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EFFECT OF *ENTEROCOCCUS FAECALIS* ON THE PUSHOUT BOND STRENGTH OF RESIN-BASED SEALER (AN IN-VITRO STUDY)

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KEYWORDS

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push out bond strength.

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ABSTRACT

Introduction: When *Enterococcus faecalis* is present in sufficient amounts, resin sealer can be degraded by esterase-like activity. *E. faecalis* has potential ability to penetrate the methacrylate-tooth interface, enter the root canal, causing secondary infections. Adhesion of the sealer to dentin and gutta percha was measured by push out bond strength using universal testing machine and adaptation using scanning electron microscope. **Aim:** This study aims to determine the effect of *E. faecalis* on push out bond strength of AH Plus epoxy resin-based sealer. **Material and methods:** A total of 40 recently extracted teeth were used in this experiment. After chemo-mechanical preparation of all samples, they were randomly classified into four groups (n=10): Group 1: Root canals inoculated with *E. faecalis*. Group 2: Root canals with no bacterial inoculation. Group 3: Root canals inoculated with dead bacteria. Group 4: Root canals inoculated with media only. Obturation was performed by single cone obturation technique. Methods of evaluation: The bond strength of the samples of all groups was calculated by dividing the load by bonding area of the filling area. The load was converted to MPa. **Results:** Control group 2 "no bacteria" and group 4 "media only" showed the highest statistically significant values of push out bond strength both at apical and coronal sections with no statistically significant difference between them. This was followed by group 3 "dead bacteria" then group 1 "with bacteria" which showed the least values of POBS with statistically significant difference between them and with other groups. **Conclusion:** *E. faecalis* demonstrated biodegradation of resin-based sealers. AH Plus sealers cannot eradicate *E. faecalis* completely.

INTRODUCTION

Root canal sealer materials tend to fill minor irregularities between the GP and the canal walls(1). It is also used as lubricant, can fill accessory canals and prevent residual bacteria from growing within the tubular space(2). The use of a root canal sealer and GP plays an important role in creating a fluid, tight, and sealed closure(3). Different types of sealers can be used in obturation(4). An epoxy resin-based sealer such as AH Plus (DentSply, Germany) is considered a benchmark sealer used with GP in a Single Cone or lateral condensation obturation techniques due to its suitable flow, sealing, adhesion and bonding properties(5). However, Epoxy resin-based sealers have good adhesion to dentin and lower rates of water solubility but it is not a bioactive material(6). *E. faecalis* is the most prevalent bacteria in cases with failed root canal treatment,

it has esterase-like activities that allow bacteria to hydrolyze resin-based chemical compounds[7]. Consequently, researchers were directed to find the type of sealer which have higher resistance to bacterial biodegradation to obtain more durable interphase between dentin and sealer[8]. Therefore, this study was conducted to evaluate the push out bond strength (POBS) of resin-based sealer regarding the presence or absence of *E. faecalis* in the treated root canals[9].

MATERIALS AND METHODS

This study was single-blinded study conducted on forty extracted maxillary single rooted teeth after approval by the research ethical committee of the faculty of Dentistry Suez Canal University (no. 153-16).

Sample size calculation:

To assess the effect of *E. faecalis* on the POBS of AH plus sealer, repeated measures analysis of variance is proposed (ANOVA). The effect size is 0.25 according to Cohen (1988), a power (1-β=0.85) of 85% at a significance probability level of $p<0.05$ partial eta squared of 0.06. According to sample size calculation a total sample size of 40 root canals were applied; each group (1, 2,3,4) was represented by 10 teeth. The sample size was calculated according to G*Power software version 3.1.9.2[10].The effect size was 0.25 using alpha (α) level of 0.05and Beta (β) level of 0.05, i.e, Power= 95%; the approximate least sample size (n) was a sum of 40 samples [10].

Collection of samples:

Forty maxillary single-rooted teeth freshly extracted for periodontal, prosthodontic or orthodontic reasons were chosen according to the following inclusion criteria: a) Maxillary anterior teeth with completely formed apex. b) No root caries. c) No internal or external resorption to avoid any loss during transverse sectioning of the roots. d) Teeth did not receive prior endodontic treatment e) Teeth with no signs of cracks to avoid fracture during instrumentation.

Randomization, allocation concealment and blinding:

The sequence generation was done for the root canals (from 1 to 40) using computer sequence generation (http://www.random.org/). Teeth were marked by a permanent marker with numbers from 1 to 40. The operator knew which sealer was used[11].

Sample preparation:

Samples were dried using sterile gauze. Each root in each group ((1, 2,3 & 4) were color-coded by different nail polish color; red, yellow, pink and blue respectively. Two layers of nail polish were applied to the external surfaces of all roots except the apical 2mm. All specimens were inserted inside a 96 well plate, closed then placed in sterilization pack and autoclaved for 20 minutes at temperature of 121°C, and pressure of 2 bars (Figure 1).

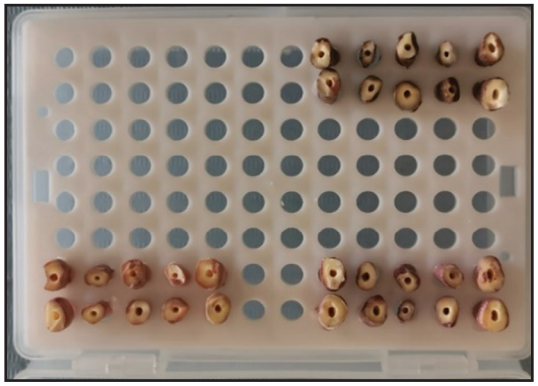

Fig. (1) A photograph showing decapitated specimens inserted in a 96 well plate.

Group 1: "Root canals inoculated with *E.faecalis*"

The apical foramina of 10 teeth "that were lately infected with *E. faecalis* ATCC 29212" were sealed with melted pink wax (Figure 2).

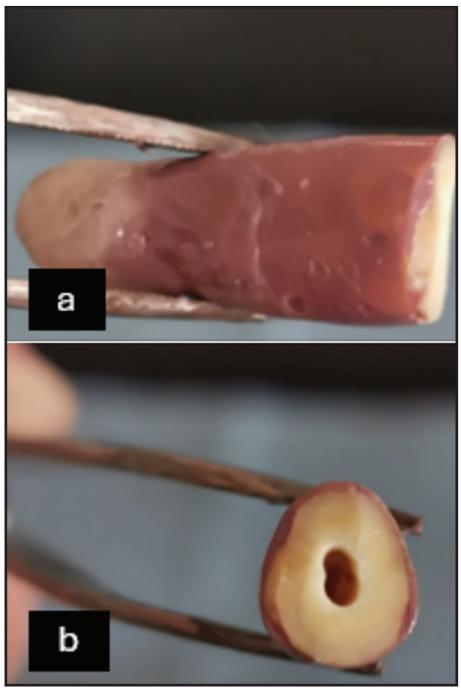

Fig. (2) Photographs a & b showing coating of samples and sealing the apex by pink wax.

Confirmation of sterilization:

After sterilization, sterile saline was injected in the root canal of each specimen then the canal was dried for 1 min. to absorb fluid inside the canal. The paper point was kept inside a test tube contains 0.5ml of brain heart infusion broth "BHI" and vortexed for 0 secs. Micro-liter was pipetted on KF Agar plates, then spreaded all over the plate using sterile swab. Plates were incubated at 37°C for 2 days. All these procedures were done inside a laminar flow hood in the lab of microbiology and immunology department at the faculty of Pharmacy in Suez Canal University to ensure complete sterilization. Absence of bacterial growth confirmed sterilization.

Root canal instrumentation:

Roots were held using moist gauze continuously to avoid dehydration, K-file #10 was introduced into the canal of each root to ensure its patency. The working length was determined by inserting a K-file size 15 in the root canal to establish the working length 1mm short of the apical foramen. Each canal was irrigated using 5 mL of 2.5% NaOCl. All selected root canals should have the same internal diameter using file size #30 K-file as an initial file. The root canals were then prepared using Protaper Universal NiTi rotary file System; 8 rotary files; 3 shaping files, SX, S1 and S2, and 5 finishers (F1 to F5). The files both shapers and finishers were used following the manufacturer's instructions using X-smart Endo motor. Shaper file SX was advanced in coronal and middle thirds of root canals until resistance was met. Consequently, S1 file was introduced until resistance using a brushing motion. This was followed by the introduction of S1 file to the full working length. The remaining files; S2, F1, F2, F3, F4 and F5 were used in sequence to achieve an apical preparation of size 50. The files were used with X-Smart motor at 250 rpm and the torque was adjusted for each file as recommended by the manufacturer. Each root canal within all the experimental groups; a volume of 5 mL of 2.5% NaOCl was used between each instrument then, 5 mL of saline solution followed by 5 mL of 17% EDTA solution was used to remove the smear layer. Finally, root canals were irrigated with 10 mL of saline solution, the canals were dried gently using sterile absorbent paper-points size 50. Forty Roots were obturated using AH Plus sealer were divided into four groups (n=10). **Group 1:** Root canals inoculated with *E. faecalis*. **Group 2:** Root canals with no bacterial inoculation. **Group 3:** Root canals inoculated with dead bacteria. **Group 4:** Root canals inoculated with media only. Cultures of *E. faecalis* ATCC 29212 grown on Kenner Fecal "KF" streptococcus agar plates were used in this study after incubation at 37°C for 48 hours.

Inoculation of root canals with E. faecalis:

Under aseptic condition, 20μl of the bacterial suspension was injected into each root canal using a sterile automatic Eppendorf micropipette, Sterile K-files #20 were used to carry the bacterial suspension to the entire root canal length, until the entire canal space was filled with fluid[11]. The coronal access of all samples was sealed with pink wax and enclosed individually within 96 well plate. Root canals inoculated with *E. faecalis* were incubated at 37ºC for 7 days to give time for bacteria to infiltrate deeply into dentinal tubules. Ten μl of fresh TSB bacterial suspension was added to the root canals at 1, 4, and 6 days after the initial inoculum to aid in bacterial growth. Group 2: Root canals with no bacterial inoculation: Ten roots were kept sterile in sterilization package with no bacterial inoculation. Group 3: Root canals inoculated with dead bacteria: Fifteen mL of TSB with *E. faecalis* was boiled for five minutes to ensure the death of bacteria. Group 4: Root caanls inoculated with media only: Fifteen mL of sterile TSB was introduced in 10 root canals without bacterial inoculation. After seven days, single GP cone size #50 .04 was introduced to the full working length and verified to a tight fit. Obturation of specimens by single cone technique: Group A "AH Plus sealer": Equal amounts of the epoxy resin sealer's AH Plus, base and catalyst were dispensed on a mixing pad, mixed according to the manufacturers' instructions using a plastic instrument till a homogenous mix was reached. Lentulo spirals were used to apply the sealer into the root canals in a counterclockwise motion, and then the master's cone tip was lightly coated with sealer and seated slowly into the root canal to the full working length. Obturation was done using complete SC technique. The working time of AH Plus sealer is about 4 hours. All specimens were kept in an incubator at 37°C for seven days to assure the complete setting of the sealers. At the end of the experiment, each root was embedded in chemically cured acrylic resin and then sectioned 90º to the long axis of the root. Sectioning was done using Isomet 4000 micro-saw (Buehler, USA) mounting a diamond disc 0.6 mm thickness at speed 2500 rpm and feeding rate 10 mm/min under water cooling[12]. Samples were cut into three sections with thickness about 5.5 mm each, and two cross sections were cut (middle of the coronal and middle of the apical), with thickness 1.5-2 mm each. Each root was divided into coronal third and apical third and were used for the POBS.

Methods of evaluation:

The filling material (GP and sealer) was loaded with a 0.9 mm diameter stainless steel plunger selected for coronal slices and 0.5mm diameter for apical slices. The plunger was mounted on the upper part of a universal testing machine (Instron universal testing machine model 3345 England, data recorded using computer software Bluehill 3 version 3.3). Then, slices were positioned in the mechanical testing machine with the cylindrical stainless-steel plunger pointed to the canal filling in an apical-coronal direction to avoid any constriction interference. Load was applied until the filling material was dislodged. The tests were conducted at a cross head speed of 0.5mm/min using a 500N load cell. The highest load value recorded was taken as the POBS. The bond strength was calculated by dividing the load by the filling area. The area under load was calculated by:

Area = 2 π r h for each section, the adhesion surface area (mm^2), the adhesion surface area (A) and sealer retention was calculated as follows; ($\pi r^1 + \pi r^2$) x h. The value of L was calculated as the square root of ($r^1 + r^2$) + h^2. Where; r^1 is the smaller canal radius in mm, r^2 is the largest canal radius in mm, π is a constant = 3.14 and h is the thickness of the section in millimeters as measured using a digital caliber.

Statistical Analysis:

By examining the distribution of the data and applying normalcy tests (Kolmogorov-Smirnov and Shapiro-Wilk tests), the data were examined for normality. A normal (parametric) distribution was seen in the data. The data were shown as mean, standard deviation (SD), mean difference, and for the difference values, 95% Confidence Interval (95% CI). Inter-observer reliability was assessed using Cronbach's alpha reliability coefficient and Intra-Class Correlation Coefficient (ICC). In addition, linear regression with regression trendline were used to assess the agreement between observer1 and 2. The significance level was set at $p \leq 0.05$. Difference between groups were assessed by ANOVA, and Paired t-test was used to compare between apical and coronal measurements. Statistical analysis was performed with IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.

RESULTS

Control group 2 "no bacteria" and group 4 "media only" showed the highest statistically significant values of POBS both at apical and coronal sections with no statistically significant difference between them. This was followed by group 3 "dead bacteria" then group 1 "with bacteria" which showed the least values of POBS with statistically significant difference between them and with other groups. ANOVA test was used to assess the difference between treatments, it was observed that the level of significance of group 1 "with bacteria" at p=0.001 was high while for control group 2 "no bacteria" and groups 3 "dead bacteria" and 4 "media only" at p=0.01 was low. Paired-t-test revealed statistically significant difference for the values of POBS between apical and coronal sections in all the experimental subgroups (Table 1. POBS in different groups, at apical and coronal sections. Differences between treatments assessed by ANOVA, Means followed by different small letters horizontally within the same row are significantly different according to DMRTs at 0.05 level. & Figure 3. Bar chart representing the POBS in different sealer type, subgroups, at apical and coronal section

Table (1) *POBS in different groups, at apical and coronal sections. Differences between treatments assessed by ANOVA, Means followed by different small letters horizontally within the same row are significantly different according to DMRTs at 0.05 level.*

Section	POBS								ANOVA
	Group 1. with Bacteria		Group 2. Control No bacteria		Group 3. Dead bacteria		Group 4. Media only		
Apical	8.61±2.48	c	35.44 1.25	a	25.46 ±1.00	b	37.85 0.57	a	<0.001***
Coronal	13.63 ± 2.49	c	38.20 1.79	a	29.87 1.89	b	41.79±0.32	a	<0.001***
p-value	0.003**		0.005**		0.017*		<0.001***		
Total	22.24 ± 4.97	c	73.64±3.04	a	55.33 ± 2.89	b	79.64 ±0.89	a	<0.001***

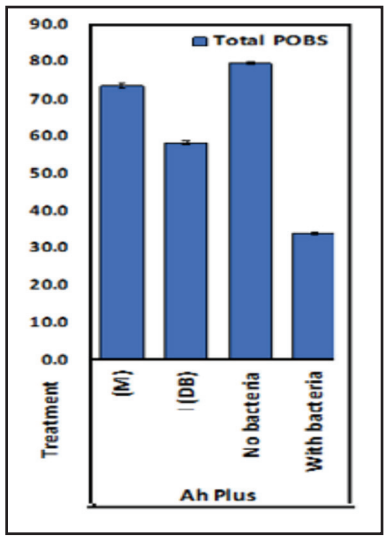

Fig. (3) Bar chart representing the POBS in different sealer type, subgroups, at apical and coronal section

DISCUSSION

Proper cleaning, shaping and obturation of the root canal system are essential for a successful endodontic procedure[13]. The need to entomb the remaining resistant bacteria and prevent the influx of tissue fluids inside the canals, which provides a source of nutrition to the latent bacteria, becomes crucial because bacteria that survive instrumentation and irrigation can jeopardize the effectiveness of endodontic treatment[8]. A good treatment outcome depends on the removal of bacteria and necrotic tissues from the root canal system[11]. This task is challenging due to the intricacy of the root canal system and the restrictions of the employed root canal irrigants[14]. Certain bacteria can persist in the hard-to-reach irregular regions of the root canal system and in the dentinal tubules, where they develop into clumps that are immersed in biofilm, an extracellular matrix[15]. Elimination of bacterial biofilms is a real challenge as they are more resistant to the commonly used irrigants because of possessing the following protective mechanisms; foremost, the extracellular polymeric substance "EPS" matrix's barrier qualities[16]. The second factor to consider is the physiological condition of biofilm microorganisms; planktonic cells develop more quickly than bacterial cells that are contained in a biofilm[17]. Third, the antimicrobial tolerance of the microorganisms within biofilm together with the inherent characteristics of the microorganisms and the nature of the microenvironment influence growth and succession of the microorganisms of the biofilm[18]. In addition, it was indicated that *E. faecalis* shows a potential for degradation of dental restorations[19]. This was observed by exposing dentin-resin interface to salivary esterase-like activity resulted in the formation of gaps that were infiltrated by bacterial biofilms[17]. These bacteria are able to cause periapical lesions when the conditions become favorable due to biodegradation of obturation materials with the consistent leakage of tissue fluids into the canals, thus providing a source of nutrition for those bacteria to nourish and multiply[6]. Thus, the purpose of this investigation was to assess how *E. faecalis* affected the POBS of an AH plus resin-based sealer[20]. The gram-positive, nonspore-forming cocci *E. faecalis* was chosen for the current investigation because to its unique properties[21]. It can be found single, in pairs, or in short chains. It is a facultative anaerobe that has evolved to survive in the harsh conditions of the gastrointestinal, vaginal, and oral cavities[22]. It has also been discovered to occur sometimes in primary root canal infections[23]. *E. faecalis* is one of the most resistant bacteria that is associated with endodontic treatment failure as it is frequently found in root filled teeth with a prevalence ranging from 30% to 90%. *E. faecalis* is commonly used as the microorganism of choice in vitro for experimental penetration into dentinal tubules as it leads to gross infections[16]. It is well colonized in root canals and forms biofilm[24]. It can penetrate deep into dentinal

tubules and is resistant to phagocytosis, antibodies, antibacterial agents, and root canal disinfecting agents[25]. Thus, it was the microorganism of choice in this study. After appropriate root canal cleaning and shaping, obturation of root canal space via core material "GP" and sealer is mandatory[26]. In order to achieve 3D hermetic seal and prevent any bacterial colonization in the root canal system, bonding of root canal sealers to both the GP core and the canal walls is very important characteristic[27]. Thus, providing ideal sealing for the root canal space to prevent microleakage. In addition, sealers should fill all irregularities of the root canal walls which cannot be filled with GP without creation of any gaps between sealer and dentinal walls or core materials[12]. Therefore, root canal fillings without sealers could potentially leak[28]. Group 1 "with bacteria" was used to study the effect of bacteria "*E. faecalis*" and its endotoxins on AH sealer. It was inoculated into the root canal before obturation. Control group 2 "no bacteria" was used to test the sealer in the absence of bacteria. Group 3 "dead bacteria" was used to exclude the effect of body of dead bacteria and group 4 "media only" was also used to exclude the effect of media "TSB" on sealing ability and biodegradation which was chosen because it was easy to make and performs equally in growing *E. faecalis* in vitro. AH Plus is a modified epoxy resin-based root canal sealer, was produced by DentSply, Germany, it is considered as a benchmark gold standard sealer because of its excellent properties such as small expansion, low solubility, adhesion to dentin and good sealing ability[11]. AH plus is composed of two pastes; Epoxide paste: Di-epoxide, Calcium tungstate, Zirconium oxide and Pigments. Amine paste: 1-adamantan amine, N,N'-dibenzyl-5, oxa-nonandiamine-1,9, TCD-Diamine, Calcium tungstate, Zirconium oxide, Aerosil and Silicon oil[11]. Epoxide paste is a polyepoxide resin, studies proved that bacteria help in degrading both natural and synthetic resins; where *E. faecalis* was isolated from degraded polymeric composite and epoxy resin[29]. During hydrolysis, extracellular enzymes secreted by microorganisms for biodegradation degrades the polymer to smaller molecule e.g oligomers, dimers and monomers[30]. Media Group and group "no bacteria" showed the highest statistically significant values of POBS at apical and coronal sections with no statistically significant difference between them due to the absence of bacterial effect. This was followed by group "dead bacteria" as this might be attributed to the enzymes secreted in the media of *E. faecalis* before dying that can withstand high killing temperature without deterioration, that can form the previous actions in biodegradation of plastics[21]. Also, it may be because of the wall components of dead bacteria which act as endotoxins (peptidoglycan and technic acid) that aids in the process of polyepoxide biodegradation[30]. This was followed by group "with bacteria" which showed the least values of POBS with statistically significant difference between them and with other groups. This can be explained as biodegradation is a complex process which is dependent on several factors, such as availability of a substrate, surface characteristics, morphology, molecular weight of the polymers and therefore, an exact definition of biodegradation is lacking[31]. Microorganisms must first excrete extracellular enzymes that depolymerize the polymers outside of the cells into smaller subunits (oligomers and/or dimers) that can be incorporated into the microbial cell and join its metabolic pathways because the polymers are insoluble in water and their molecules are too large for them to be taken up directly into the cells where most biochemical processes occur[30]. These activities ultimately yield new biomass and microbial

metabolic end-products such carbon dioxide, water, and methane (in the event of anaerobic breakdown[32]. There're many ways of degradation of resins; One way is poly-epoxide hydrolysis; it is a process that includes a reaction involving 3 amino acid residues (aspartate - histidine - serine)[30]. Aspartate: interact with histidine ring to give hydrogen bonds, while histidine ring furtherly interacts with serine by which it conducts deprotonating process with serine forming a nucleophilic alkoxide (-O) a group attacking ester bonds (polyepoxide contains ester bonds)[17]. This process results in alcohol tip and acyl enzyme complex which is furtherly attacked by water (biproduct of *E. faecalis* metabolism) to give carboxyl end and free enzyme, this free enzyme will be processed by microorganisms[33]. Other way is enzymatic degradation; In 1977 Tokiwa and Suzuki defined enzymes as catalysts with a high substrate specifity that means that a distinct enzyme only catalysis a special reaction with high efficiency[3]. The enzymatic degradation occurs in two stages: adsorption of enzymes on the polymer surface, followed by hydro-peroxidation/hydrolysis of the bonds[34]. The sources of plastic-degrading enzymes can be found in microorganisms from various environment[34]. Different levels of degradability are thought to depend on the extent of amorphous and crystalline forms, and the presence of strong C–C bonds, which are very resistant to attack by enzymes[30]. "The more the carbon bond, the more the resistance of polymer to biodegradation"[31]. Enzymes secreted by microorganism for biodegradation of plastics are of great diversity[35]. Example for enzymes are: Lipases, Proteinase K, dehydrogenase, Epoxide hydrolase, Cutinases, Carboxylesterases, Proteases H_2O_2 producing enzyme, Epoxide hydrolase, Deoxygenase (monooxygenase), Polyester hydrolase, Gelatinase, Papain and Urease. Those enzymes were proved to be able to hydrolyze ester bonds in various aliphatic polyesters and depolymerize the polymers outside the cells[3]. Group dead bacteria was the following in POBS values; this might be attributed to the enzymes secreted in the media of *E. faecalis* before dying that can withstand high killing temperature without deterioration, that can form the previous actions in biodegradation of plastics[34]. Also, it may be because of the wall components of dead bacteria which act as endotoxins (peptidoglycan and technic acid) that aids in the process of polyepoxide biodegradation[29]. Other way is the bond breakage by bacterial toxins where Bacteria breaks carbon bonds in polyepoxide C=0-0 by its toxins[36]. "Polyester/epoxy-resin structure: ring C≠0 is its chemical structure. According to an alternative idea, the term "biodegradation" of polymers usually refers to the action of microorganisms on materials based on water-insoluble polymers, such as polyepoxides, rather than the breaking down of water-soluble polymers, such as polyacrylamides and polyethyloxide[37]. Extracellular enzymes can only function on the polymer surface since they are too big to go deeper into the polymer substance, which means that the biodegradation of plastics is a traditional surface erosion process[38]. It was shown that there was a statistically significant difference between apical and coronal sections in all treatment groups where apical sections recorded lower values of POBS than coronal sections. This may be attributed to the absence of injection tip in AH plus so it can't reach apical area as much as coronal area.

CONCLUSION

E. faecalis demonstrated biodegradation of resin-based sealers. AH Plus sealers cannot eradicate *E. faecalis* completely.

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