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Investigation of the radiopacity and cytotoxicity of ALBO-DENT – novel strontium carbonate incorporated calcium silicate based dental cement

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SUMMARY

Introduction Calcium silicate (CS) dental cements have numerous clinical indications in dentistry including pulp capping, root end surgery, perforation repair and apexification/apexogenesis treatment.

Materials and methods Novel CS based dental cement with incorporation of $SrCO_3$ radiopacifier named ALBO-DENT was used as an experimental cement material while Portland cement (Aalborg, Denmark) and ProRoot MTA (Tulsa Dental, USA) were used as controls. The radiopacity evaluation was performed using digital Trophy Radiographic system with an intention to precisely determine the minimum of radiopaque agent needed to confer to ISO radiopacity requirement. Thereafter, biocompatibility of material was tested in *in vitro* conditions in mouse fibrosarcoma L929 cell culture treated with materials' extracts. Cell morphology was observed using phase-contrast microscopy, while cell viability was measured using crystal violet (CV) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays.

Results Radiopacity evaluation revealed that 30%wt addition of $SrCO_3$ was necessary to achieve satisfactory radiopacity (3.45 mm Al). Cytotoxicity analysis using CV and MTT assays revealed that pure extracts of ALBO-DENT presented superior biocompatibility when compared to PC and MTA controls while serial dilutions of experimental cements' extracts as well as that of PC and MTA did not influence L929 cell viability.

Conclusions Novel formulation of CS cement – ALBO-DENT presented satisfactory radiopacity and adequate biocompatibility.

Keywords: calcium silicate; strontium carbonate; radiopacity; dental materials biocompatibility; endodontics

INTRODUCTION

Calcium silicate (CS) dental cements have revolutionized many regenerative endodontic procedures such as root end surgery, apexification/apexogenesis, perforation repair and direct pulp capping [1]. The very first commercial CS-based dental cement - ProRoot MTA (Tulsa Dental, OK, US) has shown significant clinical outcomes [2] and it is composed of type 1 ordinary Portland cement (PC) (with fineness in the range of 4500–4600 cm^2/g) and bismuth oxide (Bi_2O_3) added for radiopacity, in the proportion of 4:1 [2]. However, it has been shown that Bi_2O_3 addition increases cement solubility, reduces its mechanical resistance and causes tooth discoloration [3]. Therefore, researchers use different alternatives aiming to meet the ISO 6876 requirement for radiopacity. The following radiopaque agents are employed previously: barium sulphate ($BaSO_4$) [4, 5], titanium dioxide (TiO_2) [4], gold (Au) [4, 5], calcium tungstate ($CaWO_4$) [6, 7], zirconium dioxide (ZrO_2) [8, 9, 10], ytterbium fluoride (YbF_3) [9], tantalum pentoxide (Ta_2O_5) [10] and niobium pentoxide (Nb_2O_5) [11, 12].

Our research group has demonstrated the satisfactory properties of two novel CS 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 mechanical properties and *in vivo* safety, after both acute and sub-chronic administration, are documented previously [13–19]. These materials have shown satisfactory setting time, increased pH value, adequate biocompatibility and enhanced neutralization of the bacterial biofilm [20, 21]. It was confirmed that CS enriched with nHA was associated with YbF_3 as radiopacifiers leading to adequate physicochemical and biological characteristics [9, 19].

This study generally served to further improve the quality of ALBO MPCA cements by incorporating the potentially bioactive radiopacifier – strontium carbonate

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($SrCO_3$). The idea is rooted in proofs of numerous beneficial effects of strontium (Sr) on bone and dental tissue, among which are: osteoproliferative and odontoproliferative effects, stimulation of bone formation and angiogenesis, inhibition of cell differentiation and activity of osteoclasts and induction of human dental pulp stem cells by promoting their odontogenic differentiation, proliferation and mineralization [22, 23, 24]. The aim of this study was to determine the minimal ratio of $SrCO_3$ capable to satisfy ISO required radiopacity standard and investigate the biocompatibility of this material in L929 cell culture.

MATERIALS AND METHODS

Synthesis of inorganic phases

The novel experimental cement – ALBO-DENT was composed of the following components: calcium silicate, zirconium oxide, strontium carbonate, magnesium silicate, mesosilica and hexaphosphate. Silicate active phase was synthesized from calcium chloride pentahydrate ($CaCl_2 \cdot 5H_2O$) (Merck, Germany) and silica sol obtained by hydrothermal treatment. Aluminium acetate ($Al(CH_3COO)_3$) was added to the mixture to provide the production of a small amount (3.01 %) of active tricalcium aluminate (C_3A) phase. Detailed procedure of used CS synthesis is given in investigations of Jokanović et al. [14, 15]. $SrCO_3$ (Sigma-Aldrich, St. Louis, Missouri, USA) was added into the mixture at 10%, 20% and 30% wt. ratio. PC (Aalborg, Denmark) and MTA+ (thereafter referred to as MTA) (Cerkamed, StalowaWola, Poland) served as control.

Specimen preparation

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, using glass mixing pad and stainless steel spatula for cement mixing. The specimens were made using polytetrafluoroethylene (PTFE) ring molds incorporating a cavity of various internal diameter and height depending on the used test. Molds were filled to a level surface with mixed cement.

Radiopacity assessments

Radiopacity was determined in accordance with ISO 6876 [25]. Specimens (n=5) measuring 8 mm in diameter and 1 mm thickness were placed alongside an aluminum step-wedge (99.6 % pure) varying in thickness from 1 to 10 mm in increments of 1 mm each and radiographed by CCD sensor and X-ray unit (Trophy Radiology, Cedex, France) operating at 65 kV, 7 mA, for 0.07 s and at the focus to target distance of 35 cm. Image J for Windows software (National Institutes of Health (NIH), Bethesda, MD, USA) was used to calculate the gray scale values of each specimen and of each aluminium step-wedge thickness. The mean grey scale values were plotted against the number of aluminum steps, the plots were linearly regressed and regressions were used to convert mean grey scale values into millimeters of aluminum.

Cell viability analysis

Preparation of the materials extracts

Cell viability was carried out in accordance with the ISO Standard 10993-5/2005 [26]. Cements were manipulated under sterile conditions. Immediately after mixing, materials were placed into pre-sterilized PTFE molds (12 mm in diameter and 2 mm thick) to set for 24 h in a humidified atmosphere. Thereafter, 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. To prepare eluents for treatment, extracts were diluted with complete culture medium that was used for cultivation of control/non-treated cells.

Cell culture and treatment

The mouse fibrosarcoma 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)). Cell viability was assessed after 24, 48 and 72 h treatment.

Cell viability assessment

The number of adherent cells was determined using crystal violet (CV) while mitochondrial dehydrogenase activity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) test. The CV assay was 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 the final concentration of 0.5 mg/ml and cells were incubated for an additional hour. Subsequently, 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 micro-plate reader at 570 nm (Sunrise; Tecan, Dorset, UK). The results were

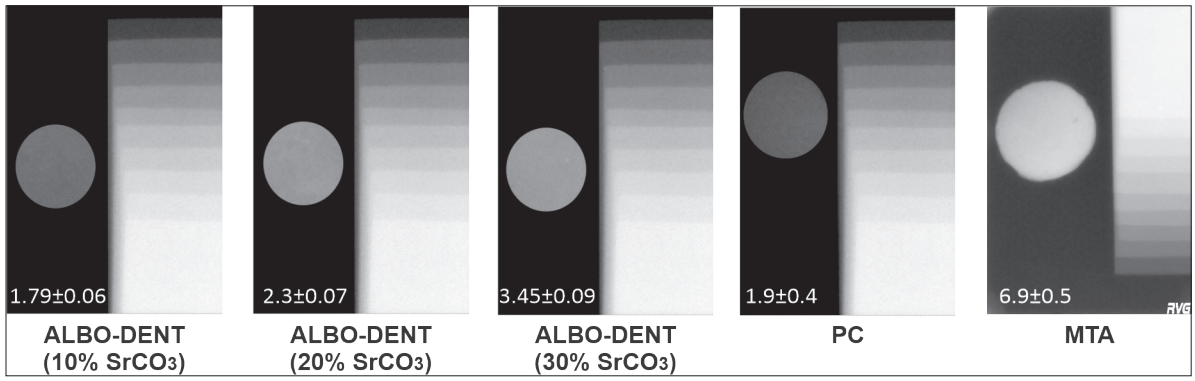

Figure 1. Radiopacity of investigated cements as measured with digital radiography. Digital radiographs of the representative disc-shaped cements' specimens alongside an aluminum step wedge ethalone. Note the increase of the radiopacity with greater percentage of $SrCO_3$ addition. $SrCO_3$ – strontium carbonate; PC – Portland cement; MTA – mineral trioxide aggregate
Slika 1. Rendgenkontrasnost ispitivanih cemenata merena digitalnom radiografijom. Digitalni radiogrami reprezentativnih uzoraka cemenata u obliku diska zajedno sa aluminijumskim etalonom. Primetiti povećanje kontrastnosti sa većim postotkom prisutnog SrCO3. SrCO3 – stroncijum-karbonat; PC – cement Portland; MTA – mineralni trioksidni agregat

presented as percentage of viability relative to untreated, control cultures, considered as 100 % viable. The experiments were performed in triplicates.

Phase contrast microscopy analysis

Morphological changes in mouse fibrosarcoma L929 cell line were observed and cells photographed under Leica DCF320 phase contrast microscope (Leica Microsystems DMIL, Wetzlar, Germany) equipped with Leica Microsystems DFC320 camera and Leica Application Suite software (version 2.8.1), with 20× magnification.

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 obtained radiopacity and cytotoxicity outcomes ($p<0.05$).

RESULTS

The Shapiro-Wilk test for normality found that data were normally distributed and thus they were subjected to one-way ANOVA analysis followed by Bonferroni test.

The results of the radiopacity evaluations are presented in Figure 1. One-way ANOVA revealed that the addition of different percentage of radiopacifiers statistically influenced the obtained values of radiopacity. The lowest value of radiopacity was found in PC that was not statistically different when compared with CS+10%$SrCO_3$ addition, while it was statistically different that all other investigated cements. On the other hand, MTA presented the greatest radiopacity value, statistically higher than in all other cements. Results revealed that 30% wt addition of $SrCO_3$ conferred the ALBO-DENT radio-density of 3.45±0.09 mm Al that was in accordance with ISO 6876 requirement, while 10 % addition and 20 % addition of $SrCO_3$ did not conform with ISO standard for 3 mm Al (1.79±0.06 mmAl, 2.3±0.07 mmAl, for 10 % and 20 %, respectively).

Cytotoxicity data are given in Figure 2 and Figure 3, while representative phase-contrast images of the cells treated with extracts of investigated materials are presented in Figure 4. For CV assay (Figure 2), one-way ANOVA showed the statistical difference among tested cements after 24 h (pure extracts, 1:2 and 1:4), 48 h (pure extracts, 1:2 and 1:4) and 72 h (pure extracts and 1:2) ($p<0.05$). For MTT assay (Figure 3), one-way ANOVA showed the statistical difference for all time points for pure, 1:2 and 1:4 dilutions ($p<0.05$), while significance was not found for 1:8, 1:16 and 1:32 dilutions ($p>0.05$). The results obtained for CV and MTT assays are highly complementary. Pure extract of ALBO-DENT presented lower cytotoxicity than PC and MTA for all time points, showed by both CV and MTT assays. For 1:2 dilution, MTA presented significant proliferative potential after 24h. Similarly, treatment with 1:2 and 1:4 dilutions of PC extract exerted statistically higher proliferative potential after 48 h. The rest of dilutions (1:8, 1:16 and 1:32) had no effect on cell viability.

Consistent with results obtained using cell viability assays, treatment of L929 cells with ALBO-DENT pure extract for 24 h had no effect on cell morphology, but slightly decreased cell proliferation. Contrary, MTA and PC pure extracts triggered morphological changes typical for cell death, cell shrinkage and rounding and detachment of cells from bottom well (Figure 4).

DISCUSSION

This study showed that $SrCO_3$ might be a radiopacifying agent in CS-based dental cement. It has been shown that 30 % wt addition of $SrCO_3$ has met ISO requirement for radiopacity and at the same time cement mixture enriched with 30 % wt $SrCO_3$ showed satisfactory biocompatibility properties.

The idea and reason behind adding $SrCO_3$ into CS-based cement formulation originate from two reasons.

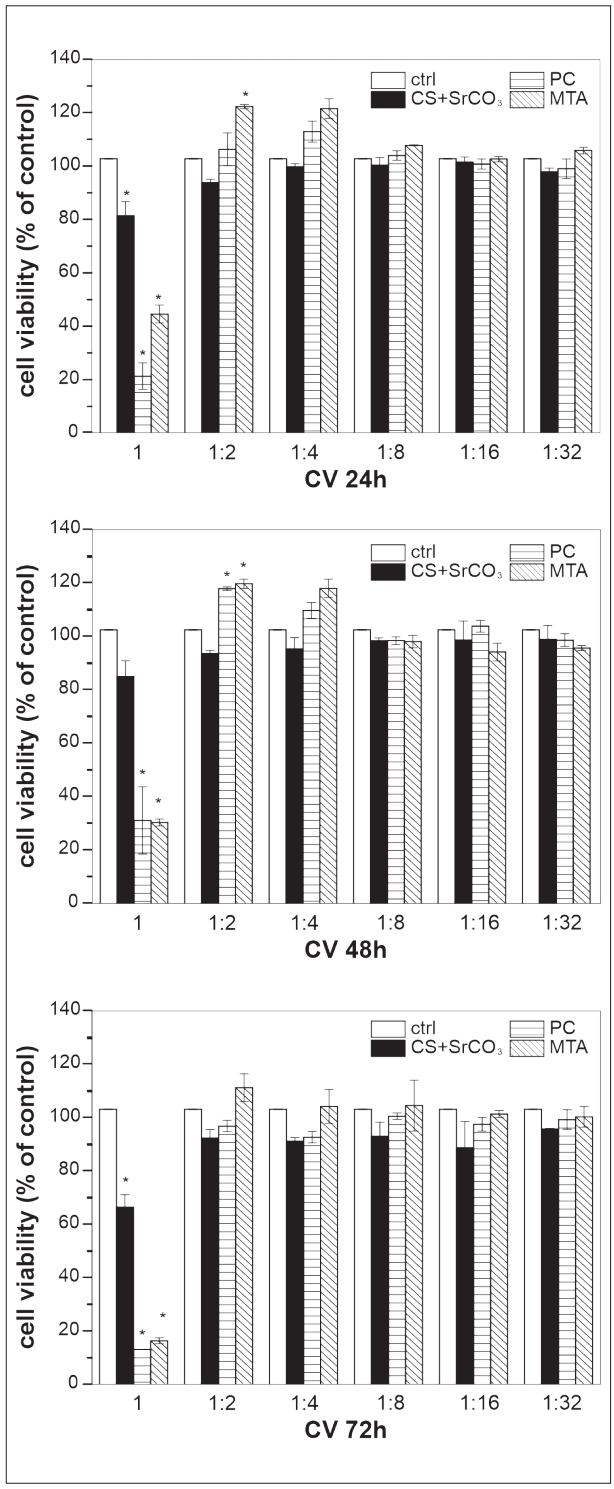

Figure 2. Cell viability (%) evaluated by the crystal violet (CV) assay after 24 h, 48 h and 72 h exposure of L929 cells to the cements' eluents – pure extract (1) and different serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32 (v:v)). The data are presented as mean ± standard deviation (SD) values of triplicates from one representative of three independent experiments. Columns with * are statistically different in comparison to control ($p < 0.05$). $SrCO_3$ – strontium carbonate; PC – Portland cement; MTA – mineral trioxide aggregate

Slika 2. Vijabilnost ćelija (%) izmerena kristal violet (KV) testom posle 24 h, 48 h i 72 h izlaganja ćelija L929 na eluate cemenata – čisti ekstrakt (1) i sa serijom razblaženja (1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32 (v : v)). Rezultati su predstavljeni kao srednja vrednost i standardne devijacije (SD) triplikata kultura iz jednog od tri nezavisna eksperimenta. Kolone sa oznakom * su statistički značajne u poređenju sa kontrolnom grupom ($p < 0,05$). SrCO3 – stroncijum-karbonat; PC – cement Portland; MTA – mineralni trioksidni agregat

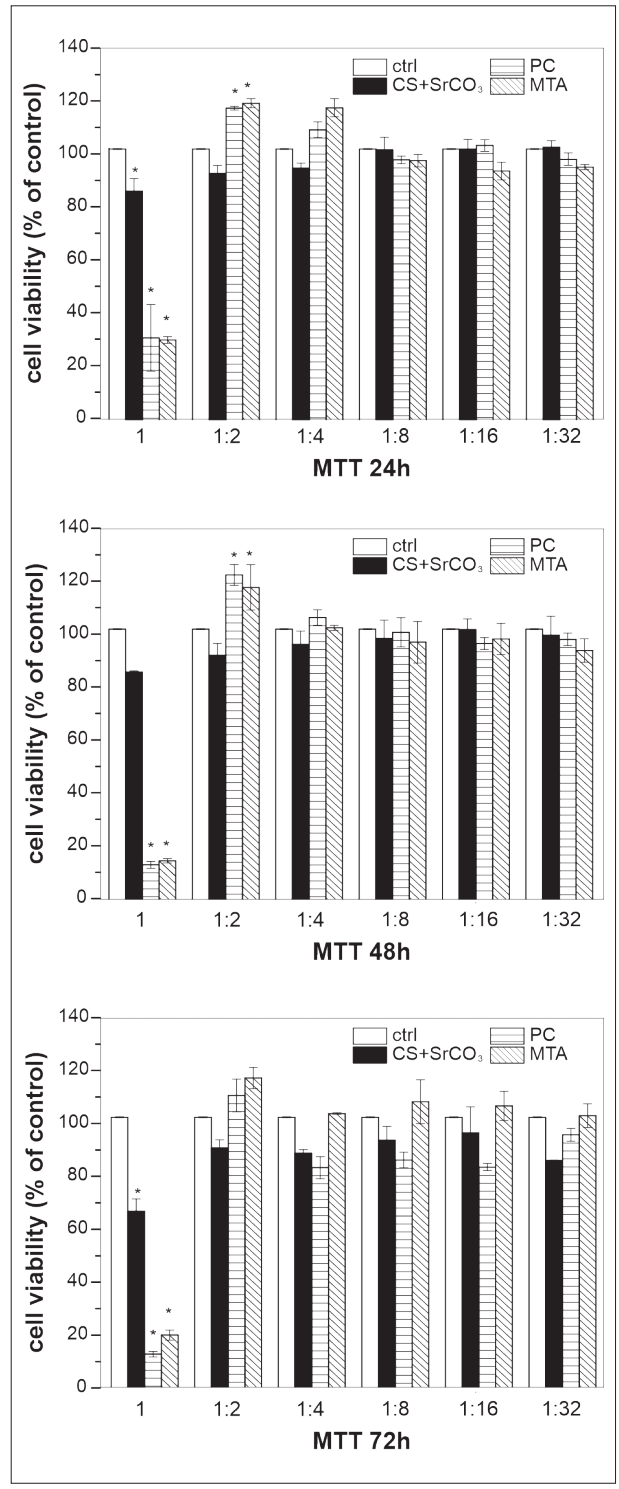

Figure 3. Cell viability (%) evaluated by the MTT test after 24 h, 48 h and 72 h exposure of L929 cells to the cements' eluents – pure extract (1) and different serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32 (v:v)). The data are presented as mean ± standard deviation (SD) values of triplicates from one representative of three independent experiments. Columns with * are statistically different in comparison to control ($p < 0.05$). $SrCO_3$ – strontium carbonate; PC – Portland cement; MTA – mineral trioxide aggregate

Slika 3. Vijabilnost ćelija (%) izmerena MTT testom posle 24 h, 48 h i 72 h izlaganja L929 ćelija na eluate cemenata – čisti ekstrakt (1) i sa serijom razblaženja (1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32 (v : v)). Rezultati su predstavljeni kao srednja vrednost i standardne devijacije (SD) triplikata kultura iz jednog od tri nezavisna eksperimenta. Kolone sa oznakom * su statistički značajne u poređenju sa kontrolnom grupom ($p < 0,05$). SrCO3 – stroncijum-karbonat; PC – cement Portland; MTA – mineralni trioksidni agregat

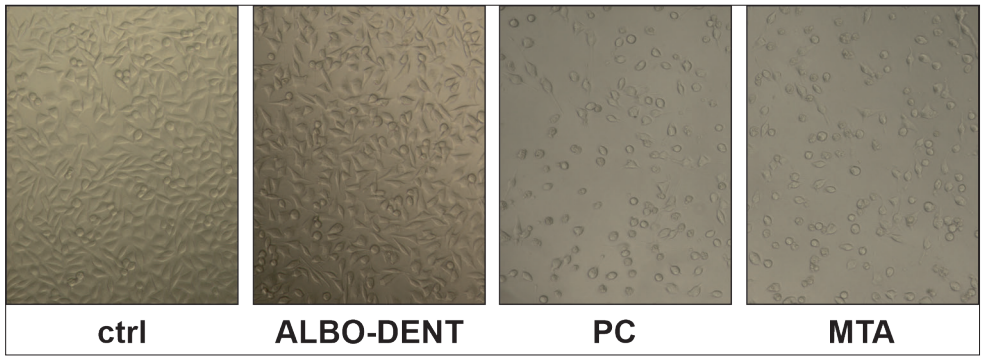

Figure 4. Effects of ALBO-DENT, MTA and PC on mouse fibrosarcoma cell line L929 morphology. L929 cell were grown in medium (A) and in presence of pure extracts of ALBO-DENT (B), MTA (C) and PC (D) for 24 h. Cell morphology was observed using phase contrast microscopy (magnification 20×).
Slika 4. Uticaj ALBO-DENTA, MTA i PC na morfologiju ćelijske linije mišjeg fibrosarkoma L929. Ćelije L929 su gajene u medijumu (A) i u prisustvu čistog ekstrakta ALBO-DENT (B), MTA (C) i PC (D) tokom 24 h. Ćelijska morfologija je posmatrana pomoću fazno kontrastnog mikroskopa (uveličanje 20×).

Firstly, Sr is nowadays accepted as a bioactive constituent of many dental materials and biomaterials used in orthopaedic surgery. In addition, modern strategies for bio-activation of the surfaces of titanium implants include their coating with Sr incorporated layers. Secondly, Sr is intentionally used in the form of carbonates since the addition of calcium carbonate ($CaCO_3$) into CS cements decreases setting time, as it was achieved in Biodentine (Septodont, France) [27].

The results of radiopacity have shown that 30 % wt addition of $SrCO_3$ was necessary to satisfy the radiopacity of ALBO-DENT. The radiopacity of ALBO-DENT was lower than previously found for CS+30%Bi_2O_3 (~11 mm Al) and CS+25%Bi_2O_3 (6.9 mm Al) [28]. The results demonstrated for radiopacity of MTA (6.9 mm Al) corroborate findings of previous studies: 4.86, 6.74, 7.0, 7.5 and 8.0mm Al [28–31]. The PC did not meet the ISO radiopacity requirement that is in line with previous studies (~0.9 mm Al) [28, 29]. The influence of $SrCO_3$ on the radiopacity of endodontic ceramics has not been previously mentioned in the literature. The variations in radiopacity come as a consequence of the difference in the atomic number between the constituents [32]. Namely, atomic number of the compounds is directly proportional to the absorption of x-rays. The atomic number of Sr (Z=40) is lower than that in Bi (Z=83) and therefore higher percentage of $SrCO_3$ is needed to meet ISO radiopacity standard. This is not playing a negative role in the case of $SrCO_3$ such as with other radiopacifiers addition (i.e. Bi) because Sr may be considered not only as biologically safe, but also biologically active constituent.

Biological safety of dental materials is of paramount importance. Therefore, *in vitro* and *in vivo* tests are routinely performed to evaluate material's biocompatibility before it can be used in clinical practice. The cytotoxicity assessment was performed for the mixture with adequate radiopacity value (30% wt addition of $SrCO_3$). ISO 10993-5 stipulates that material can be considered as not cytotoxic if it causes less than 30% cells to die in *in vitro* assays. In our study, two cytotoxicity tests were used: MTT that measures mitochondrial activity of the metabolically active cells and CV that determines the number of the adherent, viable cells. It was demonstrated that novel experimental cement ALBO-DENT performed satisfactory behavior in cell culture, comparable to that of PC and MTA. Presented results showed significantly lower percentage of viable cells in MTA/PC treatments than those found in some studies (80-150%) [7, 33, 34], but they are in agreement with other studies [35, 36]. 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 [7, 37]. The differences of cell viability results in different studies could arise from variations in specimens' size (5×3 mm [37], 5×2 mm [12] and 5×1 mm [29]). The toxic potential of PC and MTA pure elute may be a matter of debate, but it is presumably the consequence of its high alkalinity in the closed *in vitro* cell viability assessment system. It may be speculated that *in vivo*, where the constant fluid uptake is ensured, these materials may not present negative effect on the surrounding tissue. In any case, novel cement mixture ALBO-DENT presented superior characteristics than widely commercially used ProRoot MTA.

From the clinical point of view, new CS-based experimental material has certain advantages since Sr incorporation may enhance the bone healing during root end canal surgery by activating osteoblasts for improved bone synthesis [22, 23, 24]. In addition, if used for pulp capping procedures, it may stimulate odontoblasts for faster formation of tertiary dentine. These assumptions should be confirmed in the future state of the art researches.

CONCLUSION

Newly synthesized CS-based dental cement with 30% wt addition of $SrCO_3$ as radiopacifying agent meets ISO standard for radiopacity. Biocompatibility of newly synthesized cement, assessed by analysis of cell viability via measurement of the number of adherent, viable cells and viable cells with active mitochondria, is satisfactory and indicates its biological safety.

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Ispitivanje rendgenkontrastnosti i citotoksičnosti ALBO-DENTA – novog kalcijum-silikatnog cementa sa dodatkom stroncijum-karbonata

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KRATAK SADRŽAJ

Uvod Kalcijum-silikatni (KS) dentalni cementi se koriste u brojnim kliničkim indikacijama u stomatologiji koje uključuju direktno prekrivanje pulpe, retrogradnu hirurgiju korena zuba, lečenje perforacija i apeksogenezu/apeksifikaciju.

Materijali i metode U istraživanju je korišćen novosintetisani cement na bazi KS sa dodatkom $SrCO_3$ kao kontrastnog agensa ALBO-DENTA, dok su kao kontrola korišćeni cement Portland (PC, Aalborg, Denmark) i ProRoot MTA (MTA, Tulsa Dental, USA). Rendgenokontrasnost je ispitivana digitalnom radiografijom primenom aparata Trophy, sa namerom da se precizno odredi minimum kontrastnog agensa koji zadovoljava zahteve standarda ISO za rendgenkontrastnost. Biokompatibilnost materijala je ispitana *in vitro*, u kulturi ćelija mišjeg fibrosarkoma L929 tretiranoj ekstraktima ispitivanih materijala. Ćelijska morfologija je praćena upotrebom fazno-kontrastne mikroskopije, dok je vijabilnost ćelija utvrđivana kristal violet (KV) i 3-(4,5-dimetiltiazol-2-yl)-2,5-difenfl-tetrazolium bromid (MTT) esejima.

Rezultati Ispitivanje rendgenkontrastnosti je pokazalo da dodatak 30% $SrCO_3$ dovodi do zadovoljavajućeg kontrasta materijala (3,45 mm Al). Analiza citotoksičnosti KV i MTT metodom je pokazala da čisti ekstrakt ALBO-DENTA pokazuje bolju biokompatibilnost u poređenju sa PC i MTA, dok serijska razblaženja ekstrakta ispitivanog cementa, kao i PC i MTA, nisu uticala na vijabilitet ćelija L929.

Zaključci Novi cement na bazi KS – ALBO-DENT pokazao je zavodovoljavajuću rendgenkontrastnost i odgovarajuću biokompatibilnost.

Ključne reči: kalcijum-silikat; stroncijum-karbonat; rendgenkontrast; citotoksičnost; endodoncija

UVOD

Kalcijum-silikatni (KS) dentalni cementi doveli su do revolucije u mnogim endodontskim regenerativnim zahvatima kao što su endodontska apeksna hirurgija, apeksifikacija/apeksogeneza, reparacija perforacija, kao i direktno prekrivanje pulpe [1]. Prvobitno sintetisani komercijalni KS cement, ProRoot MTA (Tulsa Dental, OK, SAD) pokazao je značajne kliničke rezultate, a sastojao se od tipa 1 standardnog cementa Portland (PC) (sa finoćom u rangu od 4500 do 4600 cm^2/g) i dodatog bizmut-oksida (Bi_2O_3) kao radiokontrastnog sredstva u razmeri 4 : 1 [2]. Međutim, primećeno je da dodatak Bi_2O_3 utiče na povećanu rastvorljivost cementa, redukuje mehaničku otpornost i dovodi do diskoloracije zuba [3]. Iz tog razloga istraživači primenjuju različite alternative u cilju zadovoljavanja ISO 6876 zahteva za radiokontrast. Kao rendgenkontrasti korišćeni su barijum-sulfat ($BaSO_4$) [4, 5], titanijum-dioksid (TiO_2) [4], zlato (Au) [4, 5], kalcijum-volframat ($CaWO_4$) [6, 7], cirkonijum-dioksid (ZrO_2) [8, 9, 10], iterbijum-fluorid (YbF_3) [9], tantal-pentoksid (Ta_2O_5) [10] i niobijum-pentoksid (Nb_2O_5) [11, 12].

Naša istraživačka grupa došla je do zadovoljavajućih osobina dva nova KS preparata: jedan se sastojao od KS, nanočestičnog hidroksiapatita (nHA) i $BaSO_4$ – ALBO $MPCA_1$, dok se drugi sastojao od KS, kalcijum-karbonata ($CaCO_3$) i Bi_2O_3 – ALBO $MPCA_2$. Njihove mehaničke osobine kao i bezbednost u živom organizmu (*in vivo* safety), posle akutne i subhronične primene, dokumentovane su u prethodnim radovima [13–19]. Ovi preparati su pokazali zadovoljavajuće vreme očvršćavanja, povećanu pH vrednost, pogodnu biokompatibilnost i pojačanu neutralizaciju bakterijskog biofilma [20, 21]. Potvrđeno je da je KS obogaćen nHA uz dodatak YbF_3 kao radiokontrasta doveo do adekvatnih fizikohemijskih i bioloških karakteristika [9, 19].

Opšti cilj ove studije bio je da se dodatno poboljša kvalitet cementa ALBO MPCA dodavanjem potencijalno bioaktivnog radiokontrasta – stroncijum-karbonata ($SrCO_3$). Ideja je ukorenjena u dokazima brojnih povoljnih efekata stroncijuma (Sr) na kost i zubna tkiva, među kojima su osteoproliferativni i odontoproliferativni efekati, stimulacija formiranja kosti i angiogeneza, inhibicija ćelijske diferencijacije i osteoklastne aktivnosti, odnosno indukcija humanih stem ćelija zubne pulpe promocijom njihove odontogene diferencijacije, proliferacije i mineralizacije [22, 23, 24]. Cilj ovog istraživanja je bio da utvrdi minimalni udeo $SrCO_3$ kao rendgenkontrastnog sredstva koji zadovoljava zahteve ISO u dentalnim cementima i da proveri biokompatibilnost ovog materijala u kulturi ćelija L929.

MATERIJAL I METODE

Sinteza neorganskih faza

Novi eksperimentalni cement ALBO-DENT sačinjen je od sledećih sastojaka: kalcijum-silikata, cirkonijum-oksida, stroncijum-karbonata, magnezijum-silikata, mezosilike i heksafosfata. Aktivna silikatna faza je sintetisana od kalcijum-hlorida pentahidrata ($CaCl_2 \cdot 5H_2O$) (Merk, Nemačka) i silicijumske sol faze dobijene hidrotermalnim postupkom. Aluminijum-acetat ($Al(CH_3OO)_3$) dodat je u mešavinu da obezbedi formiranje male

količine (3,01%) aktivne trikalcijumaluminatne faze (C_3A). Detaljna procedura sinteze korišćenog KS je opisana u istraživanjima Jokanovića i saradnika [14, 15]. $SrCO_3$ (Sigma-Aldrich, St. Louis, Missouri, SAD) dodat je u mešavinu kao 10%, 20% i 30% težinski postotak. PC (Aalborg, Danska) i MTA+ (kasnije navođen kao MTA) (Cerkamed, StalowaWola, Poljska) služili su kao kontrola.

Priprema uzoraka

Svi eksperimentalni cementi i PC su ručno zamešani; odnos praha i tečnosti je bio 1 g cementa i 0,3 ml destilovane vode, dok je MTA pripremljen prema uputstvu proizvođača, metalnom špatulom za mešanje cemenata na staklenoj pločici. Uzorci su napravljeni korišćenjem politetrafluoroetilenskih (PTFE) kalupa čiji je dijametar šupljine varirao u zavisnosti od korišćenog testa. Kalupi su bili do vrha ispunjeni cementnom masom.

Određivanje rendgenkontrastnosti

Rendgenkontrastnost je utvrđivana u skladu sa standardom ISO 6876 [25]. Uzorci (n = 5) dijametra 8 mm i debljine 1 mm postavljeni su zajedno sa aluminijumskim etalonom (99,6% čistoće), čija je debljina varirala od 1 mm do 10 mm sa postepenim povećanjem od 1 mm, i radiografisani su uz pomoć CCD sensora i izvora x-zraka (Trophy Radiology, Cedex, Francuska) radeći pri sledećim parametrima: 65 kV, 7 mA, 0,07 s i rastojanju između izvora zračenja i objekta radiografisanja od 35 cm. Image J za program Windows (National Institutes of Health (NIH), Bethesda, MD, SAD) primenjen je za izračunavanje stepena sivo-bele skale svakog uzorka i svake debljine aluminijumskog etalona. Srednje vrednosti sivo-bele skale su plotovane sa debljinom aluminijuma, a izvedena linearna zavisnost i regresija korišćene su da se stepen sivo-bele skale pretvori u milimetre aluminijuma.

Analiza ćelijskog vijabiliteta

Priprema ekstrakata materijala. Analiza ćelijskog vijabiliteta je urađena u skladu sa ISO standardom 10993-5/2005 [26]. Cementi su pripremani u sterilnim uslovima. Neposredno posle mešanja materijali su stavljani u sterilisane PTFE kalupe (12 mm širine i 2 mm debljine) da očvrsnu tokom 24 sata u atmosferi zasićenoj vodenom parom. Dobijeni diskovi su zatim sterilisani ultravioletnim zračenjem u trajanju od 2 h, nakon čega su uronjeni u 1 ml kompletnog medijuma – Dulbecco modifikovani Eagle medijum (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, SAD), obogaćenog 5% fetalnim telećim serumom (FBS), 2 mM L-glutaminom i penicilinom/streptomicinom (Capricorn Scientific, Ebsdorfergrund, Nemačka) i inkubirani 24 sata na 37° C. Kako bi se pripremili eluati za tretman ćelija, ekstrakti su razblaženi kompletnim medijumom koji je korišćen za kultivaciju kontrolnih/netretiranih ćelija.

Ćelijska kultura i tretman. Ćelijska linija mišjeg fibrosarkoma L929 (Evropska zbirka animalnih ćelijskih kultura, Salisbury, UK) kultivisana je u kompletnom medijumu i održavana na 37° C, u atmosferi zasićenoj vodenom parom, sa 5% CO_2. Ćelije su pripremljene za eksperiment korišćenjem konvencionalne procedure tripsinizacije sa tripsin/EDTA i zasejavane za testove ćelijskog vijabiliteta u polistirenske ploče sa 96 bunara sa ravnim dnom u gustini 5×10^3 ćelija po bunaru. Ćelije su 24 sata posle zasejavanja tretirane čistim ekstraktom (1) i njegovim serijskim razblaženjima (1 : 2, 1 : 4, 1 : 8, 1 : 16 i 1 : 32 (v : v)). Vijabilitet ćelija je procenjen posle 24, 48 i 72 sata.

Merenje vijabiliteta ćelija. Broj adherentnih ćelija utvrđen je kristal violet (KV) testom, dok je aktivnost mitohondrijalne dehidrogenaze merena testom 3-(4,5-dimetiltiazol-2-il)-2,5 difeniltetrazolijum-bromid (MTT). Kolorimetrijski test KV se zasniva na činjenici da adherentne ćelije koje podležu ćelijskoj smrti gube sposobnost adherencije i odlepljuju se od podloge, tako da je količina boje koju ćelije u kulturi vežu direktno proporcionalna broju živih ćelija. Nakon završetka tretmana iz ploče je odliven medijum, a bunari su isprani PBS-om i na taj način su odstranjene odlepljene, mrtve ćelije. Adherentne ćelije su fiksirane metanolom u trajanju od 15 minuta na sobnoj tempeaturi, a zatim su bojene 1% rastvorom kristal violeta u PBS-u, takođe 15 minuta na sobnoj temperaturi. Boja koja se nije vezala ispirana je vodom, a boja ugrađena u ćelije je rastvorena 33% rastvorom sirćetne kiseline u vodi.

MTT je tetrazolijumska so žute boje koja se u metabolički aktivnim ćelijama redukuje pomoću mitohondrijskih dehidrogenaza do ljubičastih nesolubilnih kristala formazana. Formazan se rastvara rastvorom DMSO i intenzitet dobijene ljubičaste boje je direktno proporcionalan aktivnosti mitohondrija, što odgovara broju živih ćelija. Po završetku kultivacije ćelija medijum je odlivan i ćelije su isprane PBS-om. Ćelije su zatim inkubirane jedan sat na 37° C sa rastvorom MTT boje (0,5 mg/ml). Nakon inkubacije boja je odlivena sa ćelija, a kristali formazana su rastvoreni pomoću DMSO. Intenzitet dobijenih boja je meren na automatskom čitaču ploča za mikrotitraciju (Sunrise; Tecan, Dorset, UK) na talasnoj dužini od 570 nm. Vijabilnost tumorskih ćelija izražena je kao procenat netretirane kontrole, kojoj je arbitrarno dodeljena vrednost 100%. Eksperimenti su urađeni u triplikatima.

Fazno-kontrastna mikroskopija

Morfološke promene mišje fibrosarkomske ćelijske linije L929 su analizirane i ćelije su fotografisane nakon 24 sata tretmana pomoću mikroskopa Leica DCF320 (Leica Microsystems DMIL, Wetzlar, Nemačka) opremljenog kamerom Leica Microsystems DFC320 i softverom Leica Application Suite (verzija 2.8.1), pri čemu je izabrano uveličanje od 20×.

Statistička analiza

Softverski program SPSS (ver. 20, IBM Corp., Armonk, Njujork, SAD) primenjen je za statističku analizu. Šapiro-Vilkov test je korišćen za proveru normalnosti raspodele podataka. Nakon toga je primenjena jednosmerna analiza varijanse (ANOVA) sa Bonferonijevim post-hok testom za poređenje dobijenih vrednosti radiokontrastnosti i citotoksičnosti ($p < 0,05$).

REZULTATI

Primena Šapiro-Vilkovog testa normalnosti je pokazala da su dobijeni podaci normalno distribuirani te su podvrgnuti jednosmernoj analizi varijanse (ANOVA) praćenoj Bonferonijevim testom.

Rezultati procene rendgenkontrastnosti su predstavljeni na Slici 1. Primena ANOVA testa pokazala je da je dodavanje različitog procenata rendgenkontrastnog sredstva uticalo na stepen rendgenkontrastnosti. Najniža vrednost rendgenkontrastnosti izmerena je u PC i nije se statistički razlikovala od cementa sa dodatkom CS + 10% $SrCO_3$, ali se statistički razlikovala od svih ostalih ispitivanih cemenata. S druge strane, vrednost rendgenkontrastnosti MTA bila je statistički veća od svih ostalih cemenata. Rezultati su pokazali da je 30% težinskog dodatka $SrCO_3$ obezbedilo cementu ALBO-DENT rendgenkontrastnost od 3,45 ± 0,09 mm Al, što je u skladu sa zahtevima ISO 6876 standarda, dok dodavanje 10%, odnosno 20% $SrCO_3$ nije bilo u skladu sa zahtevima ISO standarda od 3 mm Al (1,79 ± 0,06 mm Al, 2,3 ± 0,07 mm Al, za 10%, odnosno 20%).

Rezultati citotoksičnosti prikazani su na Slici 2 (KV) i Slici 3 (MTT), a reprezentativne fotografije ćelija tretiranih čistim ekstraktima ispitivanih materijala posmatranih fazno-kontrastnom mikroskopijom prikazane su na Slici 4. Jednosmerna analiza varijanse je pokazala da za podatke dobijene KV testom postoji statistička razlika između ispitivanih materijala nakon 24 sata (čisti ekstrakti, 1 : 2 i 1 : 4), 48 sata (čisti ekstrakti, 1 : 2 i 1 : 4) i 72 sata (čisti ekstrakti i 1 : 2) ($p < 0,05$). Za podatke dobijene MTT testom ista analiza je pokazala statističku razliku u svim vremenskim tačkama za čist ekstrakt, kao i 1 : 2 i 1 : 4 razblaženja ($p < 0,05$), dok značajnost nije uočena za 1 : 8, 1 : 16 i 1 : 32 razblaženja ($p > 0,05$). Rezultati dobijeni KV i MTT testovima su izuzetno komplementarni. Čisti ekstrakt cementa ALBO-DENT je pokazao značajno manju citotoksičnost u poređenju sa PC i MTA, u svim praćenim vremenima. Razblaženje MTA 1 : 2 je pokazalo proliferativni potencijal nakon 24 sata. Razblaženje PC 1 : 2 i 1 : 4 je potenciralo proliferaciju ćelija nakon 48 sata, dok ostala razblaženja (1 : 8, 1 : 16 i 1 : 32) nisu pokazala statistički značajan uticaj na ćelijsku vijabilnost.

U skladu sa rezultatima dobijenih testova za ispitivanje ćelijskog vijabiliteta, tretman ćelija L929 sa čistim ekstraktom cementa ALBO-DENT tokom 24 sata nije uticao na morfologiju ćelija, ali je blago inhibirao ćelijsku proliferaciju. S druge strane, čisti ekstrakti MTA i PC izazvali su morfološke promene koje su karakteristične za ćelijsku smrt – ćelije su u njihovom prisustvu izgubile volumen, zaokruglile se i odvojile od podloge.

DISKUSIJA

Ova studija je pokazala da se $SrCO_3$ može koristiti kao rendgenkontrastno sredstvo kod KS dentalnih cemenata. Pokazano je da 30% težinskog dodatka $SrCO_3$ zadovoljava ISO standarde rendgenkontrastnosti i u isto vreme ova mešavina obogaćena sa 30% $SrCO_3$ pokazuje zadovoljavajuću biokompatibilnost.

Ideja i razlog dodavanja $SrCO_3$ u KS dentalne cemente proizilazi iz dva razloga. Prvo, stroncijum danas predstavlja široko prihvaćeni bioaktivni sastojak mnogih stomatoloških materijala, kao i biomaterijala u ortopedskoj hirurgiji. Takođe, savremene strategije za povećanje bioaktivnosti površina titanijumskih implantata uključuju njihovo presvlačenje filmovima na bazi stroncijuma. Drugo, stroncijum je sa razlogom odabran u formi karbonata jer dodavanje kalcijum-karbonata u KS cemente skraćuje vreme očvršćavanja, što je postignuto kod biodentina (Septodont, Francuska) [27].

Rezultati rendgenkontrastnosti su pokazali da je 30% dodatka $SrCO_3$ neophodno da se postigne željena rendgenkontrastnost ALBO-DENTA. Rendgenkontrastnost ALBO-DENTA je bila niža nego što je prethodno pronađeno za CS+30% Bi_2O_3 (~11 mmAl) i CS+25%Bi_2O_3 (6,9 mmAl) [28]. Rezultati istraživanja u vezi sa MTA (6,9 mmAl) slažu se sa prethodnim istraživanjima: 4,86, 6,74, 7,0, 7,5 i 8,0 mmAl [28–31]. PC nije ispunio ISO standard rendgenkontrastnosti, što je u saglasnosti sa prethodnim studijama (~0,9 mmAl) [28, 29]. Uticaj dodataka $SrCO_3$ na rendgenkontrastnost endodontske keramike nije do sada pominjan u literaturi. Razlike u rezultatima dobijenih vrednosti rendgenkontrastnosti potiču od različitih atomskih brojeva sastojaka [32]. Naime, atomski broj sastojaka je u direktnoj vezi sa apsorpcijom x-zraka. Atomski broj stroncijuma (Z = 40) niži je od atomskog broja bizmuta (Z = 83) i zbog toga je neophodno dodati više $SrCO_3$ kako bi se zadovoljili zahtevi ISO standarda. Ovo nema negativnu ulogu u slučaju $SrCO_3$ kao kod drugih dodatih rendgenkontrastnih sredstava (na primer bizmut) jer se stroncijum može smatrati ne samo biološki bezbednim već i biološki aktivnim sastojkom.

Biološka bezbednost stomatoloških materijala je od izuzetne važnosti. Zbog toga se *in vitro* i *in vivo* testovi rutinski primenjuju za ispitivanje citotoksičnosti materijala pre njihove upotrebe u kliničkoj praksi. Testovi citotoksičnosti su izvedeni na mešavini koja je pokazala odgovarajuću vrednost rendgenkontrastnosti (30% težinski dodatka $SrCO_3$). ISO 10993-5 propisuje da se material može smatrati necitotoksičnim ukoliko uzrokuje smrt manje od 30% ćelija u *in vitro* uslovima. U ovoj studiji korišćena su dva testa za ispitivanje citotoksičnosti: MTT, koji meri mitohondrijalnu aktivnost ćelija i KV, koji određuje broj adherentnih živih ćelija. Pokazano je da novi eksperimentalni cement ALBO-DENT pokazuje zadovoljavajuće ponašanje u ćelijskoj kulturi, uporedljivo sa PC i MTA. Dobijeni rezultati pokazuju značajno niži procenat živih ćelija kod tretmana MTA/PC-om nego u drugim istraživanjima (80–150%) [7, 33, 34], ali su u saglasnosti sa rezultatima drugih studija [35, 36]. Kod 1 : 2 i 1 : 4 razblaženja PC/MTA, KV i MTT testovi pokazali su slične rezultate i takođe su u saglasnosti sa podacima koji se navode u literaturi [7, 37]. Razlike u ćelijskoj vijabilnosti u različitim studijama mogu da proisteknu iz razlika u veličini korišćenih uzoraka (5 × 3 mm [37], 5 × 2 mm [12] i 5 × 1 mm [29]). Niže vrednosti za čiste ekstrakte PC i MTA mogu da budu predmet debate, ali su najverovatnije posledica visoke alkalnosti eluata u zatvorenom *in vitro* ćelijskom sistemu. Može se pretpostaviti da u situacijama u kojima je obezbeđen dotok tečnosti ovi materijali ne bi pokazali negativan efekat na okolno tkivo. U svakom slučaju, novosintetisani cement ALBO-DENT je pokazao superiornije osobine u odnosu na komercijalno dostupni ProRoot MTA.

Sa kliničke tačke gledišta, novi eksperimentalni materijal na bazi KS može da ima izvesne prednosti budući da dodatak stroncijuma može da poboljša zaceljenje koštanog tkiva nakon periapeksnog hirurškog zahvata aktivirajući osteoblaste [22, 23, 24]. Takođe, ukoliko se koristi za direktno prekrivanje pulpe, on može da stimuliše osteoblaste u smislu bržeg formiranja tercijarnog dentina. Ove pretpostavke bi trebalo da se potvrde upotrebom savremenih procedura koje su trenutno u toku.

ZAKLJUČAK

Novosintetisani cement na bazi KS sa 30% težinskim dodatkom $SrCO_3$ kao rendgenkontrastnog sredstva zadovoljava zahteve standarda ISO za kontrastnost. Biokompatibilnost novosintetisanog cementa na osnovu analize ćelijskog vijabiliteta merenjem broja adherentnih ćelija i aktivnosti mitohondrijalne dehidrogenaze je zadovoljavajuća i ukazuje na njegovu biološku bezbednost.

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