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CONTENT

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EFFECT OF VARIOUS RADIOPACIFIERS ON SELECTED PHYSICAL PROPERTIES AND CYTOTOXICITY OF CALCIUM SILICATE BASED DENTAL CEMENT ENRICHED WITH HYDROXYAPATITE

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ABSTRACT

This study aimed to investigate the influence of ZrO_2, Bi_2O_3 and SrF_2 added as radiopacifying agents (30wt.%) into calcium silicate/hydroxyapatite-based dental cement on its physical and biological properties. Among investigated cements, the mixture containing Bi_2O_3 had the highest values of elastic modules and toughness, similarly to control – mineral trioxide aggregate (MTA). SEM analysis of all hydrated cements has shown that bioactive calcite and tobermorite phases were formed. Crystal violet assay showed that pure (undiluted) extracts of experimental cements did not affect cell viability, while MTA exhibited an extremely cytotoxic effect on L929 cells. In 1:4 dilutions all experimental mixtures significantly increased cell proliferation potential after 72h in comparison to untreated cells and MTA, which cytotoxic effect diminished with dilutions. Further studies are needed to determine which radiopacifyer has the most desirable properties for adequate dental cement fabrication.

INTRODUCTION

Bioactive dental cements present a huge advantage in clinical practice since their interaction with host cells may provoke tertiary dentinogenesis [1]. The invention of calcium silicate-based dental materials revolutionized the treatment of pulp capping, apexification/apexogenesis, root canal perforations repair, and root end canal filling surgical procedures [2]. For instance, when used for direct pulp capping, the successful healing rate increased from ~35 % to more than 95 % [3]. Therefore, the research community has been putting significant effort to synthesize various calcium silicate-based dental cements intended for different applications. When formulating dental calcium silicate-based cement, it is imperative to add a radiopacifying component into its composition to allow its visibility on radiographs [2]. The addition of radiopacifiers to dental cements should increase their radio-visibility, but also maintain or even improve their mechanical characteristics. In our previous studies, we have enriched calcium silicate cements with nano-containing hydroxyapatite (CSC-nanoHAP), a known constituent of human bone/tooth [4]. Hydroxyapatite may be a useful compound of dental cement due to its similarity with bone tissue, but on the other side it is known as a brittle material and therefore the physical properties of resulting mixtures may be hampered [5]. Indeed, in some studies, it was shown that hydroxyapatite increases the solubility of calcium silicate/hydroxyapatite mixture [6]. We have evaluated the compressive strength of such a mixture enriched with zirconium oxide (ZrO_2), bismuth dioxide (Bi_2O_3), and strontium fluoride (SrF_2) as radiopacyfing agents [4]. This study went one step further aiming to evaluate elastic modules and the toughness of those formulations. In addition, we investigated the morphology of cement surface and their cytocompatibility.

METHODS

Material synthesis - CSC-nanoHAP was used as a basis for mixing with ZrO_2, Bi_2O_3 and SrF_2. Detailed procedure for CSC and nanoHAP synthesis is given elsewhere [4,5].

Elastic modules and toughness determination – The elastic modules and the toughness of experimental cement specimens (2 cm in length, 4 mm in depth and 4 mm wide) were determined in accordance to International Standard Organization (ISO) 6876 using the universal testing machine (Instron, Norwood, USA, loading rate 50 N/min). For the calculation of elastics modulus, the slope of the tangent to the initial straight line portion of the load deflection curve was drawn and the modulus of elasticity was calculated using the following equation:

$$E = (L^3 \times m)/(4 \times b \times d^3)$$

where E = modulus of elasticity in bending (Nm^{-2}), L = the length of the specimen (m), m = the slope of the initial straight-line portion of the load deflection curve (Nm^{-1} of deflection), b = the width of specimen (m) and d = the depth of the specimen (m).

The toughness was calculated by using area underneath the stress–strain (σ–ε) curve:

U_T = Area underneath the stress–strain (σ–ε) curve = σ × ε.

Scanning electron microscopy (SEM) analysis – Specimens measuring 5 mm in diameter and 1 mm in height were immersed in phosphate buffer saline (PBS) and placed in an incubator at 37°C in a humidified atmosphere with 5 % CO_2 for 14 days. Afterward, the specimens were dried, gold coated, and visualized under scanning electron microscopy (TESCAN, Mira3, XMU USA Inc.).

Cell viability assay – In order to evaluate a dose- and time-dependent response to potentially toxic soluble substances from investigated materials, cell viability was carried out in accordance with the ISO Standard 10993-5/2005 [7]. All materials were manipulated under sterile conditions. Mouse fibroblast L929 cell line (European Collection of Animal Cell Cultures, Salisbury, UK) was cultivated in complete medium, maintained at 37°C in a humidified atmosphere with 5 % CO_2, prepared for experiments using the conventional trypsinization procedure and seeded in 96-well flat-bottom plates (5×10^3 cells/well). Cells were treated 24 h post-seeding with pure extracts of investigated materials (1) and its serial dilutions (1:2 and 1:4 (v:v)), prepared exactly as previously described [4]. Crystal violet (CV) test, based on the inability of dead cells to remain adherent, was assessed after 24, 48 and 72 h treatment. 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. The absorbance of dissolved CV dye, corresponding to the number of adherent (viable) cells was measured in an automated microplate reader at 570 nm (Sunrise; Tecan, Dorset, UK). The results were presented as a fold increase in the number of viable cells. The experiments were performed in triplicates.

RESULTS AND DISCUSSION

CSC+HAP+ZrO_2 and CSC+HAP+SrF_2 mixtures showed significantly lower values of elastic modules (260±70 MPa and 190±40 MPa, respectively) than CSC+HAP+Bi_2O_3 (420±80 MPa) and control material – MTA (1290±90 MPa) ($p<0.05$, ANOVA and post+hoc Tukey test). Toughness analysis showed that CSC+HAP+Bi_2O_3 (0.32±0.05 J) was not significantly different than commercially available MTA (0.38±0.05 J) ($p>0.05$), while CSC+HAP+ZrO_2 (0.15±0.06 J) and CSC+HAP+SrF_2 (0.08±0.01 J) had statistically lower toughness values ($p<0.05$, ANOVA and post-hoc Tukey test). Taking into account the obtained physical data it seems that Bi_2O_3 is radiopacyfier of choice to formulate adequate dental material (**Figure 1A**).

SEM analysis of the investigated cements confirmed their bioactivity after soaking in PBS. All the investigated cements exposed the polygonal structures on surfaces, indicating deformation of

tobermoritte phase (calcium silicate hydrate-CSH) (**Figure 1B**). These results showed that hydroxyapatite addition to calcium silicate-based cement does not alter their capability to form biologically active crystals on the surfaces. The purpose of these crystals is to form a thin layer of hydroxyapatite in contact with dental tissue which in turn stimulates odontoblasts for dentinogenesis [1]. However, we believe that the experimental cements may be even more biologically active due to already incorporated hydroxyapatite. This assumption should be confirmed in further biological studies.

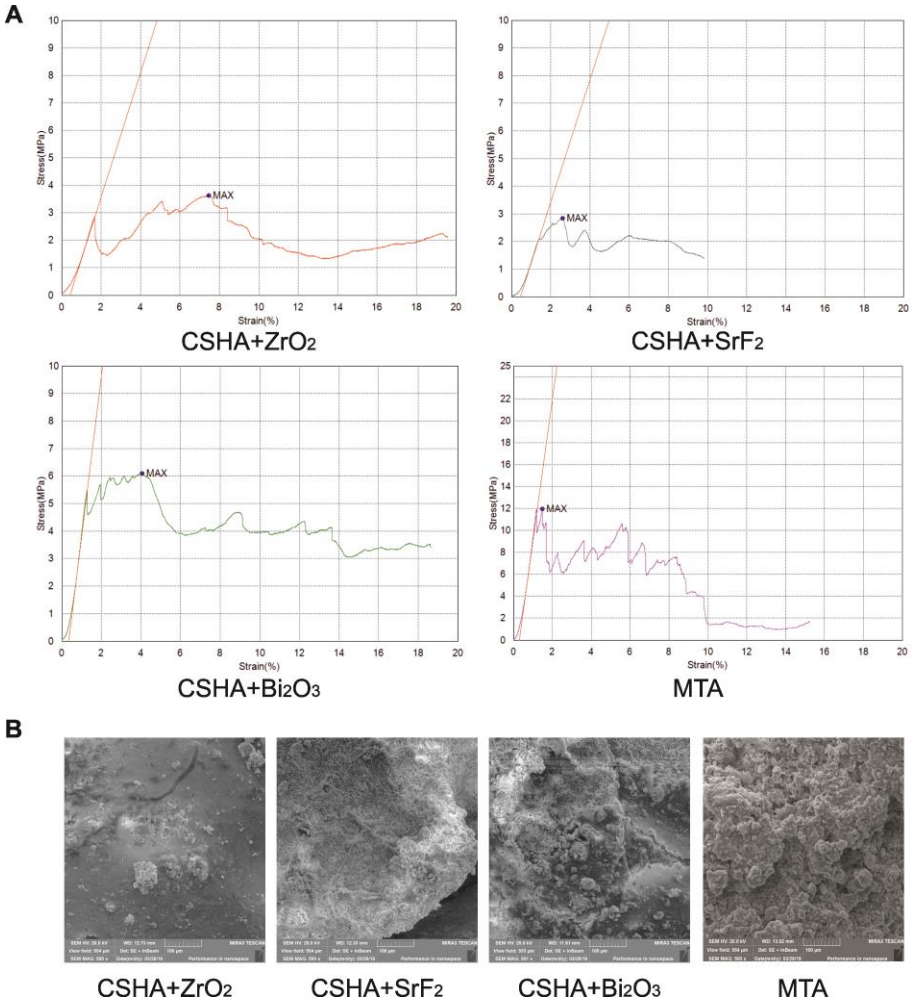

Figure 1. A) Elastic modules and toughness of cements - representative slopes of stress *versus* strain. B) SEM micrographs of the cements surface after soaking in PBS (magnification 500×).

Extracts of experimental cements had no effect on the cell viability after 24 h incubation, while pure extract of MTA exhibited extremely cytotoxic effect on L929 cells, at all time points, which diminished with dilutions. Longer incubation (48 h, 72 h) with ECHA+ZrO_2 and ECHA+Bi_2O_3 extracts (1 and 1:2) decreased cell proliferation rate, but 1:4 dilutions of all experimental cement increased cell proliferation compared to MTA and untreated cells, after 72 h treatment (**Figure 2**).

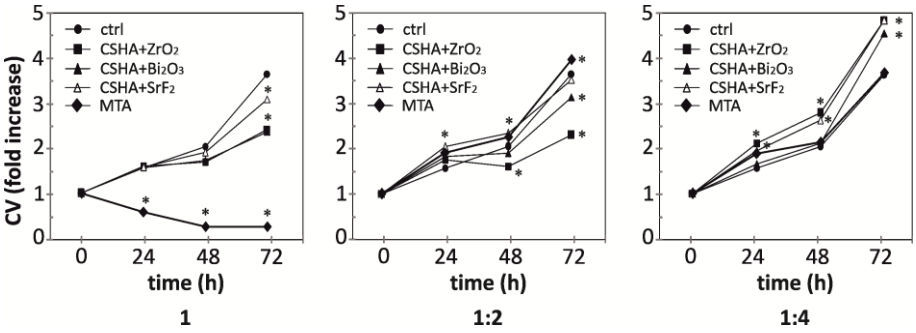

Figure 2. Cell viability (fold increase) evaluated by the crystal violet (CV) assay after 24 h, 48 h, and 72 h exposure of L929 cells to the cements' eluents - pure extract (1) and serial dilutions (1:2 and 1:4 (v:v)). The data are mean of triplicates from a representative of three independent experiments. * $p<0.05$ compared to control, untreated cells (ctrl).

CONCLUSION

Calcium silicate dental cement enriched with hydroxyapatite may be promising material in dentistry. This study has shown that, among investigated radiopacyfing agents, Bi_2O_3 may be considered as a radiopacyfier of choice due to superior physical properties (elastic modulus and toughness) when compared with ZrO_2 and SrF_2. On the other hand, when biocompatibility is taken as selection criteria, SrF_2 may be considered the most promising radiopacifying agent. Further studies should be conducted to examine other physicochemical and biological properties of manufactured cement mixtures to determine which radiopacifyer presents the most desirable properties for adequate dental cement fabrication.

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