



In vitro and *in vivo* assessment of nanoceria biocompatibility for their safe use in nervous system applications

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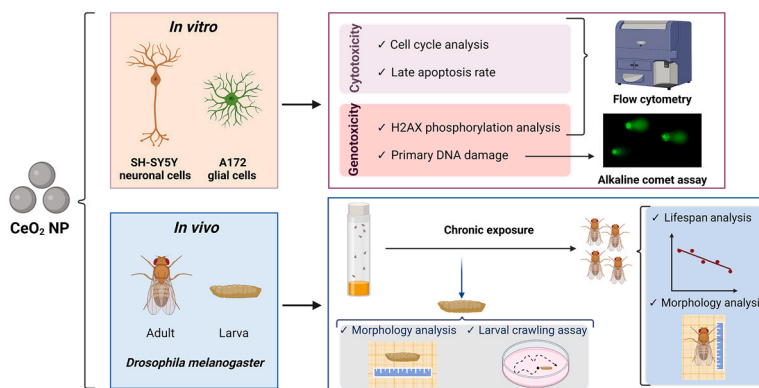
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HIGHLIGHTS

- The environmental presence of nanoceria is growing due to multiple applications.
- Nanoceria have promising biomedical uses, but their neurotoxicity is not discarded.
- This work aimed to assess the biological behaviour of CeO₂ NP *in vitro* and *in vivo*.
- Nanoceria presented a good biocompatibility with scarce cyto- or genotoxic effects.
- Effects observed *in vivo* highlight the need of further research before clinical use.

GRAPHICAL ABSTRACT

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ABSTRACT

Nanoceria, or cerium dioxide nanoparticles (CeO₂ NP), are increasingly employed in a number of industrial and commercial applications. Hence, the environmental presence of these nanoparticles is growing progressively, enhancing the global concern on their potential health effects. Recent studies suggest that nanoceria may also have promising biomedical applications particularly in neurodegenerative and brain-related pathologies, but studies addressing their toxicity, and specifically on the nervous system, are still scarce, and their potential

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Biocompatibility
Nervous system

adverse effects and action mechanism are not totally understood yet. The objective of this work was to assess the biological behaviour of CeO₂ NP *in vitro* in human nervous systems cells, and *in vivo* in *Drosophila melanogaster* to characterize their safety for exposed individuals and verify their suitability to be further employed in diagnosis and treatment of nervous system disorders. Cell cycle alterations, late apoptosis rate and DNA damage (comet and γ H2AX assays), were determined in neuronal SH-SY5Y and glial A172 cells treated with nanoceria. Moreover, the survival rate, morphological changes and behavioural alterations were analysed in *D. melanogaster* individuals chronically exposed to CeO₂ NP. The results obtained from the *in vitro* assessment showed that the nanoceria generally presented a good biocompatibility with scarce cyto- or genotoxic effects, essentially depending the exposure time and cell type, and being restricted to the longer exposure periods. Nevertheless, decrease in adult size and alterations observed in the larval crawling in the *in vivo* assays highlight the need of further investigations before establishing clinical uses of nanoceria.

1. Introduction

Cerium dioxide is one of the most abundant rare-earth elements. As a consequence, synthesis and development of functional cerium dioxide nanoparticles (CeO₂ NP or nanoceria) have prompted in last decades, being increasingly used in a variety of industrial and commercial applications. In particular, nanoceria have been extensively used as polishing materials, including electronic screen products, average optics glass and silica glass [49], to remove pollutants and metal contaminants from aqueous systems [39], as automotive diesel fuel additive-combustion catalyst [10], or as electrode materials for gas sensors in solid oxide fuel cells [25]. It was estimated that around 100–1000 tons of CeO₂ NP are being produced per year only in Europe due to their wide use in diverse fields [47]. As a consequence, the quantity of CeO₂ NP annually released into the environment via anthropogenic activities is quite significant [9].

Together with all these industrial uses, nanoceria have recently gained tremendous attention in the health field for their unique properties such as antioxidant enzyme mimetic properties, oxygen free radical scavenging activity and anti-inflammatory or antiproliferative capacities in several biological systems (reviewed in Brandão Da Silva Assis et al. [4] and Fu et al. [19]). These unique structure-dependent features, make them a promising material for potential biomedical applications, namely as an antitumour agent, for regenerative therapy, dental applications, antibacterial treatment, gene therapy, or targeted drug delivery (reviewed in S Jairam et al. [52]). Indeed, recent studies confirmed that nanoceria are particularly interesting for using in diabetic wound healing [12,55], eye disease [68], and antioxidative therapeutics [5,29]. After entering the body, CeO₂ NP can accumulate in various organs, including heart and lung, and cause systemic effects mainly involving inflammatory response [41]. Still, and despite all their uses and potential applications, their cellular uptake, potential adverse effects and action mechanism are not totally understood yet [58]. Studies carried out on cell cultures have reported contradictory results, showing that nanoceria may induce toxicity or protective effects on exposed cells mainly depending on particle size, shape, exposure time or cell type [35,64]. Furthermore, route of CeO₂ NP synthesis may play a very important role in its toxicity. Recently, Nosrati et al. [42] reviewed the different methods available to synthesized CeO₂ NP including both by chemical means and green synthesis, and their role in wound healing and their potential toxicity; and Gajbhiye et al. [20] described the influence of synthesis conditions of these NP on their physical properties and antibacterial activities.

Several recent studies suggest that nanoceria may have promising applications particularly in neurodegenerative and brain-related pathologies [14,19,51]. Many central nervous system (CNS) diseases are characterized by accumulation of reactive oxygen species (ROS), which induce severe damage to the brain tissues and irreversible neurodegeneration. CeO₂ NP have raised as a novel potential agent in the treatment of neurological diseases, mostly due to their remarkable ability to reduce oxidative stress in the damaged cells through their ROS scavenging ability, the wide range of free radicals they can scavenge, and their self-regenerating redox cycle [27,51]. Moreover, although the

exact mechanism and routes of entry remain unclear, recent studies in animal models showed that nanoceria can cross the blood-brain barrier (BBB) due to their nanoscale diameters [40,45]; this is particularly noticeable in those cases in which BBB is altered or damaged [30,65], supporting their potential as neurodrug delivery tools. Still, potential harmful effects of CeO₂ NP on nervous system cells are barely addressed in the literature and results obtained so far are inconclusive.

In vitro studies are a cost-effective and fast strategy to assess the biocompatibility/toxicity of nanomaterials. Still, sometimes results obtained by *in vitro* assays differ from those obtained *in vivo*, given their inability to entirely replicate the complex interactions that occur within an organism [37]. Even though *in vivo* NP toxicity is frequently evaluated in mice and rats, the fruit fly *Drosophila melanogaster* has been proposed and successfully used as a model organism in toxicology and nanotoxicity studies [44,61]. Among its many advantages, *D. melanogaster* shows high genetic similarity with humans, is easy to maintain and manipulate in the laboratory, has a short life cycle with different developmental stages, allows nanomaterial administration by several routes, and raises fewer ethical concerns than mammalian models [44]. Furthermore, *D. melanogaster* genome has been sequenced and annotated [1] showing that 75 % of human disease genes have related sequences in this model, including genes involved in neurological and developmental disorders and cancer [3].

The hypothesis of this study was that CeO₂ NP do not induce significant cyto- or genotoxicity in nervous system cells or adverse effects in fly organisms at biologically relevant doses which could hamper their further applications in biomedicine field. On this basis, the main objective of this work was to assess whether CeO₂ NP could induce adverse effects at cellular and genetic levels on human nervous system cells as well as to characterize their possible effects at the organism level on the *in vivo* model *D. melanogaster*, not only to eventually characterize their safety for exposed individuals but also to verify their suitability for further applications in diagnosis and treatment of nervous system disorders. To this aim, cell cycle alterations, late apoptosis rate and DNA damage, both primary (alkaline comet assay) and double-strand breaks (H2AX phosphorylation), were determined in neuronal SH-SY5Y and glial A172 cells treated with CeO₂ NP (1–100 μ g/mL) as indicative of cyto- or genotoxicity, respectively. Moreover, the survival rate, morphological changes and behavioural alterations were analysed in *D. melanogaster* individuals chronically exposed to CeO₂ NP.

2. Materials and methods

2.1. Nanoparticle dispersion and characterization

Cerium (IV) oxide nanopowder (CAS No. 1306–38–3) was obtained from Sigma-Aldrich Co.; according to the supplier, their primary particle size was < 25 nm. Nanoceria suspensions were prepared as previously described [60]. Briefly, a stock suspension of CeO₂ NP at the highest tested concentration (100 μ g/mL) was prepared in either distilled water or cell culture media. Before treatments, each suspension was ultrasonicated on ice with a 2.5 mm probe (Sonoplus mini 20, Bandelin) at 30 W for 5 min (0.5 min on and 1 min off twice, plus 2 min on), and

diluted to prepare the final CeO₂ NP concentrations tested. To determine the average hydrodynamic diameter and zeta potential of the tested nanoceria, the resulted suspensions were analysed by Dynamic Light Scattering (DLS) and mixed mode measurement phase analysis light scattering (M3-PALS), respectively, using a Zetasizer Nano-ZS (Model ZEN 3600; Malvern Instruments Ltd.). Besides, UV-Vis spectra of CeO₂ NP in water and in both cell culture media were measured in a 6850 UV/Vis Spectrophotometer JENWAY (Bibby Scientific Ltd.).

2.2. In vitro assessment

2.2.1. Cell culture

Human neuroblastoma SH-SY5Y (ECACC 94030304) and glioblastoma A172 (ECACC 88062428) cells were obtained from the European Collection of Cell Cultures and incubated in cell culture medium at 37 °C, 5 % CO₂ in a humidified atmosphere. The SH-SY5Y culture medium consisted of EMEM/F12 (1:1) medium with 1 % non-essential amino acids, 1 % antibiotic and antimycotic solution, and supplemented with 10 % heat inactivated foetal bovine serum (FBS), whereas the A172 medium was composed of DMEM medium with 1 % L-glutamine, 1 % antibiotic and antimycotic solution, and 10 % FBS. For toxicity assessment, both types of cells (80 % confluence) were detached with trypsin-EDTA, seeded in 96-well plates, and allowed to adhere in all cases for 24 h prior treatments.

2.2.2. Cytotoxicity evaluation

Cell cycle analysis was conducted as described in Valdiglesias et al. [59]. Briefly, SH-SY5Y and A172 cells were seeded in 96-well plates (2×10^4 cells/well) and incubated for 3 and 24 h at 37 °C, 5 % CO₂ with increasing concentrations (1–100 µg/mL, corresponding to 0.3–30.3 µg/cm²) of CeO₂ NP or the negative and positive controls. Negative controls used were cell culture media, and positive control was 1.5 µM mitomycin C (MMC, CAS no. 50–07–7). After exposure to nanoceria, cells were harvested, resuspended in cold phosphate buffer solution, centrifuged, and fixed with cold 70 % (v/v) ethanol (–20 °C) overnight at 4 °C. For analysis, cells were stained with propidium iodide (PI) containing RNase A and acquired by flow cytometry (FACSCalibur cytometer, Becton Dickinson). DNA content (PI signal detected by the FL2 detector) was then quantified in at least 10,000 events calculating the percentages of DNA in G0/G1, S and G2/M regions of cell cycle with the Cell Quest Pro software (Becton Dickinson). Possible interference of CeO₂ NP with flow cytometry measurements due to potential NP auto-fluorescence was discarded by using cell-free NP samples prior experiments in all cases. DNA content at the subG1 region was also quantified as indicative of late stages of apoptosis.

2.2.3. Genotoxicity evaluation

For genotoxicity experiments, 2×10^4 cells/well or 5×10^4 cells/well depending on the assay, from both cell types were incubated with a dose range of nanoceria (1–100 µg/mL) for 3 and 24 h. Negative controls used were cell culture media, and positive controls were 1 µg/mL bleomycin (BLM, CAS No. 11056–06–7) for γH2AX assay and 60 µg/mL methyl methanesulphonate (MMS, CAS No. 66–27–3) for comet assay. γH2AX assay was carried out by flow cytometry in a FACSCalibur cytometer (Becton Dickinson) following Fernández-Bertólez et al. [16], and alkaline comet assay was performed according to Fernández-Bertólez et al. [15], previously checking possible interferences of the nanoceria with the comet assay methodology, as recommended by Magdolenova et al. [38]. Parameters evaluated in these assays were the percentage of cells with phosphorylated H2AX (γH2AX) and the percentage of DNA in the comet tail (%tDNA), respectively.

2.3. In vivo assessment

2.3.1. *Drosophila melanogaster*: strain and culture medium

D. melanogaster individuals (Oregon R strain) were grown in vials at

22°C (–1/+2°C) under natural light. Specimens were grown in vials containing 1 g of Formula 4–24® Instant *Drosophila* Medium (Carolina Biological Supply) and 5 mL of yeast solution prepared as follows: 500 g of yeast are mixed with 600 mL of sterile tap water and boiled for 20 min. The mixture is then brought to 833 mL with sterile tap water. Finally, 10.4 mL of propionic acid, 112 mL of nipagin solution (11.2 g nipagin + 112 mL absolute ethanol) and 10.41 g of NaCl are added to the blend. This solution is stored at –20°C until used.

2.3.2. Experimental setup

Four conditions were tested: control (absence of CeO₂ NP), 1, 10 and 100 µg/mL of CeO₂ NP. For exposure, CeO₂ NP were incorporated in the diet: all vials contained 1 g of Instant *Drosophila* Medium and 5 mL of yeast solution that included either none (control) or the corresponding NP concentration. Prior to their addition to the yeast solution, CeO₂ NP were probe sonicated for 5 min, as described in Section 2.1. Once prepared, yeast solution was mixed vigorously, poured into the vials containing the Instant *Drosophila* Medium and let to solidify.

2.3.3. Lifespan analysis

Two different experiments were performed to assess the effect of NP exposure on lifespan: (i) exposing flies to CeO₂ NP only during the adult stage of their life cycle (first longevity experiment) and (ii) using the progeny of the flies of the first experiment, i.e. flies whose parents were exposed to CeO₂ as adults and that were exposed to CeO₂ NP during their whole life cycle (second longevity experiment). Both experiments were carried out twice, with four and three replicates for exposure condition, respectively.

For the first longevity experiment, adult individuals were anesthetized within 24 h after eclosion from the pupal case and transferred into new culture vials (5 females and 5 males per vial) containing medium with either none or one of the three CeO₂ NP concentrations tested. Medium was changed twice a week, and the number of survivors was monitored until all flies in the vial died.

For the second longevity experiment, we collected larvae from the vials of the first longevity experiment after 20 days of NP exposure and allowed them to grow into adult flies. These adults were then used to perform the experiments under the same conditions previously described for the first longevity experiments.

2.3.4. Morphology analysis

Prior to starting the second longevity experiments, the adult flies used in them—25 flies per exposure condition—were anesthetised, placed under a stereomicroscope and photographed. Their length was then determined using ImageJ software [54].

Ten third instar larvae of each condition from both longevity experiments (first and second larval morphology assay, respectively) were kept in 4 % formaldehyde for 24 h. Next, larvae were placed on a sheet of millimetre graph paper and photographed to determine their length using ImageJ software.

2.3.5. Larval crawling assay

The progeny of both the first and second longevity experiments were allowed to grow and third instar larvae of the four conditions tested were collected for the first and second larval crawling assay, respectively. In each experiment, larvae were washed with distilled water to remove any remains of food attached to their body and transferred to a Petri dish with a 2 % agarose layer at the bottom and a sheet of millimetre graph paper underneath. Each larva was let to crawl along the agarose surface of the Petri dish for 1 min and its movement recorded. The distance covered by larvae (10 larvae per exposure condition) and the number of contractions (full anterior to posterior movement = 1 contraction) of each larva during that 1 min of movement were determined.

2.4. Statistical analyses

For *in vitro* assessment, all experiments were run at least in triplicate, and differences among groups were analysed by Kruskal-Wallis test, with Mann-Whitney *U*-test for two-by-two comparisons. For lifespan analyses, results were plotted and analysed for statistical significance by means of a Log Rank test. Flies that escaped or died as a result of medium change were considered censored data. Significant differences among groups in the adult and larval morphology analysis and in the crawling assay were determined by an ANOVA test. Should the ANOVA be significant, we used the Dunnett's test to analyse differences between the control group and the treatment groups on a pair-wise basis. For data that violated the ANOVA assumptions, differences among groups were assessed by means of the Kruskal-Wallis test and on a pair-wise basis — control versus each treatment — by means of the Mann-Whitney *U*-test.

Pearson's correlation was used to investigate correlations between treatment doses and the specific variables measured in the different assays. All statistical analyses were performed using the IBM SPSS Statistics package v. 29.0 with a significance threshold of 0.05, and experimental data were expressed as mean $\pm$ standard error.

3. Results

Table 1 presents the results obtained in the physicochemical characterization (hydrodynamic diameter and zeta potential) of the CeO₂ NP used in this study, in water and in neuronal and glial cell culture media. In data obtained immediately after NP dispersion (time = 0 h), hydrodynamic diameter was comparable in the three liquid media and maintained in the nano range, indicative of no agglomeration among NP, while zeta potential values were similar and negative in the cell culture media but positive in water. In addition, to confirm the stability of the NP dispersion throughout the *in vitro* experiments in which cells were cultured up to 48 h, both parameters were measured at 3, 24, and 48 h (Table 1). The dispersion of CeO₂ NP resulted quite stable, with hydrodynamic sizes ranging between 141.0 and 154.5 nm in SH-SY5Y cell culture medium and between 139.0 and 155.6 nm in A172 cell culture medium. Zeta potential was also stable in both media, always with negative charge and values between -11.0 and -12.5 mV. The good dispersion and stability were also confirmed by UV-Vis spectroscopy by measuring the absorbance spectra of samples at room temperature over a wavelength range of 200–1000 nm. Spectra of all samples showed strong and similar absorption bands in the UV range, with single maximum absorbance peaks (λ_{max}) of 305 nm in water, 306 nm in SH-SY5Y medium, and 303 nm in A172 medium (Fig. 1). No additional time points were evaluated in water since CeO₂ NP dispersed in water were immediately incorporated to the fly food, which solidified avoiding further agglomeration of the NP with time.

Table 1
Physicochemical characterization of nanoceria in water and cell culture media.

	Hydrodynamic diameter (nm)	Zeta potential (mV)
Distilled water		
0 h	187.1 $\pm$ 1.2	14.9 $\pm$ 3.9
SH-SY5Y culture medium		
0 h	141.0 $\pm$ 2.8	-11.5 ± 0.8
3 h	136.9 $\pm$ 4.1	-11.0 ± 0.7
24 h	148.2 $\pm$ 2.3	-11.0 ± 0.7
48 h	154.5 $\pm$ 14.5	-11.2 ± 0.8
A172 culture medium		
0 h	139.0 $\pm$ 1.9	-12.1 ± 0.8
3 h	139.2 $\pm$ 2.1	-11.8 ± 0.5
24 h	162.6 $\pm$ 12.0	-12.5 ± 0.8
48 h	155.6 $\pm$ 3.0	-12.3 ± 1.0

3.1. Nanoceria cytotoxicity

Results obtained from the flow cytometry cell cycle analysis after treating neuronal and glial cells with CeO₂ NP are depicted in Fig. 2. No effects were obtained in any case in glial A172 cells, whereas slight but significant alterations were only observed in S phase of SH-SY5Y cells treated for 24 h.

In the cell cycle analyses, the subG₁ region was also evaluated since DNA fragmentation, indicative of the late stages of apoptosis, results in the appearance of PI-stained events containing subG₁ levels [17]. Results of nanoceria-induced apoptosis, measured as the percentage of events in the subG₁ region, are shown in Fig. 3. Slight increases regarding the negative control were found only in glial A172 cells treated with CeO₂ NP for 3 h at 100 $\mu\text{g/mL}$ and for 24 h at both 10 and 100 $\mu\text{g/mL}$.

3.2. Nanoceria genotoxicity

Results obtained from H2AX phosphorylation analysis showed significant increases in the percentage of neuronal cells with γH2AX at all the conditions tested and with a significant positive concentration-response relationship for the 24 h treatment ($r = 0.528$; $P < 0.05$) (Fig. 4). A similar effect but with significant concentration-dependent responses at both exposure times (3 h: $r = 0.732$; $P < 0.01$; 24 h: $r = 0.759$; $P < 0.01$) was observed in glial cells. In this case, increases in the %cells with γH2AX were found significant only at the highest concentration after 3 h exposure, and at all doses tested after 24 h.

Possible interferences of nanoceria with the comet assay protocol were discarded prior experiments in each case (Fig. 5a). The presence of CeO₂ NP did not induce modifications in the %tDNA when nucleoids were directly exposed to the NP, either in the negative or in the positive control, when compared with the absence of NP.

After discarding potential interferences, standard alkaline protocol was employed in the comet assay experiments. Results from these experiments are shown in Fig. 5b. No significant differences were observed in any case; however, slight but significant concentration-response relationships were obtained glial cells at both exposure times (3 h: $r = 0.540$, $P < 0.05$; 24 h: $r = 0.565$, $P < 0.05$).

3.3. Lifespan analysis

Fig. 6 shows the survival curves for the control and treatment groups of both *D. melanogaster* longevity experiments. No significant differences in survival were detected among the four conditions tested either for the first ($\chi^2 = 0.552$, $P = 0.907$) or for the second ($\chi^2 = 2.545$, $P = 0.467$) experiment.

3.4. Morphology analysis

The average length of the adult flies analysed for each of the three nanoceria exposure conditions are shown in Fig. 7. Both the 1 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ treatment groups showed significantly higher lengths when compared to control flies. No other morphological alterations as a result of exposure to CeO₂ NP were detected.

The average length of ten larvae of each of the four exposure conditions for the first and second larval morphology experiments is represented in Fig. 8. In both larval morphology assays, none of the nanoceria exposure conditions differed significantly from the corresponding control. Moreover, no correlation between larval length and treatment dose was detected for either experiment ($P = 0.749$ and $P = 0.564$, respectively).

3.5. Larval crawling assay

The average distance covered by larvae exposed to CeO₂ NP is shown in Fig. 9a. No significant differences in the crawling distance among

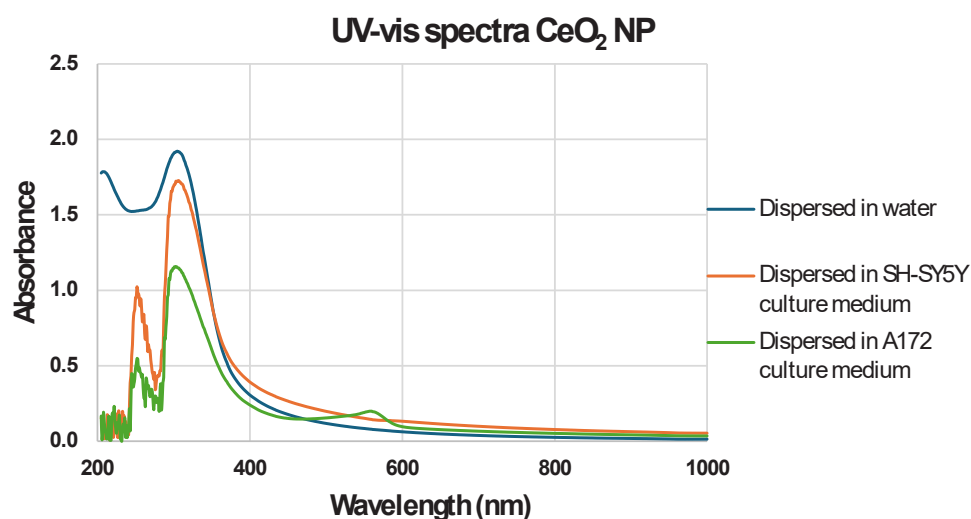


Fig. 1. UV-Vis absorption spectra of CeO₂ NP in water ($\lambda_{\max}$ =305 nm), SH-SY5Y medium ($\lambda_{\max}$ =306 nm), and A172 medium ($\lambda_{\max}$ =303 nm).

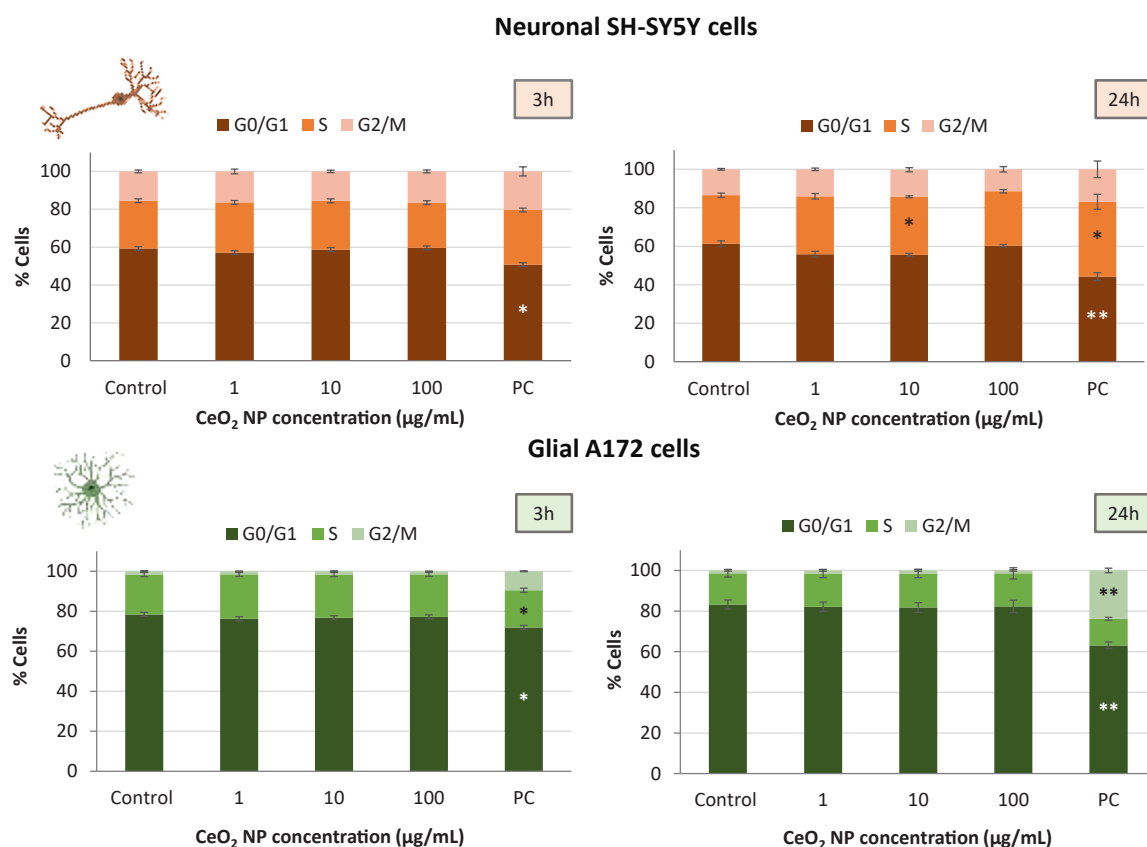


Fig. 2. Cell cycle analysis in human neuronal and glial cells treated with nanoceria for 3 (left) or 24 (right) h. Bars represent the mean $\pm$ standard error. PC: positive control (MMC 1.5 μ M). * P < 0.05, significant difference with regard to the control. Neuron and astrocyte pictures created in Biorender (BioRender.com/r22b790).

groups were detected for the first crawling assay. Nevertheless, for the second larval crawling assay, larvae exposed to 10 μ g/mL and 100 μ g/mL CeO₂ NP covered significantly lower distances than those of the control group. Furthermore, a significant correlation between distance and dose was observed for this experiment, showing a tendency for distance to decrease as the treatment dose increased ($r = -0.334$, $P = 0.035$).

Furthermore, differences among groups were detected for the first and the second experiments regarding the number of larval contractions

(Fig. 9b), with significant decreases in all nanoceria doses regarding their corresponding control in both experiments. Additionally, the two experiments showed a significant inverse dose-response relationship regarding the number of body contractions ($r = -0.715$, $P < 0.0001$ for the first experiment, and $r = -0.548$, $P < 0.0001$ for the second experiment).

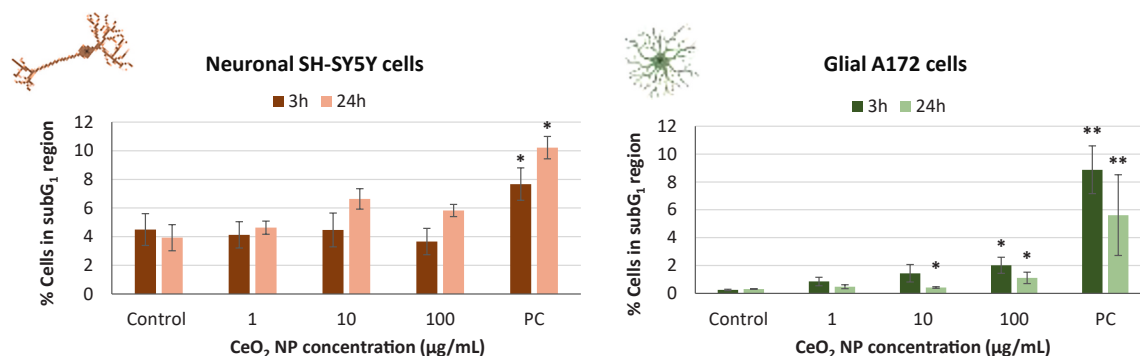


Fig. 3. Apoptosis (subG₁ region) in neuronal and glial cells treated with nanoceria for 3 or 24 h. Bars represent the mean $\pm$ standard error. PC: positive control (MMC 1.5 μ M). * P < 0.05; ** P < 0.01, significant difference with regard to the control. Neuron and astrocyte pictures created in Biorender (BioRender.com/r22b790).

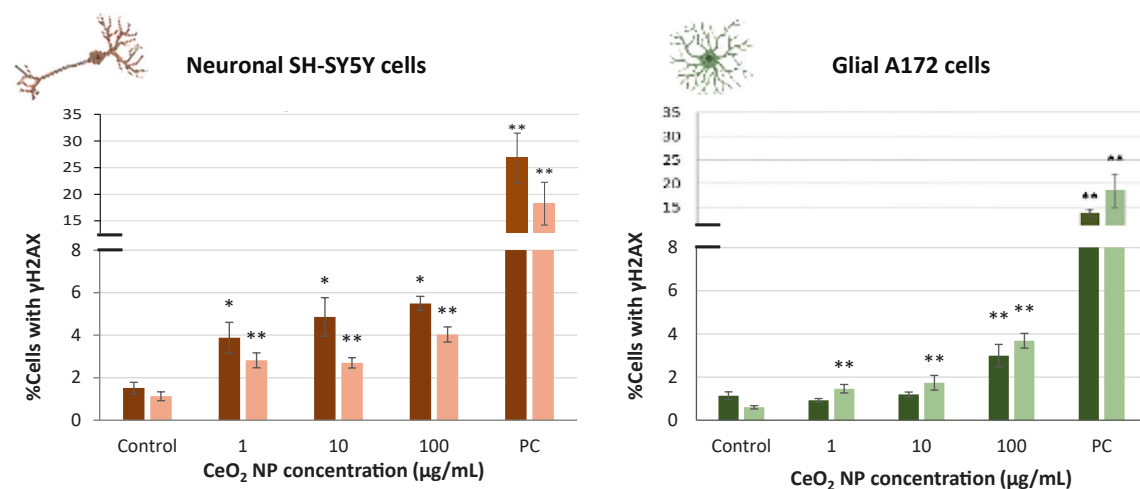


Fig. 4. Phosphorylation of H2AX histone after treatment of neuronal or glial cells with nanoceria. Bars represent the mean $\pm$ standard error. PC: positive control (BLM 1 μ g/mL). * P < 0.05, ** P < 0.01 significant difference with regard to the control. Neuron and astrocyte pictures created in Biorender (BioRender.com/r22b790).

4. Discussion

CeO₂ NP have been extensively used in a number of industrial applications and commercial products. More recently, they have received much attention in the biomedical field mainly due to their excellent catalytic activities, derived from quick and expedient mutation of the oxidation state between Ce⁴⁺ and Ce³⁺, that lead to antioxidant activity, antimicrobial properties and antitumour capacity. This extensive use and potential applications motivated CeO₂ NP to be included in the Organisation for Economic Co-operation and Development (OECD) list of high priority manufactured nanomaterials for toxicological evaluation [43]. Given the lack of information of toxicological aspects of nanoceria, in addition to the high potential of this nanomaterial to be used for biomedical purposes, we aimed to assess the biological behaviour of CeO₂ NP in human nervous system cells and the animal model *D. melanogaster* in order to detect and characterize potential adverse effects and provide useful information for further safe uses.

For the *in vitro* assessment, two well-recognized nervous system cellular models, namely SH-SY5Y neuroblastoma cell line and A172 glioblastoma cell line, were employed in this study since they are both salient models from human neurons and astrocytes, respectively, that have been routinely used in *in vitro* studies to elucidate basic neurobiological principles and mechanism of chemical action [33]. Results from cell cycle analysis of glial cells showed no effects after nanoceria exposure, and only very slight alterations were observed in exposed neuronal

cells after 24 h treatment. As for late apoptosis, mild increases were only observed in glial cells treated with nanoceria at the highest concentrations and longest exposure time. Still, the fact that the subG₁ region may also include some non-apoptotic cell death-related events, such as necrosis or mitotic catastrophe, also must be considered [67].

The lack of cytotoxic effects related to nanoceria exposure is frequent in the literature [23,6,63], being CeO₂ NP considered to show very low acute toxicity in realistic exposure levels [64]. The need of longer exposures (24 h or higher) to observe nanoceria-induced toxic responses has been also previously reported in different cell systems [21,36]. Considering the general tendency of NP to agglomerate or aggregate over time, effects observed in longer exposure periods may be also due to agglomerates rather than the effects of CeO₂ NP themselves, supporting the safety of these NP under stable conditions. Particularly in nervous system cells, 2–5 nm nanoceria at very low concentration (1.7 ng/mL) were found not to harm but to enhance survival and provide a significant neuroprotective effect in cells isolated from rat spinal cord [11]. Similarly, no cytotoxic effects – oxidative stress, lactate dehydrogenase leakage and decrease in mitochondrial function – were observed in IMR32 neuroblastoma cells treated with 25 nm CeO₂ NP at concentrations up to 50 μ g/mL [32]. Our results agree with those obtained in some previous studies that revealed cytotoxicity or drastic decreases in cell viability only at high CeO₂ NP doses (> 50 μ g/mL) and/or long exposure periods (> 24 h) [32,50,62]. Still, NP agglomeration seems not be responsible of the effects observed in our study, since hydrodynamic size

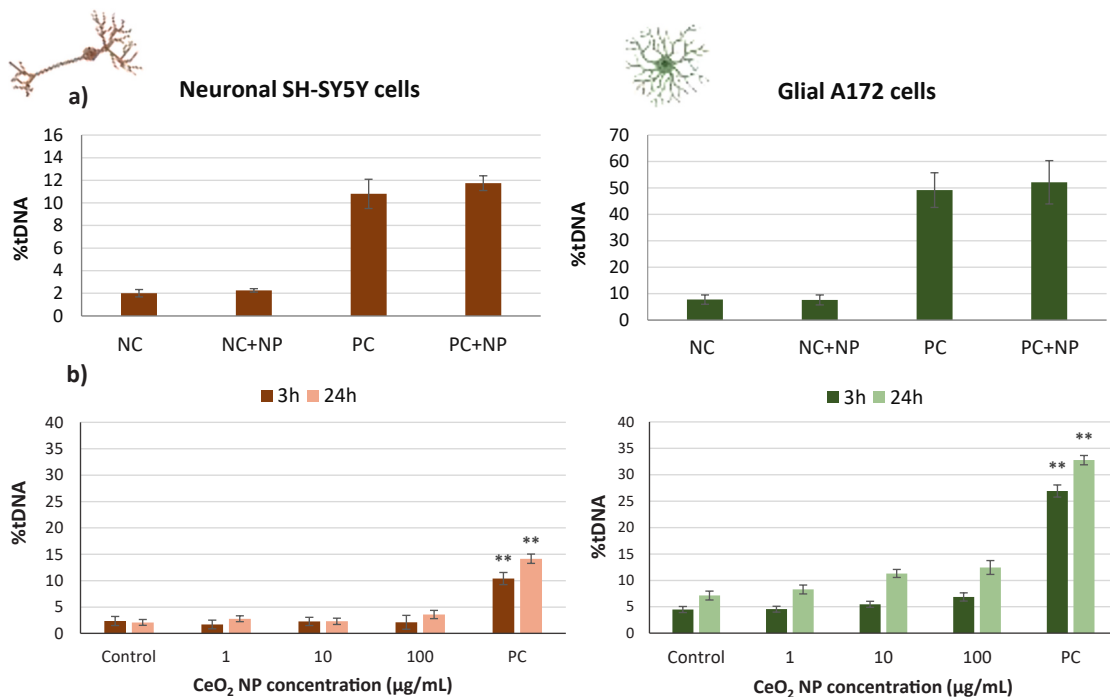


Fig. 5. Alkaline comet assay results of neuronal and glial cells treated with nanoceria. (a) Interference testing between nanoceria (100 µg/mL) and comet assay methodology, (b) evaluation of DNA damage induction. Bars represent the mean $\pm$ standard error. NP: nanoparticles, NC: negative control. PC: positive control (60 µg/mL MMS). * * $P < 0.01$ significant difference with regard to the control. Neuron and astrocyte pictures created in Biorender (BioRender.com/r22b790).

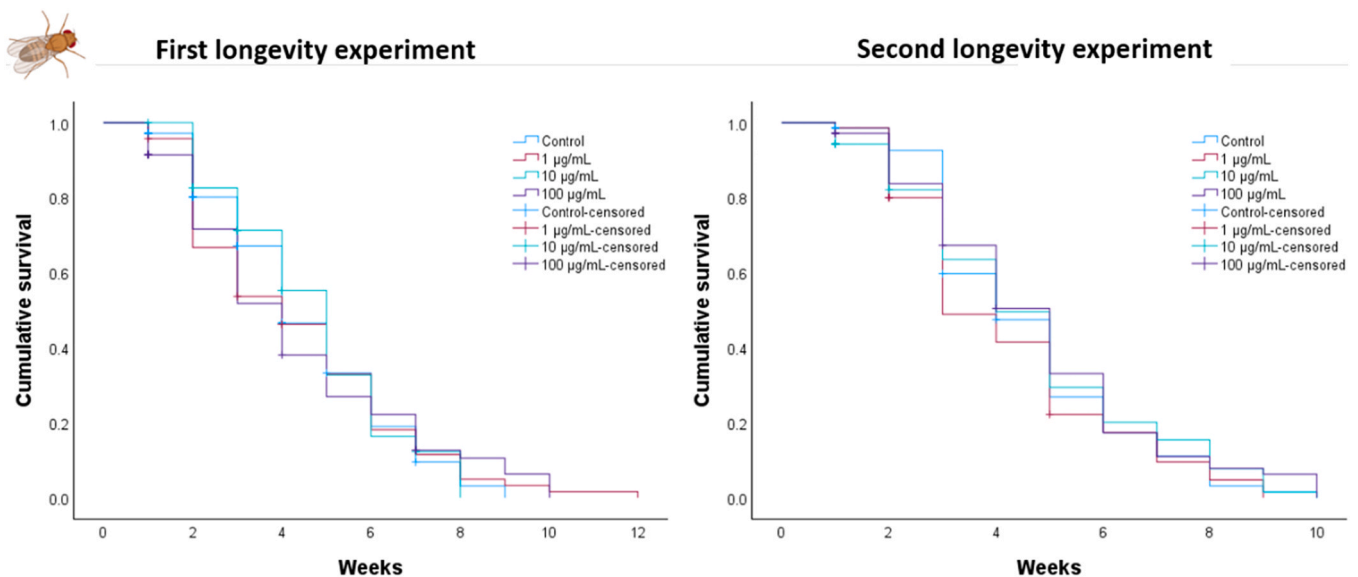


Fig. 6. Survival curves of *D. melanogaster* flies for the four conditions tested (70 flies per condition) in the first (left) and second (right) longevity experiments. Experimental points represent the cumulative survival distribution through time. Fly picture created in Biorender (BioRender.com/r22b790).

and zeta potential analyses confirmed the stability of the NP dispersion over time in both cell culture media.

Regarding genotoxic effects of nanoceria on nervous system cells, scarce DNA lesions were observed in our study. Results from comet assay evaluating primary DNA damage (alkali labile sites, single and double strand breaks, and incomplete excision repair sites) [8] showed no statistically significant induction in any case. Although significant increases of phosphorylated H2AX, indicative of DNA double strand breaks, were detected, values obtained were always lower than 6 %, supporting a scarce genotoxic potential of CeO₂ NP in both cell types tested. The lack of DNA damage observed in comet assay results despite

the significant effects detected in γ H2AX assay is likely due to the different sensitivity of each technique, being higher for the γ H2AX approach [28]. In the literature, the genotoxic effects found in different human cell types exposed to these NP are often not consistent. Franchi et al. [18] reported no significant increases in phosphorylated H2AX in fibroblasts exposed to low CeO₂ NP concentrations (10 µg/mL) for 24 h. Similarly, some previous works showed that exposure of human cells to nanoceria concentrations from 10 to 200 µg/mL did not significantly induce primary DNA damage (evaluated by the comet assay) [32,62]. On the contrary, other studies employing γ H2AX or comet assays revealed that CeO₂ NP concentrations as low as 6 µg/mL presented a

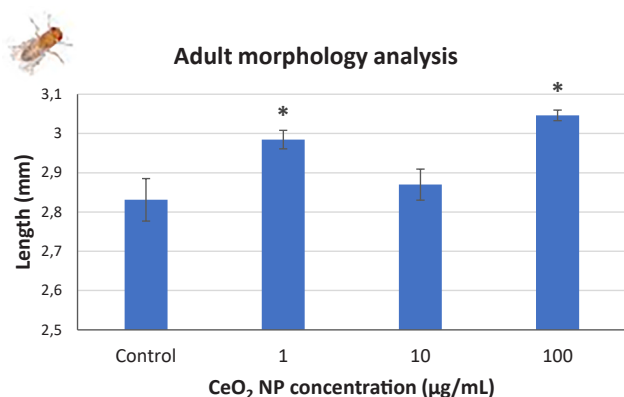


Fig. 7. Length of *D. melanogaster* adult individuals exposed to CeO₂ NP. Bars represent the mean $\pm$ standard error. * $P < 0.05$ significant difference with regard to the control. Fly picture created in Biorender (BioRender.com/r22b790).

high genotoxic potential even after 3 h exposure in human peripheral blood lymphocytes [31] or human spermatozoa [48]. Accordingly, clastogenicity was also demonstrated by micronucleus test in association with immunofluorescence staining of centromere protein A in human dermal fibroblasts exposed for 48 h to similar concentrations of nanoceria (6 – 600 µg/mL) [2]. Also, exposure to naked nanoceria (6 and 100 nm) was found to induce alterations in the expression of genes related to neurological disease, cell cycle control and growth in mouse neuronal cells, but at much higher concentration (6.9 mg/mL) in this case [34]. To the best of our knowledge, this is the first study evaluating the DNA damage in human glial cells exposed to nanoceria, and just another previous one employed human neuroblastoma IMR32 cells to evaluate genotoxicity of these NP. In that study, and agreeing with our results, DNA damage evaluated by micronucleus test and comet assay was found only at CeO₂ NP concentrations higher than 100 µg/mL or 200 µg/mL, respectively [32]. As it was previously reported that the biological behaviour of these NP would be cell type-dependent and no remarkable genotoxic or cytotoxic effects were observed in the current study at environmentally and therapeutically relevant doses (1 and 10 µg/mL) [26,66] either in glial or in neuronal cells, our findings would confirm the low toxicity profile of CeO₂ NP specifically in nervous system cells, supporting their use in this tissue.

Given that the *in vitro* effects of chemicals do not always match their impact *in vivo*, we exposed *D. melanogaster* individuals to the same three CeO₂ NP concentrations used in the *in vitro* assays, in order to investigate the long-term effects of *in vivo* exposure. *D. melanogaster* is a well established *in vivo* model organism for toxicology studies and has been successfully used in numerous occasions to assess the toxicity of several

engineered nanomaterials, including metal nanoparticles [61]. In this study, the survival rate, morphological changes and behavioural alterations were analysed in *D. melanogaster* chronically exposed to CeO₂ NP. Since the reproductive toxicity of nanoparticles is a particularly important issue as toxic effects can be transgenerational [13,24], all *in vivo* outcomes were measured not only in adult individuals exposed to nanoceria, but also in the progeny of those individuals.

No effect of NP exposure on fly survival was observed for any of the concentrations tested either as a result of CeO₂ NP exposure during their adulthood (first longevity experiment) or during their whole biological cycle (second longevity experiment). The absence of significant differences in survival after CeO₂ NP treatments contrasts with the results obtained by Sundararajan et al. [57], who also used *D. melanogaster* to study the effect of CeO₂ NP (17.2 and 172.1 µg/mL) on survival and reported a significant dose-dependent reduction after exposures longer than one month. However, increases in survival because of CeO₂ NP exposure have been reported as well. Thus, Strawn et al. [56] detected an up to 38 % extension in fly lifespan as well as an increase in survival by 200 % as a result of the administration of CeO₂ NP (doses not specified) after a lethal exposure to the herbicide paraquat, exerting a protective effect against paraquat toxicity. Also, another study reported that CeO₂ NP (doses up to 100 µg/mL) prolonged both the median and maximum lifespan in female flies, but not in male flies [7]. These discrepancies may respond to the different NP concentrations used, supporting the suggestion of *in vitro* findings that higher CeO₂ NP doses are often required to induce toxicity. Nevertheless, there are other possible factors that may underlie the reported inconsistencies, since the redox properties and cytotoxic effects of nanoceria depend not only on dosage but also on other aspects such as synthesis method, particle size and shape, dopant and surface chemistry [49,64].

We also investigated the effect of CeO₂ exposure on larval and adult morphology. Regarding adult morphology, visual examination under a stereomicroscope did not reveal any morphological alterations caused by nanoceria exposure. This result is in line with that of Parimi et al. [46], who detected no phenotypic defects in adult flies exposed to CeO₂ NP through their whole life cycle at a dose range similar to the one used in the current study. Nevertheless, two of the treated groups showed significant differences in size when compared to the control, suggesting a possible effect of nanoceria exposure on adult growth. Nevertheless, it is important to note that these groups comprise a random number of males/females. Given that *D. melanogaster* females are usually larger than their male counterparts, sex-based separation of flies is advisable for future studies.

As for the effect of CeO₂ NP on larval growth, previous studies showed contradictory results. Parimi et al. [46] reported a delay in larval growth as a result of negatively charged CeO₂ NP treatment (172.1 µg/mL). Even so, they detected no differences in length or width

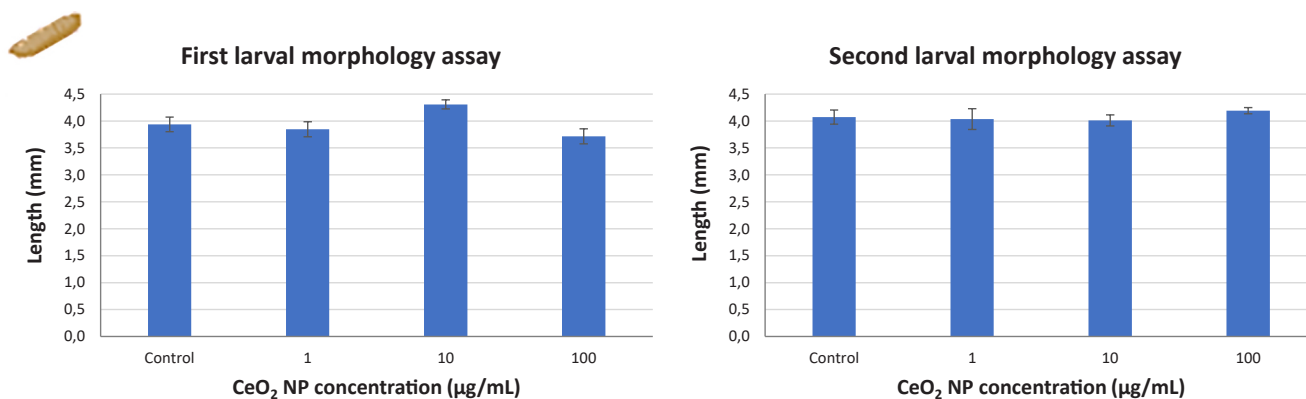


Fig. 8. Length of the *D. melanogaster* larvae exposed to nanoceria in the first (left) and second (right) larval morphology assays. Bars represent the mean $\pm$ standard error. Larva picture created in Biorender (BioRender.com/r22b790).

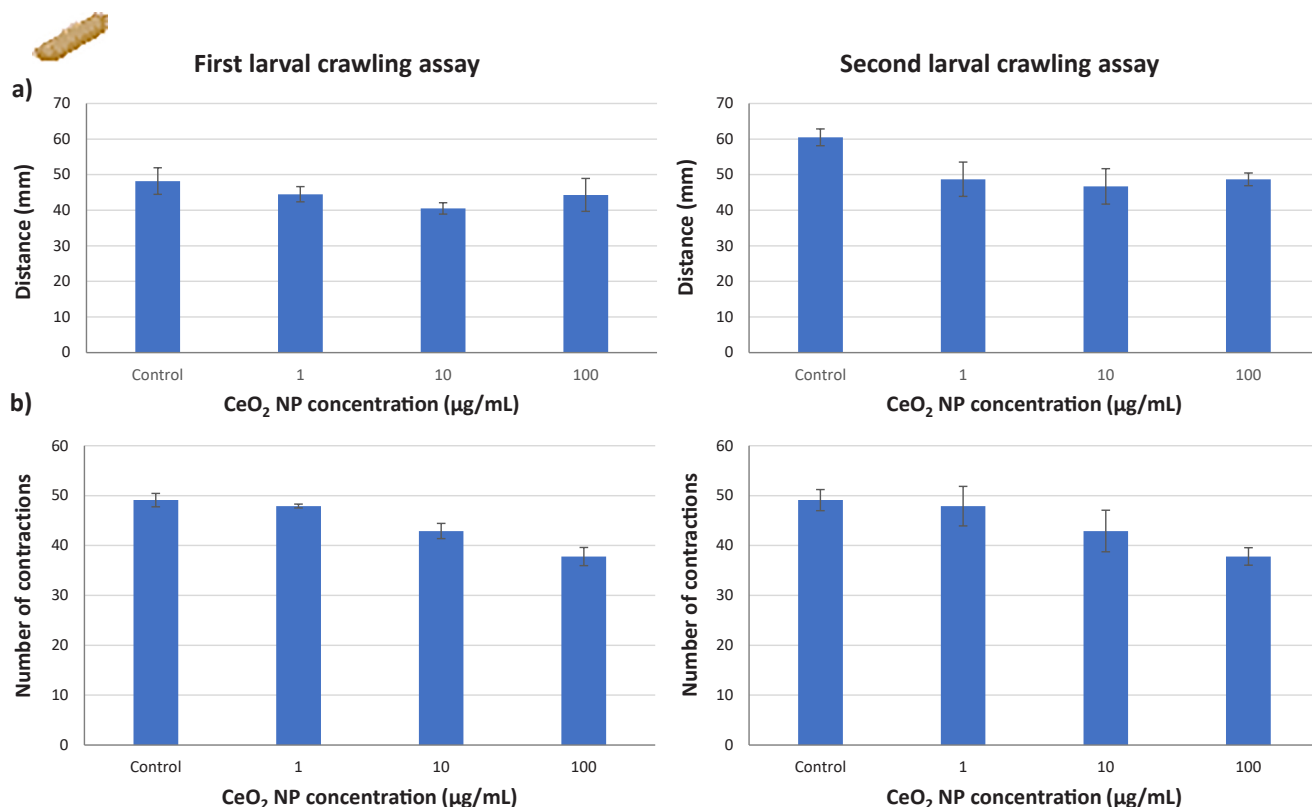


Fig. 9. Results of the crawling assay in *D. melanogaster* larvae exposed to nanoceria. a) distance covered by the larvae and b) number of contractions of the larvae in the first (left) and second (right) crawling assays. Bars represent the mean $\pm$ standard error. * $P < 0.05$, ** $P < 0.01$ significant difference with regard to the control. Larva picture created in Biorender (BioRender.com/r22b790).

once the larvae reached the third instar larval stage (one week later than the control). No effects were detected for positively charged CeO₂ NP. Other studies, however, observed that the same nanoceria concentration produced a reduction in larval length [57]. Our results showed no significant differences in larval length for any of the treatment groups when compared to the control regardless of dosage and exposure time, suggesting that oral administration of CeO₂ NP does not affect larval size.

Larval movement is the result of the peristaltic contraction of the body wall musculature. These contractions are coordinated by specific neural circuits in the CNS known as central pattern generators (CPG). CPG cause the motor neurons in the dorsal region of the ventral ganglion of the larval CNS to burst rhythmically, triggering the waves of contraction of the body segments responsible for larval locomotion [22]. Larval movement is, therefore, under neurological control, making the larval crawling assay a useful tool to assess the potential effect of NP exposure on neuronal function. This study shows that both dosage and exposure time influence the effect of CeO₂ NP on larval locomotion behaviour. On the one hand, only long-term exposures at high doses produced a decrease in crawled distances, with a dose-dependent effect. Similarly, a previous study by Sundararajan et al. [57] did not observe modifications in the crawling distance covered by larvae exposed to nanoceria (17.2 and 172.1 µg/mL) during the development of the third instar larvae. And Parimi et al. [46] reported a decrease in the crawling distance in larvae treated under similar conditions and the same doses only for negatively charged CeO₂ NP, with no effect for positively charged NP. The CeO₂ NP used in the current work were positively charged in water (suspension used for the *in vivo* treatments), and no information on surface charge is available in the study by Sundararajan et al. [57]. On the other hand, the number of body wall contractions differed significantly from the control group for all doses and exposure times tested, also in a dose-dependent manner. None of the aforementioned studies evaluated larvae contractions. To our knowledge, there is

only one previous study evaluating different neurotoxicity parameters in rodents exposed to nanoceria. Mice intranasally or intraperitoneally exposed to nanoceria (40 mg/kg) for 3 weeks resulted in oxidative damage in brain tissue, including increases in malondialdehyde and acetylcholinesterase levels, and decreases in reduced glutathione concentration, upregulation in several apoptosis-related genes and locomotor and cognitive impairment [53].

5. Conclusion

The results obtained showed that CeO₂ NP generally presented scarce cyto- and genotoxicity on nervous system cells, essentially depending on the exposure time and cell type, and being restricted to the longer exposure periods. However, behavioural effects were observed in *D. melanogaster* larvae from both the first and second generation chronically exposed to nanoceria by ingestion. These initial findings lead to consider a high biocompatibility of nanoceria, but some hints of *in vivo* effects under the experimental conditions tested support the need of further investigation before these NP can be safely used in biomedical applications. Pro-oxidant or antioxidant properties of CeO₂ NP previously reported in the literature would help explain some of these results; therefore, further investigations on this line are guaranteed. Results obtained in the current study provide a better understanding of the interaction of CeO₂ NP with cellular systems and whole organisms, and of their possible adverse effects and physiological consequences. These data may be used in risk assessment of exposure to these NP, and as a basis for establishing good practice guidelines for their management, in addition to contributing to increase the knowledge about the impact of nanoceria on human health.

Environmental implication

Cerium dioxide nanoparticles (CeO₂ NP or nanoceria) are increasingly used in a variety of industrial and commercial applications, mainly as polishing material, environmental remediation agent, automotive diesel fuel additive-combustion catalyst, and electrode material. It was estimated that around 100–1000 tons of CeO₂ NP are being produced per year only in Europe. As a consequence, the quantity of CeO₂ NP annually released into the environment via anthropogenic activities is quite significant. Still, and despite all their uses and potential applications, their potential adverse effects and action mechanism are not totally understood yet.

CRedit authorship contribution statement

Vanessa Valdíglesias: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Blanca Laffon:** Writing – review & editing, Supervision. **Carla Costa:** Writing – review & editing, Formal analysis, Conceptualization. **Assia Touzani:** Writing – review & editing, Methodology. **Lucía Ramos-Pan:** Writing – review & editing, Methodology. **Luisa Martínez:** Writing – original draft, Methodology. **Natalia Fernández-Bertólez:** Writing – original draft, Methodology.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Vanessa Valdíglesias reports financial support and article publishing charges were provided by Ministry of Science and Innovation and Xunta de Galicia. Funding for open access charge, Universidade da Coruña-CISUG. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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