



Research Article

The Expression of Antioxidant Genes and Cytotoxicity of Biosynthesized Cerium Oxide Nanoparticles Against Hepatic Carcinoma Cell Line

Ali Es-haghi^{1*}, Fatemeh Javadi¹, Mohammad Ehsan Taghavizadeh Yazdi², Mohammad Sadegh Amiri³

¹Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran

²Medical Toxicology Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

³Department of Biology, Payame Noor University, Tehran, Iran

***Corresponding author:**

Ali Es-haghi (PhD),
Department of Biology,
Mashhad Branch, Islamic
Azad University, Mashhad,
Iran
Email: eshaghi5510@
mshdiau.ac.ir; ashaghi@
gmail.com

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Abstract

Background: Drug resistance due to genetic variations renders many therapeutic methods such as surgery, radiotherapy, chemotherapy, and hormone therapy unsuccessful in eradicating cancerous cells. Nowadays, application of nanoparticles (NPs) has been promising in destroying cancerous cells without side effects on normal cells.

Objectives: This study aimed to investigate the antioxidant and anticancer effects of biosynthesized cerium oxide nanoparticles (CeO₂-NPs) on a hepatic carcinoma cell line.

Methods: MTT assay was used to determine the cytotoxicity of CeO₂-NPs in concentrations of 0, 15.6, 31.2, 62.5, 125, and 250 µg/mL after 24, 48, and 72 hours of incubation. Moreover, the expression levels of catalase (CAT) and superoxide dismutase (SOD) (the antioxidant genes) were investigated at different concentrations of CeO₂-NPs using real-time polymerase chain reaction (PCR).

Results: Our results showed a significant toxicity of the synthesized NPs against the cancerous liver cells. The IC₅₀ calculated for CeO₂-NPs was 500 µg/mL at 24 hours of incubation. In addition, the expression levels of CAT and SOD significantly ($P < 0.05$) increased upon the treatment of cells with CeO₂-NPs (500 µg/mL) compared to the untreated cells.

Conclusion: Considering the minimal effects of the biosynthesized CeO₂-NPs on normal cells and on the other hand their considerable toxicity against hepatic cancer cells, these NPs could be utilized in medicine and in the development of new drugs for cancer cells.

Keywords: Cerium oxide nanoparticles, Antioxidant gene expression, HepG2, *Ceratonia siliqua*



Background

In recent years, green chemistry methods for synthesis of nanoparticles (NPs) have become a favorite subject in nanoscience (1-3). Nanoparticle synthesis through physical and chemical methods has limitations such as toxic solvents and the remnants (4,5). Therefore, green synthesis methods using plant extracts could be beneficial in obviating such limitations (6,7). Living organisms such as plants, algae, molds, yeasts, and bacteria can be used for synthesizing NPs (8). Likewise, various physical and chemical methods have been applied in producing cerium oxide nanoparticles (CeO₂-NPs) (5).

Free radicals and reactive oxygen species (ROS) significantly affect the biological systems in medicine (9). Oxidative stress participates in the pathogenesis of various diseases such as diabetes, cancer, Alzheimer's disease, and blindness (10). Therefore, herbs containing high levels of antioxidants can beneficially protect biological systems

against these agents and improve human health (11). Free radicals are natural metabolic products which cause cellular damages, dysregulate cellular proliferation, destabilize biological molecules, and interfere with normal functions of various cells. Antioxidants neutralize detrimental free radicals and minimize their cellular damages (12,13). On the other hand, nanomedicine is the science of applying NPs (particles between 1-100 nm in size) to the diagnosis and treatment of human diseases (14). In recent years, the use of NPs as carrier systems for target drugs toward cancerous cells has made significant progress (15). CeO₂-NPs have been extensively applied in different fields of medicine (16,17). Cerium oxide (CeO₂) is a potent antioxidant that effectively scavenges ROS and can be used as a potential anticancer agent. Furthermore, synthesized CeO₂-NPs show antioxidative properties; in this respect, they have been suggested as potential new cancer therapeutics (18). In addition, synthesized CeO₂-NPs can be utilized as drug

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carriers in cancer targeted therapy (19). They have also had anti-tumor activities against cancerous cells *in vitro* while protecting normal cells by antioxidant properties (20). Hepatocellular carcinoma derived from hepatocytes is one of the most common malignancies worldwide. It is characterized by its high incidence in hepatitis B virus-associated cirrhotic liver disease and other risk factors (21). Several studies have shown that extract of *Ceratonia siliqua* shows antibacterial, antifungal, and antidiabetic properties; thus in this work, the biomedical effects of NPs were investigated (22,23).

The aim of this study was to evaluate the cytotoxicity, antioxidant, and gene expression regulation effects of CeO₂-NPs synthesized using *C. siliqua* extract on a hepatic cell line.

Materials and Methods

Chemicals and Reagents

The PCR Master Mix, SYBR green PCR master mix, RNeasy Mini Kit, and cDNA Synthesis Kit were purchased from Qiagen GmbH, Hilden, Germany. The other reagents not mentioned here were supplied from Merck (Germany).

Extract Preparation and Synthesis of Cerium Oxide Nanoparticles

In order to provide the aqueous extract, 10 g of dried *C. siliqua* leaf powder was added to the 100 mL distilled water and stirred for 24 hours. For the biosynthesis of CeO₂-NPs, 8.68 g salt of Ce (NO₃)₃.6H₂O was allowed to react with 200 mL of aqueous *C. siliqua* leaf extract. In the next step, the CeO₂-NPs were dried at 80°C for 6 hours, and eventually the purified green-synthesized CeO₂-NPs were generated by heating at 400°C for 2 hours and brownish pellets were prepared. Cells were obtained from Bu-Ali Institute of Mashhad, Iran.

MTT Assay

Cell toxicity of NPs was investigated by the MTT assay. In Brief, HepG2 cells were seeded at a density of 10 000 cells/well in a 96-well plate. Then, the plates were incubated at 37°C for 24 hours. Different concentrations of NPs (i.e., 0, 15.6, 31.2, 62.5, 125, and 250 µg/mL) were inoculated into the grown cells that contained 100 µL of medium.

During this period, after each day of incubation, 20 µL of 5 mg/mL MTT dissolved in PBS was added to each well. At the end of incubation, the medium was discarded and formazan crystals which were shaped by MTT metabolism were liquefied and dissolved through the inclusion of 100 µL of DMSO. Afterwards, the plates were shaken for 5 minutes and then the optical absorbance was measured at 590 nm.

Antioxidant Gene Expression Assay

The expressions of *CAT* and *SOD* genes were determined in the HUVEC normal cell line treated with NPs. The cells were cultured in RPMI medium at 5×10³ cells/mL in a 6-well plate and incubated with different concentrations of NPs including 0, 125, 250, and 500 µg/mL for 24 hours. At the end of incubation, the cells were washed with phosphate-buffered saline (PBS, 0.1 M, pH 7.2) twice and scraped. All the real-time polymerase chain reaction (PCR) amplifications were done in triplicate. Table 1 shows primer characteristics.

RNA Extraction

RNA was extracted from the cells after 48 hours of incubation with NPs. The extraction was done according to the kit procedure. Briefly, 1 mL of the ice-cold RNX-plus solution was added to homogenized cells and mixed by vortexing. Afterward, 200 µL of chloroform was added to the mixture and centrifuged at 12 000× g for 15 minutes at 4°C. An equal volume of isopropyl alcohol was then added to the aqueous phase and centrifuged. In the next step, 1 mL of 75% ethanol was added to the supernatant and centrifuged. The concentrations of extracted RNA were calculated using NanoDrop UV-Vis spectrophotometer and their purity was determined by gel electrophoresis on 1% agarose gel.

cDNA Synthesis

cDNA was synthesized from extracted RNA according to the manufacturer's instructions (Fermentas Kit). The mixture was incubated in the thermal cycler and the program was set as: one cycle at 37°C for 15 minutes, one cycle at 85°C for 5 seconds, and one cycle at 4°C for 5

Table 1. The Characteristics of Primers Used for the Antioxidant Gene Expression Analysis

	Sequence	Gene	Tm (°C)	Annealing Temperature (°C)
Forward	5'- AAGGCCGTGTGCGTGCTGAA-3'	SOD	64	
Reverse	5'- CAAGTCTCCAACATGCCTCT-3'	SOD	60	59
Forward	5'- TTTGGCTACTTTGAGGTCAC-3'	CAT	58	
Reverse	5'- TCCCCATTTCATTAAACCAG-3'	CAT	58	55
Forward	5'- TGCTGGTGCTGAGTATGTCG -3'	GAPDH	60	
Reverse	5'- GCATGTCAGATCCACAACGG -3'	GAPDH	62	57

minutes. Samples without RT enzymes were used for detecting contamination in the samples.

Real-Time Polymerase Chain Reaction

To assess the expression of *CAT* and *SOD* genes, SYBR green-based real-time PCR (Qiagen Rotor-Gene Q, Hilden, Germany) was used. Amplification conditions were set as: initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 56.4°C for 20 seconds, and extension at 72°C for 30 seconds. The fluorescence of SYBR green signal from 65°C to 95°C was used to obtain melting curves. Double-distilled water was used as negative control.

Statistical Analysis

All data were analyzed by SPSS software using ANOVA test. The significance was confirmed by Duncan's multiple range test. *P* value less than 0.05 was applied as the standard for a statistically significant difference. All experiments were carried out in triplicate and the findings were expressed as mean values ± standard deviation (mean ± SD).

Results

In this study, the cytotoxicity of CeO₂-NPs synthesized from *C. siliqua* extract was investigated against HepG2 hepatocellular carcinoma cells. CeO₂-NPs were morphologically spherical with the average size of 22 nm. The size range of the NPs varied from 13 to 30 nm. As shown in Figure 1, CeO₂-NPs showed dose- and time-dependent cytotoxicity against the cancerous cells. The IC₅₀ doses of CeO₂-NPs were obtained 500 µg/mL, 300 µg/mL, and 250 µg/mL at 24, 48, and 72 hours of incubation, respectively (Figure 1). CeO₂-NPs showed minimal toxicity against normal cells at the concentration of 1000 µg/mL (Figure 2).

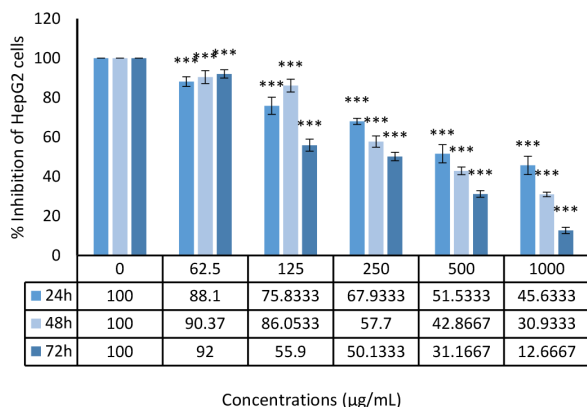


Figure 1. Cytotoxic Activity of CeO₂-NPs on the HepG2 Cell Line. Note. Results are reported as the mean ± SD (n=3) (***P*<0.01, ****P*<0.001).

Gene Expression of Catalase and Superoxide Dismutase Using Real-time PCR

The expression levels of *CAT* and *SOD* genes increased in normal cells exposed to different concentrations (125 to 500 µg/mL) of the synthesized CeO₂-NPs (Figure 3 a, b). The expression level of *CAT* gene significantly increased (*P*<0.001) upon treatment with 250 and 500 µg/mL of CeO₂-NPs compared to the untreated control cells. Only at 500 µg/mL concentration of CeO₂-NPs, the expression level of *SOD* gene was significantly (*P*<0.05) increased compared to the untreated control cells. These changes prove the antioxidant properties of the CeO₂-NPs.

Discussion

Plants have functional biochemistry groups in their structure which act as reducing agents in synthesis of NPs (24-26). In this study, the biological effects of biosynthesized NPs were investigated. Regarding the vast biological and anticancer properties of nanomaterials, CeO₂-NPs have been evaluated as anticancer agents in various studies (27, 28). Cytotoxicity against cancer cells is an important feature of anticancer drugs (20,29). Furthermore, antioxidant capabilities of CeO₂-NPs can prevent cancer development, further suggesting these materials as potential anticancer therapeutics (30). We here synthesized CeO₂-NPs and investigated their potential anticancer activities against HepG2 hepatocellular carcinoma cell line. CeO₂-NPs represent minimal toxicity against normal tissues. Synthesized by green methods, they have negligible side effects on normal cells. In the present study, the antioxidant effects of the synthesized CeO₂-NPs were shown. Oxidative stress increases the production of malondialdehyde and lactate dehydrogenase, which are markers of lipid oxidation and cell membrane damage (31-33). Our results indicated that CeO₂-NPs synthesized from *C. siliqua* extract have remarkable antioxidant

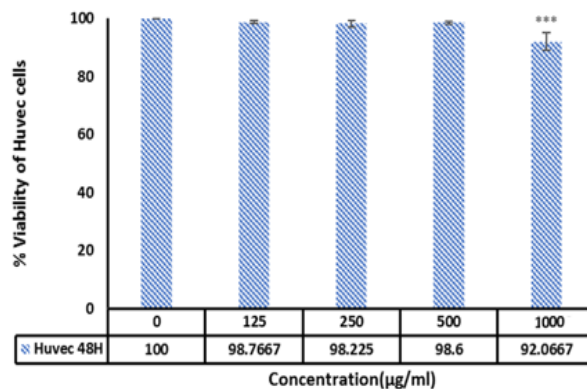


Figure 2. Cytotoxic Activity of CeO₂-NPs on HUVEC Cell Line. Note. Results are presented as the mean ± SD (n=3) (***P*<0.01, ****P*<0.001).

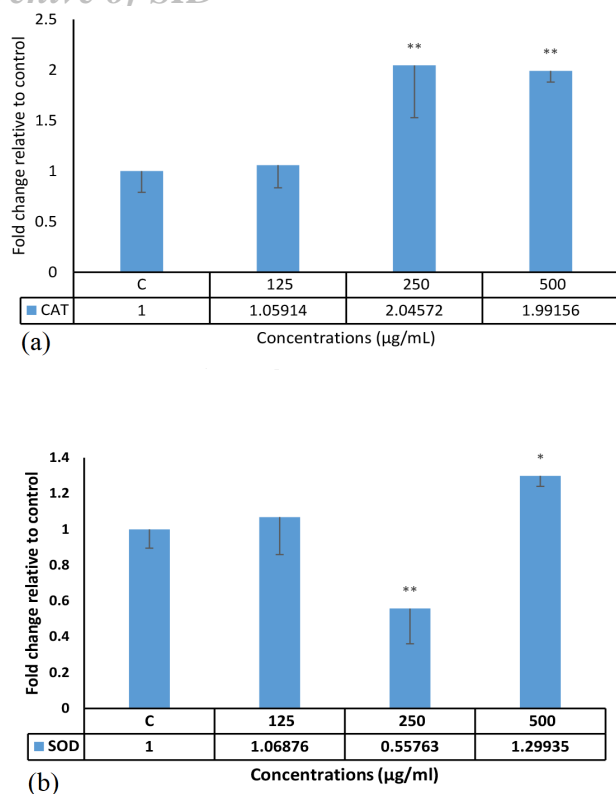


Figure 3. (a) The Expression Analysis of *CAT* Gene in HUVEC Normal Cells Upon Treatment With 0, 125, 250, and 500 µg/mL of CeO₂-NPs for 24 hours. Note. **P*<0.05 and ***P*<0.01 demonstrate significant differences in comparison to the control cells (0). (b) The Expression Analysis of *SOD* Gene in HUVEC Normal Cells Upon Treatment With 0, 125, 250, and 500 µg/mL of CeO₂-NPs for 24 hours. Note. **P*<0.05 demonstrates a significant difference in comparison to the control cells (0).

activities. This is in accordance with another study showing antioxidant properties of CeO₂ in male rats (34). CeO₂-NPs are bio-compatible and less toxic (35). Moreover, they may be considered ecofriendly and may not pose noteworthy environmental risks, in contrast to those compounds used for the chemical reduction method (36). The combination of cerium NPs with other metals may increase the anticancer effects of these NPs. In our survey, we observed that the anticancer activities of CeO₂-NPs were significantly enhanced with Ni doping which was found to be strongly correlated with the level of ROS production (37). We also demonstrated the cytotoxic effects of the synthesized CeO₂-NPs against HepG2 hepatocellular carcinoma cell line. The cytotoxic effects of NPs depend on the dose and time of incubation, as well. Further studies are needed to divulge other biological activities of these NPs against normal and cancerous cells.

Conclusion

In the present study, the biological effects of biosynthesized CeO₂-NPs were investigated. The synthesized NPs revealed antioxidant and cytotoxic properties against HepG2 hepatocellular carcinoma cell line. Therefore, these NPs

can be valuable therapeutics in treating fatal diseases such as cancer.

Authors' Contributions

Ali Es-haghi management and coordination responsibility for the research activity planning, Fatemeh Javadi: performing the experiments, Mohammad Ehsan Taghavizadeh Yazdi: writing the initial draft and statistical analysis.

Conflict of Interest Disclosures

The authors declare no potential conflicts of interest relevant to this article.

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