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Comparative study of the pure AH Plus sealer and its combination with triple antibiotic paste at different concentrations on *Enterococcus faecalis* bacteria

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ABSTRACT

Background: In this study, the effects of pure AH Plus sealer and its combination with triple antibiotic paste at different concentrations on *Enterococcus faecalis* bacteria have been investigated.
Materials and Methods: This *in vitro* study was accomplished by the means of a triple antibiotic paste combination (minocycline, metronidazole, and ciprofloxacin) at different concentrations (0%, 1%, 5%, 10%, and 25%) with AH Plus sealer on *E. faecalis* bacteria. Sealers were set in an incubator for 1 h, 1 day, 3 days, and 7 days, and then 10 μL of bacteria solution was placed on all samples except the negative control group. After drying for 1 h, 250 μL brain–heart infusion broth culture medium was added, and it was cultured in solid media. Direct contact test technique was performed, and the obtained data were analyzed by 1-way ANOVA, 2-way ANOVA, 3-way ANOVA, and *post hoc* test least significant difference. It should be noted that the data were evaluated at the significance level of $P < 0.05$.
Results: The average of colony-forming unit (CFU)/mL illustrated that there were no significant differences between fresh antibiotic-sealer combination, 1-day set, and 3-day set ($P = 0.525$), while in sealer with 7-day set, the average of CFU/mL was notably lower than other sets ($P < 0.001$). The outcomes revealed a considerable variation by passing time and the number of CFU/mL was remarkably reduced ($P < 0.05$). The data suggested that, by increasing the concentration, the average of CFU/mL was decreased, whereas the average of CFU/mL did not have significant differences in all concentrations of the antibiotic-sealer combination compared to pure sealer ($P < 0.05$).
Conclusion: The concentration of 1% triple antibiotic in combination with root canal sealer may become a crucial factor for inhibiting the growth of remaining bacteria.

Key Words: Antibiotic, *Enterococcus faecalis*, root canal sealer

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INTRODUCTION

Researchers have shown that bacteria play a significant role in the progression and spread of pulpal and periapical diseases, which lead to the creation of an inflammatory response in the periapical tissues.[1,2] Many investigations have been carried out to show that instrumentation, irrigation, and intracanal medicaments considerably decrease the

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microorganism's population, whereas due to the anatomical complexities, the microorganisms from root canal are not eliminated entirely. Hence, a good root canal-filling material with antibacterial properties will be useful in further reducing the number of residual microorganisms.[3,4] To predictably remove bacteria from the root canal, a combination of cleaning techniques along with irrigating solutions requires dissolving the organic and inorganic debris and destroying bacteria.[5,6] The usage of antimicrobial drugs during root canal obturation may allow the penetration of drugs through the surface of the dentin, irregularities of the canal system, apical foramen, and periapical tissues; therefore, it reduces the presence of bacteria and persuasion the healing process.[7-11]

Root canal sealers are applied as a thin sticky paste that acts as a lubricant agent during obturation and provides the core obturation material such as gutta-percha or other rigid materials to canal walls for filling up voids, lateral canals, accessory canals, and irregularities within the canal.[12] If the sealer does not work efficiently, the nonsurgical root canal treatment failure has occurred due to the microleakage; therefore, this process leads to the transfer of bacteria, fluids, molecules, or ions between the tooth and restorative material.[13,14] AH Plus root canal sealer is an epoxy bisphenol resin based. It has been found to have antimicrobial activity, low shrinkage, low solubility, and adhesion properties. Numerous approaches showed that AH Plus is biocompatible and silver free, which has less short-term and long-term toxicity, less genotoxicity, and does not release formaldehyde during setting compared to AH26 sealer.[15,16]

Enterococcus faecalis is an anaerobic Gram-positive coccus that is regularly isolated from infected root canals and is considered one of the etiological factors connected with failed endodontic treatments which showed high resistance to antimicrobial agents.[17-19] The research has been done by Moazzami *et al*. on the antibacterial effect of AH Plus on root canals infected with *E. faecalis* bacteria represents an increase in a number of bacteria colonies in AH Plus sealer in the period of 2 and 7 days, which indicates the inability of this sealer to remove bacteria.[20] According to the complexity of root canal infections, the utilization of a single antibiotic cannot produce predictable disinfection effects in canals. Therefore, the usage of drug combination containing several antibiotics reduces the chance of developing resistant bacteria. Hence, a triple antibiotic paste containing an equal mixture of ciprofloxacin, metronidazole, and minocycline has been carried out inside the root canal for disinfection.

Ciprofloxacin is a fluoroquinolone antibiotic which applied to treat several bacterial infections. It has great activity against Gram-negative pathogens, whereas it shows limited activity against Gram-positive bacteria.[21,22]

Metronidazole is an antibiotic with antibacterial activity against anaerobic cocci, Gram-negative and Gram-positive bacilli and is used to treat periodontal diseases.[23]

Minocycline is categorized as a group of tetracyclines, which has a broad activity on aerobic and anaerobic Gram-positive and Gram-negative bacteria.[24,25]

Adel *et al*. compared the antimicrobial efficiency of triple antibiotic and calcium hydroxide against *E. faecalis* bacteria. They reported that triple antibiotics compared to calcium hydroxide reduce the number of bacteria significantly, which is indicative of triple antibiotic can be the most effective root canal disinfectant compared to calcium hydroxide.[26] Mozayeni *et al*. studied the antibacterial effect of four substances inside the canal (calcium hydroxide, 2% chlorhexidine gel, triple antibiotic mixture, and silver nanoparticles) on *E. faecalis* bacteria. The outcomes revealed that 2% chlorhexidine gel and triple antibiotic mixture have considerable effect compared to other materials in root canal disinfection which can be used as alternative intracanal medicaments in root canal treatment.[27]

Considering the interest of this research area, we decided to investigate the antibacterial effect of the mixture of sealer AH Plus-triple antibiotic paste (including metronidazole, ciprofloxacin, and minocycline) at different concentrations (0%, 1%, 5%, 10%, and 25%), on *E. faecalis* bacteria compared to pure AH Plus sealer by direct contact test (DCT) method.

MATERIALS AND METHODS

Reagents

In this *in vitro* study, ciprofloxacin and metronidazole antibiotics were purchased from Amin Pharmaceutical Co., Iran, and minocycline antibiotic was purchased from Theopharmaco Co., brain–heart infusion (BHI broth), BHI agar, and Mitis Salivarius Agar culture

medium were all acquired by Biolife Co., Italy. The sealer used in this study was AH Plus sealer (Densply Co, Germany). In this survey, five groups of sealer mixture were considered with triple antibiotic paste (minocycline, ciprofloxacin, and metronidazole) at different concentrations (0%, 1%, 5%, 10%, and 25%), negative control group: liquid culture medium, and positive control group: *E. faecalis* ATCC29212 bacteria (American Type Culture Collection) were obtained from the microbial bank of Isfahan Islamic Azad University (Khorasgan).

Culture medium

Brain–heart infusion broth

In the beginning, 37 g of BHI broth culture medium was added to 1000 cc of distilled water and was stirred circular manner. After it was dissolved completely, a certain amount (5–6 cc) of solution was distributed in test tubes and sterilized in an autoclave for 15 min. Afterward, the 100 µL of *E. faecalis* was transferred to the BHI broth test tube and incubated at 37°C for 24 h. The turbidity in the liquid culture medium indicates bacterial growth has occurred. Fifty-two gram of BHI agar was added to 1000 cc of distilled water and autoclaved. Then, it was placed under a hood under sterile conditions into 40 sterile plates and stored in the refrigerator.

Mitis Salivarius agar culture medium

In the first step, 90 g of Mitis Salivarius agar culture medium powder was mixed with 1000 cc of distilled water and placed on a heater until it dissolved, and then it was sterilized in an autoclave. In the second step, after the solution reached to 50°C–55°C temperature, 25 cc of 1% potassium tellurite was added, and the culture medium was transferred into plates under the laboratory hood. Next, the plates were placed upside down inside the incubator at 37°C for 24 h.

Apparatus and methods

Preparation and cultivation of bacteria

A sterilized loop was inserted into the BHI broth culture medium and cultured on Mitis Salivarius agar solid medium through the streak plate technique. Then, the plates were placed in an incubator at 37°C for 24 h. The grown colonies were examined macroscopically in terms of shape. The bacterial colony had a smooth surface, round shape, medium size (about 1–2 mm), and white color. It was a positive catalase test and was resistant to β-lactam and aminoglycoside antibiotics.

Direct contact test method

DCT is a quantitative and repeatable technique that imitates the contact of the test microorganism with root canal sealers inside the root canal. Furthermore, in this measurement, the antimicrobial effect of the desired substance is measured during direct contact with the target microorganism. One of the advantages of this method is it enables to evaluate the antimicrobial effect, regardless of the solubility and diffusion of the constituent components of the sealer. Meanwhile, in this technique, the compounds with lower solubility in water are applied.

Preparation of triple antibiotic

0.1 g of each three antibiotics containing; metronidazole, ciprofloxacin, and minocycline were moved into a microtube and vortexed it until mixed completely.

Sealer-antibiotic mixture preparation at different concentrations

A sealer was made according to a mixture of two tubes with an equal ratio. It was weighed through a digital laboratory scale, and the pure sealer weights were obtained by differences between these two weights. In accordance with a ratio of 0%, 1%, 5%, 10%, and 25% of the sealer weights, a triple antibiotic was added (calculated by weight/weight percentage method). Then, the microplate was placed vertically, and an equal thin layer (about one-fourth of the circumference of a circle with a diameter of 3 mm) with a thickness of about 1 mm of the triple antibiotic-sealer mixture was placed in the wall of each microplate well. The seven groups created include five groups with sealer-triple antibiotic mixture (with a concentration of 0%, 1%, 5%, 10%, and 25%), one positive control group (*E. faecalis*), and one negative control group (pure culture medium). Afterward, each group was placed in four wells, and sealers were set in an incubator for 1 h, 1 day, 3 days, and 7 days. Then, 10 µL of *E. faecalis* bacteria solution (standardized with 0.5 McFarland concentration) was placed on all samples except the negative control group. After 1 h drying (in an incubator at 37°C), 250 µL of BHI broth culture medium was added to all seven groups.

Measurement of the number of grown colonies of *Enterococcus faecalis*

Colony-forming units (CFUs/mL) were calculated based on the following formula 1:

(Number of colony) (1/used dilution) (1/used volume) = CFU/mL (1).

Statistical analysis

Raw numbers were analyzed using SPSS 20 software (IBM Company, Chicago, USA). Statistical analysis of one-way ANOVA, two-way ANOVA, three-way ANOVA, and *post hoc* test least significant difference were carried out. It should be noted that the data were evaluated at the significance level of $P < 0.05$.

RESULTS

Measurements of sealer-triple antibiotic mixture by time and concentration

The average number of colonies (CFU/mL) in fresh, 1-day set, 3-day set, and 7-day set sealer-triple antibiotic mixture are listed in Table 1. As can be seen, a few number of colonies were decreased by increasing the triple antibiotic concentration, and significant differences were not observed ($P > 0.05$). By passing time, the number of bacteria per milliliter of the sample was reduced with a considerable difference ($P < 0.001$). The data suggest that the maximum antibacterial property was seen in 1-day later in fresh set sealer, and this activity was maintained up to 7-day later. Moreover, in 1-day set sealer-triple antibiotic mixture, the bacterial activity was stopped after 2 days. In addition, in 3-day and 7-day set sealer-triple antibiotic mixture, bypassing time the number of bacteria was decreased with significant differences.

The antibacterial effect of pure sealer with different sets over time and its comparison to the positive control group (pure bacteria) is shown in Figure 1. The outcome suggests that fresh pure sealer has the most antibacterial properties, and by increasing the setting time, the antibacterial activity will decrease ($P < 0.001$). In the positive control group, the average number of colonies were not the same on different days, while for the negative control group (pure culture medium) were zero, which implies the lack of bacterial growth.

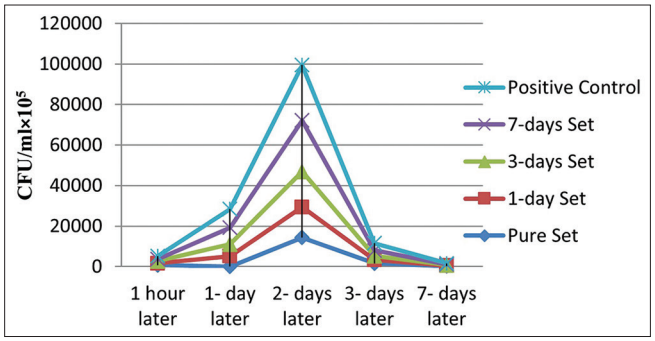

Figure 1: The average number of colonies (colony-forming unit/mL) in pure sealer and positive control group by time and concentration.

Least significant difference follow-up test measurements

1. In the sealer-antibiotic triple combination, the mean (CFU/mL) values in the sealer with the fresh, 1-day, and 3-day sets did not have a considerable difference ($P = 0.525$). In contrast, for the 7-day set, it was remarkably higher than the other sets ($P < 0.001$)
2. The mean values had a meaningful change at different times, and bypassing times, they were decreased compared to the previous time ($P > 0.05$)
3. Regarding the effect of concentration on the amount of CFU/mL, by increasing concentration, the mean value has decreased but the average (CFU/mL) between them did not differ significantly ($P < 0.05$).

Based on the obtained statistical analysis of the one-way variance test from Table 2, the mean (CFU/mL) value in pure AH Plus sealer (53,787.9) was significantly lower than the positive control group (12,372.3) ($P < 0.001$), which suggests that the growth of bacteria was decreased by 53%. Accordingly, it can be stated that the sealer-triple antibiotic 1%, 5%, and 10% mixture has a 99.1%, 99.8%, and 99.99% reduction in the number of bacteria, respectively. While the number of bacteria has decreased to 100% in the presence of sealer-triple antibiotic 25% mixture.

DISCUSSION

Since microorganisms are the principal reasons for pulpal and periapical diseases, the primary target in endodontic treatment is the elimination of bacteria. Hence, the complete removal of microorganisms from the infected root canal system (RCS), and providing these spaces, bacteria free is difficult. Due to the complicated morphological form of RCS, the mechanical preparation alone is inadequate to disinfect accessory canals, anastomoses, and fins.[28,29] Pathogens can colonize inside the dentinal tubules and cause reinfection of the RCS and periradicular tissues. As regards the gutta-percha used for root canal obturation, the material cannot stick to the dentin walls. Therefore, sealer is applied to fill the created

Table 1: Average number of colonies (CFU/mL) in fresh, 1-day set, 3-day set, and 7-day set sealer-triple antibiotic mixture

A					
Time	Concentration (%)	Colony count per mL×10^5, mean value			
		The average number of colonies (CFU/mL) in fresh sealer-triple antibiotic mixture	1-day set sealer-triple antibiotic mixture	3-day set sealer-triple antibiotic mixture	7-day set sealer-triple antibiotic mixture
1 h later	1	61	41	47.5	286
	05	20.6	15	5.8	110
	10	16.8	15.1	4.94	32
	25	12.37	0.2	0.7	3.07
Total	Total	27.69	18.25	14.7	107.77
1 day later	1	0	15	32	185.25
	5	0	2.5	0.2	6.7
	10	0	0.07	0	1.8
	25	0	0.02	0	0
Total	Total	0	4.4	8	48.44
2 days later	1	0	2.7	19.4	125.5
	5	0	0	0.2	1.3
	10	0	0	0	0.6
	25	0	0	0	0
Total	Total	0	0.6	4.9	31.8
3 days later	1	0	0	17	91
	5	0	0	0.01	0.5
	10	0	0	0	0.01
	25	0	0	0	0
Total	Total	0	0	4.2	22.8
7 days later	1	0	0	4.5	6.12
	5	0	0	0	0.76
	10	0	0	0	0.12
	25	0	0	0	0
Total	Total	0	0	1.1	1.7

SD: Standard deviation; CFU: Colony-forming units

spaces, as well as the spaces between the gutta-percha cones, thereby reducing the number of active bacteria and accelerating the process of periapical tissue recovery.[30] However, the antimicrobial properties of many sealers have been investigated on various microorganisms, the most studies are focused on the setting time which decreases after it is set. Therefore, efforts have been made to increase the antimicrobial properties of sealers along with their setting time duration.

Many investigations have shown that using local antibiotics inside the canal may be more effective and beneficial than systemic consumption. Furthermore, it has fewer side effects while higher concentrations of antibiotics can be applied.[31] The infection in the RCS is a polymicrobial. Therefore, due to its complexity, using one antibiotic alone is not sufficient for RCS disinfecting. Hence, a multiantibiotic combination is needed to dominate against microorganisms, which reduces the bacteria species resistant. The triple antibiotic paste containing metronidazole, ciprofloxacin, and minocycline were utilized, and after 24 h, no bacteria were observed on the surface of the infected dentin.

In recent years, the benefits of using a triple antibiotic combination have been investigated in the field of regenerative endodontics.[32] Furthermore, there has been a successful report achieved on the beneficial uses of triple antibiotics in necrotic permanent teeth treatment.[33] The antimicrobial effect of triple antibiotic compared to the calcium hydroxide against *E. faecalis* bacteria by Adel *et al*.[34] It has been deduced from outcomes that triple antibiotics significantly reduced the number of bacteria compared to calcium hydroxide, suggesting a functional factor in root canal disinfestation.[35]

Kangerlou *et al*. investigated the antimicrobial properties of AH26 and AH Plus sealer in combination with amoxicillin, triple antibiotic pastes,

Table 2: The statistical data from the mean and standard deviation of the pure AH Plus sealer with a triple antibiotic mixture – AH Plus sealer and the positive control considered without passing the time (1 h, 1, 2, 3, and 7 days later) and set type (fresh, 1, 3, and 7 days)

Experimental groups	Colony count per mL×10^5, mean±SD
Enterococcus faecalis bacteria (positive control group)	12,372.3±4511
Pure AH Plus sealer	5377.9±897.9
AH Plus sealer+triple antibiotic 1%	46.7±8.3
AH Plus sealer+triple antibiotic 5%	8.2±2.7
AH Plus sealer+triple antibiotic 10%	3.6±1.03
AH Plus sealer+triple antibiotic 25%	0.9±0.3

SD: Standard deviation

and nanosilver that cultured freshly after 1, 3, and 7 day with suspension of *E. faecalis* for 24 h. Like our study, adding antibiotics to the sealer increased the antimicrobial properties of the sealer. Sealers combined with amoxicillin exhibited the highest antibacterial efficacy in fresh conditions. In the set specimens, the results demonstrated that the mixture of sealers and triple antibiotic pastes exhibited the greatest antibacterial efficacy. However, in our study, different concentrations of three antibiotic pastes as well as antimicrobial properties at different times were also investigated.[36]

In this survey, the triple antibiotic was selected due to its favorable clinical effects in endodontic treatments. And it was designed considering the importance of antibacterial properties of endodontic sealers. Therefore, the antibacterial activity was investigated using sealer AH Plus in combination with different triple antibiotic concentrations. Since most of the bacteria isolated in unsuccessful root canal treatment are *E. faecalis* (80%–90%), therefore, this bacterium was applied. Hence, the presence of this pathogen in the root canal indicates a significant treatment problem. High tolerance to phosphatase conditions, biofilm formation, and the ability to attack dentin tubules protect this bacterium from drugs inside the root canal and make *E. faecalis* a resistant bacterium to endodontic treatments.[27,37]

The agar diffusion test and DCT are two common techniques for evaluating the antibacterial effect of sealers and different materials. Hence, in this study, we applied a DCT technique to investigate the antimicrobial effect of different types of sealers which many researchers have considered. According to the DCT technique, the antimicrobial activity of the substance is measured during direct contact with the target microorganism. One of the advantages of this method is regardless of the solubility and diffusion of the constituent components of the sealer, the antimicrobial activity will be evaluated. Therefore, DCT is more commonly used for compounds that have lower solubility in water.[38]

In this study, the laboratory method was selected due to the accurate investigation of pure AH Plus sealer and its combination with different concentrations of triple antibiotic paste on *E. faecalis* bacteria. Therefore, it has been done on a culture medium (not in the extracted tooth) due to the elimination of bacteria overlaps, reduction in the possibility of error, and placement of dead spaces in the root canal of the tooth as well as the easier visibility and accesses.

In this study, like the previous research, concentrations of 0%, 1%, 5%, 10%, and 25% of antibiotics were used, whereas the time set for sealers was considered fresh, 1, 3, and 7 day. Furthermore, the bacteria applied were inoculated and dried for 1 h.[39] The time for bacterial culture was measured for 1 h later and 1, 2, 3, and 7 days later.[40,41] According to a prior similar study, the standard dilution of the bacteria was performed through the light absorption of a spectrophotometer at 630 nm wavelength. 10 µL of *E. faecalis* bacteria solution and 250 µL of liquid culture medium were added to all groups.[39]

As stated by the findings of the positive control group, all the samples had 100% growth of *E. faecalis* bacteria, which suggests that the bacteria were kept during the storage period and did not disappear during the usage. Furthermore, in the negative control group, the bacterial growths were not seen on culture medium plates, which confirmed the accurate sterilizing method in the culture medium. In this survey, the one-way variance test was examined only for the variable concentration (without considering the passage of time and set type), and the two-way variance method was used to measure the two variables of concentration and time passage (regardless of set type). In addition, the three-way variance test was evaluated to analyze all three variables (set type, concentration, and time passage) simultaneously.

CONCLUSION

According to the present study, the antibacterial properties of the sealer are increased by adding the

triple antibiotic to the AH Plus sealer. Corresponding to these inspiring findings, it may be possible to add triple antibiotics on the sealer and apply it inside the canal. Clinically, it should be better to use the most effective concentration (1%) to inhibit bacteria growth. Overall, the results of this study showed that the average bacterial colony growth was reduced in pure sealer, which indicates that it has an antibacterial activity against *E. faecalis* bacteria which is consistent with other researchers. This study also stated that all concentrations (1%, 5%, 10%, and 25%) were effective in reducing bacteria growth and confirmed that the antibacterial property is raised by increasing antibiotic concentrations, whereas the average growth reduction in different concentrations (1%, 5%, 10%, and 25%) did not vary significantly.

Regarding the achieved outcomes, the triple antibiotic paste has an antimicrobial activity on *E. faecalis* bacteria in combination with AH Plus and different concentrations will improve the antimicrobial effect of sealer, and it seems that a concentration of 1% sealer-antibiotic mixture is efficient in the destruction of *E. faecalis* bacteria.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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