Title: Influence of the addition of different radiopacifiers and bioactive nano-hydroxyapatite on physicochemical and biological properties of calcium silicate based endodontic ceramic

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Abstract

The purpose of this study was to investigate the influence of different radiopacifiers on the physicochemical and biological properties of novel calcium silicate based endodontic ceramic enriched with bioactive nano-particulated hydroxyapatite – ECHA. Namely, ECHA was used as a basis for mixing with the following radiopacifiers: strontium fluoride (SrF_2), zirconium dioxide (ZrO_2) and bismuth oxide (Bi_2O_3). For comparison, Portland cement (PC) and mineral trioxide aggregate (MTA) were used. The following physicochemical characteristics were examined: the radiopacity, setting time, compressive strength, porosity, wettability and pH value. The biocompatibility of the cements was assessed by crystal violet, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and cell adhesion assays. The highest radiopacity was obtained for the ECHA+Bi_2O_3 mixture and MTA that were statistically significant in comparison to other materials ($p<0.05$). Both initial and final setting times as well as compressive strengths were statistically lower for experimental cements than for PC and MTA ($p<0.05$). The lowest total porosity was observed in the ECHA+ZrO_2 group when compared with the other two experimental cements ($p<0.05$), but not when compared with PC and MTA ($p>0.05$). Experimental cements exhibited statistically higher contact angles of glycerol than PC and MTA ($p<0.05$). For blood plasma, a statistical difference was found only between ECHA+Bi_2O_3 and PC ($p<0.05$). All investigated materials had alkalization ability. Cell viability assays revealed that the extracts of tested cements did not exhibit cytotoxic effect on L929 cells. Scanning electron microscopy had shown a high degree of cell proliferation and adhesion of cells

from apical papilla on experimental cements' surfaces. Novel endodontic ceramics with nano-hydroxyapatite addition have satisfactory biological and physicochemical properties when compared to MTA and PC controls. Considerable lower setting time of experimental cements might present a huge advantage of these synthesized materials in clinical practice. SrF_2 presents a novel promising radiopacifying agent for dental cements manufacturing.

Keywords: endodontic surgery, strontium fluoride, bismuth oxide, zirconium dioxide, radiopacifier, hydroxyapatite.

1. Introduction

The invention of calcium silicate (CS)-based endodontic ceramic (EC) has led to great progress in treatment of tooth diseases in endodontics and root-end surgery [1]. The first CS-based dental EC – mineral trioxide aggregate (MTA) was developed at Loma Linda University in the early 1990s, received acceptance by the US Food and Drug Administration (FDA) and commercialized as ProRoot MTA by Tulsa Dental, OK, USA [2]. It is composed of type 1 ordinary Portland cement (PC) with a fineness in the range of 4500–4600 cm^2/g and bismuth oxide (Bi_2O_3) added for radiopacity, in 4:1 proportion [3]. The main advantages over previously used pulp capping, retrograde root canal filling and perforation repair materials, i.e. calcium hydroxide ($Ca(OH)_2$) and super ethoxybenzoic acid (Super EBA), are its bioactivity, increased mechanical resistance and low solubility [4,5]. However, MTA showed several drawbacks such as long setting time, decreased biocompatibility owing to presence of Bi and poor handling properties that cause discomfort to the practitioner and patient [5]. Attempts have been made to circumvent some of these downsides by replacing Bi_2O_3 with alternative radiopacifiers such as barium sulphate ($BaSO_4$) [6,7], titanium dioxide (TiO_2) [6], gold (Au) [6,7], calcium tungstate ($CaWO_4$) [8,9], zirconium dioxide (ZrO_2) [8-10], ytterbium fluoride (YbF_3) [11], tantalum pentoxide (Ta_2O_5) [12] and niobium pentoxide (Nb_2O_5) [8,9,13,14].

Despite the huge progress attained in this realm, lowering the setting time of CS and finding the most appropriate radiopacifying agent remain a major issue when it comes to design an EC composition able to satisfy all

clinical needs. Recently, some products with short setting time have been developed in root-end surgery [15-17]. The problem with many radiopacifiers arises from the fact that their addition inhibits material setting reactions and bioactive behaviour [7]. Another undesirable issue related to the use of radiopacifiers is their possible toxicity in contact with human tissues [18]. Thus, the addition of radiopacifier has to be carefully balanced in order to obtain adequate radiopacity without negative influence to the CS's beneficial biological and mechanical characteristics. Our research group have demonstrated the satisfactory properties of two novel ECs formulations: one consisting of CS, nano-particulated hydroxyapatite (nano-hydroxyapatite, nHA) and $BaSO_4$ – ALBO $MPCA_1$ and another composed of CS, calcium carbonate ($CaCO_3$) and Bi_2O_3 – ALBO $MPCA_2$. Their physicochemical suitability and *in vivo* safety, after both acute and subchronic administration**,** are documented previously [19-25]. The beneficial effects of nHA addition into EC include decreased setting time, increased pH value, good biocompatibility and enhanced neutralization of the bacterial biofilm [11,19,26,27]. In previous experiments, ECHA was associated with $BaSO_4$ and YbF_3 as radiopacifiers leading to adequate physicochemical and biological characteristics [11,25]. However, the issues of solubility of $BaSO_4$ and biological safety of Yb are still under discussion [28,29].

The current paradigm in EC manufacturing advocates that the added radiopacifier has to be inert in contact with human tissues [3,4]. The present paper raises the question whether a bioactive radiopacifier can be added into bioactive ceramics for improvement of regenerative capacity of the resulting mixture. It is hypothesised that strontium fluoride (SrF_2) may simultaneously

serve as a promising radiopacifying and biologically desirable agent in newly formulated EC. The hypothesis takes into account positive proofs of osteoproliferative and odontoproliferative effects of strontium (Sr) [30]. In addition, fluoride incorporation into ECs has contributed to their advantageous properties [11,31]. The assumption is also rooted in the observations that Sr stimulates bone formation and angiogenesis, inhibits cell differentiation and activity of osteoclasts and evokes human dental pulp stem cells by promoting their odontogenic differentiation, proliferation and mineralization [32,33]. The aim of this study was to investigate the influence of the following radiopacifiers on physicochemical and biological properties of nHA-enriched endodontic ceramic: SrF_2, as potentially bioactive radiopacifier, ZrO_2, as biologically inert ceramic material and Bi_2O_3, commonly used in many CS formulations.

2. Materials and methods

2.1 Synthesis of calcium silicates containing nano-hydoxyapatite

EC with the addition of bioactive nHA (ECHA) was used as a basis for mixing with radiopaque agents. For synthesis of CS-based EC, calcium chloride pentahydrate ($CaCl_2 \cdot 5H_2O$) (Merck, Germany) and silica sol obtained by hydrothermal treatment were used. The stoichiometric quantities of $CaCl_2 \cdot 5H_2O$ (42.41 g) and silica sol (15 g of 30 % sol solution), corresponding to the ratio tricalcium/dicalcium silicate C_3S/b-C_2S=2:1 (C=CaO, S=SiO_2, C_3S=3CaO·SiO_2, C_2S=2CaO·SiO_2), were used to obtain silicate active phase (CS). Aluminium acetate ($Al(CH_3OO)_3$) was added to the mixture to provide the production of a small amount (3.01 %) of active tricalcium aluminate (C_3A) phase (C=CaO,

A=Al_2O_3, C_3A=3CaO·Al_2O_3). The nHA was produced by a hydrothermal method from the shells of chicken eggs using a two solutions procedure: $Ca(OH)_2$ (solution 1) and diammonium hydrogen phosphate $(NH_4)_2HPO_4$ (solution 2). Detailed procedure of used CS and nHA synthesis is given in the Supplement 1 and elsewhere by Jokanović and colleagues [21,22]. The nHA was added into CS mixture using C_3S to C_2S ratio of 2:1 to produce the basis of each experimental endodontic ceramic mixture (ECHA). It was composed of 34 % of CS and 66 % of nHA.

2.2 Specimens preparation

Experimental cements were manufactured by replacing 30 % of the cement powder by weight with the following radiopacifiers: SrF_2 (Sigma-Aldrich, St. Louis, Missouri, USA), ZrO_2 (Sigma-Aldrich) and Bi_2O_3 (Alfa Aesar, Karlsruhe, Germany). PC (Aalborg, Denmark) and MTA+ (thereafter referred to as MTA) (Cerkamed, Stalowa Wola, Poland) served as controls. All experimental cements and PC were hand-mixed at a powder/liquid ratio of 1 g cement/0.3 ml distilled water, while MTA preparation was performed in accordance with manufacturer's instructions. A glass mixing pad and stainless steel spatula were used for hand mixing. The specimens were made using polytetrafluoroethylene (PTFE) ring moulds incorporating a cavity of various internal diameter and height depending on the test performed. Moulds were filled to a level surface with mixed cement.

2.3 Radiopacity

The radiopacity of the specimens was determined according to ISO 6876 standard [34]. The specimens (8 mm in diameter and 1 mm thick) were

radiographed with a charge-coupled device (Trophy Radiology, Cedex, France) alongside an aluminium step-wedge, used as a reference. The step-wedge was made of aluminium plates graduated from 1-10 mm by 1 mm increments. The x-ray unit operated at 70 kV, 4 mA, distance of 33.5 mm and an exposure time of 0.077 s. Image J for Windows software (National Institutes of Health (NIH), Bethesda, MD, USA) was employed to calculate the grayscale values of each specimen and of each aluminium step-wedge thickness. The mean grey scale values of specimens were plotted against the number of aluminium steps and regressions were used to convert the grayscale values of the cements into millimetres of aluminium (mm Al) [35]. Five specimens per group were tested.

2.4 Setting time

Setting time was determined according to ISO 6876 standard using a Gilmore needle with a mass of 100±1 g and an active tip of 2±0.1 mm in diameter for measuring initial setting times and a needle with a mass of 400±1 g and active tip of 1±0.1 mm in diameter for measuring final setting times. The needles were vertically placed against the horizontal surface of the specimens (8 mm in diameter and 2 mm thick) every minute to observe indentations. The needles were cleaned between each test. The moment when the needle failed to create an indentation onto the surface of the material after being allowed to settle for 5 s was considered as an initial (100 g mass) and final (400 g mass) setting times. Three specimens per group were tested.

2.5 Compressive strength

The specimens (8 mm in diameter and 1 cm thick) were kept in phosphate buffer saline (PBS) (Sigma-Aldrich) for two weeks and thereafter

placed vertically on the lower steel plate of a universal testing machine (AUTOGRAPH AG-IS, Shimadzu, Kyoto, Japan). The upper plate was moving at a distance of 1 mm per 1 min. Specimens were subjected to compression loads until fracture. The fracture load was recorded and compressive strength was calculated in terms of megapascals (MPa) using the following formula:

$$C=4P/\pi D^2$$

where “P” represents the maximum load recorded in Newtons (N) and “D” represents the diameter of the specimens in millimeters. Five specimens per group were tested.

2.6 Porosity

The porosity of the cements was determined by scanning the specimens (5 mm in diameter and 1 mm thick) with a high-resolution micro-computed tomographic (μCT) system (SkyScan 1172 x-ray Microtomography; SkyScan, Kontich, Belgium) operated at 100 kV, 100 mA, an exposure time of 1150 ms with use of a copper/aluminium filter. Scanning was performed in 0.4º rotation steps, 10 μm isotropic resolution and 2048×2048 pixels per slice. The acquired images were reconstructed with NRecon v.1.6.9.8 software (SkyScan) using a ring artefact correction of 3 %, beam hardening correction of 40 % and no smoothing. Finally, images were analysed with CT.An 1.14.4.1 software (SkyScan) applying a global threshold of 23/255. The parameters calculated were as follows: porosity (total, open and closed), average pore size (μm) and the number of closed pores per volume ($1/mm^3$). Five specimens per group were tested.

2.7 Wettability

The cements' wettability was assessed with a contact angle (CA) analyser (Vinča Institute, Belgrade, Serbia). The CAs of the glycerol and human blood plasma were measured by placing the 2 µl liquid droplet on the specimens surface [36,37]. Since ISO 6876 does not stipulate the wettability measurements requirements, specimens measuring 10 mm in diameter and 2 mm in thickness provided enough space to succesfully place reference liquids on the cements' surfaces.The CAs were calculated using ImageJ software (NIH, Bethesda, MD, USA) by fitting the contour of the droplet placed on the surface (tangent method). Six specimens per group were tested.

2.8 pH determination

The cylinder specimens (4 mm in diameter and 6 mm thick) were immersed in distilled water and maintained at 37^{o}C for 30 min, 1 h, 3 h, 6 h, 12 h, 24 h and 7 days. At each time point, specimens were removed from the flasks and put into a new flask with 7 ml distilled water. The pH of the solutions was measured using a calibrated pH meter (Hanna precision pH meter Model pH 211, Sigma-Aldrich). Five specimens per group were tested.

2.9 Cell viability analysis

Preparation of the materials extracts. Cell viability was carried out in accordance with the ISO Standard 10993-5/2005 [38]. All materials were manipulated under sterile conditions. Immediately after mixing, materials were placed into pre-sterilized PTFE moulds (5 mm in diameter and 3 mm thick) to set for 24 h in a humidified atmosphere. Subsequently, discs were sterilized by ultraviolet irradiation for 2 h, then immersed in 1 ml complete medium –

Dulbecco's modified Eagle medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 5 % fetal bovine serum (FBS), 2 mM L-glutamine and penicillin/streptomycin (all from Capricorn Scientific, Ebsdorfergrund, Germany) and incubated for 24 h at 37°C. Subsequently, the discs were discarded and the supernatants (extracts) were collected. To prepare eluents for treatment, extracts were diluted with complete culture medium which was used for cultivation of control/non-treated cells.

Cell culture and treatment. The mouse fibroblast L929 cell line (European Collection of Animal Cell Cultures, Salisbury, UK) was cultivated in complete medium and maintained at 37°C, in a humidified atmosphere with 5 % CO_2. Cells were prepared for experiments using the conventional trypsinization procedure with trypsin/EDTA and seeded in 96-well flat-bottom plates (5×10^3 cells/well) for the cell viability assessment. Cells were treated 24 h post-seeding with pure extract (1) and serial dilutions (1:2, 1:4, 1:8, 1:16 and 1:32 (v:v)). In order to evaluate a dose- and time-dependent response to potentially toxic soluble substances from investigated materials, cell viability was assessed after 24, 48 and 72 h treatment.

Cell viability assessment. The number of adherent cells and mitochondrial dehydrogenase activity was assessed using crystal violet (CV) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) tests, respectively. The CV assay is based on the inability of dead cells to remain adherent. After treatment, the adherent, viable cells were fixed with methanol and stained with 10 % CV solution for 15 min at room temperature. CV dye was dissolved in 33 % acetic acid after rigorous washing with water. MTT test

measures mitochondrial-dependent reduction of MTT to formazan by metabolically viable cells. MTT solution was added to the cell cultures in final concentration of 0.5 mg/ml and cells were incubated for an additional hour. Thereafter, the solution was removed and cells were lysed by dimethyl sulfoxide. The absorbance of dissolved CV dye, corresponding to the number of adherent (viable) cells and the conversion of MTT to formazan, corresponding to the number of cells with an active mitochondria were measured in automated microplate reader at 570 nm (Sunrise; Tecan, Dorset, UK). The results were presented as percentage of viability relative to untreated control cultures, considered as 100 % viable. The experiments were performed in triplicates.

2.10 Cell adherence on the materials' surface

The cell adhesion assay was performed in accordance with the approval of the Ethics Committee (School of Dental Medicine, University of Belgrade, number 36/19). The cell's attachment on apical dentine and cements surface was tested using human apical papilla stem cells as previously described [39]. Apical papilla tissue was obtained after signed informed consent from the patient (18 years of age) undergoing extraction of impacted third molar for orthodontic reasons. The tooth tissue was transferred into DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 20 % FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1 % antibiotic/antimycotic solution (Gibco, Thermo Fisher Scientific, Inc.). After 30 minutes of extraction, tooth tissue was rinsed in PBS and subjected to outgrowth isolation method. The specimens were prepared by filling up the root apical thirds of the upper central incisors with cements, as previously described [36]. Root specimens were obtained with

signed informed consent from the patient undergoing extraction of mandibular central incisors for periodontal reasons. Before seeding, the sterilized specimens were immersed in a growth medium in 12 well plate for 24 h, at 37°C in 5 % CO_2, avoiding the pH variation. The next day, the growth medium was discarded from wells containing root tops. Roots were positioned in the wells vertically with the apices up, enabling cells seeding on the roots' tops. On the apical tops of the roots filled with materials, 10 µl of medium with 1×10^4 cells was carefully placed. Plates containing roots with seeded apical papilla cells were cautiously placed in the CO_2 incubator for 30 min allowing cells to attach on the apical root surface, after which 500 µl of growth medium was added into wells. Half of specimens were further cultured in the growth medium, while the other half in the osteogenic medium containing growth medium supplemented with 10 nM dexamethasone disodium phosphate, 1.8 mM monopotassium phosphate, 10 mM β-glycerophosphate and 50 µg/ml ascorbic acid (Sigma-Aldrich). The cells were cultured in growth and osteogenic medium for 21 days with the mediums being changed every 3 days. The specimens with cells grown on their surfaces were fixed with 2 vol% glutaraldehyde, dehydrated with increasing concentrations of ethanol (50 vol%, 60 vol%, 70 vol%, 80 vol%, 90 vol%, 95 vol%, and 100 vol%) and gold coated before scanning electron microscopic (SEM) evaluation (TESCAN, Mira3, XMU USA Inc.).

2.11 Statistical analysis

The SPSS software program (ver. 20, IBM Corp., Armonk, NY, USA) was employed for statistical analysis. The Shapiro-Wilk test was used to check the normality of data distribution. Afterwards, one-way ANOVA with Bonferroni post-

hoc tests was employed to compare normally distributed data while Kruskal-Wallis test followed by series of pairwise Mann-Whitney tests with Bonferroni correction test was used to compare non-normally distributed outcomes ($p<0.05$).

3. Results

3.1 Radiopacity, setting time, compressive strength, porosity, wettability and pH determination

The Shapiro-Wilk test of normality found that the radiopacity, compressive strength, total porosity, open porosity, close porosity, pore size, number of closed pores per mm^3, CA of glycerol and human blood plasma and pH data for 30 min, 1 h, 3 h, 6 h, 12 h, 24 h and 7 days were normally distributed and thus they were subjected to one-way ANOVA analysis followed by Bonferroni test. On the contrary, initial and final setting time and pH values obtained after 24 h were not normally distributed and therefore they were subjected to a Kruskal-Wallis test followed by Mann-Whitney test with Bonferroni correction for non-parametric data ($p<0.005$). One way ANOVA have found statistical difference among tested cements' radiopacity, compressive strength, porosity (all parameters) and pH values obtained after 30 min, 1 h, 3 h, 6 h, 12 h and 7 days ($p<0.05$) while Kruskal-Wallis test showed significant difference among tested cements' initial and final setting times and pH values after 24 h evaluation ($p<0.05$).

The highest radiopacity values were observed for MTA (6.9±0.5 mmAl) and ECHA+Bi_2O_3 mixture (6.7±0.1 mmAl) that were significantly higher than

those in the other three groups of investigated cements ($p<0.05$), but without statistic difference among each other ($p>0.05$) (Figure 1).

All experimental cements showed statistically much lower initial ($p<0.05$) and final ($p<0.05$) setting times as well as compressive strengths ($p<0.05$) than PC and MTA (Table 1).

The lowest total porosity was found for ECHA+ZrO_2 (1.7±0.6 %) that was statistically different compared to the other experimental cements ($p<0.05$), but not statistically different from those of PC and MTA ($p>0.05$). The highest value of open porosity was obtained for ECHA+Bi_2O_3 (8±4 %) that was statistically significant in comparison to ECHA+ZrO_2 and MTA ($p<0.05$), but statistically insignificant in comparison to ECHA+SrF_2 and PC ($p>0.05$). The lowest value of closed porosity was found for ECHA+ZrO_2 (0.3±0.1 %) that was statistically different only when compared to ECHA+SrF_2 (3±2 %) ($p<0.05$). Regarding the pore size, there were no significant differences among the investigated cements ($p>0.05$). The ECHA+Bi_2O_3 group presented the greatest number of closed pores per mm^3 $(1.5±0.3)×10^7$ and it was statistically higher than in other groups ($p<0.05$), among which the statistical difference was not noted ($p>0.05$) (Figure 2).

Experimental cements exhibited statistically higher CA of glycerol than PC and MTA ($p<0.05$). Using blood plasma as a reference liquid, the statistical difference was found only between ECHA+Bi_2O_3 and PC ($p<0.05$), while other three investigated cements were not different neither among themselves nor from ECHA+Bi_2O_3 and PC ($p>0.05$) (Figure 2).

All investigated materials had alkalization ability (Table 1). Experimental cements revealed statistically lower pH values than PC and MTA after 30 min, 1 h, 3 h, 6 h and 12 h ($p<0.05$). On the other hand, there was no significant difference between experimental cements on one side and PC and MTA on the other side neither after 24 h ($p<0.005$) nor after 7 days ($p<0.05$).

3.2 Cytotoxicity and cell adherence assay

Effects of experimental cements' eluents on L929 cell viability are presented in Figure 3. Shapiro-Wilk test of normality revealed that results of CV assay after 24 h in pure extract, 1:2 and 1:4 eluents were not normally distributed and subsequently subjected to a Kruskal-Wallis test followed by Mann-Whitney test with Bonferroni correction for non-parametric data. All data obtained by MTT assay and the rest of the results obtained by CV assay were normally distributed and they were subjected to one-way ANOVA analysis followed by Bonferroni test. For CV assay, one-way ANOVA showed the statistical difference among tested cements after 24 h (1:32), 48 h (pure extracts, 1:2 and 1:4) and 72 h (pure extracts and 1:2) ($p<0.05$). Kruskal-Wallis test showed significant difference of cell viability among tested cements after 24 h (pure extract, 1:2 and 1:4 eluents) ($p<0.05$). For MTT assay, one-way ANOVA showed the statistical difference for all time points/dilutions ($p<0.05$) except after 48 h (1:8) and 72 h (1:8, 1:16 and 1:32) ($p>0.05$).

Pure extracts of experimental cements had no effect on the cell viability after 24 h of exposure, measured by CV (Figure 3A) and MTT (Figure 3B) assays. Although longer incubation (48h, 72h) with ECHA+ZrO_2 and ECHA+Bi_2O_3 extracts induced time-dependent decrease in cell viability, the

number of adherent cells (Figure 3A) and the activity of mitochondrial dehydrogenases (Figure 3B) were significantly higher than in cultures incubated with pure extracts of MTA and PC which showed extremely high cytotoxic effect (> 60-80 %) at all time points. Further, the presence of ECHA+SrF_2 mixture eluent in 1:2 dilution potentiated proliferation of L929 cells – the number of viable (Figure 3A) and metabolically active cells (Figure 3B) was statistically higher than in all other treatments with experimental materials. Yet, in 1:2 dilution, ECHA+ZrO_2 reduced cell viability after 48 h and 72 h, while ECHA+Bi_2O_3 manifested the same effect on cell viability after 72 h. The highest number of L929 cells (Figure 3A) and mitochondrial dehydrogenase activity (Figure 3B) was detected in cell culture treated with ECHA+SrF_2 and ECHA+ZrO_2 mixture eluent in 1:4 dilution at all time points, as well as in comparison with other dilutions. Higher dillutions of all tested extracts (1:8, 1:16 and 1:32) did not affect the L929 cell viability, at all time points.

SEM analysis of cell adhesion revealed that experimental cements had properties that were able to support apical papilla stem cells and osteoblasts adhesion and increase cell proliferation (100× magnifications). Extended cytoplasmic processes and filopodia were observed to spread between coronal dentine and cement material (2000× magnification) (Figure 4).

4. Discussion

The choice of radiopacifier capable of contributing to the radio-visibility of CS-based dental ceramics is important since they should meet both the ISO standard of radiopacity and maintain adequate biological and physical

properties of ECs. In the current study, novel ECs with incorporated nHA particles were used as a basis for mixing with radiopacifiers since it was previously demonstrated that the addition of such non-stochiometric bioactive nHA significantly decreased CS's setting time [11]. All radiopacifiers were able to confer EC's ISO radiopacity requirements of 3 mmAl. The highest radiopacity value was observed in ECHA+Bi_2O_3 (6.7 mmAl). This is expected as Bi absorbs x-rays much more efficiently than Zr and Sr due to its higher atomic number (Z(Bi)=83>Z(Zr)=40>Z(Sr)=38) as absorption of x-rays is directly proportional to the third power of the atomic number of absorbing material [40]. The radiopacity of ECHA+ZrO_2 (5.3 mmAl) was slightly higher than that found recently for CS with 30 % ZrO_2 added (4.2 mmAl) [9]. The radiopacity of ECHA+Bi_2O_3 (6.7 mmAl) was lower than that previously found for CS+30%Bi_2O_3 (~11 mmAl) [6], but close to that of CS+25%Bi_2O_3 (6.9 mmAl) [41]. The result demonstrated for radiopacity of MTA (6.9 mmAl) favourably agrees with those reported previously: 4.86 [42], 6.74 [43], 7.0 [44], 7.5 [9] and 8.0 [41] mmAl. The PC did not meet the ISO radiopacity requirement which is in line with previous studies (~0.9 mmAl) [41,42]. The influence of SrF_2 on the radiopacity of EC has not been previously mentioned in the literature.

Experimental cements have shown initial setting times varying from 5 to 8.3 min, significantly lower than those of PC and MTA (46 min and 41 min, respectively). The obtained results for experimental cements indicate their fast-setting property and corroborate the results found for fast-setting RetroMTA (2.5 min) [10], CS+$CaCO_3$+30%YbF_3 mixture (6 min) [11], Endocem (11.5 min) [40] and ECHA+30% YbF_3 mixture (14 min) [11]. Literature data reported for

MTA vary between 17.8 and 50 min for initial [9,42,43,46] and between 140 and 290 min for final setting times [43,47]. Fast-setting was achieved mainly by incorporation of nano-crystalline active nHA. Such desirable behavior is expected since lower particle size increases the active surface for cement reaction with water [4]. All experimental cements had low setting time values indicating that the choice of radiopacifying agent did not alter the nHA's capability to induce the fast setting of tested materials.

From a mechanical point of view, the compressive strength of tested cements (2.7-3.8 MPa) was considerably lower than that of MTA (23 MPa) and PC (27 MPa). The data observed for MTA was slightly lower than that of recent findings: 28 MPa [46], 33 MPa [48], 43 MPA [47] and 45 MPa [43] while that found for PC correlates with previous studies: 32 MPa [47] and 51 MPa [43]. The variation of results obtained in the present and other studies might be caused by the use of greater specimens size in the current research. Namely, specimens measuring 8mm×1 cm were rather employed than usually used 4×6 mm to adjust measurements with used universal testing machine force range. The reduced compressive strength of ECHA+Bi_2O_3 mixture is in compliance with a previous study where Bi_2O_3 was added into PC [48], but in contrast to another study where excluding/eliminating Bi_2O_3 from MTA resulted in lower compressive strength [49]. Significant decrease of the compressive strength of mixtures is presumably not to a great extent the consequence of radiopacifier addition, but rather related to the addition of nHA in substantial portion (>60 %). The nHA is an inert compound that affects the CS setting reaction by interposing among its particles. Low values of compressive strength

in experimental cements are therefore not surprising and they go in line with previous studies where some other compounds replaced CS particles at high percentage: $CaCO_3$ (60-80 %) in RetroMTA or ZrO_2 (43-46 %) in Endocem [10,45]. The consequences were similar since, for instance, the addition of low-reactive/inert ZrO_2 into CS decreased its compressive strength to 10.4 MPa in Endocem [45]. In the authors' opinion, lower values of compressive strength of experimental cements do not play a major role in the clinical practice since the cements are manufactured to fulfil the most important requirement in retrograde root-end surgery – fast setting time. The same holds true for porosity. Although it is known that cements' architectural properties are important, the challenge lies in the clinical interpretation of obtained porosity data. Namely, lower values of closed porosity (ECHA+ZrO_2) mainly refers to the mechanical resistance of materials whereas lower values of open porosity (ECHA+ZrO_2 and MTA) are more likely related to the ability of the material to prevent spreading of bacterial infection in the root canal system from the area where the bacteria are not totally eliminated during instrumentation [36]. It is observed that in all experimental mixtures open porosity is significantly higher than the closed one. The results compare moderately well with previously published values demonstrating the increase of CS porosity after HA addition as well as no influence of ZrO_2 addition on CS porosity [50]. Results for radiopacifier additions to ECHA are also consistent with those reported for ECHA+30%YbF_3 (5.86 %) [11]. The outcomes found for MTA total porosity coincide with some previous reports: 1.3 % [36], 6.5 % [51], 6.9 % [52] and 9 % [53], but are lower than those reported in other studies: 14.5 % [54] and 50 % [55]. The variability of the

outputs obtained arises from different setting parameters among studies the scanning resolutions of which vary from 5 μm to 20 μm [52]. For porosity evaluation by micro-CT, specimens' size is not defined by ISO standard. The specimens' size of 5×1 mm was preferred since it provided the possibility to obtain high resolution (i.e. visualization of smaller pore sizes) having in mind that the size of the specimen directly influences the maximum scanning resolution [51-55].

The wettability experiments demonstrated the advantage of SrF_2 over ZrO_2 and Bi_2O_3 for manufacturing EC due to the higher activity of the cement surface, which is expressed as a lower CA of glycerol. Indeed, ECHA+SrF_2 and ECHA+ZrO_2 resulted in superior wettability in comparison with MTA, PC and ECHA+Bi_2O_3 after glycerol administration. Glycerol was used since researchers have documented that it mimics closely the viscosity of human blood plasma [11]. The innovative approach in this study is the use of human blood plasma for CAs calculations; here, it is the first time in the EC dental field. The higher CAs after blood plasma administration compared to glycerol administration may be related to the presence of non-polar components in the plasma, presumably proteins [37]. The wettability of dental materials has been poorly investigated and very limited contributions can be found in the literature. The CAs reported in the literature for different CS brands are as follows: Endocem=12° [45], MTA=17° [36], Biodentine=22° [36] and 41° [56] using glycerol and CeraSeal=18° [57], BioRoot=34.8° [57], Endoseal MTA=35° [58] and Thera Cal=62.3° [59] using water as a reference liquid. There are no reports for CA values of blood plasma on EC. Wettability is an important property of dental

cement since it reflects the ability of the material surface to interact with surrounding biological tissues as well as the ability of body fluids to adhesively occupy the materials surface texture, i.e. osteoblasts in the interaction with cements surface irregularities [11]. Yet, higher wettability enhances the potential of the cement to penetrate into dentinal tubules [36].

Experimental cements revealed statistically lower pH values than PC and MTA up to 12 h of evaluation. However, after 24 h and especially after 7 days of soaking in distilled water their alkalinity matched the one found in PC and MTA, having the ECHA+ZrO_2 mixture even reaching the highest pH value after 7 days of evaluation. It is noteworthy that in PC and MTA the pH values remain relatively unchanged from 30 min to 24 h time points and then decrease after 7 days of soaking. Conversely, experimental cements presented the lowest pH values after 30 min, 1 h and 3 h and then resulted in stable increase of pH values during the remaining measurements. The alkaline nature of the experimental cements is the consequence of the release of calcium ions and the formation of $Ca(OH)_2$ [5]. The slow and stable release of calcium ions and consequently increase of pH value in experimental cements might be of pertinent interest in clinical practice in those situations where long term alkalinity is desirable for complete pulp/bone healing [60]. The source of $Ca(OH)_2$ is still debatable. Some authors state that it is the product of tricalcium aluminate hydrolysis while other claim that dicalcium and tricalcium silicate in reaction with water form tobermorite ($Ca_2[SiO_2(OH)_2]_2$) and $Ca(OH)_2$ [10,61,62]. Higher alkalinity of PC group solution is expected since it contains pure CS with higher percentage of calcium-releasing components. The specimens' size of 4×6 mm

was chosen since the ISO 6876 does not propose the procedure for pH measurements. However, both experimental cements and control PC/MTA materials were made in uniform size thus providing conditions for their comparison. The pH values observed in the literature for MTA are 12.5 [43] after 30 min, 12.8 [43] after 1 h, 9.09 [19] and 10.5 [9] after 3 h, 9.9 [9] after 12 h, 8.26 [19] and 9.3 [9] after 24 h and 10.3 [9] after 7 days. The values reported for PC corroborate with present findings having the values found after 30 min (13.1) and 1 h (13.1) [43] decreased to 10.24 after 12 h and 10.2 after 24 h [63]. The results found for ECHA+ZrO_2 are also in rough agreement to those observed for CS+ZrO_2 after 12 h – (10.0) [9] and (10.21) [63] as well as after 24 h – (9.0) [9] and (10.2) [63].

The effects of experimental cements, PC and MTA on cell viability/proliferation was evaluated on mouse fibroblast L929 cell line [38], since it is easily manipulated, provides reproducible results without the individual differences [64] and the most importantly, L929 cells are more sensitive to toxic products than other cell lines [65,66]. The last feature gives L929 cells advantage when it comes to evaluation of dental materials toxicity. It is especially important for EC to precisely determine their cytotoxic potential since, in clinical practice, they are placed in a direct contact with periapical tissue for many years [67].

Cytotoxicity analysis revealed that (biologically relevant) decrease in viability of L929 cells cultured with pure extract of ECHA+ZrO_2 and ECHA+Bi_2O_3 was detected only after 72 h, while ECHA+SrF_2 did not affect cell viability at all. Opposite from the results gained with experimental cements, the

PC and MTA pure extracts displayed a strong toxicity toward L929 cells in all time points. While ECHA+ZrO_2 eluent 1:2 reduced cell viability over time, 1:4 eluent showed proliferative potential. These results are in accordance with a recent study investigating the ZrO_2 addition to CS [9]. While the ECHA+Bi_2O_3 1:2 eluent reduced viability, 1:4 eluent showed excellent effect on L929 cell proliferation. As expected, effects of experimental cements and PC/MTA on cellular viability/proliferation diminished with dilution; no significant change in number of viable and metabolically active cells were detected in 1:8, 1:16, and 1:32 eluents. Presented results showed significantly lower percentage of viable cells in MTA/PC treatments than those found in some studies (80-150 %) [9,14,68,69], but they are in accordance with other reports found for MTA after 24 h (7-37 %) [58] and 72 h (20-80 %) [63,69] and findings reported for PC after 24 h (10-25 %) [69]. The differences could arise from variations in specimens size (5x3 mm [69,70], 5x2 mm [42], 5x1 mm [13], 10 mg/ml [68], 0.5g/5 ml [9] and 1g/50 ml [14]) and their form (powder vs. set materials). Specimens measuring 5×3 mm were used in the present investigation to allow comparison to other studies where the same specimen sizes were used [69,70]. For PC/MTA 1:2 and 1:4 eluents, the CV and MTT tests showed similar outcomes and are in rough agreement with data documented in the literature [9,70]. Overall, cytotoxicity experiments revealed that results obtained by CV and MTT assays are in concordance. Pure extracts of experimental cements revealed significantly lower cytotoxic potential than PC and MTA The ECHA+SrF_2 and ECHA+ZrO_2 1:4 eluents induced significant proliferation of L929 cells (>20 %) after all time points. Finally, it should be pointed out, that ECHA+SrF_2 mixture

had no toxic effect and showed the highest ability to induce the L929 cell proliferation among tested materials.

Further, since the number of cells needed for cell adhesion assay is considerably lower than necessitated for cytotoxicity assays, osteoblasts derived from human apical papilla stem cells were used for cell adhesion experiments. This experimental set up allows to analyze cells contact with tested materials *ex vivo* and their differentiation on these materials, thus providing more clinically relevant environment. Great osteoblasts adhesion on dentine and on the surface of specimens demonstrated by SEM evaluation contributes to cements' cytocompatibility and may be of special interest for efficient periapical healing after retrograde root canal surgery. Other studies have also documented that CS-based ECs are the excellent inductors of cell adhesion, migration and proliferation on the surface of experimental cements [11,42,71,72].

Regardless of advantages of cell-based methods, there are considerable limitations in mapping results gained in the cell culture to *in vivo* conditions, due to significant simplification of *in vitro* models compared to living systems. For example, freshly mixed cements are used in the clinic, while the set ones are commonly used in the cell-based assays. Similarly, in an *in vitro* system, it is not possible to determine whether tested materials maintain their cytotoxicity for a long period of time or if their initial toxic potential declines over time [73,74]. Having in mind previous, it is not possible to assume with high certainty that absence of cytotoxicity, superior proliferative potential and good cell adhesion properties of investigated materials showed in the present study can be

translated to *in vivo* system. However, obtained results undoubtedly represent a good starting point for further investigation of their benefits *in vivo*, and aftermath in long-term clinical studies.

The novelty of this study comprises the introduction of SrF_2 as a radiopacifier in CS-based EC. Sr may be, in traces, a normal constituent of dental tissues [75]. Sr incorporation into dental tissues is an endemic issue; it occurs in regions with high Sr concentration in drinking water [75]. In addition, it is a compound of dental dentifrices and restorative materials such as glass ionomer cements [76,77]. SrF_2 addition may be interesting for dental practitioners for two reasons. Firstly, Sr-associated EC formulations may be of special interest for periapical lesions treatment taking into account that investigators in orthopaedics have documented an abundance of promising evidences that Sr-enriched biomaterials accelerate the process of bone healing [32,33]. Additionally, researchers nowadays use Sr-doped HA to coat the surface of titanium implants in orthopaedic and dental surgery to increase their bioactivity [78]. Secondly, the potentially useful contribution of fluorides for strengthening of endodontically instrumented root canals and their ability to prevent bacterial growth is widely accepted [11]. For these reasons, investigators already addressed the possibility to dope CS with fluorides in the form of sodium fluoride or YbF_3 [11,31,79]. Current experiment served to document that CS-based EC doped with both Sr and F satisfies the basic physicochemical and biocompatibility needs. What deserves to be mentioned is that SrF_2 associated mixture was the only one of the investigated materials to meet ISO 10993-5 quantitative criteria of non-cytotoxicity (maximum reduction

of cell viability by more than 30 %) for both CV and MTT assays, for all time point and in all dilutions. In addition, lower CA values in ECHA+SrF_2 than in ECHA+ZrO_2 and ECHA+Bi_2O_3 mixtures after glycerol and human blood plasma testing favour the use of SrF_2 as the radiopacifying agent. Further detailed and more sophisticated biological studies should be conducted to acknowledge the true value of promising initial outcomes.

5. Conclusion

Collective results of this study underscore the relevance of nHA addition into CS-based dental ceramics. All investigated radiopacifiers were able to increase the radio-visibility of the ECHA to meet ISO requirements. Fast setting time and excellent cell adherence as well as good biocompatibility features indicated by mitochondrial activity and the number of viable cells present valuable advantages of manufactured formulations. Further, all experimental cements exhibited satisfactory wettability and porosity. The downside of fabricated materials is their low compressive strength. This study supports the idea that SrF_2 can be a novel promising radiopacifying agent for manufacturing CS-based dental ceramics. The full spectrum of SrF_2's beneficial effects in EC formulations should be investigated in future studies.

Acknowledges

This study was supported by the Ministry of Education and Science of the Republic of Serbia (Grant No. 451-03-68/2020-14/200017 and 451-03-9/2021-14/200007) and through a bilateral PPP grant provided jointly by the Ministry of Education, Science and Technological Development of the Republic of Serbia and Ministry of Science and Technology of The Peoples Republic of China (Grant No. 451-02-818/2021-09/20). The authors thank Aleksa Milovanović and Isaak Trajković for help with compressive strength evaluation analysis. The authors' thanks are also extended to Dr Vukoman Jokanović for his useful comments and discussions.

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		ECHA+SrF_2	ECHA+ZrO_2	ECHA+Bi_2O_3	PC	MTA
Setting time (min)	**Initial**	7.7±0.6 [a]	5±0 [b]	8.3±0.6 [a]	46±7 [c]	41±4 [c]
	Final	33±1 [a]	33±7 [a]	35.3±1.2 [a]	190±10 [b]	107.3±1.6 [c]
Compressive strength (MPa)		2.5±0.9 [a]	2.7±1.3 [a]	3.8±1.9 [a]	27±8 [b]	23±17 [b]
pH	**30 min**	7.50±0.02 [a]	8.5±0.3 [b]	7.60±0.05 [a]	10.73±0.02 [c]	10.76±0.02 [c]
	1 h	8.38±0.09 [a]	8.73±0.17 [b]	8.18±0.02 [a]	10.58±0.07 [c]	10.83±0.06 [c]
	3 h	8.97±0.03 [a]	8.98±0.12 [a]	8.92±0.03 [a]	10.70±0.07 [b]	10.99±0.06 [c]
	6 h	9.33±0.06 [a]	9.47±0.05 [a]	9.67±0.03 [a]	10.57±0.12 [b]	10.82±0.19 [b]
	12 h	9.36±0.02 [a]	9.40±0.02 [a]	9.62±0.06 [a]	10.76±0.07 [b]	11.2±0.3 [c]
	1 day	9.24±0.11 [a]	9.67±0.11 [a]	9.78±0.05 [a]	10.6±0.3 [a]	10.84±0.13 [a]
	7 days	8.95±0.03 [a]	9.4±0.3 [b,c]	9.08±0.04 [a,c]	8.94±0.01 [a]	9.15±0.08 [a,c]

Table 1. Initial and final setting times, compressive strength and pH values of investigated cements. Results with different letters within the same row are statistically different (one-way ANOVA with Bonferroni post-hoc test was used for compressive strength and 30 min, 1 h, 3 h, 6 h, 12 h and 7 days pH reported data ($p<0.05$), while Kruskal-Wallis test ($p<0.05$) followed by Mann-Whitney test with Bonferroni correction ($p<0.005$) was used to compare setting time and 1 day pH data). ECHA, endodontic ceramic nano-hydroxyapatite; ZrO_2, zirconium dioxide; Bi_2O_3, bismuth oxide; SrF_2, strontium fluoride; MTA, mineral trioxide aggregate; PC, Portland cement.

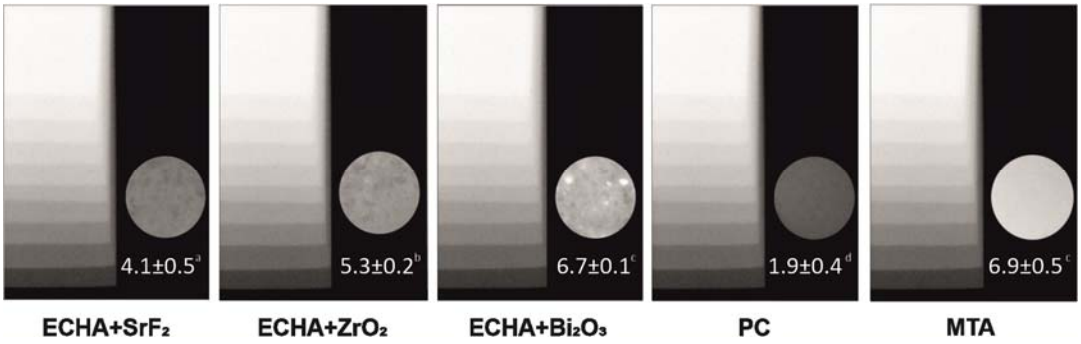
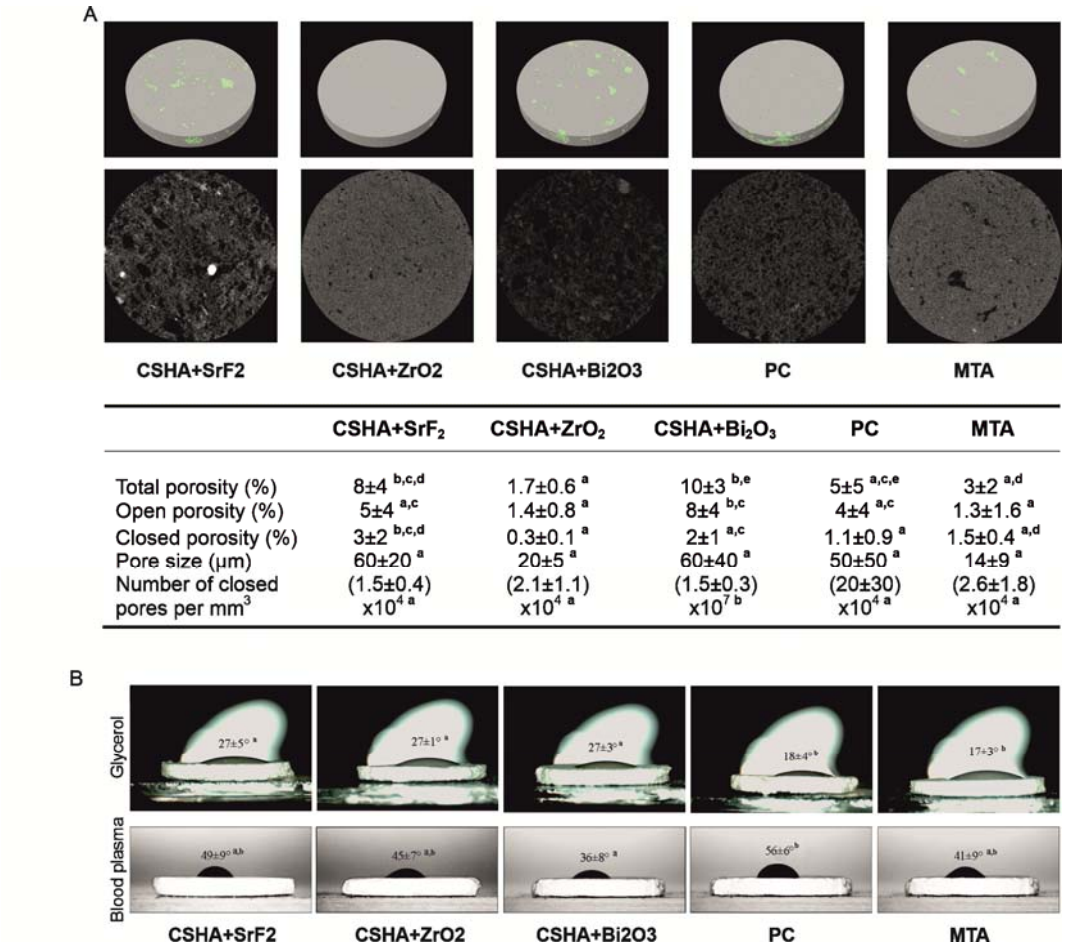


Figure 1. Radiopacity of investigated cements as measured with digital radiography. Different letters mean statistical significance among cements' specimens (one-way ANOVA followed by Bonferroni post-hoc test, $p<0.05$). ECHA, calcium silicate hydroxyapatite; SrF_2, strontium fluoride; ZrO_2, zirconium dioxide; Bi_2O_3, bismuth oxide; PC, Portland cement; MTA, mineral trioxide aggregate.



	$CSHA+SrF_2$	$CSHA+ZrO_2$	$CSHA+Bi_2O_3$	PC	MTA
Total porosity (%)	8±4 ^{b,c,d}	1.7±0.6 ^{a}	10±3 ^{b,e}	5±5 ^{a,c,e}	3±2 ^{a,d}
Open porosity (%)	5±4 ^{a,c}	1.4±0.8 ^{a}	8±4 ^{b,c}	4±4 ^{a,c}	1.3±1.6 ^{a}
Closed porosity (%)	3±2 ^{b,c,d}	0.3±0.1 ^{a}	2±1 ^{a,c}	1.1±0.9 ^{a}	1.5±0.4 ^{a,d}
Pore size (μm)	60±20 ^{a}	20±5 ^{a}	60±40 ^{a}	50±50 ^{a}	14±9 ^{a}
Number of closed pores per mm^3	(1.5±0.4) $x10^{4\ a}$	(2.1±1.1) $x10^{4\ a}$	(1.5±0.3) $x10^{7\ b}$	(20±30) $x10^{4\ a}$	(2.6±1.8) $x10^{4\ a}$

Figure 2. Physicochemical properties of investigated materials. A) Porosity of investigated materials with three dimensional (3D) reconstructions and two dimensional (2D) presentations of representative specimens. B) Wettability of investigated materials expressed as glycerol and human blood plasma contact angles. Different letters mean statistical significance among cement specimens (one-way ANOVA followed by Bonferroni post-hoc test, $p<0.05$). ECHA, calcium silicate hydroxyapatite; SrF_2, strontium fluoride; ZrO_2, zirconium dioxide; Bi_2O_3, bismuth oxide; PC, Portland cement; MTA, mineral trioxide aggregate.

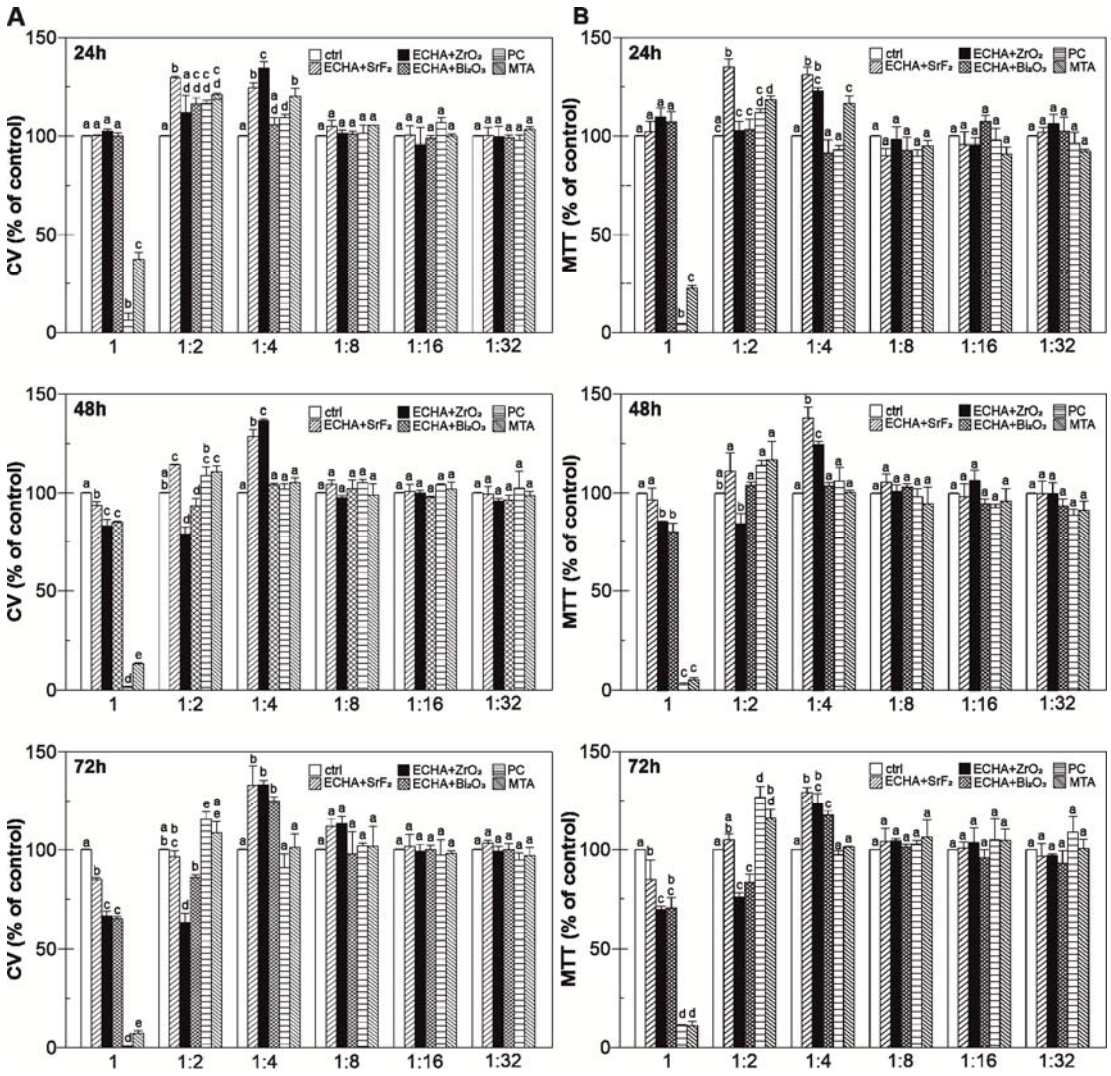


Figure 3. Cell viability (%) evaluated by the crystal violet (CV) (A) and MTT (B) assays after 24 h, 48 h and 72 h exposure of L929 cells to the cements' eluents - pure extract (1) and different serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32 (v:v)).The data are presented as mean ± standard deviation (SD) values of triplicates from one representative of three independent experiments. Different letters mean statistical significance among cement specimens (one-way ANOVA followed by Bonferroni post-hoc test, $p<0.05$, Kruskal-Wallis test ($p<0.05$) followed by Mann-Whitney test with Bonferroni correction ($p<0.005$)). ECHA, calcium

silicate hydroxyapatite; SrF_2, strontium fluoride; ZrO_2, zirconium dioxide; Bi_2O_3, bismuth oxide; PC, Portland cement; MTA, mineral trioxide aggregate.

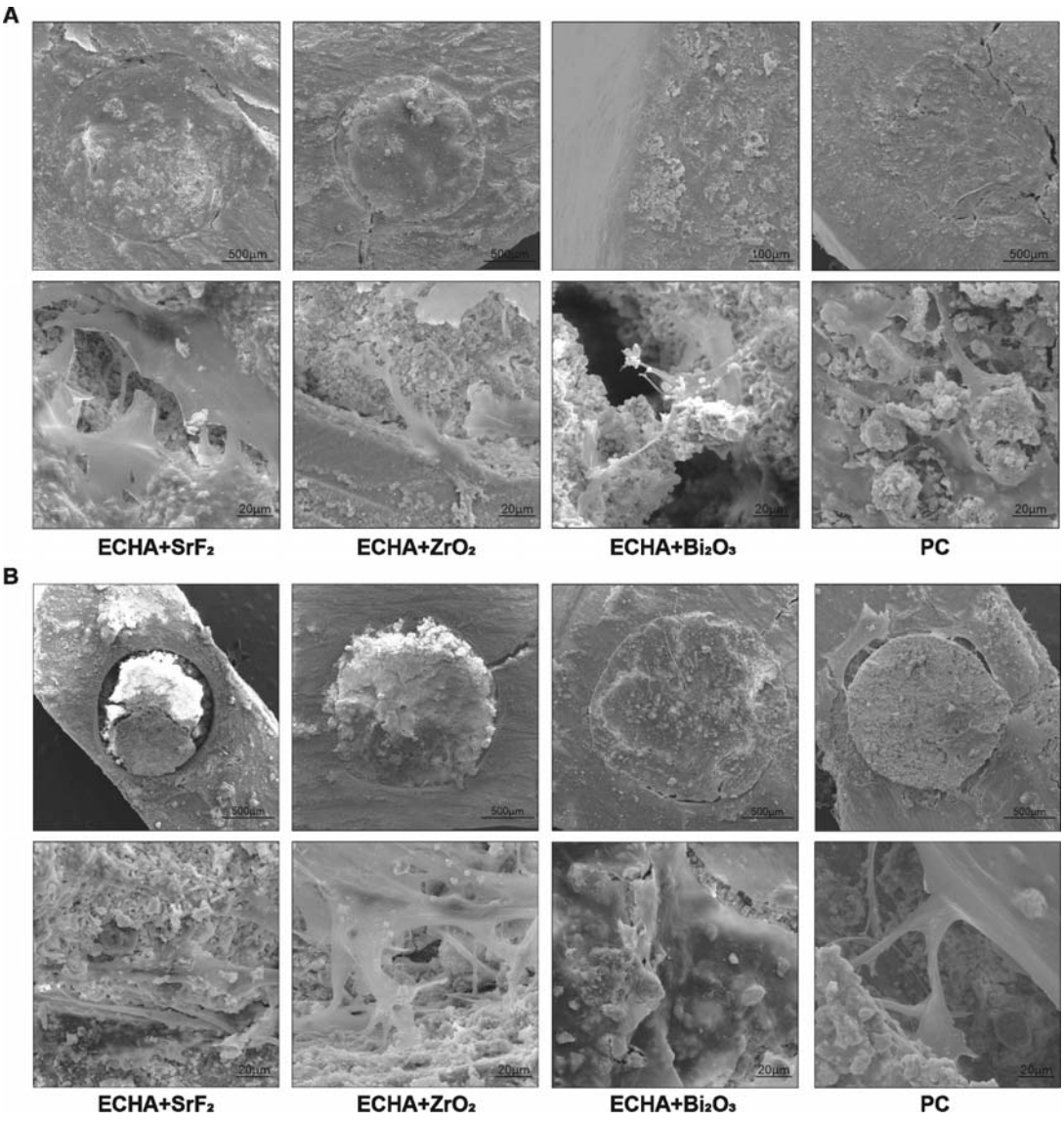


Figure 4. Representative scanning electron microphotographs of cements' specimens seeded in A) control growth medium and B) osteogenic medium for 21 days. The morphological features of osteoblast-like cells observed at lower magnification (100x) have shown flattened cells proliferated on cement and dentine surface creating the multilayers covering their surfaces and bridges at a

cement-dentine interface (upper rows). At higher magnification (2000x), numerous cell extensions that anchored well onto the cement surfaces can be observed (lower rows). ECHA, calcium silicate hydroxyapatite; SrF_2, strontium fluoride; ZrO_2, zirconium dioxide; Bi_2O_3, bismuth oxide; PC, Portland cement; MTA, mineral trioxide aggregate.

Supplement 1

Synthesis methods of obtained inorganic phases

Ammonium nitrate (NH_4NO_3), as an oxidation agent and citric acid ($C_6H_8O_7 \cdot H_2O$), as a fuel, were added to the mixture to cause a combustion reaction. The mixture of silica sol and calcium chloride pentahydrate ($CaCl_2 \cdot 5H_2O$) (corresponding to the ratio $2.3CaO/1SiO_2$) was firstly diluted in distilled water, vigorously mixed and after ten minutes dried at 80°C to obtain a gel, and then heated to 150°C to remove free and weakly bounded water inside of the mixture. In the next stage, the increase of temperature up to 680°C led to the ignition of the gel. Afterwards, the gel turned into a foam and a strong self-

propagating combustion reaction produced large volume of gases. Black ashes were obtained after auto-ignition. Rapid release of large volumes of the gaseous products during the combustion dissipated the heat and limited the temperature rise. This consequently reduced the possibility of premature local partial sintering amongst the primary particles, which is important for maintenance of the final powder activity. After high-temperature self-propagating thermal treatment, the samples were quickly cooled using copper plates, providing low crystallinity and high reactivity of obtained C_2S and C_3S (C=CaO, S=SiO_2, C_3S=3CaO·SiO_2, C_2S=2CaO·SiO_2) phases. The resulting black powder contained some carbon residues and was further calcined in the air at 650°C for 4 h to pyrolises to obtain the desired crystalline product. After thermal treatment**,** this powder was milled and final silicate phases were obtained.

The nHA was produced by a hydrothermal method. Shells of chicken eggs calcined at 900^{o}C for complete carbon removal and dissociation of $CaCO_3$ to CaO and diammonium hydrogen phosphate ($(NH_4)_2HPO_4$; Merck p.a.) were the precursors for nHA synthesis. Calcined egg shells and $(NH_4)_2HPO_4$ were dissolved in distilled water to obtain solution 1 and solution 2, respectively. Precursor solutions were further prepared using a two solutions procedure: 500 ml of 3.02 cmol solution of $Ca(OH)_2$ (solution 1) and 500 ml of 2.32 cmol of $(NH_4)_2HPO_4$ (solution 2).

The $(NH_4)_2HPO_4$ solutions were subsequently poured into $Ca(OH)_2$ solutions and vigorously mixed. Finally, 0.1 N hydrochloric acid (HCl) or ammonium hydroxide ($(NH_4)OH$) solution was added into the above given

solutions to adjust the pH value to 7.4. The solution mixture content in a glass baker covered with a flat glass pane was autoclaved under predetermined conditions (150 ^{o}C, 500 KPa and 8 h). The time needed for reaching the required temperature and pressure of the autoclaved solutions varied from 1 h to 1.5 h. After that, the solutions were kept under these conditions for 8 h. Upon hydrothermal treatment in the autoclave, precipitates were decanted, dried at 80^{o}C for 48^{o}h, disintegrated, washed with deionized water and ultracentrifuged to obtain the purest possible nHA. The powder was dried at 40^{o}C in an oven for 24 h before being ground into a fine nano-particulated powder.