Short-Term Antibacterial Efficacy of Three Bioceramic Root Canal Sealers Against Enterococcus Faecalis Biofilms

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Abstract

Objectives: The aim of the study was to evaluate the antimicrobial efficacy of three bioceramic root canal sealers against Enterococcus faecalis (E. faecalis) biofilm. Material and methods: E. faecalis bacterial suspension was grown on filter paper discs on agar plates. After the incubation period, the discs were covered with four different root canal sealers: 1) Premixing bioceramic root canal sealer (TotalFill BC Sealer); 2) Dual component bioceramic sealer (BioRoot RCS); 3) Mineral trioxide aggregate based sealer (MTA Fillapex); 4) Epoxy resin-based sealer (AH Plus). After contact time of 60 minutes, the sealers were removed, and the discs were transferred into sterile tubes containing phosphate buffered saline. After serial dilutions, the aliquots of the suspension were cultivated for 24 hours. After the incubation period, the colony forming units (CFUs) were counted. Results: There were no significant differences in antibacterial efficacy between the Total Fill BC Sealer and the AH Plus sealer (p=0.386). Both sealers showed better antibacterial efficacy compared to the BioRoot RCS and the MTA Fillapex (p<0.001). Conclusion: The Total Fill BC Sealer and AH Plus had better antibacterial efficacy than the BioRoot RCS and the MTA Fillapex sealers.

Introduction

The main goal of root canal treatment is the elimination of intracanal infection and obturation of root canal system in order to prevent reinfection (1). Although chemo-mechanical preparation reduces the number of microorganisms significantly, 40-60% of the root canals still remain positive for bacterial presence (2,3). The residual microorganisms are usually located in dentinal tubules, lateral canals and apical ramifications (4), thus preventing the healing of periapical lesion, or decreasing the long-term success of root canal treatment (5,6). Therefore, the root canal obturation needs to entomb these residual bacteria and to prevent their access to periapical tissues, and to block any other communications between the oral cavity and periapical tissues. Besides, the antimicrobial activity of root canal sealers could contribute to further reduction of viable microorganisms (7,8).

The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called Organically Modified Ceramics (OMCs). The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called OMCs. The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called OMCs. The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called OMCs. The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called OMCs. The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called OMCs. The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called OMCs. The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called OMCs. The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called Organically Modified Ceramics (OMCs). The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called OMCs. The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called OMCs. The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called OMCs. The newest generation of root canal sealers are based on tricalcium silicate or calcium phosphate and they are called
bioceramic sealers. They have attracted attention due to their alkaline pH, biocompatibility, bioactivity, non-toxicity, dimensional stability, sealing ability and potential to increase root strength after obturation (9). Their antimicrobial efficacy has been evaluated in few past in vitro studies using different microbiological methods and obtaining different results (10-12). A recent study by Wang et al. (12) revealed same efficacy of Endosequence BC Sealer as AH Plus against Enterococcus faecalis (E. faecalis) in dentinal tubules after one day, seven days and 30 days of contact time. Another in vitro study reported superiority of the epoxy resin based sealer over a bioceramic sealer (13).

The aim of this study was to evaluate the short-term antimicrobial effect of three bioceramic root canal sealers and epoxy resin based sealer against Enterococcus faecalis biofilm after 60 minutes of contact time.

Material and methods
Enterococcus faecalis cultivation
In this study, we used a wild type strain of E. faecalis, which had been isolated from a root canal of the lower first human molar with symptomatic chronic apical periodontitis. The bacteria were grown in brain heart infusion (BHI) broth at 4˚C. Subsequently, few colonies were isolated and grown up on trypticase soy broth (TSB) at 37˚C overnight. The density of the bacterial suspension was adjusted to 1.0 McFarland measured by a densitometer (Densimat, Bio Mérieux, Marcy l’Etoile, France).

For the evaluation of antimicrobial efficacy of bioceramic sealers and epoxy resin based sealer, we used the protocol with filter paper discs described by Barros et al. (14). The 10 µL of the E. faecalis suspension was grown on 81 filter paper discs (Whatman GmbH, Dassel, Germany), which were placed on Mitis Salivarius agar plates (Sigma-Aldrich, Taufkirchen, Germany). The paper discs with bacteria were incubated for 48 hours at 37˚C and 100% of humidity. The discs were randomly chosen for scanning electron microscopy (SEM) in order to confirm biofilms growth. The selected discs were fixed in 2.5% glutaraldehyde (Sigma-Aldrich, Taufkirchen, Germany) for 24h and then dehydrated in an ascending ethanol solutions (60%, 70%, 80% and 96%), for 30 minutes in each. After 24 hours of drying on air, the samples were sputter-coated with gold and observed by the SEM (Tescan Vega TS5136LS, Tescan, Brno, Czech Republic).

Placement of sealers on filter discs
The filter discs with biofilms were divided into four experimental groups (n=17/each) according to the tested sealer and the positive control group (n=10):
Group 1: Total Fill Bioceramic Sealer (TotalFill BC Sealer, FKG, Switzerland)

i naziva se biokeramičkim punilima. Pozornost su privukli zbog lužnatog pH, biokompatibilnosti, bioaktivnosti, netoksičnosti, dimenzijske stabilnosti, svojstva brtvljenja i potencijala za povećanje čvrstoće korijena nakon punjenja kanala (9). Njihova antimikrobna aktivnost ispitivana je u nekoliko istraživanja in vitro u kojima se su primijenile različite mikrobiološke metode pa su dobiveni i različiti rezultati (10 – 12). Nedavno istraživanje Wanga i suradnika (12) pokazalo je jednaku učinkovitost Endosequence BC Sealera i AH Plusa, kad je riječ o bakteriiji Enterococcus faecalis, u dentinskih tubulismima nakon jedan, sedam i 30 dana kontaktka. U drugom, pak, istraživanju in vitro istaknuta je bolja učinkovitost punila proizведенog na temelju epoksidne smole u usporedbi s biokeramičkim punilom (13).

Cilj ovog istraživanja bio je ispitati kratkoročnu antimikrobnu učinkovitost triju biokeramičkih punila i punila proizvedenog na temelju epoksidne smole na 72 sata stare biofilmove s E. faecalis nakon 60-minutnog kontakta.

Metode
Kreiranje biofilmova s bakterijama Enterococcus faecalis
U ovom istraživanju koristili smo se divljim sojem bakterije E. faecalis izoliranim iz korijenskog kanala prvoga donjeg molara sa simptomatskim kroničnim apikalnim parodontitom. Uzgojena je u moždano-srčanom bujonu (engl. brain heart infusion – BHI) na temperaturi od 4˚C. Nekoliko kolonija izolirano je uzgojeno i na mediju triptaza soj agara (engl. trypticase soy broth – TSB) preko noći na temperaturi od 37˚C. Gustoća bakterijske suspenzije podešena je denzitometrom (Densimat, Bio Mérieux, Marcy l’Etoile, Francuska) na 1,0 McFarland.

Korisili smo se protokolom s papirnatim filtar-diskovima opisanim u istraživanju Barrosa i suradnika (14), kako biti ispitali antibakterijsku učinkovitost biokeramičkih punila i punila proizvedenog na temelju umjetne smole. Količina od 10 µL suspenzije E. faecalis postavljena je na 81 papirnati filtar-disk (Whatman GmbH, Dassel, Njemačka) te su zatim položeni na agarni ploču Mitis Salivarius (Sigma-Aldrich, Taufkirchen, Njemačka). Papirnati diskovi s bakterijama inkubirani su 60 sata na 37˚C, u uvjetima 100-postotne vlaznosti. Nasumično odabrani diskovi pripremljeni su za promatranje pod pretražnim elektronskim mikroskopom (engl. scanning electron microscopy – SEM) kako bi se potvrdila prisutnost biofilmova.

Diskovi su tijekom 24 sata fiksirani u 2,5-postotnom glutaraldehidu (Sigma-Aldrich, Taufkirchen, Njemačka) te dehidrirani u sve većim koncentracijama otopine etanol (60 %, 70 %, 80 % i 96 %) 30 minuta u svakoj koncentraciji. Nakon 24-satnoga sušenja na zraku, uzorci su napareni zlatom i promatrani mikroskopom (Tescan Vega TS5136LS, Tescan, Brno, Republika Češka).

Postavljanje endodontskih punila na papirnate filter-diskove
Papirnati filtar-diskovi s biofilmovima podijeljeni su u sljedeće četiri eksperimentalne skupine (n = 17/each), ovisno o ispitivanoj tekućini u pozitivnu kontrolnu skupinu (n = 10):
Microbiological analysis of antibacterial efficacy

After 60 minutes of contact time, the sealers were carefully removed from each disc using a sterile Heidelberg instrument (Hu-Friedy Mfg. Co., Frankfurt am Main, Germany). Some minor remnants remained on the surface of few discs. Each disc was placed in a sterile tube containing 1 ml of phosphate-buffered saline (PBS) and slightly agitated for 5 seconds (Vortex mixer X-HD, Sinosource, Guangdong, China) to remove unattached bacterial cells. Then, the discs were transferred to another sterile test tube, which contained 1 ml of PBS, and agitated for 30 seconds. After 8-fold serial dilutions, 10 µL of each dilution was grown on Mitis-Salivarius agar plate (Sigma-Aldrich, Taufkirchen, Germany) and incubated for 24 h at 37°C and 100% humidity. After the incubation period, the colony forming units (CFUs) were counted and transformed into actual count based on the dilution factor.

Statistical analysis

The obtained data were analysed using the Mann-Whitney U test. All p-values lower than 0.05 were considered statistically significant. The program IBM SPSS Statistics version 23.0 (www.spss.com) was used.

Results

The SEM analysis confirmed the presence of biofilms of *E. faecalis* on filter discs (Figure 1, Figure 2). All tested materials reduced the number of CFUs significantly compared with the positive controls (p<0.001) (Table 1). There was no statistically significant difference between the Total Fill BC Sealer and the AH Plus (p=0.386), nor between the BioRoot RCS and the MTA Fillapex (p=0.931) regarding the reduction of the CFUs. The Total Fill BC Sealer and the AH Plus showed greater antibacterial efficacy than the BioRoot RCS and the MTA Fillapex (p<0.001) (Figure 3).

Skupina 1: Total Fill Bioceramic Sealer (TotalFill BC Sealer, FKG, Švicarska)
Skupina 2: BioRoot Root Canal Sealer (BioRoot RCS, Septodont, Saint Maur Des Fosses, Francuska)
Skupina 3: MTA Fillapex (Angelus, Londrina, PR, Brazil)
Skupina 4: punilo proizvedeno na temelju epoksidne smole AH Plus (Dentsply, Konstanz, Njemačka)

Testirani materijali pripremljeni su prema uputama proizvođača. Otprilike 40 µL svakog svježe pripremljenog materijala, izmjerenog žličastim ekskavatorom (Premium instruments, New York, SAD) jednakog volumena, postavljen je na kontaminiranu površinu papirnatoga filtardiska. Puni lo je na njegovu površinu razmazano mikrobiološkom ezoom (Thermo Fisher Scientific, Waltham, MA, SAD).

Kao pozitivna kontrolna skupina (n = 10) korišteni su papirnati filtardiskovi s biofilmovima koji nisu bili prekriveni punilima. Negativna kontrolna skupina bili su sterilni diskovi, na kojima biofilm ostao je na njegovom površinom sa značajnom redukcijom broja bakterija.

Mikrobiološka analiza antibakterijske učinkovitosti materijala za punjenje

Nakon 60-minutnog kontakta punila su pažljivo uklonjena sa svakog diska sterilenom Heidelbergovom štampilom (Hu-Friedy Mfg. Co., Frankfurt na Majni, Njemačka). Na dnu ostaci punila ostali su na nekoliko diskova. Svaki disk stavljen je u diskovodu koji je sadržavao 1 ml fosfatom puferiranog Šekla Monstrat (Vortex mixer X-HD, Sinostar, Šenc, Kina) kako bi se uklonile neadherentne bakterijske stanice. Uzimao se diskovi prebačeniji u drugu diskodiskovodu koji je sadržavao 1 ml fosfatom puferiranog Šekla Monstrat (Sigma-Aldrich, Taufkirchen, Njemačka) i inkubirano 30 dana na 37 °C u ambijentalnim uvjetima.

Dobiveni podaci analizirani su Mann-Whitneyjevim U testom. Sve p-vrijednosti niže od 0,05 razmatrane su kao statistički značajne. Za statističku analizu odabrano je program IBM SPSS Statistics version 23.0 (www.spss.com).

Rezultati

Analiza SEM-om potvrdila je prisutnost biofilma s bakterijom *E. faecalis* na filtru papirnatih diskova (slika 1., slika 2.). Svi ispitivanj materijali značajno su smanjili broj bakterija na filtardiskovima u usporedbi s pozitivnom kontrolnom skupinom (p < 0,001) (tablica 1.). Nije bilo statistički značajne razlike između materijala TotalFill BC i AH Plus (p = 0,386) te između BioRoot RCS-a i MTA Fillapexa (p = 0,931). TotalFill BC i AH Plus bili su antibakterijski učinkovitiji negoli BioRoot RCS i MTA Fillapexa (p < 0,001) (slika 3.).
### Table 1. Number of CFUs after 60 minutes of exposure to the tested root canal sealers and comparison to the positive control group

| Groups       | Skupine         | Mean ± SD | Minimum | Maximum  | 25th ± 25 sati | 50th (Median) ± 50th sati (sred. vrijed.) | 75th ± 75 sati | Reduction compared to positive control | Smanjenje broja u odnosu prema pozitivnoj kontroli |
|--------------|-----------------|-----------|---------|----------|----------------|------------------------------------------|---------------|----------------------------------------|--------------------------------------------------|
| TotalFill BCS|                 | 3.72E+07  | 1.20E+08| 1.00E+04 | 5.00E+08       | 1.00E+06                                | 8.00E+06      | 1.00E+07                              | 99.94%                                           |
| BioRoot RCS  |                 | 3.58E+09  | 2.56E+09| 1.00E+09 | 9.00E+09       | 1.40E+09                                | 2.90E+09      | 6.00E+09                              | 79.29%                                           |
| AH Plus      |                 | 1.78E+08  | 6.51E+08| 1.00E+05 | 2.70E+09       | 1.00E+06                                | 1.00E+07      | 4.75E+07                              | 99.93%                                           |
| MTA Filapex  |                 | 3.51E+09  | 2.50E+09| 4.00E+08 | 7.90E+09       | 1.45E+09                                | 2.80E+09      | 6.05E+09                              | 80.00%                                           |
| Positive control |             | 2.44E+10  | 2.75E+10| 2.10E+09 | 9.00E+10       | 4.75E+09                                | 1.40E+10      | 3.53E+10                              |                                                   |

### Discussion

Although all tested sealers showed significantly lower number of CFUs, there was no complete eradication of bacteria in any group. Firstly, these results can be explained due to the source of bacteria. In this study, wild-type strain of *E. faecalis* was used. Wild-type strains are less susceptible to sealers compared to most commonly used ATCC strain of *E. Faecalis*, and they have been recommended for the evaluation of antimicrobial efficacy of sealers (14). Also, *E. faecalis* has a significant role in etiology of persistent periradicular infections. In this study, wild-type strain of *E. faecalis* was used, which is less susceptible to sealers compared to ATCC strain. This makes the results more relevant for clinical practice.

### Rasprava

Iako su svi testirani materijali pokazali da smanjuju broj jedinica koje formiraju kolonije (CFU-s), ni u jednoj skupini bakterije nisu postupno eliminirane. Takvi se rezultati uglavnom vežu uz izvor bakterija. U ovom istraživanju korišten je divlji soj *E. faecalis*. Naime, divlji sojevi manje su osjetljivi u usporedbi s najčešće korištenim ATCC sojem bakterije *E. faecalis* na djelovanje punila te se preporučuju za ispitivanje njihove antimikrobne učinkovitosti (14). *E. faecalis* također je također važan u etiologiji perzistirajućih periradikularnih infekcija.
ular lesions (15) due to its virulence factors and ability to survive the conventional chemo-mechanical root canal procedures (16).

Secondly, the type of bacteria can affect antibacterial efficacy of sealers. Most previous studies evaluated the antibacterial efficacy of root canal sealers against planktonic bacteria (7, 13, 17). However, since planktonic bacteria do not represent a real clinical situation in infected root canals, the use of older bacterial biofilms which express greater resistance has been recommended (12,18,19). In this study, we used 48 hours old bacterial biofilm in a microbiological protocol described by Barros et al. (14). Therefore, the recommendation for further studies is to investigate the antibacterial efficacy of sealers on older biofilms.

Our finding of no complete eradication in all groups is consistent with few previous studies (20,21). On the contrary, Zhang et al. (7) reported complete eradication of E. faecalis after application of both, the bioceramic root canal sealer (iRoot SP Sealer) (for 2 minutes) and the AH Plus (for 5 to 20 minutes) sealer. This result could be related to the planktonic species of bacteria used in suspension. These bacteria are more susceptible than biofilms (22).

The results of this study also showed similar antibacterial activity of the TotalFill BC Sealer and the epoxy resin based AH Plus against E. faecalis biofilms (eradication of more than 99.9%) after contact time of 60 minutes. The same conclusions have been reached in a recent study by Wang et al (12), in which no difference between the bioceramic sealer (Endosequence BC Sealer) and the AH Plus was found even after longer contact time of one day, seven days and 30 days using confocal microscopy evaluation. The antibacterial efficacy of bioceramic sealers is considered to be related to the release of Ca²+ ions and high pH (7,17,22). In another study by Nirupama et al (13), the AH Plus and the bioceramic iRoot SP sealers showed statistically significant antibacterial activity of the BioRoot RCS compared with epoxy resin sealers against 18h old E. faecalis, which was explained due to its activity of the BioRoot RCS compared with epoxy resin sealers against 18h old E. faecalis (25).

An interesting finding of this study is superior results of the Total Fill BC Sealer over the BioRoot RCS since both materials are bioceramics and of similar composition. The BioRoot RCS is the newest two-component root canal sealer. The powder component is composed of tricalcium silicate, zirconium dioxide and povidone, and the liquid is composed of water, calcium chloride and polycarboxylate. There have been only few studies published so far on its antibacterial activity (25,26). Poggio et al. (25) reported lower antibacterial activity of the BioRoot RCS compared with epoxy resin sealers against 18h old E. faecalis, which was explained due to its

nih lezija (15) jer sadržava čimbenike virulencije i svojstvo da preživi konvencionalnu kemomehaničku obradu korijenskih kanal (16).

Drug, antibakterijska učinkovitost punila može ovisiti o starosnoj skupini bakterija. U većini dosadašnjih istraživanja ispitivana je antibakterijska učinkovitost punila na plankton- skim bakterijskim stanicama (7, 13, 17). No te stanice ne pokazuju stvarnu situaciju u iniciranim korijenskim kanalima i zato se preporučuje korištenje starijih bakterijskih biofilmova koji su otporniji na antimikrobna sredstva (12, 18, 19). U ovom istraživanju koristili smo se 48 sati starih biofilmovima i mikrobiološkim protokolom opisanim u istraživanju Barrosa i suradnika (14). Preporuka je da se za daljnja istraživa- vanja antibakterijske učinkovitosti upotrebljavaju punila starijih biofilmova.

Rezultati našeg istraživanja pokazuju nepotpunu elimina- ciju bakterija u svim skupinama, što se može usporediti s dosadašnjim studijama (20, 21). Suprotno su pokazali Zhang i suradnici (7) u čijem su istraživanju biokeramički materi- jal Root SP Sealer (unutar 2 minute) i AH Plus (unutar 5 do 20 minuta) potpuno eliminirali bakteriju E. faecalis. Takav se rezultat može objasniti činjenicom da su planktonske sta- nice osjetljivije od biofilmova na djelovanje antimikrobičkih sredstava (22).

Naši rezultati također su pokazali sličnu antibakterijsku učinkovitost TotalFill BC punila i AH Plusa, materijala pro- izvedenog na temelju umjetne smole, kad je riječ o biofilmovima s E. faecalis (eliminacija 99,9%) nakon 60-minutnog kontakta. Isto su zaključili i Wang i suradnici (13) u istraži- vanju u kojem su s pomoću konfokalne laserske mikrosko- pijere pokazali da nema razlike između biokeramičkog punila (Endosequence BC punila) i AH Plusa nakon duljih konta- kata (jedan, sedam i 30 dana). Smatra se da je antibakterij- ska učinkovitost biokeramičkih punila povezana s otpušta- njem Ca²+iona i visokih pH punila (7, 17, 22). U istraživanju Nirupame i suradnika (13), biokeramički materijali AH Plus i Root SP pokazali su značajnu antibakterijsku učinkovitost kad je riječ o E. faecalis. No AH Plus je bio dulje učinkovit (18 sata), vjerojatno zbog otpuštanja bisphenol-A-diglicidilnog etera tijekom polimerizacije (23). U ovom istraživanju ispitivati smo antimikrobučnu učinkovitost punila unutar 60 minuta od postavljanja materijala, a koristili smo se direktnim kon- taktnim testom (engl. direct contact test – DCT) zato što se DCT upotrebljava samo za ispitivanje kratkoročnog antimikro- kobnog učinka punila (24).

Naime, u istraživanjima u kojima se primijenio DCT, a nisu imala dentin kao podlogu, istaknuto je da punila gube antibakterijski učinak za nekoliko sati ili dana od miješanja (7, 24). I vrijeme kraće od 60 minuta pokazalo se prekratkim za početak djelovanja punila na bakterije poput E. faecalis (25).

Zanimljiv rezultat ovog istraživanja su superiorni rezultati dobiveni za Totalfill BC Sealera u usporedbi s BioRoot RCS- om, zato što su oba materijala biokeramička i imaju sličan sastav. BioRoot RCS najnovije je dvokomponentno punilo. Prah se sastoje od trikalcijeva silikata, cirkonijeva dioksida i povidona, a tekućina od vode, cirkaljeva klorida i polikarboxilata. Dosad je objavljeno nekoliko istraživanja o njegovu an- tubakterskim odobravanju (25, 26). Poggio i suradnici (25)
shorter working (minimum 10 min) and setting time (maximum 4 h) (26, 27).

Another reason could be related to smaller homogeneity of the BioRoot RCS after hand mixing when compared with the “premixing” bioceramic sealers. In the most recent study of Arias-Moliz et al. (26), the BioRoot RCS showed better intratubular antibacterial efficacy compared with the MTA Fillapex and the AH Plus.

Different results of the same materials in the above-mentioned studies could be explained due to different microbial tests used (10, 25). The most commonly used microbial tests in this kind of studies are DCT and agar diffusion test (ADT) (17, 25, 28, 29). ADT has been suitable for soluble materials but due to its limitations (dependence on diffusion and physical properties of tested materials, does not distinguish bacteriostatic or bactericidal effect of the material) (29) this test is no longer recommended (7). DCT is a quantitative and reproducible method to simulate the contact of microorganisms with endodontic sealers, providing information about bactericidal effect (7), thus being more reliable and relevant (9, 14).

In conclusion, Total Fill BCS and AH Plus presented similar antibacterial efficacy against *E. faecalis*, which was superior compared to BioRoot RCS and MTA Fillapex.

**Conflict of interest**
None declared

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