Application of green-synthesized cerium oxide nanoparticles to treat spinal cord injury and cytotoxicity evaluation on paediatric leukaemia cells

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Abstract

Injury prognosis, and treatment are one of the major objectives of nursing care during the treatment of spinal cord injury (SCI). Here, we developed a novel, biocompatible Cerium oxide nanoparticles (CeO2-NPs) through biosynthetic approach that can be used for the treatment of spinal cord injury in nursing care. CeO2-NPs were green fabricated utilizing leaf extract of Azadirachta indica. The fabricated nanoparticles still maintained the cubic-structure that was revealed by the x-ray diffraction (X-RD) analysis. Transmission electron microscopy (TEM) images displayed the sphere shape of nanoparticles having 15 nm particle size. The stretching bands of Ce–O bond were noticed at 457 cm$^{-1}$ and 451 cm$^{-1}$ from the Raman and Fourier-transform Infrared (FT-IR) spectra, correspondingly. On the other side, biological investigations demonstrated that the single dose application of CeO2-NPs at nanomolar concentration is regenerative, bio-compatible and offers a considerable neuro-protective effect on the neurons in spinal-cord of an adult rat. Neuronal function retention is showed in electro-physiological (electrography) recordings and plausibility of its uses in preventing ischemic-insult is revealed from the assay of oxidative injury. This work explored the possibility of usage of prepared CeO2-NPs for SCI treatment. Furthermore, examination of green fabricated CeO2-NPs on paediatric leukaemia (CCRF-SB) cells were found to potentiate cytotoxicity indicating their therapeutic potential for future treatment of paediatric cancer. On the other hand, the current study also highlights the potential effect of CeO2-NPs on treatment of SCI through nano-based therapy.

Introduction

A developing field of nanoscience and technology is the phytosynthesis of metal oxide and metal nanoparticles (NPs). Shape and size together play an important role in optical, electrical, chemical and physical properties of nanomaterials. In addition, CeO2 is a semiconductor having high exciton binding energy and broad band gap energy of 3.19 eV. It is utilized in plenty of applications like antibacterial activity, bio imaging, biotransformation, catalyst, sun screen cosmetics, sensor and solid oxide fuel cells [1–7]. In general, CeO2-NPs were fabricated by chemical and physical methods like co-precipitation, sol–gel, sonochemical, hydrothermal, microwave and flame spray pyrolysis [8–13]. However, majority of these approaches are time consuming, complex, hazardous and expensive. Therefore, the green chemistry methods are being developed in the phytosynthesis of metal oxide and metal nanoparticles. These methods provide a wide range of applications like pharmaceutical applications, low-cost and bulk scale industrial production. In comparison to the cell line activities of zinc oxide and titanium dioxide nanoparticles, CeO2-NPs are less toxic [14]. In recent times,
CeO₂-NPs were fabricated using egg white, extracellular compounds of fungi and honey [15–17]. The nano-crystalline nature of metal oxide nanoparticles in various morphology and sizes were produced by these biocomponents which function as reducing and stabilizing agents.

A novel emerging subject in nanoscience and technology is the photosynthesis of metal oxide and metal nanoparticles [18]. Photosynthesis of CeO₂-NPs has been recently reported using various leaf extract of plants like Acalypha indica, Gloriosa superba and Aloe vera [18–20]. In the fabrication of CeO₂-NPs, the plant extracts serve as a capping and stabilizing agent. The benefits of biofabrication techniques over mechanical and chemical approaches are cost-effectiveness, manageability, less energy and less time consumption [21] and therefore green synthetic approaches can be utilized as a valuable and economic substitute for the large-scale synthesis of non-hazardous CeO₂-NPs.

Spinal cord injury (SCI) is the loss or impairment of sensory–motor function due to disruption in the spinal nerves, consequently, resulting in paralysis and permanent disability. As the injury is an irreversible event, the mortality rate associated with SCI is high. Pneumonia and septicemia are the major reasons for augmented mortality rates. Approximately 294,000 persons from America are suffering from SCI with an incidence of 17,900 new cases each year. Among these 78% cases are male and accident is considered as one of the leading causes of SCI. Life expectancy has not improved since 1980 [22, 23]. Thus, management and non-surgical treatment has become a much tougher challenge to treat SCI.

Paediatric leukaemia (PL) is rare disease. Nonetheless, the incidence and death toll are increasing since 1975 [22]. Among the PLs, acute lymphoblastic leukaemia (ALL) is the most common type of cancer, the other being the acute myeloid leukaemia (AML) which occurs in the remaining cases. Due to rareness of this cancers, current knowledge on etiological factors of PL is not fully explained. Apart from genetics, few case-control studies report xenobiotic genes, pesticides, tobacco smoking, paints, solvents, environment, and nutrition as the altered risk factors of PL [23]. PL have been reported to spread and affect the spinal cord [24, 25]. Despite the recent advancements, there is no proper diagnostic, preventive and therapeutic measures are available to treat PL. Nano therapy is one of the promising approaches in cancer treatment with minimum side effects and non-invasive technique.

Several medicinal plants have been reported for treatment of spinal cord injury (SCI) and neuroprotective effect [26, 27]. While few studies describe the role of nanoparticles in SCI treatment by modulating the inflammatory and regenerative cellular responses [28]. In this study, leaf extract of A. indica plant was used for the fabrication of CeO₂-NPs and for the very first time their analytical studies were reported. On the other side, biological investigations of single dose application of CeO₂-NPs at nanomolar concentration are studied to know their neuro-protective effect on the spinal cord neurons of an adult rat. However, this study has to be extended to a molecular level to get insights into the mechanism of action that enhance the SCI treatment. Furthermore, cytotoxic potential of CeO₂-NP was investigated on childhood leukaemia cells.

Materials and methods

Leaf extract preparation
Leaves of A. indica plant were collected from the university campus and 10 g fine-cut leaves were added with double distilled (DD) water of 100 ml followed by boiling for 5 min at 50 °C–60 °C. The resultant extract was filtered with Whatman No. 1 filter paper and an Erlenmeyer 250 ml flask was utilized to collect the filtrate. Finally, the filtrate was stored at an ambient temperature for further use.

Fabrication of cerium oxide NPs using leaf extract of A. indica
A. indica leaf extract of 100 ml was added to cerium chloride (CeCl₃) salt of 3.72 g and on continuous stirring for 4 to 6 h at a temperature of 80 °C a white coloured precipitate was formed which changed to yellowish brown on constant stirring. Later, the precipitate was subjected to calcination for 2 h at a temperature of 400 °C in order to obtain cerium oxide nano powder, which later stored for future use.

Characterization
XRD analysis of phytosynthesized CeO₂-NPs was performed using CuKα radiation (λ as 1.54060 Å) with Ni monochromator in 2θ range of 10° to 80°. The mean crystallite size of fabricated CeO₂-NPs was obtained using Scherrer’s formula [D = 0.9λ/(βcosθ)]. TEM analysis was performed to examine the morphology of fabricated CeO₂-NPs. The samples were prepared at room temperature for performing TEM analysis by drop wise coating the NP solution on copper grids coated with carbon. Filter paper was used to remove the excess NP solution. The copper grid was dried finally under room temperature and exposed to TEM analysis using Tecnai F20 model instrument which operates at 200 kV accelerating voltage. Furthermore, the spectroscopic FT-IR analysis was performed using PerkinElmer in a wavelength range of 400 to 4000 cm⁻¹.
Coverslips surface modification for cell culture
0.1% v/v organosilane mixture in freshly distilled toluene was allowed to react with cleaned glass surface in order to form N-1 [3-(trimethoxysilyl) propyl] diethylenetriamine films. Cover slips which were coated with diethylenetriamine were heated below the boiling temperature of toluene, washed using toluene, and heated again to same boiling point and then dried in an oven. The procedure was detailed described in an earlier report [29, 30].

Separation and culturing of adult rat spinal-cord
All the experiment in animals were approved by the Institutional Animal Ethics Committee, Affiliated Hangzhou First People’s Hospital, Zhejiang University School of Medicine and the experiments were performed in accordance to their guidelines. Spinal cords were removed from the sacrificed adult rats of 3 to 4 months average age and the meninges were isolated from the spinal cords. Each spinal-cord of adult rat weighed about 1.10 g (±0.05). Then, the spinal cords were harvested followed by cutting down to tiny fragments and later collected in B27 (invitrogen), an antibiotic-antimycotic, GlutaMAX™ and cold Hibernate A [31]. Later, the tissues were digested enzymatically in papain (2 mg ml⁻¹) for half an hour. Tissues were dissociated in 6 ml cell suspension of B27, antibiotic–antimycotic, fresh Hibernate A and GlutaMAX™. The cell suspension of 6 ml was added as a layer over a step gradient of 4 ml (diluted Optipep 0.505(v/v); 0.495 v/v with GlutaMAX TM/Hibernate A/B27/antibiotic-antimycotic and later prepared to 20%, 15%, 35% and 25% v/v in GlutaMAX TM/A Hibernate/B27/antimycotic antibiotic) following 15 min centrifugation with 800 g at 41 °C temperature. The top supernatant of 7 ml was aspirated. Another 2.75 ml was collected from major band and below the band and diluted in 5 ml of GlutaMAX TM/Hibernate A/B27/antimycotic-antibiotic, which later was further centrifuged for 2 min at 600 g. Furthermore, the pellet was suspended again in GlutaMAX TM/Hibernate A/B27/antimycotic-antibiotic followed by centrifugation and then resuspended in the culture medium. Live cells of around 12,000 to 13,000 were harvested from each spinal cord of an adult rat. On every cover slip (22 × 22 mm²), a total of thousand cells were plated at 2 cells/mm². After first two days, the culture medium was completely changed and later for every four days, only half medium was changed [32, 33]. The L-3224 live/dead Assay Kit of a molecular probe was utilized for the live–dead assay [34].

Immunocytochemistry for the glial cells and neuronal cells quantification in nanoparticle and control treated culture
The glial cells and the neuronal cells were stained by using monoclonal anti-GFAP antibody of mouse (MAB360 Chemicon, diluted in ratio of 1:400) and 150 kDa of anti-neurofilament M polyclonal antibody of rabbit (diluted 1:100). The immunostaining method was described in an earlier report [32].

In vitro toxicity evaluation on PL cells
The cytotoxic effect of nanoceria was evaluated in CCRF-SB cell lines. Analysis of cytotoxicity was performed according to the methods described [35]. An initial cell density of around 50000 cells (per well) were seeded in 100 ml cell culture media containing 1 nM to 10 μM nanoceria. Assay was performed in 96-well plate and incubation was carried out at 37 °C in 5% CO₂ for 48 h. The viable cells were measured through Cell Titer-Blue reagent. Cell viability assay was performed according to the manufacturer’s instructions.

Results and discussion
Alkaloids, saponins, tannins, glycosides, flavonoids and reducing sugars are the major biomolecules from aqueous extract of neem [36]. The molecules harbouring hydroxyl and amine groups have been reported to facilitate the complexation of cerium cations (Ce³⁺) during the synthesis of cerium oxide nanomaterials. Also, the formation of electrostatic interaction between the positively charged cerium ions (Ce³⁺) and the oppositely charged biomolecules have been reported to form small and stable nanoparticles (CeO₂-NPs) with controllable growth sizes [16, 37, 38]. Besides, numerous reports have described the capping/coating and stabilizing ability of phytoconstituents from neem leaf extract (such as flavanones and terpenoids) in nanoparticles synthesis [39]. A schematic representation of neem-based CeO₂-NP synthesis is given in figure 1.

The peaks obtained in XRD analysis of the synthesized CeO₂-NPs using A. indica leaf extract were depicted in figure 2. The peaks of XRD analysis were found at 2θ- angles of 33.06°, 47.42° and 28.51°, which corresponds to (200), (220) and (111) planes of CeO₂-NPs. Furthermore, other peaks of XRD located at 2θ- angles of 78.99°, 56.30°, 76.57°, 59.09° and 69.00° are in accordance to (420), (311), (331), (222) and (400) planes of the formed CeO₂-NPs. An fcc phase of CeO₂-NPs was shown by the standard peaks of diffraction analysis.

X-ray photoelectron spectroscopy (XPS) analysis gives information about the surface functionalization composition of the formed CeO₂-NPs and it also provides the oxidation state of every element in the prepared
sample. The results of XPS demonstrate that the indexed peaks for CeO$_2$-NPs correspond to the O (1s), Ce (3d) and C (1s). The signals of C (1s) are mostly because of the organic contaminants absorption while handling or because of the residual trace amounts of the plant extract. As depicted in figure 3, the signals of Ce (3d) are split into 8 signals in the Gaussian fitting, i.e., O$_8$, O$_7$, O$_6$, O$_5$, O$_4$, O$_3$, O$_2$ and O$_1$. Furthermore, the binding energy of CeO$_2$-NPs represents 8 bands located at 921.33, 916.34, 906.56, 900.24, 897.73, 896.04, 888.25 and 882.26 eV. The binding energies of (897.73 eV & 896.04 eV) and (916.34 eV) represent 3 major peaks (O$_4$, O$_2$ and O$_3$) of Ce$^{4+}$ 3d$_{5/2}$ and Ce$^{4+}$ 3d$_{3/2}$. In addition, the O$_1$ and O$_3$ energy levels of the Ce$^{3+}$ 3d$_{5/2}$ & Ce$^{3+}$ 3d$_{3/2}$ were located...
at 882.26 eV and 900.24 eV. Therefore, on the energy levels of Ce$^{3+}$ 3d$^{5}/2$ at (921.33 eV & 888.25 eV) and Ce$^{3+}$ 3d$^{3}/2$ at 906.56 eV, the additional satellite lines O2, O8 and O6 were shown respectively.

Figure 4(A) represented the HRTEM micrograph of the synthesized CeO$_2$-NPs, that were calcined at 400°C. Approximately the particle size is obtained to be 5 nm for spherically shaped structure. Well defined diffraction rings of Debye–Scherre in the SAED pattern were due to high crystalline powder (figure 4(B)) that could be attributed to the reflections (2 0 0), (1 1 1), (2 2 0), (4 0 0), (2 2 2), (4 2 0) and (3 3 1) of cubic cerium oxide. The SAED pattern had no other rings resulting from any crystalline impurities.

The FTIR spectrum of the synthesized CeO$_2$-NPs is represented in figure 5 utilizing the method of KBr pellet in the wavelength range of 400 cm$^{-1}$ to 4000 cm$^{-1}$. The broad absorption peaks in between 3000 to 3750 cm$^{-1}$ frequency range is attributed to the O–H stretch present in water, cerium hydroxide and residual alcohol. The absorption peak for CeO$_2$-NPs was noticed at a wavelength of 3475 cm$^{-1}$ whereas the peaks of CO$_2$ were observed at 1394 cm$^{-1}$ and 2358 cm$^{-1}$ and these bands could be attributed to the CO$_2$ trapped in the air atmosphere. The stretching band of O–C–O was overlapped partly by the bending of H–O–H band which is present at 1647 cm$^{-1}$. The formation of peak owing to the Ce–O stretching frequency is generally located below 400 cm$^{-1}$ wavelength, which indicates the formation of cerium oxide [31], however, in the current study, Ce–O stretching band was noticed at a wavelength of 451 cm$^{-1}$. Similarly, one of the earlier literatures also reported that the stretching band of Ce–O was appeared at 450 cm$^{-1}$ [40].

The in vitro experiments of the as-prepared CeO$_2$-NPs were performed in an adult rat spinal cord using a model of serum free cell culture, which was exhibited to promote long term survival and growth of dissociated spinal cord neurons in an adult rat [32]. This system contains non-biological, patternable [30], organosilane substrate promoting cell growth, diethylenetriamine [34, 41–43] which is coated on surface of glass combined with a fresh serum free medium derived empirically, pre-plating methodology and a reproducible cellular isolation. Furthermore, the serum free medium contains neurobasal—A which is supplemented with neurotrophin-4, B27 [44], GlutaMAX™, heparin sulphate, acidic fibroblast growth factor, CNTF (ciliary neurotrophic factor), neurotrophin-3, neurotrophic factor derived from glial cells, brain-derived neurotrophic factor, cardiotrophin-1, antibiotic antmycotic and vitronectin.
Cell plating and cell isolation is comprehensively documented in one of the earlier studies [32]. Each coverslip was plated with cell suspension of an equal volume (1000 live cells at 2 cells mm$^{-2}$ density), in every experiment. Out of all the plated coverslips, half of them received a 10 nM dose of nanoceria during cell plating and the remaining half was utilized for control cultures. In both the cultures treated with nanoceria and control, the surviving cell types and the cell viability was quantified by conducting immunostaining assays of neuron glia and dead-live assays at different time intervals of day 15 and day 30. For statistical analysis, we utilized student’s T test.

Results were expressed in mean ± SE and n=6 where the number of coverslips was denoted by n. All the coverslips utilized for these assays were obtained from six various experiments. The assay of live-dead cells exhibited an obvious higher cell survival in the cultures treated with nanoceria on day 30 (472 ± 35) and day 15 (617 ± 34) when related with the control group cultures on day 30 (328 ± 32) and day 15 (479 ± 37), where n is considered as 6 in both the cases. Similarly, an obvious higher cell death was noticed in the control group cultures on day 30 (72 ± 8) and day 15 (110 ± 9) when related with the cultures treated with nano-ceria on day 30 (48 ± 7) and day 15 (59 ± 7), where n is considered as 6 in both the cases. The glial and neuron cells were detected by immunoreactivity for glial and neuron markers called glial fibrillary acidic protein (GFAP) and neurofilament 150 antibodies, correspondingly. In comparison with the control group cultures on day 30 (148 ± 9) and on day 15 (71 ± 26), the cultures treated with nanoceria at day 30 (221712) and day 15 (n = 6, 191740) showed a significantly greater neuronal population, where n is considered as 6 in both the cases. In addition, no substantial difference was observed in the number of glial cells or cell populations that stained in treated cultures for both glial and neurons markers when related to control group cultures at any time interval (figure 6(B)).

We hypothesize that the existence of Ce$^{3+}$ and Ce$^{4+}$ mixed valence states on the CeO$_2$-NP’s surface plays as an anti-oxidant that helps the NPs to scavenge free radicals present in the culture system. One more complex set of the chemical reaction [45] on the surface among the ions in nano-ceria and cell culture medium seems to be playing a significant role in reverse switching the oxidation state of CeO$_2$-NPs from Ce$^{4+}$ to Ce$^{3+}$. This could be an indication of auto-catalytic or cyclical regenerative reaction of the CeO$_2$-NPs. The CeO$_2$-NPs displays a mechanism where the engineered particle delivers a new material for the application of life science with pseudo infinite half-life and unprecedented antioxidant activity. The auto regenerative antioxidant property of the formed CeO$_2$-NPs seems to be the major factor for its neuro-protective activity.
In vitro toxicity evaluation on PL cells

The cytotoxic effect of various concentrations of nanoceria (ranging from 1 nM to 10 μM) was assessed in leukaemia cell lines CCRF-SB using Cell Titer-Blue reagent. The results unveiled the cytotoxic effect of nanoceria. A gradual decrease in cell viability was observed with increasing concentration of nanoceria (figure 7) and cell proliferation was significantly halted at higher concentrations of nanoceria (100 nM, 1 μM, and 10 μM).

Similar results were reported in a study from Krishnan et al.[35] in which nanoparticle coated with dexamethasone, a drug used in the treatment of PL, showed a dose-dependent cytotoxic effect in leukaemia cell lines, RS4;11 and Nalm6, through caspase-3 mediated apoptosis event. The same mechanism of action has been reported for the leaf extract of A. indica, in which the active ingredient ‘nimbolide’ from A. indica has been shown to possess anticancer property against pancreatic cancer [46]. Several other nanoparticles including silver, gold synthesized from biological materials were found to exhibit concentration-dependent activities against various cancer cell lines [47–51]. In general, it has been reported that metals or trace elements from the metals resulted in considerable level of oxidative stress which in turn induces the apoptotic event through caspase-mediated pathway. In the case of biosynthesis of nanomaterials, bioconstituents covering the nanoparticles elevate the antitumor potential of biosynthesized nanoparticles to a superior level by enhancing the oxidative stress [52]. This elevated oxidative stress consequently results in the excessive generation of reactive oxygen species (ROS) which causes damage to the biomolecules such as DNA, RNA, proteins and lipids, organelle and membrane structure [53]. Here, ROS generation also depends on the physiochemical features of generated nanoparticles including size, surface area and morphology. Eventually, ROS-mediated biotoxicity results in cell apoptosis. Hence, oxidative stress-mediated ROS generation is the main toxicity mechanism explained so far in nanoparticles-based cancer therapy [53]. However, the complete mechanism remains elusive.

Conclusion

In conclusion, CeO2-NPs were green fabricated using the leaf extract of A. indica. Transmission electron microscopy (TEM) images confirmed that the prepared NPs were spherical in shape with 15 nm particle size. The stretching bands of Ce-O were noticed at 457 cm⁻¹ and 451 cm⁻¹ from the Raman and Fourier-transform Infrared (FT-IR) spectra correspondingly. On the other side, biological investigations demonstrated that the single dose application of CeO2-NPs at nanomolar concentration is regenerative, bio-compatible and provides a considerable neuro-protective effect on the spinal cord neurons of an adult rat. Neuronal function retention is showed in electrophysiological (electrography) recordings and the assay of oxidative injury suggested the possibility of its use in preventing ischemic-insult. Furthermore, cytotoxicity assessment on PL cell lines exhibited dose-dependent cytotoxic effect on CCRF-SB cells indicating their therapeutic potential for future treatment of paediatric cancer.
Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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