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# Genotoxicity assessment of cerium oxide nanoparticles in female Wistar rats after acute oral exposure

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ABSTRACT

Cerium oxide nanoparticles ($CeO_2$ NPs; nanoceria) have demonstrated excellent potential for commercial use in various arenas, such as in biomedical industry in cosmetics and as a fuel additive. However, limited knowledge exists regarding their potential toxicity. In this study, acute oral toxicity of $CeO_2$ NPs and their microparticles (MPs; bulk) was carried out in female albino Wistar rats. The $CeO_2$ NPs and $CeO_2$ MPs were characterized utilizing transmission electron microscopy (TEM), dynamic light scattering (DLS) and laser Doppler velocimetry (LDV) for the size, distribution and surface charge respectively. The genotoxicity studies were conducted using micronucleus test (MNT), comet and chromosomal aberration (CA) assays. Results revealed that at high dose (1000 mg/kg bw) $CeO_2$ NPs induced significant DNA damage in peripheral blood leukocytes (PBL) and liver cells, micronucleus formation in bone marrow and blood cells and total cytogenetic changes in bone marrow. However, significant genotoxicity was not observed at 500 and 100 mg/kg bw of $CeO_2$ NPs. The findings from biochemical assays depicted significant alterations in ALP and LDH activity in serum and GSH content in liver, kidneys and brain only at the high dose of $CeO_2$ NPs. Tissue biodistribution of both particles was analyzed by inductively coupled plasma optical emission spectrometer (ICP-OES). Bioaccumulation of nanoceria in all tissues was significant and dose-, time- and organ-dependent. Moreover, $CeO_2$ NPs exhibited higher tissue distribution along with greater clearance in large fractions through urine and feces than $CeO_2$ bulk, whereas, maximum amount of micro-sized $CeO_2$ got excreted in feces. The histopathological examination documented alterations in the liver due to exposure with $CeO_2$ NPs only. Hence, the results suggest that bioaccumulation of $CeO_2$ NPs may induce genotoxic effects. However, further research on long term fate and adverse effects of $CeO_2$ NPs is warranted.



## 1. Introduction

Nanomaterials (NMs) have been defined as natural, incidental or manufactured materials, in different states and in the size range of 1–100 nm [1]. These nanoparticles (NPs) commonly have different physico-chemical and electronic properties including much higher specific surface area, high surface reactivity and increased quantum effects, as a function of their size from those of the corresponding microparticles (MPs; bulk) [2]. The properties of NPs also include enhanced reactivity and greater ability to penetrate tissues and cell membranes [3]. Nanotechnological progress can enhance the quality of life, but it can also raise societal concerns as unavoidable perceived risks cannot be ignored. Thus, MPs that are considered safe need to be tested when in 'nano' form for their toxic effects. Several important health risks of NPs in human tissues such as lung,

*Abbreviations:* $CeO_2$, cerium oxide; NPs, nanoparticles; MPs, microparticles; TEM, transmission electron microscopy; DLS, dynamic light scattering; LDV, laser Doppler velocimetry; OECD, organization for economic co-operation and development; MNT, micronucleus test; CA assay, chromosomal aberration assay; ANOVA, analysis of variance; MN-PCEs, micronucleated polychromatic erythrocytes; PCEs, polychromatic erythrocytes; MI, mitotic index; TA, total aberrations; ROS, reactive oxygen species; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; GSH, reduced glutathione; ICP-OES, inductively coupled plasma optical emission spectrometer.

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liver, kidneys and nervous system occur through the inhalation of nanoscale aerosols, contact with nano-structured surfaces, or by the consumption of foods with nanosized colloids [4]. Cerium oxide ($CeO_2$) is a rare earth oxide material from the lanthanide series of the periodic table which has been recently introduced for specialty applications. In $CeO_2$, a fraction of cerium (Ce) is in the $Ce^{3+}$ form. The reduction in positive charge is compensated by a corresponding number of oxygen vacancies. The $Ce^{3+}/Ce^{4+}$ redox reactions are responsible for the outstanding biological properties of $CeO_2$ NPs [5]. Owing to their large surface area to volume ratio, $CeO_2$ NPs (nanoceria) have a unique electronic structure and the reduction in the particle size results in the formation of surface oxygen vacancies, which endows it with the ability to exist in either $Ce^{3+}$ or $Ce^{4+}$ state on the particle surface [6]. The $CeO_2$ NPs are used in pharmaceutical industry [7], in nanotherapeutics [8], as polishing agents [9], UV-absorbing compounds in sunscreen [10] and UV-scattering agents in non-irritating lipsticks [11]. Nanoceria play several catalytic roles such as catalysts in the petroleum refining industry, as additives to promote combustion of diesel fuels [12], as subcatalysts for automotive exhaust cleaning [13] and as electrolytes in solid oxide fuel cells [14]. The broad range of applications of $CeO_2$ NPs increases the risk of human exposure and interactions with a variety of environmental factors with unknown health and safety implications. Organization for Economic Co-operation and Development (OECD) in the Environment Directorate added $CeO_2$ NPs in the list of 14 NMs for testing and identified it as commercially relevant to the global economic impact of nanotechnology and suggested that the most common route of $CeO_2$ exposure is likely to be through inhalation and/or ingestion [15]. There are several *in vitro* studies with $CeO_2$ NPs and their MPs in various cell lines [16–19]. Moreover, there are studies which have investigated $CeO_2$ NPs induced toxicity through different routes of exposure in rats such as inhalation [20], intratracheal instillation [21] and intravenous (iv) administration [22]. Gastrointestinal region is one of the most important portals of entry for NPs in humans and animals [3]. Hirst et al. [8] reported the biodistribution of nanoceria and their antioxidant capabilities after per-oral administration to mice. Nevertheless, there are no reports on the genotoxicity of $CeO_2$ NPs and their bulk on the animal system through oral route. Hence, this gave us the impetus to conduct an acute oral toxicity study to provide more inclusive information with regard to the fate of $CeO_2$ NPs and $CeO_2$ bulk after exposure to the gastrointestinal system and associated health effects. It is of utmost importance to investigate the physico-chemical characteristics of NMs followed by toxicological implications in the biological system. Therefore, in the present investigation we performed transmission electron microscope (TEM) analysis, dynamic light scattering (DLS) and laser Doppler velocimetry (LDV) studies to know the size, mean hydrodynamic diameter and zeta potential of $CeO_2$ NPs respectively. Acute oral toxicity study of $CeO_2$ NPs and $CeO_2$ MPs was performed following the "fixed dose method" in female albino Wistar rats. Further, genotoxicity, biochemical and biodistribution studies were conducted after acute oral treatment with $CeO_2$ NPs and its bulk. The genotoxicity studies included the most used comet assay, micronucleus test (MNT) and chromosomal aberration (CA) assay to depict the mechanisms of toxicity [23]. The comet assay is a versatile, simple and sensitive method to study the toxicity study of a broad range of compounds and is capable of measuring DNA damage in almost all organisms and cells [24]. In order to assess the clastogenic, aneugenic and epigenetic effects of compounds, the MNT is used to analyze the dysfunction of mitotic apparatus and chromosome damage. The CA test diagnoses agents that can cause numerical aberrations, structural chromosome or chromatid breaks, dicentrics and other abnormal chromosomes such as translocations which are implicated in the various human genetic diseases and cancers [23]. Therefore, the comet assay in the peripheral blood leukocytes (PBL) and liver, MNT in the blood and bone marrow and CA test in the bone marrow cells was carried out. Biochemical studies were performed to assess the activity of alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in serum for liver toxicity, loss in cell membrane integrity and cell death respectively. Reduced glutathione (GSH) is the predominant antioxidant in the aqueous cytoplasm of cells and its production is primarily in the liver [25]. Hence, GSH content in liver, kidneys and brain of the treated rats was evaluated to get an insight of the oxidative stress status of the system. The biodistribution study of NMs is essential to assess the uptake and retention of NPs that enter target tissues or sites and for determining the anatomic fate, clearance and biological effects of these substances. Therefore, the effects of the test compounds on biodistribution were analyzed in the whole blood, liver, kidneys, heart, brain, spleen, urine and feces of rats using inductively coupled plasma optical emission spectrometer (ICP-OES). In the present study, the lowest treatment dose was intended to reflect the level of potential human exposure. However, the highest dose was utilized to obtain toxicity through accidental exposure to large amounts of $CeO_2$ NPs and to obtain detectable amounts of Ce after distribution in the animal as well. There are studies on aluminum oxide, zinc oxide (ZnO) and titanium oxide ($TiO_2$) NPs in which similar high doses were used to assess the toxicological effects through different routes [26–28].

## 2. Materials and methods

### 2.1. Nanoparticles and chemicals

$CeO_2$ NPs ($CeO_2$ < 25 nm, 99.95%, CAS No. 1306-38-3), $CeO_2$ MPs ($CeO_2$ < 5 μm, 99.90%, CAS No. 1306-38-3) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Phosphate buffered saline ($Ca^{2+}$, $Mg^{2+}$ free; PBS), cyclophosphamide (CP), normal melting agarose (NMA), low melting agarose (LMA) *etc.*, were also purchased from Sigma Chemical Co. Ltd. Other chemicals were purchased from Himedia, Mumbai, India.

### 2.2. Characterization of $CeO_2$ NPs and $CeO_2$ MPs

The particles were characterized using TEM, DLS and LDV to evaluate the size of the materials, size distribution, state of dispersion and zeta potential of the NMs in the Milli-Q water. Characterization of $CeO_2$ NPs and $CeO_2$ MPs was performed to assess the size and morphology using TEM (JEM-2100, JEOL, Japan). The images were obtained from TEM with an accelerating voltage of 120 kV. The TEM was equipped with a plunge freezer and cryo transfer holder to fix specimens in the frozen state and fitted with a Gatan 2Kx2K CCD camera for acquiring high-resolution images. Particles were suspended in Milli-Q water at a concentration of 0.01 mg/ml, and one drop of suspension was placed on a carbon-coated copper TEM grid and evaporated at room temperature. The software for advanced microscopy techniques was used for the digital TEM camera. This software was calibrated for nanoscale size measurements for the accurate examination of NPs. For the size measurement, 100 particles were calculated from random fields of view and images showing the general morphology of the particles. The size and surface charge of the $CeO_2$ NPs in Milli-Q water suspension was measured through DLS and LDV using a Malvern Zetasizer Nano-ZS (Malvern Instruments, United Kingdom). This device uses a 4 mW He–Ne 633 nm laser to analyze the samples and an electric field generator for the LDV measurements. The suspensions of freshly prepared $CeO_2$ NPs and MPs in Milli-Q water at the concentration of 40 μg/ml were ultra-sonicated using a probe sonicator (UPH 100, Germany) for 10 min at 90% amplitude. Further, the suspensions were diluted and adjusted to a lower concentration to acquire enough counts per second. The prepared samples were transferred to a 1.5 ml square cuvette for DLS measurements, and 1 ml of the suspension was transferred to a Malvern Clear Zeta Potential cell for LDV measurement. The mean NPs diameter was calculated using the same software program as utilized in the NPs distribution and the polydispersity index (PdI) was used to measure the size present in the solution. The PdI scale ranges from 0 to 1, where 0 corresponds to monodispersed and 1 corresponds to polydispersed state of particles.

### 2.3. Animals

Female albino Wistar rats, aged 6–8 weeks and weighing 80–120 g, were obtained from the National Institute of Nutrition, Hyderabad, India. The animals were acclimatized for 1 week in groups of five in polypropylene cages. The animals were fed with a standard laboratory pellet diet and reverse osmosis water was provided *ad libitum* and maintained under standard conditions of humidity (55–65%), temperature (22 ± 3 °C) and light (12 h light/12 h dark cycles). The study

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