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*Proceeding Paper*

# Neuron and Glial Cells Exposed to Cerium Dioxide Nanoparticles: Results from MTT and γH2AX Assays †

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**Abstract:** Cerium dioxide nanoparticles ($CeO_2$NP) show antioxidant enzyme-like properties and reactive oxygen species (ROS) scavenging activity, making them a promising material for potential therapeutic applications in neurodegenerative diseases. The objective of this work was to assess the biological behaviour of $CeO_2$NP in human SH-SY5Y neuronal and A172 glial cells by means of MTT assay and γH2AX assay. Despite the significant dose- and time-dependent NP internalization by both cell lines, nanoceria generally presented scarce cyto- or genotoxicity, essentially restricted to highest NP doses and longest exposure time. In conclusion, a high biocompatibility of $CeO_2$NP was observed at the conditions tested.

**Keywords:** cerium dioxide nanoparticles; cellular uptake; cytotoxicity; genotoxicity; neurotoxicity; nanoceria biocompatibility; nanomedicine

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## 1. Introduction

Cerium dioxide nanoparticles ($CeO_2$NP) show antioxidant enzyme mimetic properties and oxygen free radical scavenging activity in biological systems. These unique structure-dependent features, make them a promising material for potential biomedical applications, namely as an antitumour agent, for regenerative therapy, gene therapy, or targeted drug delivery. However, their cellular uptake, action mechanism and potential adverse effects are not totally understood yet [1].

Specifically, many central nervous system (CNS) diseases are characterized by accumulation of reactive oxygen species (ROS), which induce severe damages to the brain tissues and irreversible neurodegeneration. $CeO_2$NP have raised as a novel potential agent in the treatment of neurodegenerative diseases, due mostly to their remarkable property 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 [2].

Moreover, recent studies in animal models showed that $CeO_2$NP can cross the blood-brain barrier (BBB) due to their nanoscale diameters [3].

On this basis, the main objective of this work was to assess whether $CeO_2$NP could induce adverse effects at the cellular and/or genetic level, to verify their suitability for their application in diagnosis and treatment of nervous system diseases. To this aim, the possible alterations in SH-SY5Y neuronal and A172 glial cell viability and induction of DNA double-strand breaks was determined in the presence of a wide dose range of $CeO_2$NP (1–100 μg/mL) by means of MTT assay and γH2AX assay, respectively. In a first stage, the physicochemical characterization of the $CeO_2$NP and their ability to be taken up by the cells were assessed.

## 2. Materials and Methods

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. The average hydrodynamic diameter and zeta potential in both cell culture media 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., Malvern, UK).

$CeO_2$NP internalization by both cell types was evaluated by flow cytometry, using a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) [4]. The potential antiproliferative effect of $CeO_2$NP was evaluated by MTT assay, following Mosmann (1983) [5] with some methodological modifications to avoid interference of the NP with the standard protocol [6,7], using a SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg, Germany). To evaluate the potential $CeO_2$NP genotoxicity, γH2AX assay was carried out by flow cytometry in a FACSCalibur cytometer (Becton Dickinson) [8]. For all these experiments, both cell types were incubated with a dose range of nanoceria (1–100 μg/mL) for 3, 24 and 48 h. Negative controls used were cell culture media, and positive controls were 200 μg/mL $TiO_2$NP for internalization, 1% Triton X-100 for MTT assay, and 1 μg/mL bleomycin for γH2AX assay.

Statistical analyses were performed using IBM SPSS Statistics package for Windows (version 27.0). Differences among groups were analysed by Kruskal-Wallis test, and Mann-Whitney *U*-test for two-by-two comparisons, and associations between two variables were assessed by Pearson's correlation. All experiments were run at least in triplicate. Experimental data were expressed as mean ± standard error and a *p*-value lower than 0.05 was considered significant.

## 3. Results and Discussion

Table 1 shows the main physicochemical properties of $CeO_2$NP dispersed in both cell culture media, at the highest dose used for toxicity tests. Results revealed that $CeO_2$NP were stable and did not agglomerate in any culture media, since their hydrodynamic size remained with minimal variations, and the zeta potential values showed a stable negative surface charge at all time points tested.

**Table 1.** Physicochemical description of cerium dioxide nanoparticles (100 μg/mL).

| $CeO_2$ NP | Time Point | Dispersed in A172 Cell Culture Medium [1] | Dispersed in SH-SY5Y Cell Culture Medium [1] |
|---|---|---|---|
| **Hydrodynamic diameter (nm) DLS** | 0 h | 140.1 ± 3.0 | 148.1 ± 19.8 |
| | 3 h | 142.0 ± 3.6 | 134.4 ± 4.9 |
| | 24 h | 156.2 ± 7.7 | 139.5 ± 3.7 |
| | 48 h | 166.7 ± 6.5 | 139.3 ± 3.5 |
| **Zeta potential (mV) M3-PALS** | 0 h | −11.8 ± 0.7 | −11.3 ± 0.9 |
| | 3 h | −12.1 ± 0.7 | −11.5 ± 0.5 |
| | 24 h | −12.6 ± 0.7 | −11.3 ± 1.1 |
| | 48 h | −12.4 ± 0.7 | −11.7 ± 0.7 |

DLS, dynamic light scattering; M3-PALS, mixed mode measurement—phase analysis light scattering.

Flow cytometric analysis of $CeO_2$NP internalization by both cell types showed an efficient and similar dose- and time-dependent NP uptake, although intensity was slightly higher in neuronal cells than in glial cells (Figure 1). The results obtained agree with other previous studies, employing different methodologies, that showed dose-dependent $CeO_2$NP uptake in other human cell types [9–11].

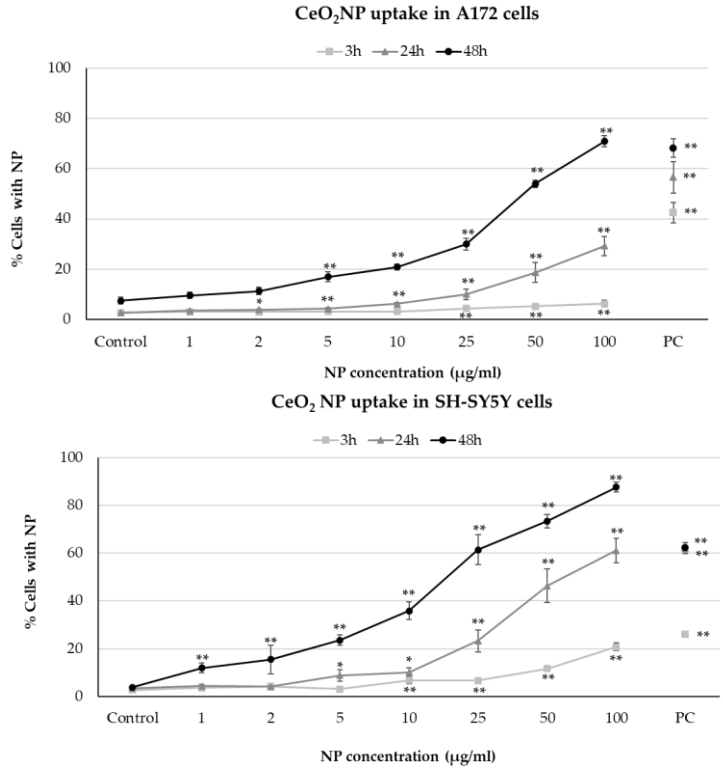


**Figure 1.** Flow cytometry analysis of $CeO_2$NP cellular uptake of in A172 glial cells (top), and SH-SY5Y neuronal cells (bottom), exposed for 3, 24 and 48 h. * $p < 0.05$; ** $p < 0.01$, significant differences regarding the corresponding control. PC, positive control (200 μg/mL $TiO_2$NP).

After cell exposure to $CeO_2$NP, viability was assessed by employing a modified version of MTT assay (Figure 2). In general, no cytotoxicity was observed at the shortest exposure times (3 and 24 h) for glial cells, since cell viability was not reduced in more than 20%. At 48 h a dose-dependent relationship was obtained, with significantly decreasing viability from concentrations over 25 μg/mL In contrast, $CeO_2$NP revealed low cytotoxicity at all times and doses tested in SH-SY5Y cells, maintaining viability values above 80% in general; hence, these NP did not induce cytotoxicity in this cell line. Our results agree with those obtained in some previous studies that revealed cytotoxicity or drastic decreases in cell viability only at high $CeO_2$NP doses (>50 μg/mL) and/or large exposure periods (>24 h) [9–11].

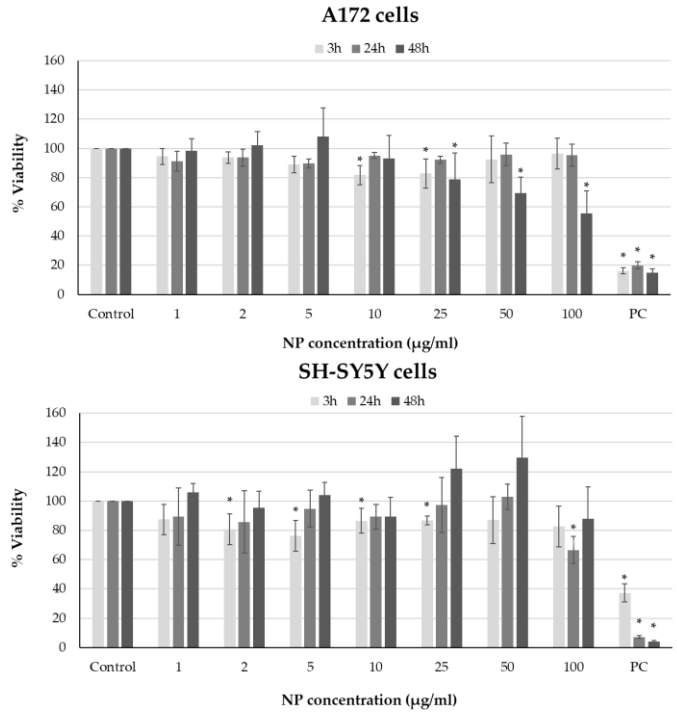


**Figure 2.** Cell viability evaluation of A172 glioblastoma cells (top), and SH-SY5Y neuroblastoma cells (bottom) exposed to $CeO_2$NP for 3, 24 and 48 h. * $p < 0.01$, significant differences with respect to the corresponding control. PC, positive control (1% Triton X-100).

Results obtained from H2AX phosphorylation analysis showed dose- and time-dependent increases in the percentage of glioblastoma cells with γH2AX, significant only at the highest concentration after 3 h exposure, and at all doses tested after 24 h (Figure 3). A similar effect, but slightly more intense (with significant increases at all tested conditions), was observed in neuroblastoma cells.

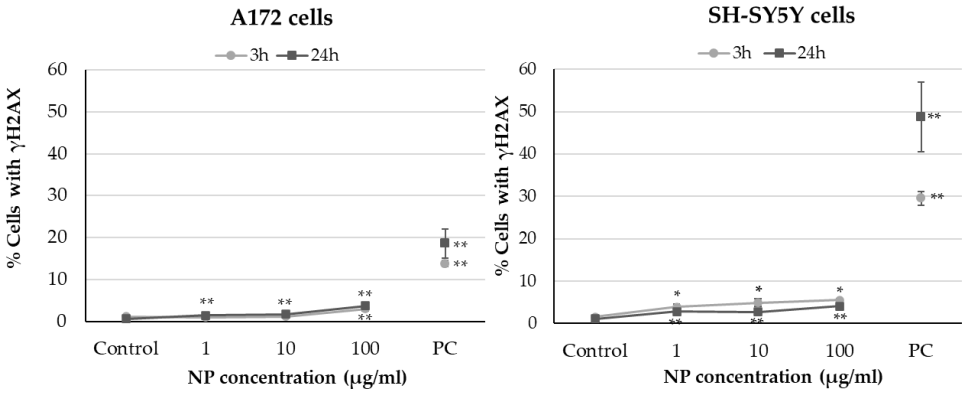


**Figure 3.** H2AX histone phosphorylation analysis after treatment of A172 cells (left) and SH-SY5Y cells (right) with $CeO_2$NP for 3 and 24 h. * $p < 0.05$, ** $p < 0.01$, significantly different regarding the corresponding control. PC, positive control (1 µg/mL bleomycin).

Despite the significant increases of phosphorylated H2AX observed in our study, values obtained were always lower than 6%, indicating a scarce genotoxic potential of $CeO_2$NP in both cell types tested. The genotoxic effects found in different human cell types exposed to these NP have been controversial. Franchi et al. [12] reported no significant increases in phosphorylated H2AX in fibroblasts exposed to low $CeO_2$NP concentrations (10 µg/mL) for 24 h. Some previous works showed that exposure of human cells to nanoceria doses from 10 to 200 µg/mL did not induce significant primary DNA damage

(evaluated by the comet assay) [10,11]. On the contrary, other studies employing γH2AX test or comet assay revealed that NP doses as low as 6 μg/mL had a higher genotoxic potential even after 3 h exposure [13,14].

## 4. Conclusions

The results obtained showed a significant dose- and time-dependent NP internalization by both cell lines. Low $CeO_2$NP induced cytotoxicity was observed in neuronal cells at all times and doses tested. Notable cytotoxicity in glial cells was restricted to 48 h treatment at concentrations over 25 μg/mL Genotoxicity obtained in glial and neuronal cells treated with $CeO_2$NP was limited, since levels of γH2AX were always lower than 6%.

In general, it is possible to consider a high biocompatibility of $CeO_2$NP under the conditions tested, except for glioblastoma cells exposed for 48 h from medium concentrations on. These results provide a better understanding of the interaction of $CeO_2$NP with cellular systems and their possible adverse effects, specifically at the level of the nervous system.

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## References

1. Thakur, N.; Manna, P.; Das, J. Synthesis and Biomedical Applications of Nanoceria, a Redox Active Nanoparticle. *J. Nanobiotechnol.* **2019**, *17*, 84. https://doi.org/10.1186/s12951-019-0516-9.
2. Rzigalinski, B.A.; Carfagna, C.S.; Ehrich, M. Cerium Oxide Nanoparticles in Neuroprotection and Considerations for Efficacy and Safety. *WIREs Nanomed. Nanobiotechnol.* **2017**, *9*, e1444. https://doi.org/10.1002/wnan.1444.
3. Pandit, R.; Chen, L.; Götz, J. The Blood-Brain Barrier: Physiology and Strategies for Drug Delivery. *Adv. Drug Deliv. Rev.* **2020**, *165–166*, 1–14. https://doi.org/10.1016/j.addr.2019.11.009.
4. Fernández-Bertólez, N.; Costa, C.; Brandão, F.; Kiliç, G.; Teixeira, J.P.; Pásaro, E.; Laffon, B.; Valdiglesias, V. Neurotoxicity Assessment of Oleic Acid-Coated Iron Oxide Nanoparticles in SH-SY5Y Cells. *Toxicology* **2018**, *406–407*, 81–91. https://doi.org/10.1016/j.tox.2018.06.003.
5. Mosmann, T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunol. Methods* **1983**, *65*, 55–63. https://doi.org/10.1016/0022-1759(83)90303-4.
6. Costa, C.; Brandão, F.; Bessa, M.J.; Costa, S.; Valdiglesias, V.; Kiliç, G.; Fernández-Bertólez, N.; Quaresma, P.; Pereira, E.; Pásaro, E.; et al. In Vitro Cytotoxicity of Superparamagnetic Iron Oxide Nanoparticles on Neuronal and Glial Cells. Evaluation of Nanoparticle Interference with Viability Tests. *J. Appl. Toxicol.* **2016**, *36*, 361–372. https://doi.org/10.1002/jat.3213.
7. Bessa, M.J.; Costa, C.; Reinosa, J.; Pereira, C.; Fraga, S.; Fernández, J.; Bañares, M.A.; Teixeira, J.P. Moving into Advanced Nanomaterials. Toxicity of Rutile TiO2 Nanoparticles Immobilized in Nanokaolin Nanocomposites on HepG2 Cell Line. *Toxicol. Appl. Pharmacol.* **2017**, *316*, 114–122. https://doi.org/10.1016/j.taap.2016.12.018.
8. Fernández-Bertólez, N.; Costa, C.; Brandão, F.; Kiliç, G.; Duarte, J.A.J.A.; Teixeira, J.P.J.P.; Pásaro, E.; Valdiglesias, V.; Laffon, B. Toxicological Assessment of Silica-Coated Iron Oxide Nanoparticles in Human Astrocytes. *Food Chem. Toxicol.* **2018**, *118*, 13–23. https://doi.org/10.1016/j.fct.2018.04.058.

9. Rubio, L.; Annangi, B.; Vila, L.; Hernández, A.; Marcos, R. Antioxidant and Anti-Genotoxic Properties of Cerium Oxide Nanoparticles in a Pulmonary-like Cell System. *Arch. Toxicol.* **2016**, *90*, 269–278. https://doi.org/10.1007/s00204-015-1468-y.
10. Vila, L.; García-Rodríguez, A.; Cortés, C.; Velázquez, A.; Xamena, N.; Sampayo-Reyes, A.; Marcos, R.; Hernández, A. Effects of Cerium Oxide Nanoparticles on Differentiated/Undifferentiated Human Intestinal Caco-2 cells. *Chem. Biol. Interact.* **2018**, *283*, 38–46. https://doi.org/10.1016/j.cbi.2018.01.018.
11. Kumari, M.; Singh, S.P.; Chinde, S.; Rahman, M.F.; Mahboob, M.; Grover, P. Toxicity Study of Cerium Oxide Nanoparticles in Human Neuroblastoma Cells. *Int. J. Toxicol.* **2014**, *33*, 86–97. https://doi.org/10.1177/1091581814522305.
12. Franchi, L.P.; Manshian, B.B.; de Souza, T.A.J.; Soenen, S.J.; Matsubara, E.Y.; Rosolen, J.M.; Takahashi, C.S. Cyto- and Genotoxic Effects of Metallic Nanoparticles in Untransformed Human Fibroblast. *Toxicol. In Vitro* **2015**, *29*, 1319–1331. https://doi.org/10.1016/j.tiv.2015.05.010.
13. Könen-Adıgüzel, S.; Ergene, S. In Vitro Evaluation of the Genotoxicity of CeO2 Nanoparticles in Human Peripheral Blood Lymphocytes Using Cytokinesis-Block Micronucleus Test, Comet Assay, and Gamma H2AX. *Toxicol. Ind. Health* **2018**, *34*, 293–300. https://doi.org/10.1177/0748233717753780.
14. Préaubert, L.; Tassistro, V.; Auffan, M.; Sari-Minodier, I.; Rose, J.; Courbiere, B.; Perrin, J. Very Low Concentration of Cerium Dioxide Nanoparticles Induce DNA Damage, but No Loss of Vitality, in Human Spermatozoa. *Toxicol. In Vitro* **2018**, *50*, 236–241. https://doi.org/10.1016/j.tiv.2018.03.013.