ORIGINAL ARTICLE

UDC: 616.314-08-74
https://doi.org/10.2298/VSP161231130T

Antibacterial effects of new endodontic materials based on calcium silicates

Antibakterijski efekti novih endodontskih materijala na bazi kalcijum silikata

Dijana Trišić*, Bojana Ćetenović†, Nemanja Zdravković‡, Tatjana Marković§, Biljana Dojčinović‖, Vukoman Jokanović†, Dejan Marković*

University of Belgrade, *Faculty of Dental Medicine, †Institute for Nuclear Sciences "Vinča", ‡Faculty of Veterinary Medicine, §Institute for Medicinal Plants Research "Dr Josif Pančić", ‖Institute of Chemistry, Technology and Metallurgy, Belgrade, Serbia

Abstract

Background/Aim. The main task of endodontic treatment is to eliminate pathologically altered tissue, to disinfect root canal space and to obtain its three-dimensional hermetic obturation. The main purpose of this study was to evaluate antimicrobial activity of new endodontic nano-structured highly active calcium silicates based materials albo-mineral plyoxide carbonate aggregate (ALBO-MPCA) and calcium silicates (CS) in comparison to mineral trioxide aggregate (MTA$^+$) and UltraCal XS (CH). **Methods.** The antimicrobial activity of materials was tested against *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 14506) strains, and following clinical isolates: *Rothia dentocariosa, Enterococcus faecalis, Staphylococcus aureus, Streptococcus anginosus* and *Streptococcus vestibularis* using a double layer agar diffusion test. The pH measurements were performed using the pH meter. Total amount of released ions was determined by inductively coupled plasma optical emission spectrometry (ICP-OES). **Results.** All tested materials showed the best antibacterial potential after 1 h of incubation. After 3h and 24h of the incubation period, the antibacterial potential of all tested materials were similar. The Agar diffusion test showed that ALBO-MPCA, CS and MTA$^+$ had similar inhibition zones ($p > 0.05$), except in the activity against *Staphylococcus aureus* where ALBO-MPCA showed better antimicrobial properties than MTA$^+$ in 3h and 24h of the incubation period ($p < 0.05$). Following 24h of the incubation, the inhibition zones were the strongest with CH against *Staphylococcus aureus* (16.67 ± 2.34 mm) followed by ALBO-MPCA (14.67 ± 1.21 mm) and the weakest with CS against *Enterococcus faecalis* (6.50 ± 1.76 mm). CH showed the highest pH, followed by ALBO-MPCA, CS and MTA$^+$. **Conclusion.** The expressed antibacterial effects indicate that materials based on nano-structured highly active calcium silicates represent effective therapeutic agents for root canal obturation in one-visit apexification treatment, therefore they are recommend for further examination and clinical trials as they are proposed for MTA substitution.

Key words:
dental pulp diseases; root canal preparation; calcium silicate; calcium hydroxide; anti-infective agents.

Apstrakt

Uvod/Cilj. Osnovni cilj endodonskog lečenja je eliminacija patološki izmenjenog tkiva, eliminacija infekcije korensko kanala i njegovo hermetičko trodimenzionalno zatvaranje. Cilj istraživanja je bio da se proceni antibakterijska aktivnost novih endodontskih nano-strukturiranih materijala na bazi visoko aktivnih kalcijum silikata *albo-mineral polyoxide carbonate aggregate* (ALBO-MPCA) i *calcium silicates* (CS) u odnosu na *mineral trioxide aggregate* (MTA$^+$) i *UltraCal XS* (CH). **Metode.** Testirana je antibakterijska aktivnost materijala protiv *Staphylococcus aureus* (ATCC 25923) i *Enterococcus faecalis* (ATCC 14506), kao i kliničkih izolata: *Rothia dentocariosa, Enterococcus faecalis, Staphylococcus aureus, Streptococcus anginosus* i *Streptococcus vestibularis* pomoću agar difuzionog testa. Merenja pH vrednosti obavljena su korišćenjem pH metra. Ukupan iznos oslobođenih jona određivan je pomoću ICP-OES. **Rezultati.** Svi testirani materijali pokazali su najbolji antibakterijski efekat nakon 1 h od inkubacije. Nakon 3 h i 24 h od inkubacije, antibakterijski efekat svih testiranih materijala bio je sličan. Agar difuzioni test pokazao je da materijali ALBO-MPCA, CS i MTA$^+$ ispoljavaju slične zone inhibicije rasta ($p > 0.05$) osim u slučaju *Staphylococcus aureus*, gde je materijal ALBO-MPCA pokazao bolje antibakterijsko dejstvo nego MTA$^+$ nakon 3 h i 24 h od inkubacije ($p < 0.05$). Nakon 24 h od inkubacije, zone inhibicije su bile najizraženije u slučaju materijala CS protiv *Staphylococcus aureus* (16.67 ± 2.34 mm), zatim ALBO-MPCA (14.67 ± 1.21 mm), a najslabije u slučaju CS protiv *Enterococcus faecalis* (6.50±1.76 mm). Materijal CH ispoljio je najveću pH vrednost, zatim ALBO-MPCA, CS i MTA$^+$. **Zaključak.** Ispoljeni antibakterijski efekti ukazuju na to da materijali na bazi visoko aktivnih

Correspondence to: Dijana Trišić, Faculty of Dental Medicine, 6 dr Subotića, 11 000 Belgrade, Serbia. E-mail: diana.trisic@hotmail.com

kalcijum silikata mogu da predstavljaju efikasnu zamenu za MTA u terapiji zuba sa nezavršenim rastom korena u jednoj poseti, te se stoga preporučuju za dalja klinička ispitivanja.

Ključne reči:
zub, bolesti pulpe; zub, lečenje korenskog kanala; kalcijum silikat; kalcijum hidroksid; antiinfektivi.

Introduction

The main task of endodontic treatment is to eliminate pathologically altered tissue, to disinfect root canal space and to obtain its three-dimensional hermetic obturation, as residual microorganisms are usually present in apical ramifications and isthmuses that are never completely filled [1]. More than 99.5 % of Gram-positive bacteria, is eliminated by a proper chemo-mechanical root canal treatment [2]. Residual microorganisms, particularly *Enterococcus* and *Streptococcus* species, are considered to be responsible for the treatment failure [3]. Moreover, *Enterococcus faecalis* has the ability to bind with the collagen fibers and survive up to 12 months in the environment without the substrate [3]. Facultative anaerobes and Gram-positive species, revealing a heterogeneous profile of polymicrobial infection are frequently isolated from the root canals following an unsuccessful endodontic treatment [2].

An ideal material for root canal obturation must prevent both, apical and coronal leakage. It has to be biocompatible, noncancerous and nongenotoxic, dimensionally stable and insoluble in tissue fluids. Considering the ability of residual microorganisms to provoke periapical irritations, it is preferable for sealing materials to possess antibacterial activity [4].

So far, the sealers based on calcium hydroxide proved to be the most efficient against a range of pathogenic microorganisms [5]. Their major advantage is a high pH which is toxic to bacterial cells, leading most likely to protein denaturation and damages of cytoplasmic membrane or DNA [6]. However, it is also proved that the calcium hydroxide based sealers have a limited antimicrobial effect on *Enterococcus faecalis* [6].

In the early 1990, different commercial products of mineral trioxide aggregate (ProRoot MTA, WMTA Angelus, GMTA Angelus) were synthesized. Initially, MTA was recommended as a root-end filling material, while today it is used in a number of endodontic procedures, particularly as an apical barrier in teeth with incomplete root development [7]. MTA is composed of hydrophilic particles which, in the presence of water, form a colloidal gel that is transformed into solid cement [8]. When mechanically mixed, MTA based materials achieve better marginal adaptation, and consequently possess better sealing property [9]. The high pH value achieved during the setting suggests a potential antibacterial activity of the material [10]. Due to variations in the chemical composition of MTA based materials, and the grain size, differences in hydration rate, flowability, consistency and setting time can be expected [8]. Incorporation of the hydrosoluble polymer can reduce dry consistency of MTA based materials and thus to improve the material handling [8]. Several attempts were made to improve the MTA manipulation characteristics which complicate its use during the orthograde canal filling procedures [11–13]. Similar to MTA, new nano-structured materials, calcium silicates (CS) and albo-mineral polyoxide carbonate aggregate (ALBO-MPCA), with the reduced setting time and morphology which provides a distinct activity after their placing into vital tissues, were introduced [14, 15].

The aim of this study was to evaluate pH, ion release and the antimicrobial effects of two new endodontic materials based on nano-structured highly active calcium silicates (ALBO-MPCA and CS) in comparison to MTA^{+} and Ultra-Cal XS (CH).

Methods

The study was carried out at the University of Belgrade: Faculty of Dental Medicine, Institute for Nuclear Sciences "Vinča", Faculty of Veterinary Medicine and Institute of Chemistry, Technology and Metallurgy. Prior to conducting this study, informed written consent was obtained from the patients. The study was designed in accordance with the guidelines of the Declaration of Helsinki and approved by the Ethics Committee.

The isolation of microorganisms

All clinical isolates used in the experiment were obtained at the University Clinic, from the patients' infected root canals, using the endodontic needles. The endodontic needle samples were taken in pairs (for aerobic and anaerobic cultivation) and collected in thioglycollate broth (Institute of Virology, Vaccines and Sera-Torlak, Belgrade, Serbia) and brain heart infusion agar (BHI, Becton, Dickinson and Company, Sparks, USA) and left for 24 h at 37°C. The overnight cultures were streaked on the appropriate media for cultivation; aerobic cultures on Columbia agar with 5% sheep blood (COS, bioMérieux, Marcy-l'Étoile, France) and MacConkey agar (Becton, Dickinson and Company, Sparks, USA) and incubated in the aerobic atmosphere overnight, while anaerobic ones on Columbia agar with 5% sheep blood and incubated in a jar under the anaerobic conditions using GasPack (GasPak™ EZ Gas Generating Container Systems, Becton, Dickinson and Company, USA), at 37°C for 2 to 5 days. The grown bacterial colonies from the anaerobic conditions were put on Columbia agar with 5% sheep blood at 37°C overnight to determine demand for obligatory anaerobiosis in such bacteria. Preliminary identification of clinical isolates was done by the Gram stain, hemolysis on chitodigosaccharide (COS), catalase, oxidase (Oxidase Reagent Droppers Becton, Dickinson and Company, USA) and coagulase tests (Rabbit plasma, Veterinary Medicine Institute Inc., Zemun, Serbia). In order to confirm the identification of the Gram positive bacteria, the BD BBL Crystal™ Identification Systems Gram–Positive ID (Becton, Dickinson and Company, Sparks, USA) was conducted.

Materials

For the synthesis of two new nano-structured materials based on the active silicate systems calcium silicates (CS) and albo-mineral polyoxide carbonate aggregate (ALBO-MPCA), mixture components were prepared [14, 15]. Briefly, calcium silicate phases, 2β-$CaSiO_4$ (β-C_2S) and Ca_3SiO_5 (C_3S), were synthesized using stoichiometric quantities of $CaCl_2 \times 5H_2O$ and silica sol by hydrothermal treatment, in the following ratio: $C_3S : C_2S = 2 : 1$. $Al(C_2H_3O_2)$ was added to allow the production of an active C_3A phase. Calcium chloride tetrahydrate was used as the precursor for production of $CaCO_3$, while sulfonyl dodecyl sulfate was added as an antiagglomeration agent. The final mixture was made by mixing $CaCO_3$ with calcium silicate phases (C_3S and β-C_2S) in the case of CS, while the monoclinic Bi_2O_3 was added in case of as ALBO-MPCA as a radiocontrast agent.

As the control materials, mineral trioxide aggregate (MTA^+, Cercamed, Stalowa Wola- Poland), consisting of calcium hydroxide and silicon, iron, aluminium, sodium, potassium, bismuth, magnesium oxides and calcium phosphate as well as calcium hydroxide based paste (UltraCal XS, UltraDent, South Jordan, USA) were used.

Agar diffusion test

The antimicrobial activities were examined against the following bacterial strains: *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 14506 and clinical isolates: *Enterococcus faecalis*, *Staphylococcus aureus*, *Rothia dentocariosa*, *Streptococcus anginosus* and *Streptococcus vestibularis*. After activation form the stock culture, microorganisms were maintained as the overnight cultures on Cation Adjusted Mueller-Hinton Broth (CAMHB, Becton, Dickinson and Company, Sparks, USA) and seeded on Cation Adjusted Mueller-Hinton agar (CAMHA, Becton, Dickinson and Company, USA) and COS at 37°C for 24 h before use.

The examination of antimicrobial activity of endodontic materials was conducted by the double layer agar diffusion test (ADT) on the 90 mm sterile Petri plates. The base layer was made of 10 mL sterilized CAMHA. After 24 h, four uniform wells (5 mm in diameter), each one corresponding to a single tested sealer, were made by the sterile plastic tubes and filled with the freshly mixed materials. The seeding layer that was put over the base, consisted of 10 ml sterile CAMHA inoculated to achieve 10^8 (CFU)/mL of tested bacteria, which corresponds to the 0.5 McFarland scale. The plates were left at room temperature for 2 h, in order to allow prediffusion of materials, and after that they were incubated for 1 h, 3 h and 24 h, at 37°C. Aliquots of 5 mL of triphenyltetrazolium chloride (TTC) prepared with 0.05% of TTC and 1% CAMHA were added for optimization. After solidification of CAMHA+TTC, the plates were incubated for 30 min at 37°C. Negative control was conducted using the same method without placing the materials. The diameters of inhibition zones of bacterial growth were measured in above mentioned time intervals. All tests were done in sixtiplicate, except the positive controls which were done in triplicate.

pH measurements

All pH values were repeatedly measured (three times), using the pH-meter (pH-vision Microcomputer 6071, JENCO Electronics Ltd., Linkou Shiang, Taiwan) combined with the HI-type electrode 1131 (Hanna Instruments WTW GmbH, Woonsockets, RI-USA). The calibration of pH-meter was performed using biftalato (pH = 4.01) and phosphate buffer (pH = 7.00) (Carlo Erba Reagents SpA, Rodano, Italy). Suspensions of 50 mg/mL of each tested material into deionized water were prepared, then shaken on vortex for 30 min and centrifuged for 15 min at 4000 rpm. Readouts of the pH measurements were carried out after 1 h, 3 h and 24 h. The solutions of deionized water were used as controls (5.76 ± 0.51).

Inductively coupled plasma-optical emission spectroscopy (ICP-OES) analysis

Investigated materials were prepared according to the manufacturers instruction and placed into the plastic molds (5 mm in diameter and 5 mm high) to set. After the setting, the discs of each investigated materials were placed into 20 mL of deionized water (n = 3). Deionized water was changed after 1 h, 3 h and 24 h and the concentrations of ions were measured using the Thermo Scientific iCAP 6500 Duo ICP (Thermo Fisher Scientific, Cambridge, UK) spectrometer equipped with the RACID86 Charge Injector Device detector, concentric PTFE nebulizer, quartz torch and alumina injector. The ICP-OES measurements for each sample were carried out three times. Quantifications of released ions into deionized water were performed at the adequate emission wavelength of light.

Statistical analysis

Data analysis was performed using the ANOVA Repeated Measures test, and post hoc Tukeys' test. The level of significance was set at $p < 0.05$ and the data were processed using the statistical software IBM SPSS 20.

Results

The data obtained in the ADT for each of the investigated materials are presented in Figures 1–7. The CH had the largest inhibitions zones against all bacterial strains (Figure 8). The inhibition zones of tested materials, 24 h following the incubation, were the largest with the CH against *Staphylococcus aureus* (16.67 ± 2.34 mm) followed by the ALBO-MPCA (14.67 ± 1.21 mm), and the weakest with the CS against *Enterococcus faecalis* (6.50 ± 1.76 mm). *Streptococcus anginosus* did not exhibit any growth after 1 h. The statistically significant differences were observed between the CH and other investigated materials with respect to: *Streptococcus anginosus* and *Enterococcus faecalis*; *Enterococcus faecalis* ATCC and *Streptococcus vestibularis*, except between CH (24 h following the incubation) and ALBO-MPCA (1 h following the incubation). The statistically significant

differences concerning antibacterial activity against *Staphylococcus aureus* were also registered between: the CH and MTA$^+$, in all observation periods; the CH and CS (3 h and 24 h following the incubation); the MTA$^+$ (3 h and 24 h following the incubation) and ALBO-MPCA (1 h and 3 h following the incubation).

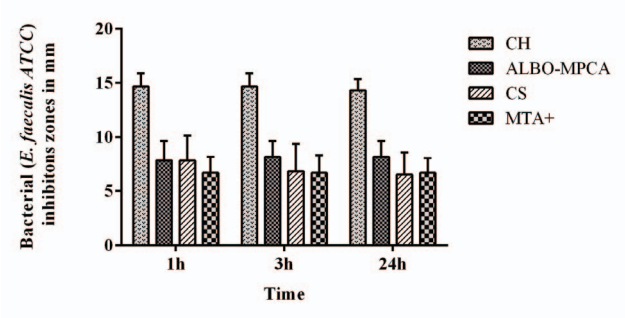

Fig. 1 – Inhibition zones of *Euterococcus faecalis* (ATCC14506) determined by the double layer agar diffusion test in different time periods.
CH- UltraCal XS; MTA- mineral trioxide aggregate, MTA$^+$; ALBO-MPCA- calcium silicate based material with Bi_2O_3; CS- calcium silicate based material without Bi_2O_3.

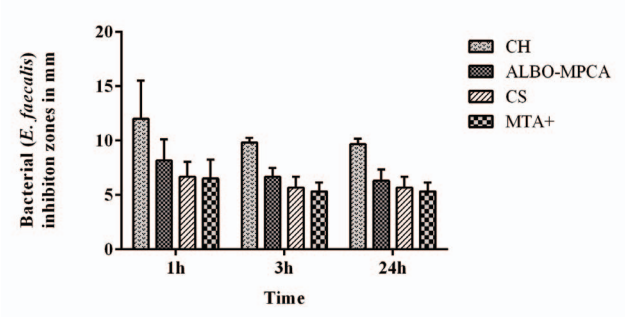

Fig. 2 – Inhibition zones of *Euterococcus faecalis* determined by the double layer agar diffusion test in different time periods.
CH- UltraCal XS; MTA- mineral trioxide aggregate, MTA$^+$; ALBO-MPCA- calcium silicate based material with Bi_2O_3; CS- calcium silicate based material without Bi_2O_3.

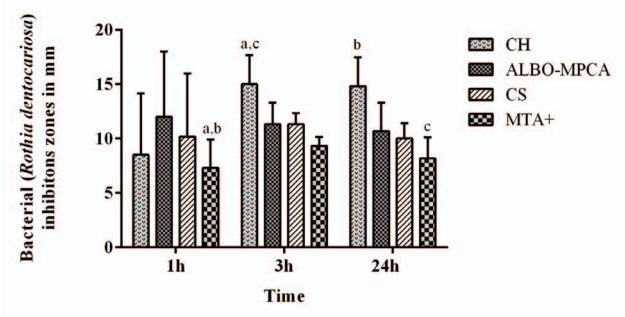

Fig. 3 – Inhibition zones of *Rothia dentocariosa* determined by the double layer agar diffusion test in different time periods.
CH- UltraCal XS; MTA- mineral trioxide aggregate, MTA$^+$; ALBO-MPCA- calcium silicate based material with Bi_2O_3; CS- calcium silicate based material without Bi_2O_3. Different small letters indicate the statistically significant differences between the tested materials ($p < 0.05$).

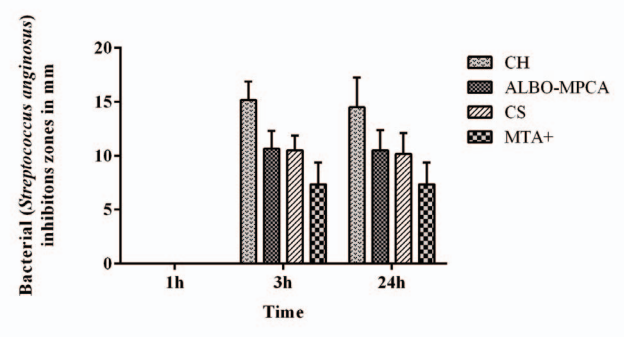

Fig. 4 – Inhibition zones of *Streptococcus anginosus* determined by the double layer agar diffusion test in different time periods.
CH- UltraCal XS; MTA- mineral trioxide aggregate, MTA$^+$; ALBO-MPCA- calcium silicate based material with Bi_2O_3; CS- calcium silicate based material without Bi_2O_3.

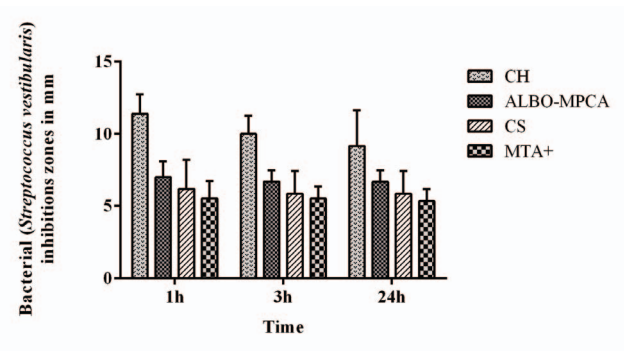

Fig. 5 – Inhibition zones of *Streptococcus vestibularis* determined by the double layer agar diffusion test in different time periods.
CH- UltraCal XS; MTA- mineral trioxide aggregate, MTA$^+$; ALBO-MPCA- calcium silicate based material with Bi_2O_3; CS- calcium silicate based material without Bi_2O_3.

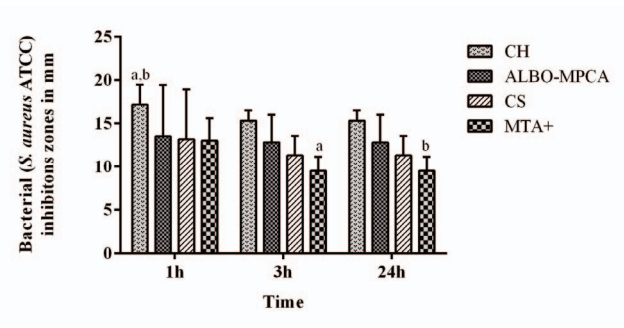

Fig. 6 – Inhibition zones of *Staphylococcus aureus* (ATCC 25923) determined by double layer agar diffusion test in different time periods.
CH- UltraCal XS; MTA- mineral trioxide aggregate, MTA$^+$; ALBO-MPCA- calcium silicate based material with Bi_2O_3; CS- calcium silicate based material without Bi_2O_3. Different small letters indicate the statistically significant differences between the tested materials ($p < 0.05$).

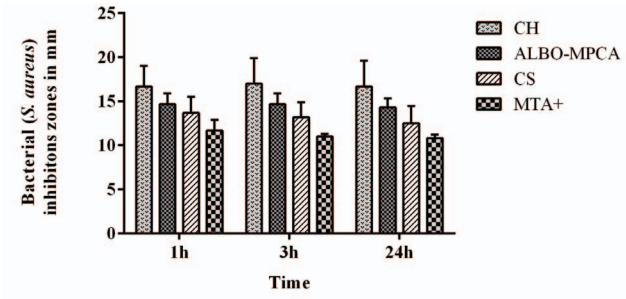

Fig. 7 – Inhibition zones of *Staphylococcus aureus* (in mm) determined by the double layer agar diffusion test in different time periods.
CH- UltraCal XS; MTA- mineral trioxide aggregate, MTA^+; ALBO-MPCA- calcium silicate based material with Bi_2O_3; CS- calcium silicate based material without Bi_2O_3.

The values of inhibitions zones decreased over time in most tested bacterial strains and incubation periods, but increased or remained in size in the certain cases: ALBO-MPCA against *E. faecalis* ATCC 14506 (8.17 ± 1.47); CH (14.83 ± 2.64) and MTA^+ (8.17 ± 1.94) against *Rothia dentocariosa*; and CH (16.67 ±2.94) against *S. aureus*. Although without observed statistical differences, the investigated materials in our study seem to show better antibacterial activity against clinical isolates in comparison to *S. aureus* ATCC 25923 strain, with an exception in case of the CH and the MTA^+ 1h following the incubation. On the contrary, the smaller inhibition zones concerning clinical isolates of *E. faecalis* were observed, then the referent strain.

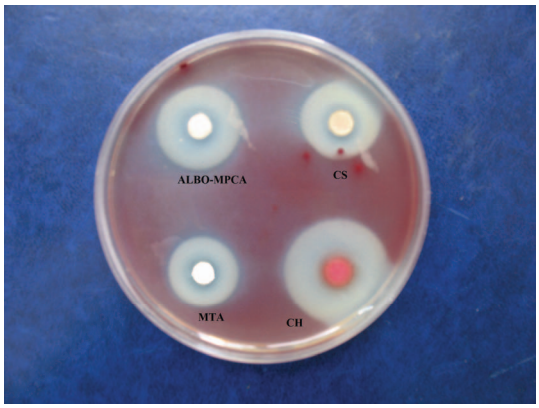

Fig. 8 – Representative inhibitions zones of a clinical isolate *Staphylococcus aureus* determined by the double layer agar diffusion test, after 1 hour.
CH- UltraCal XS; MTA- mineral trioxide aggregate, MTA^+; ALBO-MPCA- calcium silicate based material with Bi_2O_3; CS- calcium silicate based material without Bi_2O_3.

The mean pH values of investigated materials are presented in Table 1. All of them acquired the pH values above 11, with an increasing trend during time, except in the case of the MTA^+. The pH values for the MTA^+ were the lowest (8.23 ± 0.01), but still alkaline.

Table 1

Mean values and standard deviations of pH in different time intervals

Materials	1 h	3 h	24 h
CH	12.42 ± 0.01	12.35 ± 0.06	12.40 ± 0.01
ALBO-MPCA	11.54 ± 0.01	11.70 ± 0.01	12.13 ± 0.15
CS	11.19 ± 0.01	11.30 ± 0.01	11.75 ± 0.01
MTA^+	10.68 ± 0.01	9.04 ± 0.01	8.23 ± 0.01

Note: There are no statistically significant differences among tested materials ($p > 0.05$).
CH – UltraCal XS; ALBO-MPCA – albo-mineral polyoxide carbonate aggregate; CS – calcium silicates; MTA – mineral trioxide aggregate; h – hour.

Table 2

Cumulative ion release (mean value) by the investigated materials into deionized water (ppb)

Materials*	Time	Al	Ca	K	Mg	Na	P	Si
MTA^+	1 h	755	44,570	1,997	161	1,775	5	0
	3 h	2,144	80,900	2,506	338	3,233	8	615
	24 h	4,164	115,900	3,313	473	4,548	11	4,546
CS	1 h	255	25,820	11	423	6,356	5	22
	3 h	1,441	67,870	345	728	7,696	9	104
	24 h	2,101	118,780	702	895	8,452	10	3,520
ALBO-MPCA	1 h	936	25,820	3,859	170	1,657	4	717
	3 h	1,437	62,890	5,354	759	10,605	11	2,792
	24 h	3,644	131,990	7,003	1,510	19,867	12	12,556
CH	1 h	0	46,100	940	207	1,498	0	0
	3 h	0	92,820	1,074	383	2,620	0	0
	24 h	0	145,610	1,333	618	4,437	0	0

***For abbreviations see under Table 1; ppb- parts per billion.**

Table 2 represent ion releases by investigated materials into deionized water. The calcium ion release increased over time with regard to all tested materials, except the MTA^+, where the release kept declining. Unlike the cumulative aluminium ion release (MTA^+>ALBO-MPCA>CS>CH), the values for the cumulative release of calcium were as follows: CH>ALBO-MPCA>CS>MTA^+. Although weaker antibacterial performance, AMBO-MPCA had multiple larger potassium, magnesium and sodium ion release compared to CH.

Discussion

The ADT is a widely used method for the determination of antibacterial activity of soluble materials. The results obtained by this method may depend on solubility of tested materials, their ability to diffuse in agar and cell medium [16]. The diffusion ability of materials may be influenced by numerous factors, such as: agar type, contact between material and agar, molecular mass, size and form of antibacterial agent, load and concentration of tested material, agar viscosity, ion concentration in relation to medium, used microorganisms, agar quantity, incubation time, etc. [16]. One of the major limitations of the ADT method is that it is not capable of determining whether material possesses bacteriostatic or bactericidal effect [17].

So far, many researchers reported conflicting results on the antibacterial effects of a range of sealers and their different forms, weather they were freshly mixed or completely set [16, 18]. Nevertheless, sealers may have the ability to release constituents with the antibacterial effects even after their complete setting [19]. Since the sealing materials are commonly applied freshly mixed in everyday clinical practice, in our study we investigated the antibacterial effects of materials in such a form. We left Petri dishes for 2 h at the room temperature to rest in order to achieve prediffusion of the tested materials, which is an important step in demonstrating the antibacterial effects, as previously observed [20]. Optimization with 0.05% TTC was performed in order to differentiate the exact growth of bacterial colonies [20]. Special attention was paid to pre-diffusion and optimization with the (TTC) 2, 3, 5-triphenyltetre zoliumchloride procedures, which allowed us to precisely determine zones of bacterial growth inhibition, and avoid possible misinterpretation with a diffusion capacity of the materials.

Up to now, little attention has been devoted to investigation of antibacterial efficacy of materials similar to MTA against bacterial clinical isolates [21]. With regard to that, a major part of our experiment was conducted using the clinical isolates collected from the infected teeth of patients. Since similar materials previously showed the highest and the lowest antibacterial effects against *E. faecalis* and *S. aureus* [10, 22], in our experiment, we compared the results of ADT using the clinical and ATCC strains of the same two important bacteria. Though without statistical differences and with exceptions in cases of the CH and MTA^+ following 1h of incubation, the investigated materials in our study appeared to possess better antibacterial activity against clinical isolates in comparison to *S. aureus* ATCC strain(s).

The antimicrobial activity of calcium hydroxide based sealers is linked to the release of hydroxyl ions as strong free radicals, and the capacity to absorb carbon dioxide [23]. The similar mechanism may be proposed for the MTA based materials, taking into account their setting process [8]. It is quite familiar that the pH values above 12 inhibit the growth of many microorganisms, including *E. faecalis* [24]. Despite the high pH, even 7-day period time appears to be insufficient for CH pastes to kill bacterial cells in biofilm [25]. Limited antibacterial efficacy against *E. faecalis* for calcium hydroxide based sealers which have pH beyond 12, put this particular bacteria in closer scope. Evens et al. [6] suggest that the main reason for *E. faecalis* resistance lies in proton pumps that exist in its cell membrane. Our results are in agreement that the material solo property extremely high pH value was not sufficient and that apart from it, some other factors also interfere with bacterial growth.

All materials tested in our study had the highest antibacterial effects against *S. aureus* and the lowest against *E. faecalis,* which is in accordance with results of some previous studies [10, 22]. In addition, the MTA based materials may also fail to inhibit the growth of *E. faecalis* [26], but inhibited the growth of caries-associated bacteria [27, 28]. An increase in inhibition zones exceeding 10%, between 1h and 24h, was observed in the case of CH and MTA^+ against *E. faecalis* ATCC 14506, and in the case of CH, MTA^+ and CS against *Rothia dentocariosa*. Our results obviously do not support previously reported ones stating that an increase in duration of incubation leads to a decrease in effectiveness of tested materials [22], which is probably due to differences in the methodology applied (the authors of this study compared 24 hours and 7 days samples), chemical composition of tested materials and bacterial strains. It is known that the CH is formed during hydration reaction of the MTA based materials, but for the complete maturation of different phases the time should be sufficient [8]. This might be a possible reason for acquiring conflicting results, in addition to the fact that the measured pH values may not necessary match the ones achieved during the complex process of MTA setting and thus do not depict *in vivo* conditions.

Tanomaru-Filho et al. [10] showed that the MTA based materials possess the antimicrobial activity against *S. aureus* and *E. faecalis,* although the sealers based on zinc oxide and eugenol made larger inhibition halos. Asgary and Kamrani [29] also tested antibacterial activity of gray GMTA and WMTA, CH and a new endodontic cement (NEC) on the same bacteria species and confirmed the antibacterial activity of all tested materials, with significant differences observed between the CH and NEC in comparison to the MTAs. The conclusions reported by Holt et al. [30] and Sipert et al. [31] were similar, beside that the antibacterial activity may be increased by the aerobic conditions (created by inducing reactive oxygen species) [32]. In contrast to the previous studies, Yasuda et al. [33] and Miyagak et al. [34] concluded that the ProRoot MTA had no antimicrobial activity against any investigated species (*S. aureus, E. faecalis, C. albicans, S. mutans* and *S. sanguinis*), while the AH plus exhibited the highest antimicrobial activity out of all tested materials.

Previous studies showed that aluminium ions possess antibacterial effects [35]. Investigated material MTA$^+$ showed largest aluminum cumulative ion release. Regarding the correlation between aluminium ion release and antibacterial effects, results of our study seem to be not enough conclusive, meaning that the individual impact of other factors had to be further investigated. The CH showed highest cumulative calcium ion release after 24h (145610 ppb), and though an initial calcium release was high with respect to the MTA$^+$ (44570 ppb), it declined over time, but only in the case of this material. The CH also exhibited the smallest sodium cumulative ion release. The above stated information contributes to understanding their antibacterial efficacy and longevity. While sodium is a vital nutrient for many oral *Streptococci,* calcium is alkaline metal with relatively high atomic mass which diffuses slowly [36].

Conclusion

Calcium hydroxide pastes have been considered as a "golden standard" for the treatment of immature teeth for decades, but the risk of tooth fractures, potential reinfections, incomplete calcifications difficulties and consequently therapy duration remains. Considering the fact that materials based on nano-structured highly active calcium silicates possess the favourable physicochemical properties, biocompatibility and as shown in this study, express the satisfactory antibacterial effects, they are the effective therapeutic agents for root canal obturation in one-visit apexification treatment and thus significantly decrease duration of therapy. The microbiological properties of new-age nano-structured highly-active materials CS and ALBO-MPCA suggest further investigations in clinical aspect and they may substitute the MTA materials in dental medicine of the future.

Acknowledgements

This study was founded by the Ministry of Education, Science and Technological Development of the Republic of Serbia (project No: 172026 and TR31079).

R E F E R E N C E S

1. *Vera J, Siqueira JF Jr, Ricucci D, Loghin S, Fernández N, Flores* B et al. One-versus two-visit endodontic treatment of teeth with apical periodontitis: a histobacteriologic study. J Endod 2012; 38(8): 1040−52.
2. *Endo MS, Ferraz CC, Zaia AA, Almeida JF, Gomes BP.* Quantitative and qualitative analysis of microorganisms in root-filled teeth with persistent infection: monitoring of the endodontic retreatment. Eur J Dent 2013; 7(3): 302−9.
3. *Peciuliene V, Reynaud AH, Balciuniene I, Haapasalo M.* Isolation of yeasts and enteric bacteria in root-filled teeth with chronic apical periodontitis. Int Endod J 2001; 34(6): 429−34.
4. *Heyder M, Kranz S, Völpel A, Pfister W, Watts DC, Jandt KD* et al. Antibacterial effect of different root canal sealers on three bacterial species. Dent Mater 2013; 29(5): 542−9.
5. *Mohammadi Z, Dummer PM.* Properties and applications of calcium hydroxide in endodontics and dental traumatology. Int Endod J 2011; 44(8): 697−730.
6. *Evens M, Davies JK, Sundqvist G, Fridor D.* Mechanisms involved in the resistance of Enterococcus faecalis to calcium hydroxide. Int Endod J 2001; 35(3): 221−8.
7. *Cetenovic B, Markovic D, Petrovic B, Peric T, Jokanovic V.* Use of mineral trioxide aggregate in the treatment of traumatized teeth in children–Two case reports. Vojnosanit Pregl 2013; 70(8): 781−4.
8. *Camilleri J, Sorrentino F, Damidot D.* Investigation of the hydration and bioactivity of radiopacified tricalcium silicate cement, Biodentine and MTA Angelus. Dent Mater 2013; 29(5): 580−93.
9. Sisli SN, Ozbas H. Comparative Micro-computed Tomographic Evaluation of the Sealing Quality of ProRoot MTA and MTA Angelus Apical Plugs Placed with Various Techniques. J Endod 2017; 43(1): 147−51.
10. *Tanomaru-Filho M, Tanomaru JM, Barros DB, Watanabe E, Ito IY.* In vitro antimicrobial activity of endodontic sealers MTA-based cements and Portland cement. J Oral Sci 2007; 49(1): 41−5.
11. *Flores-Ledesma A, Barceló Santana F, Bucio L, Arenas-Alatorre JA, Faraji M, Wintergerst AM.* Bioactive materials improve some physical properties of a MTA-like cement. Mater Sci Eng C Mater Biol Appl 2017; 71: 150−5.
12. *Duarte MA, Alves de Aguiar K, Zeferino MA, Vivan RR, Ordinola-Zapata R, Tanomaru-Filho M* et al. Evaluation of the propylene glycol association on some physical and chemical properties of mineral trioxide aggregate. Int Endod J 2012;45(6): 1-6.
13. *Khalil I, Naaman A, Camilleri J.* Properties of Tricalcium Silicate Sealers. J Endod 2016; 42(10): 1529−35.
14. *Jokanovic V, Colovic B, Mitric M, Markovic D, Cetenovic B.* Synthesis and properties of a new endodontic material based on nanostructured highly active calcium-silicates and calcium carbonates. Int J Appl Ceram Techol 2014; 11(1): 57−64.
15. *Opacic-Galic V, Petrovic V, Zivkovic S, Jokanovic V, Nikolic B, Knezevic-Vukcevic J,* et al. New nanostructural biomaterials based on active silicate systems and hydroxyapatite: characterization and genotoxicity in human peripheral blood lymphocytes. Int Endod J 2012; 46(6): 506−16.
16. *Leonardo MR, Silva LA, Tanomaru-Filho M, Bonifacio KC, Ito IY.* In vitro evaluation of antimicrobial activity of sealers and pastes used in endodontics. J Endod 2000; 26(7): 391−4.
17. *Zhang H, Pappen FG, Haapasalo M.* Dentin enhances the antibacterial effect of Mineral Trioxide Aggregate and BioAggregate. J Endod 2009; 35(2): 221−4.
18. *Eldeniz AU, Hadimli HH, Ataoglu H, Orstavik D.* Antibacterial effect of selected root end filling materials. J Endod 2006; 32(4): 345−9.
19. *Vivan RR, Zapata RO, Zeferino MA, Bramante CM, Bernardineli N, Garcia RB,* et al. Evaluation of the physical and chemical properties of two commercial and three experimental root-end filling materials. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010; 110(2): 250−6.
20. *Begue WJ, Kline RM.* The use of tetrazolium salts in bioautographic procedures. J Chromatogr 1972; 64(1): 182−4.
21. *Lovato KF, Sedgley CM.* Antibacterial activity of endosequence root repair material and ProoRoot MTA against clinical isolates of Enterococcus faecalis. J Endod 2011; 37(11): 1542−6.
22. *Mohammadi Z, Giardiono L, Palazzi F, Shalavi S.* Antibacterial activity of a new mineral trioxide aggregate-based root canal sealer. Int Dent J 2012; 62(2): 70−3.

23. *Kim D, Kim E*. Antimicrobial effect of calcium hydroxide as an intracanal medicament in root canal treatment: a literature review-Part I. In vitro studies. Restor Dent Endod 2014; 39(4): 241−52.
24. *McHung CP, Zhang P, Michalek S, Eleazer PD*. pH required to kill Enterococcus faecalis in vitro. J Endod 2004; 30(4); 218−9.
25. *Zancan RF, Vivan* RR, *Milanda Lopes MR, Weckwerth PH, de Andrade FB, Ponce JB*, et al. Antimicrobial Activity and Physicochemical Properties of Calcium Hydroxide Pastes Used as Intracanal Medication. J Endod 2016; 42(12): 1822−8.
26. *Kim* RJ, *Kim MO, Lee KS, Lee DY, Shin JH*. An in vitro evaluation of the antibacterial properties of three mineral trioxide aggregate (MTA) against five oral bacteria. Arch Oral Biol 2015; 60(10): 1497−502.
27. *Bhavana V, Chaitanya KP, Gandi P, Patil J, Dola B, Reddy RB*. Evaluation of antibacterial and antifungal activity of new calcium-based cement (Biodentine) compared to MTA and glass ionomer cement. J Conserv Dent 2015; 18(1): 44−6.
28. *Özyürek Tand, Demiryürek EÖ*. Comparison of the antimicrobial activity of direct pulp-capping materials: Mineral trioxide aggregate-Angelus and Biodentine. J Conserv Dent 2016; 19(6): 569−72.
29. *Asgary S, Kamrani FA*. Antimicrobial effect of five different root canal sealing materials. J Oral Sci 2008; 50(4): 469−4.
30. *Holt DM, Watts D, Beeson TJ, Kirkpatrick TC, Rutledge RE*. The anti-microbial effect against enterococcus faecalis and the compressive strength of two types of mineral trioxide aggregate mixed with sterile water or 2% chlorhexidine liquid. J Endod 2007; 33(7): 844−7.
31. *Sipert CR, Hussne RP, Nishiyama CK, Torres SA*. In vitro antimicrobial activity of Fill Canal, Sealapex, Mineral Trioxide Aggregate, Portland cement and EndoRez. Int Endod J 2005; 38(8): 539−43.
32. *Ribeiro CS, Scelza MF, Hirata JR, de Oliveira LM*. The antimicrobial activity of gray-colored mineral trioxide aggregate (GMTA) and white-colored MTA (WMTA) under aerobic and anaerobic conditions. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010; 109(6): e109−12.
33. *Yasuda Y, Kamaguchi A, Saito T*. In vitro evaluation of the antimicrobial activity of a new resin-based endodontic sealer against endodontic pathogens. J Oral Sci 2008; 50(3): 309−13.
34. *Miyagak DC, de Carvalho EM, Robazza CR, Chavasco JK, Levorato GL*. In vitro evaluation of the antimicrobial activity of endodontic sealers. Braz Oral Res 2006; 20(4): 303−6.
35. *Mukherjee A, Sadiq IM, Prathna TC, Chandrasekaran N*. Antimicrobial activity of aluminium oxide nanoparticles for potential clinical applications. Formatex Res Center 2012; 1: 245−51.
36. *D'Amato RF, Thornsberry C*. Calcium and Magnesium in Mueller-Hinton Agar and Their Influence on Disk Diffusion Susceptibility Results. Curr Microbiol 1972; 2(3): 135−8.

Received on December 31, 2016.
Revised on April 29, 2017.
Accepted on June 28, 2017.
Online First September, 2017.