International Journal of Nanomedicine

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ORIGINAL RESEARCH

Antioxidant and toxicity studies of biosynthesized cerium oxide nanoparticles in rats

This article was published in the following Dove Medical Press journal:
International Journal of Nanomedicine

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Purpose: The purpose of this study was to investigate the acute toxic potential of cerium oxide nanoparticles (CNPs) synthesized by pullulan in adult male Wistar rats.

Patients and methods: Thirty male Wistar rats randomly were divided into five experimental groups of six animals each. The animals were received 50, 100, 200, and 400 mg/kg CNPs for 14 consecutive days. At the end of the experiment, the rats were euthanized and histopathological evaluation of the liver and renal tissues, as well ass, the markers of serum oxidative stress including thiobarbituric acid reactive substances, total sulfhydryl content, and antioxidant capacity (using ferric reducing/antioxidant power assay) were assessed. Hematological parameters and the activity of liver function enzymes were also measured.

Results: The results of this study showed that CNPs caused no significant changes in the activity of liver enzymes, hepatic and renal histopathology and hematological parameters, while significantly improved serum redox status.

Conclusion: Acute administration of pullulan-mediated CNPs is safe and possess antioxidant activity.

Keywords: green synthesis, metal oxide, sol-gel, toxicology, radical scavenger

Introduction

In the last few decades, nanotechnology has been employed throughout biomedical research studies.[1–3] Cerium oxide nanoparticles (CNPs), consisting of cerium atoms linked by oxygen atoms, have been used in commercial, industrial, and biological applications.[4–6] CNPs are considered as one of the most interesting nanomaterials due to their catalytic attributes and seem to be practical in therapy applications.[7,8] Furthermore, these nanoparticles are extensively employed for producing oxygen sensors,[9] automotive catalytic converters,[10] and so on. Pullulan, a unique natural material, is a water-soluble polysaccharide that could be used in various biomedical/bioengineering applications due to its non-toxic, non-immunogenic, non-mutagenic, and non-carcinogenic nature.[11,12] In our pervious study, we have prepared ceria nanoparticles by using pullulan as a capping agent that could affect the morphology of CNPs. In fact, the presence of hydroxyl groups in the backbone would provide a medium that would interact effectively with cationic cerium ions.[13] It was illustrated from the cytotoxicity results that CNPs have no toxicity against Neuro2A cells of <125 µg/mL.[14] The disease progression of many neurodegenerative conditions is strongly related to the oxidative damage that is caused by increasing the generation of reactive oxygen species (ROS).[15] Nowadays, the antioxidant properties of CNPs have been discovered to be throughout the $Ce3^{+}/Ce4^{+}$ redox reaction on the surface of nanoparticles,[4,16] which could be beneficial in biomedical applications and protect cells from damage due to radiation, oxidative stress, or inflammation.[17] Since CNPs are capable of stimulating

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http://dx.doi.org/10.2147/IJN.S194192

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the catalytic potency of superoxide dismutase (SOD),[18] it could be applied as a potent antioxidant. Redox properties of CNPs could also detoxify the existing free radicals for prolonged time intervals by maintaining its bioactivity within the tissues.[19] The main objective of this work was to evaluate the effects of biosynthesized CNPs on oxidative stress status and biochemical and hematological parameters, along with the histopathological alterations in adult male Wistar rats.

Materials and methods

CNP synthesis and its cytotoxicity

CNPs were prepared from a previously reported method.[14] Briefly, to prepare CNPs, $Ce(NO_3)_3{\cdot}6H_2O$ (10.1 g) was dissolved in double distilled water (ddH_2O, 25 mL) and stirred for 30 minutes. Then, dissolved pullulan (1.5 g in 50 mL ddH_2O) was added at 60°C with string for 8 hours. Finally, the obtained resin was calcined (rate: 3°C/min) at 500°C for 2 hours. The CNPs were characterized by FTIR (Figure S1), FESEM (Figure S2), and X-ray diffraction (XRD) (Figure S3). Cell viability of CNPs was determined by MTT assay against Neuro2A cells as described in the literature.[14] The detailed procedure is provided in the Supplementary materials. The absorbance of the amount of formazan formation was measured by a plate reader (statfax303) at λ_{max} =570 and 620 nm (Figure S4).

Animals and experimental procedure

We obtained male Wistar rats (weighing 200–230 g) from the Animal Center of School of Medicine, Mashhad University of Medical Sciences (MUMS), Mashhad, Iran. Then, they were provided with accommodations, which included housing and constant temperature (22°C±2°C), as well as the standard conditions of 12 hour light/dark cycle and free access to food pellets and tap water. The rats had been allowed to acclimatize for 1 week prior to the testing process. All the designated rats were handled in conformity with the National Institutes of Health Guidance for Care and Use of Laboratory Animals, while the Animal Ethics Committee of MUMS had authorized their usage for this particular project. The procedure was performed in five separate groups, each comprising six rats divided randomly. Animals in the experimental groups have been treated intraperitoneally with CNPs in order to obtain 50, 100, 200, and 400 mg/kg in 14 consecutive days. The negative control group has been injected with normal saline for 14 days.

Laboratory analyses

The anesthetizing process has been carried out by using ketamine and xylazine on the rats subsequent to the passage of the 14-day experiment in animal laboratory of MUMS, Faculty of Medicine. The blood samples were collected from the abdominal aorta and were accumulated and kept within sterile tubes with an anticoagulant (K3-EDTA) in order to investigate the hematological parameters. Non-anticoagulant was used in biochemical testing procedures such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), glucose, urea, creatinine (Cr), and direct and total bilirubin (Bill-D/T) by using the related commercial kits and a Biotecnica BT-3500 chemistry analyzer (Diamond Diagnostics, Holliston, MA, USA).[20] After blood serums were separated by centrifuge, they were analyzed by Biotechnica BT-3500 chemistry analyzer (Diamond Diagnostics; USA) using glucose, Cr, Bill-D/T, urea, AST, ALT, ALP and LDH reagents. The experimental assays were performed at a commercial laboratory (Laboratory of Modern Sciences and Technology Department at Mashhad University of Medical Sciences) using Man company kits in Iran under license of ELItech group, France. The detailed protocols could be obtained from the Man Company website (http://mancompany.com/en/; in biochemistry products). Routine hematological parameters including white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hb) concentration, hematocrits (HTC), mean corpuscular volume (MCV), platelet (PLT) count, and RBC distribution width (RDW) have been determined by the application of a blood cell counter (Sysmex KX-21N™; Sysmex, Hyogo, Japan). Serum was used for evaluating malondialdehyde (MDA), SOD activity, total sulfhydryl (SH) groups, catalase activity, and ferric reducing/antioxidant power (FRAP). The results were analyzed by one-way ANOVA using GraphPad Prism version 6.01 (GraphPad Software, Inc., La Jolla, CA, USA). Data were expressed as mean ± SEM, and values were considered statistically significant at $P<0.05$. The protocol was confirmed by the Animal Use and Care Advisory Committee of MUMS.

Histopathological examination

After collecting the blood samples, the rats were euthanized and the liver and kidney tissues were removed and fixed in 10% formalin. After mounting, a 5 μm section of each tissue was prepared and then stained with H&E. Histopathological changes were then investigated and blindly scored by a pathologist.[21,22]

Measurement of MDA

The levels of MDA, as the final product of lipid peroxidation process, were determined using thiobarbituric acid (TBA), as the reagent.[23,24] Briefly, 100 μL of serum was mixed with 2 mL of TCA-TBA-HCl reagent (15% trichloroacetic acid, 0.67% TBA, and 0.25 N HCl) and heated for 45 minutes in a boiling

water bath. After cooling, the mixture was centrifuged at 3,000 rpm for 10 minutes. The supernatant was collected, and the absorbance was read against the blank reagent at 532 nm. The amount of MDA produced was calculated in μmol/L.[23]

Determination of SOD activity

SOD activity in serum was evaluated using a spectrophotometric method.[25] Briefly, the sample was incubated in a solution containing Tris-HCl buffer (pH 8.2, 50 mM) and EDTA (1 mM). Reaction was initiated by the addition of 0.2 mM pyrogallol. Oxidation of pyrogallol was measured at 420 nm for 10 minutes, at intervals of 1 minute. The percentage inhibition of pyrogallol autoxidation was determined using the following equation:

$$\text{\% inhibition of pyrogallol autoxidation} = \left[1 - \left(\frac{\Delta A}{\Delta A_{max}}\right)\right] \times 100$$

where ΔA stands for absorbance change due to pyrogallol autoxidation in the sample reaction system, and ΔA_{max} represents the absorbance change that is induced by pyrogallol autoxidation in the control (without homogenate sample). A 50% inhibition was interpreted as one unit (U) of SOD activity.[25]

Assay of total SH groups

Total SH groups were measured using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB).[26] Briefly, 1 mL of Tris-EDTA buffer (pH 8.6) was added to 50 μL of serum and 50 μL of the sample. The sample absorbance was observed at 412 nm against Tris-EDTA buffer (A1). Afterward, 20 μL of DTNB reagent (10 mM in methanol) was added to the mixture and, after 15 minutes, the sample absorbance was read again (A2). Moreover, the absorbance of DTNB reagent was read as a blank (B). Total thiol concentration (mM) was determined using the following equation:

$$\text{Total thiol concentration (mM)} = (A2 - A1 - B) \times \frac{1.07}{0.05} \times 13.6$$

Assay of catalase activity

The decomposition of hydrogen peroxide (H_2O_2) by catalase can be measured directly by the decrease in H_2O_2 absorbance at 240 nm. The difference in absorbance per unit time is a measure of catalase activity. The results obtained for the sample containing 500 μL of serum and 500 μL of the substrate solution (10 mM H_2O_2 prepared in 50 mM phosphate buffer, pH 7.0) were compared with those of a blank containing 500 μL of phosphate buffer, instead of substrate solution, and 500 μL of sample. The reaction was initiated by adding the substrate solution and the solution was incubated at 20°C for 1 minute. Catalase activity was manifested as mmol H_2O_2/min/mg protein (U/L). An enzyme unit has been defined as the quantity of enzymes that can catalyze the release of 1 μmol of H_2O_2 per minute at the temperature of 20°C. Specific activity has been measured with reference to units per milligram of protein.[27]

Assay of FRAP

The serum antioxidant (reducing) power was measured using the FRAP assay.[26] Briefly, 50 μL of serum was added to 1.5 mL of freshly prepared and pre-warmed (37°C) FRAP reagent (300 mM acetate buffer, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ in the ratio of 10:1:1) in a test tube and incubated at 37°C for 8 minutes. The optical density of the blue colored complex was read against the blank reagent at 593 nm. The data were expressed as μmol of ferric ions reduced to ferrous form per liter (FRAP-value).[26]

Results

Effect of CNPs on hematological parameters

As it is illustrated in Table 1, there has been no significant changes in the WBC, RBC, HCT, Hb, PLT, MCV, and RDW indices in all of the experimental groups in comparison with the control group ($P>0.05$ for all).

Effect of CNPs on biochemical markers

According to the obtained data (Table 2), significant differences in serum BS, urea, Cr, Bill-D, and Bill-T levels, as well as the ALP, LDH, AST, and ALT activities were seen throughout the experimental groups compared to the control ($P>0.05$ for all).

Histopathological examinations

Liver

The liver tissue was investigated for the existence of any pathological changes such as inflammatory reactions, necrosis, apoptosis, and fibrosis in different liver zones, ports, and regional centrilobular space. Light microscopic evaluation of liver tissue in all the doses of CNPs did not display any notable variation in comparison with the control (Figure 1A, $P>0.05$).

Kidney

Structural changes in the renal glomerular tubules and vessels were also investigated. The analyses of the kidneys revealed

Table 1 Effect of CNPs on hematological parameters in male Wistar rats

Parameters	Control	CNPs 50	CNPs 100	CNPs 200	CNPs 400	*P*-value
WBC ($\times10^3$/μL)	5.30±0.41	6.32±0.43	6.84±0.36	7.06±0.27	7.00±0.41	NS
RBC ($\times10^6$/μL)	8.18±0.08	7.50±0.22	7.60±0.19	7.30±0.15	7.43±0.25	NS
HCT (%)	54.3±0.16	43.8±1.27	44.2±0.65	42.6±0.98	42.9±1.45	NS
Hb (g/dL)	14.7±0.06	14.2±0.29	14.3±0.21	13.8±0.27	13.8±0.51	NS
PLT ($\times10^3$/μL)	262.6±24.8	223.6±20.98	251±42.67	254.6±24.47	249.2±23.02	NS
MCV (fL)	53.5±0.22	56.2±2.29	57.2±0.35	58±0.38	57.42±0.34	NS
RDW (fL)	13.18±0.10	13.82±0.84	13.60±0.16	14.32±0.48	13.08±0.27	NS

Abbreviations: CNPs, cerium oxide nanoparticles; WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin concentration; HTC, hematocrits; MCV, mean corpuscular volume; PLT, platelet count; RDW, RBC distribution width; NS, not significant.

no significant pathological changes ($P>0.05$) between the CNP-treated group and the control group (Figure 1B).

Effect of CNPs on MDA level

Figure 2A shows that that CNP administration at doses of 50, 100, 200, and 400 mg/kg significantly ($P<0.001$ for all) decreased the MDA level to 1.5±0.02, 1.5±0.03, 1.5±0.03, and 1.5±0.02 μmol/L, as compared to control (1.9±0.02 μmol/L), respectively.

Effect of CNPs on SOD level

As illustrated in Figure 2B, administration of CNPs resulted in significant increase in SOD activity in CNP-treated rats (27.4±0.4 U/L at 50 mg/kg, $P<0.05$; 28.0±0.6 U/L at 100 mg/kg, $P<0.01$; 29.4±0.4 U/L at 200 mg/kg, $P<0.001$; and 29.8±0.6 U/L at 400 mg/kg, $P<0.001$), as compared to the control (24.7±0.5 U/L).[28]

Effect of CNPs on SH level

As shown in Figure 2C, administration of CNPs at doses of 50, 100, 200, and 400 mg/kg increased the total SH content to 15.6±0.3 mM ($P<0.05$), 15.6±0.8 mM ($P<0.05$), 16.0±0.9 mM ($P<0.01$) and 16.3±0.5 mM ($P<0.01$) respectively, in comparison with control (12.3±0.5 mM).

Effect of CNPs on CAT level

As shown in Figure 2D, the administration of CNPs at doses of 50, 100, 200, and 400 mg/kg has caused an increase in the CAT level toward 30.6±2.4, 33.1±0.5, 32.9±1.2, and 33.0±4.0 U/L, as compared to the control (23.0±0.7 U/L), respectively ($P<0.05$ for all).

Effect of CNPs on FRAP level

We have perceived a notable increase within the FRAP level following the administration of CNPs with doses of 50 (653.3±22.12), 100 (659.8±12.55), 200 (659.0±9), and 400 mg/kg (657.8±21.48) in comparison with the control (586.6±12), respectively ($P<0.05$ for all) (Figure 2E).

Discussion

Investigation of the effects of nanomaterials on cellular function is necessary to confirm the safety of utilization

Table 2 Biochemical serum profile of male Wistar rats after 14 days exposure to CNPs

Parameters	Control	CNPs 50	CNPs 100	CNPs 200	CNPs 400	*P*-value
Glucose (mg/dL)	85.2±3.5	89.4±5.8	78±5.5	80±5	92.8±7.4	NS
Urea (mg/dL)	38.8±1.50	39.2±1.65	40.8±1.8	38.20±1.35	40±1.22	NS
Cr (mg/dL)	0.68±0.026	0.72±0.02	0.7±0.03	0.7±0.028	0.7±0.022	NS
Bill-D (mg/dL)	0.036±0.005	0.034±0.005	0.034±0.005	0.30±0.003	0.032±0.004	NS
Bill-T (mg/dL)	0.25±0.02	0.22±0.03	0.24±0.024	0.21±0.018	0.22±0.023	NS
ALP (IU/L)	77.4±6.30	81.8±9.2	83.4±9.68	71.6±6.20	80.2±6.12	NS
LDH (IU/L)	81.2±8.96	84.6±7.1	80.8±10.20	71.6±6.20	88.8±7.99	NS
AST (IU/L)	62.6±2.62	61.8±3.9	59.2±5.94	55.6±4.67	63.2±4.88	NS
ALT (IU/L)	26.4±1.03	27.8±1.2	22.8±1.77	26.2±1.53	24.6±1.54	NS

Note: All values are expressed as mean ± standard error of the mean.

Abbreviations: CNPs, cerium oxide nanoparticles; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; Cr, creatinine; Bill-D/T, direct and total bilirubin; NS, not significant.

of these materials in medical applications.[10] Biochemical parameters have a significant role in determining toxicity because changes in their levels are considered as one of the best general indicator of the toxicity.[29–31] Moreover, these particular biochemical tests can be easily carried out in most of the diagnostic laboratories.[32] On the other hand, CNPs were known as antioxidants because of their dual redox states on the surface.[33–35] Antioxidants could be harmful per se, because of pro-oxidant activity.[36–38] For example, Srinivas et al suggested that acute exposure of CNPs through inhalation route may induce cytotoxicity via oxidative stress and may lead to a chronic inflammatory response.[39] Adebayo et al also showed that administration of 100–300 mg/kg CNPs thrice in a week for 5 consecutive weeks to mice induces testicular dysfunction via disruption of antioxidant/oxidant balance and endocrine suppression.[40]

In compliance with our previous studies, CNPs were characterized by FTIR, FESEM, and XRD. The results of characterization were in good agreement with our previous report.[14] Nanoparticles can be potentially hazardous to the central nervous system because they can readily bypass or cross the blood–brain barrier.[41–43] One of the limitations of our study is the lack of brain pathology data following CNP administration to rat. Therefore, Neuro2A cells (as neuroblastoma) was

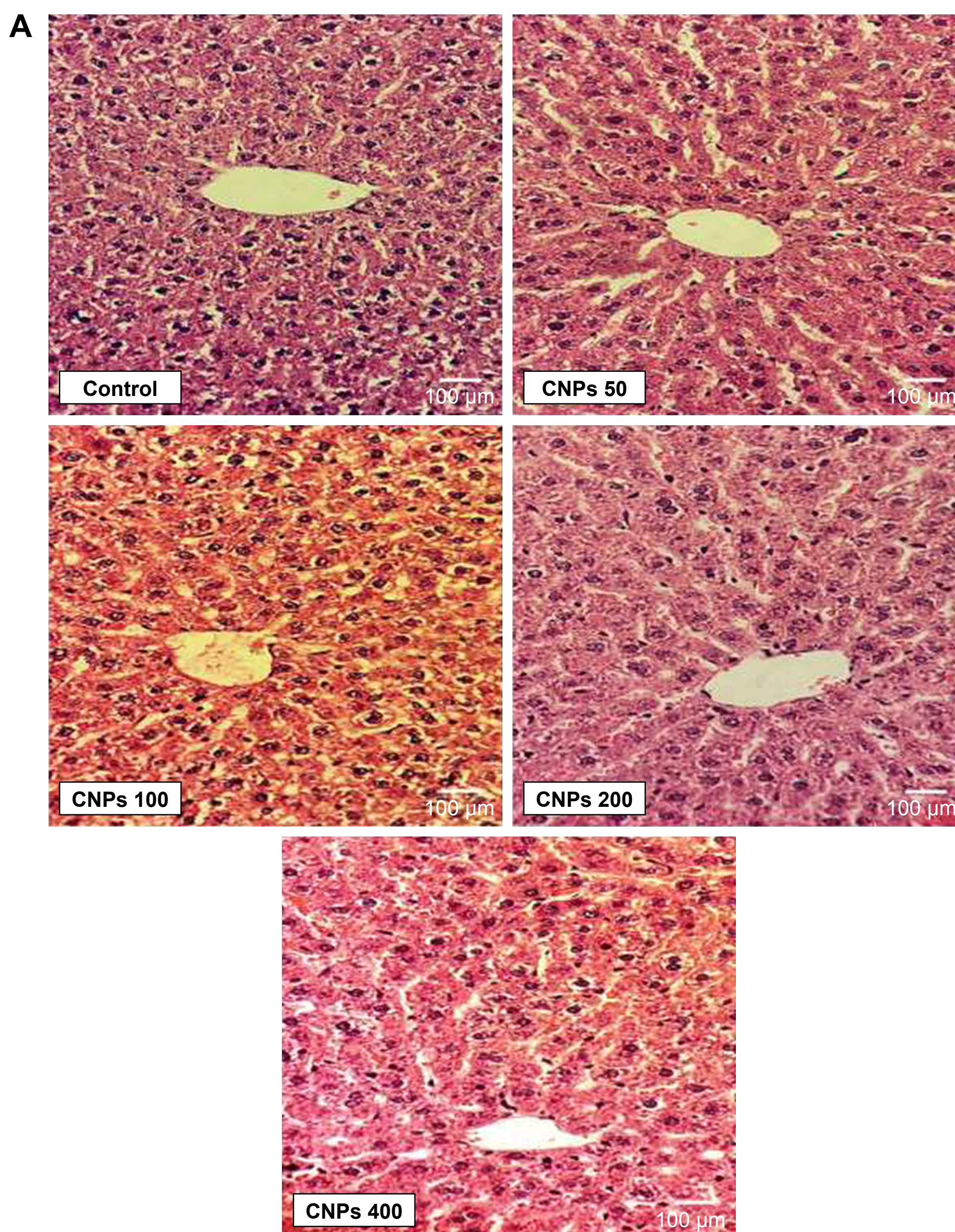

Figure 1 (*Continued*)

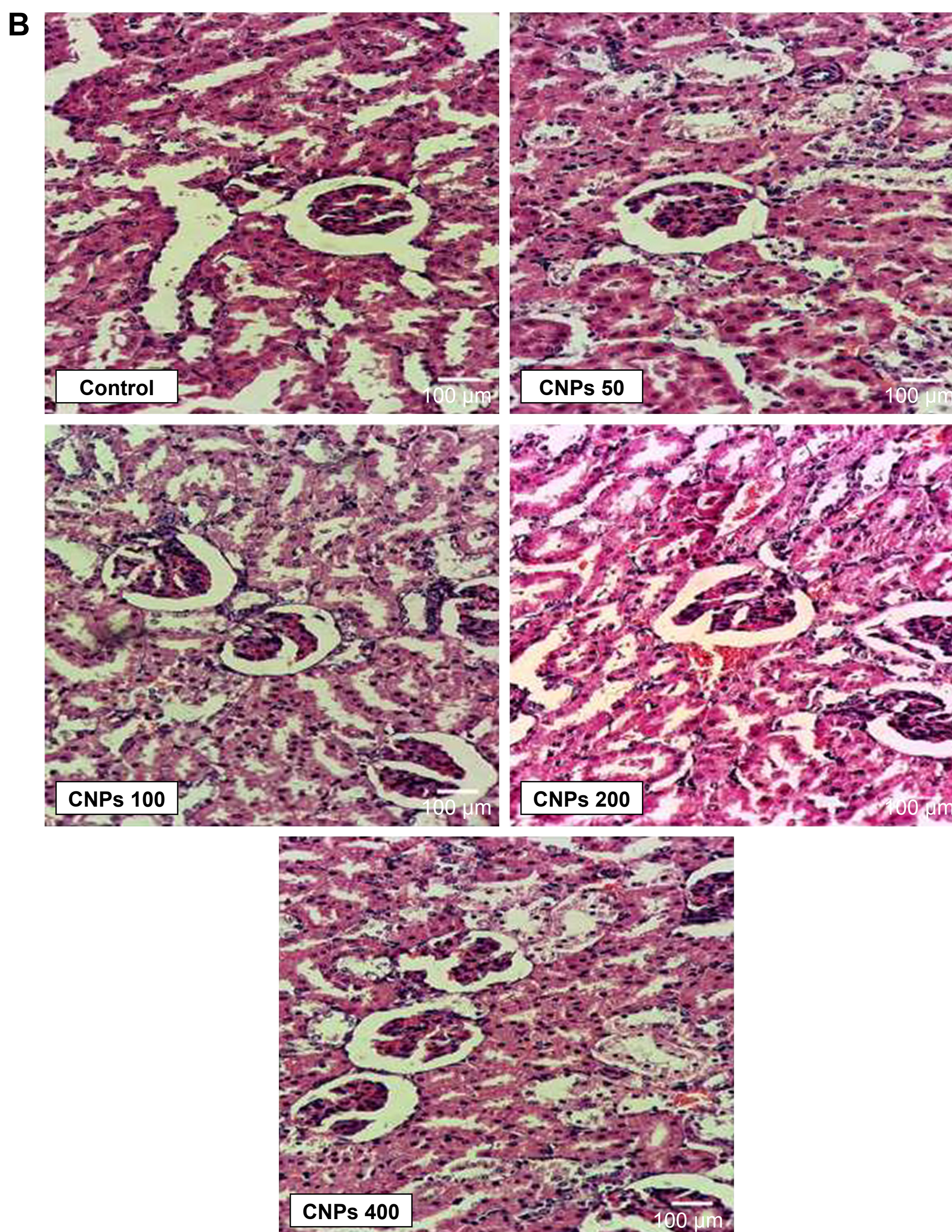

Figure 1 (**A**, **B**) Histopathological findings in liver and kidney of male Wistar rats after treatment with CNPs for 14 days (H&E, 400×).
Abbreviation: CNPs, cerium oxide nanoparticles.

used for the evaluation of possible toxicity of CNPs, which showed low dose-toxicity under 500 µg/mL. Intraperitoneal administration is a commonly used technique to administer large quantity of medicine in small animals. Although it is parenteral route, but unlike intravenous injection, the drug delivery is much slower.[44] Therefore, intravenous injection usually uses lower doses of nanoparticles that is usually under 5 mg/kg for CNPs,[45–47] but in intraperitoneal injection, it is safer if higher doses were used.[48] Herein, we want to show that CNPs are safe following acute administration. To evaluate the toxicity of the substances in vivo, CNPs were administered to rat and then some toxicological parameters such as histopathological parameters were evaluated and hematotoxicity analyses were performed. CNPs did not cause any vital alteration throughout the hematological and biochemical parameters of experimental groups relative to negative controls. In addition, histopathological examination of liver and kidney tissues, which were performed by using light microscopy technique, has not pointed out any notable pathological changes. This seems to be in parallel with the obtained normal results from biochemical parameters and partly confirms the safety of CNPs. In support of

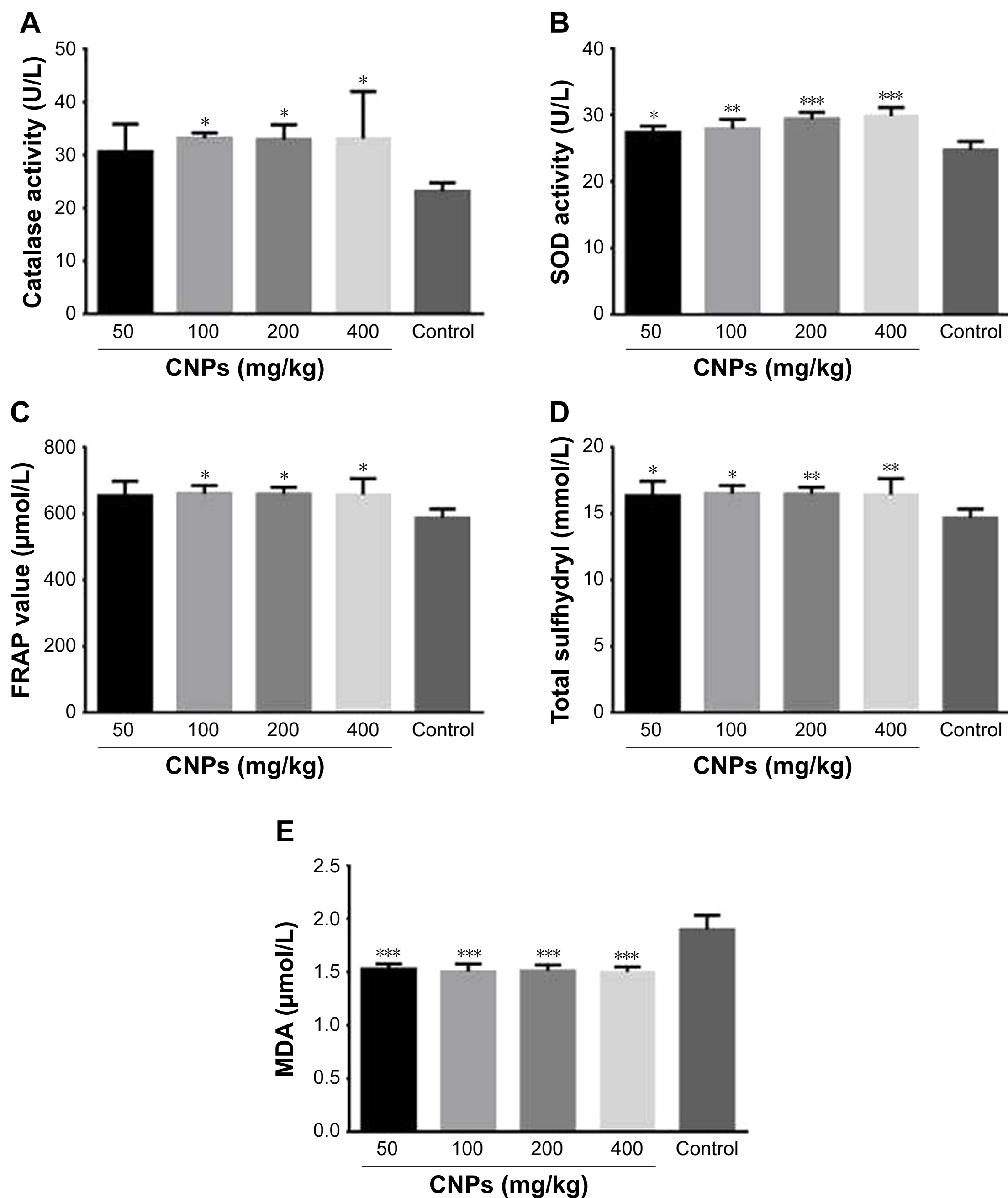

Figure 2 Effects of CNPs on serum oxidant/antioxidant status of male Wistar rats after treatment with CNPs for 14 days. (**A**) Effect of CNPs on MDA level. (**B**) Effect of CNPs on SOD activity. (**C**) Effect of CNPs on total SH level. (**D**) Effect of CNPs on CAT activity. (**E**) Effects of CNPs on FRAP values are expressed as mean ± SEM (n=7). *$P<0.05$, **$P<0.01$, ***$P<0.0001$ as compared with the control group.

Abbreviations: CNPs, cerium oxide nanoparticles; MDA, malondialdehyde; SOD, superoxide dismutase; SH, sulfhydryl; CAT, catalase; FRAP, ferric reducing/antioxidant power.

our results, Srinivas et al also reported that CNPs did not cause any harmful effects on liver and kidney functions or hematology or blood biochemistry of rats exposed to CNPs at 14-day post exposure period.[39] Furthermore, the measures of serum oxidative/anti-oxidative indices including MDA, SOD activity, SH groups, and the catalase (CAT) indices among all experimental doses in treated rats have a significant change in comparison with the control group, which have indicated that they can considerably effect on the potency of antioxidant. Antioxidant effects of CNPs may not be very obvious in healthy non-injured animals (ie, physiological conditions), but it has been reported that protective antioxidant effects are found under pathological conditions.[49] On the other hand, according to the FRAP test on serums obtained from rats that had received CNPs, a stronger antioxidant activities in contrast to the control group was observed. These data are in

agreement with other studies indicating the ability of CNPs to bind to oxygen under reducing and oxidizing circumstances, which plays a key role in neutralizing a variety of ROS.[50,51] CNPs have been so far applied for ameliorating the signs of several oxidative stress-based animal model's diseases, including Alzheimer's, cardiomyopathy, and cancers.[10,52,53] Indeed, the balance between the oxidant production and antioxidant content is pivotal for a correct cell function.[54] Therefore, anti-oxidant agents might have toxic or non-toxic effects. In general, CNPs exhibited protective effects against oxidative stress, in vitro and in vivo.[55,56] The current research was carried out to exactly evaluate the safety/toxicity of CNP (synthesized with pullulan) administration, as an anti-oxidant agent. The scientific databases revealed that there are several studies about the effects of CNPs on different types of cells. For instance, a study performed on the rat retina cells showed that nanoceria was found to be non-toxic.[57] Similarly, the results of another study stated the non-toxicity of 2,000 mg/kg body weight per day of CNPs that was administrated orally.[58] In contrast, cerium oxide was toxic to bronchial epithelial lung fibroblasts in culture,[59] but non-toxic to mammary epithelial cells,[60] macrophages,[61] immortalized keratinocytes,[62] or immortalized pancreatic epithelial cells.[56] They are not toxic in normal cells, while the physiological pH is suitable for enabling canonical radical scavenging by CNPs.[63,64]

Conclusion

Based on the findings demonstrated here, it was found that pullulan-mediated CNPs is safe following acute administration. In this work the safety and antioxidant ability of CNPs have been studied by evaluating several markers of serum oxidative stress, hematological parameters, and some liver enzymes. It could be concluded that CNPs have no damage to liver enzymes, liver, and renal tissues. CNPs seem safe to be used for medical applications and have a protective behavior against the oxidative stress. Of course, further studies, including more doses, are required to prove its safety in subacute/chronic situations. At the same time, further research studies should also be performed on pullulan-mediated CNPs to further develop its potential as an effective antioxidant for the management of illnesses caused by uncontrolled oxidative stress.

Acknowledgment

We would like to thank the Vice Chancellery for Research and Technology, MUMS for financial support (grant no 930954) and facilities.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

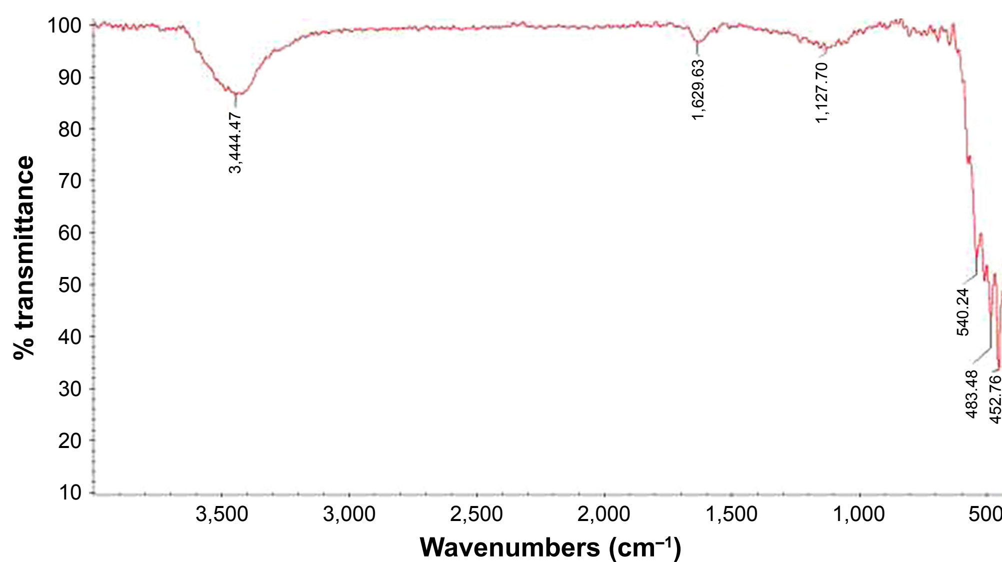

Figure S1 FTIR of CNPs.
Abbreviation: CNP, cerium oxide nanoparticle.

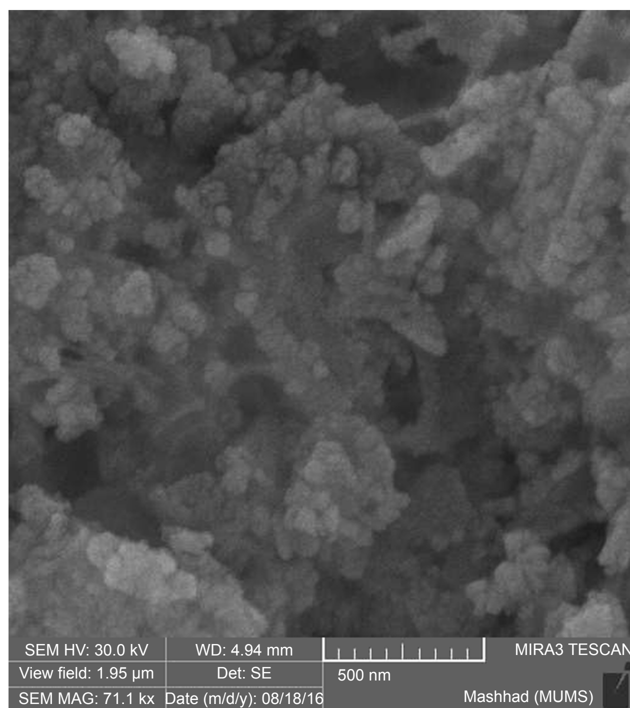

Figure S2 FESEM image of CNPs.
Abbreviation: CNP, cerium oxide nanoparticle.

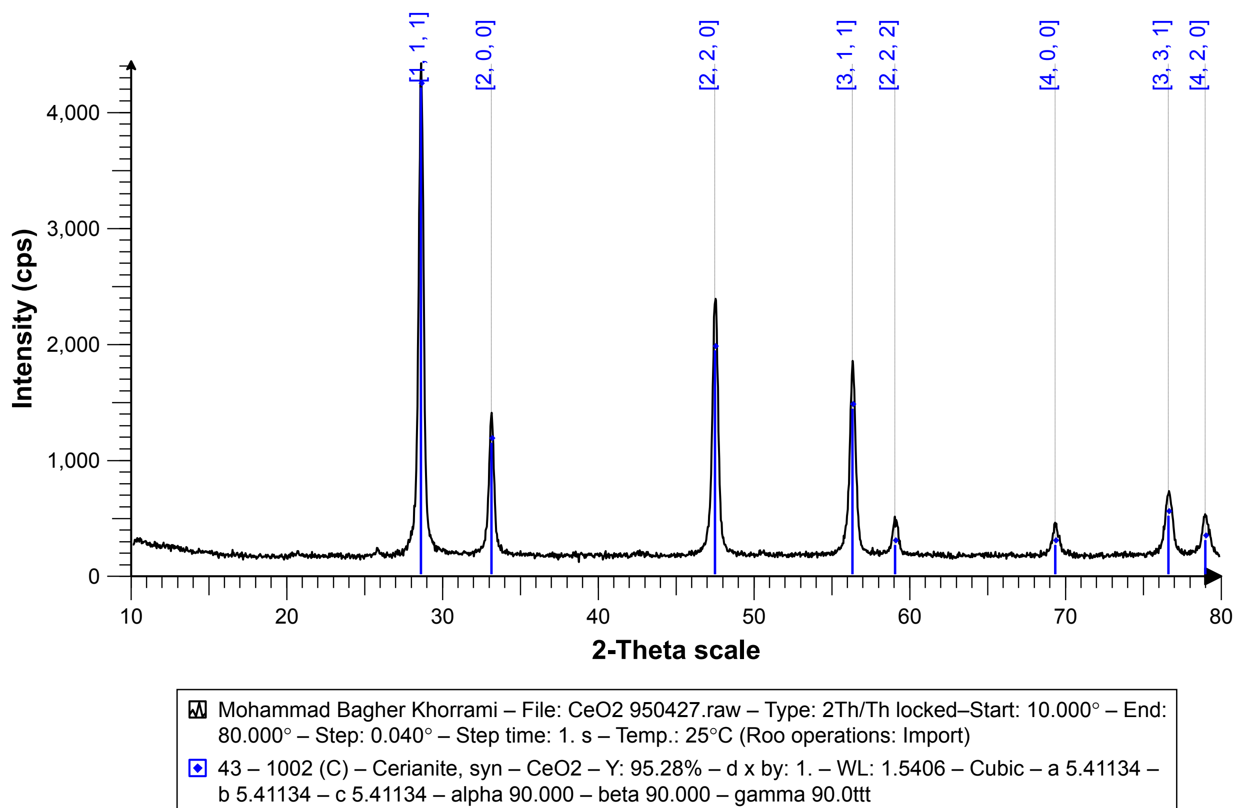

Figure S3 XRD pattern of CNPs.
Abbreviations: CNP, cerium oxide nanoparticle; XRD, X-ray diffraction.

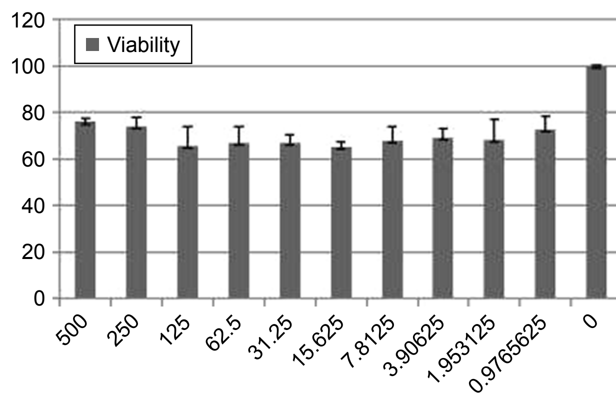

Figure S4 MTT assay of CNPs.
Abbreviation: CNP, cerium oxide nanoparticle.

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