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ORIGINAL ARTICLE

Oxidative Stress Biomarkers in Hepatic and Cardiac Toxicity Induced by Copper Oxide Nanoparticles in Mice

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ABSTRACT: Copper oxide nanoparticles (CuO-NPs) are used in products such as semiconductors, heat transfer fluids, lubricants, gas sensors, field emission emitters, catalysts, solar cells and lithium batteries, and antimicrobial equipment. Since oxidative stress is a key mechanism for cellular damage by nanoparticles, the present study investigated the oxidative stress induced by CuO-NPs in the liver and heart of adult male mice. Animals were randomly divided into 5 groups (n=15/group) including negative control (intact), pseudo-control (sham, receiving normal saline as a vehicle without nanoparticles), and three experimental groups received 1 ml of doses 10, 20 and 40 mg kg^{-1} b.w. of CuO-NPs intraperitoneally (IP), every other day for 21 days. Blood samples were collected to measure changes in the levels of hepatic necrosis biomarkers (ALT&AST) in the sera. Liver and heart homogenates were made to determine changes in the reactive oxygen species (ROS) level, malondialdehyde (MDA/LPO) content, activities of catalase (CAT) and glutathione peroxidase (GPx), along with total antioxidant capacity (TAC) value. Cardiac and hepatic samples were also examined histologically. No significant differences were observed between negative control and sham groups in all experiments. Data figured out the changes in oxidative stress biomarkers as illustrated by an increase in the ROS levels, MDA content, and CAT activity; a reduction in the activity of GPx and TAC value; and an elevation in the blood levels of ALT and AST. Tissue damages were also observed in the cardiac and hepatic samples. In brief, the aforementioned treatments exerted cardiac and hepatic toxicity, and it might be due to the induction of oxidative stress and related damages.

INTRODUCTION

Nanotechnology is a rapidly growing field to use nanomaterials for new products. With the increase in knowledge about nanotechnology, it is necessary to evaluate the unwanted effects of these materials on environmental organisms. Nanotoxicology, a field unraveled by the need to assess the environmental safety of nanomaterials, has evolved over recent years into a fully developed area of toxicology. In this context, it is intended to determine whether and to what extent nanoscaled materials cause threats to the health of organisms. Nanoparticles (NPs) are generally used on a scale of 1-100 nm for a wide range of commercial products. The NPs may leak into the environment in their production, consumption, and disposal processes [1].

The NPs can cause continuous toxicity when accumulate in biological systems without any proper excretion. The toxicity of NPs is connected with their physiochemical characteristics such as size, shape, dose, surface structure, solubility, etc. Owing to mentioned unique properties, NPs act via markedly different toxicological mechanisms than the bulk counterparts of the same materials. For instance, the high aspect ratio (surface to volume) of NPs is the main feature that results in their different and/or enhanced properties over their bulky

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equivalents. On the basis of this ratio, NPs hallmark a higher proportion of surface atoms to inner atoms. Due to their high chemical reactivity, the possibility of their cell uptake and interactions with biomolecules is augmented. The size of NPs directly affects the distribution, diffusion, and cellular physiological responses. Owing to a tiny size, NPs can cross tissue junctions and biomembranes where they induce structural and/or functional damages to the mitochondria and nucleus resulting in DNA abnormalities, and ultimately cell death. Additionally, it is well known that smaller NPs can pass cell membranes by translocation, whereas larger NPs enter the cells by processes like phagocytosis and nonspecific translocation. According to the kinetic studies, organ distribution of NPs is exceedingly size-dependent [1, 2]. Previous studies have demonstrated that a high aspect ratio makes NPs highly reactive to biomolecules. Accordingly, NPs can easily penetrate through biological membranes, and become more toxic to organisms. Meanwhile, there are evidences that nonspherical NPs can be internalized by cells at faster rates and in larger quantities than spherical ones. Besides size and shape, surface chemistry (zeta potential) remarkably affects toxicity. It is documented by other studies that NPs with positive zeta potential are have more tendency to exert toxicity than those with negative charges. The induced cytotoxicity by NPs is explained via direct cell damage by interaction with cellular components and/or indirect damage due to the production of reactive oxygen species (ROS). The creation of ROS by the presence of metallic NPs in the biological samples is mediated by the Fenton and Harber-Weiss reactions, reducing ferric into the ferrous ions. During the reactions, in the first step of ROS generation, hydroxide radical (˙OH) as the most potent oxidant in biological systems is produced. It is revealed that excessive amounts of ROS can induce the polymerization of proteins resulting in the mutation of the DNA. It is also figured out that the higher surface area of NPs is linked with increased number of reactive sites on the surface which result in higher chemical reactivity to elevation in the ROS generation. However, the disruption of redox balance by upgraded ROS generation and downgraded activities of antioxidants results in oxidation of macromolecules and cell toxicity. NPs can pass through the epithelial cells of the small intestine and after entering the bloodstream, penetrate tissues such as the brain, kidneys, and liver. NPs may bind with mediators which can activate inflammatory responses [2, 3].

Copper (Cu) is an essential element in the animal's body and acts as a cofactor in some redox enzymes in many metabolic processes, body homeostasis, and its lack gives rise to various diseases. Toxicity endpoints such as excessive generation of free radicals and histological changes were detected in the hepatic and intestinal cell lines overloaded with Cu ions induces [4]. Amongst NPs, copper oxide nanoparticle (CuO-NPs) is a type of metal oxide that is produced on a large scale for industrial purposes such as gas sensors, semiconductors, heat transfer nanofluids, solar cells, lithium batteries, antimicrobial equipment, etc. Studies have demonstrated that the release of metal oxide NPs such as copper and zinc oxides causes toxicity in the living organisms. Release of Cu^{2+} from CuO-NPs can disrupt the homeostasis of cellular metal cation, giving rise to cell toxicity [5]. The ROS is one of the mechanisms of NPs toxicity that can cause oxidative stress, inflammation, and consequently damage to macromolecules, cell membranes, and genomes. ROS assault can induce DNA fragmentation, and alter gene expression as well [6]. As soon as NPs enter the cell, oxidative stress triggers by causing imbalance between oxidative and antioxidant processes, may give rise to cell death. ROS can initiate and propagate lipid peroxidation (LPO) process as initial phase in cell membrane damage. Malondialdehyde (MDA) is a highly reactive end-product of the LPO process which reflects the extent of LPO in the biomembranes. MDA has been shown to induce cross-links in phospholipids and proteins, leading to failure in the cell survival. Oxidative stress is the cause of many disorders such as cardiovascular, pulmonary, and autoimmune diseases as well as aging [7]. The toxicity of CuO-NPs depends on their solubility, which is dependent on the temperature and pH of the relevant media. Release of Cu^{2+} from CuO-NPs following accumulation in lysosomes, where the solubility parameter increases due to acidic pH leads to introducing highly cytotoxic Cu (II) ions into the cytoplasmic compartments [8]. Administration routes (oral, inhalation, subcutaneous,

injection, etc.) of NPs are also having significant effects on their toxicities in mammals. For instance, in rats, it is reported that CuO-NPS following oral exposure caused significant toxicity to the liver that was attributed to the induction of oxidative stress [9]. Moreover, CuO-NPs have been marked as an inducer of oxidative DNA damage and cell death via signaling pathway, copper ion-mediated P38 MAPK, in the vascular endothelial cells [10]. Histological studies have shown that exposure to different doses of CuO-NPs can cause severe histopathological changes in the heart, such as blood clotting, necrosis, and infiltration of inflammatory cells via induction of oxidative stress with an increase in the dose of NPs [11].

Despite Cu necessity in human life, chronic overexposure to Cu exerts some deleterious effects in animals. Humans are quite resistant to Cu and acute toxicity manifestation appears at quite high doses. In industrial workers, occupational exposure to Cu may result in Cu toxicity with symptoms like nausea followed by vomiting and diarrhea. Under chronic Cu toxicity, Cu is gradually accumulated in the liver without any obvious symptoms. Beyond capacity of hepatic Cu storage, hepatocellular lesions and the liberation of Cu from the liver into the bloodstream are occurred that triggers pathophysiological alterations like hemolysis, jaundice, and renal insufficiency [2, 8, and 9].

The extreme usage of CuO-NPs brings challenges to the environment and humans. We need more toxicity data to ensure the safe use of CuO-NPs in the future, especially the application as the drug sources of antibacterial, antitumor, and feed additives. In this regard, the current study investigated the alterations in oxidative stress biomarkers in the liver and heart of adult male mice induced by CuO-NPs, upon constant laboratory conditions.

MATERIALS AND METHODS

Nanoparticles

CuO-NPs, with 40 nm average particle size determined by X-ray diffraction technique, and spherical shape determined by scanning electron microscopy (SEM), were purchased from Fanavaran Mehregan Chemistry Ltd. Co., Tehran, Iran. For experiments, the required solutions were prepared from the stock solution of CuO-NPs in normal saline (Figure 1).

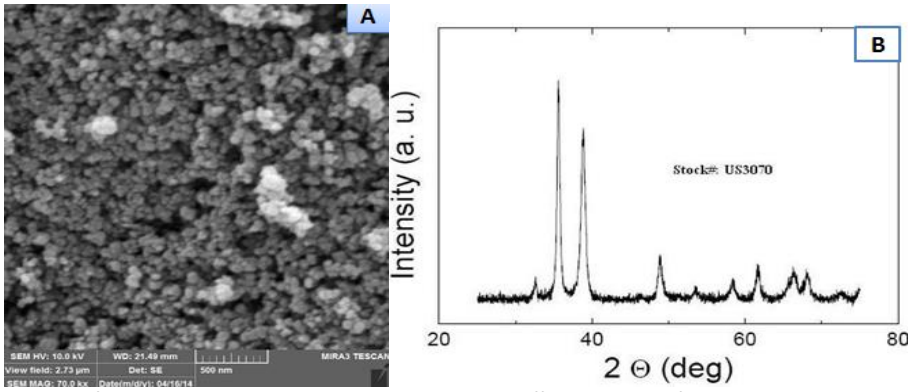

Figure 1. Characterization of CuO-NPs: (A) SEM micrograph and (B) the X-ray diffraction pattern of CuO-NPs (according to the brochure of Co.).

Experimental design

Utilized animals were 75 adult male BALB/c mice, weighing 31±2 gr, supplied by Dante Animal Group in Karaj, Iran. Mice hold for 14 days to accommodate laboratory conditions at 20±2 ºC, 12-hour light/dark cycles, and 60-65% humidity, and were subjected to free access to standard food and water (*ad libitum*) throughout the experiments. After acclimatization**,** animals were randomly divided into five groups having 15 mice each, including group 1: negative control (intact), group 2: pseudo-control (sham, receiving normal saline as a vehicle without NPs), and groups 3-5: three experimental groups (receiving CuO-NPs). During a 21-day treatment period, 1 ml of determined doses (receiving 10, 20, and 40 mg kg^{-1} b.w.) of CuO-NPs solutions were administered intraperitoneally (IP) every other day. At the end, mice were anesthetized deeply

with chloroform, and blood, cardiac and hepatic samples were gently collected.

The blood samples were collected via cardiac puncture, and centrifuged at 3000 rpm for 15 min. The resulting blood sera were used to determine the levels of the hepatic necrosis biomarkers (ALT and AST). The liver and heart tissues were separated, and dissected, and then related homogenates were made in chilled 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA solution and 0.16 mg/ml heparin to remove any clots. To evaluate the changes in the oxidative stress biomarkers, liver, and heart homogenates were divided into two parts, i) for measuring MDA level and total protein content, and ii) centrifuged at 10,000 rpm at 4˚C for 10 min to obtain the post-mitochondrial supernatant for enzymatic assays including catalase (CAT) and glutathione peroxidase (GPx) activities [12]. Formalin-fixed cardiac and hepatic samples were also used for histological evaluations.

Detection of ROS level

The liver and heart samples were smashed, filtered through a 350-mesh nylon membrane, centrifuged at 600 ×g for 5 min. By adding phosphate-buffered saline (PBS) solution, final cell density of 1.0×10^6 cells/ml was achieved. A volume of 300 μl of cell suspension was stained with 2′-7′- dichlorofluorescin diacetate (DCFH-DA; 10 μM) at 37 °C for 20 min. Then, the cells were washed with PBS and centrifuged at 600 ×g at 37 °C for 5 min. To end, after resuspention in 0.5 ml PBS, cells were counted using a BD FACSCalibur flow cytometer [13].

Measurement of malondialdehyde (MDA/LPO)

The LPO chain-reaction is assessed by the measurement of MDA as its low molecular weight by-product. A 500 µl aliquot of heart and liver homogenates was diluted with 1.0 ml of 10 % (w/v) cold trichloroacetic acid (TCA) solution and then centrifuged at 2500 ×g at 37 °C for 25 min. The resulting supernatant was diluted with 1.0 ml of 10 % thiobarbituric acid (TBA) solution, incubated at 90°C at 37°C for 10 min. Finally, the test tubes were cooled at room temperature and the optical density of MDA was recorded at 535 nm using a UV-1700 spectrophotometer (Shimadzu, Japan). MDA values were then calculated using a molar extinction coefficient (156×10^3 $M^{-1}cm^{-1}$) and finally expressed as µM MDA per mg protein [14].

Measurement of catalase (CAT) activity

CAT (EC 1.11.1.6) activity was measured by a decrease in absorbance at 240 nm for 30 s, measuring every 10 s, due to the decomposition of hydrogen peroxide (H_2O_2). Briefly, 2 ml of 10 % supernatants (in 50 mM potassium phosphate buffer, K_3PO_4, pH=6.5) were added to a quartz cuvette and placed in spectrophotometer for 3 min to reach temperature equilibrium with the surrounding environment. Finally, 1 ml of 30 mM H_2O_2 in potassium phosphate buffer was added to the cuvette, and changes in the absorbance were recorded with UV-1700 Spectrophotometer (Shimadzu, Japan). The values were expressed as nM H_2O_2 decomposed per mg protein. One unit of CAT activity is defined as the amount of enzyme required to consume 1 micromole of H_2O_2 per min at pH 7.0 and 25°C [15].

Measurement of glutathione peroxidase (GPx) activity

Lawrence and Paglia's (1976) method was used to measure changes in the activity of the antioxidant enzyme GPx. The assay mixture contained 0.5 ml of 50 mM potassium phosphate buffer, 0.4 ml of 2 mM EDTA, 0.1 ml of 6 IU glutathione reductase (GR), 0.1 ml of 2 mM sodium azide, 0.1 ml of 10 mM glutathione, 0.1 ml of 2.5 mM NADPH and 0.1 ml of supernatant. The mixture was transferred to the cuvette at 37 °C and placed in a spectrophotometer for 3 min to reach temperature equilibrium with the surrounding environment. The main reaction was started by addition of 2.5 mM hydrogen peroxide solution to the assay mixture in the cuvette. Finally, changes in optical density were recorded at 412 nm for 2 min (measuring every 2 s) [16].

Measurement of hepatic necrosis biomarkers (ALT&AST) levels

The commercial standard kits were obtained from Sigma-Aldrich Company to measure the level of hepatic necrosis markers ALT/GPT (#MAK052) and AST/GOT

(#ERMAD457IFCC) in the blood sera using a BT3000 autoanalyzer.

Total antioxidant capacity (TAC) value

The TAC values in supernatants of heart and liver homogenates were determined using ferric reduction antioxidant power (FRAP) assay.

Reagents: i) 300 mM acetate buffer (pH 3.6): sodium acetate trihydrate in glacial acetic acid; ii) 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mM HCl; iii) 20 mM $FeCl_3.6H_2O$. The fresh working FRAP reagent was prepared by mixing i, ii & iii solutions (10:1:1).

Procedure: 1.5 ml FRAP solution was incubated at 37°C for 5 min, mixed with 0.1 ml of diluted supernatants in chilled 50 mM potassium phosphate buffer (pH=6) (1:10) and incubated at 37°C for 10 min. A blue color was developed after formation Fe^{2+}-TPTZ as an end product in the reaction mixture. The changes in absorbance of developed blue color was recorded at 593 nm for subsequent 4 min. Five concentrations of $FeSO_4.7H_2O$ (0.1, 0.4, 0.8, 1, 1.12, 1.5 mM) were used for the construction of the calibration curve. Data were expressed as μM Fe (II) equivalent extrapolated from the standard curve [17].

Content of total protein

The method of Bradford was performed using bovine serum albumin (BSA) as standard at 595 nm. Results were calculated and then expressed as mg protein per ml of organ homogenate [18].

Histological examinations

Small pieces of cardiac and hepatic samples were immersed in a 10% formalin solution. After 24 hr, formalin was replaced. After dehydration in graded alcohol concentrations, samples embedded in paraffin blocks and serially cut using a rotary microtome. Finally, slides stained with hematoxylin-eosin (H&E) technique and evaluated with a conventional light microscope (Leica DM-2500 biosystem) for assessment of histological changes.

Statistical analysis

All statistical analyses were performed in SPSS 24.0 (SPSS, Chicago, IL, USA). All data were examined for the normality and homogeneity of variances using the Kolmogorove-Smirnov test and Levene's test. Multiple comparisons were performed using one-way ANOVA, followed by Tukey's posthoc tests at a 95 % confidence level ($P<0.05$), using SPSS software (2019 v26) to determine the individual comparisons between the CuONPs-exposed groups and the control group. Data are expressed as mean ± standard deviation (SD) (n=15/group).

RESULTS

Throughout the experiments, differences between the negative control and sham groups were not significant.

ROS level

As shown in Figure 2, the levels of ROS production were increased significantly in the 20 and 40 mg kg^{-1} CuO-NP treated groups after 21 days in liver and heart samples, compared to the relative control groups ($P<0.05$).

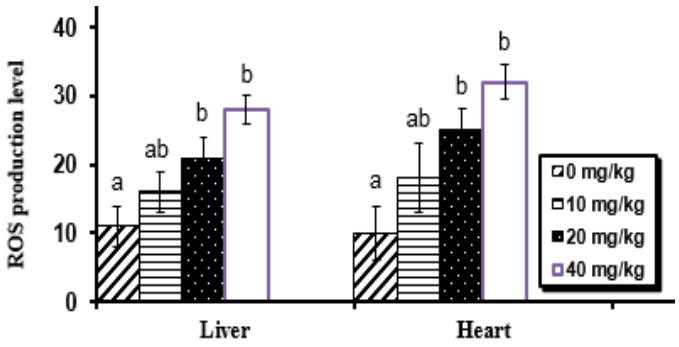

Figure 2. The levels of reactive oxygen species (ROS) in the liver and heart of mice exposed to CuO-NPs. [a,b]: data not sharing a common letter are significantly different (P<0.05) from control between the same tissues.

MDA/LPO level

The MDA level exhibited significant elevation among CuO-NP exposed groups in the liver and heart tissues compared to the related control groups (P<0.05) in a concentration-dependent manner. There was no significant difference between the three different concentrations of CuO-NPs in terms of hepatic and cardiac MDA levels (P>0.05). The highest MDA level was related to the 40 mg kg^{-1} CuO-NP treated group in the heart (109.97%) and liver (82.82%) tissues in comparison to related control (intact; zero concentration on CuO-NP) ones (P<0.05) (Figure 3).

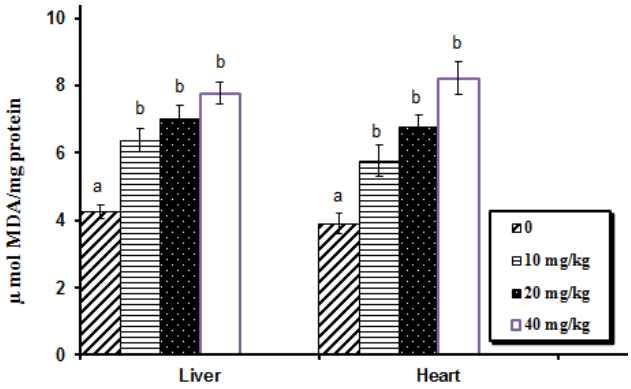

Figure 3. The level of malondialdehyde (MDA/LPO) in the liver and heart of mice exposed to CuO-NPs. [a,b]: data with no common letter are significantly different (P<0.05) from control between the same tissues.

CAT activity

According to the results depicted in Figure 4, the CAT activity exhibited an elevated in 20 and 40 mg kg^{-1} CuO-NPs treated groups compared to respective controls concentration-dependently (P<0.05). The highest increase in CAT activity was belonged to the 40 mg kg^{-1}-treated groups in the heart and liver (75.813% and 60.16%, respectively), compared to the related controls (P<0.05).

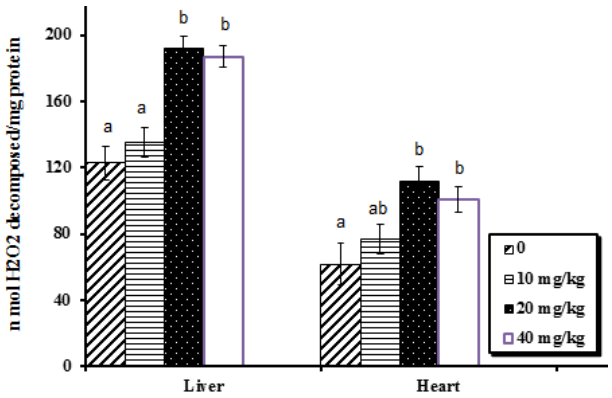

Figure 4. The activity of catalase (CAT) in the liver and heart of mice exposed to CuO-NPs. [a,b]: data with no common letter are significantly different (P<0.05) from control between the same tissues.

GPx activity

Figure 5 shows a downward trend in the activity of GPx after exposure to CuO-NPs in both heart and liver samples. This trend was observed with an increase in concentrations of NPs. The most reduction in GPx activity was observed at the highest concentration of CuO-NPs (40 mg kg^{-1}) in the heart and liver samples by 23.28% and 24.20%, respectively, as compared to relative controls (P<0.05).

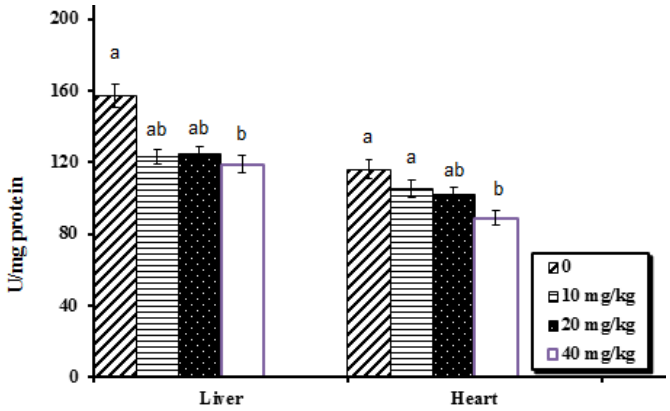

Figure 5. The activity of glutathione peroxidase (GPx) in the liver and heart of mice exposed to CuO-NPs. [a,b]: data with no common letter are significantly different (P<0.05) from control between the same tissues.

ALT & AST levels

As depicted in Figure 6, CuO-NPs induced hepatotoxicity as illustrated by elevated levels of ALT and AST in the serum (P<0.05). The highest levels of ALT/GPT and AST/GOT were observed in the treatment with 40 mg kg^{-1} CuO-NPs in the heart and liver samples by 199.71% and 65.65%, respectively, compared to the related control groups (P<0.05).

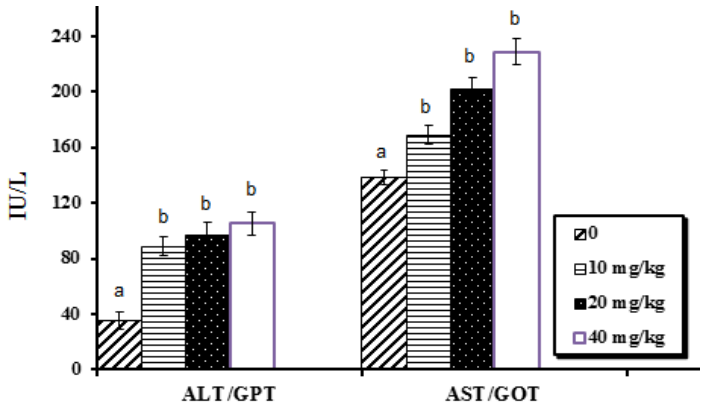

Figure 6. The levels of hepatic necrosis biomarkers (ALT/GPT&AST/GOT) in blood sera of mice exposed to CuO-NPs. [a,b]: data with no common letter are significantly different (P<0.05) from control between the same tissues.

TAC values

TAC values decreased with increasing CuO-NPs concentrations which were significant at 20 and 40 mg kg^{-1} compared to controls (P<0.05). The lowest TAC value was recorded following exposure to 40 mg kg^{-1} of CuO-NPs in heart and liver samples (33.34% and 35.22%, respectively) as compared to the relative controls (P<0.05)(Figure 7).

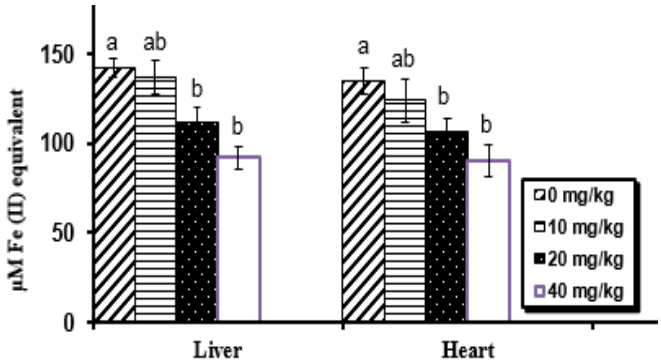

Figure 7. The value of total antioxidant capacity (TAC) in the liver and heart of mice exposed to CuO-NPs. [a,b]: data with no common letter are significantly different (P<0.05) from control between the same tissues.

Heart histological changes

Our results as detected histological changes were based solely on visual observations. Figures 8A-8F are dealing with the normal heart architecture and histological changes in the heart samples treated with different concentrations of CuO-NPs. Observations revealed sporadic hyperemia (an excess in blood supply) in cardiac tissue in different treatments with CuO-NPs particularly at higher concentrations of 40 mg kg^{-1} which was more prominent compared to the control group where these changes were not present.

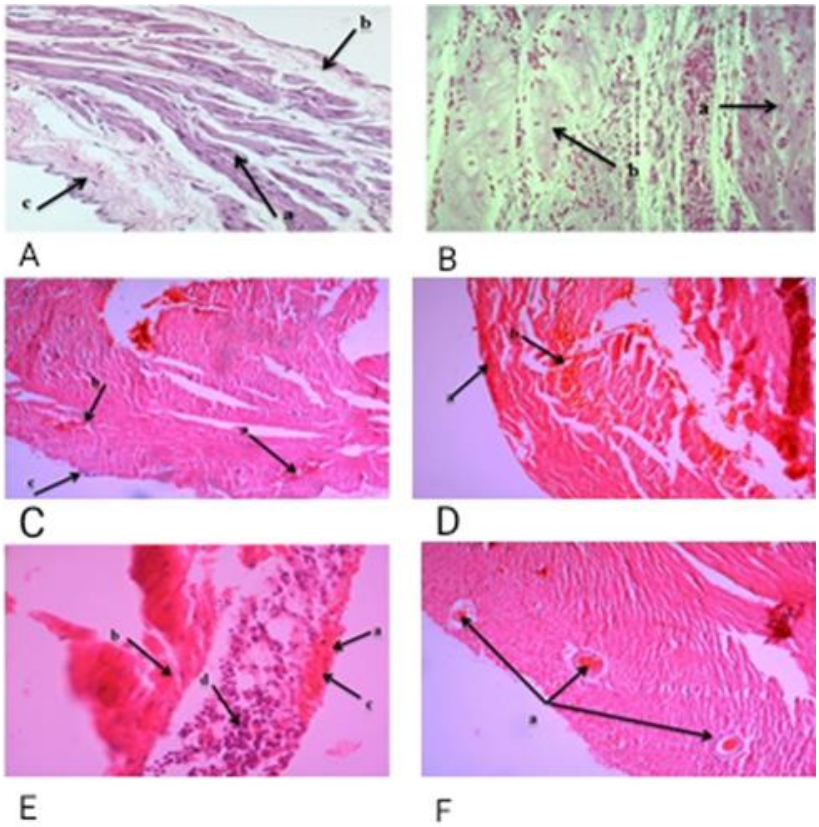

Figure 8. Histological changes in microscopic view of heart tissue section (A, 10×; B 40×, H&E) in the negative control group with normal architecture (a: myocardium, b: pericardium, c: endocardium). Treatment of 10 mg kg^{-1} CuO-NPs (C, 10×) (a,b: scattered hyperemia in cardiac tissue and c: inflammatory cell infiltration in the pericardium). Treatment of 20 mg kg^{-1} CuO-NPs (D, 10×) (a: hyperemia in pericardium, b: hyperemia in cardiac tissue). Treatment of 40 mg kg^{-1} CuO-NPs (E, 40×) (a & c: severe pericardial hyperemia, b: severe cardiac hyperemia, d: inflammatory cell infiltration in the pericardium. Treatment of 40 mg kg^{-1} CuO-NPs (F, 10×) (a: hyperemia in cardiac tissue). There were no differences between the negative control and sham groups.

Liver histological changes

Histological changes were recorded via visual observations. Figures 9A-9E show normal liver architecture and the results of hepatic tissue changes in mice treated with CuO-NPs. The observations showed hyperemia in the hepatic sinusoids, interlobular inflammatory cell infiltration around the central vein, hepatocytes with duplicated cell nuclei, an increase in kupffer cells, and necrosis at different concentrations of CuO-NPs.

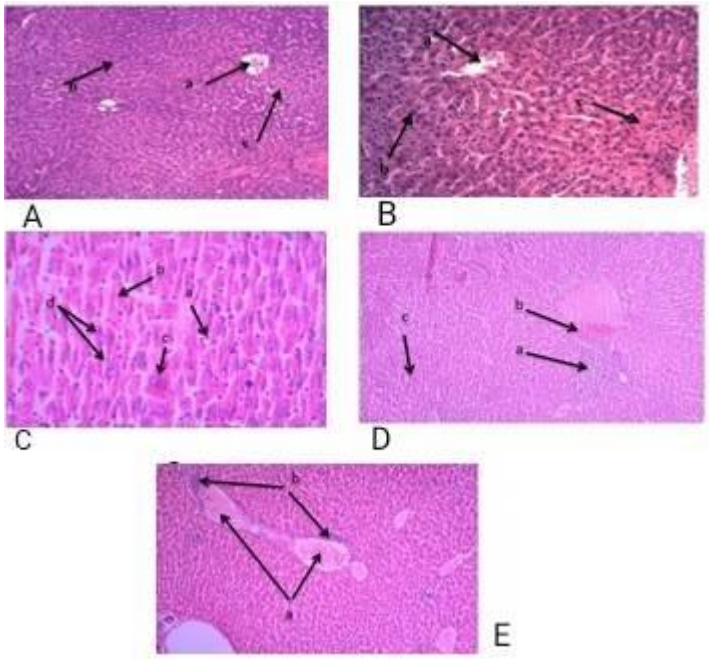

Figure 9. Histological changes in microscopic view of liver tissue section (H&E) in the control group with normal architecture (A, 40× and B, 100×, a: central vein, b: hepatocytes, c: sinusoid). Treatment of 10 mg kg^{-1} CuO-NPs (C, 100×) (a: increased kupffer cells, b: loss of cell nucleus and necrosis, c: cell nuclei duplication, d: cell nuclei condensation). Treatment of 20 mg kg^{-1} CuO-NPs (D, 100×) (a: inflammatory cell infiltration around the central vein, b: central venous hyperemia, c: increased inflammatory cells in sinusoids). Treatment of 40 mg kg^{-1} CuO-NPs (E, 100×) (a: aggregation in the central vein, b: inflammatory cell infiltration around the central vein). There were no differences between the negative control and sham groups.

DISCUSSION

Today, using NPs for the production of various products, playing an important role in human life, has increased significantly. Investigations showed that the release of NPs into the environment might have negative impacts on organisms [19]. Some studies have found that copper ions and CuO-NPs are capable to induce oxidative stress in different biosystems [9, 20]. In mice, hepatocytes are defined as primary target cells for Cu toxicity. Several studies have exhibited that Cu exposure induces ROS creation, oxidative stress, and ultimately apoptosis in hepatocytes [21, 22]. However, the underlying mechanism remains unclear. This study assessed the extent of changes in the oxidative stress biomarkers induced by CuO-NPs in the heart and liver of adult male mice. To constitute an animal model of CuO-NP-induced hepatic and cardiac damages, the experimental mice were exposed to graded levels of CuO-NP (10, 20, and 40 mg kg^{-1} b.w.) via IP route for 21 days.

ROS level

ROS are defined as oxygen metabolites that emerge during cell life in an oxygenated environment and include hydroxyl radicals, superoxide anions ($^{\cdot}O_2^-$), and H_2O_2. Once NPs are encountered to the acidic environment of lysosomes or interact with oxidative organelles such as mitochondria, they can induce ROS directly. Owing to their large specific surface area, these particles can interact with biomolecules that provides CuO-NPs with high reactive activity [23]. The imbalance between ROS generation and function of the antioxidant defense system leads to oxidative stress and related tissue damage. The overproduction of ROS and the subsequent induction of oxidative stress are known as predominant mechanisms leading to nanotoxicity, including genomic damages, cell death, unregulated cell signaling, altered cell motility, and cancer initiation [24]. CuO-NPs caused strong hepatic toxicity by inducing oxidative stress and inflammation [25]. Our findings indicated that CuO-NPs could induce the overproduction of ROS in the mouse liver and heart samples in a concentration-dependent manner. Several mechanisms have been intended to explain Cu-induced cytotoxicity. One of the most accepted is ROS overproduction, which results in oxidative damages to cellular macromolecules such as proteins and lipids, leading to cell dysfunction [26]. It has been demonstrated that the upgraded ROS levels and oxidative stress process under CuO-NP treatments can

activate related regulation pathways resulting in increased expression of proinflammatory cytokines and cellular dysregulation [27]. In line with our results, it is revealed that ROS overproduction is occurred even when only small amounts of CuO-NPs entered the cells [28]. Meanwhile, with increase in ROS creation, NPs can cause oxidative DNA damage and increase in gene expression of the death receptor leading to apoptosis. This type of damage is believed to be linked with biological mechanisms such as cancer, gene mutations, and aging-mediated diseases in humans [29]. According to changes observed in ROS levels, it can be concluded that CuO-NP imposes a greater pressure stimulation effect on ROS production in mice.

MDA/LPO level

In the biochemical assessments, several parameters are assumed as the biomarkers of oxidative stress, including the modified lipids in the biological membranes and associated LPO by-products such as MDA and hydroxynonenals. Evaluating the MDA extent plays a remarkable role in the determination of toxicological effects of the pollutants such as metals and xenobiotics in humans and animals. In this regard, it is clear that during the oxidative processes, upgraded ROS production induces negative effects such as lipid modification, protein cleavage, DNA damage, and even cell death [9, 30]. The capability of NPs to generate free radicals is the main mechanism of cellular toxicity [31], which results in oxidative stress, inflammation, and change in the cell membrane and DNA stability [31, 32]. The toxic effects of CuO-NPs disturb the biological activities of cell membranes, mainly due to the induction of oxidative stress and the increase in LPO in biological membranes [33, 34]. The resulting data from this study showed that CuO-NPs at all concentrations during a 21-day treatment period caused increases in the level of MDA/LPO in the liver and heart samples compared to the relative control ones. As MDA is a biomarker for inducing oxidative stress, it can be concluded that treatments with CuO-NPs induced oxidative stress marked by MDA elevation in the heart and liver of mice. On the other hand, it can be stated that CuO-NPs have the potential to damage cardiac and hepatic tissues via an increase in free radical generation. In consistency with our results, Goma et al. (2021) claimed that CuO-NPs could induce oxidative stress, and increase MDA level and content of protein oxidation [35]. Moreover, in 2015, it was found that the application of different concentrations of CuO-NPs caused damage to the function and structure of cardiac tissue [11]. In agreement with our results, a study on the effect of CuO-NPs on liver and kidney functions in rats showed an increase in ROS-mediated oxidative stress along with severe histopathological changes in tested tissues [36]. In addition, Feng et al. (2022) recently suggested that Cu^{2+} exposure induces oxidative stress and apoptosis in crab *E. sinensis*, and the toxicity may be associated with the activation of the molecular pathways including AMPK, ERK, and TLR2-MyD88-NF-kB [37].

CAT activity

Tissues possess a battery of antioxidant defense systems including the enzyme CAT which is constructed to inhibit harmful oxidative damages following an increase in the level of ROS [30]. Our results in the present study declared that CAT activity was elevated in all CuO-NP-treated liver and heart samples. In line with our results, a study by Abdelazeim et al. (2020) showed that exposing male albino rats to CuO-NPs could develop oxidative stress and increase the MDA level and CAT activity of liver samples. This elevation in the CAT activity is beneficial to reverse the oxidative action of CuO-NPs [38]. Meanwhile, increased expression of the CAT gene in the liver and gills of fish *Oreochromis niloticus* was reported as an efficient hallmark for inducing oxidative stress by CuO-NPs [39]. In addition, in 2020, it was demonstrated that treatments with CuO-NPs caused an increase in the LPO rate and CAT activity in the renal homogenates of mice which was ameliorated by the use of curcumin as a flavonoid antioxidant [40]. Conversely, i*n vitro* and *in vivo* studies showed that cells exposed to CuO-NPs exhibited oxidative damage and reduction in the CAT activity [9, 41]. The noted variations across species might be due to different doses and shapes of used nanoparticles, route of administration, sex differences, mode of study (i*n vitro*/*in vivo*), lab conditions, etc. However, it can be assumed that CuO-NPs under different physiological conditions and concentrations, and routes of exposure exhibit different influences on CAT activity.

ALT & AST levels

According to our results, the extent of LPO as a marker for losing cell membrane integrity was increased in the CuO-NP- intoxicated mice. In animal models, blood biochemical parameters are important markers for determination of physiological status. In most cases, loss of cell membrane integrity results in the release of cytosolic enzymatic systems such as ALT and AST into the blood circulation. Upsurge in the levels of these biomarkers is a potential key indicator for diagnosing various hepatic injuries [42]. Knowing enzymes ALT and AST as hepatic necrosis biomarkers can confirm with histopathological examinations of the liver that exhibited tissue injuries under certain physiological disorders [43]. The liver is one of the main target organs for NPs intoxication after they gain entry into the body via possible routes. Studies have shown cytotoxic responses of CuO-NPs in liver cells [44]. It was reported by Anreddy (2018) that 14-day oral exposure to CuO-NPs in rats could result in mild toxicity to the liver that might be due to the induction of oxidative stress [9]. Furthermore, liver toxicity assessments in rats showed that exposure to CuO-NPs increased serum ALT and AST levels significantly, further promoting hepatic oxidative stress, inflammatory responses, and relative histopathological changes [45]. Our results explain that the hepatotoxicity via CuO-NP mediated necrotic changes led to an increase in cell membrane permeability and ultimately release of ALT and AST into the blood. Several previous studies are in line with our findings regarding hepatotoxicity induced by CuO-intoxication in the liver along with elevation in the levels of serum ALT and AST [19, 38, and 46].

GPx activity

In the present study, GPx activity showed a downward trend in the cardiac and hepatic tissues upon treatment with CuO-NPs. In 2018, Anreddy explained that hepatotoxicity with CuO-NPs in rat liver represents induction of oxidative stress and dysregulation of some enzymatic antioxidants such as CAT and SOD [9]. It has been revealed that CuO-NPs treatments could decrease intracellular levels of reduced glutathione (GSH) resulting in oxidative stress in the kidney cells of human fetuses and mice leading to renal dysfunction [47, 48]. Recently in 2022, it was indicated that CuO-NPs exerted toxicity in the human placental cells as documented by induction of LPO, production of high levels of hydrogen peroxide, and reduction in the GPx activity [49]. As GSH is required for appropriate activity of GPx, then it can be deduced that in our study CuO-NPs toxicity causes a reduction in the GPx activity via depletion of GSH molecules which are necessary for proper GPx function in the liver and heart samples. Conversely, GPx gene expression exhibited a rising tendency after CuO-NPs exposures in the liver and gills of *Oreochromis niloticus* and in the kidney of rats, which is itself a sign of induction of oxidative stress by tested NPs [39, 40]. However, differences between GPx responses can be explained by different used species, exposure time, and concentration of tested NPs.

TAC value

Generally, antioxidants are substances that protect molecules and whole organism from oxidation by oxidants via chain reactions, dampen or repair the damage. The amount and nature of antioxidant defense systems may differ from cell to cell and tissue to tissue. Antioxidative molecules encounter oxidants in some way. Non-enzymatic antioxidants including albumin, reduced glutathione (GSH), the vitamins C and E along with enzymatic antioxidants such as CAT, GPx, SOD, etc. serve protective mechanisms versus oxidants and oxidative stress. Natural and/or synthetic antioxidants can stop and/or delay the cellular damage caused by ROS and unstable ions and molecules, etc. Antioxidants devote electrons to free radicals and neutralize them to limit the extent of cell damage. ROS overproduction along with weakening of antioxidant protection leads to oxidative stress and corresponded oxidative damage to cellular macromolecules like DNA, proteins, and lipids [50, 51]. Nanotoxicity is heavily influenced by oxidative stress. Most of NPs function as oxidants and have a tendency to exert negative effects on living systems via oxidative stress. Entry of NPs into the cells results in increased levels of ROS [51]. TAC value attributes to the antioxidative power of a biofluid like blood, and includes

synergistic interactions between different redox-active molecules [52]. Our findings reveal that serum TAC value in CuO-NP-treated mice was decreased in a concentration-dependent pattern which can be explained by the ability of CuO-NP to consume the power of antioxidant defense systems in blood sera. In line with our results, Arafa et al. (2017) showed a reduction in the TAC value of liver homogenate of CuO-NP-intoxicated mice along with elevation in blood ALT and AST levels, and DNA fragmentation detected as comets [53]. Other NPs like Al_2O_3-NPs and ZnO-NPs could exert a decline in the TAC value of rat brains. [54]. Hence, it can be assumed that by-products of oxidative stress upon CuO-NP intoxication in liver and heart samples might be the cause for the depletion of antioxidant power detected as lowered TAC value.

Histological examinations

Oxidative stress and related abnormal increase in ROS levels are among the main causes of many diseases and physiological disorders [34]. Our results showed that CuO-NPs treatments led to the propagation of LPO in both cardiac and hepatic samples. An explanation for that would be the induction of oxidative stress due to an imbalance between the abnormal generation of ROS production and antioxidant defense function in animal tissues. Studies revealed that free radicals are capable to induce protein oxidation, i.e., unfolding or alteration of protein structure in cells, resulting in tissue damage [55]. In addition, one of the negative impacts of CuO-NPs on cells is the conversion of cells to autophagic type, which through stimulating autophagic lysosomes and causing cell death in cells, causes the appearance of necrotic regions in various tissues [56]. As noted earlier, ROS overproduction causes oxidative damage to macromolecules including lipid, protein, and DNA peroxidation leading to cytotoxicity. Besides, oxidative stress induced by many toxic compounds disrupts several major metabolic pathways within target organs [57]. Accordingly, in the present study, the same mechanism is probably used to cause tissue damage in the liver and heart of mice treated with CuO-NPs.

Regarding the effect of CuO-NPs on intercellular junctions and their role in maintaining tissue integrity, in 2018, it was shown that the intoxication of NPs reduced intercellular junctions and cell adhesion molecules, resulting in induced cell death and inhibited cell proliferation [58]. Therefore, it can be assumed that in the present study, CuO-NPs may have caused some tissue damage in the cardiac and hepatic structures by disturbing intercellular junctions. Meanwhile, in 2015, it was shown that CuO-NPs, in a concentration-dependent manner, caused functional disorders and accumulation of inflammatory immune cells in the heart of mice [11]. In the liver of rats treated with CuO-NPs, tissue damage including obstruction in the central vein and bile ducts, accumulation of inflammatory cells, destruction of sinusoids, fibroplasia in the portal triad, and tissue necrosis were also observed [48]. According to the above-mentioned results and our results, different concentrations of CuO-NPs had negative effects on tissue structure and function in the liver and heart of tested mice via ROS overproduction and subsequently induction of oxidative stress.

CONCLUSIONS

Our study has indicated that the aforementioned CuO-NPs treatments could impose oxidative damages in the liver and heart of male mice as represented by the alterations in the oxidative stress biomarkers including increases in ROS and MDA levels, CAT activity, hepatic necrosis biomarkers (ALT&AST) levels; and reduction in the GPx activity and TAC value along with histological damages, particularly at high concentrations.

ETHICAL CONSIDERATION

All experiments have been complied with the ARRIVE guidelines 2.0 and carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and also with Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes was adopted on 22 September 2010. This study was approved by the Ethics Committee of the Shahrekord University (Approval No. IR.SKU.REC.1393.95).

Conflict of interest

The authors declare that there is no conflict of interest.

Authors'contributions

MA designed and supervised study and drafted original manuscript. FS performed experiments, analyzed data. All authors read and approved the final manuscript.

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