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Anti-apoptotic and Antioxidant Effect of Cerium Oxide Nanoparticles on Cyclophosphamide-Induced Hepatotoxicity

ORIGINAL ARTICLE

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ABSTRACT

Objective: Cyclophosphamide (CP), as an anticancer agent has side effects, especially hepatotoxicity. Cerium oxide nanoparticles [nanoceria (NC)], which act as antioxidants, have a protective effect against oxidative stress-induced toxicity. This study evaluated the preventive effect of NC in hepatotoxicity induced by CP.

Materials and Methods: Thirty-two mice were randomly distributed into four groups: control (received only normal saline), NC (received NC 100 µg/kg for 3 days, intraperitoneally), CP (received CP 200 mg/kg single dose on the third day of the study, intraperitoneally), and NC+CP (NC 100 µg/kg and CP 200 mg/kg). Two days after the final treatment, oxidative stress marker (MDA and GSH) and alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels were measured, and histopathological and immunohistochemical examinations were performed for determining the effects of NC on hepatotoxicity.

Results: CP-induced hepatotoxicity was evident by the significant elevation of liver enzyme levels and alteration in oxidative stress marker levels. Additionally, histopathological changes and apoptosis were markedly increased. NC decreased MDA level, increased GSH level and decreased liver enzyme levels in CP-treated mice. In addition, NC pretreatment could be alleviated by the immunoreactivity of caspase-3 in CP-treated mice compared with the CP group.

Conclusion: NC showed a potent antioxidant effect in hepatotoxicity induced by CP. The study suggested that NC through its antioxidant and anti-apoptotic properties has a protective effect against hepatotoxicity induced by CP.

Keywords: Cyclophosphamide, nanoceria, hepatotoxicity, oxidative stress, caspase-3

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INTRODUCTION

Cyclophosphamide (CP), as an alkylating mediator for chemotherapy and immunosuppressor, often has deleterious side effects, especially hepatotoxicity (1). Phosphor amide mustard and acrolein are two major metabolites of CP that are produced in the liver by microsomal cytochrome P450 (2). These metabolites with alkylation of nucleophilic sites in DNA, RNA, and protein cause cellular toxicity and genotoxicity (3). CP, with the generation of free radicals and induction of oxidative stress, results in organ damage (2), such as hepatotoxicity (4). In CP therapy, various liver injury enzymes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and *alkaline phosphatase* (ALP) , and Lactate dehydrogenase (LDH) are released from the hepatocytes into blood circulation (4). However, despite the severe toxicity of CP in experimental animal and human studies (5, 6), clinicians have been prescribing it as the first-line treatment for patients with cancer. Several studies have confirmed that exogenous antioxidants with pre, post, or co-treatment could attenuate the side effects induced by CP and other chemotherapy drugs (7-9).

Due to the widespread use of cerium oxide nanoparticles [Ceo2 NPs; nanoceria (NC)] in the industry and their application in medicine and biology, recently, researchers have been interested in evaluating their antioxidant properties (10). NC with free radical scavenging (11) has a protective effect against oxidative stress. This element along with a reduction in the accumulation of reactive oxygen species (ROS) inhibits apoptosis due to antioxidant effects (12). NC, as one of the most important classes of nanomaterial and with the redox capability, have antioxidant properties that scavenge free radicals (13). The protective effect of NC in *in vitro* and *in vivo* studies has been demonstrated (13, 14). However, this effect on hepatotoxicity induced by CP has been not established. The present study mainly aimed to determine the protective effect of NC on hepatotoxicity induced by CP in a mice model. The extent of the protective effect of NC was determined by assessing oxidative stress marker levels, liver injury indexes (ALT, AST, and ALP), and histopathological and immunohistochemical assays in mice.

MATERIALS and METHODS

Animals and Materials

Thirty-two male BALB/c mice weighing 25-30 g were prepared by the Institutional Animal Care, and their use was approved by the Ethics Committee of the Medical science of Mazandaran University, Sari, Iran (ID: IR.MAZUMS.REC.1395.S222). For adapting to the experiment environment, the animals were housed in a 12-h light and dark cycle at 20°C-25 °C for 1 week. They had free access to food and water throughout the study period. CeO_2 NPs were obtained from Neutrino Co. (Tehran, Iran). CP was purchased from Baxter Company (Unterschleißheim, Germany).

Study Design

In this study, the animals were randomly divided into four groups (8 mice/group): control group (mice received normal saline; same volume as in other groups); NC group [mice received NC at a dose of 100 µg/kg intraperitoneally (IP) for 3 consecutive days]; CP group (mice received 200 mg/kg of CP by IP on the third day); and NC+CP group (mice received NC and CP at the same dose as that in the NC and CP groups;

The dose of NC was selected according to the pilot study, and that of CP was selected according to previous study (15). NC was suspended in distilled water and CP was dissolved in normal saline. After two days, serum biochemical, histochemical, histopathological, and immunohistochemical assays were evaluated.

Specimen Collection

Mice were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) 2 days after drug administration. Blood samples were collected from the heart. Serum was separated from the coagulated blood by centrifugation at 3000 ×g for 15 min and stored at −20°C for evaluating serum liver enzyme levels. Then, animals were sacrificed, and the liver was immediately removed. It was divided into two pieces, washed with cold phosphate buffer saline (PBS), and weighed. For biochemical evaluation, one of the two pieces was freshly used. The other pieces were fixed with buffered formalin (10%) for histopathological and immunohistochemical assessment.

Measurement of Malondialdehyde (MDA)

MDA as a lipid peroxidation (LPO) marker was measured according to the previous standard method. The liver sample was homogenized, and 0.1 mL of this sample, 1 mL of 0.6% 2-thiobarbituric acid, 3 mL of 1% phosphoric acid, and 0.1 mL of distilled water were mixed. After 45 min of boiling in a water bath, the mixture was cooled, and then n-butanol (4 mL) was added to extract the cold thiobarbituric acid reactants. Next, the samples were centrifuged for separating the butanol layer. The optical density of the N-butanol layer was determined by spectrophotometry. MDA concentration was expressed as µg/mg protein.

Measurement of Glutathione (GSH) Content

Glutathione level in the liver was determined with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as an index at 412 nm by spectrophotometer (UV-1601 PC, Shimadzu, Japan) and expressed as µM.

Serum Biochemical Assay

The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and and alkaline phosphatase (ALP) were determined by a Chemistry Analyzer (*Tokyo Boeki Prestige 24i)* using the quantitative detection kit Pars Azmoon (Iran) for AST (Cat. No. 1 400 018), ALT (Cat. No. 1 400 019), and ALP (Targa BT 3000, Cat. No. 12201).

Histopathological Assay

For the histopathological assay and to determine the effect of CP and NC on the liver, samples were fixed in 10% (w/v) buffered formalin for 24 h. After processing and embedding in paraffin using a standard protocol, sections with 5-µm thickness were stained with hematoxylin and eosin (H & E) for the evaluation of liver damage. Sample sections were evaluated using 40× magnification for the assessment of the extent of liver injury by a histologist who

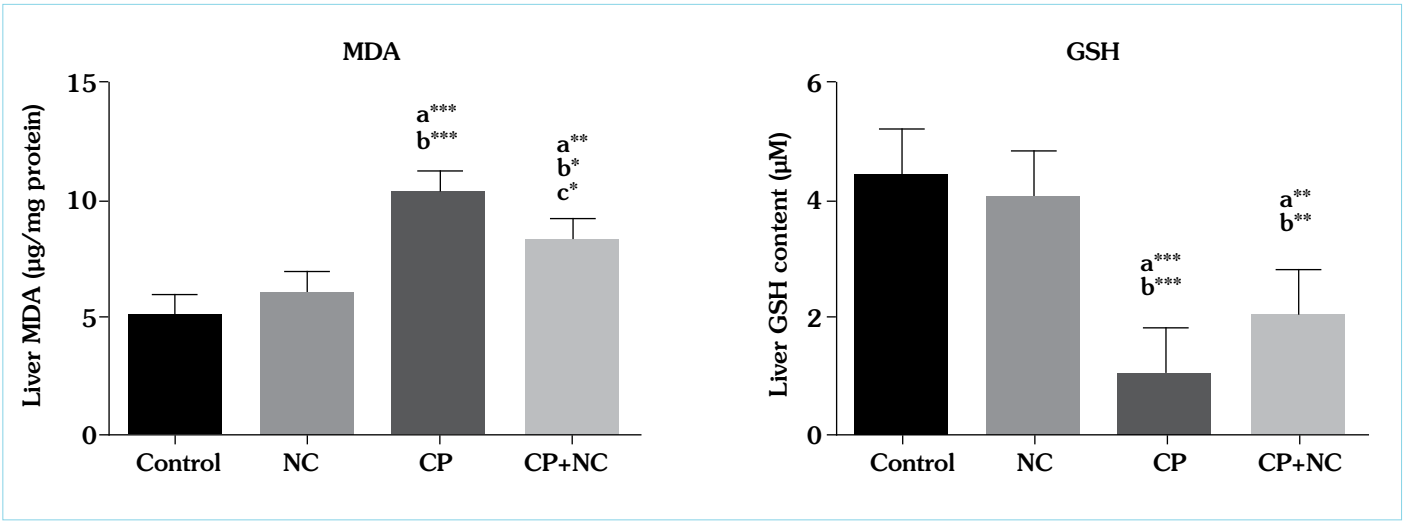

Figure 1. Effect of NC on CP-induced liver damage assessed using MDA and GSH levels. MDA levels increased and GSH levels decreased in CP-treated mice compared with the control group ($p<0.05$). Treatment with NC in CP-treated mice significantly decreased MDA levels and increased GSH levels in the liver tissue ($p<0.05$) compared with the CP group. All the values are expressed as the mean±SD. a, significant vs control; b, significant vs NC; c vs CP groups; *, $p<0.05$; **, $p<0.01$, and ***, $p<0.001$. CP; cyclophosphamide, NC; nanoceria.

was blinded to the treatment groups. For semiquantitative analysis, histological micrographs were assessed by a scoring system. According to the severity, sinusoidal dilatation, inflammatory cell infiltration, congestion, degeneration, and cytoplasmic vacuolation were scored as 0 (normal), 1 (mild), 2 (moderate), and 3 (severe).

Immunohistochemical Assay

The immunohistochemical procedure was performed according to the guidelines of the kit manufacturer (Abcam Company, USA). First, tissue sections were deparaffinized with xylene and then rehydrated in alcohol series. Next, they were incubated in 0.3% H_2O_2 in methanol for 15 min to block the endogenous peroxidase activity. Then, the tissue sections were incubated with a protein blocker for 10 min. After incubation with primary antibodies (anti-caspase 3 rabbit polyclonal antibody, 1:100 in PBS, v/v, Abcam, Lat: GR224831-2) at 4°C overnight, secondary antibody (mouse- and rabbit-specific HRP/DAB, Abcam, Lat: GR2623314-4) for 20 min, and diaminobenzidine tetrahydrochloride for 5 min (16). Then, the slides were dehydrated in alcohol series. Finally, all the slides were assessed under a light microscope with 40× magnification. For quantitative investigation, immunohistochemical micrographs were evaluated using ImageJ software (MacBiophotonics, version 1.41a) by densitometry. The positive staining severity was determined as the ratio of the stained area to the entire field area.

Statistical Analysis

Data were analyzed by The Statistical Package for the Social Sciences (SPSS) version 19 (IBM Corp.; Armonk, NY, USA) and Prism (GraphPad, version 6.07, USA) software. All the data are expressed as the mean±SD. Different groups were compared using one-way ANOVA and Tukey tests. A p-value of <0.05 was considered statistically significant.

RESULTS

Effects of NC on Oxidative Stress in CP-Treated Mice

MDA, which is the final product of LPO, and GSH levels in the liver tissue are presented in Figure 1. MDA level was significantly

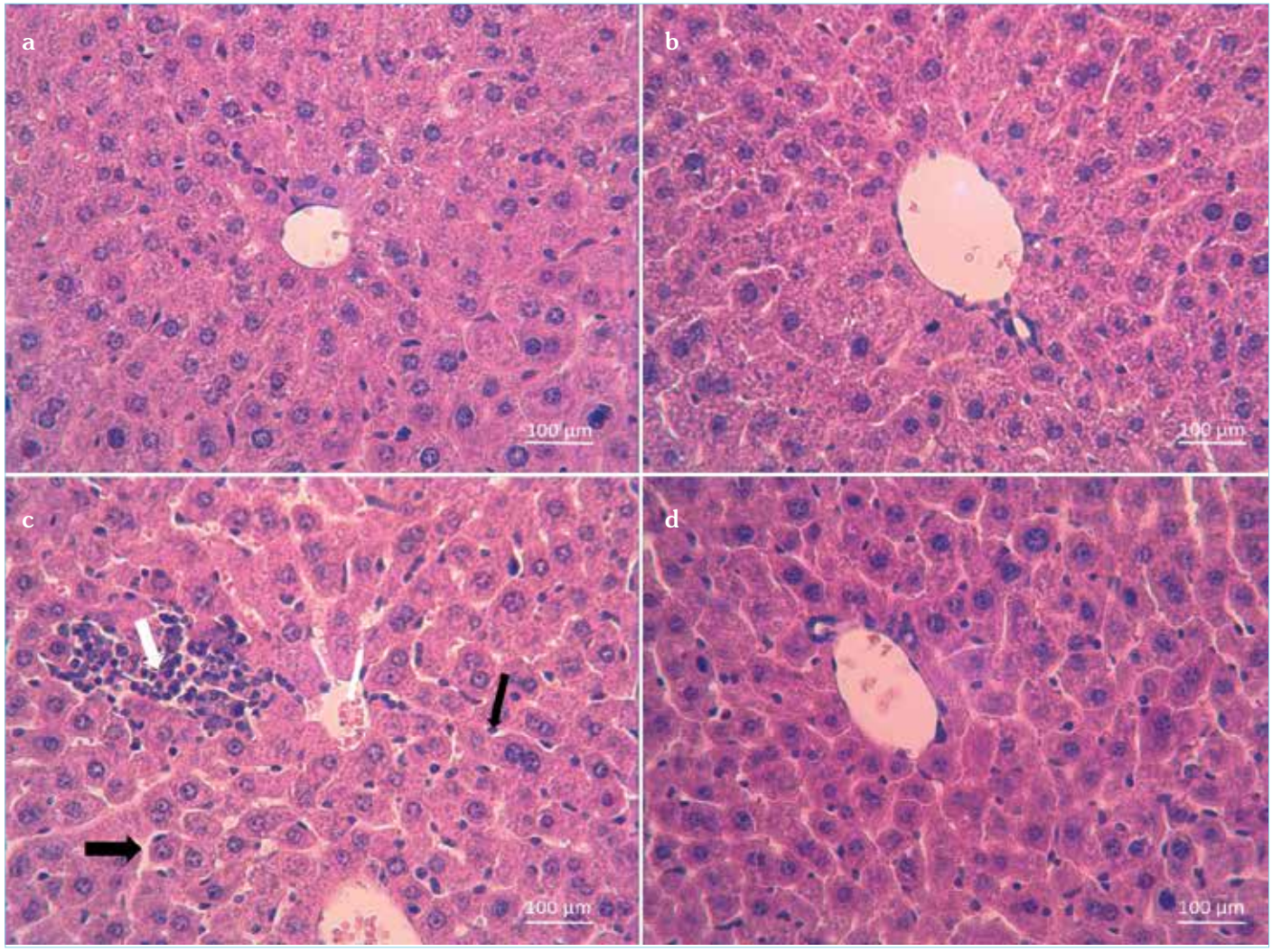

Figure 2. a-d. Photomicrographs showing the effect of NC and CP on the histological architecture of the liver in the groups. Control (a), NC (b), CP (c), and NC+CP (d) groups. Normal structure in control and NC groups, hepatic tissue disorganization, dilation sinusoids (thick black arrow), hyperplasia in Kuppfer cells (narrow black arrow), hemorrhage in the central vein (narrow white arrow), congestion and vacuolization, granulomatous formations (thick black arrow) in the CP group. Treatment with NC improved these changes. (H & E staining; magnification: 40×; scale bar=100 µm). CP, cyclophosphamide; NC, nanoceria.

Table 1. Effect of NC on serum marker enzymes of hepatic injury (AST, ALT, and ALP) in CP-treated mice in all the groups.

Groups	AST	ALT	ALP
Control	56.50±3.42	40.60±8.96	154.4±41.4
NC	67.75±5.85	49.80±8.04	173.8±31.34
CP	128.3±18.46^{a***b***}	66±10.80^{a**b*c**}	302.2±46.64^{a***b***}
CP+NC	86.25±16.21^{a*c**}	44.60±4.78^{c**}	240.8±16.07^{a**b*}

Values are expressed as the mean±SD. Mice treated with CP showed a decrease in the levels of serum marker enzymes. Pretreatment with NC significantly increased these serum marker levels. All the values are expressed as the mean ± SD. a, significant vs control; b, significant vs NC; c vs the CP groups; *, p<0.05; **, p<0.01 and ***, p<0.001. CP: cyclophosphamide; NC: nanoceria; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase.

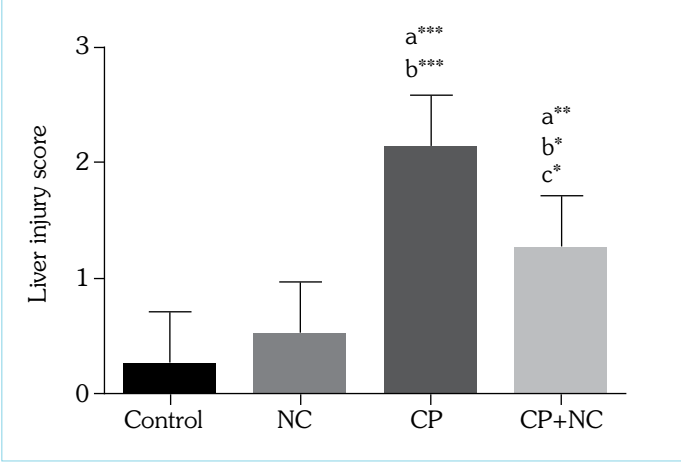

Figure 3. Liver injury score in the liver tissue. The highest score was for the CP group. NC decreased liver injury score in the NC+CP group. Data are presented as the mean ± SD. a, significant vs control; b, significant vs NC; c vs the CP groups;*, p<0.05;, p<0.01, and***, p<0.001. CP; Cyclophosphamide, NC; nanoceria.**

increased and GSH level was decreased in CP-treated mice compared with the control group. In contrast, NC administration in CP-treated mice significantly decreased the MDA level compared with the CP and control groups, whereas the GSH level was significantly increased compared with the control groups.

Effects of NC on Serum Enzyme Levels in CP-Treated Mice

Cyclophosphamide administration in mice significantly increased serum hepatocellular injury biomarker levels (AST, ALT, and ALP) as presented in Table 1. Furthermore, the administration of NC alone changed the levels of serum marker enzymes of hepatic damage but not significantly. Co-administration of NC with CP significantly alleviated the elevated levels of serum marker enzymes of the liver, but this decline was not statistically significant for ALP compared with that in the CP group.

Effect of NC on the Histopathology of the Liver in CP-Treated Mice

The micrographs of the liver in all the groups are shown in Figure 2. Normal histoarchitecture of the liver with normal hepatocytes, Kupffer cells, and sinusoidal space with normal hepatic lobules were seen in the control group (A). The structure of the liver in mice treated with only NC (B) was similar to that in the control group. Liver sections in the CP-treated group showed periportal leucocyte infiltration, dilation in sinusoidal space (thick black arrow), focal necrosis with pyknotic nuclei, hepatocyte vacuolation, hyperplasia in Kuppfer cells (narrow black arrow), congestion, granulomatous formation (thick black arrow), and hemorrhage in the central vein (narrow white arrow) (C). Conversely, NC administration in CP-treated mice regressed the pathological changes compared with those in the CP-treated group (D). The mean liver injury scores of all the groups are shown in Figure 3. Liver injury scores increased in CP-treated mice. Liver injury scores in the NC+CP group were significantly alleviated compared with the CP group.

Effect of NC on the Expression of Caspase-3 in CP-Treated Mice

Immunohistochemical photomicrographs of the liver are shown in Figure 4. The section of the liver in the control group did not show apoptosis. Immunoreactivity of caspase 3 was approximately similar in the control (Figure 4a) and NC (Figure 4b) groups. Increased immunoreactivity of caspase-3 was observed in CP-treated mice. Immunoreactivity staining was seen in the hepatocytes, especially in the periportal area (Figure 4c). Immunoreactivity staining of caspase 3 was weak in the NC+CP group (Figure 4d) compared with that in the CP group.

Semiquantitative investigation of caspase 3 staining is shown in Figure 5. The most severe immunoreactivity of caspase 3 was seen in CP-treated mice (22.1±5.92) compared with other groups (p<0.05). NC administration in CP-treated mice mitigated the severity of immunoreactivity of caspase 3 (13.03±3.93). Immunoreactivity of caspase 3 in the NC group was approximately similar to that in the control group.

DISCUSSION

Cyclophosphamide, a chemotherapeutic agent, has side effects, especially hepatotoxicity. Currently, NC, which has antioxidant properties, is an appropriate candidate for the reduction of oxidative stress due to medicines (17). To appraise NC as the potential therapeutic antioxidant, we investigated serum biochemical, histochemical, histopathological, and immunohistochemical measurements in hepatotoxicity induced by CP. Our data showed that NC reduces oxidative stress, histopathological change, and apoptosis induced by CP. Oxidative stress occurs due to an imbalance between oxidants and antioxidants status and can be determined by the evaluation of oxidative stress parameters. GSH as an important antioxidant in different redox species (18) plays a role in cytoprotection against oxidative injury. In clinical applications, CP produces oxidative stress that has a significant role in the occurrence of hepatotoxicity. CP can inhibit antioxidant enzyme activities and reduce GSH levels (19). In the present study, the administration of CP had a direct effect on oxidative marker levels. The administration of CP caused a significant increase in MDA levels and decrease in GSH levels, which was confirmed by the histochemical assay. In addition, NC administration in CP-treated mice improved oxidative stress marker levels, possibly due to the antioxidant effects of NC. Nrf2 is a key protein regulating the expression of antioxidant

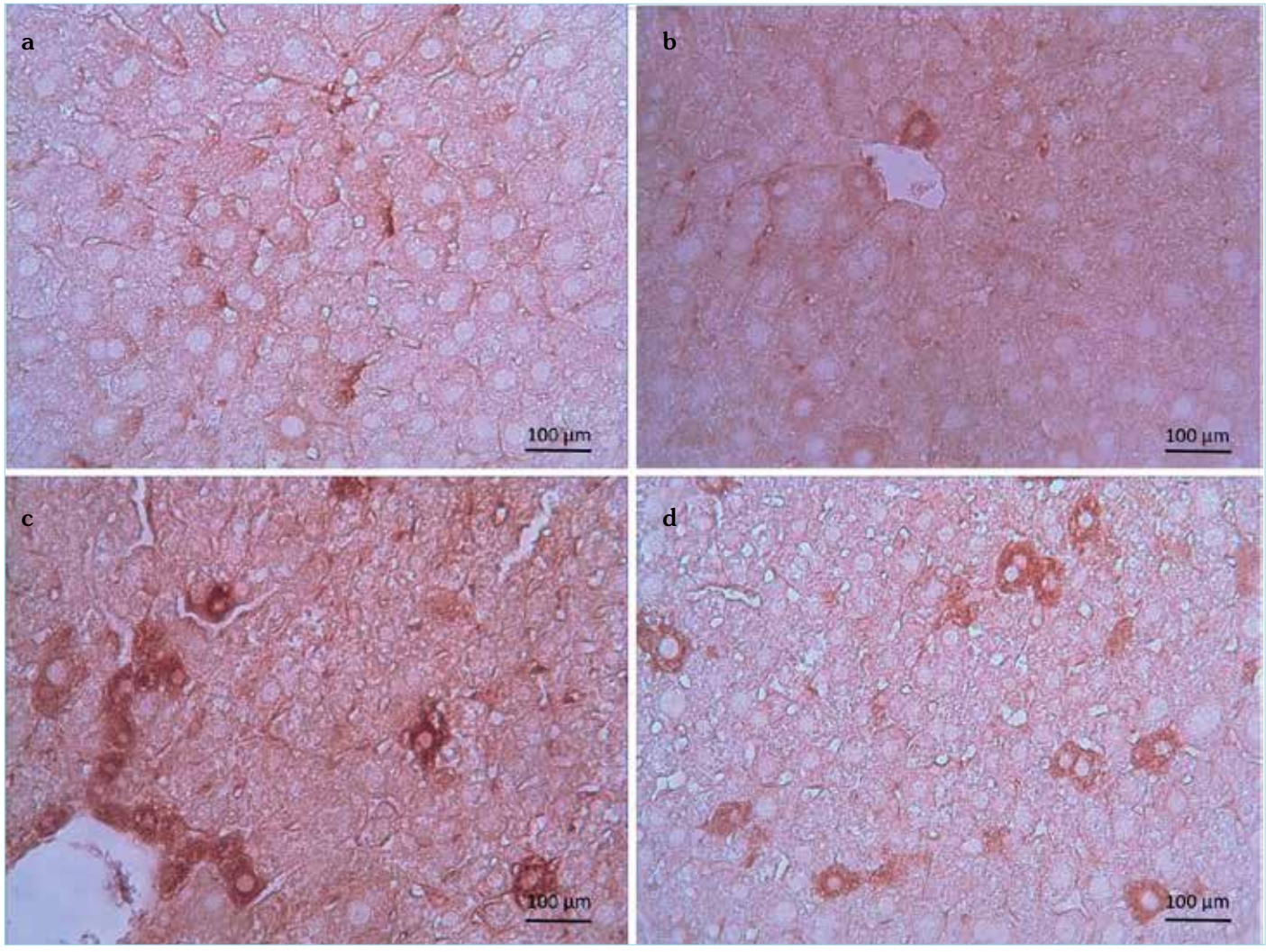

Figure 4. a-d. Immunohistochemical staining. Caspase 3-positive cells are shown with brown color. Immunohistochemical staining in (a) control and (b) NC groups showed no caspase 3 immunoreactivity. (c) Immunohistochemical staining displayed caspase 3 immunoreactivity in the CP group in the hepatocytes of the pericentral vein (d). NC treatment decreased caspase 3 immunoreactivity in CP-treated mice. CP; cyclophosphamide, NC; nanoceria.

proteins. NC decreased the transfer of Nrf-2 from the cytoplasm to the nucleus and subsequently reduced the gene expression of HO-1. Thus, NC can increase GSH, GPX1, SOD, and catalase levels and act as a powerful antioxidant to protect organs against oxidative injury (20).

Nanoceria switches between Ce^{3+} and Ce^{4+} states and scavenges free radicals (21). Cerium can easily, rapidly, and reversibly release oxygen. A reduction in ROS levels can decrease oxidative stress and subsequently reduce tissue injury. NC is accumulates in the plasma membrane and mitochondrial outer membrane (21) and effectively protects mammalian cells against ROS-induced oxidative stress injury (22). A study found that NC, with accumulation in the hepatocyte membrane, protected hepatotoxicity induced by monocrotaline (23). Furthermore, it can increase cellular resistance to exogenous oxidative stress. A previous article reported the protective effect of NC against radiation-induced oxidative damage (24). In addition, NC showed cardioprotective effect against monocyte chemo-attractant protein 1 in transgenic mice (25). Our results are consistent with other study results that demonstrated hepatoprotection by NC (20). Differences in size, shape, dose, method of preparation, and surface charges during the synthesis process may affect the study findings (26). Biodistribution of NC is mainly in the spleen and liver (27). On the other hand, processing of NC and its transformation in the liver has been demonstrated in a previous study. NC after transformation has increased ROS-scavenging ability (28). Our study confirmed that NC with anti-oxidative properties has a protective effect against oxidative stress and free radicals.

In animal and human studies, CP-induced hepatotoxicity and increased enzymes marker levels have been observed (4). ALT, AST, and ALP are biomarkers of hepatic damage and toxicity. Elevation in these marker levels could be attributed to the release of these enzymes from the cytoplasm of hepatocytes into blood circulation (3). In the present study, we demonstrated serum hepatic enzyme marker (AST, ALT, and ALP) levels to be increased in CP-treated mice. This is in agreement with other previous studies (4). However, treatment with NC improved the hepatic marker enzyme lev-

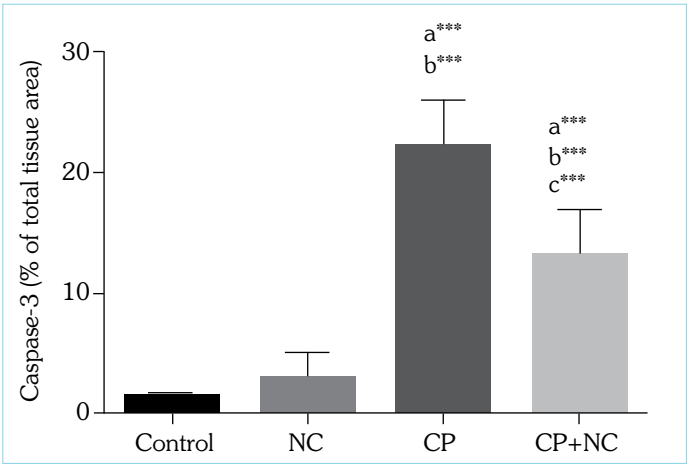

Figure 5. Densitometry investigation of caspase 3 expression. Immunoreactivity of caspase-3 increased in CP-treated mice. NC significantly decreased apoptosis. Immunoreactivity of caspase-3 in the control and NC groups was similar. Data was showed as the ratio of the stained area to the entire field area as the mean ± SD. a, significant vs control; b, significant vs NC; c vs the CP groups; ***, p<0.001. CP; Cyclophosphamide, NC; nanoceria.

els toward normal, thus indicating hepatoprotective effects of NC against toxicity induced by CP.

Histopathological changes in the liver were determined by light microscopy. Hepatic enzyme marker levels confirmed liver damage. Parenchymal degeneration, central vein dilatation, congestion, edema, sinusoidal dilatation, neutrophil infiltration, necrosis, vacuolization in hepatocytes, and cell proliferation in the periportal area were seen in the CP group. The results were consistent with the findings of other studies that found that acrolein has a role in inflammatory response, hemorrhage, and necrosis (19). NC could inhibit liver damage and preserve the liver histoarchitecture. It has been previously shown that NC has a hepatoprotective effect in D-GALN/LPS-induced hepatotoxicity in a mice model (20).

Nanoceria inhibits the programmed cell death pathway With the effect on the production of free radicals , thereby inhibits apoptosis (29). The important result of this research is that NC significantly prevents apoptosis via decreasing the immunoreactivity of caspase 3 in CP-induced apoptosis. CP, with generation of oxidative stress, crosslinks in DNA–DNA and DNA–protein, cause DNA damage (3). Kwon et al. (30) found that NC is mainly localized in the mitochondria, mitigates oxidative stress and subsequent mitochondrial damage, and suppresses cell death. Furthermnore, NC down-regulates the expression of genes involved in apoptosis signaling pathways, thereby having a protective effect on the tissue (11). The activation of caspases induces apoptosis which cleaves intracellular proteins (31). NC can decrease caspase 8 mRNA expression and caspases 9 and 3 activities (11). In the present study, CP significantly increased apoptosis in hepatocytes, especially in the periportal area, whereas treatment with NC significantly decreased apoptosis. These findings clearly indicated the potential role of NC in the inhibition of apoptosis.

In a pilot study, NC induced severe toxicity at a dose of 5 mg/kg for 7 days. While Khaksar et al. (32) showed that NC (35 mg/kg daily for 2 weeks) has a protective effect against diazinon-induced pancreatic damage. They also showed that NC decreases oxidative stress and apoptosis (caspase 3 and 9). Serebrovska et al. (33) showed that NC at a dosage of 0.6 mg/kg four times had an anti-inflammatory and antioxidant effect. They administered NC with an orogastric catheter, whereas we used NC 100 µg/kg for three times via intraperitoneal administration.

Chemotherapeutic effects of NC have been shown in human melanoma cells during doxorubicin treatment. Furthermore, NC enhances the antitumor activity of doxorubicin and increases apoptosis in cancer cells. NC also has cytotoxic effects; however, co-treatment of cancer cells with doxorubicin and NC showed synergistic effects (34). In our pilot study, we observed the cytotoxic effect of NC at a dose of 5 mg/kg, and co-treatment of NC and CP markedly decreased oxidative stress marker levels, liver injury enzyme levels, histopathological changes, and apoptosis in the liver (not published). Akhtar et al. (35) investigated the effect of NC with 20, 50, 100, and 200 µg/ml concentrations on oxidative stress induced with H_2O_2 *in vitro*. They showed that NC has no effect on cell death. Furthermore, NC maintained cell viability by increasing glutathione (GSH) levels.

CONCLUSION

In summary, we reported the protective effect of NC on CP-induced hepatotoxicity. NC with antioxidant and anti-apoptotic properties inhibited the degeneration in the liver tissue. Thus, NC is potentially useful as a chemoprotective agent in patients with cancer who are treated with CP.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of the Medical science of Mazandaran University, Sari, Iran (ID: IR.MAZUMS.REC.1395.S222).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Conceived and designed the experiments or case: FTA, SJH. Performed the experiments or case: MH, SYB. Analyzed the data: FTA. Wrote the paper: FTA, SJH. All authors have read and approved the final manuscript.

Conflict of Interest: The authors have no conflict of interest to declare.

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