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Cerium and Yttrium Oxide Nanoparticles Against Lead-Induced Oxidative Stress and Apoptosis in Rat Hippocampus

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Abstract Due to numerous industrial applications, lead has caused widespread pollution in the environment; it seems that the central nervous system (CNS) is the main target for lead in the human body. Oxidative stress and programmed cell death in the CNS have been assumed as two mechanisms related to neurotoxicity of lead. Cerium oxide (CeO_2) and yttrium oxide (Y_2O_3) nanoparticles have recently shown antioxidant effects, particularly when used together, through scavenging the amount of reactive oxygen species (ROS) required for cell apoptosis. We looked into the neuroprotective effects of the combinations of these nanoparticles against acute lead-induced neurotoxicity in rat hippocampus. We used five groups in this study: control, lead, CeO_2 nanoparticles + lead, Y_2O_3 nanoparticles + lead, and CeO_2 and Y_2O_3 nanoparticles + lead. Nanoparticles of CeO_2 (1000 mg/kg) and Y_2O_3 (230 mg/kg) were administered intraperitoneally during 2 days prior to intraperitoneal injection of the lead (25 mg/kg for 3 days). At the end of the treatments, oxidative stress markers, antioxidant enzymes activity, and apoptosis indexes were investigated. The results demonstrated that pretreatments with CeO_2 and/or Y_2O_3 nanoparticles recovered lead-caused oxidative stress markers (ROS, lipid peroxidation, and total thiol molecules) and apoptosis indexes (Bax/Bcl-2 and caspase-3 protein expression). Besides, these nanoparticles reduced the activities of lead-induced superoxide dismutase and catalase as well as the ADP/ATP ratio. Interestingly, the best recovery resulted from the compound of these nanoparticles. Based on these outcomes, it appears that this combination may potentially be beneficial for protection against lead-caused acute toxicity in the brain through improving the oxidative stress-mediated programmed cell death pathway.

Keywords Lead · Hippocampus · Oxidative stress · Programmed cell death · Cerium and yttrium oxide nanoparticles

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Introduction

Lead is a toxic heavy metal, widely distributed in the environment due to numerous industrial applications, and lead poisoning is an important health issue in many countries in the world [1]. As a neurotoxic agent, lead damages the central nervous system (CNS), but the accurate mechanisms are still unclear. Perturbation of pro- and antioxidant balance can be one possible molecular mechanism involved in lead neurotoxicity, which can contribute to brain injury via increase of reactive oxygen species (ROS) and oxidative stress [2]. Oxidative stress is an imbalance between higher cellular levels

and ROS and cellular antioxidant defense. ROS acts as a subcellular messenger in complex processes such as mitogenic signal transduction, gene expression, and regulation of cell proliferation, when it is generated excessively or when enzymatic and nonenzymatic defense systems are impaired [3, 4]. On the other hand, ROS plays a significant role in the pathogenesis of many diseases, particularly in neurological diseases due to CNS vulnerability to oxidative stress [5]. While many extra- and intracellular molecules may be involved in neuronal injury and cell apoptosis, accumulation of oxidative stress due to excessive generation of ROS appears to be a possible cause of cell damage and death [6]. Programmed cell death is a gene-regulated phenomenon, among which Bax and Bcl-2 are the major proteins acting as apoptotic inducer and an inhibitor, respectively [7]. Documents indicate that lead exposure can cause a higher ratio of Bax/Bcl-2 protein expression, which promotes lead-induced neurotoxicity in the brain [8]. Hippocampus, the controller of cerebral activities and vital behaviors, gets affected by lead [9–11]. For this reason, we selected hippocampus as the target region in the present study. There is no doubt that neurotoxicity is one of the serious toxicological events, because damage of even a low number of neurons can be a menace to life. Therefore, coming up with an approach for the prevention of lead-induced brain damage is basically required. There are various antioxidant systems to scavenge ROS in the body, the majority of which is glutathione peroxidase; but, due to the low glutathione content, this system is inefficient in the brain [3]. In this study, cerium oxide (CeO_2) and yttrium oxide (Y_2O_3) nanoparticles were chosen because of their free radical-scavenging activity [12, 13]. CeO_2 is a nonstoichiometric compound in which the cerium atom is specified by both +4 and +3 oxidation states. This dual oxidation state means that CeO_2 nanoparticles have oxygen vacancies. Deprivation of oxygen and reduction of Ce^{4+} to Ce^{3+} are accompanied by emergence of an oxygen vacancy. The interesting redox chemistry exhibited by CeO_2 nanoparticles is due to this feature; thus, they resemble a free radical scavenger or an antioxidant [13]. Nanoparticles made up of other metal oxides such as Y_2O_3 were also considered for their potential scavenger behaviors. Y_2O_3 is notable because of its high free energy of oxide formation from elemental yttrium among known metal oxides [10]. According to evidences, we recently hypothesized that CeO_2 and Y_2O_3 nanoparticles, particularly their combination, cope against oxidative stress to limit the amount of ROS required for cell apoptosis [14, 15]. The purpose of the present study was to investigate the possible ameliorating and neuroprotective effects of CeO_2 and Y_2O_3 combination in acute lead-caused head injury, through measuring biochemical and molecular pathways of oxidative stress-mediated programmed cell death.

Materials and Methods

Animals

A total of 30 male Wistar albino rats (2–3 months old, weighing 200–250 g) were used and housed in individual stainless steel cages under controlled environmental conditions (25 °C and a 12-h light/dark cycle). The rats were handled daily and provided with food and water ad libitum. The protocol of this study was approved by an institutional review board, code 90-02-118-13956, and all the ethical concerns for the use and care of laboratory animals were carefully adhered.

Experimental Treatment Protocols

All the rats were randomly divided into five groups with six rats in each. The rats in the first group (control group) were injected with physiological saline [100 μL, intraperitoneal (IP)] for 5 days. In the second group (poisoned group), they received physiological saline (100 μL, IP) for 2 days and then were injected with lead acetate (25 mg/kg, IP) for 3 days [16]. Rats of the third group (Nan Cer Ox group) received CeO_2 nanoparticles (0.5 lethal dose$_{50}$ [LD_{50}]=1000 mg/kg, IP) for 2 days and then were injected with lead acetate (25 mg/kg, IP) for 3 days. Rats of the fourth group (Nan Ytt Ox group) received Y_2O_3 nanoparticles (0.5 LD_{50}=230 mg/kg, IP) for 2 days and then were injected with lead acetate (25 mg/kg, IP) for 3 days. In the fifth group (Nan Cer Ox + Nan Ytt Ox group), they received IP injections of the same doses of CeO_2 nanoparticles and Y_2O_3 nanoparticles for 2 days and then were injected with lead acetate (25 mg/kg, IP) for 3 days. Nanoparticle doses were optimized with various concentrations of CeO_2 (0.1, 0.3, and 0.5 LD_{50}) and Y_2O_3 (0.1, 0.3, and 0.5 LD_{50}) nanoparticles to achieve an effective dose (0.5 LD_{50}). During the experiments, animals had free access to the standard lab diet and were observed twice daily for any symptoms of severe lead poisoning such as seizure or incident of destruction.

Sample Preparation

Twenty-four hours after the final injection, the animals were completely anesthetized by inhalation of chloroform and blood samples were collected through cardiac puncture into heparinized tubes. The collected blood samples were centrifuged for 10 min at 1200*g* at 4 °C; then, the plasma was isolated and frozen at −80 °C until the next analysis. The brain

was rapidly removed after decapitation, and the hippocampus was dissected out and frozen in liquid nitrogen until used. Afterwards, the hippocampus was homogenized in sucrose medium (1 mM EDTA, 0.32 M sucrose) by a homogenizer (Hielscher UIS250V, Germany) and centrifuged at 1000*g* for 10 min; the supernatant was used for further testing.

Analysis of Lead Concentration in the Plasma and Hippocampus

For lead determination in the plasma and hippocampus, tissue samples of the hippocampus were dried in oven at 60 °C and combusted at 450 °C for 24 h. Thereafter, the combusted samples were dissolved in a hot solution of HNO_3 1 % and then the sample volumes were adjusted to 1 mL with HNO_3 1 %. After that, 10 μL of this mixture was analyzed using atomic absorption spectrophotometer (AA-680, Graphite Furnace GFA-4B, Shimadzu Corporation, Kyoto, Japan) with Zeeman background correction. The samples were examined at 283.3 nm. The principal concentration in each sample was estimated by analysis of the calibration curve standards with a concentration range of 50 ppb. The mean value of the two absorbance readings for each sample and the mean value of the three absorbance readings for the standards were used to calculate the lead concentration [17].

Measurement of Oxidative Stress Indexes in the Hippocampus

Reactive Oxygen Species

Intracellular ROS in the hippocampus was measured using 2′-7′-dichlorofluorescein diacetate (DCF-DA), a nonfluorescent cell-permeating compound which is converted into highly fluorescent DCF by cellular peroxides. The hippocampus, homogenized in sodium phosphate buffer, with pH adjusted to 7.4 using 140 mM KCl, was treated with DCF-DA (10 μM) at 37 °C for 30 min. ROS generation was measured every 6 min for 60 min using an ELISA F-2000 fluorescence spectrometer at the excitation wavelength of 488 nm and emission wavelength of 525 nm [18]. Values are obtained as a unit of fluorescence per milligram protein.

Lipid Peroxidation

Lipid peroxidation (LPO) was determined by the reaction of thiobarbituric acid (TBA) with malondialdehyde. Briefly, 200 μL of each sample was mixed with TCA (20 %) and the produced precipitate was dispersed in H_2SO_4 (0.05 M). After addition of TBA (0.2 % in sodium sulfate), the sample was heated in a boiling water bath for 30 min. Afterwards, the LPO adducts were extracted by *n*-butanol and the absorbance was measured by ELISA microplate reader (Synergy, BioTek Instruments Inc., Bad Friedrichshall, Germany) at 532 nm [19].

Total Thiol Molecules

Total thiol molecules were measured using DTNB as the reagent. Briefly, for each sample, 10 μL of the supernatant of tissue homogenate was mixed with 0.2 mL Tris-EDTA buffer (Tris base 0.25 M, ethylenediaminetetraacetic acid 20 mM, pH 8.2) and then mixed with 10 μL of 5.5′-dithiobis-2-nitrobenzoic acid (10 mM in pure methanol). After 15–20 min, the color appeared and absorbance of the supernatant was determined against a blank by ELISA microplate reader at 412 nm [20].

Measurement of Antioxidant Enzyme Activity in the Hippocampus

Superoxide Dismutase Activity

To determine the superoxide dismutase (SOD) activity, a Cayman assay kit (Cayman Chemical, Ann Arbor, USA) was used. According to the kit protocol, the superoxide radicals generated by xanthine and xanthine oxidase react with 2-(4-iodophenyl)-3-(4-nitrophenol) 5-phenyltetrazolium chloride (INT) and create a red formazan dye. The SOD activity was determined by the degree of inhibition of this reaction. A single unit of SOD causes 50 % inhibition of the INT reduction rate. The color reaction was evaluated at 505 nm by ELISA microplate reader. The results are shown as unit per milligram of protein.

Catalase Activity

Enzyme activity was determined by observing the initial rate of hydrogen peroxide (H_2O_2) disappearance in a spectrophotometer at 240 nm. Briefly, the reaction mixture (100 μL of supernatant and 10 μL of alcohol ethanol) was vortexed and kept in ice water bath for 10 min. Then, 10 μL of Triton X-100 was added and the mixture was vortexed in room temperature. Afterwards, 100 μL of phosphate buffer containing H_2O_2 0.66 M was added to the reaction mixture, and decrease of absorbance was observed at 240 nm against a blank using spectrophotometer (Cecil CE7250-7000 series, Milton Technical Centre, Cambridge, UK). The answers were reported as micromoles of H_2O_2 metabolized per milligram protein per minute [21].

Apoptosis Assay

Preparation of Tissue Extract and Western Blot Analysis To detect Bax, Bcl-2, and caspase-3 protein expression, the frozen hippocampus was homogenized in lysis buffer; the

supernatant was removed and conserved after centrifuge at 15,000*g*. The total protein was assessed using Bradford reagent at 595 nm. For immunoblotting, equal amounts of proteins (50 μg from each sample) were loaded into 15 % sodium dodecyl sulfate-polyacrylamide gel, and after electrophoresis, they were transferred to a polyvinylidene difluoride membrane. The blot was blocked for 1 h at room temperature with 2.5 % nonfat milk in phosphate-buffered saline (PBS) with 0.1 % Tween 20. Afterwards, the membrane was incubated with primary antibodies (rabbit polyclonal anti-Bax, 1:200; mouse monoclonal anti-Bcl2, 1:200; rabbit polyclonal anti-caspase 3, 1:1000) and a secondary antibody (anti-mouse IgG and anti-rabbit antibody conjugated with horseradish peroxidase, 1:2000). Bands were visualized using ECL detection system (Amersham Pharmacia Biotech), quantified by densitometric analysis (Total Lab Software, Wales, UK), and normalized against β-actin protein [11].

Analysis of ADP/ATP Ratio in the Hippocampus This assay was carried out using high-performance liquid chromatography (HPLC) according to a method that was previously set up in our laboratory [22]. In brief, the frozen hippocampus was homogenized in ice-cold 6 % perchloric acid and centrifuged at 12,000*g* for 15 min at 0.5 °C, and the supernatant was neutralized to a pH of 6–7; then, it was injected into HPLC. Detection was performed by the HPLC system (Waters Chromatography Division, Milford, MA, USA), consisting of Waters 510 pump and solvent delivery system, column (SUPELCOSIL™ LC-18-T) with guard column holder, and Waters 486 UV-Vis Detector. The protocol consisted of isocratic elution with TBAHS (4 mM) in potassium phosphate buffer (0.1 M; pH=5.5) and methanol (85:15 *v*/*v*). The flow rate was 1 mL/min for 20 min at 254 nm. The levels of ATP and ADP were quantified, after creating the standard curve, and the concentrations of ATP and ADP were expressed as micrograms per milliliter per milligram of tissue; the energy changes were reported as the ADP/ATP ratio.

Statistical Analysis Values were reported as mean±SE for the six rats in each group, and the significance of the differences between the mean values was determined using one-way ANOVA and Tukey's post hoc tests by StatsDirect version 2.7.8. The significance level was set at $P<0.05$.

Results

Lead Level in the Blood and Hippocampus

As shown in Table 1, lead administration to the rats produced high blood levels of lead with high concentration ranges. Blood lead levels were much higher in the lead-exposed rats than the controls ($P<0.001$). A significant penetration of lead from the blood to the brain was noticed, and the level of lead in the hippocampus of the exposed rats was much higher than that in the controls ($P<0.001$).

Oxidative Stress Indexes of the Hippocampus

Table 2 shows the pretreatment effects of the Nan Cer Ox and/or Nan Ytt Ox on oxidative stress indexes of the hippocampus in the lead-treated rats. A substantial increase in hippocampus ROS was noted in the Pb-treated rats compared with the controls ($P<0.001$), while pretreatments with a Nan Cer Ox and Nan Ytt Ox showed a significant recovery ($P<0.01$ and $P<0.05$, respectively). The best recovery occurred in ROS with the combination of Nan Cer Ox + Nan Ytt Ox ($P<0.001$).

Lead exposure induced a substantial growth in hippocampal thiobarbituric acid substances (TBARS) as compared with the controls ($P<0.01$). Pretreatments with Nan Cer Ox or the combination resulted in significant reversal of lead-induced increase in TBARS ($P<0.05$ and $P<0.01$, respectively).

Lead exposure induced a substantial reduction in hippocampal total thiol molecules (TTM) as compared with the controls ($P<0.01$). Administration of Nan Cer Ox or combination before lead exposure resulted in a substantial recovery of TTM ($P<0.05$ and $P<0.01$, respectively).

Antioxidant Enzyme Activity in the Hippocampus

Figures 1 and 2 show the results of lead exposure and pretreatments with Nan Cer Ox and/or Nan Ytt Ox on antioxidant enzymes in the hippocampus. A substantial increase in SOD and catalase (CAT) activities were noted in lead-exposed animals compared with the controls ($P<0.001$). The animals that received Nan Cer Ox or Nan Ytt Ox pretreatments showed significant protection against changes of SOD and CAT ($P<0.01$ and $P<0.05$, respectively, for both enzymes). On the other hand, combination pretreatment led to a more pronounced protection of these enzymes ($P<0.001$).

Table 1 The lead levels in blood and hippocampus of the control and lead-treated rats in the acute model of toxicity

Sample	Lead level	
	Control group	Lead-treated group
Blood, μg/dL	123.20±40.02	148.50±348.62[aaa]
Hippocampus, μg/g *w.w.*	24.48±3.58	187.16±22.91[aaa]

Data are expressed as the mean±SEM of three independent measurements performed on samples, derived from six animals in each group

[aaa] $P<0.001$, significantly different from the control group

Table 2 Effects of Nan Cer Ox and/or Nan Ytt Ox pretreatments on biochemical variables, indicative of oxidative stress in the hippocampus of lead-treated rats

	Con	Lead	Nan Cer Ox + lead	Nan Ytt Ox + lead	Nan Cer Ox + Nan Ytt Ox + lead
ROS (unit of fluorescence/mg protein)	62.00±6.00	110.00±8.00^{aaa}	72.00±5.00^{bb}	77.00±6.00^{b}	63.00±5.00^{bbb}
TBARS (μM/μg protein)	71.01±8.38	111.62±8.61^{aa}	76.90±8.53^{b}	90.97±7.76	71.73±7.01^{bb}
TTM (μM)	117.70±6.40	74.30±6.70^{aa}	102.90±7.30^{b}	82.40±6.10^{a,c}	111.40±6.70^{bb}

Values are presented as mean±SEM (n=6)

Con control, *Nan Cer Ox* nano cerium oxide, *Nan Ytt Ox* nano yttrium oxide, *ROS* reactive oxygen species, *TBARS* thiobarbituric acid substances, *TTM* total thiol molecules

^{aaa} Difference between the control and other groups is significant at $P<0.001$

^{aa} Difference between the control and other groups is significant at $P<0.01$

^{a} Difference between the control and other groups is significant at $P<0.05$

^{bbb} Difference between the lead-treated and other groups is significant at $P<0.001$

^{bb} Difference between the lead-treated and other groups is significant at $P<0.01$

^{b} Difference between the lead-treated and other groups is significant at $P<0.05$

Expressions of Hippocampal Bax, Bcl-2, and Caspase-3 Proteins

Western blot analysis of few apoptotic markers in the rat hippocampus is shown in Figs. 3, 4, and 5. In the lead-treated hippocampus, the expression of Bax (pro-apoptotic) was enhanced compared with the control, whereas Bcl-2 decreased in these conditions (Fig. 3). Therefore, the Bax/Bcl-2 relative expression ratio was found to be significantly upregulated after densitometric analysis in the lead group in comparison with the control ($P<0.001$) (Fig. 4). As indicated in Figs. 3 and 4, pretreatments with Nan Cer Ox and/or Nan Ytt Ox prevented the lead-caused increase of the Bax/Bcl-2 ratio ($P<0.001$). Interestingly, the best recovery was noted with the combination as compared with a Nan Cer Ox or Nan Ytt Ox alone ($P<0.05$ and $P<0.01$, respectively). In addition, a significant growth in expression of caspase-3 was observed following lead exposure, as compared with the control ($P<0.001$), which demonstrated significant recovery following pretreatments with a Nan Cer Ox or Nan Ytt Ox ($P<0.01$) as well as further recovery with the combination ($P<0.001$) (Figs. 3 and 5).

ADP/ATP Levels in the Hippocampus

Figure 6 shows the pretreatment effects of Nan Cer Ox, Nan Ytt Ox, and their combination on the ADP/ATP level in the rat hippocampus exposed to lead. Acute lead exposure resulted in a significant increase in hippocampal ADP/ATP level compared with the control group ($P<0.001$). Administration of Nan Cer Ox and/or Nan Ytt Ox resulted in a significant recovery in the hippocampal ADP/ATP level ($P<0.001$). However, the best recovery was observed with the

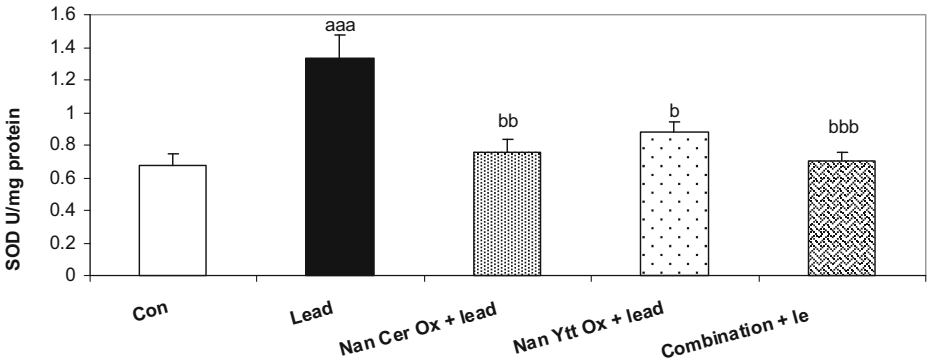

Fig. 1 Effects of Nan Cer Ox and/or Nan Ytt Ox pretreatments on SOD activity of the hippocampus on lead-treated rats. Values are presented as mean±SEM (n=6). The difference between the control and other groups is significant at $P<0.001$ (^{aaa}). The difference between the lead-treated and other groups is significant at $P<0.001$ (^{bbb}), $P<0.01$ (^{bb}), and $P<0.05$ (^{b}). *Con* control, *Nan Cer Ox* nano cerium oxide, *Nan Ytt Ox* nano yttrium oxide. Combination: nano cerium oxide + nano yttrium oxide

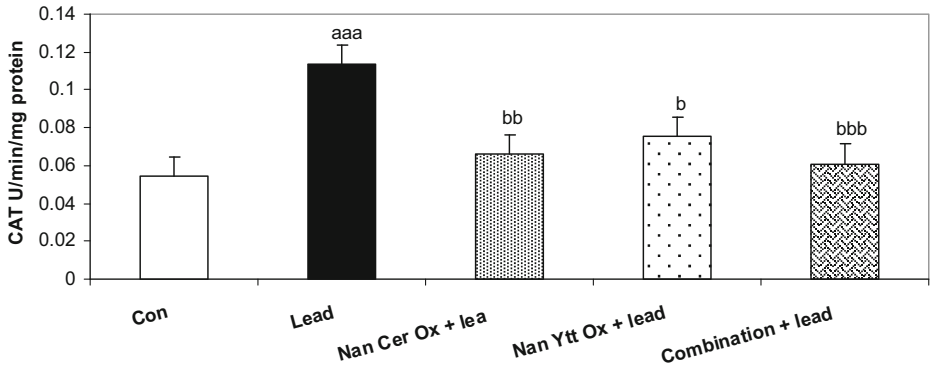

Fig. 2 Effects of Nan Cer Ox and/or Nan Ytt Ox pretreatments on CAT activity of the hippocampus on lead-treated rats. Values are presented as mean±SEM (n=6). The difference between the control and other groups is significant at P<0.001 (^{aaa}). The difference between the lead-treated and other groups is significant at P<0.001 (^{bbb}), P<0.01 (^{bb}), and P<0.05 (^{b}). *Con* control, *Nan Cer Ox* nano cerium oxide, *Nan Ytt Ox* nano yttrium oxide. Combination: nano cerium oxide + nano yttrium oxide

combination compared with Nan Cer Ox or Nan Ytt Ox alone (P<0.01 and P<0.001, respectively).

Discussion

Lead is a major public health problem that concerns all industrialized countries, and it may be dangerous for those who are occupationally or accidentally exposed. Among all the organs, the brain is the most sensitive to toxic effects of lead. Lead exposure in vivo caused many changes in petrochemical parameters, alterations in brain structure, and deficits in mental retardation, learning impairment, and behavioral abnormalities [23, 24]. There is no doubt that neurotoxicity is one of the serious toxicological events, because damage to even a small number of neurons can be a threat for the whole organism. Therefore, finding an approach for the prevention of lead-induced brain damage is essential.

Over the last decade, the hippocampus was of primary concern as a target for lead. Hippocampus is functionally related to intellectual activities and vital behaviors such as learning and memory that are inhibited by lead with different mechanisms. So, understanding the mechanisms of lead neurotoxicity can provide the basis for developing new therapeutic strategy aimed at preventing learning and memory abnormalities induced by lead poisoning [9, 11]. Oxidative stress and cell apoptosis are two mechanisms assumed for lead neurotoxicity.

Nowadays nanoparticles comprise a new generation of free radical scavengers such as CeO_2 and Y_2O_3 nanoparticles. The chemistry of CeO_2 and Y_2O_3 nanoparticles supports their potential usage as biological free radical scavengers, antioxidants, and anti-apoptotics.

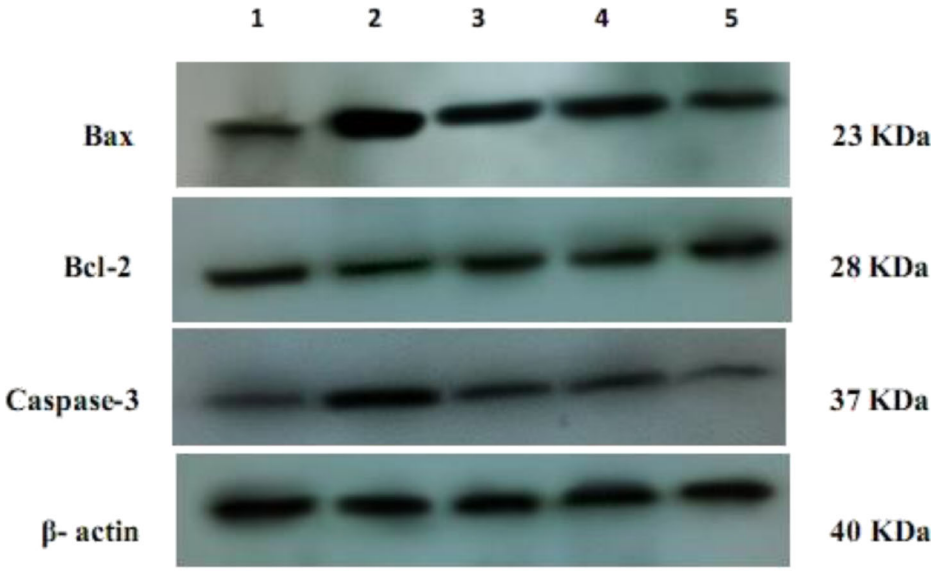

Fig. 3 Effects of Nan Cer Ox and/or Nan Ytt Ox pretreatments on Bax, Bcl-2 and Caspase-3 proteins expression of hippocampus in lead-treated rats (Western blot results) β-actin was used as an internal control. Control (*1*), lead (*2*), nano cerium oxide + lead (*3*), nano yttrium oxide + lead (*4*) and nano cerium oxide + nano yttrium oxide + lead (*5*)

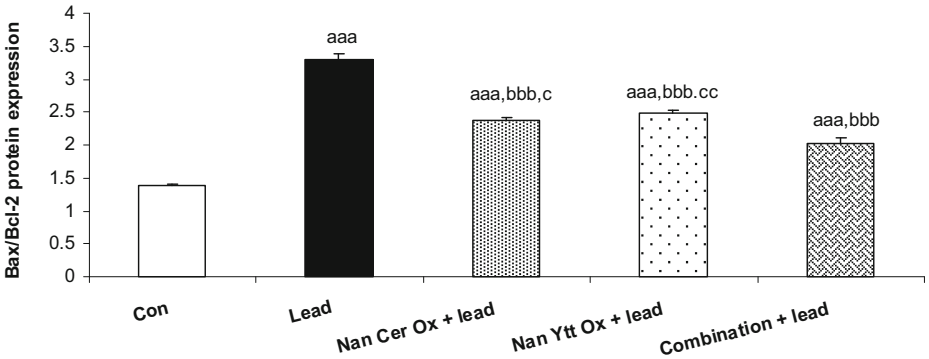

Fig. 4 Effects of Nan Cer Ox and/or Nan Ytt Ox pretreatments on Bax/Bcl-2 protein expression of hippocampus in lead-treated rats (quantitative expression intensity). Values are presented as mean±SEM (n=6). The difference between the control and other groups is significant at P<0.001 (^{aaa}). The difference between the lead and other groups is significant at P<0.001 (^{bbb}). The difference between the combination and other groups is significant at P<0.01 (^{cc}) and P<0.05 (^{c}). *Con* control, *Nan Cer Ox* nano cerium oxide, *Nan Ytt Ox* nano yttrium oxide. Combination: nano cerium oxide + nano yttrium oxide

In this study, we have examined the oxidative stress, apoptosis, and antioxidant system status in the hippocampus of rats exposed to acute levels of lead. The results showed a toxic effect of lead in the hippocampus through increase of oxidative stress and apoptosis as mechanisms of lead neurotoxicity. Likewise, we noticed improvements in these factors, using CeO_2 and/or Y_2O_3 nanoparticles and interestingly, the best recovery was noted by using the compound of these nanoparticles. Indeed, this research indicated that these nanoparticles may represent a novel therapeutic regenerative material which prevents the hippocampus damage caused by acute lead exposure. CeO_2 nanoparticles have more advantages than other antioxidants: first, they offer many active sites for free radical scavenging because of the mixed valence states for unique redox chemistry as well as their large surface/volume ratio [25]. Second, the free radical scavenging property of CeO_2 nanoparticles is regenerative, because these nanoparticles reconstitute their catalytic function by spontaneously moving between oxidation and reduction states [25]. Third, these nanoparticles are more likely to enter the cell because they are so small; for example, when administered systemically, they cross the blood-brain barrier, thereby allowing for treatment of neural damage or disease [26]. Recent studies about the effects of CeO_2 nanoparticles in both in vitro and in vivo models such as diabetic rats, adult rat spinal cord neurons, transgenic murine models of cardiomyopathy, radiation-induced damage in human colon cells (CRL 1541), radiation-induced pneumonitis, radiation-induced cellular damage, and retinal degeneration have supported the idea that these nanoparticles are capable of reducing harmful free radicals and thus protecting the cells against oxidative stress-induced damages [27–33]. Y_2O_3 is famous for its high free energy of oxide formation from elemental yttrium among known metal oxides. Since yttrium behaves similarly to lanthanide elements including cerium, there are similarities between the biological activities of Y_2O_3 and CeO_2 nanoparticles [13]. On the other side, the Ce^{3+}/Ce^{4+} cycles allow CeO_2 nanoparticles to react catalytically with hydrogen peroxide and superoxide, mimicking the action of the two key antioxidant enzymes CAT and SOD [25]. Since CAT and SOD

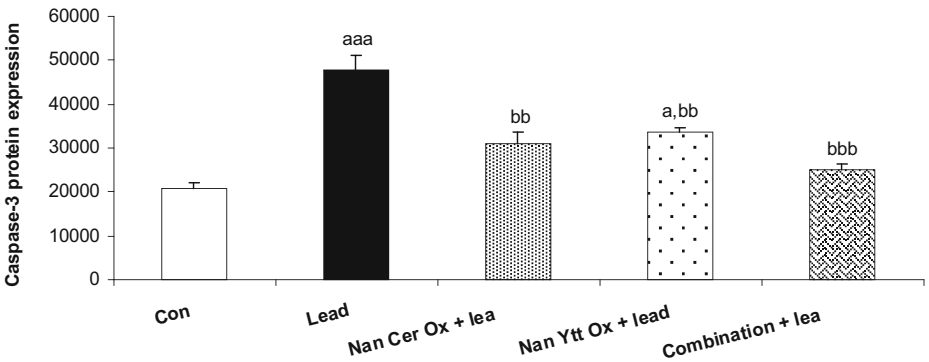

Fig. 5 Effects of Nan Cer Ox and/or Nan Ytt Ox pretreatments on Caspase-3 protein expression of hippocampus in lead-treated rats (quantitative expression intensity). Values are presented as mean±SEM (n=6). The difference between the control and other groups is significant at P<0.001 (^{aaa}) and P<0.05 (^{a}). The difference between the lead and other groups is significant at P<0.001 (^{bbb}) and P<0.01 (^{bb}). *Con* control, *Nan Cer Ox* nano cerium oxide, *Nan Ytt Ox* nano yttrium oxide. Combination: nano cerium oxide + nano yttrium oxide

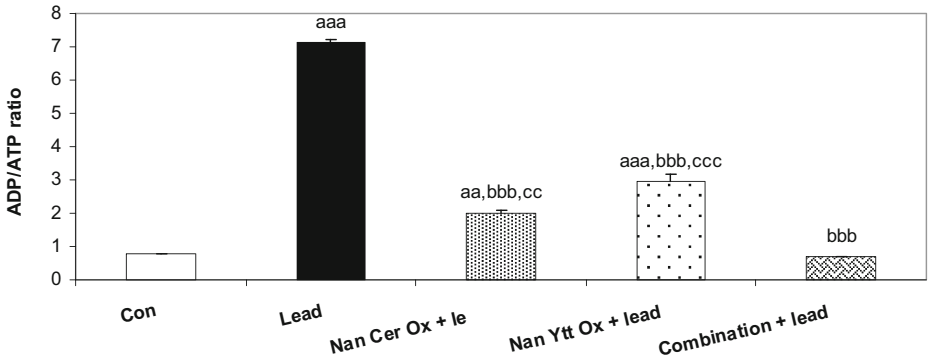

Fig. 6 Effects of Nan Cer Ox and/or Nan Ytt Ox pretreatments on ADP/ATP level of hippocampus in lead-treated rats. Values are presented as mean±SEM. The difference between the control and other groups is significant at $P<0.001$ (^{aaa}) and $P<0.01$ (^{aa}). The difference between the lead and other groups is significant at $P<0.001$ (^{bbb}). The difference between the combination and other groups is significant at $P<0.001$ (^{ccc}) and $P<0.01$ (^{cc}). *Con* control, *Nan Cer Ox* nano cerium oxide, *Nan Ytt Ox* nano yttrium oxide. Combination: nano cerium oxide + nano yttrium oxide

mimesis are performed by the reduction of Ce^{4+} and oxidation of Ce^{3+}, respectively, the coexistence of superoxide and H_2O_2 in cells renders CeO_2 nanoparticles a potential self-regenerating biological antioxidant [34]. SOD and CAT are important enzymes in the antioxidant system and their increase suggests the enhancement of the antioxidant potential of the brain to reduce oxidative stress. The increased activity of SOD and CAT can protect against oxidant impairment [35]. The previous works showed that CAT modulated oxidative stress-induced neural cell damage [36]. Our results indicated that the activities of SOD and CAT in the hippocampus of lead-exposed rats significantly increased. The previous studies reported a similar increase in SOD and CAT activities in rats under lead-induced oxidative stress [9, 35]. This increase is expectable as the body attempts to encounter with overwhelming oxidative stress through the output of more SOD and CAT in hippocampus [35]. Interestingly, the changes implemented in SOD and CAT due to lead treatment improved by administration of CeO_2 and/or Y_2O_3 nanoparticles. According to the above descriptions, this improvement by using nanoparticles due to their SOD- and CAT-like activities is not surprising.

Generally, CeO_2 and Y_2O_3 nanoparticles are relatively nontoxic and are able to rescue cells from oxidative stress-stimulated cell death. There are some alternative explanations which prove that the CeO_2 and Y_2O_3 combination protects cells from oxidative stress [13]. They act through blocking the production of ROS and inhibition of programmed cell death pathway. In addition, exposure of cells to particulate materials causes a form of preconditioning which induces low levels of ROS [37]. Therefore, the potency of this combination in improving lead neurotoxicity is not surprising. Two mechanisms have been suggested for lead neurotoxicity including oxidative stress [1, 8, 38] and cell apoptosis [10, 11, 39, 40], which are now supported by the present findings. Brain and hippocampal neurons in particular are more susceptible to oxidative stress due to brain high usage of inhaled oxygen, high rate of oxidative metabolic activity, and low level of antioxidant enzymes in hippocampal neurons [41, 42]. An array of cellular defense systems includes non-enzymatic and enzymatic antioxidants existing in the brain to counterbalance ROS. These systems cut down the concentration of free radical species and repair oxidative cellular damages [23]. Furthermore, in previous studies, interference of oxidative stress and apoptosis in hippocampal neuron damage was proved and the use of antioxidants for protection against these pathways was suggested [43]. In this study, the ROS level significantly increased in the hippocampus of lead-exposed rats. Surveys suggested that generation of high ROS levels after lead exposure might lead to depletion of intrinsic antioxidant defenses in cells [8]. Result showed that lead-induced ROS increase improved by using CeO_2 and/or Y_2O_3 nanoparticles due to antioxidant effect as well as ROS scavenging of these nanoparticles. The concentration of TBARS, which is a reflection of endogenous cell lipid peroxidation, was also more eminent in the hippocampus of lead-exposed rats in this subject area. Numerous surveys in the past have indicated that lead increases lipid peroxidation in the brain [44, 9], which confirms our results. As expected, CeO_2 and/or Y_2O_3 nanoparticles for their ROS scavenging effect could reduce lipid peroxidation in the hippocampus of lead-exposed rats. The potential of lead in irreversible complexion with free SH groups of antioxidant enzymes or proteins [21, 45, 46] also explains the reduction of TTM observed in the present study. The thiol redox system antioxidants display neuroprotective activities, but thiol groups are too susceptible to modification by free radicals [4]. Therefore, it seems that improvement

of TTM by CeO_2 and/or Y_2O_3 nanoparticles can be through ROS scavenging effect of these nanoparticles.

Additionally, the present study confirmed the induction of programmed cell death by lead as well as its prevention by both CeO_2 and Y_2O_3 and their compounding. Programmed cell death is a gene-regulated phenomenon happening via a number of anti- and pro-apoptotic genes which express homologous proteins of Bcl-2 and Bax. Construction of these proteins is known as a marker of cell apoptosis. Additionally, caspases are a family of proteins that execute terminal steps of apoptosis and regulate upstream induction of cell destruction. Among the caspases, caspase-3 plays an important role in mitochondrial dysfunction after the release of cytochrome C [21, 35, 45–47]. Previous studies indicated that lead-induced cytotoxicity in the hippocampus can be mediated through an increase of Bax/Bcl-2 ratio and caspase-3 activity [10, 41, 47], as confirmed by the present study too. Therefore, a higher level of Bax/ Bcl-2 protein expression can turn to an important index of apoptotic cell death, suggesting that lead-induced neurotoxicity may be due to facilitation of apoptosis, and improvement of this factor by CeO_2 and/or Y_2O_3 nanoparticles is indicative of anti-apoptotic effects of these nanoparticles [34, 48].

Disrupted energy metabolism is another mechanism caused by lead, resulting in behavioral abnormalities and brain dysfunction. Induction of oxidative stress by lead causes mitochondrial dysfunction which results in disruption of the respiratory chain as the source of ATP synthesis. This process results in depletion of ATP. In previous studies, lead reduced cellular ATP levels in rat brain synaptosomes [49]. In addition to oxidative stress and apoptosis, voltage-dependent anion channels (VDAC) show altered expression levels in the presence of lead. VDAC located on mitochondria plays a central role in regulating energy metabolism in neurons through ATP synthesis. Studies showed reduced expression of VDAC in lead toxicity has been associated with decreased ATP synthesis [50]. Our results indicated that acute lead exposure caused a substantial growth in the ADP/ATP ratio in the hippocampus of rats exposed to lead, whereas CeO_2 and/or Y_2O_3 nanoparticle pretreatments restored this change. In reinforcement of this determination, the potential of CeO_2 and/or Y_2O_3 nanoparticles in increasing the amount of ATP in Langerhans islets has recently been found [51]. It seems that increase of ATP by CeO_2 and/or Y_2O_3 nanoparticles can be through decrease of oxidative stress and thereby improvement of mitochondrial function and respiratory chain.

In summary, since oxidative stress and apoptosis are two potential mechanisms in lead-induced neurotoxicity, we require a strong antioxidant and anti-apoptotic mixture to protect the hippocampus. The present study showed the usefulness of CeO_2 and Y_2O_3 nanoparticles in this respect. The synergistic result of this combination is also noteworthy and can be the base of further researches.

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References

1. Wang J, Wu J, Zhang Z (2006) Oxidative stress in mouse brain exposed to lead. Ann Occup Hyg 50:405–409
2. Kermanian F, Mehdizadeh M, Nourmohammadi I (2010) Effects of vitamin C supplementation on lead-induced apoptosis in adult rat hippocampus. Neural Regener Res 5:364–367
3. Eren I, Naziroğlu M, Demirdaş A (2007) Protective effects of lamotrigine, aripiprazole and escitalopram on depression-induced oxidative stress in rat brain. Neurochem Res 32:1188–1195
4. Nazıroğlu M, Senol N, Ghazizadeh V, Yürüker V (2014) Neuroprotection induced by N-acetylcysteine and selenium against traumatic brain injury-induced apoptosis and calcium entry in hippocampus of rat. Cell Mol Neurobiol 34:895–903
5. Dilek M, Naziroğlu M, Baha Oral H et al (2010) Melatonin modulates hippocampus NMDA receptors, blood and brain oxidative stress levels in ovariectomized rats. J Membr Biol 233:135–142
6. Nazıroğlu M (2011) TRPM2 cation channels, oxidative stress and neurological diseases: where are we now? Neurochem Res 36:355–366
7. Cory S, Adams JM (2002) The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2:647–656
8. Lu X, Jin C, Yang J et al (2013) Prenatal and lactational lead exposure enhanced oxidative stress and altered apoptosis status in offspring rats' hippocampus. Biol Trace Elem Res 151:75–84
9. Bokara KK, Brown E, McCormick R, Yallapragada PR, Rajanna S, Bettaiya R (2008) Lead-induced increase in antioxidant enzymes and lipid peroxidation products in developing rat brain. Biometals 21:9–16
10. Sharifi AM, Baniasadi S, Jorjani M, Rahimi F, Bakhshayesh M (2002) Investigation of acute lead poisoning on apoptosis in rat hippocampus in vivo. Neurosci Lett 329:45–48
11. Sharifi AM, Mousavi SH, Jorjani M (2010) Effect of chronic lead exposure on pro-apoptotic Bax and anti-apoptotic Bcl-2 protein expression in rat hippocampus in vivo. Cell Mol Neurobiol 30:769–774
12. Chung D (2003) Nanoparticles have health benefits too. New Scientist 179:2410–2416
13. Schubert D, Dargusch R, Raitano J, Chan SW (2006) Cerium and yttrium oxide nanoparticles are neuroprotective. Biochem Biophys Res Commun 342:86–91
14. Hosseini A, Baeeri M, Rahimifar M et al (2013) Antiapoptotic effects of cerium oxide and yttrium oxide nanoparticles in isolated rat pancreatic islets. Hum ExpToxicol 32:544–553
15. Hosseini A, Abdollahi M (2012) Through a mechanism-based approach, nanoparticles of cerium and yttrium may improve the outcome of pancreatic islet isolation. J Med Hypotheses Ideas 6:4–6
16. Struzyñska L, Bubko I, Walski M, Rafałowska U (2001) Astroglial reaction during the early phase of acute lead toxicity in the adult rat brain. Toxicology 165:121–131

17. Abdel Moneim AE (2012) Flaxseed oil as a neuroprotective agent on lead acetate-induced monoamineric alterations and neurotoxicity in rats. Biol Trace Elem Res 148:363–370
18. Bernardi C, Tramontina AC, Nardin P et al. (2013) Treadmill exercise induces hippocampal astroglial alterations in rats. Neural Plast 2013:709732.Epub 2013 Jan 17
19. Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358
20. Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77
21. Flora SJ, Gautam P, Kushwaha P (2012) Lead and ethanol co-exposure lead to blood oxidative stress and subsequent neuronal apoptosis in rats. Alcohol Alcohol 47:92–101
22. Hosseini A, Sharifzadeh M, Rezayat SM et al (2010) Benefit of magnesium-25 carrying porphyrinfullerene nanoparticles in experimental diabetic neuropathy. Int J Nanomedicine 5:517–523
23. Struzyńska L (2000) The protective role of astroglia in the early period of experimental lead toxicity in the rat. Acta Neurobiol Exp (Wars) 60:167–173
24. Han JM, Chang BJ, Li TZ et al (2007) Protective effects of ascorbic acid against lead-induced apoptotic neurodegeneration in the developing rat hippocampus in vivo. Brain Res 1185:68–74
25. Korsvik C, Patil S, Seal S, Self WT (2007) Superoxide dismutase mimetic properties exhibited by vacancy engineered ceria nanoparticles. Chem Commun 10:1056–1058
26. Rzigalinski BA, Meehan K, Davis RM, Xu Y, Miles WC, Cohen CA (2006) Radical nanomedicine. Nanomedicine (Lond) 1:399–412
27. Pourkhalili N, Hosseini A, Nili-Ahmadabadi A et al (2011) Biochemical and cellular evidence of the benefit of a combination of cerium oxide nanoparticles and selenium to diabetic rats. World J Diabetes 2:204–210
28. Das M, Patil S, Bhargava N et al (2007) Auto-catalytic ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons. Biomaterials 28:1918–1925
29. Niu J, Azfer A, Rogers LM, Wang X, Kolattukudy PE (2007) Cardioprotective effects of cerium oxide nanoparticles in a transgenic murine model of cardiomyopathy. Cardiovasc Res 73:549–559
30. Colon J, Hsieh N, Ferguson A et al (2010) Cerium oxide nanoparticles protect gastrointestinal epithelium from radiation-induced damage by reduction of reactive oxygen species and upregulation of superoxide dismutase 2. Nanomedicine 6:698–705
31. Colon J, Herrera L, Smith J et al (2009) Protection from radiation-induced pneumonitis using cerium oxide nanoparticles. Nanomedicine 5:225–231
32. Tarnuzzer RW, Colon J, Patil S, Seal S (2005) Vacancy engineered ceria nanostructures for protection from radiation-induced cellular damage. Nano Lett 5:2573–2577
33. Chen J, Patil S, Seal S, McGinnis JF (2006) Rare earth nanoparticles prevent retinal degeneration induced by intracellular peroxides. Nat Nanotechnol 1:142–150
34. Celardo I, De Nicola M, Mandoli C, Pedersen JZ, Traversa E, Ghibelli L (2011) Ce^{3+} ions determine redox-dependent anti-apoptotic effect of cerium oxide nanoparticles. ACS Nano 5:4537–4549
35. She JQ, Wang M, Zhu DM et al (2009) Monosialoanglioside (GM1) prevents lead-induced neurotoxicity on long-term potentiation, SOD activity, MDA levels, and intracellular calcium levels of hippocampus in rats. Naunyn Schmiedebergs Arch Pharmacol 379:517–524
36. Nazıroğlu M (2012) Molecular role of catalase on oxidative stress-induced Ca(2+) signaling and TRP cation channel activation in nervous system. J Recept Signal Transduct Res 32:134–141
37. Becker S, Soukup JM, Gallagher JE (2002) Differential particulate air pollution induced oxidant stress in human granulocytes, monocytes and alveolar macrophages. Toxicol In Vitro 16:209–218
38. Soltaninejad K, Kebriaeezadeh A, Minaiee B et al (2003) Biochemical and ultrastructural evidences for toxicity of lead through free radicals in rat brain. Hum ExpToxicol 22:417–423
39. Dribben WH, Creeley CE, Farber N (2011) Low-level lead exposure triggers neuronal apoptosis in the developing mouse brain. Neurotoxicol Teratol 33:473–480
40. Kiran Kumar B, PrabhakaraRao Y, Noble T et al (2009) Lead-induced alteration of apoptotic proteins in different regions of adult rat brain. Toxicol Lett 184:56–60
41. Flora SJ, Saxena G, Mehta A (2007) Reversal of lead-induced neuronal apoptosis by chelation treatment in rats: role of reactive oxygen species and intracellular Ca(2+). J Pharmacol Exp Ther 322:108–116
42. Al-Majed AA (2011) Probucol attenuates oxidative stress, energy starvation, and nitric acid production following transient forebrain ischemia in the rat hippocampus. Oxid Med Cell Longev 2011:471590
43. Ghazizadeh V, Nazıroğlu M (2014) Electromagnetic radiation (Wi-Fi) and epilepsy induce calcium entry and apoptosis through activation of TRPV1 channel in hippocampus and dorsal root ganglion of rats. Metab Brain Dis 29:787–799
44. Bennet C, Bettaiya R, Rajanna S et al (2007) Region specific increase in the antioxidant enzymes and lipid peroxidation products in the brain of rats exposed to lead. Free Radic Res 41:267–273
45. Adewole SO, Ayoka AO (2009) Beneficial role of Quercetin on developmental brain of rats against oxidative stress-induced poisoning. Pharmacol Online 2:1171–1184
46. Bagchi D, Vuchetich PJ, Bagchi M et al (1997) Induction of oxidative stress by chronic administration of sodium dichromate [chromium VI] and cadmium chloride [cadmium II] to rats. Fre Radic Biol Med 22:471–478
47. Xu J, Ji LD, Xu LH (2006) Lead-induced apoptosis in PC 12 cells: involvement of p53, Bcl-2 family and caspase-3. Toxicol Lett 166:160–167
48. Clark A, Zhu A, Sun K, Petty HR (2011) Cerium oxide and platinum nanoparticles protect cells from oxidant-mediated apoptosis. J Nanopart Res 13:5547–5555
49. Rafałowska U, Struzyńska L, Dabrowska-Bouta B, Lenkiewicz A (1996) Is lead toxicosis a reflection of altered energy metabolism in brain synaptosomes? Acta Neurobiol Exp (Wars) 56:611–617
50. Prins JM, Park S, Lurie DI (2010) Decreased expression of the voltage-dependent anion channel in differentiated PC-12 and SH-SY5Y cells following low-level Pb exposure. Toxicol Sci 113:169–176
51. Pourkhalili N, Hosseini A, Nili-Ahmadabadi A (2012) Improvement of isolated rat pancreatic islets function by combination of cerium oxide nanoparticles/sodium selenite through reduction of oxidative stress. Toxicol Mech Methods 22:476–482