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# The Effects of Rod and Round-Like Nanohydroxyapatites on *Allium cepa* Root Meristem Cells

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**Highlights**

- Nanohydroxyapatites stimulated the growth of *A. cepa* root tips.
- Particle size makes a difference in the genetic damage potential of nanoparticles.
- Round-like nano-hydroxyapatites cause genetic damage on *A. cepa* root meristem cells.

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**Abstract**

Biomaterials are engineered products that are widely used in many areas of medicine fields such as orthopaedic applications, facial and maxillofacial surgery, artificial heart parts, metal parts, and implantable devices. These materials are widely used in medicine because they are biocompatible with the organism, non-allergic, and are resistant to physical and chemical factors. Hydroxyapatites are bioactive calcium phosphate ceramics that are compatible with tissues. Nano-sized hydroxyapatite has been produced to increase their bioactivity. Although there are advantages to the use of nanoparticles in medicine and therapy, the potential toxicity of these compounds on the ecosystem and human health are of concern. One of the key issues to be investigated is whether the different forms of the same nanoparticle will cause differences in genotoxicity. Herein, the potential genotoxic effects of rod and round forms of nano-sized hydroxyapatites (nHAs) were evaluated using the *Allium cepa* Single Cell Gel Electrophoresis (SCGE) method. Results had shown that the round form of nHA in the *A. cepa* meristem root tip cells caused statistically significant genotoxicity at 25 µg/mL concentration in terms of tail intensity and tail moment. This study indicated small-sized-nanohydroxyapatite-induced genotoxicity and cell death in *A. cepa*. This study has shown that the physical properties of nanoparticles affect potential toxicity mechanisms.

## 1. INTRODUCTION

Hydroxyapatite (HA) is accepted as the most suitable material in terms of biocompatibility in orthopaedic and dental applications [1]. Over the last decade, nano-hydroxyapatite (nHA) has been used to repair damaged calcified tissues such as bones and teeth, to compose scaffolding, and to deliver drugs [2]. In the nanotechnology industry, which has developed rapidly in recent years, new materials are being developed and delivered to consumers every day in medicine, textiles, cosmetics, and many other areas. Nanoparticles (NPs) use is causing concern for human and environmental health [3]. The nanoscale size of NPs permits them to cross the plasma membrane, reach the nucleus, enter through nuclear pore,s and cause DNA damage by interacting with DNA [4]. With exception to the size, the shape, is an important factor in NPs entry into the cell and their potential toxic effect [5]. Several studies have shown that NPs might induce DNA damage and oxidative stress [6]. *In vitro* studies revealed oxidative stress, cytotoxicity and apoptosis in V79, BEL-7402, MC3T3, HK-2 cell lines [7-11]. Besides medical applications, nHAs have been administrated to eliminate heavy metals from aquatic and terrestrial systems [12]. For this reason, nanohydroxyapatite

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applications are being carried out in plants to assess its potential toxicity in the environment [13-15]. However, it should be considered that this material or its residues pose a potential risk to organisms considering that the nanoparticles are also an environmental pollutant [16, 17].

Ecotoxicological evaluations of nanomaterials in plants is required to determine their accumulation in plant cells as a first step in food chain assessment [18]. Oxidative stress and genotoxicity can arise from anthropogenic (plant protection products, air pollution) or natural (UV, drought, pathogens) sources. Nanoparticles can cause ROS production in the plant, damage to mitochondria and the photosynthetic, and genotoxicity [19-20]. In 1938, Levan used *Allium cepa* to record disturbances in the mitotic cycle after colchicine exposure [21]. Nowadays, *A. cepa* has been used by many researchers to assess the genotoxic potential of toxic substances as a bioindicator of environmental contamination [22-24].

In this study, FTIR and TEM were used to characterise spherical and rod-shaped NHAs produced by the sol-gel method. Single-cell gel electrophoresis is a fast, reliable, and robust assay to assess DNA breaks production after xenobiotics exposure. The DNA damage potential of nHA in root tip meristem cells was determined by SCGE in an alkaline medium. In addition, the growth rate of *A. cepa* root tips and cell damage were also analysed by the trypan blue test, which was used to determine living and dead cells in root tips. The potential toxicity of nHAs was evaluated in *A. cepa* root tips using various parameters.

## 2. MATERIAL METHOD

### 2.1. Chemicals

Ammonium dihydrogen phosphate [$NH_4H_2PO_4$] (CAS:7722-76-1), diammonium dihydrogen phosphate [(NH4)2HPO4] (CAS: 7783-28-0), distilled water (MilliQ), calcium nitrate tetrahydrate [$Ca(NO_3)_2.4H_2O$] (CAS: 13477-34-4) were purchased from Merck; ethanol absolute (CAS: 64-17-5), ethidium bromide (EtBr) (CAS: 1239-45-8), ethylenediamine tetraacetic acid ($Na_2EDTA$) (CAS: 6381-92-6), N-Phenylthiourea (CAS:103-85-5), phosphate buffer (PBS) (CAS: 7758-11-4), N-Lauryl sarcosine sodium salt (CAS: 137-16-6), sodium chloride (NaCl) (CAS: 7647-14-5), sodium hydroxide (NaOH) (CAS: 1310-73-2 ), Triton-X (CAS: 9036-19-5 ), trizma base bioultra (CAS: 77-86-1 ), trypan blue (CAS No: 72-57-1) were purchased from Sigma Aldrich; low melting agar and low melting agar "DNA grade" were purchased from AppliChem. All chemicals were used in analytical grade and without further purifications.

### 2.2. Nanoparticle synthesis and characterization

#### 2.2.1. Synthesis of round-like hydroxyapatite particles

Round-like hydroxyapatite particles were synthesized according to our previous study [25]. The reaction was performed by keeping the atomic ratio to 1.67 for Ca/P. Firstly, 78.88 g $Ca(NO_3)_2.4H_2O$ and 129.6 mL ethanol were stirred until they became a homogeneous solution. Then, $NH_3$ was used to adjust the pH of the solution (pH=10). Later, 23.2 g $NH_4H_2PO_4$ and 186.5 mL distilled water was stirred to gain a homogenous phosphate solution. Following this, the pH of the solution was set at 10 by $NH_3$. Next, the calcium solution was added (2.4 g/min) into the phosphate solution. The final solution was aged at 22 hours at 25°C and later centrifuged (5000 rpm/5 min). The obtained particles were washed with distilled water (6x), dried (70°C/24 hrs), followed by calcination process (750°C/4 hrs).

#### 2.2.2. Synthesis of rod-like hydroxyapatite particles

Rod-like hydroxyapatite particles were produced using a modified chemical precipitation technique [26] by setting the atomic ratio to 1.67 for Ca/P. Briefly, 17.712g $Ca(NO_3)_2.4H_2O$ and 5.940g $(NH_4)_2HPO_4$ were dissolved in 32.288 mL and 44.06 mL distilled water, respectively, to prepare a homogeneous calcium and phosphate solution. Then, $NH_3$ was used to adjust the pH of the solution (pH=10). Later, the calcium solution was included (2.4 g/min) to a stirring phosphate solution. The stirring process was performed for 1 h. The final solution was centrifuged (5000 rpm/5 min) then the obtained particles were calcined (500°C/5 hrs).

### 2.2.3. FT-IR analyses

Fourier Transform Infrared (FT-IR) spectroscopy (Bruker-Tensor 27) was used to examine the chemical analysis of hydroxyapatite particles, both round and rod-like, in transmission mode. The analysis covered a range from 400 to 4000 $cm^{-1}$ with a resolution of 4 $cm^{-1}$.

### 2.2.4. TEM analyses

The morphology and size of the synthesized hydroxyapatite particles were characterized by Transmission Electron Microscopy (TEM, ZEISS LEO906).

### 2.3. Exposure System

*A. cepa* (2n: 16) (18-25 mm and 2-4 g) is a model organism frequently used for genotoxicity studies. Five bulbs were used for each application. After root tip elongation (2-3 cm), onions were exposed to liquid solutions of nano hydroxyapatites for 18-24 hours in a dark environment at 25-26 °C. Initially, a wide concentration range between 10 µg/mL and 1000 µg/mL was chosen to determine the concentrations used in the study. Intense precipitation was observed during the application of nHAs at concentrations of 400, 800 and 1000 µg/mL. For this reason, the highest concentration was set at 200 µg/mL to ensure that the onion roots could be exposed to sufficient and maximum levels of nHA. Concentrations of 100, 50, and 25 µg/mL were then added. Distilled water, the solvent for the chemicals, was considered as a negative control in all experiments.

### 2.4. Cell Viability Test

The cell viability test can be measured on *A. cepa* root tips using trypan blue or Evans blue dyes [27]. *A. cepa*, whose root tips were cut with a razor blade and germinated with distilled water, were exposed to the different concentrations of nHAs for 24 hours. A cell viability assay was performed according to Arya and Mukherjee (2014) with minor modifications [28]. Root tips were cut to 2 cm using a razor blade. The cut tips were stained with trypan blue (0.4%) for 30 min. After staining, they were washed with distilled water for 10 min and several repetitions were conducted for this step. After washing, for each application, 10 root tips of equal length were incubated with 3 mL N, N-dimethylformamide at 24°C for 1 hour. The amount of trypan blue released in the solution was measured at 600 nm absorbance in the spectrophotometer (Thermo Scientific Skan It).

### 2.5. Root Growth Test

*Allium* root growth test was performed according to previously described method with slight modifications [29]. For each control group and nHAs concentrations 35 root tip lengths (7 root tips from each bulb) were measured with a mechanical caliper. The percentage of root tip growth was calculated in relation to the data of the negative control group.

### 2.6. Single Cell Alkaline Gel Electrophoresis (SCGE) Test in *A. cepa* Root Tip Cells

In the SCGE assay, 4 mM ethyl methanesulfonate (EMS) was used as the positive control, [30]. The isolated root meristem nuclei have been used in the SCGE assay. The SCGE assay proves to be a highly sensitive and dependable method for detecting single-strand DNA damage [31-33]. The genotoxic effects of nano-hydroxyapatite on *A. cepa* were assessed according to Kaya et al. (2015) [25]. Onion root tips (from five onion bulbs) were gently and rapidly cut with a razor blade in ice-cold Tris solution (400 mM Tris buffer (pH 7.5)) and nuclei were isolated. The cells were mixed with low-melting agar (LMA) and plated on slides coated with normal melting agar (NMA). The slides were kept in cold electrophoresis buffer (1 mM $Na_2EDTA$and 300 mM NaOH, pH ≥13) and electrophoresis was performed at 300 mA and 25 V. After electrophoresis, the slides were kept in neutralisation solution (400 mM Tris buffer (pH 7.5) for 5 minutes. This procedure was repeated 2 times. After neutralisation, the slides were washed in distilled water for 5

minutes. Slides were coverslipped after staining with 50 µl EtBr (0.4 µg/mL). Fifty cells were counted for each slide and DNA tail intensity (%) and tail moment were used as evaluation parameters. Slides were measured at 40x magnification on a 590 nm barrier-filtered fluorescence microscope (Nikon Eclipse E200) with charge-coupled device (CCD) camera attachment using Comet Assay IV (Instem UK). The results of the experiments were evaluated with by analysis of variance (ANOVA-Dunnet Test) using the SPSS software v.23 [34-35].

## 3. RESULTS AND DISCUSSION

### 3.1.TEM Analyses

TEM images show that the hydroxyapatite particles have a nearly round (Figure 1a) and rod-like (Figure 1b) shape. The round-like nHA has a diameter range of 35-60 nm and the rod-like nHA has a rod length of 45-90 nm.

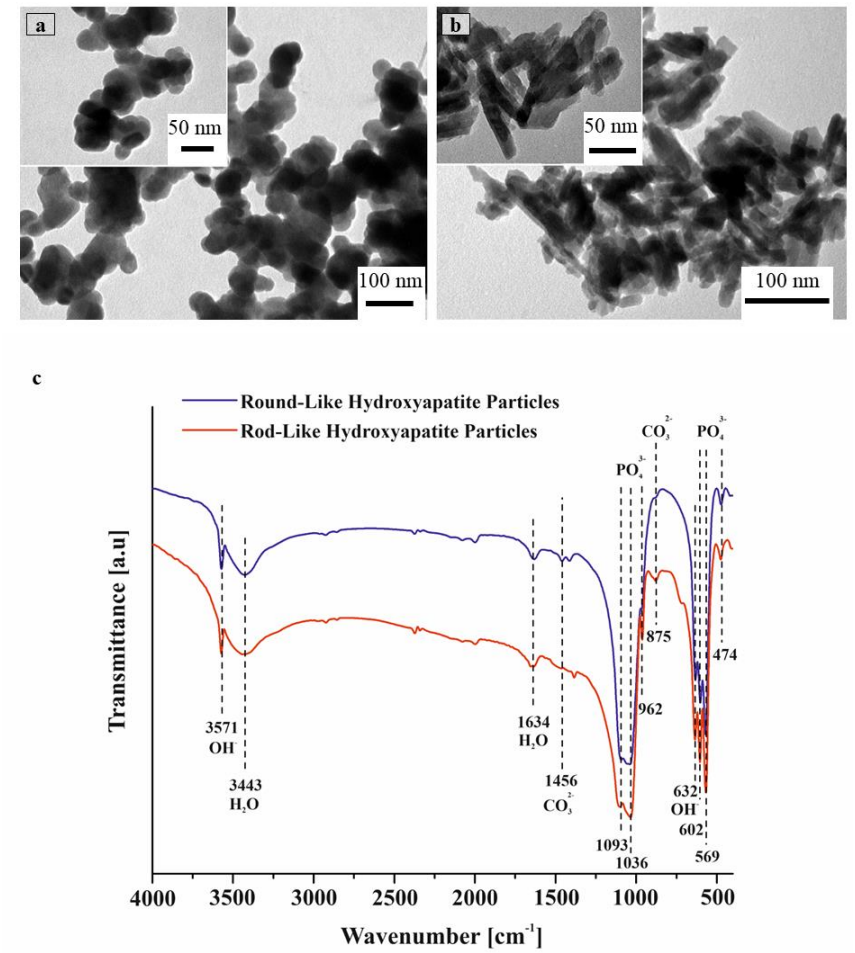


***Figure 1.*** *Nanoparticle characterization. TEM images of different shapes of hydroxyapatite particles round-like (a) and rod-like (b). FT-IR spectra of round-like and rod-like hydroxyapatite particles (c)*

### 3.2. FT-IR Analyses

Round-like and rod-like hydroxyapatite particles' FT-IR spectra as shown in Figure 1c. The FT-IR spectra illustrate the hydroxyapatite's functional groups. Typical $PO_4^{3-}$ bands are seen at 472, 569, 602, 962, 1036, and 1093 $cm^{-1}$. On the other hand, the bands at 1634 and 3443 $cm^{-1}$ correspond to adsorbed water ($H_2O$) The typical hydroxyl groups (OH-) in the crystal structure of hydroxyapatite appeared at 632 and 3571 $cm^{-1}$ [36-37]. In addition, the weak bands at 875 and 1456 $cm^{-1}$ are attributed to carbonate ($CO_3^{2-}$) [38-39]. The observed carbonate in the structure may be due to the carbon dioxide which comes from the atmosphere

during the reaction as reported in previous studies [40]. This result confirms that both hydroxyapatite particles were effectively synthesized.

### 3.3. Cell Viability Test

To minimize heterogeneity in the application suspension, nHA exposure of onions was carried out on a magnetic stirrer for all experiments. Figure 2 shows the cell death induced by rod and round-like nHAs in *A. cepa* root meristem cells after the release of trypan blue into N, N dimethylformamide solution. Rod-like nHAs induced cell death at all concentrations, while round-like nHAs induced cell death only at 100 µg/mL.

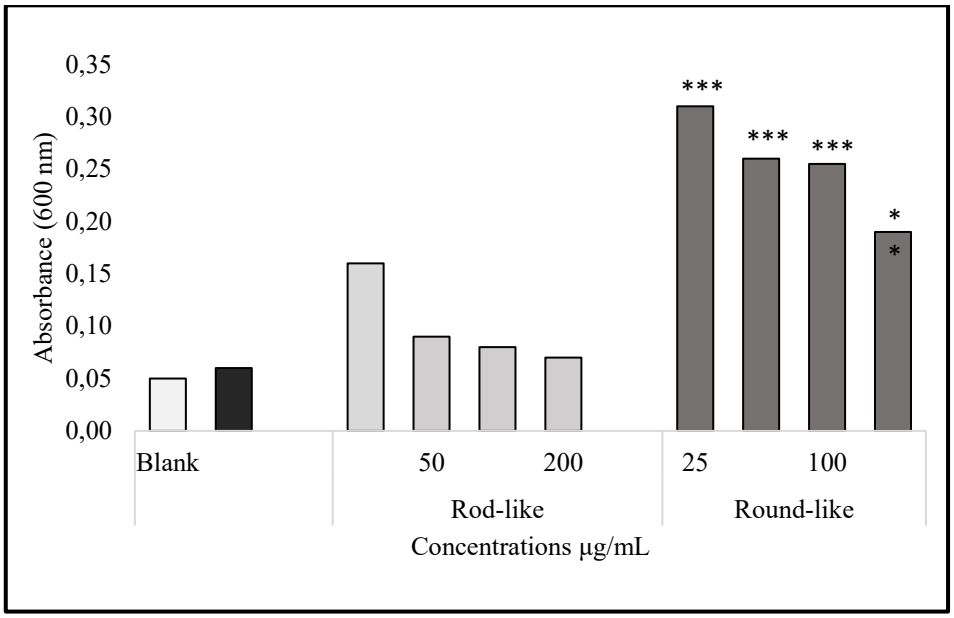


***Figure 2.*** *Cell death induced by rod and round-like nHAs on A. cepa root meristem cells (*** p<0.000, **p<0.01)*

Methylene blue and trypan blue are dyes that have been used to stain biological environments since the beginning of the 20th century, are also used in clinical diagnosis [41]. Sinitha and Thoppil (2016) found that *Amomum pterocarpum* extract induced apoptosis in *A. cepa* root tip meristem cells in a concentration-dependent manner with the Evans blue test [42]. Arya and Mukherjee (2014) determined the presence of apoptosis in *A. cepa* root tip cells as a result of cadmium toxicity [28]. In a study investigating the toxicity of arsenic-contaminated groundwater, a concentration-dependent increase in cell death was detected in *A. cepa* root tips using Evans blue dye [43]. In this study rod-like nHAs induced apoptosis only at 100 µg/mL. Round-like nHAs cause a dose-dependent decrease in cell death.

### 3.4. Root Growth Test

The root growth test results are presented in Table 1. Both rod and round-like nHAs induced root length that is statistically significant compared to the negative control. The root growth was accelerated by rod-like nHA with 50-75% and round-like nHA with 39-62%.

***Table 1.*** *Results of the root growth test on A. Cepa*

| Concentrations | | Average length ± SE (cm) # | Growth (%) |
|---|---|---|---|
| Control | | 2.11 ± 0.22a | 100.00 |
| Rod-like nHA | 25 µg/mL | 3.46 ± 0.18 bc | 163.80 |
| | 50 µg/mL | 3.61 ± 0.14 c | 170.97 |

| | | | |
|---|---|---|---|
| | 100 µg/mL | 3.18 ± 0.11 bc | 150.53 |
| | 200 µg/mL | 3.71 ± 0.27 c | 175.70 |
| Round-like nHA | 25 µg/mL | 2.89 ± 0.22 b | 136.65 |
| | 50 µg/mL | 3.39 ± 0.23 bc | 160.49 |
| | 100 µg/mL | 3.43 ± 0.25 bc | 162.32 |
| | 200 µg/mL | 3.49 ± 0.25 bc | 165.15 |

# Means with the same letter do not differ statistically ($p$<0.05).
SE; Standard error.

Growing plant roots in a calcium-rich environment contributes positively to the plant development. Calcium acts both as one of the cell wall elements and as an intracellular secondary messenger in all parts of plants from roots to pollen tubes [44]. It was observed that the yield obtained from onions living in soil rich in Ca increased by 25-100% [45]. In a study on *Lupinus havardii*, an increase in weight was observed in the roots and bulbs of the plant grown in Ca-enriched media [46]. In our study, the roots were elongated compared to the control group. In addition, when root morphologies were examined, it was observed that onion roots exposed to nHA were rigid and thick. Calcium ions could be released in the environment due to apatites, this could explain the obtained results.

### 3.5. Single Cell Alkaline Gel Electrophoresis (SCGE) Test in *A. Cepa* Root Tip Cells

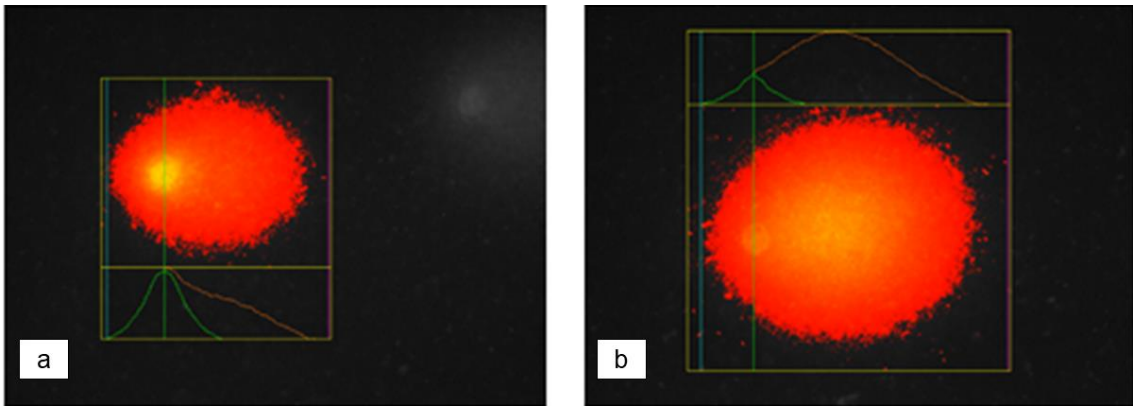


***Figure 3.*** *Comet images. Distilled water (a). 4 mM EMS(b)*

Comet images of *A. cepa* root cells are shown in Figures 3a and 3b. In the *A. cepa* SCGE experiment, concentrations of 25, 50, 100 and 200 µg/mL of rod-like and rounded nanohydroxyapatite were used. According to the results obtained, nHA in rod-like form showed the lowest genotoxic effect in all parameters at a concentration of 25 µg/mL. Concentrations of 50, 100 and 200 µg/mL of rod-like nHA showed no significant genotoxic effect compared to the negative control group (Figure 4). Rod-shaped nHA showed genotoxicity only at a concentration of 25 µg/mL. The genotoxic effect of rod-like nHA decreased with increasing dose.

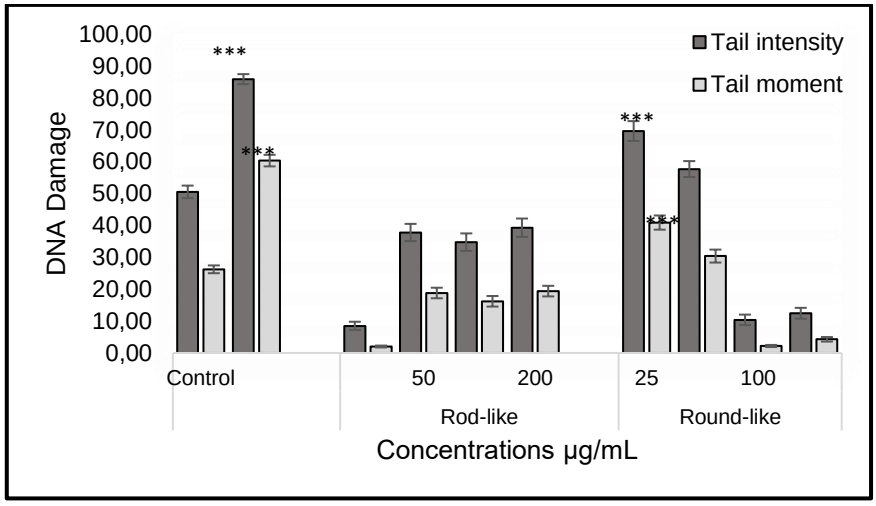


***Figure 4.*** *Effect of nHAs on DNA damage in A. cepa (*** p<0.000)*

The effects of NPs on plants are evaluated considering that they play a key role in ecotoxicology due to their inactivity of plants in ecological systems, having root networks, and functioning as primary producers in ecosystems [47]. Kaya et al. (2015) reported that the micro (>100 nm)-and nano (15 nm)-sized silicon dioxide particles in *A. cepa* root meristem cells using the SCGE assay had significant genotoxic effects at all studied concentrations (1-, 10, 100 µg/ mL) of nanoparticles and the highest concentration of microparticles (100 µg/mL) [30]. Liman et al. (2015) analyzed the genotoxicity of the herbicide Imazethapyr by using the *A. cepa* SCGE assay and observed an increase in DNA damage at the concentrations of 20 and 40 µg/mL at 24, 48, 72, and 96 hours after exposure [48]. Ghosh et al. (2010) evaluated the genotoxic effects of ionic and nanoform $TiO_2$ using the *A. cepa* SCGE assay, and while ionic $TiO_2$ at concentrations of 1.25, 1.5, 1.75, and 2 mM caused significant DNA damage, nanoform $TiO_2$ (100 nm) induced DNA damage only at a concentration of 250 µM [49]. Demir et al. (2014) evaluated the genotoxicity of 50 nm ZnO and $TiO_2$ nanoparticles (10, 100, and 1000 µM) by using *A. cepa* SCGE assay and observed significant DNA damage in a concentration-dependent manner [50]. Liman et al. (2019) investigated the effects of CeO micro (4 µm) and nanoparticles (20 nm) by (micronucleus) MN and SCGE assays in *A. cepa* and observed a concentration-dependent increase in genotoxicity [51].

In the SCGE assay performed on *A. cepa* in this study, the data obtained from both rod-like and round-like forms showed similar results for the tail intensity and tail moment. Tail intensity and tail moment increased depending on the concentration in rod-like form, and in both parameters, the damage rate decreased depending on the concentration in round-like form. It is thought that the genotoxic effect decreases as a result of the lack of aggregation or entry into the cell at high concentrations of 100 and 200 µg/mL of round-like nHAs. The irregular shape structure, higher surface area, and variable subunits of nanoparticles that aggregate outside the cell also affect their entry into the cell [4]. Kumari et al. (2011) observed that the mitotic index decreased and MN and chromosomal aberrations increased in a study where 25, 50, 75, and 100 g/mL ZnO NP (100 nm) were applied in *A. cepa* stem cells [52].

As a consequence of the increased production and consumption of NPs, NPs release would increase in the environment [53, 54]. Although studies on the effects of these materials on human health have been accelerated in the last 10 years, there are still many questions that need to be answered and gaps in the literature that need to be addressed [55]. Biomaterials science involves the development, evaluation, and application of special substances for use in biology and medicine research [56]. The fact that HAs are nano-sized allows the material to have a larger surface area, thus reducing the fragility and improving its mechanical properties and bioactivity [57].

In addition to the toxic effects that nHAs may cause due to their size, it is also important to investigate their toxic potential in different shape forms. In an *in vitro* study cytotoxicity and apoptosis of rat osteoblast cell line increased after exposure to 4 different nHA species (needle, round, thin rod, and long rod form) (10-40 nm) (20-100 mg/L) depending on the concentration. In addition, it was observed that the greater toxic

effect occurred on the needle-shaped particles and the lowest toxic effect was recorded on the long rod-like nanoparticles [58]. In osteoblast primary cell culture, it has been determined that nanohydroxyapatite in short rod and micro-sized round forms increase ROS production and cause DNA damage in the SCGE assay [59]. Rao et al. (2019) observed an increase in cytotoxicity, ROS and lactate dehydrogenase levels at all concentrations between 63-500 μg/mL of hydroxyapatites in the round, needle, rod, and plate forms in kidney epithelial cells (HK-2) [60]. Deterioration of cell morphology and necrosis were detected. In the present study, the round-like form caused higher toxicity in *A. cepa* stem cells than the rod-like form. NPs properties including size and shape are one of the important factors influencing the toxicity mechanism [61]. Accordingly, the small size of the round-like form can be seen as a triggering factor for toxicity mechanisms.

Plant model organisms such as *A. cepa* are excellent bioindicators for investigating the potential hazards of nanomaterials, due to their high sensitivity to detecting cytotoxic and genotoxic effects of environmental pollutants [47]. *A. cepa* is an important model organism used to assess the genotoxicity potential of various pollutants due to its characteristics such as high number of dividing cells, uniform chromosome size and ease of culture in the laboratory environment [62]. Various cellular pathways can be affected in a direct or indirect manner after the gradual demineralization or biotransformation of NPs once they enter the roots [63]. Ma et al. (2011) showed that NPs penetrate living tissues and are transported from the infiltrated tissue to other tissues of the plants by the roots and via vascular systems [64]. These processes differ depending on the chemical composition, shape and diameter of the NPs and plant anatomy [47]. The fact that *A. cepa* is a vascular plant makes it a suitable model organism for the detection of mutagens in different environments in the assessment of environmental contaminants [65-66].

## 4. CONCLUSIONS

The genotoxic effects of two different nanoforms of hydroxyapatites, which have an important place in regenerative medicine, were evaluated in *A. cepa* in this study. In our study, root tips were elongated, but cell and DNA damage was also detected. It is observed that the damage is mostly in small-size nanoparticles. These results indicate to us that the nano-size growth promoting substances may bring some negative side effects. While the nanotechnology industry introduces new products every day to improve human and environmental health, it is crucial to evaluate the potential toxicity of these products. Since biomaterials have important areas of use for human beings, there is a need for studies to expand biomedical application areas, develop new biomaterials, or increase their usability and reliability by manipulating the structure and composition of existing biomaterials. The molecular mechanisms underlying the genotoxic effects of nHAs should be elucidated with different model organisms and more studies should be conducted at an advanced molecular level.

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## CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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