



ORIGINAL RESEARCH ARTICLE

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Hepatic function and structure in feral pigeons (*Columba livia domestica*) exposed to zinc oxide nanoparticles

Mehran Arabi^{1*} and Hamid-Reza Naseri¹

Abstract

Background Broad applications of nanoparticles have invoked the concerns about their impacts on living organisms. This study aimed to evaluate the possible changes that could take place in the liver of feral pigeons (*Columba livia domestica*) after oral ingestion of zinc oxide nanoparticles (nano-ZnO).

Methods Pigeons were acclimatized to the laboratory conditions with a photoperiod of 12:12 h at 20 ± 2 °C for 14 days and maintained on seed mixture and clean tap water ad libitum. The birds were randomly assigned to five groups of 10, including one control group and four experimental groups orally receiving 0, 30, 50, and 75 mg/kg b.w. of nano-ZnO through oral gavage for 7 and 14 consecutive days.

Results The oxidative stress (OS) biomarkers, namely lipid peroxidation content and catalase activity in the liver samples, and the level of hepatic necrosis markers (ALT, AST, and ALP) in the blood sera were increased in a concentration-dependent manner. Meanwhile, the total antioxidant capacity of liver samples measured by FRAP test was reduced. Histopathological changes revealed inflammatory cell infiltration, swelling area, vacuolization, and expansion of interstitial spaces in liver samples exposed to 75 mg/kg nano-ZnO.

Conclusions Nano-ZnO induced obvious hepatotoxicity in the liver of pigeons, where the OS pathway may be the potential mechanism underlying this toxicity.

Keywords ZnO nanoparticles, Pigeon, Oxidative stress, Hepatotoxicity

Background

For many years, birds because of their phylogenetic distance from man have not been used as suitable models for toxicological evaluations; however, these animals and man have a virtual food relationship, and therefore, environmental factors affecting the health of bird populations have the potential of an impact on the food safety of man [1]. Nowadays, there is a growing need to use nanoparticles (NPs) in biomedical and industrial applications.

More studies have indicated that manufactured NPs due to high surface area to volume ratio and abundant reactive sites on the surface are toxic to organisms [2]. The NPs may leak into the environment in their life cycles including production, consumption, and disposal processes. Of all the NPs, metallic NPs (MNPs) have considerable commercial interest due to unique properties such as small size and greater surface area to volume; entering the body via the skin, inhalation, and gastrointestinal tract; and circulating into the body, eventually accumulating in various organs [3]. There is a lack of information regarding the magnitude of released MNPs and their impact on living organisms in the terrestrial systems. Therefore, there is an urgent need for information on the ecological risks of MNPs [4].

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Zinc oxide nanoparticles (nano-ZnO) as MNPs have attracted attention owing to their applications in veterinary sciences due to their wound healing, antibacterial, and angiogenic properties, etc. [3]. Once nano-ZnO is absorbed, the elevated level of Zn^{2+} can be recorded in the liver, adipose tissue, and pancreas [2]. Common provenances of zinc toxicity in birds include the coating on galvanized wire cages, food, and water dishes, etc. [5]. In Aves, plasma zinc concentrations above 4 ppm are suggestive of toxicosis. However, birds may be clinically affected without high plasma zinc concentrations [6]. The MNPs may stimulate the reactive oxidative species (ROS) generation by modifying the cellular metabolism [6] or damaging the antioxidant defense system, resulting in oxidative damage to the proteins, lipids, and DNA [7, 8].

The biomarkers are determined as molecular, biochemical, physiological, or histological indicators of contaminant effects [9, 10]. The use of biomarkers can be a complementary approach to standard toxicity tests because it provides more information about the organism's stress response to individual and/or combined toxicants [10]. The antioxidant systems, enzymatic and non-enzymatic antioxidants, play a vital role in defense against cellular oxidative damage caused by ROS [11, 12]. Oxidative stress (OS) is defined as an imbalance between the excessive ROS generation and its quenching by antioxidant defense systems. Once OS occurs, the lipid peroxidation (LPO) process and its by-product of malondialdehyde (MDA) are the initial steps of cellular membrane damage and are considered to be valuable indicators of oxidative damage of cellular components. Excessive accumulation of MDA changes the permeability of cell membranes and disrupts DNA and proteins resulting in cell death [12, 13].

Biochemical methods of monitoring chemical pollution have been widely used with avian models, and some of the methods may have direct application to health effects [1]. The feral pigeons (*Columba livia domestica*) are appropriate animal models in ecotoxicology studies. Due to their commercial importance, these animals must be protected from environmental pollutants [14]. Birds exposed to NPs show adverse alterations in the function of certain tissues [15, 16], but the ecotoxicological data on nano-ZnO are just emerging and scanty.

The liver as a main organ in the body possesses abilities of detoxification, storage, secretion, synthesis, etc. MNP intoxication may lead to liver structural changes and dysfunction. Markers such as a significant increase of alkaline phosphatase (ALP) with aspartate aminotransferase (AST) in the blood serum indicate liver injury. These markers mainly manifested in liver structural changes

causing metabolic dysfunction. MNPs could cause liver toxicity via mechanisms including inflammation, OS, and different types of cell death [17]. Given their diffusion, there is insufficient information in the consulted literature regarding the toxicity of nano-ZnO in birds and requires further investigation. Therefore, this study aimed to evaluate the hepatic function and structure in feral pigeons (*Columba livia domestica*) exposed to zinc oxide nanoparticles under laboratory conditions.

Methods

Chemicals and animal model

The nano-ZnO white powder with a diameter size of ≤ 20 nm was purchased from Nano-shop Company, Tehran, Iran. According to the company brochure, the shape and purity of tested nano-ZnO were spherical and 99%, respectively. Other chemicals in reagent grade were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The nano-ZnO suspension was made using deionized water to minimize ROS generation during sonication, and the cold water bath was used to minimize the heating of the particles. Then, the suspension was sonicated for 20 min in a bath sonicator (SONOPULS HD 2070.2, BANDELIN electronic GmbH & Co. KG, Germany) and was vortexed for 1 min before every administration [1].

Healthy feral pigeons (*Columba livia domestica*), with a mean weight of about 300–350 g, were purchased from a local bird market in Shahrekord, Iran. Birds reared in clean wooden cages with metal nets in the Animal Physiology Laboratory, Department of Animal Sciences, University of Shahrekord, Iran. Thereafter, all birds were acclimatized to the laboratory conditions with a photoperiod of 12:12 h at $20 \pm 2^\circ\text{C}$ for 14 days. Seed mixture and tap water were provided ad libitum.

Experimental design

After acclimatization, birds were randomly divided into five groups having 10 pigeons each, including one control and four experimental groups (in triplicate). The experimental groups received 30, 50, and 75 mg/kg b.w. of nano-ZnO solutions for 7 and 14 consecutive days (1 ml/bird) through an oral gavage cannula (Fig. 1). The control group received tap water for the same time periods. During the experimental period, pigeons were monitored for clinical and physical symptoms.

Biochemical assays

The blood samples, about 2–3 ml, were collected via puncturing from the brachial vein. The samples were then transferred to the test tubes without any anticoagulant



Fig. 1 Nano-ZnO administration through oral gavage cannula

agent and centrifuged at 3000 rpm for 15 min [18]. The resulting blood sera were used to determine the activities of the hepatic necrosis markers AST, alanine aminotransferase (ALT), and ALP by related commercial kits. Then, the pigeons were euthanized by cervical dislocation. The liver samples were taken out and, after perfusion with normal saline, were homogenized in chilled 50-mM potassium phosphate buffer (1:8, w/v; pH 7.0) containing 0.5 mM EDTA. The resulting homogenate was divided into two parts: (i) for measuring MDA and protein content and (ii) for centrifugation at 10,000 rpm for 10 min at 4°C to obtain the post-mitochondrial supernatant for CAT assay [12].

MDA level

The TBARS assay [19] was performed to determine the MDA/LPO level in the supernatant of liver homogenates. In brief, 500 µl of aliquots was mixed with 1 ml of chilled 30% trichloroacetic acid (TCA) and centrifuged at 5000 × g for 10 min. The resulting aliquot reacted with thiobarbituric acid (TBA) and MDA absorbance was recorded at 532 nm, using a UV-1700 spectrophotometer (Shimadzu, Japan). The MDA level was expressed as *n* moles MDA per mg protein min.

CAT activity

The changes in CAT activity in the assay mixture containing H₂O₂ phosphate buffer and supernatant of liver homogenate were recorded at 240 nm. The activity was expressed as IU H₂O₂ decomposed per mg protein min [20].

Total protein

To measure total protein content, the Bradford method was used using bovine serum albumin (BSA) as standard at an absorbance of 595 nm. Results were expressed as milligram protein per milliliter of whole organ extract [21].

Hepatic necrosis markers

The commercial standard kits were obtained from Sigma-Aldrich Company to measure the level of hepatic necrosis markers namely ALT (#MAK052), AST (#ERMAD457IFCC), and ALP (#AP0100) in the pigeon blood sera.

Total Antioxidant Capacity (TAC) content

Ferric reducing ability of plasma (FRAP) was performed to measure changes in the TAC of liver samples. During the assay, yellow ferric tripyridyltriazine complex (Fe (III)-TPTZ) is reduced to blue ferrous complex (Fe (II)-TPTZ) by the action of electron-donating antioxidants in liver samples. At the end of the experiment, the resulting blue color is linearly related to the total reducing capacity of antioxidants in liver samples. Briefly, FRAP reagent (1.5 ml) was incubated at 37 °C for 5 min. Then, this solution was mixed with 0.1 ml of diluted liver sample (1:10) and incubated at 37 °C for 10 min. The end product ferrous tripyridyltriazine complex (Fe²⁺-TPTZ) had a blue color. The absorbance of the reaction mixture was measured at 593 nm for 4 min. The standard was FeSO₄·7H₂O in methanol. The absorbance values obtained were expressed as *m* moles Fe²⁺ produced per mL [22].

Histopathological examinations

For the histopathology of the liver samples, after the narcotization and decapitation, the pigeons were dissected. The liver tissues were taken and placed into 10% of formalin. Then, it was dehydrated in graded alcohol concentrations and embedded in paraffin. Sections of 5 microns were prepared, stained with hematoxylin and eosin (H&E) technique, and evaluated with a light microscope (Leica DM-2500 biosystem) for assessment of histopathological changes.

Statistical analyses

Data were presented as mean ± standard deviation (SD) (*n* = 10/group). The SPSS software (2019 v26) was used to perform statistical analyses using standard ANOVA techniques, followed by Tukey's HSD post hoc test with *p* < 0.05 as a significant probability level. Pearson's correlation coefficient analysis was conducted to determine the strength of association among parameters.

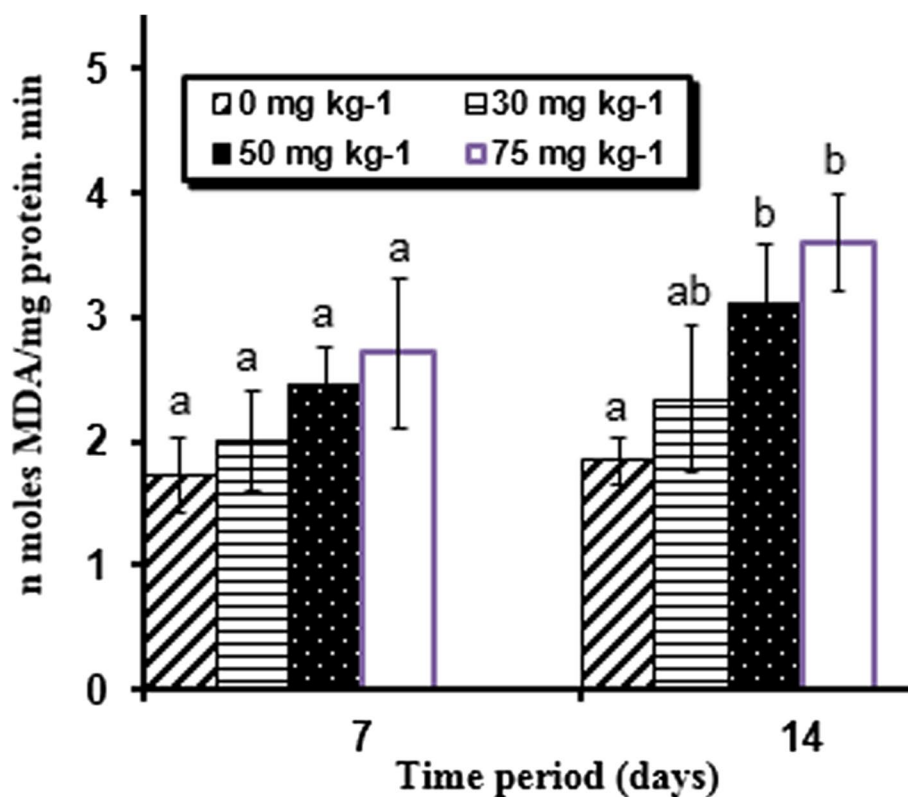


Fig. 2 The level of malondialdehyde (MDA/LPO) in feral pigeons exposed to nano-ZnO after 7 and 14 days. **a, b** Data not sharing a common letter are significantly different ($p < 0.05$) between treatments in the same exposure time

Results

During the experimental period, no clinical and physical symptoms were monitored in the tested pigeons.

MDA level

An increase was recorded in the MDA/LPO level over a time exposure of 7 and 14 days, compared with the controls (Fig. 2). The MDA level increased in a concentration-dependent manner. The highest MDA level was obtained in 75 mg/kg of nano-ZnO-treated groups after 14 days by 95.65% ($p < 0.05$). The elevated MDA level was greater in groups with 14 than 7-day exposures (Fig. 2). A positive correlation was observed between nano-ZnO increasing concentrations and elevated MDA level ($r = 0.81$, $p < 0.05$) after a 14-day exposure to nanoparticles.

CAT activity

According to Fig. 3, the CAT activity was elevated in all treated groups compared to controls in a concentration-dependent trend. The elevation was found to be

significant only in 75 mg/kg groups after 14 days (24.14%, $p < 0.05$). The correlation between nano-ZnO increasing level and elevated activity of CAT was $r = 0.87$ ($p < 0.05$) (Fig. 3) following a 14-day exposure to nano-ZnO.

Hepatic necrosis markers

As shown in Fig. 4, the level of ALT increased concentration dependently in the blood sera samples. This elevation was significant ($p < 0.05$) in all treated groups except 30 mg/kg after 7 days. The highest ALT level was obtained in 75 mg/kg nano-ZnO-treated groups after 14 days by 58.04% ($p < 0.05$). A strong correlation ($r = 0.91$, $p < 0.001$) was observed between increased ALT levels and elevated nanoparticle concentrations after a 14-day exposure.

Following 7 and 14 days, the level of AST in all groups exposed to nano-ZnO was found to be increased significantly ($p < 0.05$). The highest level obtained for AST was in 75 mg/kg nano-ZnO-treated groups after 14 days by 39.31% ($p < 0.05$). A positive value of correlation between increasing nano-ZnO concentrations and

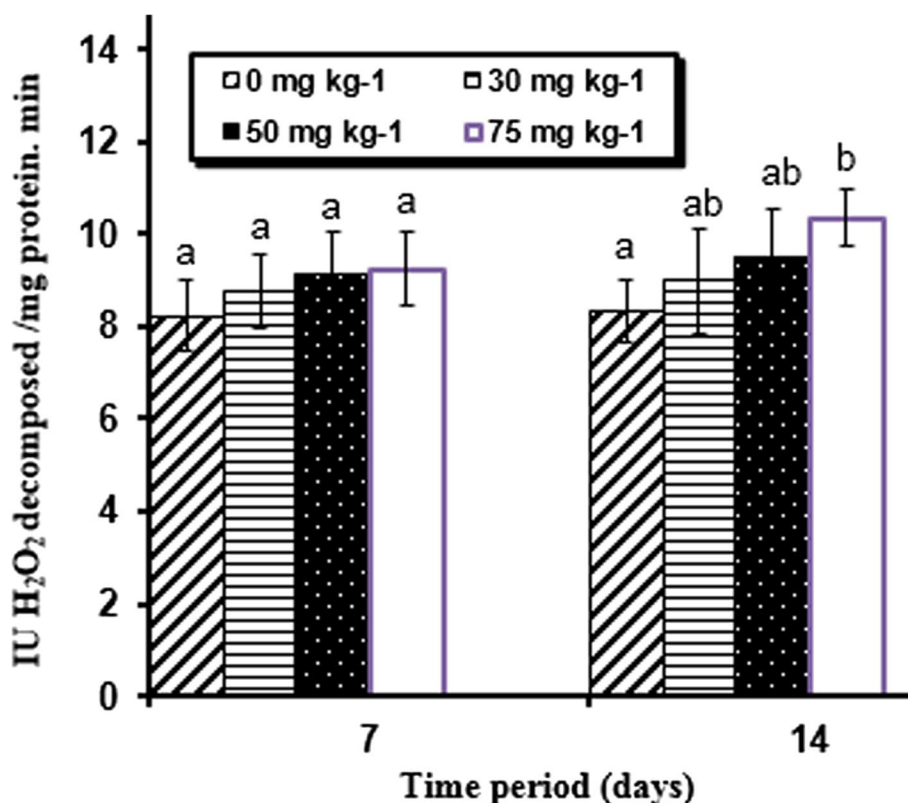


Fig. 3 The activity of catalase (CAT) in feral pigeons exposed to nano-ZnO after 7 and 14 days. **a, b** Data not sharing a common letter are significantly different ($p < 0.05$) between treatments in the same exposure time

elevated AST level was observed ($r = 0.93$, $p < 0.001$) after a 14-day exposure (Fig. 5).

The results showed that there was a clear concentration–response relationship between nano-ZnO treatments and elevated levels of ALP in the pigeon blood sera (Fig. 6). It was found that the increase in ALP level was greater in 14 than in 7-day exposed groups. In addition, the highest level of ALP was observed in the 75 mg/kg nano-ZnO-treated group after 14 days by 36.73% ($p < 0.05$). The correlation between MDA increasing level and elevated ALP level was $r = 0.82$ ($p < 0.05$) following a 14-day exposure (Fig. 6).

TAC content

According to the results in Fig. 7, the total antioxidant capacity (TAC) values of liver samples in feral pigeons exposed to nano-ZnO after 7 and 14 days were found to be reduced upon an increase in the nanoparticle concentrations. It was found that the reduction in TAC values was greater in 14 than in 7-day exposed groups. In

addition, the lowest values of TAC of liver samples were observed in 75 mg/kg nano-ZnO-treated groups after 14 days by 44.74% ($p < 0.05$) (Fig. 8). A negative correlation between TAC value and increasing nano-ZnO concentrations was observed in liver samples by $r = -0.91$ ($p < 0.001$) following a 14-day exposure (Fig. 7).

Histopathological examinations

Figure 8A–D deals with the histopathological observations in pigeon liver samples. Normal architecture in parenchyma and the well-arranged nature of hepatocytes without any inflammatory cell (leukocytes) infiltrations in the interstitial spaces are depicted in Fig. 8A as the control group. Our results indicate that tissue damages in the pigeon liver samples were only seen in pigeons treated with higher concentrations of nano-ZnO viz. 75 mg/kg after 14 days of exposure including inflammatory cell infiltration, swelling area, vacuolization, and expansion of interstitial spaces (Fig. 8B, C, D). These tissue changes, somewhere less, and somewhere more are

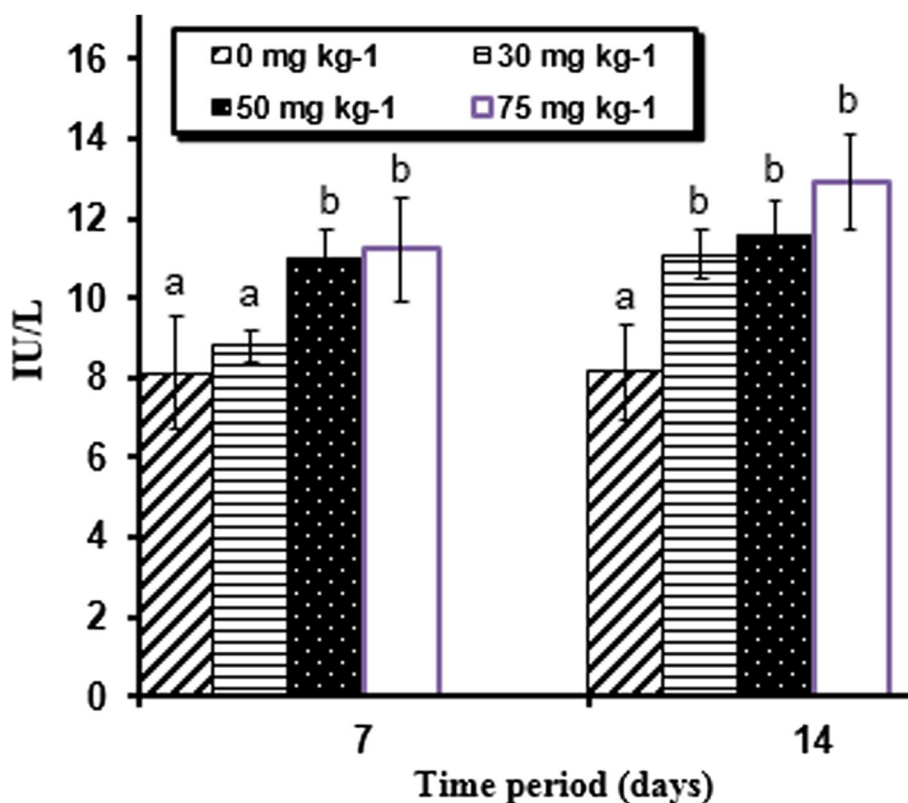


Fig. 4 The level of alanine aminotransferase (ALT) in feral pigeons exposed to nano-ZnO after 7 and 14 days. **a, b** Data not sharing a common letter are significantly different ($p < 0.05$) between treatments in the same exposure time

observed in all specimens of feral pigeons treated with a higher concentration of nano-ZnO compared to the control group where these changes were not present.

Discussion

It is inevitable that during their use, engineered MNPs will be released into the environment. Despite the greater attention on MNPs' increased ecotoxicological risks for terrestrial organisms, evidence regarding their toxic impacts on feral pigeons (*Columba livia domestica*) is still insufficient, and in this research, we defined the hepatotoxicity of nano-ZnO in feral pigeons. NP-mediated ROS generation initiates a sequence of pathological events, including inflammation, genotoxicity, and carcinogenesis, and is modulated by physicochemical features of NPs, such as size, charge, surface area, and chemical structure [3, 23]. Interestingly, in bacteria, *Streptococcus pyogenes*, findings from Liang et al. [24] evidenced that nano-ZnO induces significant ROS generation, LPO propagation, and cell wall disruption with concomitant thiol depletion.

MDA level

The MDA/LPO level is used as a biomarker of OS which reflects indirectly the degree of damage from ROS insult [12]. It has been indicated that exposure to nano-ZnO leads to ROS generation and activation-specific signal transduction pathways in target cells. An imbalance in the ROS production and antioxidant defense system of cells results in interferences with normal cellular processes which cause several different outcomes leading to cell death [25]. There are some reports that attribute the toxicity of nano-ZnO to particle dissolution and subsequent release of Zn^{2+} ions that lead to ROS-mediated injuries such as lysosomal and mitochondria damage and ultimately cell death [26]. Our results also indicate an elevation in the MDA level, in a concentration-dependent manner. Augmented MDA level reflects the induction of LPO chain reaction followed by oxidative damages in pigeon liver samples exposed to nano-ZnO. Accumulation of MDA in organism's tissues results in changes in the permeability of bio-membranes leading to cell dysfunction [11]. In agreement with our results, it

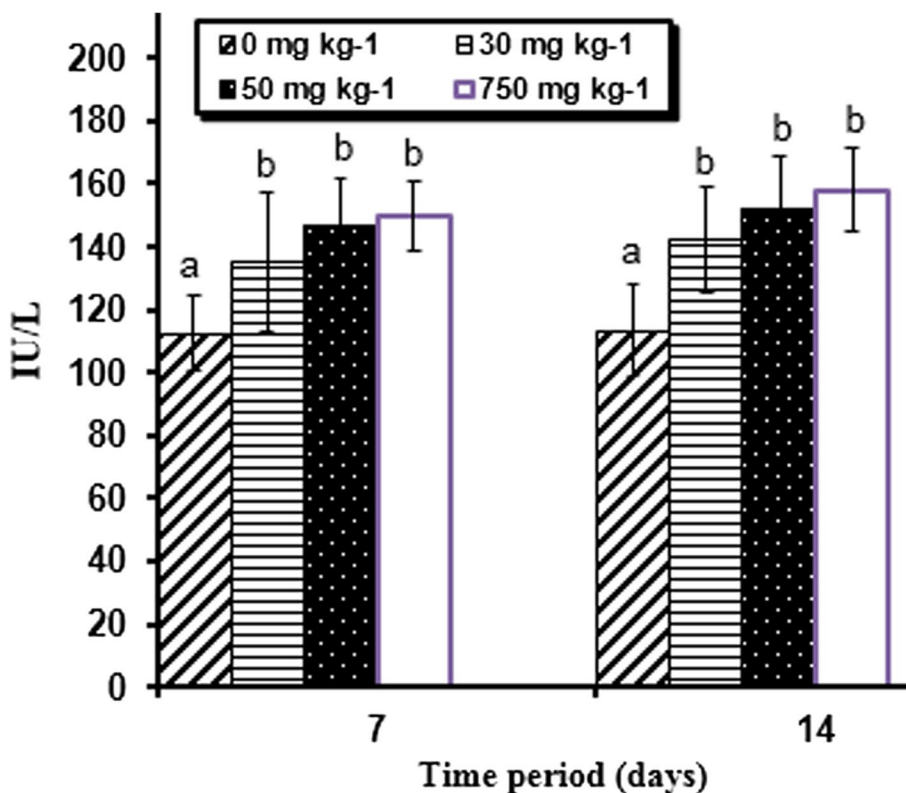


Fig. 5 The level of aspartate aminotransferase (AST) in feral pigeons exposed to nano-ZnO after 7 and 14 days. **a, b** Data not sharing a common letter are significantly different ($p < 0.05$) between treatments in the same exposure time

has been demonstrated that nano-ZnO caused a significant increase in the splenic and thymic MDA levels in rats [1]. In addition, nano-ZnO consumed antioxidants in the liver and induced ROS to affect the structure and function of the endoplasmic reticulum of mouse hepatocytes, which is believed to be related to apoptosis and autophagy [27].

CAT activity

In the present study, the activity of the antioxidant enzyme CAT was found to be elevated which clearly indicated that nano-ZnO was able to induce OS and related damages. CAT is an enzymatic antioxidant that decomposes H₂O₂ and protects the tissue from highly reactive hydroxyl radicals and reduction of the cell membrane fluidity [28]. However, we can assume that such an increase in the CAT activity of liver samples may reflect the detoxification reactions in nano-ZnO-treated pigeons. El-Bahr et al. (2020) demonstrated that in Japanese quails upon stimulation with nano-ZnO to eliminate oxidative damage due to their toxic substances, the CAT activity was increased. They also found that the liver retained a higher

nano-ZnO concentration than that of brain tissue [29]. On the basis of Mittler’s ROS theory, it can be postulated that nano-ZnO exposure resulted in the production of excessive H₂O₂ and lipid radicals in pigeons. In order to adapt to the oxidative status, the CAT activity was increased [30]. Other researchers also explained the role of nano-ZnO in enhancing the antioxidant status in chickens [31].

Hepatic necrosis markers

Blood biochemistry parameters are important markers of physiological status in animal models. The current study demonstrated the increases in the level of ALT, AST, and ALP as pathological indicators. These are considered as hepatic necrosis markers, upsurge in their levels is a potential key indicator for diagnosing various hepatic injuries such as acute alcoholic hepatitis and cirrhosis [32]. Results from our study clearly indicate that the hepatotoxicity may be via necrotic changes induced by nano-ZnO, which in turn leads to increased cell membrane permeability and release of these cytoplasmic enzymes into the pigeon blood

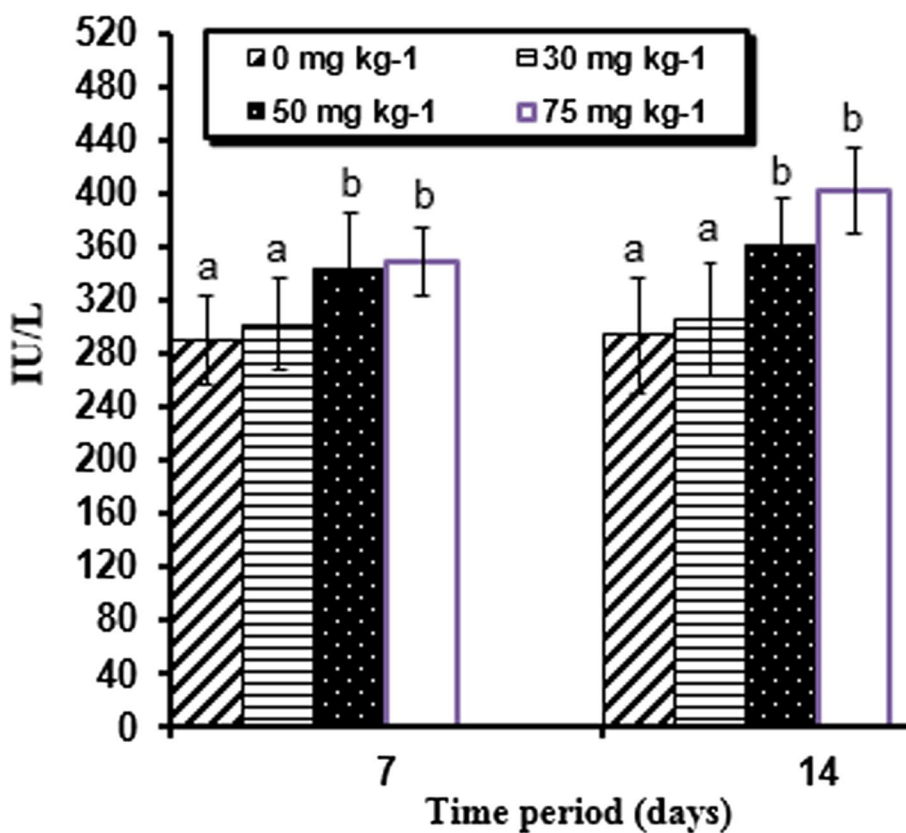


Fig. 6 The level of alkaline phosphatase (ALP) in feral pigeons exposed to nano-ZnO after 7 and 14 days. **a, b** Data not sharing a common letter are significantly different ($p < 0.05$) between treatments in the same exposure time

circulation. Knowing these enzymes as hepatic injury markers was confirmed by close histopathological examination of the hepatic organ that exhibited hepatic tissue injuries and enhanced serum enzyme activities linked with abnormal physiological ailment [33]. This depiction is met with our results and findings of Fazlati et al. [34] who observed the elevation in ALT and AST with the exposure to Zn²⁺ as a trace metal in the Japanese quail. It is reported that continuous exposure to nano-ZnO had toxicity in rats leading to an increase in liver enzymes, such as ALT, AST, and ALP at high doses [35]. On the other hand, the mRNA expression of proinflammatory cytokines such as IL-1 β , IL-10, and TNF- α was found to be potentiated following nano-ZnO supplementation in rats [1]. Increased TNF α may be attributed to the OS induced by nano-ZnO poisoning which is evidenced by a significant increase in MDA level and decreased reduced glutathione (GSH) level in the blood serum of exposed birds [36]. Regarding nano-ZnO toxicity, it is also reported that these NPs dissolve

in the lysosomes which leads to the reduction of pH level. Reduced pH affects lysosomal enzymes involved in protein digestion and increases intra-lysosomal Zn²⁺ level, which leads to lysosomal destabilization and cell membrane instability [37].

TAC content

The TAC value refers to the antioxidative status of an organism and includes the synergic and redox interactions between the different molecules present in the biological fluids [38]. Because the antioxidants do not work alone, the cooperation among the different antioxidants may provide greater protection against ROS insults. Thus, TAC may give more biologically relevant information than that obtained from individual antioxidants [39]. To counteract the oxidative damage, the organism's body is well-equipped with antioxidant defense mechanisms [40]. Cellular non-enzymatic antioxidant defense potentially includes GSH, vitamins C and E, etc. Amongst them, the GSH plays a vital role in the protection of cells against oxidative injury. The

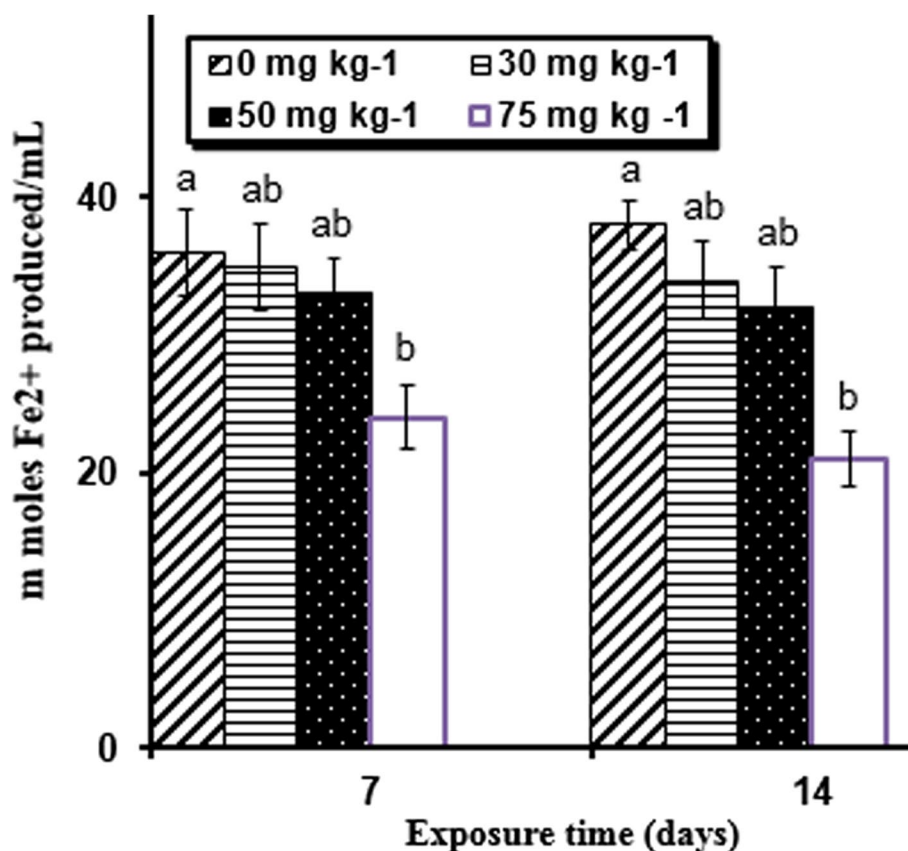


Fig. 7 Total antioxidant capacity (TAC) of the liver in feral pigeons exposed to nano-ZnO after 7 and 14 days. **a, b** Data not sharing a common letter are significantly different ($p < 0.05$) between treatments in the same exposure time

depletion of GSH by toxicants could be due to the direct conjugation of their metabolites with GSH leading to OS [41]. Our results indicate that the TAC value in liver samples of treated pigeons with nanoparticles was decreased in a concentration-dependent manner which can be attributed to the nano-ZnO ability to lower the power of antioxidant defense systems in the liver samples. Hence, it can be concluded that in our study, induction of OS and excessive ROS production in the pigeon's body might be the reason for the antioxidant power depletion (lowered TAC value) in the liver samples under the stress of nano-ZnO intoxication which come due to imbalance in homeostasis between oxidants and antioxidants.

Histopathological examinations

The histologically based endpoints have been shown to be sensitive indicators of the sub-lethal toxicity of chemicals [42]. The liver has the critical job of maintaining the body's metabolic homeostasis. Our results indicate that the liver samples of pigeons treated with

higher concentrations of nano-ZnO viz. 75 mg/kg were found to have different tissue damages. Resulting damages may lead to hepatic dysfunction and the release of hepatic necrosis markers (ALT, AST, and ALP) into the blood circulation as well. Apparently, the histopathological changes in the liver samples were the result of tissue response to the induction of OS signaling pathways due to an imbalance between ROS production and antioxidant defense function [7, 43]. We assume that the liver is a sensitive organ to nano-ZnO toxicity, as evidenced here by elevated hepatic necrosis markers and alterations of other biochemical parameters, such as LPO and enzymatic antioxidant activities after nano-ZnO oral ingestion. Our findings about the histology of the liver of feral pigeons are in accordance with the results of Dashti et al. [44] and Wang et al. [45]. In broiler chicks, exposure to high doses of ZnO resulted in pronounced changes in the liver such as connective tissue hyperplasia, liver cell hypertrophy, and accumulation of inflammatory cells [46].

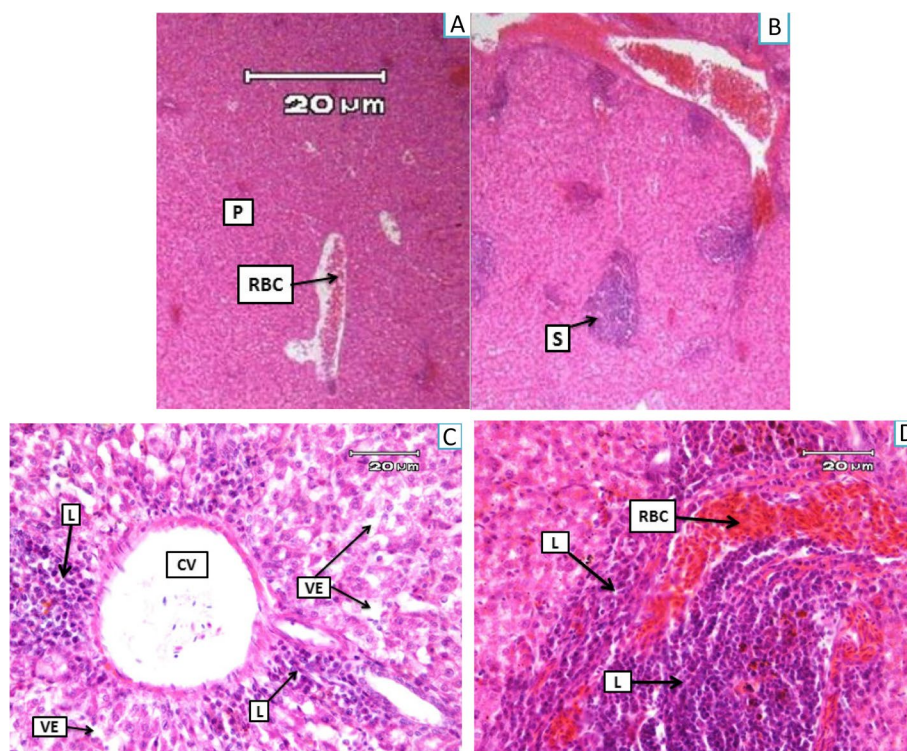


Fig. 8 Histopathological changes in microscopic view of the liver of feral pigeon in control group with normal structure (A, 10×) and treated with 75 mg/kg of nano-ZnO (B 10×, C, D 40×) stained with H&E. CV central vein, L leukocyte infiltration (inflammation), P normal hepatic parenchyma, RBC red blood cells, S hepatocyte swelling area, VE vacuolization and expansion of interstitial spaces

Conclusion

The findings of the present study call to reconsider the potential effects of nano-ZnO on pigeons. We showed that the nano-ZnO is injurious to the pigeon's health at different concentration levels. Overall, our findings demonstrated that nano-ZnO could induce OS and have adverse effects on liver function and structure in the exposed pigeons. However, studies regarding the effect of nano-ZnO in pigeons are insufficient and require further investigation.

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Authors' contributions

MA designed and supervised the study and drafted the original manuscript. HRN did the investigation. Both authors read and approved the final manuscript.

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Availability of data and materials

Data are available with the corresponding author to be presented upon request.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Research Ethical Committee of Shahrekord University, Iran; SKU-FBS/1389-91. All animal experiments have 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 September 22, 2010.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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