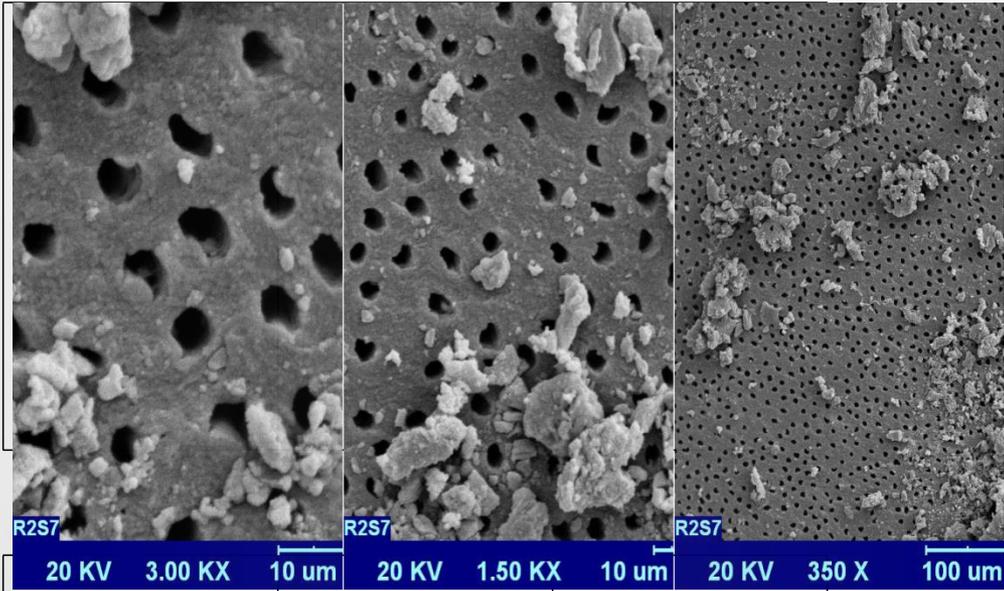


- A complete accordance was observed between reduction in the CFUs per ml and destruction of the biofilms; At concentrations of 50 MIC and above, a notable difference in biofilm structure was observed between group 3 (Fig. 3C) and group 5 (Fig. 3D), but not between group 2 and group 4, even at concentration of 1000 MIC (Fig 3A for the former and Fig 3B for the latter).

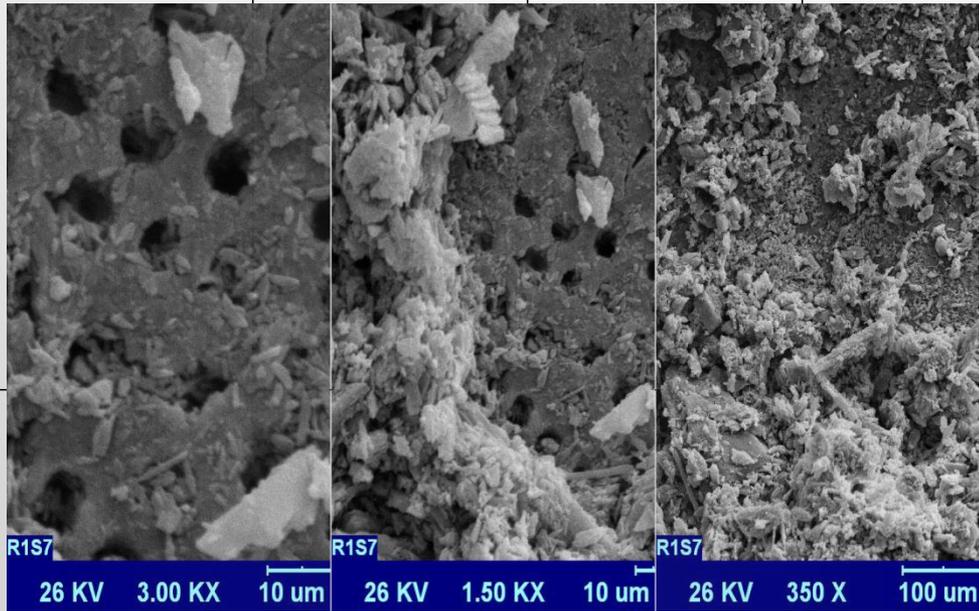
- Figure 3. Scanning electron microscopic micrographs of *E. faecalis* colonizing root canals at 30 days. Roots medicated for 7 days with
 - (A) ciprofloxacin at concentration of 1000 MIC,
 - (B) ciprofloxacin at concentration of 1000 MIC plus 8 mg/ml of N-acetylcysteine,
 - (C) levofloxacin at concentration of 50 MIC,
 - (D) levofloxacin at concentration of 50 MIC plus 8 mg/ml of N-acetylcysteine.
- Magnifications are 3000× for the left column, 1500× for the middle column, and 350× for the right column. The cells were coated with gold palladium and SEM was done at 25 kV.

Figure3

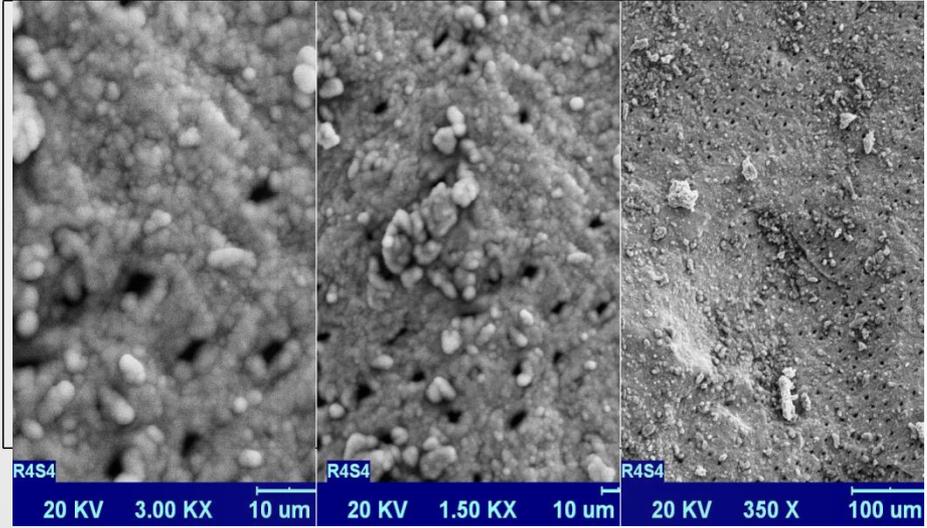
A



B



C



D

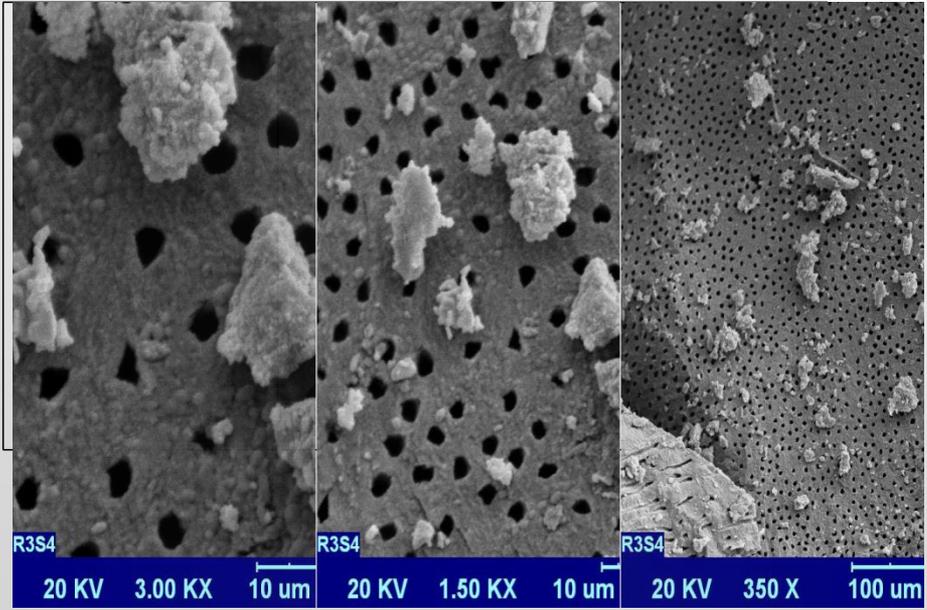


Table 1. Eight Groups of Roots, Including Five Medication Groups, Control Group for Medication Experiments, Negative and Positive Controls for Biofilm Formation Experiments.

No. of group	Name of group	No. of roots in each group	Incubation with <i>E. faecalis</i> for 30 days	Intracanal medication for seven days
1	Ca(OH) ₂	14	Yes	Calcium hydroxide (Sigma-Aldrich, St Louis, MO) at seven different concentrations
2	CIP	14	Yes	Ciprofloxacin (Sigma-Aldrich, St Louis, MO) at seven different concentrations
3	LEV	14	Yes	Levofloxacin (Sigma-Aldrich, St Louis, MO) at seven different concentrations
4	CIP + NAC	14	Yes	Ciprofloxacin at seven different concentrations plus 8 mg/ml of N-acetylcysteine (Sigma-Aldrich, St Louis, MO)
5	LEV + NAC	14	Yes	Levofloxacin at seven different concentrations plus 8 mg/ml of N-acetylcysteine
6	Control for medication	4	Yes	Water of standardized hardness
7	Positive control for biofilm formation	4	Yes	None
8	Negative control for biofilm formation	4	No bacterial inoculum	None



Discussion

- This *in vitro* study aimed to develop a 30-day old *E. faecalis* biofilm onto root canal dentin and evaluate the antimicrobial activity of CIP and LEV alone and combined with NAC against the bacteria in the biofilms.
- *E. faecalis* is commonly detected in asymptomatic, persistent endodontic infections with a high prevalence ranged from 24% to 77% (5), which mainly stems from its ability to form biofilms in root canals (9). Although Ca(OH)₂ has been considered the intracanal medication of choice (13), concern has more recently arisen about its limited antimicrobial efficiency against several bacterial species in infected root canals (14), including *E. faecalis* (9, 15, 16).

- In our study, in agreement with some studies (15, 16) but contradicting the others (28, 29), Ca(OH)_2 failed to eliminate biofilm-producing *E. faecalis* from the infected roots. Incubation of root dentin with *E. faecalis* for 77 days compared with 2 days led to a well-developed biofilm which was highly resistant against Ca(OH)_2 dressing (9). Similarly, it can be speculated that mature biofilm (30 days) was the reason of Ca(OH)_2 inefficiency in our study. This might be also because we used Ca(OH)_2 in paste form which has been shown to be largely lacking in both hydroxyl ion release and antimicrobial activity compared to its aqueous suspension (30). All of the chemotherapeutic agents showed significantly more antibacterial activity than Ca(OH)_2 .

- We used CIP and LEV as the antibiotics of choice, firstly due to their ability to penetrate into bacterial biofilms (18, 19, 21) and secondly, based on previous studies that tested the antimicrobial susceptibility of intracanal and periodontal *E. faecalis* (17, 20). CIP was also previously found to be significantly more effective than Ca(OH)_2 in elimination of biofilm-producing *E. faecalis* inside the root canal system (17). Although in that study, as in this study, a 30 days-old biofilm was developed, CIP applied at MIC concentration led to 80% negative cultures (17) while in our study, CIP caused no reduction in the number of CFU/ml unless at concentrations of 50 MIC and more. This difference may be because Saber and El-Hady added saline to the antibiotic powder until achieving a thick paste (17), but we prepared the antibiotic solutions with defined concentrations then mixed them with sufficient and equal amount of dental powder.

- The combined use of antimicrobial agents (*i.e.* CIP and LEV) and an agent of proven mucolytic activity such as NAC (22, 23) may theoretically lead to an increased efficacy against biofilm bacteria. We used NAC at a concentration of 8 mg/ml which previously showed the highest anti-biofilm activity compared with lower concentrations (22). Although the difference in the antimicrobial effectiveness among the medication groups other than Ca(OH)₂ was statistically non-significant, LEV at concentrations of 50 MIC and more plus 8 mg/ml NAC showed significantly better antimicrobial properties than LEV alone. This finding coincides with a recent *in vitro* study reporting that NAC effectively eradicated *E. faecalis* biofilms from dentin (24). However, similar to our observation about CIP plus NAC, combined use with NAC was not seen to enhance alexidine activity against biofilm-producing *E. faecalis* (31).

- Recent studies have provided us with a better understanding of *E. faecalis* and the mechanisms involved in persistent endodontic infections. Despite the facts that an infected root canal usually contains more than one species of pathogen (1) and even in the case of *E. faecalis* biofilms, distinct ultrastructural and physiochemical properties were demonstrated on root canal wall by changes in the environment (8) which put the clinical relevance of *in vitro* antimicrobial susceptibility testing under question, based on our *in vitro* results, we conclude that intracanal medication with CIP and/or LEV alone or combined with NAC could be highly more effective than paste of Ca(OH)₂ in elimination of biofilm-producing *E. faecalis* from dental apparatus.

Highlights

30 days-old *E. faecalis* biofilms were formed on root canal of extracted human teeth.

Antibacterial efficacy of five different intracanal medicaments at seven increasing concentrations was evaluated.

Ciprofloxacin and levofloxacin were significantly more effective intracanal medicaments than calcium hydroxide.

Intracanal medication with ciprofloxacin or levofloxacin combined with N-acetylcysteine was significantly more effective than that with calcium hydroxide.

There was no significant difference between combined treatment with N-acetylcysteine and single antibiotic treatments.

Levofloxacin at concentrations of 50 MIC and above plus 8 mg/ml of N-acetylcysteine showed significantly higher anti-*E. faecalis* activity than levofloxacin alone.

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Thanks