




Long-Term Effects of Unmodified 50 nm ZnO in Mice

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Abstract

Nanometer zinc oxide (nano-ZnO) is widely used in many kinds of fields. However, information about the toxicity and toxic mechanism of nano-ZnO is limited. The aims of this study were to investigate the long-term toxic effects of unmodified 50 nm ZnO administered by gavage in mice. After 90 days, hematological parameters, hepatic and renal functions, and oxidative and anti-oxidative status were measured. Pathological damages in livers, kidneys, and other tissues were also examined by hematoxylin and eosin (H&E) staining. The results showed that oral nano-ZnO exposure induced anemia and damages to liver and kidney, influenced the antioxidant system, and impacted functions of liver and kidney in mice after a 90-day exposure. The main cause for oxidative stress *in vivo* induced by nano-ZnO might be hydroxyl free radical. The lowest observed adverse effect level (LOAEL) was 40 mg/kg-bw, and the livers, kidneys, lungs, pancreas, and gastrointestinal tracts are the target organs.

Keywords Nanometer zinc oxide · Sub-chronic toxicity · LOAEL · Target organs

Introduction

Nanometer zinc oxide (nano-ZnO), with size between 1 and 100 nm, is one of the most commonly utilized nanometer materials. Nano-ZnO possesses many unique physicochemical characteristics, including semiconducting; electrical, optical, catalytic, magnetic, and ultraviolet shielding properties; heat resistance; deodorizing effects; and antibacterial properties. Nowadays, nano-ZnO has been applied in paints, pigments, metallurgy additives, rubbers, alloys, ceramics, chemical fibers, electronics, catalysts, cosmetics, and sunscreens. Nano-ZnO has also been used in the food industry as additives, in agriculture as fungicides, in biomedicine as anticancer drugs [53], in fertilizers and animal feed as a source for the micronutrient Zn [16], and in animal feed to replace antibiotics

and other drugs [25]. Being added into animal feed, nano-ZnO could improve antioxidant capacity, animal production performance, animal growth, and product quality; enhance immunity and abilities of resistance; increase animal reproductive performance; and reduce diarrhea rate of piglet [24, 26].

However, negative impacts, human risk, and toxicity mechanism of nano-ZnO are ignored and still not well-known. ZnO is generally considered to be a kind of low toxicity material, because Zn is an essential trace element for both animals and humans. However, normally harmless substance will alter to be toxic and show potential hazard when making it into nanoscale ultrafine grain.

The toxic effects of nanometer particles have been reported in the respiratory, nervous, digestive, immune, and circulatory systems and to easily induce pulmonary inflammation and damage, vascular obstruction, myocardial inflammation, and degeneration symptoms after being absorbed and accumulated in organs [9, 18, 19, 33, 41, 44]. Results of a series of studies showed that ZnO nanoparticles have toxic effects on the mammalian cells, including calcium dyshomeostasis [51], membrane damage, inflammation, DNA damage, apoptosis, mitophagy [10, 12, 49], alteration in DNA methylation [34], complex cell-cell and cell-matrix interactions, changes in hormones, and autophagic dysfunction [55].

Because of the well dispersion in solvents and air, nano-ZnO could easily be absorbed through gastrointestinal and respiratory system; then reach blood and organs such as the

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liver, kidney, and lung; and induce lesions in those organs [15, 31, 56]. Esmaïllou et al. [7] reported that exposure of ZnO nanoparticles for 5 days could injure the liver, kidney, and lung. Ben-Slama et al. [3] reported that consecutive 5 days oral exposure to ZnO nanoparticles could induce a marked increase of AST and ALT. Hong et al. [13] reported that nano-ZnO could induce histopathologic lesions in the liver, kidney, lung, spleen, and pancreas and elevation of liver dysfunction factors. Kim et al. [20, 21] reported that continuously oral administration of different size and charged ZnO nanoparticles for 14 days could induce in vitro and in vivo immunotoxicity, of which nature was an immunosuppression.

However, observations regarding nanotoxicity of nano-ZnO from short-term exposure studies are limited to assess the safety of nano-ZnO. In addition, the information related to biological effects of nano-ZnO on cells and animals are still limited and often controversial [2]. Therefore, long-term exposure studies are extremely required to detect potential sub-chronic and chronic toxicity of nano-ZnO.

In order to further understand the toxicity and provide the basic toxicity data of nano-ZnO, the sub-chronic oral toxicity of 50 nm unmodified nano-ZnO was evaluated in accordance with OECD method for testing of chemicals with a little modification. Histopathological changes, oxidative and anti-oxidative status, and Zn contents in organic tissues were examined. The present study was expected to provide valuable information on general toxic effects and target organs of 50 nm unmodified ZnO at relatively long-term repeated oral exposure, which could provide references for risk assessment of nano-ZnO.

Materials and Methods

Subjects

Unmodified nano-ZnO white powder was purchased from Jingcai Co. The purity of the nano-ZnO is 99.5%. The average particle size is 50 nm. The characteristics of nano-ZnO are shown in Table 1. The administered particles were suspended in 1% sodium carboxymethyl cellulose (Na-CMC) and dispersed by ultrasonic vibration for 15 min before intragastric administration. In order to avoid aggregation of the particles, the suspension was stirred on vortex agitator before intragastric administration.

Animals and Treatments

In the present study, animal experiment was conducted under the guidelines of the European Union (Directive 2010/63/EU) with a little modification and was approved by the local ethics committee. The 3-week-old healthy Kunming mice, 12 ± 2 g, were supplied by the Experimental Animal Center of

Table 1 The properties of nano-ZnO

Property	Value
Appearance	White powder
Whiteness	94–96
Average grain size	50 nm
pH value	5.5–6.5
Specific surface area	20–30 m ² /g
Bulk density	0.2–0.3 g/mL
Loss on drying (105 °C)	0.5–1.0%
Loss on ignition (850 °C)	1.0–1.8%
ZnO content	99–99.9%

Zhengzhou University. The animals were housed in clean polypropylene cages and maintained in an air-conditioned animal house at 20 ± 2 °C, 50–70% relative humidity, and 12 h light/dark cycle. There were 6 mice per cage. The animals were provided with commercial mouse pellet diet (Experimental Animal Center of Zhengzhou University, Zhengzhou, Henan) and deionized water ad libitum and acclimated for 5 days before the initiation of the study. The feed formula is shown in Table 2. The mice were divided into 6 groups: (1) a negative control (distilled water) group; (2) a vehicle control (1% Na-CMC) group; (3) a 40 mg/kg-bw nano-ZnO group; (4) a 80 mg/kg-bw nano-ZnO group; (5) a 160 mg/kg-bw nano-ZnO group; and (6) a 320 mg/kg-bw nano-ZnO group. There were 12 male mice and 12 female mice in each group.

Distilled water, 1% Na-CMC, and nano-ZnO suspensions were administered daily to the mice by gavage on the basis of 0.2 mL liquid per 10 g body weight, respectively. The mice were kept fasting over night before treatment. After the administration, mice were kept fasting for 3 h, but drinking water was unconstrained. The dose levels in the 90-day oral toxicity study were determined based on the results of a previously

Table 2 Composition of the diet

Ingredients	Formulation (%)
Corn flour	27
Wheat bran	19
Rice	16
Soybean cake	16
Fish meal	13
Calcium powder	3
Bone meal	3
Yeast	2.3
Salt	0.5
Vitamins premix	0.1
Mineral premix	0.1
Total	100

study of LD50 measured in accordance with the Karber's method. The symptoms of central nervous system, motor nerve system, vegetative nervous system, respiratory system, digestive system, urinary system, and changes of the eyes, skin, clothing hairs and death situations were continuously observed and recorded during the experiment. Special attentions were paid on the clinical signs of toxicity including tremor, convulsion, salivation, nausea, vomiting, diarrhea, lethargy, and coma. Body weights were weighed at grouping and once per week after the first administration.

Necroscopy and Organ Weights

After 90 days, all the mice were sacrificed by cervical dislocation. Necroscopy was performed to observe the pathological lesions in all organs of the chest cavity, the abdominal cavity, and the cranial cavity. The tissues and organs, such as livers, kidneys, lungs, hearts, spleens, and brains, were removed and weighed accurately to determine the wet weight of each organ. The organ indexes were then calculated for each organ.

Hematology Analysis

Blood samples were withdrawn from the eye vein by removing the eyeball quickly and collected in a complete blood count collection tube containing ethylenediaminetetraacetic acid dipotassium (EDTA-2K) as anticoagulants. Hematological parameters, including total leukocyte counts, total erythrocyte counts, hemoglobin (Hb) concentrations, monocytes, lymphocytes, and neutrophils, were analyzed using an automatic hematology analyzer (Sysmex XT 2100i, Sysmex, Lujiazui, Shanghai).

Serum Biochemistry Analysis

Blood samples were withdrawn from the eye vein by removing the eyeball quickly and collected in a centrifuge tube. Serum was harvested by centrifuging the blood at 3000 rpm for 15 min after blood clotting at room temperature for 2 h. The serological parameters, including aspartate transaminase (AST), alanine aminotransferase (ALT), creatinine (Crea), and blood urea nitrogen (BUN), were measured by an automatic biochemical analyzer (Hitachi 7600, Hitachi, Tokyo).

The Contents of Zn in Tissues and Organs

Zn concentrations in organs including hearts, livers, spleens, lungs, and kidneys after removal of the fatty tissue portions were determined by graphite furnace atom absorption spectrophotometry (Varian Spectr AA 220Z, Varian, Palo Alto, CA) to analyze the distribution of the 50 nm unmodified nano-ZnO.

Determination of Oxidative and Anti-oxidative Status in Blood Serum

Superoxide dismutase (SOD) activities were detected by the xanthine oxidase method. Malondialdehyde (MDA) contents were detected by the thiobarbituric acid (TBA) colorimetric method. Catalase (CAT) activities were detected by the ammonium molybdate method. Hydrogen peroxide (H₂O₂) contents were detected by the colorimetric method. Hydroxyl free radical inhibition abilities were detected based on the Fenton reaction by colorimetric method. The above biomarkers were determined via corresponding commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Histopathological Examination

After 90 days, all the animals were sacrificed by cervical dislocation and then dissected. The tissues and organs, including hearts, livers, kidney, lungs, spleens, brains, testes, ovaries, pancreas, and small intestines, were stripped out and weighed accurately. Two small blocks of the above organic tissues in both males and females from each group were fixed in 10% formalin for further histopathological examinations. The tissues of organ samples were embedded in paraffin blocks, then sliced and placed onto glass slides. After hematoxylin and eosin (H&E) staining, the histopathological changes were observed on an optics microscope.

Statistical Analysis

The data were expressed as mean \pm standard deviation. For statistical analysis, the experimental values were compared between groups. A one-way analysis of variance (ANOVA) in the SPSS software (Version 20.0) was used to illustrate the significant difference between groups. The significant difference was considered to be $p < 0.05$.

Results

Particle Properties of Nano-ZnO

The average sizes of nano-ZnO particles were measured as 50 nm by TEM and the particles were close to spherical or elliptical shape (Fig. 1). The characteristics of nano-ZnO are shown in Table 1.

Clinical Symptoms, Body Weights, and Organ Indexes

Clinical symptom including piloerection and slight head swinging was observed in the 160 mg/kg and the 320 mg/kg groups. One male mouse died in the 320 mg/kg group. Lesion

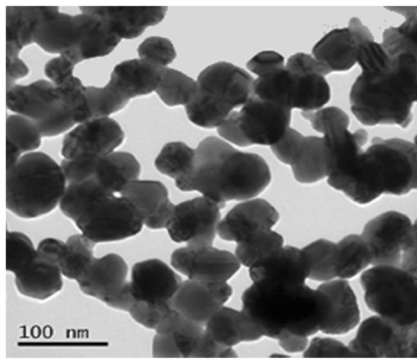


Fig. 1 TEM image of the unmodified 50 nm ZnO

in the stomachs, small intestines, livers, hearts, spleens, and lungs were found. The stomachs and small intestines of dead mice were inflated. The kidneys, lungs, hearts, and spleens were tumescent. Blood vessels in the small intestines and lungs were congested. The intestinal walls became thinner. There were petechiae at the surface of livers, hearts, and kidneys.

Body weights of mice were weighed before the administration and then weighed every week. The results showed that the body weights of mice in the 40 mg/kg·bw group were significantly higher than those of other groups ($p < 0.05$) on the day 35. No statistically significant differences were observed among groups at other times. The results are shown in Table 3.

The liver indexes of the 320 mg/kg·bw group were significantly higher than those of other groups ($p < 0.01$). The liver indexes of the 40 mg/kg·bw and the 160 mg/kg·bw group were significantly higher than those of the control group and the Na-CMC group ($p < 0.05$). The kidney indexes of the

320 mg/kg·bw group were significantly higher than those of the 80 mg/kg·bw and the 160 mg/kg·bw group ($p < 0.05$). The heart indexes of the 160 mg/kg·bw group were significantly lower than those of the control group and the Na-CMC group ($p < 0.01$). The heart indexes of the 40 mg/kg·bw group were significantly higher than those of other groups ($p < 0.01$). There were no significant differences in the lung indexes, spleen indexes, and brain indexes among groups. The results are shown in Table 4.

Zn Contents in Organs

Zn contents in major organs including hearts, livers, spleens, lungs, and kidneys were determined by graphite furnace atom absorption spectrophotometry. The results showed that Zn concentrations in the livers and kidneys of the 4nano-ZnO groups were significantly higher than those of the Na-CMC group ($p < 0.05$) and the control group ($p < 0.05$). No statistically significant differences of Zn contents in hearts, lungs, and spleens were observed among the groups treated with nano-Zn, Na-CMC group, and control group. The results are shown in Table 5.

Hematological Analysis

Hematological parameters were analyzed using an automatic hematologic analyzer. The results showed that the amounts of red blood cells (RBC) of the 40 mg/kg·bw group, the 160 mg/kg·bw group, and the 320 mg/kg·bw group were significantly decreased ($p < 0.05$) compared with the Na-CMC group and the control group. The leukocyte amounts of the 40 mg/kg·bw group and the 320 mg/kg·bw group were significantly higher

Table 3 The influence of nano-ZnO on the body weights of mice (g)

Time	Control group	Na-CMC group	40 mg/kg·bw group	80 mg/kg·bw group	160 mg/kg·bw group	320 mg/kg·bw group
0 day	12.76 ± 2.12	12.69 ± 2.23	12.71 ± 1.97	13.02 ± 2.39	12.93 ± 2.26	12.81 ± 2.03
7 days	13.76 ± 2.35	13.68 ± 2.41	15.56 ± 2.06	14.69 ± 2.97	14.75 ± 2.36	14.86 ± 2.52
14 days	14.88 ± 2.23	15.17 ± 2.25	20.89 ± 3.68	19.15 ± 2.82	17.06 ± 4.08	16.98 ± 3.03
21 days	15.95 ± 2.83	15.97 ± 2.82	21.98 ± 3.51	21.26 ± 3.18	18.02 ± 3.19	18.08 ± 2.82
28 days	17.09 ± 3.83	16.96 ± 3.88	22.06 ± 2.57	21.69 ± 3.09	19.81 ± 3.07	19.63 ± 3.51
35 days	17.91 ± 4.62	18.02 ± 4.94	23.35 ± 3.86*	19.38 ± 3.01	17.04 ± 4.06	16.98 ± 2.91
42 days	19.39 ± 5.09	19.74 ± 5.31	23.22 ± 4.32	21.42 ± 3.25	20.36 ± 3.38	19.83 ± 5.16
49 days	20.38 ± 4.56	20.86 ± 4.83	24.37 ± 2.87	24.54 ± 3.22	22.14 ± 3.63	22.02 ± 5.09
56 days	22.82 ± 3.07	22.19 ± 3.89	25.90 ± 2.85	25.20 ± 3.17	23.02 ± 3.45	22.81 ± 5.18
63 days	23.51 ± 4.06	23.41 ± 3.63	26.06 ± 2.87	25.56 ± 2.87	24.04 ± 3.96	23.09 ± 5.26
70 days	24.04 ± 3.40	24.16 ± 3.58	26.75 ± 2.51	26.06 ± 2.68	24.95 ± 4.37	23.62 ± 4.83
77 days	24.86 ± 3.15	24.79 ± 3.26	27.01 ± 2.35	26.23 ± 3.16	25.08 ± 4.38	24.06 ± 4.38
84 days	25.37 ± 2.72	25.41 ± 3.17	27.38 ± 2.99	26.58 ± 2.86	25.65 ± 4.58	24.25 ± 4.07
90 days	26.16 ± 3.13	26.04 ± 2.69	27.98 ± 3.16	26.89 ± 3.06	25.87 ± 3.86	24.98 ± 4.39

On the top right corner of the same line, there are the “*” which stand for conspicuous diversity ($p < 0.05$), and there are no “*” which stand for inconspicuous diversity ($p > 0.05$)

Table 4 The influence of nano-ZnO on the organ indexes of mice (%)

Organ	Control group	Na-CMC group	40 mg/kg·bw group	80 mg/kg·bw group	160 mg/kg·bw group	320 mg/kg·bw group
Liver	3.51 ± 0.09 ^{bbB}	3.62 ± 0.11 ^{bbB}	4.42 ± 0.59 ^{abB}	3.73 ± 0.45 ^{bbB}	4.18 ± 0.47 ^{abB}	5.38 ± 0.98 ^A
Kidney	1.33 ± 0.21	1.28 ± 0.19	1.26 ± 0.15	1.19 ± 0.18 ^b	1.09 ± 0.88 ^b	1.53 ± 0.45 ^a
Heart	0.53 ± 0.06 ^B	0.52 ± 0.02 ^B	0.63 ± 0.03 ^A	0.55 ± 0.08 ^B	0.44 ± 0.08 ^C	0.52 ± 0.09 ^{CB}
Lung	1.31 ± 0.99	1.28 ± 0.89	0.76 ± 0.16	0.79 ± 0.28	0.69 ± 0.16	0.81 ± 0.23
Spleen	0.61 ± 0.16	0.65 ± 0.09	0.45 ± 0.21	0.58 ± 0.26	0.49 ± 0.25	0.59 ± 0.29
Brain	1.58 ± 0.13	1.55 ± 0.21	1.69 ± 0.33	1.71 ± 0.18	1.54 ± 0.26	1.49 ± 0.23

On the top right corner of the same line, there are different miniscules which stand for conspicuous diversity ($p < 0.05$), and there are the same miniscules which stand for inconspicuous diversity ($p > 0.05$). On the top right corner of the same column, there are different capital alphabet which stand for conspicuous diversity ($p < 0.01$), and there are the same alphabet which stand for inconspicuous diversity ($p > 0.05$)

than those of the 80 mg/kg·bw group. The hemoglobin contents of the 320 mg/kg·bw group were significantly lower than those of the Na-CMC group and the control group ($p < 0.01$). There were no significant differences in the percentages of monocytes, lymphocytes, neutrophils, eosinophilic granulocyte, and basophilic granulocyte among groups. The results are shown in Table 6.

Serum Biochemical Analysis

The serological parameters of AST, ALT, Crea, and BUN were measured. The results showed that the ALT activities of the 320 mg/kg·bw group were significantly higher than those of the Na-CMC group ($p < 0.05$), the control group ($p < 0.05$), and the 160 mg/kg·bw group ($p < 0.01$). The AST activities of the 80 mg/kg·bw group and the 320 mg/kg·bw group were significantly higher than those of the Na-CMC group and the control group ($p < 0.05$). The serum Crea levels of the 40 mg/kg·bw group, the 80 mg/kg·bw group, and the 320 mg/kg·bw group were significantly higher than those of the Na-CMC group and the control group ($p < 0.01$). The serum Crea levels of the 40 mg/kg·bw group and the 320 mg/kg·bw group were significantly higher than those of the 160 mg/kg·bw group ($p < 0.05$). There were no significant differences on BUN levels among the 6 groups. The results were shown in Table 7.

Determination of Oxidative and Anti-oxidative Status in the Blood Serums

The oxidative and anti-oxidative biomarkers were determined. The results showed that the serum CAT activities of the 80 mg/kg·bw group were significantly higher than those of the 40 mg/kg·bw group, the 160 mg/kg·bw group, and the 320 mg/kg·bw group ($p < 0.01$). The SOD activities of the 4nano-ZnO groups were significantly higher than those of the Na-CMC group and the control group ($p < 0.01$). The MDA contents of the 320 mg/kg·bw group were significantly higher than those of the Na-CMC group and the control group ($p < 0.01$). The abilities of hydroxyl free radical ($\cdot\text{OH}$) inhibition in the serum of the 40 mg/kg·bw group, the 80 mg/kg·bw group, and the 320 mg/kg·bw group were significantly lower than those of the Na-CMC group and the control group ($p < 0.01$). There were no significant differences in serum H_2O_2 contents among groups. The results are shown in Table 8.

Histopathological Examination

The typical histopathological changes in livers, kidneys, lungs, spleens, hearts, small intestines, and pancreas are shown in Fig. 2. Congestion and inflammatory cell infiltration in livers were observed in mice exposed to nano-ZnO, which are shown in Fig. 2a. Glomeruli atrophy and inflammatory cell infiltration in kidneys were found, which are shown in

Table 5 The influence of nano-ZnO on concentration of Zn in organs of mice (%)

	Liver	Kidney	Heart	Lung	Spleen
Control group	12.76 ± 4.02 ^b	15.63 ± 5.75 ^b	10.02 ± 8.15	12.25 ± 5.67	16.54 ± 6.17
Na-CMC group	11.45 ± 6.96 ^b	16.25 ± 4.69 ^b	9.62 ± 6.59	13.46 ± 7.55	15.51 ± 8.65
40 mg/kg·bw group	15.83 ± 8.05 ^a	23.75 ± 11.52 ^a	9.49 ± 4.83	14.73 ± 6.19	16.01 ± 10.12
80 mg/kg·bw group	16.34 ± 7.38 ^a	25.62 ± 9.49 ^a	11.75 ± 6.82	13.54 ± 4.89	18.01 ± 9.82
160 mg/kg·bw group	18.69 ± 9.33 ^a	32.18 ± 15.83 ^a	10.85 ± 5.67	15.73 ± 7.63	17.01 ± 8.16
320 mg/kg·bw group	20.46 ± 8.94 ^a	35.74 ± 14.31 ^a	13.39 ± 7.36	14.73 ± 8.29	20.01 ± 13.67

Means followed by different lowercase letters within the column are significantly different ($p < 0.05$)

Table 6 The effects of nano-ZnO on the hematological parameters of mice

	Control group	Na-CMC group	40 mg/kg·bw group	80 mg/kg·bw group	160 mg/kg·bw group	320 mg/kg·bw group
Erythrocyte ($\times 10^{12}/L$)	11.48 \pm 0.03 ^a	11.29 \pm 0.09 ^a	9.97 \pm 0.15 ^b	10.42 \pm 0.91	10.12 \pm 1.02 ^b	10.09 \pm 0.83 ^b
Leukocyte ($\times 10^9/L$)	6.91 \pm 3.04	7.03 \pm 3.11	7.92 \pm 0.56 ^a	4.25 \pm 0.38 ^b	7.02 \pm 2.45	8.55 \pm 3.03 ^a
Hemoglobin (g/L)	163.02 \pm 2.06 ^A	160.10 \pm 1.99 ^A	155.18 \pm 13.02	152.35 \pm 7.68	157.06 \pm 12.03 ^a	137.32 \pm 11.68 ^{BB}
Monocytes (%)	26.17 \pm 16.68	26.08 \pm 15.93	27.59 \pm 11.05	17.07 \pm 5.71	26.89 \pm 13.92	21.03 \pm 6.87
Lymphocytes (%)	54.26 \pm 17.37	53.38 \pm 17.62	59.02 \pm 10.06	68.87 \pm 8.03	61.25 \pm 6.05	55.22 \pm 18.09
Neutrophil (%)	19.68 \pm 1.66	19.27 \pm 1.53	16.18 \pm 3.91	18.56 \pm 1.12	18.69 \pm 1.42	19.11 \pm 1.55
Eosinophilic granulocyte (%)	0.95 \pm 0.55	1.02 \pm 0.62	0.98 \pm 0.73	0.65 \pm 0.43	1.10 \pm 0.56	1.26 \pm 1.16
Basophilic granulocyte (%)	0.15 \pm 0.05	0.16 \pm 0.06	0.14 \pm 0.05	0.13 \pm 0.08	0.14 \pm 0.06	0.17 \pm 0.07

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Fig. 2b, c. The thickened alveolar septum and inflammatory cell infiltration in the pulmonary interstitial tissue were observed, which are shown in Fig. 2d. Acinar cell apoptosis and inflammatory cell infiltration in the pancreas were found, which are shown in Fig. 2e, f. Rupture of myocardial fibers were observed, which are shown in Fig. 2g. The intestinal walls became thinner and the structures of villus in small intestines were incomplete, which are shown in Fig. 2h, i. Neuronophagia was observed and are shown in Fig. 2j. The white pulps of spleens were increasing, which are shown in Fig. 2k.

Discussions

The present 90-day oral repeated-dose study of unmodified 50 nm ZnO was performed to investigate the sub-chronic toxicity and determine the target organs in mice. The body weights of mice in each group were weighed before administration and then every week. After 90 days, there were no significant changes observed on body weights among groups. Nevertheless, clinical signs including piloerection and slight head swinging were observed in the 160 mg/kg group and the 320 mg/kg group. And one male mouse died in the 320 mg/kg group. Results of other studies showed that fur loss were

observed after repeated oral administration of surface charge modified 20 nm and 100 nm ZnO for 90 days [20, 21, 33]. Particle size and surface charge modification might affect the toxicity of nano-ZnO and induce different clinical symptoms.

The liver indexes of the 320 mg/kg·bw group were significantly higher than those of other groups ($p < 0.01$). And liver indexes of the 40 mg/kg·bw and the 160 mg/kg·bw group were significantly higher than those of the control group and the Na-CMC group ($p < 0.05$). The results were similar with a previous study [20, 21]. The results indicated that nano-ZnO might be metabolized in the livers and might also induce lesions in livers.

Overdosing of metallic zinc or zinc salt in humans could result in hemorrhage, which could reduce critical blood markers, and finally lead to gastrointestinal damage [30]. Moreover, long-term supplementation of zinc in humans can cause anemia, which results from copper deficiency [35]. Yan et al. [50] reported that high zinc contents in diet could lead to iron deficiency anemia, which was related to a decrease in the levels of Hb, hematocrit (HCT), mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH). Seok et al. [38] reported that treatment with 40 nm ZnO nanoparticles resulted in significant changes in anemia-related parameters. Male rats treated with 536.8 mg/kg ZnO nanoparticles showed significant decreases in levels of Hb, HCT, MCV, and MCH.

Table 7 Effects of nano-ZnO on hepatic function and renal function of mice

	Control group	Na-CMC group	40 mg/kg·bw group	80 mg/kg·bw group	160 mg/kg·bw group	320 mg/kg·bw group
ALT (U/L)	82.36 \pm 20.19 ^b	81.62 \pm 19.83 ^b	108.52 \pm 17.61	96.51 \pm 7.65 ^b	65.51 \pm 6.93 ^B	163.74 \pm 85.26 ^{AA}
AST (U/L)	149.87 \pm 35.01 ^b	151.28 \pm 34.96 ^b	213.05 \pm 17.18	254.02 \pm 78.06 ^a	225.34 \pm 57.09	261.89 \pm 57.16 ^a
Crea (μ mol/L)	27.12 \pm 0.64 ^B	26.89 \pm 0.76 ^B	35.92 \pm 3.49 ^{AA}	33.17 \pm 3.97 ^A	28.96 \pm 1.62 ^b	35.46 \pm 4.83 ^{AA}
BUN (μ mol/L)	12.63 \pm 1.03	13.07 \pm 1.15	17.05 \pm 7.83	12.18 \pm 1.62	13.17 \pm 2.83	13.89 \pm 1.78

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Table 8 The effects of nano-ZnO on the oxidative and anti-oxidative status in blood serum of mice

	Control group	Na-CMC group	40 mg/kg-bw group	80 mg/kg-bw group	160 mg/kg-bw group	320 mg/kg-bw group
CAT (U/mL)	25.12 ± 11.82	24.86 ± 12.28	10.06 ± 3.49 ^B	30.06 ± 14.15 ^A	5.17 ± 3.26 ^B	16.22 ± 8.17 ^B
SOD (U/mL)	12.85 ± 0.46 ^E	11.92 ± 0.37 ^E	21.06 ± 1.17 ^C	24.18 ± 0.25 ^A	22.08 ± 1.21 ^B	19.16 ± 1.94 ^D
MDA (nmol/mL)	4.65 ± 2.83 ^B	4.87 ± 3.06 ^B	6.08 ± 1.57	6.42 ± 1.35	4.08 ± 1.15 ^B	7.75 ± 1.04 ^A
H ₂ O ₂ (mmol/L)	74.26 ± 30.25	75.38 ± 31.22	77.91 ± 23.05	56.67 ± 28.26	59.16 ± 23.52	51.17 ± 16.28
Inhibition of ·OH (U/mL)	63.85 ± 10.57 ^A	62.53 ± 9.68 ^A	43.72 ± 9.54 ^B	45.51 ± 9.28 ^B	60.08 ± 9.38 ^A	46.22 ± 8.06 ^B

On the top right corner of the same line, there are different miniscules which stand for conspicuous diversity ($p < 0.05$), and there are the same miniscules which stand for inconspicuous diversity ($p > 0.05$). On the top right corner of the same column, there are different capital alphabet which stand for conspicuous diversity ($p < 0.01$), and there are the same alphabet which stand for inconspicuous diversity ($p > 0.05$)

In female rats treated with 536.8 mg/kg ZnO nanoparticles, the levels of MCV and MCH were significantly decreased, whereas the platelet (PLT) counts were significantly increased. Park et al. [33] reported that oral exposure to nano-ZnO significantly decreased the amount of Hb, HCT, MCV, MCH, and mean corpuscular hemoglobin concentration (MCHC) in the male 250 and/or 500 mg/kg groups and in the female 500 mg/kg group. Results of the present study showed that exposure to nano-ZnO could lead to significant reduction of the levels of Hb and RBC which is similar to previous studies. The results indicated that long-term oral exposure to nano-ZnO might induce anemia.

However, Wang et al. [48] reported that oral administration with 250 mg/kg 40 nm nano-ZnO significantly increased the neutrophil count and Hb content as compared to the control group ($p < 0.05$) and did not affect the RBC count ($p > 0.05$), which is inconsistent with the present study. In the study of Wang et al., 40 nm ZnO was dispersed in deionized water. In the present study, 50 nm ZnO was dispersed in 1% Na-CMC solution. The inconsistent results indicated that particle size and media might affect the toxicity of nano-ZnO and induce different effects on hematological parameters.

Blood biochemical parameters including ALT, AST, Crea, and BUN are very useful biomarkers in diagnosis of lesions in the liver and kidney. Nanomaterials could lead to liver and kidney dysfunction in animals. Ben-Slama et al. [3] reported that sub-acute exposure to ZnO nanoparticles induced a marked increase of AST and ALT. However, levels of uric acid, Crea, and glucose were not modulated by ZnO nanoparticle administration. Abbasalipourkabir et al. [1] reported that intraperitoneal injection of 150 mg/kg and 200 mg/kg ZnO daily for 10 days led to a significant increase in ALT activity in comparison with the control group. Wang et al. [48] reported that glutamic-pyruvic transaminase (GPT) activities in the nano-ZnO (250 mg/kg) and zinc sulfate groups were significantly higher than those in the control group ($p < 0.01$). The results of the present study showed that 90 days oral exposure of 50 nm unmodified nano-ZnO could increase the activities of ALT and AST in mice, which is in accordance with the above studies. Moreover, the Crea levels increased after

90 days oral exposure of 50 nm unmodified nano-ZnO. The results indicated that exposure of nano-ZnO might impact functions of the liver and kidney and induce damage to the liver and kidney.

However, Kim et al. [20, 21] reported that compared with the control groups, the group of male rats receiving 31.25 mg/kg of negative charged 100 nm ZnO showed significantly decreased BUN levels while there was no significant difference of AST and ALT activities between groups. Park et al. [33] reported that none of the investigated parameters (ALT, AST, Crea, and BUN) differed significantly between the negatively charged ZnO nanoparticle-treated groups and the control group. Ryu et al. [37] reported that dermal exposure of negatively charged 20 nm ZnO had no significant effect on activities of serum ALT and AST and levels of Crea and BUN of rats. The results of the present study are inconsistent with the above two studies. The inconsistent results indicated that surface charge might affect the toxicity of nano-ZnO and induce different effects on biochemical parameters.

Nano-ZnO could distribute into different tissues and organs, including the brain, lung, heart, kidney, spleen, liver, intestine, stomach, and blood [39, 42]. Lee et al. [22] reported that orally administered ZnO nanoparticles were mainly distributed to the liver and kidney within 72 h following administration. Wang et al. [48] reported that 250 mg/kg nano-ZnO increased the zinc concentrations of the liver, kidney, and serum. Cho et al. [4] reported that 13 weeks of repeated oral administration of ZnO nanoparticles significantly increased Zn concentrations in the liver and kidney compared with the vehicle control. In the present study, Zn concentrations were significantly increased in the kidney and liver, which is in accordance with previous studies. The results indicated that nano-ZnO might be metabolized in both the liver and kidney.

The antioxidant enzymes including SOD, glutathione peroxidase (GSH-Px), and CAT work together to eliminate reactive oxygen species (ROS) and prohibit the harmful effects of oxidant molecules on cells and tissues. Small deviations in physiological concentrations of these enzymes may result in defect of body defense system and vulnerability of biomolecules to oxidative damages [29]. Nano-ZnO could significantly

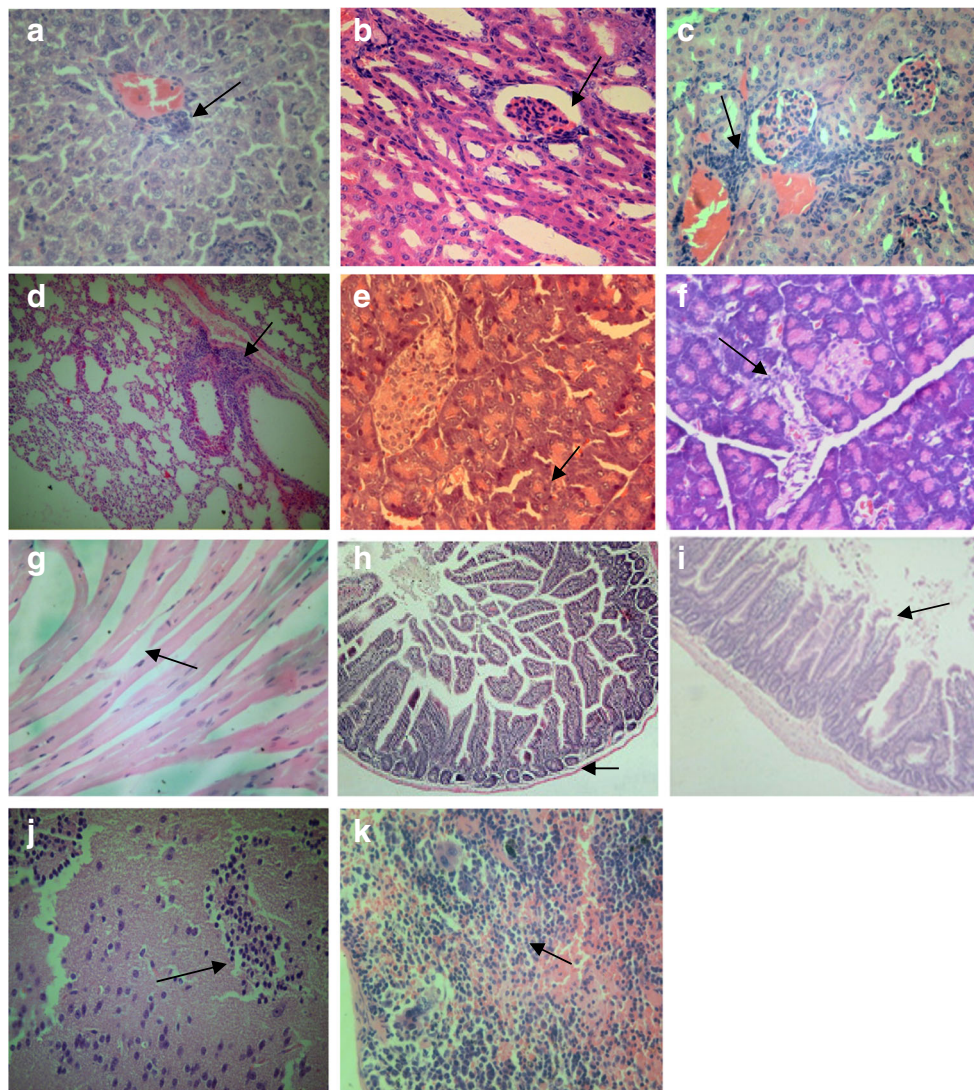


Fig. 2 **a** Histopathological changes in livers after treatment with nano-ZnO ($\times 400$). The arrow represents inflammatory cell infiltration. **b** Histopathological changes in kidneys after treatment with nano-ZnO ($\times 400$). The arrow represents glomeruli atrophy. **c** Histopathological changes in kidneys after treatment with nano-ZnO ($\times 400$). The arrow represents inflammatory cell infiltration. **d** Histopathological changes in lungs after treatment with nano-ZnO ($\times 400$). The arrows represent inflammatory cell infiltration. **e** Histopathological changes in pancreas after treatment with nano-ZnO ($\times 400$). The arrow represents acinar cell apoptosis. **f** Histopathological changes in pancreas after treatment with nano-ZnO ($\times 400$). The arrow represents inflammatory cell infiltration. **g**

Histopathological changes in hearts after treatment with nano-ZnO ($\times 400$). The arrow represents rupturing myocardial fibers. **h** Histopathological changes in small intestines after treatment with nano-ZnO ($\times 100$). The arrow represents thinner intestinal wall. **i** Histopathological changes in small intestines after treatment with nano-ZnO ($\times 100$). The arrow represents incomplete intestinal villus. **j** Histopathological changes in brains after treatment with nano-ZnO ($\times 400$). The arrow represents neuronophagia. **k** Histopathological changes in spleens after treatment with nano-ZnO ($\times 400$). The arrow represents the increasing white pulps of the spleen

reduce the mitochondrial membrane potential, increase the production of ROS, trigger endoplasmic reticulum stress, lead to cell damage, and eventually induce cell apoptosis [11, 54]. Abbasalipourkabir et al. [1] reported that an increment was observed in the SOD and GSH-Px activities in all experimental groups which were intraperitoneally injected daily for 10 days compared to the control group. Treatment with 150 mg/kg and 200 mg/kg nano-ZnO showed a significantly increased MDA level in comparison with the control group and other nano-ZnO groups. The 200 mg/kg nano-ZnO group showed a

significant increase in total oxidant status (TOS) and a significant reduction in total antioxidant capacity (TAC) in comparison with the control group. In the present study, results showed that the SOD activities of the 4 nano-ZnO groups were significantly higher than those of the Na-CMC group and the control group ($p < 0.01$). The MDA contents of the 320 mg/kg·bw group were significantly higher than those of the Na-CMC group and the control group ($p < 0.01$). The $\cdot\text{OH}$ inhibition abilities of the 40 mg/kg·bw group, the 80 mg/kg·bw group, and the 320 mg/kg·bw group were significantly lower than

those of the Na-CMC group and the control group ($p < 0.01$). The results indicated that nano-ZnO could induce oxidative stress in mice, which is similar to the above studies.

SOD plays a critical role in neutralizing of ROS and is tightly associated with zinc. SOD possesses the largest catalytic efficiency of any known enzyme [29]. The enzyme catalyzes the partitioning of the toxic superoxide radical with high proficiency [45]. Nazarizadeh and Asri-Rezaie [29] reported that treated with ZnO nanoparticles could raise the levels of SOD activities of RBC in diabetic rats. Muthuraman et al. [28] reported that exposure of different concentrations of ZnO nanoparticles could induce a dose-dependent increase in the gene expression and activity of SOD in adipocytes. The results suggested that treatment with nano-ZnO might result in increasing ROS, which could stimulate the activity of SOD to cope with the increased oxidative stress.

Hydroxyl radical produced from the decomposition of hydroperoxides (ROOH) is one of the most important and highly reactive ROS. Hydroxyl radical cannot be eliminated by enzymatic reaction which makes it a very dangerous compound to the organism [36]. Irradiated ZnO nanoparticles could produce reactive $\cdot\text{OH}$ [17, 23, 51, 52]. The results of the present study showed that the serum $\cdot\text{OH}$ inhibition abilities of mice exposed to nano-ZnO were significantly ($p < 0.01$) reduced compared with the control group, which is similar with previous studies. The results indicated that nano-ZnO might induce oxidative stress via the increased $\cdot\text{OH}$. ZnO nanoparticles or powders in aqueous solution can produce singlet oxygen or superoxide anion [8]. Singlet oxygen and superoxide anion could react with H^+ catalyzed by SOD and then produce H_2O_2 . While nano-ZnO had no stimulating effect on CAT activity, the produced H_2O_2 could not be removed completely by CAT directly. Moreover, H_2O_2 could react with $\text{O}_2^{\cdot-}$ and then produce $\cdot\text{OH}$ and cause intracellular accumulation of ROS [57] especially $\cdot\text{OH}$. The accumulated ROS could induce oxidative stress and cell damage and even cause apoptosis of cells.

Exposure of nano-ZnO through gastrointestinal tract, respiratory tract, and dermal administration might induce organ damages in animals. A series of in vivo acute and sub-acute toxicity studies of nano-ZnO demonstrated histopathologic lesions in livers, kidneys, lungs, spleens, and pancreas and elevations of liver dysfunction factors. Wang et al. [46] reported that the neutrophils increased in the serosa layer of the stomach in mice treated with 20 nm ZnO. Furthermore, significant hydropic degeneration in the hepatocytes, the chronic inflammatory cells in the pancreas, slight enlargement of the splenic corpuscles, and fatty degeneration in cardiovascular cells were found. The results indicated that the target organs for nano-ZnO after oral administration were the liver, spleen, heart, pancreas and bone. Esmacillou et al. [7] reported that cellular necrosis, congestion, and glycogen aggregation in livers, glomeruli segmentation, hydropic degeneration in epithelial cells, necrosis of epithelial cells in tubules, and swelling

in epithelial cells of proximal tubules in all kidney tissues, serous inflammation, severe hyperemia in alveoli, and edema in lungs were observed in all mice exposed to ZnO nanoparticles. The target organs for nano-ZnO after oral administration were the liver, kidney, and lung. Sharma et al. [42] reported that the livers revealed hepatocellular necrosis and accumulation of mixed inflammatory cells around the necrotic area. The kidneys depicted cystic dilation of tubules along with hypertrophied tubules. In the present study, congestion in livers, glomeruli atrophy, thickening of alveolar septum, rupture of myocardial fiber, increasing spleen white pulp, and inflammatory cell infiltration in livers, kidneys, and pulmonary interstitial tissues were observed. The results are similar as the above researches. ROS-mediated pathways are involved in hepatocytes apoptosis [6, 43]. It is speculated that nano-ZnO could induce lesions in the liver, kidney, lung, and other organs and tissues via ROS-mediated pathways, which need to be further studied.

However, several long-term toxicity studies of nano-ZnO reported different lesions in organs. Seok et al. [38] reported that mild to moderate pancreatitis with focal lymphocyte infiltration and mild acinar apoptosis were found after 13 weeks exposure of ZnO nanoparticles. And, there were no significant pathological changes in other organs. Park et al. [33] reported that exposure of $\text{ZnO}^{\text{SM20(-)}}$ nanoparticles could prompt acinar cell apoptosis and ductular hyperplasia, stimulate periductular lymphoid cell infiltration and excessive salivation, and increase the numbers of regenerative acinar cells in the pancreas. In addition, lesions in stomachs and retinal atrophy were observed. Kim et al. [20, 21] reported that squamous cell hyperplasia and vacuolation in nonglandular stomach, intracytoplasmic hyaline droplet, sub-mucosal edema and inflammatory cell infiltration, and mucous cell hyperplasia in glandular stomach were found. In addition, acinar cell apoptosis and chronic inflammation in pancreas were found in the groups receiving 500 mg/kg of 100 nm zinc oxide nanoparticles. The suppurative inflammation in the prostate gland in males receiving 500 mg/kg of 100 nm zinc oxide nanoparticles and retinal atrophy in the eyes in both sexes receiving 500 mg/kg of 100 nm zinc oxide nanoparticles were also observed. In the present study, acinar cell apoptosis, thinner intestinal wall, incomplete small intestinal villus structure, and inflammatory cell infiltration in the pancreas were found. It is in accordance with the above long-term toxicity studies of nano-ZnO. In addition, in the present study, lesions in other organs including rupture of myocardial fibers, neuronophagia, increasing white pulp in spleens, and inflammatory cell infiltration in livers, kidneys, lungs, and interstitial tissues were observed, which are inconsistent with previous long-term toxicity studies of nano-ZnO. Combined with the zinc content in organs and blood biochemical parameters, it could be speculated that the target organs for long-term oral exposure of 50 nm unmodified ZnO in mice were the liver, kidney, gastrointestinal tract, lung, and pancreas.

Physicochemical properties including size, surface area, shape, aspect ratio, surface charge, surface coating, and surface roughness could affect the toxicity of nano-ZnO [27, 32, 40, 47]. Different particle sizes might induce different lesions in organs. Particle sizes of the administered nano-ZnO in the previous long-term toxicity studies were 40 nm, 20 nm, and 100 nm, respectively. In the present study, the particle size of nano-ZnO was 50 nm. Solvents and media that nano-ZnO suspended in could also affect the toxicity of nano-ZnO. Colvin [5] reported that the toxic effects of nanoparticles showed variation depending upon the medium composition in which the nanoparticles were suspended. Hou et al. [14] reported that the same nanoparticles exhibited different toxic manifestations when dissolved in different mediums. In the research of Seok et al., 40 nm unmodified ZnOs suspended in water were administered. In the research of Park et al., surface charge modified 20 nm ZnO with negative charges suspended in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was applied. And in the research of Kim et al., surface charge modified 100 nm ZnO with negative charges or positive charges suspended in 20 mM HEPES was applied, whereas, in the present study, 50 nm unmodified ZnO suspended in 1% Na-CMC was administered. The different histopathological changes of organs might be induced by the particle sizes, surface charges, and suspending medias. The mechanisms of the effects of the size, surface charge, and suspending agent on the *in vivo* toxicity of nano-ZnO need to be further studied.

Conclusions

In the present study, unmodified 50 nm nano-ZnO at doses of 40, 80, 160, and 320 mg/kg-bw were repeatedly administered by gavage for 90 days in mice. The effects induced by nano-ZnO including inflated stomach and small intestine; tumescent kidney, lung, heart, and spleen; and congested blood vessels in the small intestine and lung. The intestinal wall became thinner. There were peptic ulcers on the surface of livers, hearts, and kidneys. Histopathologic lesions including acinar cell apoptosis, thinner intestinal wall, incomplete small intestinal villus structure, and inflammatory cell infiltration in the pancreas were found. And lesions in other organs, including rupture of myocardial fibers, neuronophagia, increasing white pulp in spleen, and inflammatory cell infiltration in the liver, kidney, lung, and interstitial tissue were observed. Thus, the target organs and tissues for the unmodified 50 nm nano-ZnO were considered to be the liver, kidney, lung, gastrointestinal tract, and pancreas. Significant toxic effects were observed at doses greater than 80 mg/kg. Therefore, the LOAEL of unmodified 50 nm nano-ZnO was considered to be 40 mg/kg for both sexes.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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