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GENOTOXIC EFFECT OF NEWLY SYNTHESIZED NANOMATERIALS FOR POTENTIAL DENTAL APPLICATION

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Abstract: Introduction: Biocompatibility is the property of a material to fulfill its function in the body without causing harmful side effects. To confirm biocompatibility, all newly synthesized materials must undergo numerous in vitro and clinical tests. The starting point is cytotoxicity and genotoxicity tests. This study aimed to determine the genotoxicity of newly synthesized nanomaterials based on calcium aluminates and calcium silicates with additional hydroxyapatite.
Material and methods: The research was conducted at the Institute of Oncology in Belgrade. An alkaline version of the Comet test was used, following the recommendation of the international standard ISO / DIS 10993-3, and MRC-5 (normal human lung fibroblasts) was used as a cell line. Cells were grown in monolayer culture, in a complete nutrient medium, at a temperature of 37 °C in air enriched with 5% CO_2 and saturated water vapor. The genotoxicity of calcium aluminate and mixtures of hydroxyapatite and calcium silicate was investigated. and untreated cells were used as controls. A 40x lens on a fluorescence microscope was used to visualize the DNA damage. Comet Assay IV (Perceptive Instruments) was used to statistically process the results.
Results: ALBO-CA extract did not show a genotoxic effect, that is, the percentage of DNA damage at the highest applied concentration of cement of 100 mg / mL, was 9.6%. ALBO-CS-HA extract showed genotoxic potential at concentrations ≥ of 25 mg / mL
Conclusion: Nanostructured calcium aluminate did not show genotoxic potential on human lung fibroblasts, unlike ALBO-CSHA, with recommendations for further studies.

Keywords: genotoxicity, nanomaterials, calcium aluminates, calcium silicates

1. INTRODUCTION

Calcium aluminate cement has been successfully used for over a hundred years in the construction industry. The advantages of these materials to calcium silicate cement are abrasion resistance, initial strength, and resistance to various chemical agents [1]. In the last 20 years, more intensive synthesis and application of calcium aluminate-based biomaterials in restorative dentistry, and endodontics, but also as a carrier of materials for drug delivery has begun. Accordingly, numerous in vitro and clinical studies have been conducted to examine their biocompatibility, bioactivity, and antibacterial and mechanical properties [2-7]. Aluminous cement is composed of Al_2O_3 (43%), CaO (19%), water (15%), and ZrO_2 (19%), while in smaller concentrations they contain magnesium, silicon, iron, and titanium [7]. Due to the reduced porosity, the materials show good mechanical properties, compressive strength values range from 50-250 MPa, while the thermal properties are close to the properties of dental tissues [2]. However, some studies indicate

material solubility and water absorption of calcium aluminate-based nanostructured material was significantly higher compared to calcium silicate (CS) and MTA [3]. The binding reaction of calcium aluminate cement is started by mixing powder and water, and like calcium silicates, they are included in hydraulic cement. By changing the powder/liquid ratio and the addition of certain additives, the setting time of these materials can be controlled from short periods (several hours) to significantly longer ones (day and week), and rheological properties and radiocontrast can also be improved [8-14].

Calcium silicate cement is a material known for its exceptional biological and adequate physical properties. However, these materials have a long setting time and rinsing from the application site.

Calcium phosphate cement is also a much-researched material, however, although biocompatible they have poor mechanical properties, which prevents their independent endodontic application [15,16]. Nanostructured biomaterials based on calcium aluminate and calcium silicate hydroxyapatite were created to overcome the known shortcomings of calcium silicate cement [6, 17].

This study aimed to examine the genotoxicity of recently synthesized nanomaterials based on calcium aluminate and calcium silicate hydroxyapatite intended for potential dental use.

2. MATERIAL AND METHODS

The research was conducted at the Institute of Oncology in Belgrade. The Comet test was applied, following the recommendation of the international standard ISO / DIS 10993-3 (Test for genotoxicity, carcinogenicity, and reproductive toxicity). (Normal human lung fibroblasts) Cells were grown in monolayer culture, in a complete nutrient medium, at a temperature of 37 °C in air enriched with 5% CO_2 and saturated water vapor. Comet or agarose gel single-cell electrophoresis is a rapid and sensitive method for the examination of DNA integrity at the level of a single cell. It is a technique for rapid detection of damage and monitoring of repair in the DNA molecule. It is used on all cell types except erythrocytes and is most often tested on peripheral blood lymphocytes. In practice, neutral and alkaline versions of the Comet test are performed. For our research, we applied the alkaline version, adhering to the protocol according to *Singh et al. (1988)* [18].

2.1. Tested Materials

We examined the genotoxicity of calcium aluminate systems (CA) and mixtures of hydroxyapatite and calcium silicate systems (HA-CS).For the synthesis of the active calcium aluminate system, it was necessary to first synthesize individual components: calcium aluminate ($CaOAl_2O_3$), calcium carbonate ($CaCO_3$), and monocyclic Bi_2O_3 or $BaSO_4$ (as an X-ray contrast). The calcium aluminate phase was synthesized using $CaCl_2$ * $5H_2O$ and the aluminate salt (AlOOH) was obtained by hydrothermal treatment. Aluminum2-butoxide is dissolved in a mixture of ethanol and water (in a ratio of 1: 4). This mixture was then heated to 85 °C with vigorous stirring. After 2 hours of heating, the solution was cooled to room temperature, and H_2SO_4 was added. The resulting solution was mixed with a stoichiometric amount of $CaCl_2x5H_2O$ (Merck, Germany) and transferred to an autoclave, where it remained for the next 5 hours at a temperature of 150 ° C and a pressure of 5 bar. The resulting gel was dried at 150 °C for evaporation of water. Calcium chloride tetrahydrate ($CaCl_2x4H_2O$) (Sigma-Aldrich, St. Louis, MO) was used as a precursor in the synthesis of calcium carbonate. The amount of 5 mmol $CaCl_2x4H_2O$ is dissolved in 50 ml of ethylene glycol (Sigma-Aldrich) by ultrasound at 40 °C (Elmasonic S30H). Then, 10 mmol of $NaHCO_3$ was dispersed in 50 ml of ethylene glycol dropwise over 30 minutes with mechanical stirring. The resulting dispersion was then heated at 30 °C for 30 min. Calcium carbonate was then separated from the murky solution by centrifugation (3.4 g, 30 min), washed several times in a mixture of water and ethanol (1: 4), and finally, only in water. Sulphonyl dodecyl sulfate (0.5%) was added as an agglomeration agent. The resulting nanoparticles were then exposed to ultrasound for 30 min, with strong mechanical stirring for 5 hours. The resulting powder, after drying at 120 °C for 5 hours, was heated to 500°C for one hour to give a calcium carbonate phase. Monocyclic Bi_2O_3 was produced by calcination Bi $(NO_3)_3$ (Chemical, Croatia) at 450 ° C for 20 hours. This procedure was performed to obtain a stable tetragonal Bi_2O_3 phase, saturated with oxygen. In addition to Bi_2O_3, $BaSO_4$ is also used as a contrast agent. The new calcium aluminate called ALBO-CA was obtained by mixing $CaCO_3$, and Bi_2O_3 or $BaSO_4$ with a calcium aluminate phase in a ratio of 2: 2: 1. This mixture was mixed with water (water: powder 1: 2) to obtain

the consistency of the paste. For the synthesis of the active calcium silicate system, the individual components were first synthesized: calcium silicate phase 2β-$CaSiO_4$ (βC2S) and $Ca_3SiO_{5,}$ (C3S) and calcium carbonate ($CaCO_3$). The synthesis of calcium carbonate proceeded in the manner described above. The calcium silicate phase was synthesized using $CaCl_2$ x $5H_2O$ (Merck, Germany), and the silica salt was obtained by hydrothermal treatment. Stoichiometric amounts of $CaCl_2$ x $5H_2O$ (35.59 g), silica salt (15 g 30% salt solution), and C2S: C3S in a ratio of 2: 1 were used to obtain the silicate active phase (40% contained in the mixture). 4.55 g of Al ($C_2H_3O_2$) was added to this mixture to provide small amounts of the active C3A phase (3.01%). As an oxidizing agent, to initiate the combustion reaction, 71.3 g of ammonium nitrate (NH_4NO_3) and 53.51 g of citric acid ($C_6H_8O_7CH_2O$) were added to the mixture, which was used as fuel during the combustion reaction. After drying at 80 °C to obtain the gel, all the samples were subsequently dried at a temperature of 150 °C to remove all the water between silica particles. In the next phase, the increase in temperature to 180°C caused the gel to burn. The gel gradually turned into a foam, in the end, there was strong self-sufficiency of the reaction of combustion with the release of a large number of gases. The rapid release of large quantities of gas products during combustion led to the dissipation of the heat of combustion and the temperature growth threshold, which reduced the possibility of early, partial synthesis of the primary particles, which is important for maintaining the final activity of the mixture. After such a high temperature and self-expansion of the fraction, the samples were quickly dried using copper plates to minimize the crystallization phase and obtained high reactivity of the obtained β-C2S and C3S phases. The freed black powder contained carbon residues and its calcification was continued at 650°C for 4 hours to finally produce a product with a small crystallite size. After this thermal treatment, the powder is further ground to obtain the silicate phases to be used in the final cement mixtures. In addition to the β-C2S and C3S phases, which comprised 60% of the total amount of the mixture, additional components were used, such as calcium carbonate ($CaCO_3$) or dehydrated gypsum in the amount of 20% and BaSO4 (Merck, Germany) also in an amount of 20% due to the X-ray contrast of the mixture. The composition of ALBO-CSHA was: 40% hydroxyapatite 20% mixture of C2S and C3S in a ratio of 2:1, 20% calcium carbonate ($CaCO_3$), and 20% $BaSO_4$ (Merck, Germany). This mixture, after grinding, and better homogenization is mixed with water (water: powder in a ratio of 1: 2) to obtain cement paste.

2.2. Reagent Preparation

Reagents:
Disodium EDTA
Ethidium Bromide
PBS (Ca ++, Mg ++ free)
Sodium chloride (NaCl)
Sodium hydroxide (NaOH)
Triton X-100
Trisma Base
Procedure:
1. Lysis buffer: per 1000 mL: 2.5 M NaCl 146.1 g
100 mM EDTA 37.2 g
10 mM Trisma base 1.2 g

All ingredients were added to 700 mL of distilled H2O. After stirring the mixture thus obtained, 88 mg of NaOH was added and left for about 20 min to dissolve. The pH was adjusted to 10, using concentrated HCl, and then made up to 990 mL with distilled H2O. Storage at room temperature followed.

Final lysis buffer: Fresh 1% Triton X-100 was added and left in the refrigerator for 30 minutes before treating the samples on slides.

2. Electrophoresis buffer
(300 mM NaOH / 1 mM EDTA):
Stock solutions:
1. 10 N NaOH (200 g / 500 mL dH_2O)
2. 200 mM EDTA (14.89 g / 200 mL dH_2O, pH 10)
They were stored at room temperature.

For 1X buffer (which was prepared fresh before each electrophoresis): per 1 liter, 30 mL of NaOH and 5.0 mL of EDTA were added, made up to 1000 mL with vigorous stirring. Before use, we made sure to check the pH of the buffer, which had to be> 13.

3. Neutralization buffer: 0.4 M Tris - 48.5 g was added to 800800 mL of distilled H2O, the pH was adjusted to 7.5 with concentrated (> 10 M) HCl: q.s. to 1000 mL with distilled H_2O and stored at room temperature.

4. Staining solution:

Ethidium Bromide (EtBr; 10X stock - 20 µg / mL): 10 mg in 50

mL of distilled H_2O and kept at room temperature. For 1X stock - 1 mL of 10X stock was mixed with 9 mL of distilled H_2O.

EtBr has been handled with special caution, as it is a known carcinogen.

2.3. Preparation of Slides
For Scge / Comet Test

Material:
Normal spot agarose (NMA)
Low Melting Point Agarose (LMPA)
Methanol
Cover glass (No. 1, 24 x 60 mm)
Microcentrifuge tubes
Micropipette
Microscopic slides
Microgel electrophoresis (MGE) slides
Koplin jars (opaque)
Horizontal gel electrophoresis apparatus
Power Supply Electrophoresis

Preparing slides

1. First prepared 1% (500 mg in 50 ml PBS) and 0.5% LMPA (250 mg in 50 ml PBS) and 1.0% NMA (500 mg in 50 ml in Milli Q water). This was followed by heating them in the microwave oven almost to boiling until the agarose dissolved.

2. LMPA samples of 5 mL each were stored in the refrigerator, until the moment of their use when they were dissolved in the microwave oven. Thereafter, these samples were placed in a water bath at 37 ºC, until temperature stabilization was achieved.

3. The slides were immersed in methanol, and then we passed over the burner to remove any remaining oil and dust.

4. While the agarose was warm, the slides were immersed in a third of the polished part and gently removed. We wiped their underside to remove agarose, placed them on a flat surface to dry, and then stored them at room temperature.

2.4. Cell Treatment

Cell culture (monolayer culture)

The comet test was performed on the MRC-5 cell line (normal human lung fibroblasts). Immediately after removal of the medium, 0.005% trypsin was added (high trypsin concentration increases DNA damage). The cells were kept at a temperature of 37 ° C for 5 minutes until they separated or detached from the medium. FCS medium was then added to stop the enzymatic action of trypsin and the density was adjusted to 1x106 cells per mL.

5–10 µl of cells were mixed with 75 mL of LMPA, and further work was continued according to the protocol for the Comet test.

2.5. Microgel Slide Electrophoresis

Electrophoresis was performed under alkaline conditions (pH> 13). The following procedure was performed:

• Incubate for 2 hours at 4 ° C, then gently remove the slides from the lysis buffer and place them in the tub as close to each other as possible.

• Fill the tub tank with fresh electrophoresis buffer so that the liquid completely covers the slides.

• Leaving the slides for 20 minutes an alkaline electrophoresis buffer, led to the unfolding of DNA at the sites of alkaline labile damage.

• Voltage was set to ~ 0.74 V / cm and current to 300 and electrophoresis was performed for 30 minutes.

• After the power is turned off, the slides are gently removed and placed obliquely in the neutralization tray. The neutralization buffer was pipetted and left for 5 minutes. This was followed by a repeat of the neutralization, two more times.

• Slides were stained with 80 µL 1X ethidium bromide. The paint was left for 5 minutes, after which the slides were immersed in cold distilled water, to remove excess paint. Then, a cover plate was placed on the slide, and scoring was done immediately.

• The slides were drained, kept for 20 min in ice-cold 100% ethanol, air-dried, and stored in a dry place.

• The slides were rehydrated with cold distilled water and stained with ethidium bromide, as in step 6. After scoring, the cover plate was removed, and the slide was washed in 100% alcohol, dried, and preserved for archiving.

Steps 1-4 were performed under dim yellow light, to prevent DNA damage by white fluorescent light.

2.6. Evaluation of Dna Damage

A 40x lens on a fluorescent microscope was used to visualize the DNA damage.

Although many image analysis systems are suitable for quantifying SCGE data, we used the Comet 5 image analysis software (Kinetic Imaging, Ltd. Liverpool, UK) connected to a CCD camera to quantitatively and qualitatively assess DNA damage, and measure DNA migration length and migrated percentage. DNA. In general, 50-100 randomly selected cells were analyzed per sample.

2.7. Statistical Data Processing

Comet Assay IV (Perceptive Instruments) was used to statistically process the results of the Comet test.

3. RESULTS

The Comet test shows the genotoxic effect of substances on cells, ie. the percentage of DNA damage to cells at different concentrations of substances. In this study, calcium aluminate cement (ALBO-CA) and calcium silicate hydroxyapatite (ALBO-CS-HA) were examined, whose genotoxicity was analyzed on MRC-5 line cells, i.e. cells of normal human lung fibroblasts. The genotoxicity of the tested substances was expressed by the percentage of the "tail" length that is created from the spherical shape of the fibroblast cell in the electrophoretic gel (Figure 1).

An alkaline version of the comet test with a black-and-white image display variant was applied. The white field on the micrographs is a hydro blast cell, the comet-shaped tail is a damaged DNA mole-

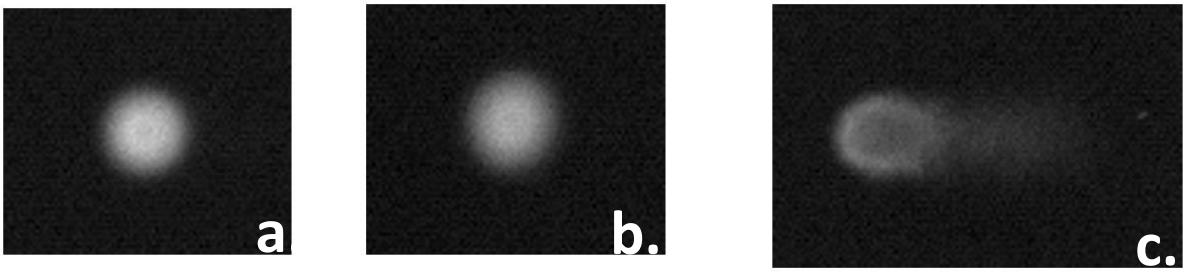
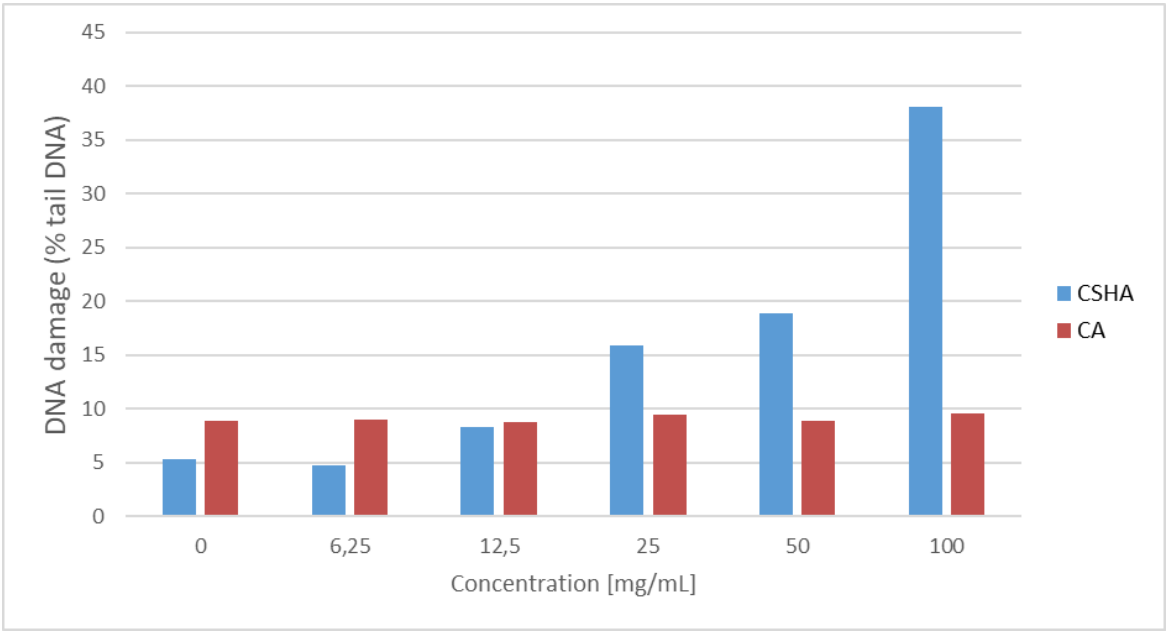


Figure 1. *Micrographs of snail fibroblast cells: a. control, b. treated with ALBO-CA substance and c. treated with ALBO-CS-HA substance. Comet test, 200x magnification*



Graph 1. *Percentage of DNA damage (% DNA in comet tail) depending on the applied concentration of material*

cule, and the background is black. Calcium aluminate cement extract ALBO-CA did not show a genotoxic effect, that is, the percentage of DNA damage, even at the highest applied cement concentration of 100 mg / mL, was 9.6% (Figure 1b, Table 1, Graph 1). It remained at the level of DNA damage percentage as in untreated control cells of normal lung fibroblasts ≤ 10% (Figure 1a). Calcium silicate hydroxyapatite cement extract ALBO-CS-HA showed genotoxic potential at concentrations ≥ 25 mg / mL (Figure 1c, Table 1, Graph 1): at 25 mg / mL, it was 15.9%, at 50 mg / mL it was 18 .9% and at 100 mg / mL is 38.1%. Because the percentage of DNA damage is higher than 10% at concentrations ≥ 25 mg / mL, the substance ALBO-CS-HA has a genotoxic effect on lung fibroblast cells.

Table 1. DNA damage (% DNA in comet tail)

C(mg/mL)	ALBO-CS-HA	ALBO-CA
0	5,3	8,8
6,25	4,7	9
12,5	8,3	8,7
25	15,9	9,4
50	18,9	8,9
100	38,1	9,6

4. DISCUSSION

The absence of genotoxicity is one of the most important features of biocompatibility. Genotoxic damage does not necessarily lead to cell death. They can damage the cellular genome and consequently significantly reduce tissue reparation capacity, which in the long run will result in the development of neoplasms [19].

As part of the study of the genotoxic potential of calcium aluminate systems, we opted for the application of the Comet test, thanks to its many advantages over other genotoxic tests.

The comet test or agarose gel single-cell electrophoresis is a fast and sensitive method for examining the integrity of DNA at the single-cell level. It is a test that allows us to quickly detect damage and monitor reparations in the DNA molecule. It is used on all cell types, except erythrocytes, which is very important, because genotoxic and gene-protective effects can be specific for a certain type of cells and tissues [19,20].

The advantages of this test are fast analysis, the simplicity that does not require a large number of cells per sample (<10,000), high sensitivity of the test, good price, applicability to almost all eukaryotic cells (proliferating and non-proliferating); flexibility, ie the possibility of modifying the protocol to detect different types of DNA damage: by changing the conditions of electrophoresis and using different lesion-specific enzymes. Namely, it is known that in addition to single-stranded, double-stranded breaks, and purine sites, there are other types of DNA damage such as oxidized bases and UV-induced dimers [20].

Initially, a neutral Comet test was performed that could only be used to analyze double-stranded DNA breaks. *Singh et al. (*1988) developed an alkaline version of the Comet test (the alkaline single-cell gel electrophoresis), which allowed the analysis of both single-stranded and double-stranded DNA breaks, as well as alkaline labile sites (basic sites that lag after removal of damaged bases) [18].

When DNA damage occurs, the noose relaxes. During electrophoresis, an electric field pulls out loose loops and broken ends toward the positively charged anode. Determining the relative amount of DNA that has traveled along the gel allows simple measurements of DNA breaks at the level of individual cells [21]. Former cell nuclei and broken fragments stretched in an electric field form the "head" and "tail" of a comet, hence the name Comet Test [21].

When measuring comets, an epifluorescence microscope is used, which is connected to a computer, and with the help of special software, certain parameters are measured: tail length and% of DNA in the tail [19-21]. The length of the tail is determined by the length of DNA migration and is directly related to the size of the DNA fragment, ie the degree of damage. The comet test does not provide insight into the size of DNA fragments, because the fragments were not separated during electrophoresis. However, increasing the number of DNA breaks leads to greater relaxation of the DNA loop, and as a result, more free ends can migrate and a larger amount of DNA travels to the comet's tail. The length of a comet's tail is calculated from the middle of the head to the end of the tail and indicates the amount of DNA fragment, which directly points to the part of the genome that is affected by the damage. The percentage of DNA in the tail is the most reliable parameter of the Comet test [19-21].

When examining the genotoxic potential of a material, the DNA is first exposed to its action and, if DNA damage is found, its quantification is then approached. If the genetic damage is small, the DNA molecule stretches during electrophoresis. As the number of lesions increases, parts of the DNA molecule travel freely and build the comet's tail, until an extreme case (apoptosis) in which the comet's head and tail are separated [19-21].

The genotoxic potential of calcium aluminate cement (CA) and calcium silicate hydroxyapatite (CS-HA), a material that previously exhibited cytotoxic activity, was examined using an alkaline version of the Comet assay on the MRC-5 cell line (normal human fibroblasts).

Experimental nanostructured biomaterial based on CA did not show genotoxic potential, ie the percentage of DNA damage at all applied concentrations is at the level of untreated control cells, which is in line with a previous study by *Janković et al. (2020)* which the same material confirmed cytocompatibility using MTT test on human lung fibroblasts [4], as well as with the findings of *Čolović et al.* (2019) who confirmed the absence of genotoxicity of nanostructured calcium aluminate cement using the Comet test [2].

Fernandez et al. (2018) evaluated the genotoxicity and hemocompatibility of the new calcium aluminate-based cement, EndoBinder (EB) (Binderware, São Carlos, SP, Brazil) compared to MTA and AWMTA (Angelus, Soluções Odontológicas, Londrina, PR, Brazil). To assess genotoxicity, a comet assay was applied to Chinese hamster ovary (CHO) cells grown for 24 h in Dulbecco's modified eagle medium incubated with each of the cement for 24 h at 37 ° C. The percentage of DNA damage in the head and tail was analyzed. Both materials showed higher hemolytic activity than that established by international standards, while induction of DNA damage was not observed [22].

The results, which are somewhat comparable to the findings of this study, when it comes to CA cement, were derived from an extensive study by *Pameier et al. (2008).* In the complex research of these scientists, among other things, the mutagenicity of calcium aluminate cement Ceramir® Crown & Bridge (formerly XeraCem ™) was performed using the Ames test (reversible mutation test). According to the results of their study, Ceramir C&B did not show mutagenicity. The absence of the mutagenic effect of this calcium aluminate cement may be due to its composition, because Ceramir® Crown & Bridge is a hybrid material composed of calcium aluminate and glass ionomer cement, and the biocompatibility of glass ionomer cement has long been known [23].

Brazilian scientists *Kido WH. et al. (2014)* using the Comet test proved the absence of the genotoxic effect of aluminate scaffold with the addition of bioglass (BG) and hydroxyapatite (HA) on blood, liver, and kidney cells, and after the incorporation of this material into rat bone tibia defects. The authors explain this result by the increased degradation of BG and HA, which consequently stimulated osteogenesis by attracting osteoprogenitor cells and creating an osteoblastic matrix, and thus new bone [24].

Ashraf A. Eid et al. (2013) evaluated changes in mRNA levels by monitoring gene expression (RUNX2, OCN, BSP, and DMP1) associated with osteogenic/dentinogenic differentiation and mineralization, alkaline phosphatase production, and extracellular matrix mineralization (Alizarin red S). There was no statistically significant difference between the experimental calcium aluminosilicate cement (Quick-set) and WMTA. Such a good result of calcium aluminosilicate cement (Quick-set) is probably due to the method of its production during which the cationic surfactant was removed from the liquid gel component, which was considered to interfere with biocompatibility [25].

In an experimental in vitro study, *Aminozarbian et al. (2012)* compared the genotoxicity of three calcium aluminate cement: α-calcium-aluminate cement (CAAC), α-plus calcium aluminate cement (CAAC plus), mixtures of wollastonite and CAAC cement (WOLCA), and MTA using the Comet test on human gingival fibroblasts. MTA had the lowest values of DNA damage compared to others, although CAAC cement showed DNA damage similar to MTA. CAAC Plus and WOLCA had significantly higher genotoxicity than MTA, however, the values obtained for CAAC cement were comparable to MTA suggesting its possible use in vivo [26].

Interestingly, in two studies, alumina, one of the constituents of the calcium aluminate cement tested here, showed a genotoxic effect.

Balasubramanyam et al (2009) examined genotoxic effects on peripheral blood cells of rats exposed to alumina. Their results indicated that the alumina compound, in vivo, showed dose-depen-

dent genotoxicity. Also, Al can affect the structure of chromatin and lead to DNA damage. The second mechanism is supported by observations that the interaction of cells with Al can lead to the formation of ROS. In addition, A1 can improve the permeability of the lysosomal membrane and inhibit the lysosomal proton pump, which can lead to the release of DNA from the lysosome. DNA introduced into the cytoplasm by electroporation is a potent inducer of cytogenetic damage. All may induce genotoxicity by one or more mechanisms. Further studies are certainly needed, but the authors suggest that the genotoxicity of alumina compounds is more pronounced at higher doses [27].

The Hashimoto et al study (2015) negatively evaluated the biological responses of cultured macrophages (RAW264) to alumina nanoparticles (Al2O3NPs) and silica nanoparticles (SiO2NPs) by cytotoxicity analysis (WST-8). They concluded that concentrations of aluminum and silicon oxide above 200 g / mL were large enough to induce cytotoxic and genotoxic effects on cells [21]. The size of the alumina and silica nanoparticles (13 nm) used in this study is smaller than those used in other cytotoxicity studies in the literature. The authors point out that there is a size-dependent induction of nuclear NP penetration, which penetrates the pores to a greater extent than larger NPs, leading to the promotion of cytotoxicity and genotoxicity. Also, the NP concentrations in this study (200 and 400 g / mL) are relatively high, which allowed for easy obtaining of cytotoxic and genotoxic effects and micromorphological changes of cells, but it is certainly necessary to do additional research measuring the effect of NP on cells in low concentrations (below 100 g / mL) [21].

Aluminum oxide is, it is true, only one of the ingredients that are part of the calcium aluminate cement tested in this research. It should be borne in mind that for the synthesis of the active calcium aluminate system, which was the subject of this study, it was necessary to first synthesize individual components: calcium aluminate ($CaOAl_2O_3$), calcium carbonate ($CaCO_3$), and monocyclic Bi_2O_3 or $BaSO_4$ (as X-ray contrast). The calcium aluminate phase was synthesized using $CaCl_2x5H_2O$, and the aluminate salt (AlOOH) was obtained by hydrothermal treatment. Monocyclic Bi_2O_3 was produced by calcination of Bi $(NO_3)_3$ (Kemika, Croatia) at 450 ° C for 20 hours. This procedure was performed to obtain a stable tetragonal Bi_2O_3 phase, saturated with oxygen. The explanation for the absence of genotoxic activity of the investigated nanostructured CA could lie precisely in its chemical composition and the way of its synthesis by the process of nanotechnology, the combination of the sol-gel method, and the self-burning wave method. The salt gel method enables the target product to be obtained on a specific surface of 7 to 120 m^2 g-1, improves the properties of the material, and contributes to the reduction of mass, and the increase of functionality and stability of the material [28]. According to *Chen et al. (2011),* materials obtained by sol-gel processes are more bioactive compared to materials obtained by other synthetic methods [29].

Experimental CS-HA, unlike CA, exhibited genotoxic activity. When using the Comet test in CS-HA, only concentrations of 5 and 10 mg / mL caused DNA damage at the control level, while increasing concentrations resulted in significantly greater damage. The genotoxicity of CS-HA was somewhat expected, given that this material exhibited a cytotoxic effect, during a previously performed MTT assay.

Furthermore, the dependence of DNA damage on the applied concentration of CS-HA material was established. As expected, the highest percentage of DNA damage (38.1%) was recorded at the highest applied concentration of material (100 mg / mL), while the lowest percentage of DNA damage (4.7%) was found at a CS-HA concentration of 5 mg / mL.

In the study, Opačić Galić et al. (2013.) experimental CS-HA, composed of CS (34%) and HA (66%) and produced by hydrothermal sol-gel method showed the genotoxic effect on donor 2 lymphocytes. The authors examined the genotoxic potential of the material using the Comet test on human peripheral blood lymphocytes. Genotoxicity of CS-HA on donor 2 lymphocytes was confirmed at all tested concentrations except the lowest (0.01 mg / mL). Interestingly, the genotoxic effect of CS-HA was absent on donor lymphocytes 1, and even the highest applied concentration of CS-HA (10 mg / mL) resulted in a decrease in the percentage of DNA damage in donor lymphocytes 3. The reason for these differences in genotoxic expression The effect of CS-HA, according to the authors, could be the different sensitivity of cells obtained from three different donors, which have different genetic bases, and thus different DNA repair capacities [30].

Khalil and Eid (2013.) investigated and compared the systemic toxicity of BioAggregate, trical-

cium silicate cement with the addition of hydroxyapatite and MTA on the liver and kidneys, after 7 and 30 days. They concluded that MTA has significantly more serious harmful effects on the liver and kidneys than BioAggregate but without permanent damage. As a possible explanation, the authors state that MTA contains heavy metals and releases arsenic. During the surgery, arsenic came into contact with the blood and reached the liver and kidneys through the bloodstream. It is known that in case the liver fails to metabolize arsenic, it becomes toxic and causes damage, even in sublethal concentrations. Another interpretation of the systemic negative effect is the production of cytokines such as IL-1 and IL-6 in the subcutaneous tissue that can enter the circulation, bind to hepatocytes, and cause inflammatory reactions [31].

On the other hand, HA, which is part of CS-HA, did not show genotoxic potential on the L1210 cell line (leukemia cells), as well as on MG63 scaffolds and mesenchymal stem cells. The explanation for the excellent biocompatibility of HA could be in its surface layer, which has a crucial role in achieving the connection between cellular tissues and this biomaterial [32].

The diversity of genotoxic responses in the examination of the toxicity of dental materials on various cell lines has been confirmed by studies [2,19-22,26,33].

There is a high probability that the final results of the assessment of the genotoxic potential of a substance are influenced by the genetics of the person from whom the cells are taken, the type of selected cell line, but also the conditions under which the experiment is performed.

The results of the genotoxic potential of the calcium aluminate cement primarily tested certainly encourage further research into this material in the future and other experimental studies.

4. CONCLUSION.

Nanostructured biomaterial based on calcium aluminate did not show genotoxic potential on human lung fibroblasts. The percentage of DNA damage at all applied concentrations was at the level of untreated control cells.). Calcium silicate hydroxyapatite cement extract ALBO-CS-HA showed genotoxic potential at concentrations ≥ 25 mg / mL.

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ГЕНОТОКСИЧНО ДЕЈСТВО НОВОСИНТЕТИСАНИХ НАНОМАТЕРИЈАЛА ЗА ПОТЕНЦИЈАЛНУ СТОМАТОЛОШКУ ПРИМЈЕНУ

Сажетак: Увод: Биокомпатибилност је својство неког материјала да испуњава своју функцију у организму не узрокујући при томе штетне нуспојаве. Како би потврдили биокомпатибилност сви новосинтетисани материјали морају проћи бројна *ин витро* и клиничка тестирања. Полазну тачку представљају тестови цитотоксичности и генотоксичности. Циљ ове студије је био утврдити генотоксичност новосинтетисаних наноматеријала на бази калцијум алумината и калцијум силиката са додатком хидроксиапатита .

Материјал и методе: Истраживање је реализовано на Институту за онкологију у Београду. Примијењена је алкална верзија Комет теста, у складу са препоруком међународног стандарда ISO/DIS 10993-3, а као ћелијска линија примијењена је MRC-5 нормални хумани плућни фибробласти. Ћелије су узгајане у једнослојној култури, у комплетном хранљивом медијуму, на темпаратури 37 °C у ваздуху обогаћеном са 5% CO_2 и засићеном воденом паром. Испитивана је генотоксичност калцијум алумината и мјешавине хидроксиапатита и калцијум силиката, а као контрола су послужиле нетретиране ћелије. За визуелизацију ДНК оштећења је употријебљен 40x објектив на флуоресцентном микроскопу. За статистичку обраду резултата Комет теста је кориштен Comet Assay IV (Perceptive Instruments).

Резултати: Екстракт ALBO-CA није испољио генотоксични ефекат, то јест, проценат оштећења ДНК и при највећој примијењеној концентрацији цемента од 100 mg/mL, износио је 9,6 %. Екстракт ALBO-CS-HA је показао генотоксични потенцијал при концентрацијама ≥ 25 mg/mL

Закључак: Наноструктурни ALBO-CA није показао генотоксични потенцијал на хуманим плућним фибробластима, за разлику од ALBO-CSHA, уз препоруке за даљњим испитивањима.

Кључне ријечи: генотоксичност, наноматеријали, калцијум алуминати, калцијум силикати.

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