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In Vitro Antimicrobial Effects of Endoseal MTA and AH Plus Sealers on *Enterococcus Faecalis* and *Candida Albicans* Mature Biofilms

Elham Khoshbin[1], Forough Nikzad[2], Mohammad Reza Arabestani[3], Maryam Farhdian[4], Hamed Karkehabadi[1*]

1. Endodontics Department, Dental Research Center, Avicenna Institute of Clinical Sciences, Avicenna Health Research Institute, Hamadan University of Medical Sciences, Hamadan, Iran
2. Endodontics Department, School of Dentistry, Kashan University of Medical Sciences, Kashan, Iran
3. Department of Medical Microbiology, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
4. Department of Biostatistics, School of Public Health and Research Center for Health Sciences, Hamadan University of Medical Sciences, Hamadan, Iran

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**** Corresponding author:***
Endodontics Department, School of Dentistry, Hamadan University of Medical Sciences, Hamadan, Iran

Email:
Hamed_karkehabadi@yahoo.com

A B S T R A C T

Objectives: This study compared the antimicrobial effects of Endoseal MTA and AH Plus sealers on *Enterococcus faecalis* (*E. faecalis*) and *Candida albicans* (*C. albicans*) mature biofilms on dentin blocks.

Materials and Methods: In this in vitro study, the root canals of 207 dentin blocks sectioned from the roots of extracted teeth were inoculated with *E. faecalis* and *C. albicans*, and incubated for 3 weeks. The specimens were evaluated in 12 groups (n=17) for random root canal filling with AH Plus, Endoseal MTA, or no sealer, and assessment after 24 hours and 2 weeks. Dentin chips were cultured on blood agar, and the colonies were counted. One random specimen from each group was assessed under a scanning electron microscope (SEM). Data were analyzed using the Kruskal-Wallis and Mann-Whitney tests with Bonferroni adjustment at 0.05 level of significance.

Results: At 24 hours, Endoseal MTA significantly decreased the *E. faecalis* count compared with the positive control (P<0.008). At 2 weeks, the colony count of both microorganisms was significantly lower in the AH Plus group compared with the control group (P<0.008). The *C. albicans* colony count in the Endoseal MTA group was significantly lower than that in the control group at 2 weeks (P<0.008). AH Plus caused significantly greater reduction in *C. albicans* colony count at 2 weeks compared with Endoseal MTA (P<0.008).

Conclusion: AH Plus showed a higher antimicrobial activity over time against both *E. faecalis* and *C. albicans*. Endoseal MTA only showed short-term antibacterial effect.

Keywords: Root Canal Therapy; Enterococcus Faecalis; Candida Albicans; Anti-Infective Agents

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INTRODUCTION

Microorganisms and their byproducts remaining in the root canals are the primary cause of endodontic treatment failure [1]. *Enterococcus faecalis* (*E. faecalis*) and *Candida albicans* (*C. albicans*) are among the most commonly isolated microorganisms from the infected root canals [1]. *E. faecalis* is the

dominant pathogen in endodontic infections. It is resistant to irrigating solutions and intracanal medicaments [2]. *E. faecalis* can invade the dentinal tubules, penetrate deep into the tubules, resist the process of cleaning and shaping, and remain viable in filled canals with no support from other bacteria. Also, it can colonize the accessory canals, canal communications, and ramifications, and cause flare-ups [3].

Some fungal species are also responsible for endodontic treatment failure, and *C. albicans* is among the most commonly isolated fungi in endodontic treatment failures [4]. It is compatible with a wide range of pH values, and is resistant to intracanal medicaments such as calcium hydroxide [5]. Also, similar to E. faecalis, *C. albicans* can form a microbial biofilm even under nutrient deprivation [6]. *C. albicans* is known as a dentinophilic microorganism [7].

Microorganisms present in the root canal system are often organized in the form of biofilm, and biofilm microorganisms are more resistant than planktonic bacteria to antimicrobial agents [2]. Biofilm is an assemblage of microbial colonies that are attached to each other or the underlying structure. Biofilm protects the bacteria against antimicrobial agents [8].

Complete elimination of all microorganisms from the root canal system is impossible [9]. Moreover, none of the root filling materials could provide a hermetic seal, and micron-scale gaps always remain between the root filling material and canal walls. Thus, microorganisms can colonize these spaces and lead to reinfection. Therefore, it is highly important for the root filling materials to possess antimicrobial activity [1].

An ideal sealer should have optimal biocompatibility and no polymerization shrinkage. It should be insoluble in oral and tissue fluids and prevent microbial growth, while it should not be cytotoxic for the human tissues. Root canal sealers with antimicrobial activity can contribute to the success of endodontic treatment especially in cases with recurrent or refractory infections [10]. Bioceramic sealers have attracted many attentions due to their optimal biocompatibility, alkaline pH, bioactivity, non-toxicity, optimal dimensional stability and sealability, and the potential to reinforce the root structure [9, 11]. Endoseal MTA has higher amounts of sodium oxide, magnesium oxide, aluminum oxide, sulfur oxide, and iron oxide than other sealers, and is claimed to have higher antimicrobial activity [12]. Its manufacturer also claims that due to the presence of calcium silicate in the composition of this sealer, it has high antimicrobial activity in alkaline conditions [13]. However, no study is available on the antibacterial effects of Endoseal MTA on bacterial and fungal biofilms formed in dentinal tubules.

Bacteria present in the form of biofilm in the oral environment are more resistant than the planktonic form to antimicrobial agents [2, 14]. Use of dentin blocks for biofilm formation can help in better simulation of the clinical setting. Thus, this study aimed to compare the antimicrobial effects of Endoseal MTA bioceramic sealer and AH Plus epoxy resin sealer on *E. faecalis* and *C. albicans* mature biofilms formed on human dentin blocks.

MATERIALS AND METHODS

This in vitro, experimental study was conducted on sound single-rooted human teeth (incisors and premolars) that had been extracted due to orthodontic treatment or periodontal disease. The study was approved by the ethics committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1400.568).

The sample size was calculated to be 16 in each group (a total of 96) assuming the expected mean difference in colony count to be 8 x 105 colony forming units (CFUs)/mL, standard deviation of 8 x 105 CFUs/mL, 95% confidence interval, and 80% study power.

The inclusion criteria were sound single-rooted teeth with no caries, cracks, or anatomical irregularities, no history of previous endodontic treatment, and no calcification as confirmed on buccolingual and mesiodistal radiographs. A total of 207 single-rooted extracted teeth were collected and stored in saline until the experiment. Teeth with fracture, root resorption, and open apex were excluded.

Preparation of dentin cylinders:

The study methodology was adopted from a study by Haapasalo and Ørstavik [15]. Tooth crowns were cut at 1mm below the cementoenamel junction by a diamond bur

(Diatessin, Switzerland) with 0.6mm thickness at 1000rpm speed under water irrigation. Dentin cylinders with 4mm thickness were prepared from the roots, and the canals were prepared by a #2 peeso reamer (Maillefer Instruments, Ballaigues, Switzerland). The specimens were placed in an ultrasonic bath containing 5.25% sodium hypochlorite (Morvabon, Iran) and subsequently in 17% EDTA (Morvabon, Iran), each for 4 minutes. One specimen was inspected under a scanning electron microscope (SEM) to ensure complete smear layer removal [16]. The remaining 206 specimens were rinsed with sterile water for 10 minutes, and autoclave-sterilized at 121°C for 20 minutes. The external surface of the specimens was coated with two layers of nail varnish to seal the tubules [15].

Inoculation with E. faecalis:

E. faecalis (ATCC29212) was cultured on blood agar plates, and incubated at 37° in an aerobic condition overnight to form bacterial colonies. A 0.5 McFarland bacterial suspension was then prepared and added to a microtube along with tryptic soy broth (TSB) and a dentin cylinder.

Totally, 103 dentin cylinders were incubated in TSB at 37° in an aerobic condition for 3 weeks. The culture medium was refreshed every 48 hours to eliminate the dead bacteria and ensure the viability of the remaining bacteria [17]. After 3 weeks of incubation, one specimen was inspected under a SEM to ensure biofilm formation on dentin cylinders. Of the remaining 102 specimens, 68 dentin cylinders were randomly selected for the two experimental groups (to use the two sealers) and 34 dentin cylinders were randomly assigned to the positive control group (without sealer).

Inoculation with C. albicans:

C. albicans (ATCC10231) suspension along with 2 mL of Sabouraud dextrose agar culture medium and a dentin cylinder were placed in a microtube. Totally, 103 dentin cylinders were incubated in Sabouraud dextrose agar at 37° in an aerobic condition for 3 weeks. The culture medium was refreshed every 48 hours. After 3 weeks of incubation, one specimen was inspected under a SEM to ensure biofilm formation on dentin cylinders. Of the remaining 102 specimens, 68 dentin cylinders were randomly selected for the two experimental groups (for application of the two sealers) and 34 dentin cylinders were randomly assigned to the positive control group (without sealer).

Sealer application:

Infected dentin specimens were rinsed with sterile water for 1 minute. In the *E. faecalis* group, 34 canals were randomly filled with Endoseal MTA, and 34 other canals were filled with AH Plus using the plastic tip of the syringe as instructed by the manufacturers (Table 1); 34 specimens remained without sealer to serve as the positive control group [16]. The same was repeated for the *C. albicans* group. The specimens were then incubated at 37°C The following 12 groups were evaluated:

Groups 1 and 2 (positive control): Dentin cylinders inoculated with E faecalis with no sealer for assessment after 24 hours (n=17) and 2 weeks (n=17).

Group 3 and 4: Dentin cylinders inoculated with *E. faecalis* with AH Plus sealer for assessment after 24 hours (n=17) and 2 weeks (n=17).

Groups 5 and 6: Dentin cylinders inoculated with *E. faecalis* with Endoseal MTA for assessment after 24 hours (n=17) and 2 weeks (n=17).

Table 1. Composition of the sealers used in this study

Material	Composition	Manufacturer
AH Plus	Paste A: bisphenol-A epoxy resin, bisphenol-F epoxy resin, calcium tungstate, zirconium oxide, silica, iron oxide pigments Paste B: dibenzyldiamine, aminoadamantane, tricyclodecane diamine, calcium tungstate, zirconium oxide, silica, silicone oil	Dentsply De Trey GmbH, Konstanz, Germany
Endoseal MTA	Calcium silicates, calcium aluminates, calcium aluminoferrite, calcium sulfates, radiopacifier, thickening agents	Endoseal, Maruchi, Seoul, Korea

Groups 7 and 8 (positive control): Dentin cylinders inoculated with *C. albicans* with no sealer for assessment after 24 hours (n=17) and 2 weeks (n=17).

Groups 9 and 10: Dentin cylinders inoculated with *C. albicans* with AH Plus sealer for assessment after 24 hours (n=17) and 2 weeks (n=17).

Groups 11 and 12: Dentin cylinders inoculated with *C. albicans* with Endoseal MTA for assessment after 24 hours (n=17) and 2 weeks (n=17).

It should be noted that the number of cultured specimens in each group was 16, and one sample from each group underwent SEM assessment.

Sealer removal:

The specimens were assessed after 24 hours and 2 weeks. At each time point, a #2 and then a #5 peeso reamer were used with up-and-down movement to remove the sealer from the root canal system until the canal surface was exposed [18]. Dentin chips removed from the canal after using the peeso reamers were collected in a sterile Petri dish placed under the specimens, and 1ml of TSB was added to each Petri dish with a sterile universal pipette [18]. Next, the universal pipette was used for aspiration of the samples and their transfer to a small test tube. The tubes were shaken for 10 seconds, and the contents were cultured on blood agar plates. The plates were incubated at 37°C for 24 hours, and after incubation, visible colonies were counted. The colony count (number of CFUs) was multiplied by 10 to calculate the number of bacteria as CFUs/ml The selected specimens for SEM assessment underwent SEM assessment after sealer removal at both 24 hours and 2 weeks.

Preparation of specimens for SEM assessment:

Two grooves were created, one in the buccal and one in the lingual surface of dentin cylinders by a diamond bur (Diatessin, Switzerland) with 0.6mm thickness at a speed of 1000rpm. These grooves were extended close to the canal. Next, a spatula was used to split the cylinders in half through the grooves by the wedging effect. One half was randomly selected for inspection under a SEM. The specimens were prepared for SEM inspection according to the standard technique described by Brown and Brenn [19]. For this purpose, dentin cylinders were fixed in 2% glutaraldehyde (Daejung, Korea) for 1 hour. They were then rinsed with phosphate buffered saline and subjected to ascending concentrations of ethanol for complete dehydration. After drying, they were placed on aluminum stubs and gold sputter-coated for inspection under a SEM.

Statistical analysis:

Data were analyzed using SPSS version 18 by the Kruskal-Wallis and Mann-Whitney tests with Bonferroni adjustment at 0.05 level of significance.

RESULTS

The results showed the significant effect of sealer type on *E. faecalis* colony count at both 24 hours and 2 weeks, and the significant effect of sealer type on *C. albicans* colonies at 2 weeks ($P<0.05$). Pairwise comparisons of the groups by the Mann-Whitney test and Bonferroni adjustment (Table 2) showed that after 24 hours, Endoseal MTA caused a greater reduction in *E. faecalis* and *C. albicans* colony counts compared with the control group; however, this difference was only significant for *E. faecalis* ($P<0.008$). Although the colony count of both *E. faecalis* and *C. albicans* in the AH Plus group was lower than that in the control group at 24 hours, this difference was not significant ($P>0.05$).

Table 2. Pairwise comparisons of the groups at different time points regarding the colony count

Groups	Time	P- value		
		Control–AH Plus	Control–Endoseal MTA	AH Plus–Endoseal MTA
E. faecalis	24 Hours	0.136	0.002*	0.031
	2 Weeks	<0.001*	0.299	0.009
C. albicans	24 Hours	0.113	0.113	0.692
	2 Weeks	<0.001*	0.001*	0.002*

*Significant based on Mann-Whitney test

In comparison of sealers, the number of both *E. faecalis* and *C. albicans* colonies was lower in the Endoseal MTA group than the AH Plus group at 24 hours, but not significantly (P>0.05).

At 2 weeks, the colony count of both *E. faecalis* and *C. albicans* in the AH Plus group was significantly lower than that in the control group (P<0.008). Comparison of the control and Endoseal MTA groups at 2 weeks showed lower count of both *E. faecalis* and *C. albicans* in the Endoseal MTA group than the control group; but this difference was only significant for *C. albicans* (P<0.008).

In comparison of sealers, the number of both *E. faecalis* and *C. albicans* colonies was lower in the AH Plus group than Endoseal MTA at 2 weeks; but this difference was only significant for *C. albicans* (P<0.008).

Within-group comparisons at 24 hours and 2 weeks:

AH Plus: Within-group comparisons in the AH-Plus group showed a significant reduction in colony count in 2 weeks compared with 24 hours for both *E. faecalis* (P<0.05) and *C. albicans* (P<0.05).

Endoseal MTA: Within-group comparisons in the Endoseal MTA group showed a slight increase in both *E. faecalis* and *C. albicans* counts at 2 weeks compared with 24 hours; however, this increase was not significant for any microorganism (P>0.05).

Control: Within-group comparisons in the control group showed a slight reduction in *E. faecalis* count at 2 weeks compared with 24 hours, which was not significant (P.0.05). However, a significant increase was found in the colony count of *C. albicans* at 2 weeks compared with 24 hours (P<0.05). Table 3 presents the mean colony counts in the groups at different time points.

Results of SEM analysis:

Figure 1 shows the SEM micrograph taken to ensure complete elimination of smear layer prior to the experiment. As shown, the dentinal tubule openings were free from bacteria. Figures 2A and 2B present confirmation of biofilm formation after 3 weeks of incubation of dentin cylinders with microorganisms. As shown, microorganisms had invaded the dentinal tubules such that the tubule openings were completely obstructed with the microbial biofilm. SEM micrographs of the no-sealer control groups at 24 hours and 2 weeks (Figs. 3A, 4A, 5A, and 6A)

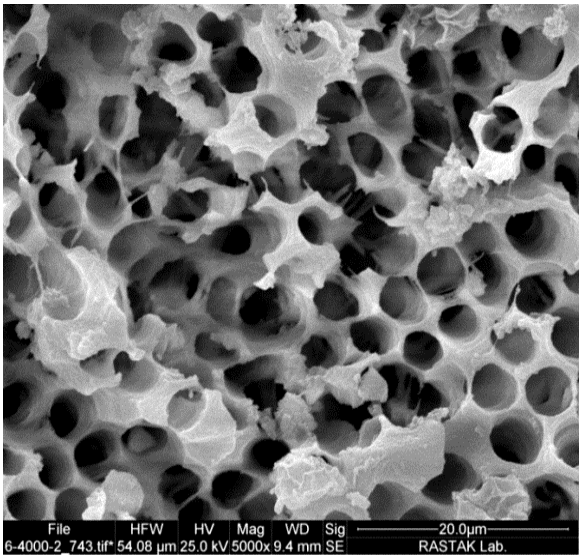

Fig 1. SEM photomicrograph of root dentin surface with open dentinal tubules after smear layer removal by EDTA and sodium hypochlorite (x5000 magnification).

Table 3. Mean colony counts of *E. faecalis* and *C. albicans* in the groups at different time points (n=16)

Groups	Time	Mean± Standard Deviation (CFUs/mL)			P-value
		Control	AH Plus	Endoseal MTA	
E. faecalis	24 Hours	164.4±107.3	139.4±161.3	66.9±74.6	0.004*
	2 Weeks	141.9±95.0	23.1±30.0	92.5±92.9	0.001*
	P-value	0.180	<0.001*	0.394	-
C. albicans	24 Hours	758.1±499.1	615.6±764.5	483.8±344.7	0.178
	2 Weeks	1570.0±721.9	211.3±457.4	616.3±539.9	<0.001*
	P-value	0.002*	0.026*	0.678	-

* Statistical significance: p < 0.05.

indicate intact thick structure of biofilm covering the openings of dentinal tubules. Multicellular colonies penetrating into the tubules can be seen as well. Figures 3B and 3C, 4B and 4C, 5B and 5C, and 6B and 6C show the disrupted structure of biofilm, some open dentinal tubules, decreased thickness of biofilm, and lower number of microbial colonies following the application of sealers.

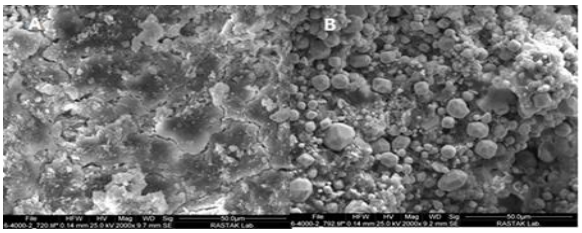

Fig 2. SEM photomicrograph of root dentin surface with mature biofilm after 3 weeks, completely obstructing the dentinal tubule openings (x2000 magnification): (A) E. faecalis; (B) *C. albicans*.

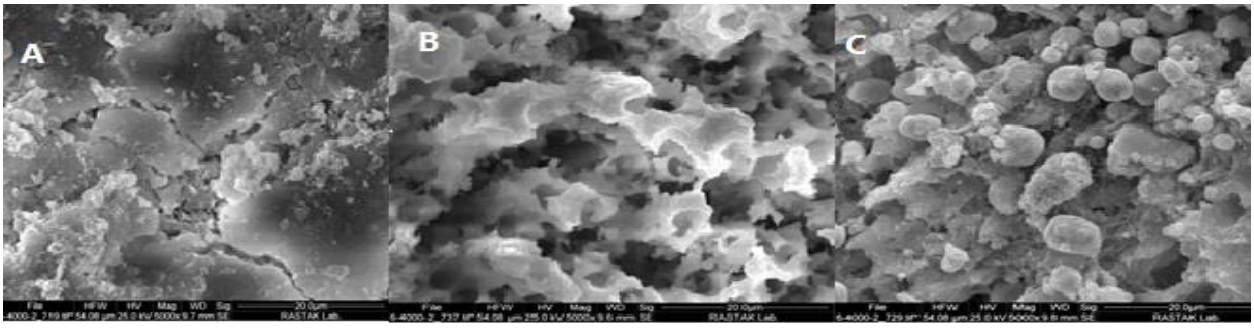

Fig 3. SEM photomicrographs of root dentin surface inoculated with *E. faecalis* without sealer in the control group (A), after the application of AH Plus (B), after the application of Endoseal MTA (C) after 24 hours (x5000 magnification)

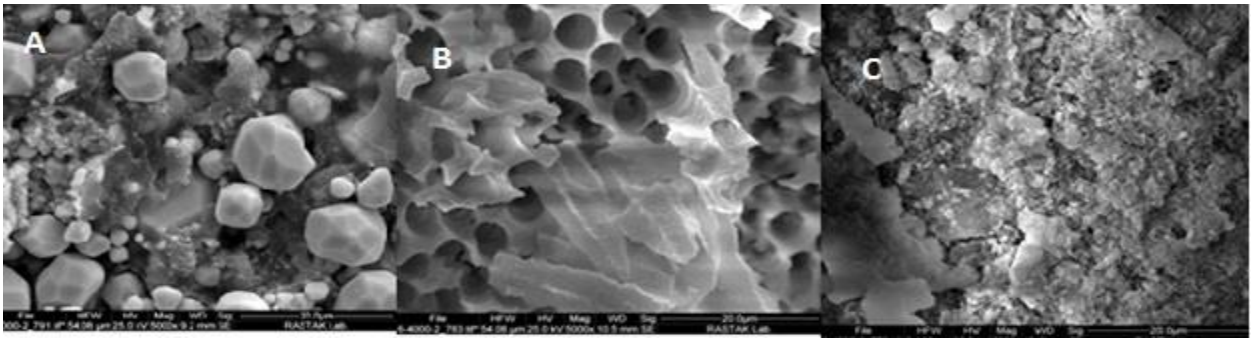

Fig 4. SEM photomicrographs of root dentin surface inoculated with *C. albicans* without sealer in the control group (A), after the application of AH Plus (B), after the application of Endoseal MTA (C) after 24 hours (x5000 magnification)

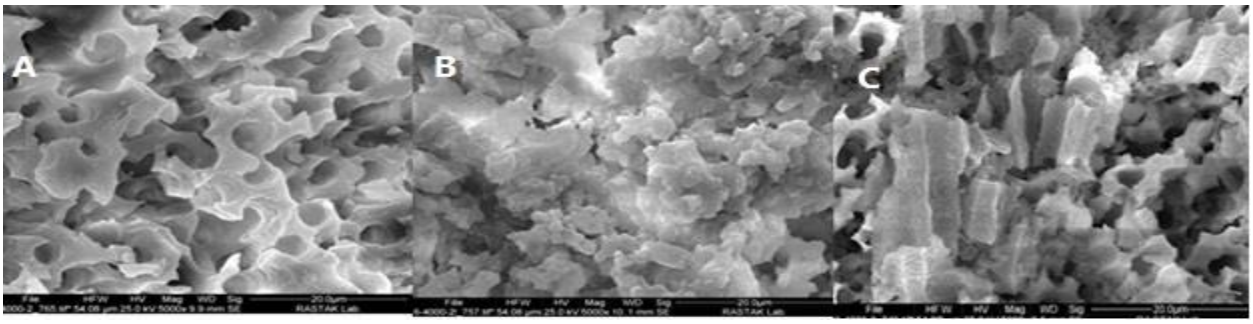

Fig 5. SEM photomicrographs of root dentin surface inoculated with *E. faecalis* without sealer in the control group (A), after the application of AH Plus (B), and after the application of Endoseal MTA (C) after 2 weeks (x5000 magnification)

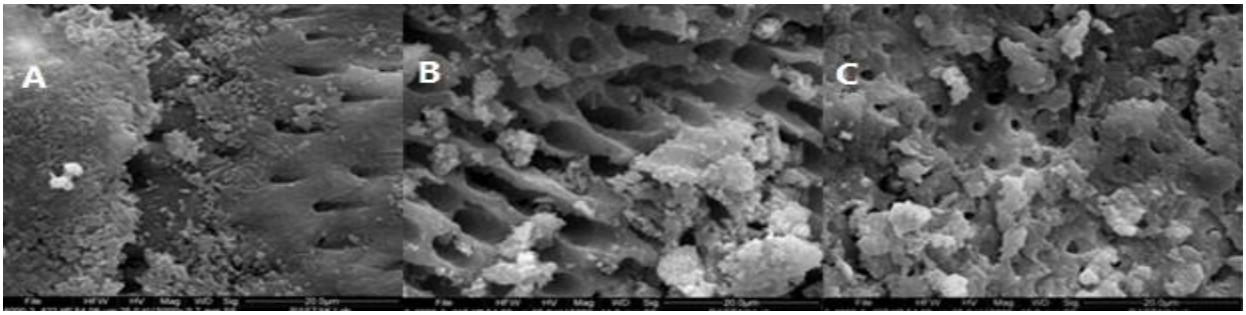

Fig 6. SEM photomicrographs of root dentin surface inoculated with *C. albicans* without sealer in the control group (A), after the application of AH Plus (B), and after the application of Endoseal MTA (C) after 2 weeks (x5000 magnification)

DISCUSSION

This study compared the antimicrobial effects of Endoseal MTA bioceramic sealer and AH Plus epoxy resin sealer on *E. faecalis* and *C. albicans* mature biofilms formed on human dentin blocks. The results showed that after 24 hours, Endoseal MTA decreased the *E. faecalis* and *C. albicans* colony counts compared with the control group; however, this difference was only significant for E. faecalis. This result was in line with the findings of Zhang et al [9]. They showed that the bioceramic sealer had a significant antibacterial effect after 24 hours Consistent with the present results, Gurel et al. [20] found that a bioceramic sealer had the highest antifungal effects after 24 hours, compared with other groups. The short-term antimicrobial effects of bioceramic sealers can be attributed to the combined effects of a highly alkaline pH and active release of calcium silicate, calcium phosphate, and calcium hydroxide [9, 21], which may decrease over time. Release of these materials increases the pH over 9, which can reversibly/irreversibly inactivate the cell membrane enzymes, impairing the biological activity of the microorganisms [22].

The present results revealed that AH Plus caused a reduction in *E. faecalis* and *C. albicans* colony counts at 24 hours compared with the control group; although this reduction was not significant. Similar results were reported by Alsubait et al [23]. They evaluated the antimicrobial effects of several sealers on dentin blocks using confocal microscopy, and found that AH Plus caused a significant reduction in *E. faecalis* count compared with the control group at 24 hours. Özcan et al, [24] and Pizzo et al. [25] found that AH Plus had high antimicrobial activity immediately after mixing, but this effect was no longer significant after 24 hours. Their results were in line with the present findings. The present results, however, were different from the findings of Kayaoglu et al, [26] who reported that after 24 hours, AH Plus had a significant effect on bacterial count compared with the control group, which may be due to the absence of mature biofilm, using a filter paper for sampling, and not simulating the clinical conditions in their study.

In the present study, the results indicated that after 2 weeks, AH Plus caused a significant reduction in colony count of both *E. faecalis* and *C. albicans* compared with the control group. This result was similar to the findings of Candeiro et al, [27] who showed that number of counted *E. faecalis* colonies at 7 days was lower than that at 1 hour. Saleh et al. [28] reported an increase in antibacterial effects of AH Plus over time. Gomes et al. [29] evaluated the antimicrobial effects of five sealers on bacteria and fungi at 24 and 48 hours and 7 days, and found that AH Plus sealer decreased fungal colonies after 7 days, which was in accordance with the present results.

AH Plus is an epoxy resin sealer, which releases lower amounts of formaldehyde than AH 26 [30]. Thus, the antimicrobial activity of AH Plus sealer may be due to the fact that unset epoxy resin along with amine in its structure can be toxic; the residual unpolymerized compounds in this sealer can

also have toxic effects. Moreover, bisphenol diglycidyl ether is recognized as a mutagenic agent in the composition of resin-based materials [31]. All these factors can explain the significant reduction in bacterial colony count by AH Plus sealer after 2 weeks.

Slutzky-Goldberg et al. [32] reported results contrary to the present findings. They reported that AH Plus sealer had no antimicrobial activity against *E. faecalis* after 2 weeks, which may be due to the fact that they did not use a mature bacterial biofilm, and dentinal tubules were not present in their study.

In the present study, an increase in bacterial and fungal count occurred over time in the Endoseal MTA group; although it was not significant. This result was in line with the findings of Zhang et al, [9] who found that the antimicrobial effect of a bioceramic sealer significantly decreased after 7 days. In another study, Candeiro et al. [27] demonstrated that the antimicrobial effect of a bioceramic sealer remained constant after 24 hours and 7 days.

In the present study, AH Plus caused a significant reduction in both bacterial and fungal counts over time. The setting time is 8 hours for AH Plus and maximally 13 minutes for Endoseal MTA at 38°C according to the manufacturers. Longer antimicrobial activity of AH Plus may be due to its longer setting time as well. Delayed setting of sealer can affect its biocompatibility and its potential to release toxic byproducts prior to final setting, and may cause a reduction in the number of bacterial and fungal colonies [33]. The colony count did not experience a significant reduction after 7 days in the Endoseal MTA group, which may be due to its short setting time and short-term pH rise, or reversible activity of the microbial cell wall enzymes.

In the present study, pairwise comparisons of the effects of sealers on *E. faecalis* did not reveal any significant difference between Endoseal MTA and AH Plus at 24 hours or 2 weeks. Mak et al, [34] and Shin et al, [35] also used Endoseal MTA and AH Plus. However, in contrast to the present study, they showed that Endoseal MTA had a greater antimicrobial activity than AH Plus at both time points; although the difference was not significant. Using the planktonic form of bacteria in their study and application of X-ray fluorescence for assessment of antimicrobial activity of sealers may explain the variations in the results.

Pairwise comparisons of the effects of sealers on *C. albicans* in the present study revealed that after 2 weeks, the colony count in the AH Plus group was significantly lower than that in the Endoseal MTA group; this difference may be due to the high antimicrobial activity of AH Plus as explained earlier. Moreover, it should be noted that *C. albicans* is a eukaryote and has a cell membrane and a cell wall; while, *E. faecalis* also has a bacterial capsule in addition to a cell membrane and a cell wall. Bacterial capsules serve as a defense mechanism and their absence in *C. albicans* may explain the greater antifungal effect of AH Plus on *C. albicans* colonies.

In the present study, SEM was used for assessment of microbial biofilm and debris according to Estrela et al [36]. The SEM micrograph taken after smear layer removal revealed open dentinal tubules without biofilm, known as the wormhole pattern [37]. The sealer groups revealed impaired integrity of the biofilm, indicating the antimicrobial effect of sealers. However, in the control groups, the mature biofilm had completely covered the dentinal tubule openings. SEM micrographs confirmed the results of colony counting.

To the best of the authors' knowledge, this study is the first to compare the antimicrobial effects of Endoseal MTA and AH Plus on dentin blocks coated with mature 3-week biofilms of *E. faecalis* and *C. albicans* by the colony counting method modified by Haapasalo and Ørstavik [15]. It has been demonstrated that mature biofilm has a higher resistance to root canal disinfecting agents, compared with a fresh biofilm [38].

Not using a confocal laser microscope was a limitation of this study. Also, mono-species biofilm was evaluated in this study, which does not completely simulate the polymicrobial nature of endodontic infections [39]. The antimicrobial effects of sealers may vary on polymicrobial environments due to synergistic effects of multi-species biofilms [40]. Future studies are required on polymicrobial biofilms to better simulate endodontic infections in the clinical setting.

CONCLUSION

AH Plus showed higher antimicrobial activity over time against both *E. faecalis* and *C. albicans*. Endoseal MTA only showed short-term antibacterial effect. The difference between the two sealers was only significant on *C. albicans* at 2 weeks.

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CONFLICT OF INTEREST STATEMENT

None declared.

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