ISSN (Online) : 2320-9682
ISSN (Print) : 2320-9674

In vitro cytotoxicity studies of cerium oxide nanoparticles

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Abstract

Background/Objectives: Cerium oxide nanoparticles (CeO2-NP), a member of the lanthanide series of rare metal oxide, have beneficial property like radicals scavenging and being examined as a potent candidate for many biomedical applications. The aim of the study is to investigate certain key *in vitro* cytotoxicity endpoints using CeO2-NP.
Methods/Statistical analysis: The techniques applied include, characterization, MTT assay, LDH release, ATP measurement and Caspase 3/7 activity assay. The results were subjected to null hypothesis testing to determine its significance.
Findings: CeO2-NPshowed mild cytotoxicity with a loss of MTT reactivity and depletion in ATP production. CeO2-NP treatment also resulted in an increase in Caspase3/7 activity and leakage of LDH in the tested cell line suggesting that it induces apoptosis via release of reactive oxygen species or through the physical damage of the cell membrane.
Improvements/Applications: Thefindings add base line information to the biocompatibility and toxicity data sheet on CeO2-NP. The results will also be beneficial in risk assessing CeO2-NP based medical devices.

Keywords: Cerium oxide nanoparticles, Cytotoxicity, L-929 cells

1. Introduction

Cerium is a member of the lanthanide series of metals and is the most abundant of the rare-earth elements in the earth's crust and it is separated out from the other rare-earth elements through oxidation in the form of Cerium oxide (CeO_2) [1,2]. Cerium oxide nanoparticles (CeO2-NP or Nanoceria) have recently received attention from the scientific community due to their unique free radicals scavenging property in biological system, both *in vitro* and *in vivo*[3]. It decreases hepatic reactive oxygen species (ROS) levels linked to the progression of diabetes [4], inhibits myocardial oxidative stress [5] and thus proving to be an effective antioxidant agent [6]. CeO2-NP is also well suited for applications in nanobiology and regenerative medicine for its promising efficacy against endometriosis related pathogenesis [7], diseases associated with chronic oxidative stress and inflammation [8], in neurodegenerative disorders [9] and the delay it brings in the progression of retinal degeneration and as anti-angiogenic agent in rodent models [10]. Studies have shown CeO2-NP to be toxic to cancer cells, inhibit invasion, and sensitize cancer cells to radiation therapy [11]. Despite these advantages of nanoceria over other therapeutic strategies, there are toxicity issues to be considered [12]. Presently only minimal biocompatibility information are available on nanoceria to declare their safety.

In this article, we probed biocompatibility of nanoceria in line with well-established ISO 10993-1 standards. Specifically we investigated cytotoxicity using four different *in vitro* assays which are some vital biocompatibility parameters for any material of medical applications (ISO 10993-1, 2010) [13]. Many guidelines on the safety assessment of nanomaterials to be used in food/feed, cosmetics and medical devices have been published by European Food Safety Authority (EFSA), Scientific Committee on Consumer Safety (SCCS) and Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) as Scientific Committees of the European Commission. While, International Organization for Standardization (ISO) ISO 10993 standards have no specific guidelines on testing the biocompatibility of materials in the nanoscale range [14], we therefore adopted these wide principles and combined them with the common techniques used in nanotoxicology to assess the biocompatibility of nanoceria. It should be mentioned here that ISO is presently evolving guidance on evaluating nanoparticles employed in medical devices (ISO/NP TR 10993-22under development) and our findings may incorporate data into this process. Moreover our results will be supportive in evaluating the safety of medical device technologies using CeO2-NP as a raw material. Increasing interest in the biocompatibility of nanomaterials [15-17] necessitated the need to undertake this work to scale down the research gaps that exist with reference to toxicity and health risk assessment of CeO2-NP employed in medicine.

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2. Materials and methods

2.1 Characterization of cerium oxide (IV) nanoparticles (CeO2-NP)

CeO2-NP was obtained from Strem Chemicals Inc, USA. The certificate of analysis provided from the manufacturer specified the purity as 99.99%. The impurities listed were in negligible amounts and therefore unlikely to affect the results obtained. To confirm that the taken test material is in nanosize, the morphology of the sample was inspected using Scanning Electron Microscopy (SEM) with an accelerating voltage of 20kV (Quanta 200 FEG). The chemical composition of the sample was determined by Energy Dispersive X-ray Spectroscopic analysis. The crystal structure of the CeO2-NP was characterized by X-ray Diffraction (XRD) studies with Cu Kα radiation at a scanning rate of 0.02s-1 using PANalytical (X'pert PRO) X-Ray Diffractrogram instrument.

2.2 Cytotoxicity assessment

It comprised of MTT assay to measure mitochondrial activity, LDH release to measure membrane integrity, ATP levels to measure the biochemical status of the cell and Caspase 3/7 activity assay (apoptosis induction). The tests were carried out from battery of cytotoxicity assays described in ISO 10993-1, 2009/Cor 1:2010 and ISO 10993-5, 2009 [13,18].

2.3 Cell lines and culture conditions

L-929 cells of mouse origin with identification NCTC clone 929 [L cell, L-929, derivative of Strain L] (ATCC® CCL-1™) were obtained from ATCC (American Type Culture Collection, Manassas, VA). The Maintenance Frozen stocks were stored in liquid nitrogen. Laboratory stock cultures were maintained in ATCC-formulated Eagle's minimum essential medium supplemented with 10% newborn calf serum, 4 mM glutamine and penicillin/streptomycin at 37 ± 1°C in an atmosphere of 5% (v/v) CO_2 in air. Cells were passaged regularly to avoid overgrowth. Five different concentrations of CeO2-NP in the range from 0.2mg/mL to 1.0 mg/mL were used in the investigation. The test substance were added to the tissue culture wells with appropriate cell number in 100 µl media per well. Positive control and negative control were applied in each assay to ascertain the validity of the experiment. Commercially available kits were used to evaluate the MTT assay (Sigma Aldrich, UK), LDH release (Roche Applied Sciences, Germany), ATP levels (Sigma Aldrich, UK) and Caspase 3/7 activity (PromegaBioSciences, USA).

2.3.1 Culture set-up

Cells were cultured at 37 ± 1°C in a humidified atmosphere of 5% (v/v) CO_2 in air. Cultures were re-fed with appropriate culture medium and sub-cultured (with trypsinization) until required for assay treatment. Near confluent cultures were harvested by trypsinization and re-suspended in the culture medium. The cell number in the suspension prepared was determined using a haemocytometer. Aliquots (100 µl) of the culture medium were dispensed into all peripheral wells (blanks) of the appropriate number of 96-well plates. The cell suspension was diluted to give a final concentration of $1x10^5$ cells/mL and 100 µL was pipetted into the appropriate number of wells (i.e. 10^4 cells/well). At least six wells were assigned for negative control, positive control and each test article dose treatment. Plates were incubated at approximately 37°C for up to 48 hours in a humidified atmosphere of approximately 5% (v/v) CO_2 in air to achieve approximately 70% confluent monolayers. The absorbance for each assay was measured using recommended wavelength and all measurements were recorded using the THERMOmax™ plate reader linked to a personal computer using SoftMax Pro software. Programming of the plate reader included setting up a template to which blanks, controls, empty and test article wells were assigned. The raw data were transferred and statistical analysis performed using Microsoft Excel.

Table 1. Results of MTT assay in L-929 cells - Cell Viability (OD_{540})

Cerium oxide	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Mean	SD	CV
Untreated	0.6742	0.6461	0.7546	0.7145	0.6527	0.6833	0.6876	0.0409	0.0594
0.2mg/mL	0.7464	0.5253	0.5261	0.6536	0.6852	0.6537	0.6317	0.0888	0.1406
0.4mg/mL	0.4857	0.6126	0.6931	0.4685	0.5437	0.6648	0.5781*	0.0935	0.1617
0.6mg/mL	0.3755	0.4162	0.2841	0.3246	0.2418	0.2153	0.3096***	0.0776	0.2505
0.8mg/mL	0.2412	0.1637	0.1574	0.1462	0.1475	0.1682	0.1707***	0.0356	0.2086
1.0mg/mL	0.1052	0.1361	0.1433	0.1154	0.1263	0.1427	0.1282***	0.0155	0.1207
Phenol 1 mg/mL	0.0162	0.0251	0.0364	0.0218	0.0215	0.0162	0.0229***	0.0075	0.3273

SD- Standard deviation, CV-Coefficient of Variation

*-P<0.05, ***- P<0.001 compared to untreated control using T-test

Phenol 1 mg/mL was used as positive control

Table 2. Results of ATP level depletion in L-929 cells - Total cellular ATP (Relative fluorescence Unit)

Cerium oxide	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Mean	SD	CV
Untreated	46728	45975	46426	53396	48375	47457	48059	2746	0.0572
0.2mg/mL	46428	44314	45853	46854	48416	45983	46308	1344	0.0290
0.4mg/mL	48753	46582	44457	46386	43541	44542	45710	1903	0.0416
0.6mg/mL	36356	38528	39723	38506	38733	37461	38217***	1161	0.0304
0.8mg/mL	35276	33784	35226	39648	36532	35490	35992***	1994	0.0554
1.0mg/mL	36623	32541	33759	36362	36575	36755	35435***	1816	0.0513
Paracetamol 7.5 µg/mL	22631	17648	19431	23935	22846	23587	21679***	2541	0.1172

SD- Standard deviation, CV- Coefficient of Variation

***- P<0.001 compared to untreated control using T-test

Paracetamol 7.5 µg/mL was used as positive control

Table 3. Results of LDH release in L-929 cells - LDH in medium after 24 hours treatment ($OD_{490} – OD_{650}$)

Cerium oxide	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Mean	SD	CV
Untreated	0.0726	0.0471	0.0638	0.0524	0.0485	0.0742	0.0598	0.0121	0.2023
0.2mg/mL	0.0164	0.0279	0.0716	0.0974	0.1158	0.1026	0.0720	0.0413	0.5743
0.4mg/mL	0.0562	0.0628	0.0564	0.0613	0.0475	0.0685	0.0588	0.0072	0.1219
0.6mg/mL	0.0474	0.0683	0.0748	0.0524	0.0252	0.0653	0.0556	0.0180	0.3245
0.8mg/mL	0.3722	0.3935	0.2428	0.2673	0.4731	0.3346	0.3473	0.0849	0.2446
1.0mg/mL	0.5865	0.0426	0.3718	0.6256	0.5342	0.5672	0.4547	0.2201	0.4841
Tween 20 (1%)	1.3742	1.2536	1.5782	1.4315	1.5623	1.5748	1.4624	0.1329	0.0909

SD- Standard deviation, CV- Coefficient of Variation

Table 4. LDH in remaining viable cells after 24 hours treatment ($OD_{490} – OD_{650}$)

Cerium oxide	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Mean	SD	CV
Untreated	1.4273	1.7512	1.6328	1.5846	1.6924	1.5473	1.6059	0.1141	0.0711
0.2mg/mL	1.8355	1.7563	1.8425	1.8823	1.8292	1.7543	1.8167	0.0510	0.0281
0.4mg/mL	1.7215	1.7481	1.6433	1.7327	1.7293	1.6926	1.7113	0.0380	0.0222
0.6mg/mL	1.7473	1.6328	1.6485	1.4732	1.9982	1.9463	1.7411	0.2002	0.1150
0.8mg/mL	1.5365	1.5168	1.4035	1.5284	1.4238	1.4763	1.4809	0.0564	0.0381
1.0mg/mL	1.1854	1.4842	1.2537	1.1462	1.1341	1.1626	1.2277	0.1326	0.1080
Tween 20 (1%)	0.2431	0.4368	0.1634	0.1475	0.1326	0.1238	0.2079	0.1200	0.5773

SD- Standard deviation, CV- Coefficient of Variation

Table 5.Percentageof LDH release into medium (% cytotoxicity)

Cerium oxide	Total cellular LDH	% LDH released (% cytotoxicity)	Interpretation
Untreated	1.6657	3.5900	Non cytotoxic
0.2 mg/mL	1.8887	3.8121	Non cytotoxic
0.4 mg/mL	1.7701	3.3218	Non cytotoxic
0.6 mg/mL	1.7967	3.0945	Non cytotoxic
0.8 mg/mL	1.8282	18.9963***	Mildly cytotoxic
1.0 mg/mL	1.6824	27.0268***	Moderately cytotoxic
Tween 20 (1%)	1.6703	87.5531***	Cytotoxic

***- P<0.001 compared to untreated control using T-test

1% Tween 20 was used as positive control

Table 6.Results of Caspase 3/7 activation in L-929 cells - Caspase 3/7 activity (Relative fluorescence Unit)

Cerium oxide	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Mean	SD	CV
Untreated	2368	2257	2294	2463	2361	2452	2366	82.34	0.0348
0.2mg/mL	2162	2385	2441	2486	2387	2413	2379	112.82	0.0474
0.4mg/mL	2416	2452	2641	2296	2515	2347	2445	123.30	0.0504
0.6mg/mL	2572	2746	2683	2856	2793	2641	2715***	103.80	0.0382
0.8mg/mL	3756	3629	3886	3592	3626	3752	3707***	111.66	0.0301
1.0mg/mL	4281	3943	4275	4107	4221	4129	4159***	128.41	0.0309
Stauroporine 0.1 µg/mL	5684	5527	5586	5764	6105	5663	5722***	204.89	0.0358

SD- Standard deviation, CV- Coefficient of Variation

***- P<0.001 compared to untreated control using T-test

Stauroporine 0.1 µg/mL was used as positive control

ISSN (Online) : 2320-9682
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3. Results and Discussion

Cerium oxide nanoparticles (CeO2-NP) have been employed as potent free-radical scavengers with neuroprotective, radioprotective, and anti-inflammatory properties. These properties of cerium oxide nanoparticles can open new vistas in medicine [19]. Testing the biocompatability of (CeO2-NP) is the utmost need before its applications in curative science. It should be noted here that ISO 10993 standard is not specifically designed for assessing devices containing nanoparticles. Nanoparticles may have a different toxicity profile because of their small size and relatively high surface area, making them highly reactive. A new standard, ISO 10993 parts 33 is being developed for this purpose[14].

In the present study, we performed a series of assays to look at various cytotoxicity endpoints. In the routinely used MTT assay, CeO2-NP exhibited cytotoxicity from 0.6 to 1.0 mg/mL (Table 1). This indicates that the CeO2-NP interferes with the cellular metabolism and this reduction in cellular process is also reflected in the decrease in ATP levels at the corresponding concentrations (Table 2). This suggests that CeO2-NP probably produces a generalised decrease in biochemical processes in the cell. CeO2-NP also showed some compromise in the cell membrane function as measured by LDH release. Treatment of cells with CeO2-NP resulted in the leakage of LDH from 0.8 mg/mL upwards (Table 3, 4&5). This is most probably due to the direct piercing effect of rigid nanoparticles on the cell membrane which results in its loss of function. It is also possible that CeO2-NP may show a loss of MTT reactivity and decrease in ATP production, due to this membrane disruption. From this investigation it is still not clear whether the CeO2-NP has any direct effects on the mitochondria and further studies are required to address this issue. It is interesting to note that CeO2-NP treatment resulted in a definite increase in Caspase3/7 activity (Table 6) and these enzymes are involved in the initial steps of the apoptotic pathways. This suggests that CeO2-NP may someway activate the apoptotic mechanism either through reactive oxygen radical generation or may be even due to the cell or mitochondrial membrane damage. This is the first time such exhaustive data on various cytotoxicity mechanisms are being investigated for CeO2-NP and the findings suggests that CeO2-NP is cytotoxic, most probably due to its physical nature.

4. Conclusion

We investigated the *in vitro* cytotoxicity of CeO2-NP using globally harmonised key test procedures, to generate validated toxicity data. CeO2 has varied uses in many industrial and medical applications and the material safety and biocompatibility data sheet indicates presence of toxicity data gaps with reference to cytotoxicity. Our furnished data narrows down the research gaps in the toxicity profile of CeO2-NP and provide guidance on evaluating nanoparticles employed in medical applications. As CeO2-NP is gaining attention in nanomedicine, further research from this point will aid in better understanding of this particle to be applied *in vivo*.

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The Publication fee is defrayed by Indian Society for Education and Environment (iSee). www.iseeadyar.org

Citation:

V.Kalyanaraman, T.S Kumaravel, S.S Murugan. *In vitro* cytotoxicity studies of cerium oxide nanoparticles . *Indian Journal of Nanoscience.*2016; 4(1), January.