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Research Paper: Preparation of Cerium Oxide Nanoparticles and Their Cytotoxicity Evaluation *In vitro* and *In vivo*

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ABSTRACT

Background: Nanotechnology plays a significant role in medicine, especially in diagnosis and treatment as a carrier to drugs and vaccinology. Several studies were conducted using nanoparticles as an adjuvant. The main aim of this study was *in vivo* and *in vitro* toxicity evaluation of synthesized Cerium Nanoparticles (CeNPs).

Methods: In the present study, cerium nanoparticles were prepared using the wet chemical method. The formation of cerium nanoparticles was confirmed by scanning electron microscopy, transmission electron microscopes, x-ray diffraction analysis, dynamic light scattering. *In vivo* and *in vitro* toxicity of synthesized nanoparticles was evaluated in three different amounts of cerium nanoparticles (30 µg, 50 µg, & 100 µg) in mice and human fibroblast cell lines, respectively.

Results: Cerium nanoparticles were successfully synthesized, and the identity was confirmed by x-ray diffraction analysis. The shape and size of nanoparticles were spherical and <100 nm, respectively. The prepared nanoparticles had a charge of -26.6 mV and a hydrodynamic radius of 446 nm. MTT assay indicated that none of the concentration of cerium was toxic, and *in vivo* toxicity also clarified the safety of cerium nanoparticles in mice; no significant un-normal behavioral and physical symptoms were observed in mice after CeNP administration

Conclusion: Cerium nanoparticles have special properties, especially low toxicity, unique capabilities in stimulating the immune system. Cerium nanoparticles can be considered an effective and safe candidate in vaccines.

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1. Introduction

Nanotechnology is among the most attractive industrial innovations of the 21st century. Metal nanoparticles are widely used in medicine, especially as carriers for drugs and vaccines, especially in diagnosis and treatment. These particles have adjuvant properties and can increase cell uptake, activate antigens, produce cytokines and induce humoral immune responses. Due to the charge transfer, these particles have also been shown to cross-react with critical biological molecules, such as Cerium Nanoparticles (CeNPs), forming strong bonds with histidine molecules [1].

In the periodic table, cerium as the first element of the lanthanide series is a rare-earth metal. The 4f orbitals of cerium are shielded by 5p and 4d electrons, which gives interesting catalytic properties to cerium [1]. Both 3+ and 4+ states are observed for cerium. Therefore, cerium oxide can observe in both cerium dioxide (CeO_2) and Ce_2O_3 states [2, 3]. At the nanoscale, cerium oxide has existed a combination of the 3+ and 4+ forms on the nanoparticle surface. The number of 3+ sites on the surface rise and reduction in nanoparticle diameter causes a high oxygen vacancy [1].

Cerium dioxide nanoparticles have unique biochemical properties that are particularly promising for medical applications. In general, studies highlighted that cerium nanoparticles have low toxicity. CeO_2 has also been revealed to mimic Superoxide Dismutase (SOD) activity at a constant catalytic rate greater than biological enzyme activity [4]. CeO_2 nanoparticles, as antioxidants, can neutralize free radicals, can regenerate, and cross the brain-blood barrier on a nanoparticle scale [5]. D'Angelo et al. suggested that cerium nanoparticles protect nerve cells against cell death due to Alzheimer's damage [6]. Studies reported that metastasis and tumorigenesis of ovarian cancer mice models are inhibited by treating cancer cells via CeO_2 NPs. In these cancerous cells, angiogenesis is also observed as decreased in mice treated with CeO_2 nanoparticles [7]. It has also been shown that angiogenesis and tumor cell formation is lessened by the interaction of folic acid and CeO_2 nanoparticles [4]. Furthermore, oxidative stress signaling pathways and inflammatory cytokines levels such as IL-1β (Interleukin 1 beta) and TNF-α (Tumour Necrosis Factor α) was inhibited and decreased respectively in carbon tetrachloride-induced liver fibrosis mice by administrating CeO_2 nanoparticles [8].

Metal nanoparticles develop the innate immune response by affecting phagocytes, neutrophils, mast cells, and natural killer cells. Also, these particles activate acquired immunity by affecting T and B cells [9]. Cerium oxide nanoparticles are widely employed in engineering and biological applications, including pharmaceutical, catalytic materials, and solar cells. This study synthesized cerium nanoparticles using a simple wet chemical method. *In vitro* and *in vivo* cytotoxicity of the nanoparticles was evaluated on human L929 fibroblasts cells and outbred mice, respectively.

2. Materials and Methods

Cell line and materials

L929 cell line was purchased from the national cell bank (Pasteur Institute of Iran, Iran). DMEM medium, fetal bovine serum, and penicillin-streptomycin solution were obtained from Invitrogen, USA. DMSO, ammonium hydroxide, and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma, USA. Cerium nitrate hexahydrate [98.5%, $Ce(NO_3)3.6H_2O$] was obtained from Merck, USA. All other chemical reagents like for the preparation of phosphate buffer saline were purchased from Sigma, USA.

CeO_2 nanoparticles synthesis

CeO_2 nanoparticles were prepared according to the method described by Chelliah et al. [10]. In this study, cerium nitrate hexahydrate [Ce $(NO_3)3.6H_2O$; 98.5%, Merck] and ammonium hydroxide were used as a precursor of cerium and a precipitating agent, respectively. Initially, 125 mL of 1 M sodium nitrate solution was poured into a beaker and sonicated the solution. Then, 25% ammonium hydroxide diluted with water was added slowly to increase the pH of the solution to 9.

The mixture's temperature at 80°C was controlled during the sonication process and the pH of the solution at approximately 9. The solution sonication process was continued for 2 hours. Next, the product was washed with distilled several times with water and centrifuged. Finally, the product was placed in an oven at 100°C for 24hr.

The characterization and identification of Cerium Nanoparticles (CeNPs)

Morphological and structural characterization of structures was done by Scanning Electron Microscopy (SEM), Transmission Electron Microscopes (TEM), X-ray Diffraction Analysis (XRD), Dynamic Light Scattering (DLS) to investigate the structure of the nanoparticle.

X-Ray Diffraction (XRD)

X-ray diffraction was used to identify the crystallographic structure of cerium nanoparticles. In the current study, X-ray diffraction analysis was performed by XRD instrument with CuKa (Copper anticathode) lamp radiation in the angle range of 20–80 degrees.

Scanning Electron Microscope (SEM)

Scanning electron microscope images and point-to-point study were used to evaluate the size and morphology of nanoparticles after coating with gold. The nanoparticles were dissolved in some water, and the suspension was imaged on the gold grade by SEM.

SEM evaluated the morphology of CeNPs. The powder samples were coated with the gold film for loading the dried particles on the SEM instrument. The gold coating was performed by a Sputter Coater model SCD005 made by BAL-TEC (Pfäffikon ZH, Switzerland), and the images were taken at desired magnification.

Transmission Electron Microscopy (TEM)

To assess the morphology and confirm the size of cerium nanoparticles, a drop of the suspension sample containing nanoparticles was placed on a carbon film grade. After drying at laboratory temperature, it was imaged using a transmitting electron microscope.

Zeta potential and the hydrodynamic radii by DLS method

The synthesized nanoparticles' size and charge were measured using a zeta sizer (Malvern Zetasizer). The synthesized nanoparticles were dissolved in 1 mL of water; the resulting suspension was sonicated for 5 minutes. The resulting suspension was then poured into a special cuvette. Finally, the size and charge of the synthesized nanoparticles were measured.

In vitro toxicity assay of the synthesized CeNPs

L929 cell line was cultured in DMEM supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. These cell lines were purchased from the cell bank (Pasteur Institute of Iran). For MTT assay, 104 cells/well were cultured in a 96-well plate (Thermo Fisher Scientific) and incubated overnight at 37°C with 5% CO_2. According to previous studies, 100µL of different concentrations of CeNPs (15.625, 31.25, 62.5, 125, 250, & 500 ug/mL) were added to each well in triplicates, and cells were incubated for 24 h at 37°C [11]. Then, one hundred µL (0.5 mg/mL) of MTT (Sigma-Aldrich, Germany) was added to each well and incubated for four h at 37°C. The supernatants were removed, 100 µL DMSO (Sigma, USA) was added to each well, and the reaction was read by a microplate reader (DNM-9602G) at 570nm. Data were analyzed by SPSS software using the Independent t-Samples t-test.

Outbred mice

The Ethics Committee approved all the research protocols used in the Tehran University of Medical Sciences experiment, and the laws, norms, and regulations dealing with international animal ethics were followed (IR.TUMS.SPH.REC.1398.283).

In this study, 20 Naval Medical Research Institute (NMRI) female mice weighing an average of 11-14 g (21 days) were used, purchased from the laboratory animal center of the Pasteur Institute of Iran. Animal feed was supplied from the Pasteur Institute of Iran, tablets with a standard formula. The animals had free access to food and drinking water through bottles. The daily feed intake was estimated at 5 to 10 g per mouse.

The study animals were randomly divided into 7 groups with 7 members apiece: (1) PBS (Phosphate-Buffered Saline) control group (control) and (2) to (4) CeNPs treated groups (test). CeNPs were administered to the peritoneal cavity of test groups mice in three doses: 30, 50, and 100 ug/mL. The Control group mice were vaccinated with 100 mL PBS.

After intraperitoneal administration of the corresponding sample, toxicity sign, animal behavior, body weight, and lethal response were followed daily for 14 days.

Statistical analysis

SPSS was used for statistical analysis. The significance of statistical comparisons was calculated using one-way Analysis of Variance (ANOVA). LSD and Duncan's methods compared variables. $P<0.05$ was considered significant. All values were expressed as Means±SD.

3. Results

The characterization of the synthesized CeNPs

XRD, SEM, TEM, and DLS techniques were used to determine the identity, size, and shape of the synthesized CeNPs.

XRD analysis

XRD analysis was used to evaluate of composition and purity of CeNPs. The X-ray scattering pattern of the syn-

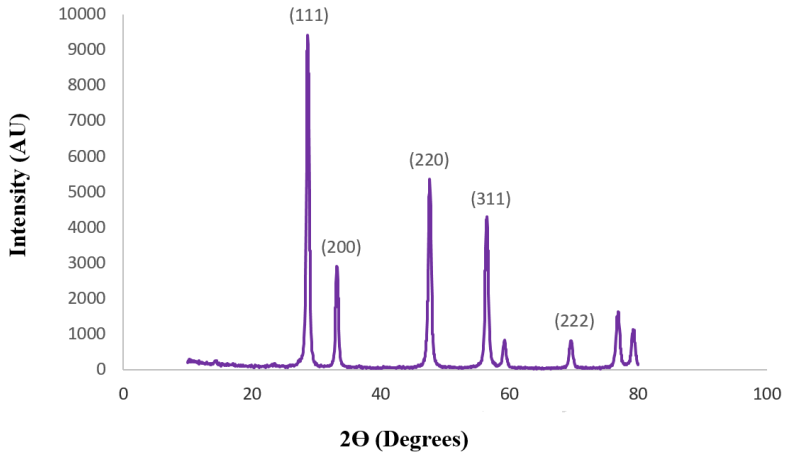

Figure 1 XRD pattern of green synthesized CeNPs

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thesized cerium nanoparticles is shown in Figure 1. The obtained characteristic diffraction peaks in this XRD pattern were observed at 2θ, 28.53, 33.06, 47.42, and 56.33, which are assigned to the 111, 200, 220, and 311 crystallographic planes of face-centered cubic structure for the cerium powder sample.

Scanning Electron Microscope (SEM)

The image obtained from the SEM electron microscope (Figure 2) illustrates the three-dimensional structure of the synthesized nanoparticles. As per Figure 2, the three-dimensional structure of cerium nanoparticles was spherical, and the sizes of the nanoparticles were different and <100 nm.

Transmission Electron Microscopy (TEM)

The obtained image from TEM (Figure 3) indicates the confirmation of the synthesis of cerium nanoparticles with the desired diameter (less than 50 nm).

Hydrodynamic radius and zeta potential of synthesized nanoparticles

The nanoparticles were dissolved in some water, and the resulting suspension was sonicated for 5 minutes. The sonicated suspension was then poured into a special cuvette, and the hydrodynamic radius and zeta potential of the synthesized nanoparticles were measured. The synthesized nanoparticles had a charge of -26.6 mV and a hydrodynamic radius of 446 nm (Figure 4).

The toxicity of cerium nanoparticles on L929 cell line

Following Figure 5 demonstrates the effects of different concentrations of cerium nanoparticles on L929 cells and the viability of these cells. As per the Figure 5, no significant cytotoxic effects were observed in any tested concentrations.

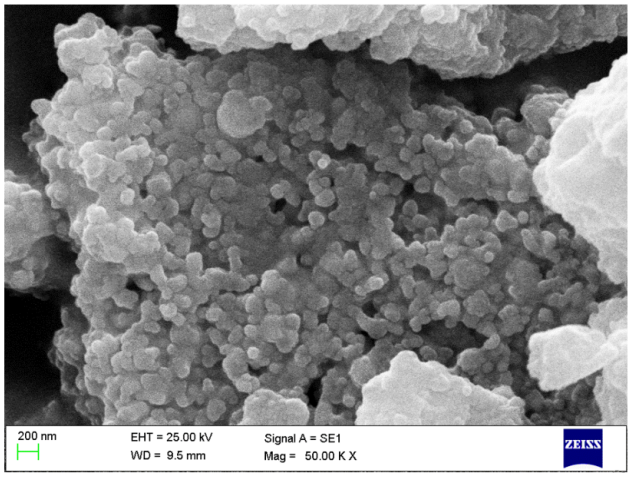

Figure 2. SEM imaging of synthesized CeNPs

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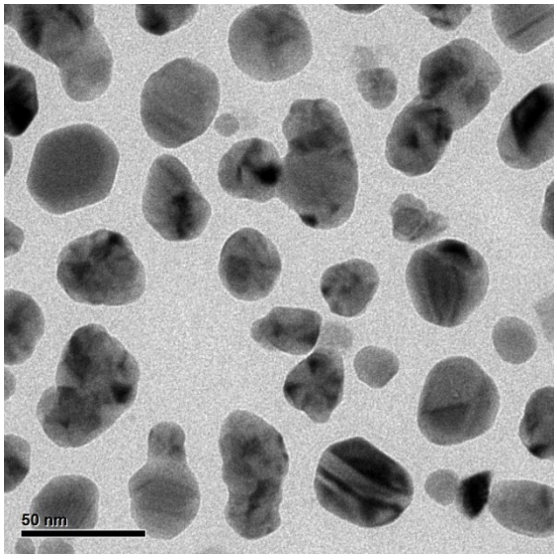

Figure 3. TEM imaging of synthesized CeNPs

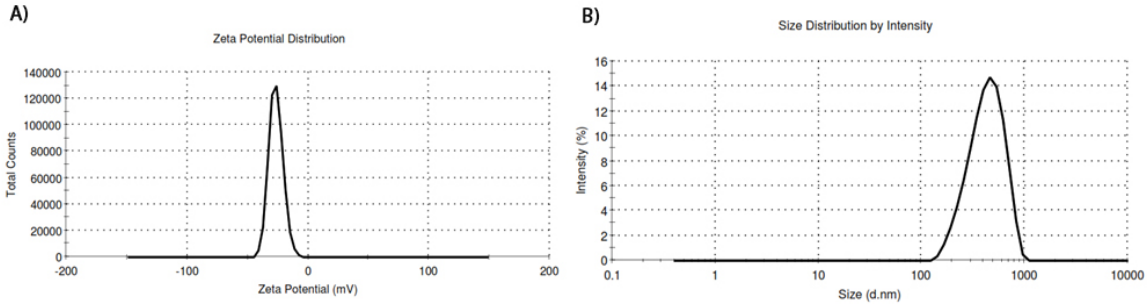

Figure 4. (A) Zeta potential of synthesized nanoparticles and (B) hydrodynamic radius of synthesized nanoparticles

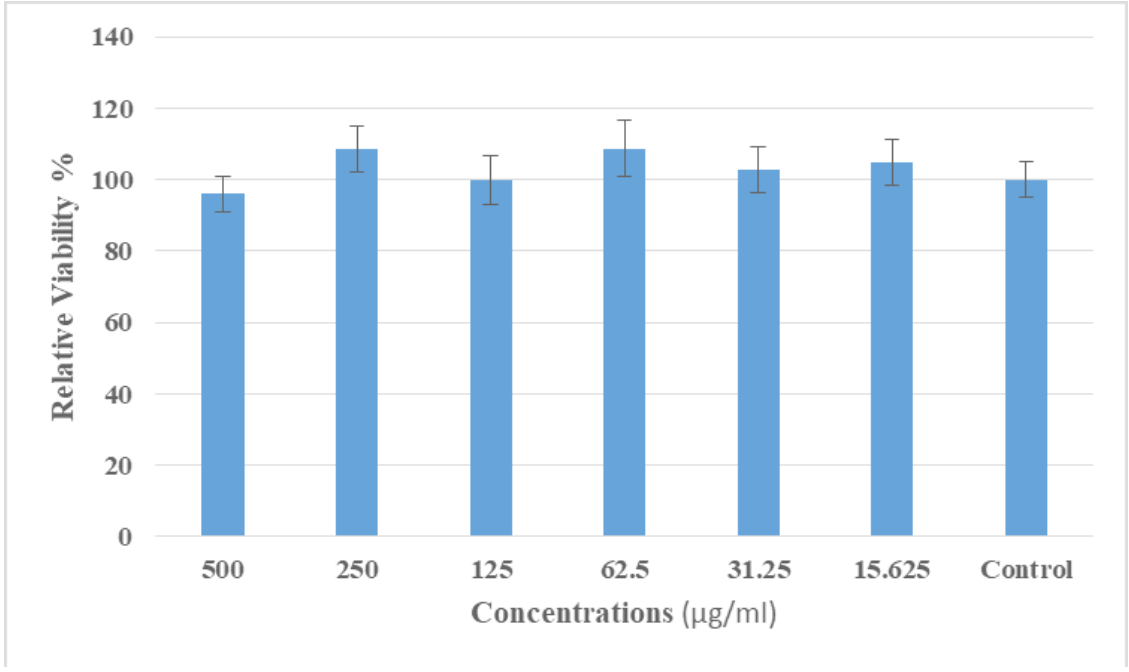

Figure 5. Investigating the effects of cerium nanoparticles on the L929 cell line

Table 1. Behavioral and physical symptoms observation of mice after CeNP administration

Concentration CeNP (ug/mL)	Symptom (% of Mice)	Day												
		2	3	4	5	6	7	8	9	10	11	12	13	14
30	Drowsiness*	0	10	0	0	0	0	0	0	0	0	0	0	0
	Tremor	0	0	0	0	0	0	0	0	0	0	0	0	0
	Hypoactivity	10	10	20	0	0	0	0	0	0	0	0	0	0
	Piloerection	10	20	0	0	0	0	0	0	0	0	0	0	0
50	Drowsiness	0	0	0	0	0	0	0	0	10	0	0	0	0
	Tremor	0	0	0	0	0	0	0	0	0	0	0	0	0
	Hypoactivity	20	0	0	0	0	0	0	0	0	0	0	0	0
	Piloerection	10	20	20	20	10	10	0	0	0	0	0	0	0
100	Drowsiness	0	0	0	0	0	0	0	0	0	0	0	0	0
	Tremor	0	0	0	0	0	0	0	0	0	0	0	0	0
	Hypoactivity	10	0	0	0	0	0	0	0	0	0	0	0	0
	Piloerection	10	20	40	50	40	10	0	0	0	0	0	0	0

* All behavioral changes were self-limiting after a few days.

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Follow-up

During the 14 days following mice, no significant un-normal behavioral and physical symptoms were observed in mice after CeNP administration (Table 1).

4. Discussion

The current investigation showed that the cytotoxicity of CeO_2-NPs was minimal at evaluated concentrations; however, at high concentrations, the symptoms of poisoning such as hypoactivity and piloerection appeared in mice that were self-limiting after a few days. Nanotechnology has attracted a great deal of medical research interest because of the needs and applications of nanomaterials in many fields, such as drug delivery, diagnostic, imaging, antimicrobial techniques, cell repair, protein and peptide delivery, molecular imaging, and therapy [12].

Cerium is a lanthanide series of metals [13], metal oxide, and metal nanoparticles. Considering the high surface area and a high fraction of atoms and their interesting properties can be antimicrobial agents in biological fields [14]. Furthermore, cerium oxide nanoparticles were produced with various preparation methods, such as thermal decomposition, sol-gel, flame spray pyrolysis, microemulsion methods, solvothermal oxidation, and microwave-assisted solvothermal process [15].

In some studies, the green synthesis of CeO_2 nanoparticles was reported using various biological derivatives, such as microbial and plant extracts, for different applications [16]. Cerium nanoparticles were successfully produced using a low-cost and easy method in the current research. The chemical precipitation of ammonium hydroxide and its intraperitoneal toxicity profile was evaluated *in vitro* and *in vivo*. The characteristics of the cerium nanoparticles were studied by XRD, SEM, TEM, and DLS techniques. Comparing XRD results with the Joint Committee on Powder Diffraction Standards (JCPDS) files confirmed compound identity, and TEM studies confirmed Debye Scherrer's formula's calculated particle size value [17]. Comparing our synthesis results with previously published studies indicates that [18-20] our synthesized product has the same results as them.

Some investigations compared the toxicity of nanoceria within cells. Darroudi et al. evaluated the toxicity of synthesized CeO_2 nanoparticles in neuro2A cell line by MTT assay; their research revealed the concentration-dependent toxicity of cerium oxide nanoparticles with the non-toxic effect of concentration under 10 mg/mL after 24h incubation. Their study does not include any *in vivo* investigation on CeO_2 [21]. Soltani et al. assessed the effect of two nano and bulk forms of cerium oxide in 3 cell lines, including C_2Cl_2 (ATCC mouse skeletal muscle cell line), SKBR3 (human breast cancer cell line),

and A431 (human epidermoid carcinoma cell line) cells. They reported different toxicity of cerium oxide in both nano and bulk forms. Moreover, they found that the toxicity of cerium oxide in higher levels of concentrations after 24, 48, and 72 hours was more on cancerous cells than normal cells [19].

Another study suggested that the toxicity of the nano form of cerium oxide is more than the equimolar bulk form [22]. Fabianne Ribeiro et al. evaluated the effect of CNPs at a low dose on cell survival, migration, and the proliferation of L929 fibroblast cultures under UVA (Ultraviolet A-Ray)-induced oxidative redox imbalance. They found that CNPs can decline fibroblast death induced by UVA via cell redox restoration leading to the modulation of signal-regulated protein kinases 1 and 2 (ERK 1/2) that control cells proliferation and survival [23].

Compared to previous studies, the particle size of the nanoparticles produced in this research is similar to the other syntheses [24]. However, an autoclave-assisted hydrothermal process and microwave-mediated hydrothermal synthesis created the new-generation CeNP with the upper-range crystalline size [25, 26].

We evaluated cerium nanoparticles toxicity in six different doses, including 15.625 ug/mL, 31.25, 62.5, 125, 250, and 500 ug/mL on L929 on cell line and in any of the tested concentrations. We also followed up mice which cerium nanoparticles in different 30, 50, and 100 ug/mL administrated intraperitoneal. No significant cytotoxic effects were observed during the 14-day follow-up.

In some metal nanoparticles such as silver, gold, and copper, the toxicity increases with decreasing nanoparticle size [27]. Other physicochemical properties such as elemental composition, surface charge, shape, crystallinity, solubility also affect the toxic potential of the compounds [11, 12]. Therefore, metal-based nanoparticles should not be considered a homogeneous population with basic toxic properties since they act individually to elicit various biological reactions. The concentration and dimensions of nanoparticles determine their biological functions. According to previous literature, Ce nanoparticles have therapeutic effects; due to the physical and chemical properties and low toxicity of cerium dioxide nanoparticles, they can be considered suitable candidates for adjuvant in vaccines. To better understand the acute and subchronic toxicity study results, we recommend performing a detailed dose metric analysis parallel with the *In vitro* tests to establish the NP dose delivered over the exposure period and then corroborating the biological findings with these results.

5. Conclusion

Recently, nanotechnology has led to various applications, especially in producing specific sizes and shapes of metal nanoparticles. Cerium metal has special properties, especially low toxicity, unique capabilities in stimulating the immune system. CeNPs can be considered an effective and safe candidate in vaccines.

Ethical Considerations

Compliance with ethical guidelines

The Ethics Committee approved all the research protocols used in the Tehran University of Medical Sciences experiment, and the laws, norms, and regulations dealing with international animal ethics were followed (IR.TUMS.SPH.REC.1398.283).

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Author's contributions

Data collection: Milad Zandi, Maryam Fazeli, Rouzbeh Bashar, Razieh Bigdeli, Vahid Asgari and Reza Ahangari Cohan; Data analysis: Maryam Fazeli, Reza Ahangari Cohan and Shohreh Shahmahmoodi; Funding acquisition and Resources: Maryam Fazeli and Shohreh Shahmahmoodi.

Conflict of interest

The authors declared no conflict of interest.

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