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ORIGINAL ARTICLE

Patho-biochemical Markers of Renal Toxicity in Homing Pigeons (*Columba Livia f. Urbana*) Induced by Nano-ZnO Particles

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KEYWORDS

Birds;
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ABSTRACT: Nanotechnology, the main technology in the twenty-first century, is the perception and control of matter at the dimensions 1-100 nanometers which revolutionized many aspects of modern life. Birds have not been used as commonly animal models in experimental toxicology but have proven useful in monitoring of environmental quality. This study aimed to evaluate zinc oxide nanoparticle (nano-ZnO) toxicity on kidneys in the homing pigeons (*Columba livia f. urbana*). The experimental groups orally received doses of 0, 30, 50, and 75 mg kg^{-1} b.w. of nano-ZnO (1 ml per bird, everyday) for time periods of 7 and 14 days. The lipid peroxidation (MDA/LPO) content, catalase (CAT) activity, and carbonyl protein (CP) content were increased and the total antioxidant capacity (TAC) level was lowered in kidney samples. The plasma levels of uric acid, urea, and creatinine were also slightly elevated ($p>0.05$). Histopathological examinations showed glomerular nanoparticle aggregation and tubular necrosis lines in the kidney parenchyma. In brief, nano-ZnO affected kidney function and structure in homing pigeons through the induction of oxidative stress.

INTRODUCTION

Nanotechnology, the main technology in the twenty-first century, is the perception and control of matter at the dimensions 1-100 nanometers which revolutionized many aspects of modern life. Nanotechnologies are based on the manipulation and integration of atoms and molecules to form different structures and systems at the nanoscale. Nanoparticles (NPs) can enter the environment from natural sources and anthropogenic activities. On the basis of high surface area to volume ratio of NPs, they have known as toxic materials to organisms. During life cycles, NPs may release into the surrounding environment from synthesis and consumption processes [1, 2]. Induction of oxidative stress (OS) as a possible mechanism for damaging cells by NPs is well evidenced. Due to the high chemical reactivity, NPs cause an upgraded creation of reactive oxygen species (ROS) that is believed as a pivotal cause for their intoxication resulting in the related damages lipids, proteins, and genome [2]. The physiological level of ROS is balanced by the function of antioxidant molecules such as reduced glutathione (GSH), vitamin E, catalase (CAT), glutathione S-transferase (GST), etc. Following the occurrence of OS, elevated lipid peroxidation (LPO) chain-reaction and its by-product namely malondialdehyde (MDA) are deleterious to the cellular membrane integrity. Any accumulation of MDA in cells leads to disruption in the cell membrane [3, 4]. The metallic NPs such as nano-Zinc oxide particles (nano-ZnO) are attractive because of their wide range of uses. Nano-ZnO is used generally in veterinary applications due to their antimicrobial and angiogenic effects in the wound healing. The nano-ZnO dissolves in

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the extracellular region, which in turn increases the intracellular [Zn^{2+}] level. Cytotoxicity, increased ROS and OS, decreased mitochondrial membrane potential, and apoptosis, are accounted as the main symptoms following exposure to nano-ZnO in cells. Nano-ZnO can induce the production of ROS in addition to that nano-ZnO harms DNA and induces apoptosis, as nano-ZnO are characterized by excitation of toxicity with easy penetration and accumulation in the organism. Nano-ZnO penetrates the cell membrane via ion channels in the cell membrane and mitochondrial membrane, specific receptors, and endocytosis. The more nano-ZnO internalizes in the cell, the more distributed in cell organelles [5].

Generally, birds may respond to environment contaminants in many ways ranging from tissue accumulation to decline in their population. Apart from the species, the factors including sex, age, feeding and migratory habits, etc. may affect the accumulation of contaminants in the tissues, fluids, and bird products [6]. Zinc toxicity is frequently seen in pet birds [7,8]. The pigeon for many centuries is one of the avian species used for human nutrition. The homing pigeon (*Columba livia f. urbana*) is one of the recommended model organisms that are used to assess environmental pollution [9]. NPs-exposed birds show alterations in the normal function of their tissues [10], but the ecotoxicological assessments on nano-ZnO are limited. The avian renal system is central to body water and solute homeostasis. Generally, avian kidneys comprise 1-2.6 % of body weight compared to an average of 0.5 % of body weight in mammals [11].

There is little data concerning the toxicological effects of nano-ZnO on renal system in the homing pigeons (*Columba livia f. urbana*). Thus, the current study aimed to evaluate these effects in the homing pigeons under the standard conditions.

MATERIALS AND METHODS

Chemicals and experimental design

The spherical nano-ZnO with purity level: 99 %, and size: ≤ 20 nm was obtained from Nano-shop Company, Tehran, Iran. Other chemicals were purchased from Sigma-Aldrich Chemical CO. (St. Louis, MO, USA). The nano-ZnO was suspended in deionized water and sonicated for 20 min and then vortexed for 1 min before every administration [12].

Homing pigeons (*Columba livia f. urbana*), with a weight range of 300-350 gr were adopted to the laboratory conditions under a photoperiod of 12:12 hr at 20±2 ˚C for 14 days. Food and water were available *ad libitum*. Then, birds were randomly divided into the three treated groups, in triplicate, and orally received 0, 30, 50, and 75 mg kg^{-1} b.w. doses of nano-ZnO (1 mL per bird) for 7 and 14 following days (Figure 1).

Figure 1. Oral administration of Nano-ZnO by gavage.

Preparation of kidney samples

The cervical dislocation method was used to euthanize pigeons. The kidney samples were removed and then properly homogenized in fresh 50 mM potassium phosphate buffer (1:8, w/v) at pH 7.0. The homogenates were divided into two portions, one for estimation of MDA, TAC, CP, and protein levels, and second part centrifuged (10,000 rpm, 4˚C) for 10 min to collect the post-mitochondrial supernatant for assay of CAT activity [4].

Lipid peroxidation (LPO/MDA) content

In this assay, MDA as a reliable marker of LPO reacted with thiobarbituric acid (TBA) forming TBA-reactive substances (TBARS). The fresh 30 % trichloroacetic acid

(TCA) was added to supernatants and then centrifuged (5000 ×g, 10 min). Then, the aliquot reacted with TBA forming TBARS, and the optical density of formed MDA recorded at 532 nm by use of a UV-1700 spectrophotometer (Shimadzu, Japan). Results were expressed in terms of nmoles MDA. mg protein per min using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ [13].

Catalase (CAT) activity

A spectrophotometric method was applied to measure the breakdown rate of hydrogen peroxide (H_2O_2) in assessing the changes in CAT (EC 1.11.1.6) activity at 240 nm. To start the reaction, 50 mM potassium phosphate buffer (pH 7.0) with H_2O_2 was added to 10 % supernatants. Any decrease in the optical density of the assay mixture (H_2O_2 degradation) was recorded for 3 min (20 s intervals) at 240 nm. The activity of CAT is defined in units equals the amount of CAT that is needed to decompose H_2O_2 (1 mM/min). The calculated activity of CAT was expressed as IU H_2O_2 decomposed. mg protein. min, using absorption coefficient for H_2O_2 of 0.04 $mmol^{-1}$ cm^{-1} [14].

Carbonylated proteins (CP) content

The supernatant was added to 10 mM DNPH (2, 4-dinitrophenylhydrazine) and incubated for 60 min. For stopping the reaction, 10 % trichloroacetic acid (TCA) was added and centrifuged (3,000 ×g, 20 min). The pellet was solubilized in guanidine and potassium phosphate, the resulting solution was incubated at 37 °C for 15 min. The changes in the concentrations of carbonylated proteins were measured at 370 nm and expressed in terms of nmol CP. mg protein [15].

Total antioxidant capacity (TAC) value

A ferric reducing ability of plasma (FRAP) test was used to determine TAC value. During the test, the yellow developed ferric tripyridyltriazine (Fe (III)-TPTZ) complex is reduced to ferrous (Fe (II)-TPTZ) complex in blue. FRAP reagent was added to diluted kidney sample (1:10) and incubated at 37°C for 10 min. The absorbance of blue color as antioxidative power in the kidney samples was recorded at 593 nm for following 4 min and expressed as mmoles Fe^{2+} produced per mL [16].

Blood plasma biochemistry

The blood specimen collection was obtained from a brachial vein and centrifuged (10000 ×g, 10 min, 4˚C) in test tubes containing 0.02 ml heparin/ml blood. The resulting plasma was stored at −80˚C. The possible changes in urea, uric acid, and creatinine levels were measured by a Roche Hitachi Model 917 Multichannel Analyzer using related commercial kits.

Histopathological examinations

The kidney tissues have been taken and placed into formalin 10%. Consequently, the prepared 5 microns serial sections were stained with H&E method. The structural changes of the sections were assessed by optical microscopy.

Statistical analyses

The one-way ANOVA with Tukey's HSD was used to compare the means of groups in SPSS software. A value of $P<0.05$ was taken as significant level of statistical analyses. Data were depicted as Mean ± Standard deviation (n=10/group). Pearson's correlation was used to measure the strength of linear relationships between two variables.

RESULTS

LPO/MDA content

LPO/MDA content increased against controls in a concentration-dependent manner following 7 and 14 days (Figure 2). The highest level of MDA was seen in the 75 mg kg^{-1} nano-ZnO treated groups after 14 days (143.37 %, $P< 0.05$). A correlation ($r = 0.92$, $P<0.01$) was observed between increased nano-ZnO concentrations and level of MDA after 14-day exposure to NPs.

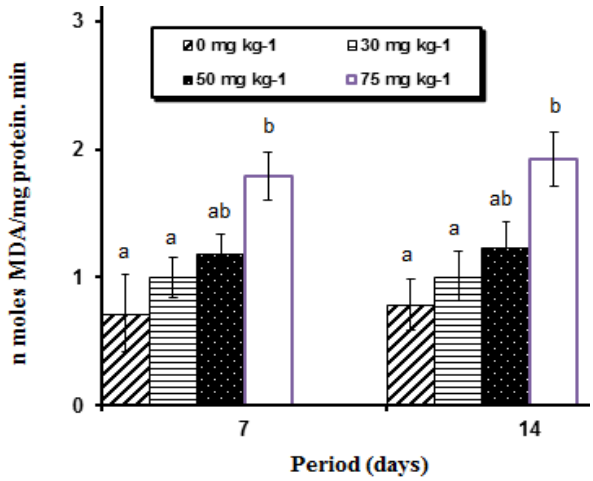

Figure 2. The changes in the lipid peroxidation (MDA/LPO) content in the kidney of homing pigeon exposed to nano-ZnO after 7 and 14 days.
[a,b]: Data without common letters are significantly different (P<0.05).

CAT activity

In Figure 3, the CAT activity showed a significant elevation concentration-dependently in all treated groups except in the 30 mg kg^{-1} groups. The highest elevation was seen in the 75 mg kg^{-1} groups after 14 days by 62.81% (P<0.05). There was a correlation (r = 0.90, P<0.05) between increased nano-ZnO level and upgraded CAT activity after 14-day exposure to nano-ZnO.

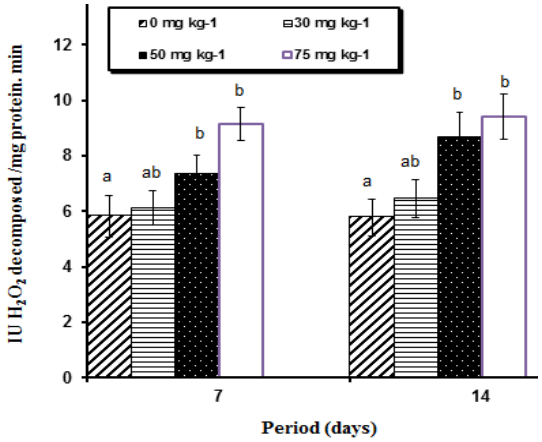

Figure 3. The changes in the catalase (CAT) activity in the kidney of homing pigeons exposed to nano-ZnO after 7 and 14 days.
[a,b]: Data without common letters are significantly different (P<0.05).

CP content

The CP content was elevated with increases in nano-ZnO concentrations compared to controls concentration-dependently (Figure 4). The highest and most significant CP value was seen in the 75 mg kg^{-1} nano-ZnO treated groups after 14 days (91.62 %, P<0.05). There was a correlation (r = 0.89, P<0.05) between the increment in CP content and increased nano-ZnO concentrations.

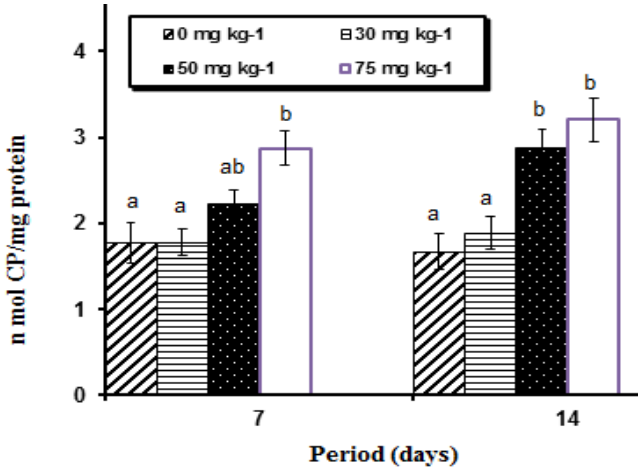

Figure 4. The changes in the carbonylated proteins (CP) content in the kidney of homing pigeons exposed to nano-ZnO after 7 and 14 days.
[a,b]: Data without common letters are significantly different (P<0.05).

TAC Value

The TAC values of kidney samples were found to be lowered with increases in nano-ZnO concentrations following both exposure times (Figure 5). The lowest TAC value was observed in the 75 mg kg^{-1} nano-ZnO treated group after 14 days by -40.02 % (P<0.05). It was observed a negative correlation between lowered TAC values and increasing nano-ZnO concentrations by r = -0.87 (P<0.05) after 14-day exposure.

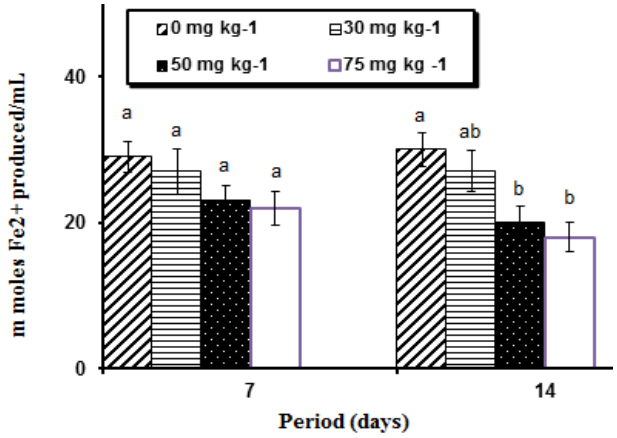

Figure 5. The changes in the total antioxidant capacity (TAC) value in the kidney of homing pigeons exposed to nano-ZnO after 7 and 14 days.
[a,b]: Data without common letters are significantly different (P<0.05).

Blood plasma biochemistry

In Table 1, plasma urea, uric acid, and creatinine levels showed non-significant elevations with increases in nano-ZnO concentrations following 7 and 14 days. The highest values for plasma urea, uric acid, and creatinine were obtained following treatment with 75 mg kg^{-1} nano-ZnO after 14 days by 31.74 %, 12.63 %, and 15.83 %, respectively (P<0.05).

Table 1. Alterations in plasma urea, uric acid, and creatinine of nano-ZnO exposed homing pigeons after 7 and 14 days.

nano-ZnO (mg kg^{-1})	Parameter	Exposure time (days)	
		7	14
0	U	0.61 ± 0.03	0.63 ± 0.04
	UA	281 ± 83	285 ± 79
	C	21.32 ± 0.23	22.49 ± 0.29
30	U	0.68 ± 0.02	0.72 ± 0.03
	UA	293 ± 81	295 ± 91
	C	23.67 ± 0.26	24.98 ± 0.31
50	U	0.70 ± 0.02	0.81 ± 0.05
	UA	301 ± 85	302 ± 87
	C	24.332 ± 0.25	24.67 ± 0.21
75	U	0.78 ± 0.04	0.83 ± 0.05
	UA	305 ± 81	321 ± 79
	C	25.39 ± 0.34	26.05 ± 38

Units: U: urea (mmol L^{-1}), UA: uric acid (µmol L^{-1}), C: creatinine (µmol L^{-1}).

Histological examinations

Figure 6A shows liver section of control group with normal parenchyma and well-arranged urinary tubules without any defects. Our findings showed that tissue damage in the pigeon kidney samples were only observed in pigeons exposed to the 75 mg kg^{-1} nano-ZnO following 14 days including aggregation of nano-ZnO in the glomerulus and tubular necrosis lines (Figure 6B). It was found more or less pathological tissue changes in all kidney samples compared to controls.

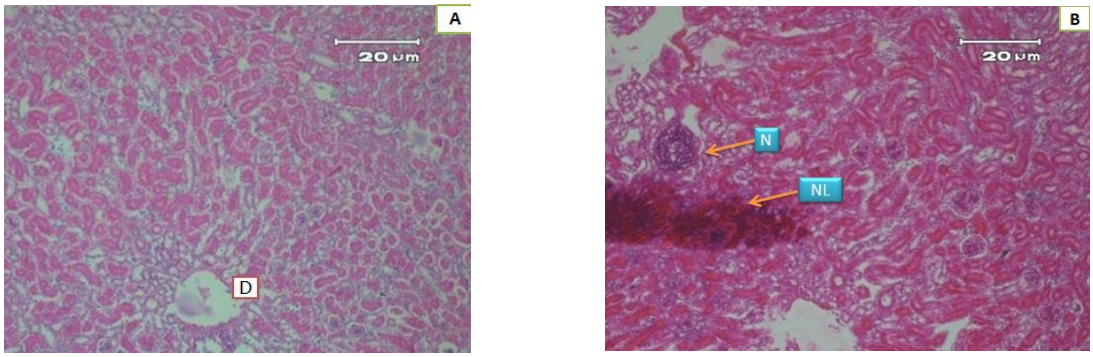

Figure 6. Histology of the kidney of homing pigeons in the control group (A, 10×) and exposed to the 75 mg kg^{-1} of nano-ZnO after 14 days (B 40×) (H&E). D: duct, N: nanoparticle aggregation in the glomerulus, NL: tubular necrosis line.

DISCUSSION

The organisms are exposed to nano-ZnO through dermal, inhalational, and oral routes. Emerging reports have indicated various types of toxicity such as mitochondrial dysfunction and genotoxicity related to these NPs [1, 17].

On contrary, antioxidant, antimicrobial, and anti-inflammatory properties of nano-ZnO have also been reported [17]. According to previous results, the accumulation of nano-ZnO is high in certain tissues including the liver, kidneys, lungs, brain, and spleen [5]. However, evidence regarding the nano-ZnO toxicological effects on birds is limited and the current study was performed to evaluate the renal toxicity induced by nano-ZnO in the homing pigeons.

LPO/MDA content

It is known that the generation of ROS and induction of OS has been defined to be the major contributing factor in nano-ZnO mediated toxicity [18]. In our study, elevated level of MDA was assumed as a marker for induction of the LPO process followed by oxidative damage in pigeon kidney samples. In the line with our results, Heidai-Moghadam et al. (2019) revealed that OS was increased in the kidney by nano-ZnO through enhancing MDA content and reducing activities of SOD [19]. Exposure to nano-ZnO results in ROS creation and altered antioxidant defense systems in cells that interfere with normal cellular processes [20]. MDA is a relatively reactive and toxic molecule that can rapidly interact with thiols and amino groups forming stable adduct proteins and ultimately leads to cell inactivation [21]. In the previous work from our team, it was shown that nano-ZnO could affect the biochemical biomarkers in blood of pigeons, where oxidative damages may be the key mechanism involved in this intoxication. We also found that nano-ZnO is capable of augmenting the MDA level dose-dependently in the blood samples of the homing pigeons following 14-day treatment [21].

CAT activity

According to what emerged from the results, increased CAT activity indicates that nano-ZnO had the potency to induce oxidative damage in the pigeon's kidney. CAT is an antioxidant enzyme that decomposes H_2O_2 in the oxidative stress status and protects the tissue from related oxidative damages [3]. CAT may detoxify nano-ZnO toxic effects in the treated pigeons. In nano-ZnO exposed Japanese quails, the CAT activity was elevated to eliminate the ROS-induced oxidative damage [22]. According to ROS theory, it may assume that nano-ZnO exposure caused excessive production of H_2O_2 and lipid radicals in pigeons and the CAT activity was increased to cope with this oxidative status [23]. In the previous investigation, we also demonstrated that nano-ZnO at similar doses could cause an increase in the activity of CAT in the blood samples indicating the propagation of oxidative damages in the blood compartments [21].

CP content

Here, we showed increased MDA and CP content in nano-ZnO-treated kidneys in pigeons. The occurrence of CP has been assumed as a hallmark of ROS-induced protein oxidation processes in animal models exposed to the environmental contaminants. The reaction of MDA with the amino group of amino acids forms reactive carbonyl residues that reflects protein modification altering the cellular activity [13]. Thus, any increase in MDA concentration may lead to protein damage likely occurring due to the elevated CP levels. Jenni-Eiermann et al. (2014) found lower concentrations of PC during the night in resting birds than in flying birds due to higher metabolic rate and ROS generation in the latter [24]. Meanwhile, an increase in LPO accompanied by enhancement of the CP content was detected in the muscles of pigeons living in areas with different levels of pollution [25].

TAC value

The TAC value attributes to the antioxidant power in organism's body and includes the synergistic interactions between redox-active components present in the biologic fluids [26]. To counteract the harmful effects of ROS and related oxidative damage, antioxidant (enzymatic and non-enzymatic) defense mechanisms are present in the organism's body. The reduced glutathione (GSH), vitamin E, urates, albumin, etc. are potentially non-enzymatic antioxidants. Amongst, the depletion of GSH by ROS/oxidants insult could be due to the direct conjugation of their metabolites with GSH leading to OS [3]. On the basis of our findings, the TAC value in the kidneys of nano-ZnO-treated pigeons was concentration-dependently decreased which can be attributed to the nano-ZnO intoxication in lowering the antioxidative

power of defense systems. Hence, it is possible to conclude that the induction of OS and excessive ROS production in the pigeon's body might be the reason behind the depletion of antioxidant power in the kidneys upon the stress imposed by nano-ZnO toxicity. In the line with these results, we earlier showed that the antioxidative power of blood plasma samples of pigeons was lowered upon nano-ZnO administration marked by reduced TAC value [21].

Blood plasma biochemistry

Blood biochemistry analyses are valuable tools for the early diagnosis and correction of metabolic disorders before occurring serious symptoms. In our results, nano-ZnO administration caused an elevation in plasma urea, uric acid, and creatinine levels which proved to be non-significant increases. Evaluation of plasma urea and uric acid levels have been assumed as inappropriate parameters to assess renal activity in birds, but their elevated amounts in blood are associated with severe renal diseases [27]. Also, the elevated level of creatinine in blood is consequently a sign of impaired kidney function. Reduced glomerular filtration can lead to an increased plasma creatinine concentration [28]. Here, although their concentrations did increase in response to nano-ZnO stress, the concentrations did not exceed significantly the border line for these variables. In other animals, administration of nano-ZnO caused marked increases in the urea, uric acid, and creatinine levels in the blood samples [19].

Histological examinations

Due to the abundance of long-chain polyunsaturated fatty acids (PUFAs) in the composition of renal lipids, the kidneys are highly vulnerable to ROS-mediated damages [29]. Our results show NPs aggregation in the glomerulus along with tubular necrosis lines in the kidney samples of homing pigeons treated with the 75 mg kg^{-1} of nano-ZnO for 14 days. An explanation for that would be an induction of OS due to an imbalance between excessive generation of ROS and function of antioxidant systems in animal tissues. ROS are capable of inducing some protein modifications, i.e., protein unfolding as an alteration of protein structure in the cells [30], resulting in the tissue damage. In most cases, ROS production leads to cytotoxicity and oxidative damage to macromolecules including proteins, lipids, and genomic materials. Besides, the overwhelmed OS induced by toxicants block certain metabolic pathways within kidneys as target organs [31]. In agreement with our results, AbdAlaziz and Albaker (2021) demonstrated that nano-ZnO treatments cause swelling, degeneration, and necrosis of the renal epithelial cells; hemorrhage between the renal tubules and within the glomerulus, with infiltration of inflammatory cells upon examination of microscopic sections [32]. Concerning with the role of nano-ZnO in nephrotoxicity in other animal models, Yan et al. (2012) reported that nano-ZnO could disturb the energy metabolism via impairments in mitochondria and cell membrane integrity in kidneys leading to nephropathy in rats [33]. In mice, it was shown that nano-ZnO toxicity cause degeneration of proximal and distal tubules as tubular necrosis and infiltration of inflammatory cells around glomerular capillaries [34].

CONCLUSIONS

We demonstrated that the nano-ZnO has deleterious effects on pigeons at higher concentrations. Nano-ZnO affected kidney function and structure in homing pigeons through the induction of oxidative stress. However, further investigation regarding nano-ZnO stress in pigeons is required.

ACKNOWLEDGEMENTS

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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

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

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