Antibacterial effects of new endodontic materials based on calcium silicates

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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 phoxide 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, Staphylococcus aureus, Streptococcus anginus 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.

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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. More than 99.5% of Gram-positive bacteria, is eliminated by a proper chemo-mechanical root canal treatment. Residual microorganisms, particularly Enterococcus and Streptococcus species, are considered to be responsible for the treatment failure. Moreover, Enterococcus faecalis has the ability to bind with the collagen fibers and survive up to 12 months in the environment without the substrate. 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.

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 periradicular irritations, it is preferable for sealing materials to possess antibacterial activity.

So far, the sealers based on calcium hydroxide proved to be the most efficient against a range of pathogenic microorganisms. 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. However, it is also proved that the calcium hydroxide based sealers have a limited antimicrobial effect on Enterococcus faecalis.

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. MTA is composed of hydrophilic particles which, in the presence of water, form a colloidal gel that is transformed into solid cement. When mechanically mixed, MTA based materials achieve better marginal adaptation, and consequently possess better sealing property. The high pH value achieved during the setting suggests a potential antibacterial activity of the material. 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. Incorporation of the hydrosoluble polymer can reduce dry consistency of MTA based materials and thus to improve the material handling. Several attempts were made to improve the MTA manipulation characteristics which complicate its use during the orthograde canal filling procedures. 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.

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 UltraCal XS (CH).

Methods

The study was carried out at the University of Belgrade: Faculty of Veterinary Medicine and Institute of Nuclear Sciences “Vinca”, 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 thiglycollate broth. Needle samples were cultured aerobically and anaerobically after 37°C overnight to determine demand for obligatory anaerobiosis. Preliminary identification of the bacterial colonies from the anaerobic conditions was done by the Gram stain, hemolysis on sheep blood (COS, bioMérieux, Marcy-l’Étoile, France) and incubated in a jar under the anaerobic conditions using GasPack (GasPak™ EZ Gas Generating Container Systems, 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 chitidogosaccharide (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.

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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. Briefly, calcium silicate phases, 2β-CaSiO₄ (β-CS) and Ca₃SiO₅ (C₃S), were synthesized using stoichiometric quantities of CaCl₂ × 5H₂O and silica sol by hydrothermal treatment, in the following ratio: C₃S : C₂S = 2 : 1. Al(C₂H₅O₂)₃ was added to allow the production of an active C₂A phase. Calcium chloride tetrahydrate was used as the precursor for production of CaCO₃, while sulfonyl dodecyl sulfate was added as an antiagglomeration agent. The final mixture was made by mixing CaCO₃ with calcium silicate phases (C₃S and β-CS) in the case of CS, while the monoclinic Bi₂O₃ was added in case of ALBO-MPCA as a radiopaque agent.

As the control materials, mineral trioxide aggregate (MTA⁷, Cercedam, 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 10⁸ (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 sixtriplicate, 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 bifaralate (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 (3.76 ± 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 inhibition 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).

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**Fig. 1** – Inhibition zones of *E. 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\(_2\)O\(_3\); CS- calcium silicate based material without Bi\(_2\)O\(_3\).

**Fig. 2** – Inhibition zones of *E. 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\(_2\)O\(_3\); CS- calcium silicate based material without Bi\(_2\)O\(_3\).

**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\(_2\)O\(_3\); CS- calcium silicate based material without Bi\(_2\)O\(_3\). Different small letters indicate the statistically significant differences between the tested materials (\(p < 0.05\)).

**Fig. 4** – Inhibition zones of *S. 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\(_2\)O\(_3\); CS- calcium silicate based material without Bi\(_2\)O\(_3\).

**Fig. 5** – Inhibition zones of *S. 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\(_2\)O\(_3\); CS- calcium silicate based material without Bi\(_2\)O\(_3\).

**Fig. 6** – Inhibition zones of *S. 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\(_2\)O\(_3\); CS- calcium silicate based material without Bi\(_2\)O\(_3\). Different small letters indicate the statistically significant differences between the tested materials (\(p < 0.05\)).
The values of inhibitions zones decreased over time in most tested bacterial strains and incubation periods, but increased or remained in size in 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.

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.

| 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    | 25,200 | 38.1  | 207  | 1,198 | 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.

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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\textsuperscript{1}, where the release kept declining. Unlike the cumulative aluminium ion release (MTA\textsuperscript{1}→ALBO-MPCA→CS→CH), the values for the cumulative release of calcium were as follows: CH→ALBO-MPCA→CS→MTA\textsuperscript{1}. 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\textsuperscript{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.\textsuperscript{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\textsuperscript{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\textsuperscript{16, 17}. Nevertheless, sealers may have the ability to release constituents with the antibacterial effects even after their complete setting\textsuperscript{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 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.\textsuperscript{10} showed that the MTA based materials possess the antimicrobial activity against \textit{S. aureus} and \textit{E. faecalis}, although the sealers based on zinc oxide and eugenol made larger inhibition halos. Asgary and Kamrani\textsuperscript{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.\textsuperscript{30} and Sipert et al.\textsuperscript{31} were similar, beside that the antibacterial activity may be increased by the aerobic conditions (created by inducing reactive oxygen species)\textsuperscript{32}. In contrast to the previous studies, Yasuda et al.\textsuperscript{33} and Miyagak et al.\textsuperscript{34} concluded that the ProRoot MTA had no antimicrobial activity against any investigated species (\textit{S. aureus}, \textit{E. faecalis}, \textit{C. albicans}, \textit{S. mutans} and \textit{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. Investigated material MTA showed largest aluminium 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.

**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 apicification 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.

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