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RESEARCH ARTICLE

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Genotoxic assessment of cerium and magnesium nanoparticles and their ionic forms in *Eisenia hortensis* coelomocytes by alkaline comet assay

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Abstract

The present study aimed to evaluate the genotoxic potential of cerium oxide (CeO_2), magnesium oxide (MgO) nanoparticles and their ionic forms by alkaline comet assay. *Eisenia hortensis* were exposed to different series of concentrations (25, 50, 100, 200, and 400 μg/ml) of chemicals for 48 h to find LC_{50}. The LC_{50} for MgO and CeO_2 NPs were 70 and 80 μg/ml. Whereas, the LC_{50} for their ionic forms were 50 and 70 μg/ml. To assess the potential DNA damage caused by the chosen chemicals, *E. hortensis* was further exposed for 48 h to the following concentrations, based on their respective LC_{50s}: $LC_{50/2}$, LC^{50}, and $2xLC_{50}$. Comet scores demonstrated the significant increase ($p < 0.05$) in DNA damage at all concentrations, both for NPs and ionic forms in a concentration-dependent manner. Findings of the present study revealed the genotoxic effects of CeO_2 NPs, MgO NPs and their ionic forms on *E. hortensis*.

KEYWORDS
cerium oxide, DNA damage, ecotoxicology, *Eisenia hortensis*, genotoxicity, magnesium oxide

Research Highlights

- Genotoxic assessment of CeO_2 and MgO NPs and their ionic forms was conducted.
- Characterization of NPs through electron microscopy and alkaline comet assay was performed on *E. Hortensis*.
- Highest DNA damage of CeO_2 and MgO NPs was observed on earthworm.

1 | INTRODUCTION

Earthworms are some of the most suitable model organisms for measuring the soil toxicity and have also been identified as ecosystem engineers due to their central role in terrestrial ecosystems (Lee et al., 2007; Shoults-Wilson et al., 2011). Earthworms are involved in the breakdown of organic matter in the terrestrial ecosystem and the formation of the soil (Scott-Fordsmand & Weeks, 2000). They are widely used in toxicological evaluation of various chemicals and nanoparticles (Brami et al., 2017; Ciğerci et al., 2016; Ciğerci et al., 2018; Eom et al., 2007; Lin et al., 2014; Patricia et al., 2017; Zhang et al., 2014). They have been proven to be a useful tool in the evaluation of terrestrial ecotoxicology and hazardous wastes. Their permanent contact with the soil particles through the permeable skin and the digestive system allows them to be used as a good model organism for monitoring soil pollution (Roubalova et al. 2015).

DNA strand breaks are an important cellular component to find environmental stress in both aquatic and terrestrial organisms (Frenzilli et al., 2004). They have been proposed as an important sensitive biomarker (Reinecke & Reinecke, 2004). Coelomocytes play an important physiological role in the immune system of worms and considered as most suitable cells in evaluating genotoxic damage (Du et al., 2015).

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The commercial value of nanotechnology is estimated to be 75 billion dollars with a production amount of 58,000 tons by 2020 (Bai et al., 2017; Deng et al., 2017). The nanotechnology industry develops highly efficient products, but it has significant uncertainties and lack of data about the reliability of these materials (Gonzalez et al., 2008). The fact that NPs individually or combination in aquatic and terrestrial environments have drawn the attention of environmentalists (Bundschuh et al., 2018). When NPs interact with existing pollutants and dissolved organic matter, their environmental distribution and interactions with organisms are affected (Bundschuh et al., 2018; Deng et al., 2017). NPs, which are aggregated, are less mobile and can be included in the food chain through bottom feeders and filter feeders (Nowack & Bucheli, 2007). Nanoscale materials can interact with cell components and may exhibit severe adverse effects for aquatic and terrestrial organisms at their cellular levels (Keller & Lazareva, 2014; Lead et al., 2018). Increased surface area, antimicrobial behavior, high protective properties have led to extensive use of NPs, but researchers are concerned about the potential harmful effects of nanomaterial on humans and the environment (Pulido-Reyes et al., 2017).

Cerium (Ce) is a rare earth element in the lanthanide series on the periodic table (Arnold et al., 2013). Ce is used as polishing material in various glass coatings and ornamental stones. Cerium oxide nanoparticles (CeO_2 NPs) are mostly used as diesel fuel components (Cassee et al., 2011). Ce, which acts as a catalyst in the fuel, increases the efficiency of the fuel and reduces the amount of particulate matter between 0.2 and 2.5 mm (Snow et al., 2014). Because of its antioxidant effects, Ce is used in pharmacology for neurodegenerative pathologies, autoimmune disorders, diabetes, and oxidative stress-related disorders such as cancer (Celardo et al., 2011; Li et al., 2016). Magnesium is used in many industries, especially in transportation due to its lower weight compared to other metals (Dieringa, 2018; Paramsothy et al., 2011; Sankaranarayanan & Gupta, 2015). Mg protects the cell from oxidative damage in biological systems due to its antioxidant properties (Ścibior et al., 2018). The food development authority (FDA) indicates that MgO can be used in food applications due to its antibacterial effect (Swaroop & Shukla, 2018). However, genotoxic potential of CeO_2 NPs and nanosized MgO has also been observed in different model systems (Dunnick et al. 2017; Koehlé-Divo et al., 2018; Mangalampalli et al., 2017).

Various studies have been reported the genotoxic effects of NPs on different model systems (Ciğerci et al., 2018; Liman et al., 2020; Liman, Ali, et al., 2021). But no study has been conducted on the genotoxic potential of CeO_2 and MgO NPs and their inoic forms on the *Eisenia hortensis* earthworms. So, in this study, genotoxicity studies of these NPs and their ionic forms were carried out using *E. hortensis* as a model organism.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Cerium oxide NP (Ceria = CeO_2 NP) (Cas No: 1306-38-3, particle size: <25 nm), cerium oxide (Ceria = CeO_2) ionic form (> 99%) (Cas No: 1306-38-3), magnesium oxide NP (MgONP) (Cas No:1309-48-4, Particle size: <50 nm), magnesium oxide (MgO) ionic form (> 98%) (Cas No: 1309-48-4), ethyl methanesulfonate (EMS) (Cas No: 62–50-0). Other chemicals used in the comet assay were purchased from Sigma Aldirich and were of analytical grade.

2.2 | Characterization of nanoparticles

CeO_2 and MgO NPs were characterized for their size distribution and measurement of zeta potentials (Malvern Zetasizer Nano-ZS) in the Department of Chemistry of Akdeniz University. Transmission Electron Microscope (TEM) (FETEM / JEOL JEM2100F) was used to determine size and morphology at the Central Laboratory of the Middle East Technical University.

2.3 | Earthworms collection and the experimental procedure

Adult earthworms (400 and 600 mg) were collected from a pollution-free area, Latitude: 38 ° 48′ 8.3844″ N, Longitude: 30 ° 16′ 49.5912″ E, very far from the industries and population. Species identification was confirmed through the specified key of taxonomic classification (Sims, 1985). The worms were kept in humid stock soil at 24 ± 2°C in dark. Stock soil was collected from the same area from where the earthworms were collected. Genotoxicity assay was performed in aqueous-solution and different concentrations (25, 50, 100, 200, and 400 μg/ml) of NPs and ionic forms were used to find LC_{50}. The MgO NP and its ionic form and the ionic form of CeO_2 NP purchased in powder form. Nominal stock concentration of 400 μg/ml was prepared for each chemical by dissolving in distilled water. It was then kept in the sonicator (Bandelin Sonorex, 35 kHz, 80/320 W) for 1 h at room temperature. Nominal concentrations of MgO NP, CeO_2 NP, and their ionic forms were measured anlaytically by inductively coupled plasma-mass spectrometry (Thermo Scientific ICAP RQ ICP-MS,USA), which were 290 ± 10, 255 ± 08, 319 ± 16, and 310 ± 16 μg/ml for MgO NP, CeO_2 NP, and their ionic forms, respectively.

In each group of concentration, ten earthworms were used. Earthworms were exposed in aqueous-based solution of chemicals in plastic petri dishes, having 15 ml capacity by volume. Petri dishes were placed in well aerated dark area and further tiny holes were made on the covers of petri dishes for the exchange of air. Moreover, solution of each group was changed on daily basis. Each earthworm was touched on head after 48 h with small wooden stick to take response. The earthworms which were not responding were considered dead. EPA Probit program (ver. 1.5) was used to determine the 48 h LC_{50}. Negative control was the distilled water, having an equal number of earthworms. Experiment was run in triplicate.

2.4 | Alkaline comet assay for earthworms

The concentrations of $LC_{50/2}$, LC_{50}, and $2\times LC^{50}$ were used for 48 h to find the genotoxic effects of NPs and ionic form in *E. hortensis*

through comet assay. Comet assay was carried out as described by the Ciğerci et al. (2016) and Reinecke and Reinecke (2004). Briefly, coelomic fluid was extracted through extrusion buffer (EDTA 1.091 g, 9.09 g Guaiacol glycerol 2 ether, and NaCl 4.161 g added in 950 ml distilled water, 50 ml absolute ethanol added additionally to make it 1 L. pH: 7.5). Coelomoycte solution was collected in centrifuge tube and centrigued at 2000 rpm for 5 min. Coelomocytes (10 μl) were mixed with LMP agarose (100 μl; 0.8%) and spread over the precoated NMP (1%) agarose slides. Then, slides were kept in chilled lysis solution for 1 h and electrophoresis was performed in 20 min without running, and then at 25 V (1 Vcm^{-1}) for 20 min. Finally, slides were stained with ethidium bromide (20 μg/ml). One hundred comets were scored under fluorescent miscroscope visually as belonging to one of five classes (0: undamaged, 1: mild damage, 2: moderate damage, 3: severe damage, 4: complete damage) (Collins, 2004). To express the degree of DNA damage, the Arbitrary Unit (AU) was calculated according to the following equation.

$$\text{Arbitrary unit} = \sum_{i=0}^{4} Ni \times i$$

Ni = Number of cells in i degree; i = degree of damage (0, 1, 2, 3, 4).

2.5 | Statistical analysis

Analysis of variance (ANOVA) was used by using SPSS (Version, 15.0) for performing statistical analysis of comet assay findings. Duncan test was further applied to compare the statistically significant difference ($p < 0.05$) among different concentrations of groups and with that of negative control.

3 | RESULTS AND DISCUSSION

Surface and shape of nanoparticles play an important role in penetrating the living cell. Therefore, the characterization of nanoparticles is inevitable to explore its action (Elsaesser & Howard, 2012; Singh et al., 2009). The surface load of nanoparticles is one of the factors that affect stability and cell-nanoparticle interaction. Under the electric current, the motion of the nanoparticles is measured by the zeta potential (Das et al., 2017). In the analyzes, the mean particle size of CeO_2 NPs was determined as 22 nm with a zeta potential of 1.72 mV (Figure 1) while MgO NPs mean particle size was 11 nm with a zeta potential of 0.738 mV (Figure 2).

The LC_{50} for CeO_2 and CeO_2 NPs were found at 70 and 80 μg/ml, respectively. Whereas, it was found 50 and 70 μg/ml, for MgO and MgONPs, respectively. There was a concentration-dependent increase in the DNA damage at all concentrations of CeO_2 and CeO_2 NPs (Table 1). Similar results were found for the MgO and MgO NPs (Table 2). The highest significant DNA damage was observed at the highest concentration ($2\times LC_{50}$) of all NPs and their ionic forms. Similarly, the lowest DNA damage was observed at the lowest concentrations ($LC_{50/2}$) of all the treatments. There was also a statistical difference compared to the control group for all concentrations. Moroever, ionic forms of cerium and magnisium oxide found more DNA damage compared to their NPs.

Comet assay is widely used test to detect genotoxic effects of various chemicals, mutagens, carcinogens and plant extracts etc., on different models systems (Ali et al., 2022; Ali & Cigerci, 2017; Ali & Ciğerci, 2019a; Ali & Ciğerci, 2019b; Ciğerci et al., 2022; Liman, Acikbas, et al., 2021; Liman et al., 2022; Liman, Ali, et al., 2021). Environmental pollution due to NPs may be caused by their uses in various processes such as agricultural activities, industrial processes and waste removal (Barrick et al., 2017). Bioaccumulated NPs or complex structures formed by the contaminants in the environment have the potential to penetrate food chains, therefore, its biological effects need to be comprehensively understood (Deng et al., 2017). Although some studies on the toxicity of metal oxide nanoparticles have been carried out, but the studies focusing on eco-toxicological threats are limited (Mukherjee & Acharya, 2018).

CeO_2 NPs, are frequently used in automotive and optics. Recently, these are being used in manipulating the various cellular processes such as antioxidant activity, cell membrane stability, and enzymatic reactions (Koehlé-Divo et al., 2018). Changes in the surface coating of CeO_2 NPs can affect the pro- or antioxidative properties of these NP's interaction with the cells (Ould-Moussa et al., 2014). These antioxidant scavenging properties of CeO is used in chemotherapy for the cancer treatment and anticancer drugs delivery systems (Rubio et al., 2018). CeO_2 NPs can be used in biological applications by forming biocompatible surface coatings due to their clinical applications and regenerating antioxidant properties (Das et al., 2017).

In the A549 cell line, CeO_2 NPs at different concentrations (01–75 μg/cm^2) did not show the significant cytotoxic effect with Alamar Blue test at 3 and 24-h exposure (El yamani et al. 2017). In the Caco-2 cell line, 70 nm of CeO_2 NPs did not cause genotoxic or oxidative damage (Vila et al., 2018). In a study describing the micro and nano-scale CeO_2NPs in *Daphnia magna*, only the lowest concentration (0.05 g/L) of CeO_2 NP showed an increase in death rate (Gaiser et al., 2012). MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl Tetrazolium Bromide Assay) and comet assays were performed in a human neuroblastoma cell line and it was observed that CeO_2 NP was more toxic than CeO_2 ionic form (Kumari et al., 2014). In *D. Magna* and *Chironomus riparius*, CeO_2 NPs reported the DNA damage in comet assay (Lee et al., 2009). In human fibroblast cells, it was observed that CeO_2 NPs are clustered near mitochondria and endoplasmic reticulum. In addition, 10 μg/ml CeO_2 was found to cause oxidative stress-induced DNA damage in fibroblast cells (Franchi et al., 2015). Oxidative-induced damage by CeO_2 NPs toxicity has been evaluated with various biomarkers (Eom & Choi, 2009). Gormley et al. (2016) reported that CeO_2 NP (30 nm) did not cause an increase in oxidative stress markers in rat plasma. However, in the mouse tumor macrophage cell line (RAW264.7), various concentrations (15, 30, 60 and 120 μg/ml) of rod and spherical shaped CeO_2 NPs increased the LDH, TNF-a and reactive oxygen species (ROS) levels (Forest et al., 2017). In male Sprague–Dawley rats, intratracheal administration of 0.5 and 1 mg/kg for 1, 7, and 84 days showed that lactate dehydrogenase levels increased on days 1 and 7, but on the 84th day, the level of

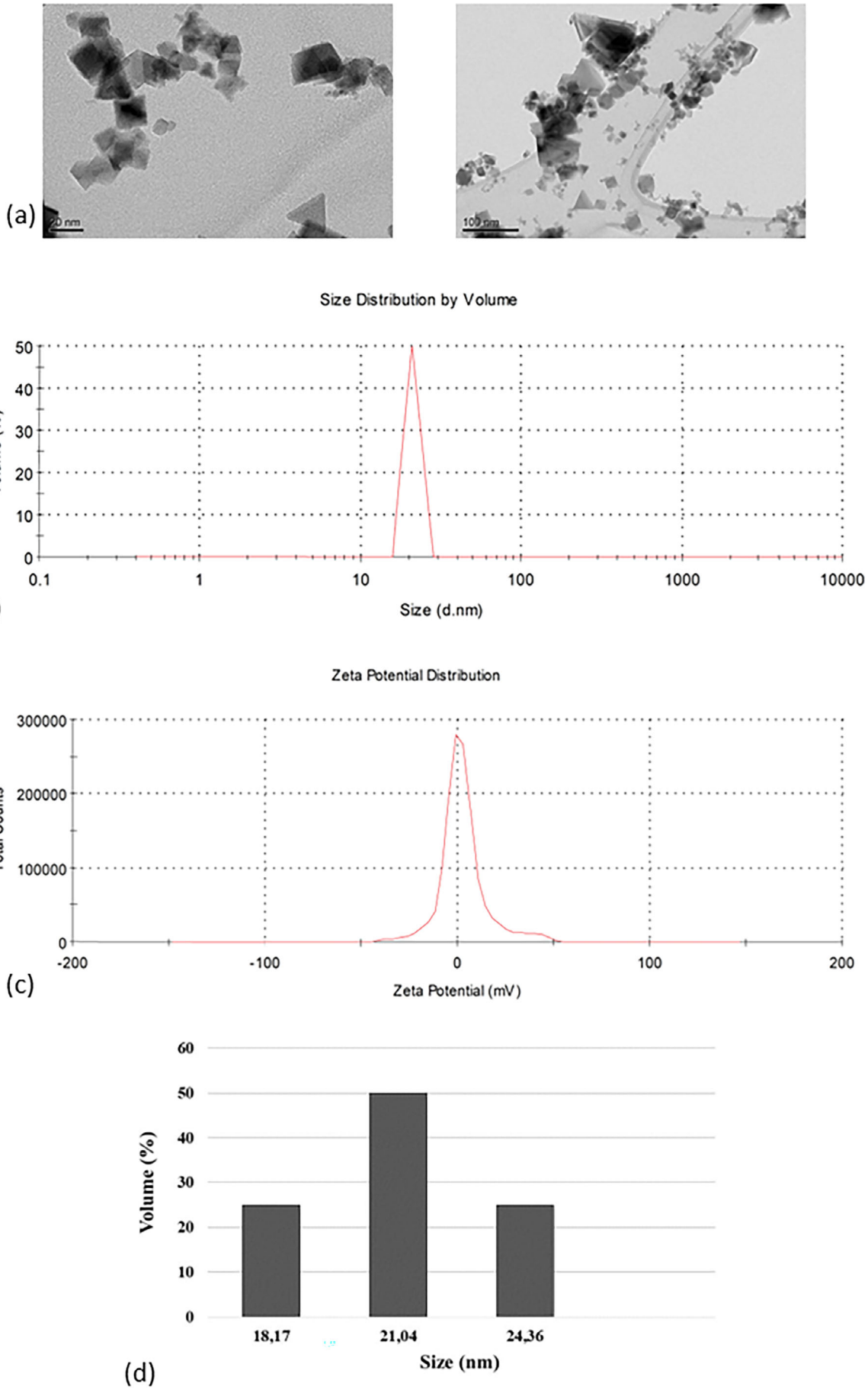


FIGURE 1 Characterization of CeO_2 NPs. (a) TEM image; (b) particle size; (c) zeta potential; (d) percentage by volume of particle size

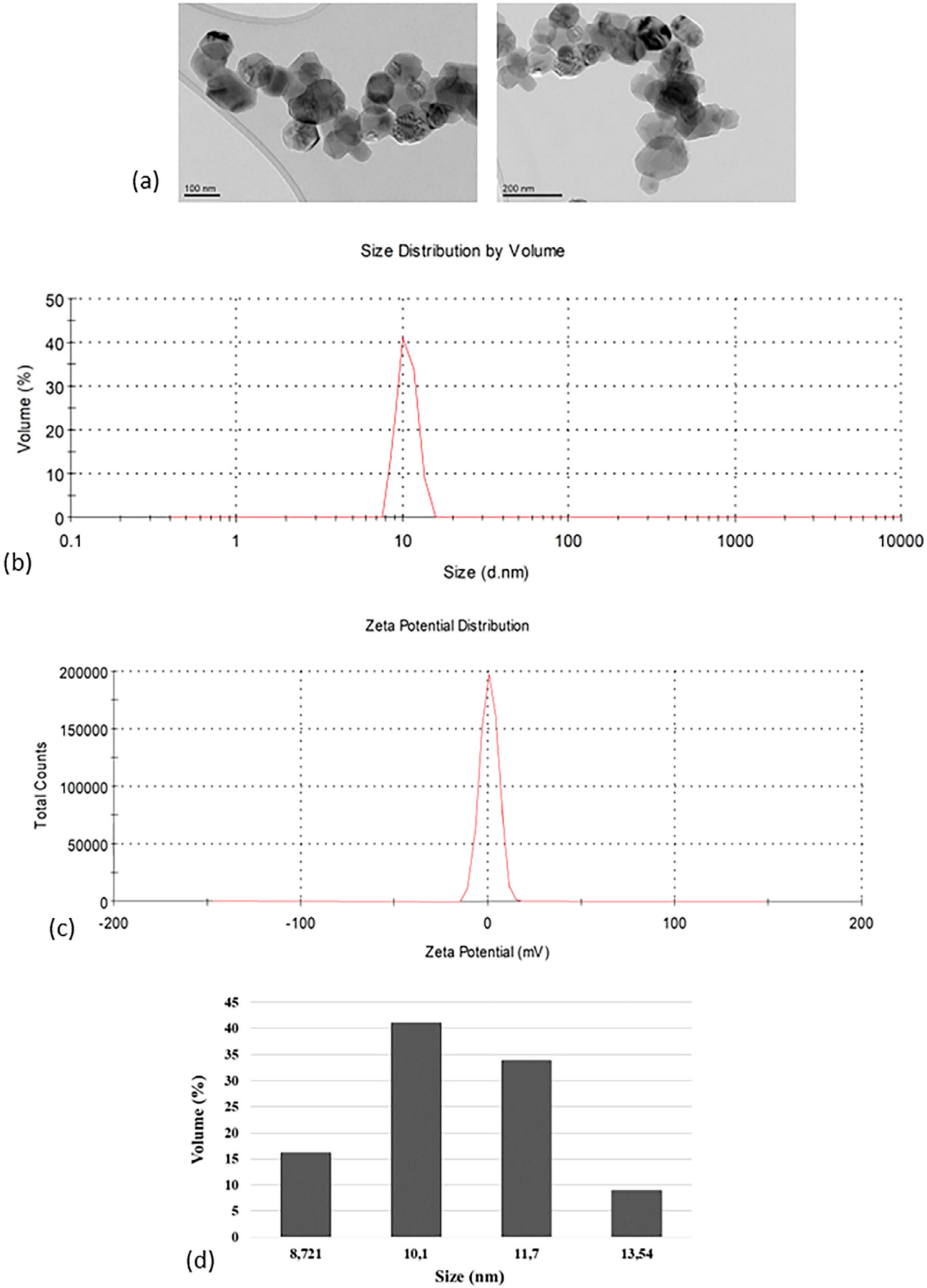


FIGURE 2 Characterization of MgO NPs. (a) TEM image; (b) particle size; (c) zeta potential; (d) percentage by volume of particle size

TABLE 1 DNA damage scores in the coelomocytes of *E. hortensis* earthworms treated with CeO_2 ionic form and CeO_2 NPs

Compounds	Concentrations (μg/ml)	DNA damage score (AU)
Negative control		38 ± 1.56
CeO_2	$LC_{50/2}$	56 ± 1.52[a]
	LC_{50}	80 ± 1.40[c]
	$2\times LC_{50}$	86 ± 2.4[c]
CeO_2 NPs	$LC_{50/2}$	50 ± 1.52[a]
	LC_{50}	60 ± 3.40[b]
	$2\times LC_{50}$	80 ± 2.4[c]

TABLE 2 DNA damage scores in the coelomocytes of *E. hortensis* earthworms treated with MgO ionic form and MgONPs

Compounds	Concentration (μg/ml)	DNA damage score (AU)
Negative control		38 ± 1.56
MgO	$LC_{50/2}$	60 ± 4.2a
	LC_{50}	77 ± 4.08c
	$2xLC_{50}$	82 ± 2.2c
MgONPs	$LC_{50/2}$	56 ± 3.2a
	LC_{50}	66 ± 4.08b
	$2xLC_{50}$	78 ± 2.2c

damage was decreased. In addition, no inflammatory response or lipid peroxidation was observed (Dunnick et al., 2016).

Knowledge about the accumulation of CeO_2 NPs in water and soil is very limited (Johnson & Park, 2012). The solubility of CeO_2 NPs varies according to the amount of dissolved organic matter to the soil structure and the surface properties of NPs (Malev et al., 2017). There is also evidence that CeO_2 NP produces antioxidant-free radical scavenging action, cellular damage and oxidative stress. It is thought that oxidative stress may be caused by the reduction of Ce (IV) to Ce (III). In addition, protective or toxic effects are also related to exposure duration and concentration of CeO_2 NPs (Rollin-Genetet et al., 2015).

MgO has been found to neutralize toxic and biological agents and it is used to provide broad-spectrum protection in decontamination systems (Sellik et al., 2017). It is recommended that MgO NPs are used in drug transport systems, in water treatment, in human and environmental health (Mazaheri et al., 2019). However, nanosized metal oxides with antibacterial properties can cause oxidative stress, lipid peroxidation and cell membrane damage (Kadiyala et al., 2018). The genotoxic and oxidative damage-inducing potential of MgO NPs in four different cell lines (HepG2, NRK-52E, Caco-2, A549) demonstrated the oxidative damage in HepG2 cells and but no DNA damage was detected in this cell line. In addition, DNA damage was observed at higher doses (150 and 300 μg/ml) of MgO NPs in NRK-52E, Caco-2 and A549 cells, while no significant difference was observed in MDA, 8-OHdG, and PC protein levels (Abudayyak et al., 2016). It has been revealed that MgO NP decreases ROS and lipid peroxidation and increases total antioxidant capacity at lower doses (100 μg/ml) in Wistar rat pancreatic cells (Moeini-Nodeh et al., 2017). In male and female Wistar rats, comet assay with MgO NP showed increased DNA damage in peripheral blood lymphocytes, liver, and tissues in dose-dependent manner (500 and 1000 mg/kg). In addition, there was an increase in MDA and LDH levels and a decrease in GSH and CAT levels (Mangalampalli et al., 2018). Since nanoparticles affect the amount of antioxidant or reactive oxygen species, it has been used in cancer treatments and studies on cancer cell lines (Akhtar et al., 2012; Gao et al., 2014). In the HeLa, AGS, and SNU-16 cell lines, MgO NP exposure resulted in increased lipid peroxidation and ROS content, resulting in the induction of apoptosis (Krishnamoorthy et al., 2012). MgO NP-induced caspase-3 enzyme activity and DNA damage were observed in HT 29 cells (Alqahtani & Alomar, 2016). In fresh water snail *Radix leuteola L.* nanosized MgO showed genotoxic effects at 51 μg/ml on 4th day of its exposure whereas, antioxidant enzymes were decreased at 34 μg/ml of MgO NPs nanoparticles and were increased at 51 μg/ml of its exposure along with exposure time (Ali et al., 2016).

4 | CONCLUSIONS

It was concluded that higher concentrations of CeO_2 and MgO NPs and their ionic forms had the potential genotoxicity on *E. hortensis*. Ionic forms of CeO_2 and MgO NPs were observed more genotoxic compared to their NPs. Earthworm's coelomocytes showed a sensitive biomarker to find enivronmental pollution in terrestrial ecosystems. Genotoxic effects of these NPs and their ionic forms showed that these NPs release into the environment and could cause the ecotoxicology.

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CONFLICT OF INTEREST

No conflict of interest has been found.

DATA AVAILABILITY STATEMENT

The data that support the finding of this study is available inside the manuscript.

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