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ORIGINAL ARTICLE

The Influence of Zinc Oxide Nanoparticles on Blood Markers in Domestic Pigeons (*Columba livia*)

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KEYWORDS

ZnO nanoparticles;
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ABSTRACT: The analysis of blood provides a minimally invasive way to gain an insight into the health status of an organism. Organisms that have become closely associated to human housing are at greatest risk of being affected by anthropogenic pollutants. The purpose of this study was to determine the alterations in blood markers of domestic pigeon (*Columba livia*) after oral administration of zinc oxide nanoparticles (n-ZnO). The birds were acclimatized to standard conditions with a photoperiod of 12:12 hr at 20±2ºC for 14 days. Birds were randomly assigned to one control and three experimental groups (in triplicate). The experimental groups orally received 30, 50, and 75 mg kg^{-1} b.w. of n-ZnO (1 ml/bird, daily) through oral gavage for 7 and 14 consecutive days. The oxidative stress (OS) biomarkers namely LPO/MDA level and CAT activity in the blood plasma samples with the activity of LDH were increased in a concentration-dependent manner. Meanwhile, total antioxidant capacity (TAC) of blood plasma and blood δ-ALAD activity were found to be lowered. Collectively, n-ZnO could affect the blood markers of pigeons, where oxidative damages may be the potential mechanism underlying this intoxication.

INTRODUCTION

Anthropogenic pollution in ecosystems has become a great problem today in many countries. Therefore, pollution monitoring and assessment is of paramount value. Avian species are useful organisms for this purpose, as they are representative of every polluted area and accumulators of environmental pollutions. Harmful chemicals released into the atmosphere by factories and other human activities contaminate our climate, food and water resources. Birds are abundant in ecosystems and present in large numbers and various forms. As these organisms have become closely associated with human activities, they are at high risk of being contaminated. One such species is the feral pigeon (*Columba livia*), which is closely associated with urbanized areas and is considered an indicator organism for monitoring pollutants in urban and industrial environments [1, 2]. Because of their commercial importance, these animals must be protected from environmental pollutants [3].

The pressure upon pollution of nanoparticles (NPs) that anthropogenic activities exert on the environment substantially affects the function and stability of an ecosystem. Moreover, growing urbanization irreversibly alters the foundation and ecological processes of natural habitats. NPs possess a high surface-to-volume ratio, and

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large number of reactive sites on the surface exerts a mild and/or severe toxicity to organisms [4]. Among NPs, metallic ones (MNPs) mainly include metal and metal oxide nanoparticles. MNPs have been included in a wide variety of products and fields, such as electronic devices, cosmetics, paints, additives in food, and biological and medical systems [5]. These particles have garnered great interest, because they can enter an organism's body through different routes such as skin, inhalation, and the gastrointestinal tract and eventually accumulate in various tissues [6]. Therefore, there is an urgent need for information on the ecological risks of MNPs. Earlier, it waas demonstrated that MNPs are capable of inducing excessive generation of reactive oxidative species (ROS) through modifications in cellular reactions [7] or alterations in the antioxidant defense system, resulting in oxidative damages to cellular compartments [8,9].

Zinc oxide nanoparticles (n-ZnO) as MNPs are well known as antibacterial agents because of ROS production in microorganisms. Therefore, their release into the environment is expected to raise major concern about toxicity in terrestrial organisms. The n-ZnO has high reactivity and is responsive with high absorptivity [10]. Once n-ZnO is absorbed, elevated levels of Zn^{2+} can be recorded in different tissues of animals [4]. Jiang et al. [11] demonstrated that with the quenching of free radicals, the antimicrobial effect of n-ZnO is suppressed. Zinc toxicity results from chronic and/or repeated exposure. If dietary exposure is excessive and homeostatic mechanisms fail, zinc toxicity can occur. In birds, concentrations beyond 4 mg kg^{-1} of zinc in blood plasma are suggested as in the range of toxicose. However, with zinc contamination, birds may be clinically affected without elevated concentrations in plasma [7].

According to the WHO, a biomarker is the subset of measurable indicators which may be chemo-physical and/or biological in nature, and the measurement may be functional, physiological, biochemical, cellular, or molecular. The biomarkers reflect an interaction between a biological system and an environmental agent like NPs. Unlike other tissues, blood may be used for non-destructive bio-monitoring. The analysis of blood provides a minimally invasive way to gain insight into the health status of an individual [12]. Blood sampling is often an essential part of biological investigations. In birds, blood sample evaluations are used to respond to questions about the alterations in plasma hormonal levels, molecular evolutionary studies, metabolic status, clinical examinations, migratory tracking, etc. [13].

Oxidative stress (OS) is a common mediator in pathogenicity of diseases, defined as an imbalance between the production of ROS and scavenging ability of antioxidants, leading to a disruption of redox signaling and damages to cellular components. The reactivity of ROS varies a lot; some are more corrosive and selective in their reactivity with biological molecules, e.g., hydroxyl and superoxide radicals, than others. In particular, lipid peroxidation (LPO) is considered a hallmark of OS in which ROS interact with membrane lipids leading to the formation of lipid by-products such as malondialdehyde (MDA), which then cause damages to components of cell membrane and other macromolecules such as proteins and DNA. MDA is a highly reactive dialdehyde generated mainly by the peroxidation of membrane polyunsaturated fatty acids. MDA as a by-product of the LPO process causes structural modifications in the macromolecules which alter the membrane permeability and electrolyte balance in cells. Antioxidant are defined as any mechanism, structure and/or substance that prevents, delays, removes or protects against oxidative non-enzymatic chemical modifications to a target molecule. The antioxidant defense systems, enzymatic and non-enzymatic agents have a key role in the defense against oxidative damages caused by ROS in cells [14-16]. The LPO process and MDA are the initial steps of cellular membrane damage and are considered valuable indicators of oxidative damage to cellular components. Excessive accumulation of MDA changes the permeability of cell membranes and disrupts DNA and proteins resulting in the cell death [15].

Birds are considered as befitting and excellent fauna for assessing the induced toxic effects of different pollutants as well as very important responsive bio-indicators of contamination and their effluence in the surrounding. They rank relatively high on the food chain and are important structural components of the ecosystem [17]. Birds exposed to n-ZnO show adverse alterations in the function of certain tissues leading to different complications [18]. Despite the growing literature on NP applications, the information about the toxicological effects of n-ZnO in birds is still scanty. The current study aimed to evaluate the impact of n-ZnO on blood markers in domestic pigeon (*Columba livia*) under constant laboratory conditions.

MATERIALS AND METHODS

Chemicals and experimental design

The n-ZnO with a diameter size of ≤ 20 nm, spherical shape, and 99% purity, was purchased from Nano-shop Company, Tehran, Iran. The other chemicals were obtained in reagent grade from Sigma-Aldrich Chemical CO. (St. Louis, MO, USA). First, the n-ZnO suspension was well mixed in a sonicator (SONOPULS HD 2070.2, BANDELIN Electronic GmbH & Co KG, Germany) for 20 min. For every administration, the suspension was again mixed by vortex set for 1 min.

Healthy domestic pigeons (*Columba livia*), with average body weights of 300-350 gr, were purchased from local bird market, Shahrekord, Iran. Birds were reared in clean wooden cages with metal nets in the animal physiology laboratory, Department of Animal Sciences, University of Shahrekord, Iran. Thereafter, birds were allowed to acclimate to the facilities with a 12:12 hr photoperiod at 20±2 ˚C with 50% humidity for 14 days prior to the experiment was begun. Seed mixture and tap water were provided *ad libitum*. After acclimatization, pigeons were randomly divided into two subgroups, designated one control (0 mg kg^{-1} b.w. of n-ZnO) and three experimental groups (in triplicate) having 10 pigeons each. The experimental groups received 30, 50, and 75 mg kg^{-1} b.w. of n-ZnO solutions daily for 7 and 14 consecutive days. A total volume of dosing solution (1 ml per bird) was orally gavaged into the esophagus (Figure 1). The control group received tap water for the same time periods. Toxicity symptoms and possible mortalities were recorded twice daily throughout the study.

Blood plasma samples

The blood samples, about 2-3 ml, were collected by puncturing the brachial vein with a heparinized syringe and were then placed into test tubes containing 0.02 ml heparin/ml blood for centrifugation (10000 ×g, 4˚C for 10 min). The resulting blood plasma was used to determine changes in the blood markers throughout the study. The plasma samples were stored at −80 ˚C until further analyses.

Malondialdehyde (MDA) level

The LPO/MDA assay [19] was performed according to the reaction with thiobarbituric acid (TBA) in an acidic pH at 90 ˚C. In the test reaction, MDA reacts with TBA and produces a pink chromophore with maximum optical density at 532 nm. The blood samples were joined with butylated hydroxytoluene (BHT) and TCA, then incubated at room temperature for 10 min and centrifuged (4000 ×g, 10 min). The supernatants reacted with a mixture of TBA and TCA in a boiling water bath for 20 min. After cooling, the absorbance was read at 532 nm using a UV-1700 spectrophotometer (Shimadzu, Japan). The results were calculated using an index of absorption for MDA of 1.56×10^5/M/cm.

Catalase (CAT) activity

The activity of CAT (EC. 1.11.1.6) was determined according to the procedure explained by Luck [20]. The enzyme activity in the reaction mixture containing H_2O_2 phosphate buffer and blood plasma samples were recorded spectrophotomerically at 240 nm.

Lactate dehydrogenase (LDH) activity

LDH activity can be quantified using the NADH produced during the conversion of lactate to pyruvate to reduce a second compound in a coupled reaction into a product with properties that are easily quantitated. Briefly, blood plasma samples were incubated at 37 ˚C with 2, 4-dinitrophenylhydrazine (DNPH) and 0.4 N NaOH solutions and brownish color was developed. Sodium pyruvate was used as the standard and the absorbance of the samples was recorded at 440 nm [21].

δ-aminolevulinic acid dehydratase (δ-ALAD) Activity

ALAD (EC. 4.2.1.24) activity was estimated according to the method of Berlin and Schaller [22]. The principle of this method was based on incubation of the enzyme (whole blood) with substrate δ-aminolevulinic acid (ALA) for 50 min at 37 ˚C. ALAD activity was evaluated by measuring the amount of porphobilinogen (PBG) formed under the assay conditions. The reaction was stopped by the addition of TCA 10% containing $HgCl_2$ 0.05 M. The produced PBG, was mixed with a modified Ehrlich's reagent, and the developed color was measured spectrophotometrically at 555 nm against a blank.

Determination of total protein concentration

Bradford's method was applied, with bovine serum albumin (BSA) and diluted in 100 m mol L^{-1} Tris HCl buffer (pH = 7.4), as standard at absorbance of 595 nm. Results were expressed as mg protein per ml of blood plasma [23].

Total antioxidant capacity (TAC) content

The TAC value of blood plasma samples was measured using the FRAP (Ferric reducing ability of plasma) test [24]. In this method, yellow ferric tripyridyltriazine complex (Fe (III)-TPTZ) is reduced to blue ferrous complex (Fe (II)-TPTZ) by the action of plasma antioxidant power. First, 1.5 ml of FRAP reagent was incubated at 37 ˚C for 5 min. Then, 0.1 ml of blood plasma sample was added and incubated at 37 °C for 10 min till blue ferrous tripyridyltriazine complex (Fe^{2+}-TPTZ) developed. The optical density was read at 593 nm for 4 min. The $FeSO_4.7H_2O$ in methanol was used as the standard.

Statistical analyses

Data were presented as Mean±Standard Deviation (SD) (n=8 per group). SPSS software (2020) was used for statistical analyses using standard ANOVA techniques with Tukey's HSD post hoc test. Pearson's correlation coefficient analysis was also conducted to determine the strength of association among parameters. A value of $P<0.05$ was considered statistically significant.

Figure 1. n-ZnO administration through oral gavage using a cannula.

RESULTS

Toxicity signs

At the end of experiments, no toxicity signs and/or mortalities among the tested pigeons were recorded.

MDA level

Shown in In Figure 2, MDA level is increased in a concentration-dependent trend. The highest value for MDA level was seen in groups exposed to 75 mg kg^{-1} n-ZnO after 14 days by 64.13% (P<0.05). The augmented MDA level was greater in groups with 14 days of exposure than in the 7 day group. The correlation between increases in n-ZnO concentrations and augmented MDA level was r = 0.84 (P<0.05) after 14 days of exposure to n-ZnO.

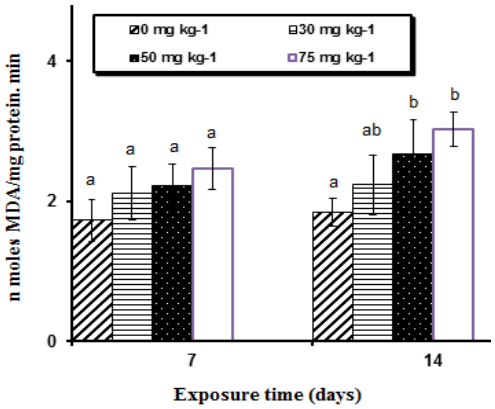

Figure 2. Llevel of malondialdehyde (MDA/LPO) in blood plasma of domestic pigeons exposed to n-ZnO after 7 and 14 days. [a,b]: data not sharing a common letter are significantly different (P<0.05) between treatments with the same exposure time.

CAT activity

As shownd in Figure 3, CAT activity increased in all treated groups in a concentration-dependent manner. The elevation was found to be significant only in 50 and 75 mg kg^{-1}-treated pigeons following 14 days by 26.52% and 29.56%, respectively (P<0.05). The correlation between n-ZnO level and increased activity of CAT was r = 0.85 (P<0.05) (Figure 3) following exposure to n-ZnO for 14 days.

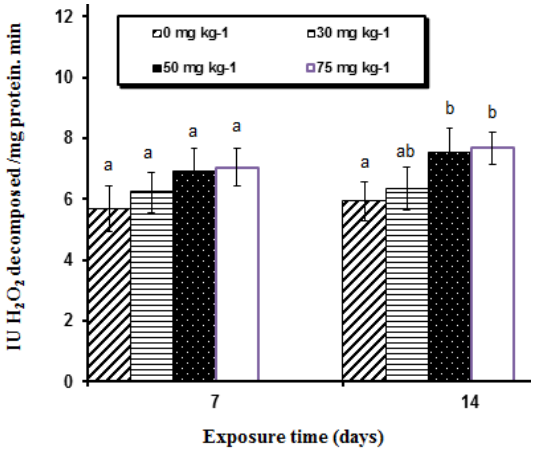

Figure 3. Catalase (CAT) activity in blood plasma of domestic pigeons exposed to n-ZnO after 7 and 14 days. [a,b]: data not sharing a common letter are significantly different (P<0.05) between treatments with the same exposure time.

LDH activity

As Figure 4 shows, the activity of LDH increased with increases in n-ZnO concentration. The significant increase was recorded only in the group expose to 75 mg kg^{-1} n-ZnO following 14 days by 22.48% (P<0.05). The correlation between increased LDH level and elevated n-ZnO concentrations was found to be r = 0.81 (P<0.05) after 14 days of intoxication.

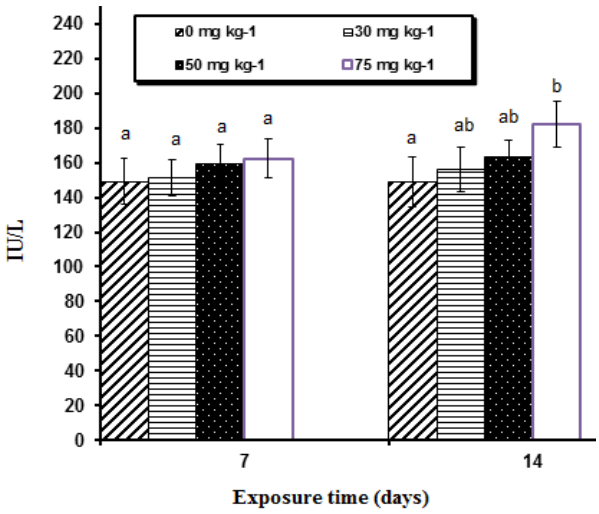

Figure 4. Lactate dehydrogenase (LDH) activity in blood plasma of domestic pigeons exposed to n-ZnO after 7 and 14 days. [a,b]: data not sharing a common letter are significantly different (P<0.05) between treatments with the same exposure time.

δ-ALAD Activity

According to Figure 5, decreases in δ-ALAD activity in all groups exposed to n-ZnO were not significant (P>0.05). The lowest value obtained for δ-ALAD activity was seen in the group treated with 75 mg kg^{-1} n-ZnO after 14 days by -11.14% (P>0.05). The correlation between increasing n-ZnO concentrations and lowered enzyme activity was r = -0.77 (P<0.05), after 14 days exposure.

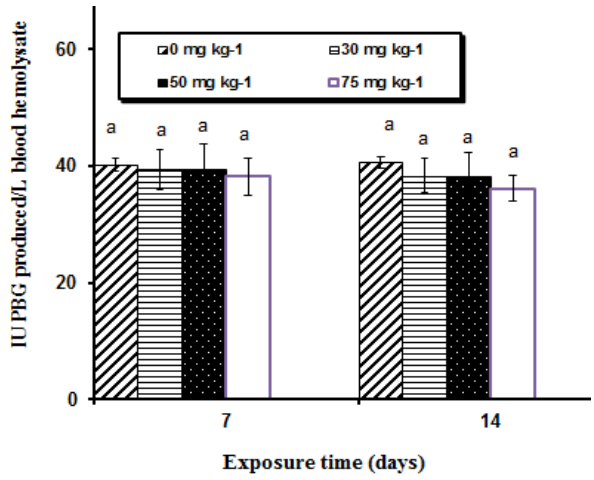

Figure 5. δ-aminolevulinic acid dehydratase (δ-ALAD) activity in blood plasma of domestic pigeons exposed to n-ZnO after 7 and 14 days. [a,b]: data not sharing a common letter are significantly different (P<0.05) between treatments in the same exposure time.

TAC content

On the basis of results shown in Figure 6, the TAC values of blood plasma in domestic pigeons were reduced in a concentration-dependent trend after 7 and 14 days. The reduction in TAC values was more remarkable in the 14 day than 7 day treated groups. The lowest value of TAC was obtained in the group exposed to 75 mg kg^{-1} n-ZnO after 14 days by 41.82% ($P<0.05$). A negative correlation between TAC value and increasing n-ZnO concentrations was observed by r = -0.93 ($P<0.001$) after 14 days of exposure to nanoparticles (Figure 6).

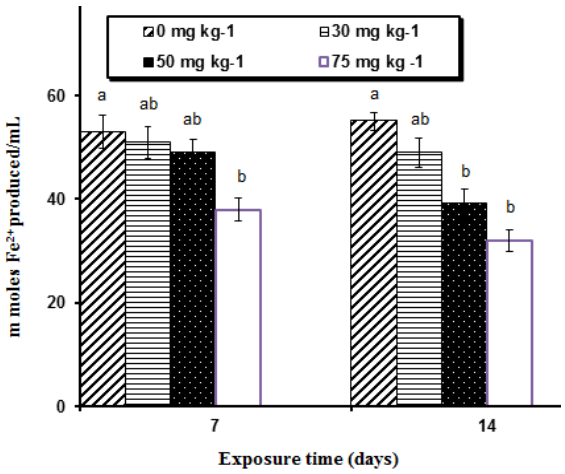

Figure 6. Total antioxidant capacity (TAC) content of blood plasma in domestic pigeons exposed to n-ZnO after 7 and 14 days. [a,b]: data not sharing a common letter are significantly different ($P<0.05$) between treatments with the same exposure time.

DISCUSSION

In modern life, environmental pollution as a result of anthropogenic activities has greatly augmented the global availability and distribution of NPs. As commercial interest in NPs is growing, research efforts have invested in assessing the potential deleterious impacts of nanomaterials in living systems. NPs are capable to generate ROS by direct and indirect chemical reactions. The pro-oxidant effects of NPs have been reported to use a series of intracellular chemical and physical reactivities which contribute to a diverse range of cellular responses [25]. Blood biochemistry parameters are important markers of physiological status in animal models. Data regarding the influence of n-ZnO on blood biochemistry in domestic pigeon (*Columba livia*) are still insufficient and the current study defined the toxicity of these NPs on the blood markers in these birds.

MDA level

LPO plays a prominent role in the pathogenesis of many diseases and tissue injuries induced by several toxic substances. Many examples of NPs including MNPs are able to induce ROS production as one of the principal cellular mechanisms of their toxicity. Once NPs gain access to the mitochondria, they stimulate ROS through impaired electron transport chain, structural damage, activation of NADPH-like enzyme system, and depolarization of the mitochondrial membrane [25]. Singh et al. [10] evidenced that n-ZnO induced significant ROS generation, protein oxidation and DNA damage with concomitant thiol depletion leading to OS. Determination of MDA level is used as an OS biomarker which reflects indirectly the degree of damages from ROS-induced LPO in blood and other tissues. The MDA is a moderately toxic and reactive molecule which gently interacts with thiols and amino groups leading to stable adduct proteins, which cause cell inactivation, so if it is not removed, it can promote pathological processes. A basal level of LPO may

contribute to the formation of this adduct, and an elevated level of MDA would be indicative of a pathological condition [15, 26]. It is well known that n-ZnO administration leads to a subsequent release of Zn^{2+} ions causing ROS-mediated injuries such as cell membrane and mitochondrial damages and ultimately apoptosis [27]. In the current study, it is shown that concentration-dependent elevations in MDA level reflects oxidative damages in the blood plasma samples of pigeons exposed to n-ZnO. It was assumed that the augmented MDA level is related to increased oxidation rate in tissues following the presence of n-ZnO. MDA-heamoglobin covalent cross links may lead to RBC deformity which, in turn, may lead to impaired oxygen supply and accelerated aging of RBCs. Following RBC deformities, the function of the RBC in removing toxic metabolites from the cell is also impaired [28]. MDA may also contribute to inflammation by activating several pro-inflammatory cytokines and adhesion molecules [29]. As a result of the oxidative damages to the cell membrane components such as proteins and lipids, changes in membrane permeability occur, leading to hemoglobin leakage [28]. In other tissues, it has been documented that n-ZnO could cause a marked elevation in splenic and thymic MDA levels in rats [4].

CAT activity

In addition to oxidative modifications in lipids, proteins, and nucleic acids, other variables such as the activities of antioxidant defense enzymes are used as biomarkers of OS [25]. Antioxidants have the ability to scavenge free radicals and reduce and/or inhibit oxidative damages caused by ROS. These defensive substances are important indicators of ROS insult. In the first line of defense against ROS insult, antioxidants such as CAT function as scavenging enzymes. In the aerobic cells, CAT is expressed mainly in peroxisomes and shows its activity on RBCs and other tissues by disintegrating H_2O_2 to prevent the accumulation of methemoglobin [26, 30]. In the present study, the activity of CAT was increased which clearly indicated that n-ZnO is capable of inducing OS and related damages. However, it can be postulated that this increase in the CAT activity of blood plasma samples may reflect the detoxification reactions in n-ZnO treated pigeons. In Japanese quails, El-Bahr et al. [31] demonstrated that after exposure to n-ZnO, oxidative damages and increased CAT activity occurred in blood samples. On the basis of Mittler's ROS theory, it can be postulated that at n-ZnO exposure resulted in the production of excessive H_2O_2 and lipid radicals in pigeons. In order to adapt to the oxidative status, the CAT activity was increased [32]. Under elevated OS status, a general presumption is that any increase in the activities of antioxidant systems such as CAT indicates cellular damages following oxidative chain reactions [33].

LDH activity

Plasma enzymes are of diagnostic importance in toxicological studies. Increases in the plasma concentrations of these enzymes are proportional to the expanse of tissue damage. Moreover, this increase is indicative of a loss of functional integrity of tissues cell membranes and consequent leakage into the blood circulation. The current study demonstrated the increase in LDH levels as a pathological indicator in blood plasma samples of pigeons. In energy production, the enzyme LDH catalyzes the conversion of lactate to pyruvate into the cells. Some of the organs relatively rich in LDH are the RBCs, kidney, liver, muscle tissue, etc. Following cell death with expansion of cell membrane permeability, LDH is released into the blood stream and utilized as a sensitive indicator of cellular damages lasting for a long period [34]. Accordingly, results from the current study clearly indicate that the toxicity of n-ZnO may occure damage to different tissues, particularly muscles, leading to an increase in cell membrane permeability, allowing LDH to enter the pigeon blood stream. Meanwhile, it is also reported that these NPs affect lysosomes, which leads to lysosomal destabilization and cell membrane instability, allowing the release of enzymes into the blood [35].

δ-ALAD activity

Chemicals with the potency to disturb the heme biosynthetic pathways have been used for many years as biomarkers for detecting the sub-lethal toxicity of organic and inorganic toxicants in animal models. The δ-aminolevulinate dehydratase (δ-ALAD) (EC. 4.2.1.24), also known as porphobilinogen synthase, catalyzes the first common reaction in the biosynthesis of all tetrapyrrole pigments, namely the asymmetric condensation of two molecules of δ-aminolevulinic acid (δ-ALA), yielding to the mono-pyrrole product porphobilinogen. δ-ALA as a thiol-containing enzyme can be oxidized under OS condition resulting in inactivation. Therefore, δ-ALA-D can play an important role as a marker of OS and in the impairment of metabolic processes [36]. Therefor, the evaluation of ALAD activity could be a diagnostic bioassay to assess anemia induced by environmental exposure to NPs. In line with this, during the current study, it was revealed that the activity of δ-ALAD was reduced insignificantly in all treated groups. Once OS is increased, the activity of δ-ALAD may be reduced, leading to the accumulation of δ-ALA, which, in turn, may aggravate the production of ROS, contributing to more OS. Therefore, this enzyme may be proposed as an indirect marker of oxidative processes, because the modulation in the activity of δ-ALAD contributes to the global level of OS [37, 38]. It is demonstrated that the cysteinyl residues in δ-ALAD are highly sensitive to ROS and pro-oxidant conditions that enhances formation of disulfide cross-bridges and enzyme inhibition as well. Therefore, the sulfhydryl enzyme δ-ALAD can play an important role as a useful marker for oxidative impairments in metabolic processes [39]. Thus, it can be assumed that the inhibition of δ-ALAD activity in n-ZnO-treated pigeons may be a consequence of oxidation of essential cysteinyl residues of δ-ALAD by ROS insult, resulting in decreased activity and, ultimately, anemia. Furthermore, δ-ALAD activity correlates negatively with blood metal concentration and can be used as a specific biomarker for lead and copper monitoring in pigeons [1].

TAC content

The precise antioxidant power of an organism can be determined by the measurement of TAC content [40]. The current results indicate that the TAC values in blood plasma samples of treated pigeons with n-ZnO was lowered in a concentration-dependent manner, which may be attributed to the n-ZnO capability of reducing the capacity of antioxidant defense systems in blood plasma. It is well known that the FRAP method is as a novel method for assessing TAC content, and a useful indicator of the body's antioxidant status to overcome ROS-induced oxidative damages. The advantage of the FRAP method is in being sensitive in the measurement of total antioxidant power of biological fluids with highly reproducible results [40]. TAC content considers the cumulative effect of all antioxidants present in blood and other body fluids. TAC test is the measure of the amount of ROS scavenged by a bio-fluid like blood plasma. TAC value is the result of many variables such as redox status of the compounds present in the test solution, cumulative and synergistic interactions, type of stressor, nature of pro-oxidants and oxidants, antioxidant localization, etc. [41]. The antioxidants work in synergy and help the body be protected against oxidative damages. The non-enzymatic antioxidant defense system potentially includes GSH, vitamins C and E, etc. Of all these, GSH plays a prominent role in cellular defense to combat oxidative damages. Thus, more depletion of GSH pool results in more OS status [42]. It is notable that any increases in the antioxidant capacity of blood plasma may not necessarily be a desired condition if it reflects a response to increased OS. Similarly, any decreases in the blood plasma antioxidant capacity may not necessarily be an undesirable condition if the measurement reflects decreased production of ROS [43]. However, it can be concluded from the current study that the induction of OS and excessive ROS production in a pigeon’s body might explain the antioxidant power depletion as reduced TAC content in the blood plasma samples under the stress of n-ZnO.

CONCLUSIONS

Findings in the present study identified the harmful effects of n-ZnO on domestic pigeons. The results showed that n-ZnO is injurious to pigeon's health and capable of inducing OS in pigeon's blood plasma. n-ZnO intoxication reduced the antioxidant power in the exposed pigeons. Furthermore, n-ZnO may alter the enzymatic chain of heamoglobin synthesis leading to anemia. For detailed elucidation of n-ZnO-related deteriorations in pigeons, however, further research is needed.

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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.

Conflict of interest

The authors declare that there is no conflict of interest

Authors'contributions

M A designed and supervised study and drafted original manuscript. HR N performed experiments. All authors read and approved the final manuscript.

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