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Evaluation of Long-Term Toxicity of Oral Zinc Oxide Nanoparticles and Zinc Sulfate in Mice

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Abstract The toxicological effects of zinc oxide nanoparticles (nano-ZnOs) are related to their dissolution and interference with zinc ion homeostasis. High-soluble zinc sources may produce more severe and acute toxicity; however, the evaluation of potential toxicity of long-term exposure to nano-ZnOs and high-soluble sources of zinc remains obscure. This study aimed at evaluating effects of nano-ZnOs and zinc sulfate on development, serum and hematological parameters, and mineral concentrations in selected tissues and intestinal microbiota in mice via gastrointestinal administration for 7 weeks. Results indicated that 250 mg/kg nano-ZnOs reduced the body weight from weeks 8 to 11, increased serum glutamic-pyruvic transaminase activity, and increased the zinc concentrations of the serum, liver, and kidney while did not affect the relative organ weight, intestinal microbiota, and other mineral concentrations (Fe, Cu, and Mn) in the kidney, liver, and thigh muscle. Oral administration with 250 mg/kg zinc sulfate seemed to show more severe and acute toxicity since mice in zinc sulfate group exhibited reduced body weight from weeks 5 to 11, decreased relative pancreas weight, and increased serum glutamic-oxalacetic transaminase activity and intestinal enteric group.

Keywords Zinc oxide nanoparticle · Zinc sulfate · Development toxicity · Microbiota · Mineral concentration

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Introduction

With the rapid development of nanotechnology, more concerns have been spurred over the potential detrimental effects of manufactured nanomaterials on human health and environment [1–3]. The potential toxicity of zinc oxide nanoparticles (nano-ZnOs) is receiving increasing attention since nano-ZnOs are widely used, such as in cosmetics, sunscreens, plastics, and rubber [4]. Owing to the high antibacterial activity and stability, nano-ZnOs are becoming the extremely attractive materials in fields of food additives, packing and agriculture, and biomedicine [5, 6], which leads to even more concerns about human health and environment.

Although several studies had been conducted on the toxicity of nano-ZnOs, most of these studies focused on the acute toxicity, respiratory toxicity, or cytotoxicity. Wang et al. [7] found the acute oral toxicity of high doses of nano-ZnOs (1–5 g/kg body weight; size of 20 and 120 nm) via the damaged liver, spleen, and pancreas in mice. Yan et al. [8] reported that oral treatment with nano-ZnOs for 14 days can disturb energy metabolism and cause mitochondria and cell membrane impairment in rat kidney via inducing nephrotoxicity. It was also identified that nano-ZnOs could produce prenatal development toxicity after the 15-day oral treatment [9, 10]. Wang et al. [11] found that inhalation exposure to nano-ZnOs for 3 days led to severe damage in the liver and lung, which was similar to the results reported by Beckett et al. [12]. Yang et al. [13] found that nano-ZnOs could elevate the lactate dehydrogenase (LDH) leakage and cause genotoxicity to primary mouse embryo fibroblasts. Lin et al. [14] confirmed that nano-ZnOs resulted in intracellular oxidative stress, cell membrane leakage, and DNA damage in human lung epithelial cells, while long-term effects of oral exposure to nano-ZnOs on mice were rarely reported.

The possible underlying mechanism of nano-ZnO toxicity has been proposed that nanoparticles could cross the cell membrane, enter the cytoplasm, and induce oxidative stress for cell damage [13, 15], while recently, studies indicated that zinc ion played important roles in the toxicity of nano-ZnOs. George et al. [16] reported that decreasing the nano-ZnO dissolution rate could slow zinc ion release and reduce the toxicity. Kao et al. [17] confirmed that nano-ZnOs interfered with zinc ion homeostasis to cause cytotoxicity. It could be hypothesized that high-soluble sources of zinc, such as zinc sulfate, may produce more severe and acute toxicity than nano-ZnOs. However, the evaluation of potential toxicity of long-term exposure to nano-ZnOs and high-soluble sources of zinc remains obscure. Therefore, this study was conducted via gastrointestinal administration for 7 weeks to investigate the long-term effects of nano-ZnOs and zinc sulfate on development, serum and hematological parameters, and mineral concentrations in selected tissues and intestinal microbiota in mice.

Materials and Methods

Experiments were approved and conducted under the supervision of the Institutional Animal Care and Use Committee of Nanjing Agriculture University, China.

Preparation of Nano-ZnO Suspension

The nano-ZnOs were provided by Hangzhou King Techina Technology Co., Ltd (Zhejiang, China). The nano-ZnO exhibited nearly spherical geometry with an average diameter about 40 nm as determined with a transmission electron microscope (TEM, Hitachi H-7500, Japan), as shown in Fig. 1. These results were similar to those of our previous report as examined by using Malvern Autosier and TEM [18]. The nanoparticles were dispersed in the deionized water by ultrasonic vibration for 30 min, and the suspension of the nano-ZnO was stirred on the vortex agitator before use.

Animal and Experimental Design

A total of 24 CD-ICR male mice (3-week old) were randomly divided into three groups, eight mice per group. Mice in each group were housed in four polypropylene cages (each cage two mice). The mice and their pellet diet were provided by the Experimental Animal Center, Yangzhou University (Jiangsu, China). The house was kept at 21 ± 3 °C and in a 12 h/12 h light/dark cycle. After the adaptation periods for 7 days, mice in the three groups were gastrointestinally administered once a day: (1) control group: 0.2 ml deionized water; (2) nano-ZnO group: 0.2 ml nano-ZnO suspension at a dose of 250 mg/kg body weight; and (3) zinc sulfate group: 0.2 ml zinc sulfate solution at a dose of 250 mg/kg body weight. During this 7-week experiment, the food and water supply, behavior patterns, and clinical signs of toxicity (lethargy, coma, tremors, nausea, vomiting, etc.) will be checked at 8:00 and 18:00 each day. Once clinical sign occurred, mice would have been raised separately. When mice became unconscious, they would have been sacrificed humanely with carbon dioxide. The body weight was recorded every week and no mice died.

Sample Collection

At the end of this feeding trial, mice were fasted for 6 h prior to sacrifice. The blood was taken by removalling eyeball. Tubes with EDTA-Na were used to collect the whole blood for hematological parameters, while the serum was obtained by centrifugation at 3000×*g* for 15 min at 4 °C and stored at −80 °C until for serum parameter analysis. The organic tissues of the liver, kidney, spleen, pancreas, brain, and testis were weighed to calculate relative organ weight. The liver, kidney, and thigh

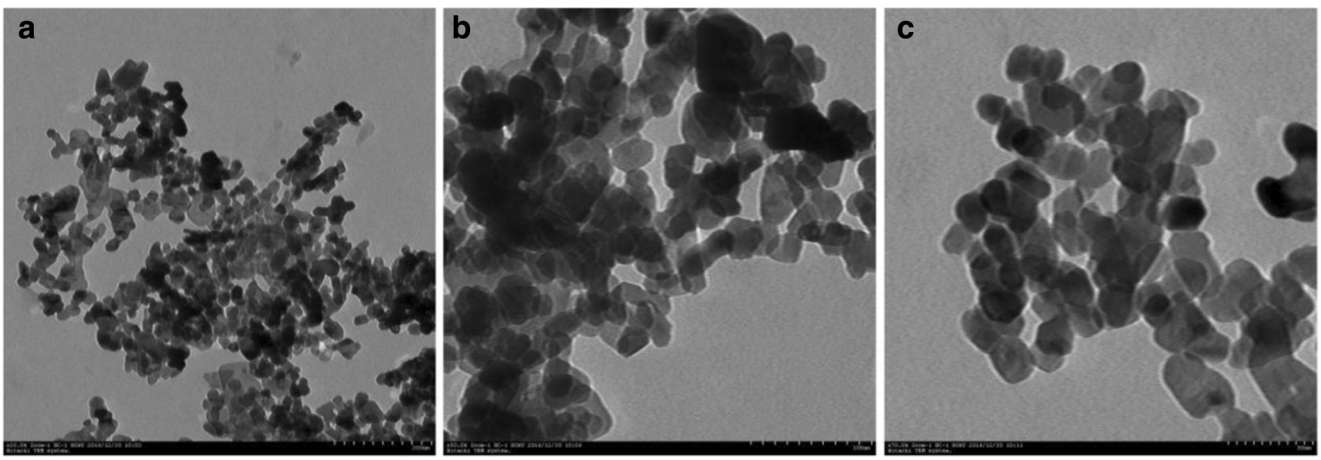

Fig. 1 TEM images of nano-ZnOs with low (**a**), middle (**b**), and high (**c**) magnification

muscle (rectus femoris muscle and vastus medialis muscle) were stored at −20 °C.

Analysis of Serum and Hematological Parameters

The Zn concentration and activities of glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and LDH in serum were determined by corresponding commercial kits as the recommended procedure. Kits were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The whole-blood samples were stored at 4 °C and were analyzed within 48 h. The hematological parameters were analyzed by an automatic hematology analyzer, including white blood cells (WBC), red blood cells (RBC), neutrophil count, lymphocyte count, monocytes, hemoglobin (HGB), and platelets (PLT).

Determination of Mineral Concentration in Selected Tissues

The mineral concentrations (Zn, Fe, Cu, and Mn) of the liver, kidney, and thigh muscle were determined as described by Demirbaş [19]. Briefly, tissues were digested using an acid mixture (HNO_3:$HClO_4$ = 4:1, *v*:*v*). The digest was brought to a volume of 25 ml with demineralized water. Blanks and standard solutions were prepared. After that, external matrix-matched standard curves (standards added to 4% HNO_3 solution as for samples) were used to analyze the mineral concentrations via inductively coupled plasma mass spectrometry (ICP-MS, USA).

Microbiological Analysis

The bacteria were quantified with the method of fluorescent in situ hybridization and flow cytometry as described by Xie et al. [20] and Vaahtovuo et al. [21]. Briefly, the cecal digesta were suspended in phosphate buffer solution (1:10 *w*/*v*). After incubated at 4 °C, the suspension was centrifuged at 120×*g* for 60 s to remove debris. After the supernatant was fixed in 4% paraformaldehyde solution, bacteria were obtained by centrifugation at 12,000×*g* for 5 min. The bacteria were stored in 50% ethanol/phosphate buffer solution at −20 °C until for analysis. The 16S rRNA-based oligonucleotide probes included Enter 1432 [22] and Bif 164 [23], which targeted to enteric group and bifidobacterium, respectively. The bacteria were hybridized with the probes in the hybridization buffer at 50 °C for 16 h. The hybridization solution was diluted with phosphate buffer solution and analyzed using the BD FACSCalibur™ flow cytometer (Becton Dickinson, USA).

Statistical Analysis

All the data were expressed as mean ± SE, which were analyzed by Tukey's multiple range test of SPSS statistical package for Windows (version 20.0, SPSS Inc., Chicago, IL). The cage was used as the experimental unit for the data of body weight, and individual mouse was the unit for the other parameters. Treatment difference of $P < 0.05$ was considered significant, and $0.05 < P < 0.10$ was considered a tendency.

Results

Body Weights

At the duration of this animal trial, the body weights were recorded weekly and are presented in Fig. 2. As shown in Fig. 2, the body weight of the mice in the zinc sulfate group was significantly lower than that of the control mice from weeks 5 to 11 ($P < 0.05$). However, there was no significant difference between the control and nano-ZnO groups from weeks 5 to 7 ($P > 0.05$), and the body weight was significantly decreased by oral administration with nano-ZnOs as compared to that of the control mice from weeks 8 to 11 ($P < 0.05$). There was no significant difference between nano-ZnO and zinc sulfate groups ($P > 0.05$).

The Relative Organ Weight

Effects of nano-ZnOs and zinc sulfate on the relative organ weight are shown in Table 1. Results indicated that oral nano-ZnOs and zinc sulfate did not affect the relative organ weights of the liver, spleen, brain, and testis ($P > 0.05$). However, the

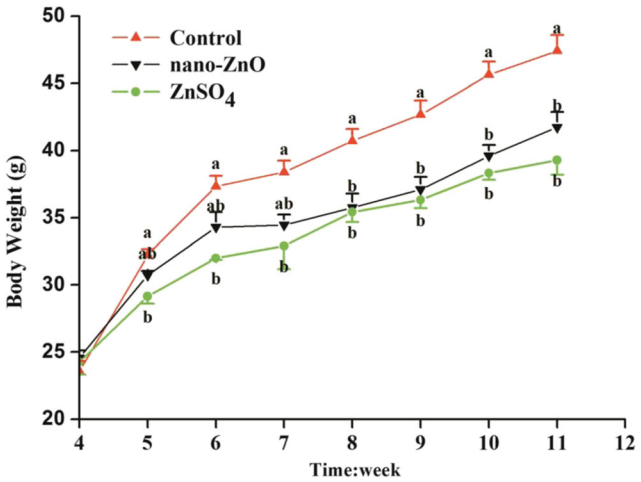

Fig. 2 Mean body weights of the mice in control, nano-ZnO, and $ZnSO_4$ groups from weeks 4 to 11. Data were expressed as means ± SE ($n = 4$). Means at the same age with *different superscripts* were significantly different ($P < 0.05$)

Table 1 Effects of nano-ZnOs and zinc sulfate on the relative organ weight (%)

Item	Control	Nano-ZnO	$ZnSO_4$	*P*
Liver	4.42 ± 0.04	4.48 ± 0.16	4.40 ± 0.10	0.88
Pancreas	0.78 ± 0.05^{a}	0.99 ± 0.09^{ab}	1.19 ± 0.09^{b}	0.01
Spleen	0.27 ± 0.01	0.25 ± 0.03	0.24 ± 0.02	0.57
Brain	0.71 ± 0.02	0.78 ± 0.02	0.81 ± 0.05	0.12
Testis	0.50 ± 0.02	0.53 ± 0.03	0.56 ± 0.03	0.30

Means in a row with different superscripts were significantly different ($P < 0.05$). Data were expressed as mean values ± SE ($n = 8$)

relative weight of the pancreas was decreased in the mice of the zinc sulfate group as compared to the control ($P = 0.01$).

Serum Parameters

The effects of nano-ZnOs and zinc sulfate on the serum parameters are shown in Table 2. Results indicated that the serum zinc concentration and GPT activity in the nano-ZnO and zinc sulfate groups were significantly higher than those in the control group ($P < 0.01$). In addition, the serum GOT activity of mice in the zinc sulfate group was significantly higher than that of the control mice ($P < 0.05$), while there was no significant difference between nano-ZnO and zinc sulfate groups ($P > 0.05$). The oral administration of nano-ZnOs and zinc sulfate tended to increase the serum LDH activity ($P = 0.07$).

Hematological Parameters

As shown in Table 3, the oral administration with nano-ZnOs significantly increased the neutrophil count and HGB content as compared to the control group ($P < 0.05$). However, the oral administration with nano-ZnOs or zinc sulfate did not affect the other hematological parameters, including the count of WBC, lymphocyte, monocytes, RBC, and PLT ($P > 0.05$).

Mineral Concentrations of Selected Tissues

Effects of nano-ZnOs and zinc sulfate on the mineral concentrations of selected tissues are presented in Table 4. Results indicated that oral nano-ZnOs and zinc sulfate for 7 weeks did not affect the mineral (Fe, Cu, and Mn) concentrations in the liver, kidney, and thigh muscle ($P > 0.05$). Compared to the control group, oral nano-ZnOs and zinc sulfate significantly increased the zinc concentrations in the liver and kidney ($P < 0.05$) while did not affect the zinc concentration in the thigh muscle ($P > 0.05$).

Table 2 Effects of nano-ZnOs and zinc sulfate on the serum parameters

Item	Control	Nano-ZnO	$ZnSO_4$	*P*
Zn (μmol/l)	24.82 ± 1.97^{b}	31.99 ± 1.32^{a}	37.04 ± 2.43^{a}	<0.01
GPT (U/l)	16.61 ± 1.09^{b}	22.57 ± 0.87^{a}	24.67 ± 1.49^{a}	<0.01
GOT (U/l)	54.96 ± 3.18^{b}	62.69 ± 1.98^{ab}	67.28 ± 1.80^{a}	0.01
LDH (U/dl)	51.59 ± 2.42	58.04 ± 2.14	64.98 ± 5.80	0.07

Means in a row with different superscripts were significantly different ($P < 0.05$). Data were expressed as mean values ± SE ($n = 8$)

Composition of Intestinal Bacterial Population

As shown in Table 5, there was no significant difference in the proportion of bifidobacterium among the three groups ($P > 0.05$). Compared to the control group, oral zinc sulfate significantly increased the proportion of enteric group ($P < 0.05$), while there was no significant difference between the nano-ZnO and control groups ($P > 0.05$).

Discussion

Zinc is an essential trace element for human and animals, and zinc oxide (ZnO) is widely used as a nutritional or medical additive [5, 9]. It is well known that dietary added pharmacologic concentrations of ZnO (2000–4000 mg/kg) alleviate post-weaning diarrhea, enhance the immunity, and improve growth performance in weaning piglets [24–27]. Owing to the multiple properties and easy synthesis [28], nano-ZnOs exhibit great potentials in many fields, including food additives, biomedicine, and feed additives [5, 6, 29]. Recently, it had been documented that the toxicological effects of nano-ZnOs were related to their dissolution and interference with zinc ion homeostasis. High-soluble zinc sources may produce more severe and acute toxicity; however, the evaluation of potential toxicity of long-term exposure to nano-ZnOs and high-soluble sources of zinc, such as zinc sulfate, remains obscure.

Previous studies verified that body and organ weights are important and sensitive indicators for toxicity studies on animals [30–32]. In our present study, the growing mice were used to investigate the long-term effects of nano-ZnOs and zinc sulfate via gastrointestinal administration for 7 weeks. Results indicated nano-ZnOs decreased the body weight after the treatment for 3 weeks while mice in the zinc sulfate group exhibited lower body weight after the treatment for only 1 week. At the same time, nano-ZnOs did not affect the relative organ weights, including the liver, spleen, brain, testis, and pancreas, while zinc sulfate decreased the relative weight of the pancreas. These results suggested that oral 250 mg/kg zinc sulfate might show much more acute and severe toxicity in mice than nano-ZnOs. Similar to our results, Jo et al. [33] showed that the body weight gain of the treated rats by gavage 500 mg/kg nano-ZnOs was about up to 16% lower than that of the control rats, and no obvious detrimental effects on the testes, epididymides, and ovaries were found. Ko et al. [34] reported that gavage 500 mg/kg nano-ZnOs for 2 weeks did

Table 3 Effects of nano-ZnOs and zinc sulfate on hematological parameters

Item	Control	Nano-ZnO	$ZnSO_4$	*P*
WBC (10^9/l)	3.60 ± 0.45	4.60 ± 0.49	4.68 ± 0.50	0.24
Neutrophil count (10^9/l)	1.57 ± 0.17^a	2.33 ± 0.20^b	1.98 ± 0.17^{ab}	0.03
Lymphocyte count (10^9/l)	2.07 ± 0.30	1.88 ± 0.40	2.15 ± 0.36	0.86
Monocytes (10^9/l)	0.20 ± 0.08	0.33 ± 0.08	0.23 ± 0.06	0.43
RBC (10^{12}/l)	7.92 ± 0.40	8.90 ± 1.20	8.22 ± 0.70	0.71
HGB (g/l)	100.00 ± 4.80^b	129.00 ± 7.55^a	113.17 ± 7.79^{ab}	0.03
PLT (10^{11}/l)	6.93 ± 0.60	7.00 ± 0.55	7.50 ± 0.61	0.76

Means in a row with different superscripts were significantly different ($P < 0.05$). Data were expressed as mean values ± SE ($n = 6$)

WBC white blood cells, *RBC* red blood cells, *HGB* hemoglobin, *PLT* platelets

not affect the rat body weight while rats in 1000- and 2000-mg/kg groups had lower body weight gain and decreased feed consumption. Prenatal rat oral exposure to 15-day repeated 400 mg/kg nano-ZnOs showed reduced food consumption, increased adrenal gland weight, and decreased body and liver weight [9]. The different toxicity of nano-ZnOs in these studies could be due to differences in the experimental duration, animal types, and quality of nano-ZnOs.

Activities of GOT, GPT, and LDH in serum were determined to evaluate the liver injury. Results indicated gastrointestinal administration with 250 mg/kg nano-ZnOs and zinc sulfate for 7 weeks increased serum GPT activity and tended to increase the serum LDH activity in mice. In agreement with our present results, Wang et al. [35] verified that oral 5 g zinc/kg body weight as nano-ZnOs for 14 days significantly increased serum activities of GOT, GPT, and LDH. Piao et al. [36] reported that intraperitoneal administration of 8 mg/kg zinc acetate damaged the liver and decreased serum GOT activity in rats. Sharma et al. [37] also found that oral exposure to 300 mg/kg nano-ZnOs elevated alanine aminotransferase and alkaline phosphatase serum levels and pathological lesions in the liver. The authors proposed that the liver damage could be attributed to the accumulation of nanoparticles, which further induced oxidative stress, DNA damage, and apoptosis [37]. However, oral administration with 250 mg/kg zinc sulfate also led to liver damage in our present study, which indicated that the damage might be due to the accumulation of zinc. The forms of accumulated liver zinc (nano-ZnO or other zinc forms) have not been determined in the present study, which should be further investigated in the future.

Wang et al. [35] reported that oral 5 g/kg body weight nano-ZnOs significantly decreased the blood HGB and hematocrit and increased the red cell distribution width corpuscular volume and PLT in mice, suggesting heavy anemia might occur. Some other researchers documented that excessive dietary zinc could induce deficiencies of copper and iron and then cause anemia [38–40]. However, our results indicated that nano-ZnOs and zinc sulfate did not affect blood PLT. Moreover, nano-ZnOs significantly increased the blood HGB and neutrophil count, which suggested long-term treatment with nano-ZnOs might alter the immune function in mice [41].

The zinc level in serum and mineral concentrations (Zn, Fe, Cu, and Mn) in selected tissues had been determined in the present study. Results indicated that oral administration with

Table 4 Effects of nano-ZnOs and zinc sulfate on the mineral concentrations of selected tissues

Item	Control	Nano-ZnO	$ZnSO_4$	*P*
Liver				
Zn (mg/kg)	38.11 ± 2.44^a	54.04 ± 2.43^b	59.64 ± 3.87^b	<0.01
Fe (mg/kg)	141.22 ± 18.07	160.31 ± 30.69	174.35 ± 13.57	0.57
Cu (mg/kg)	5.27 ± 0.51	6.23 ± 0.49	6.23 ± 0.37	0.26
Mn (mg/kg)	2.44 ± 0.40	3.28 ± 0.33	2.95 ± 0.26	0.22
Kidney				
Zn (mg/kg)	32.37 ± 3.20^a	47.40 ± 2.85^b	44.17 ± 3.01^b	0.01
Fe (mg/kg)	140.69 ± 6.95	146.63 ± 8.21	153.67 ± 10.09	0.57
Cu (mg/kg)	4.56 ± 0.56	3.40 ± 0.23	4.06 ± 0.53	0.23
Mn (mg/kg)	2.95 ± 0.45	3.86 ± 0.14	3.16 ± 0.33	0.15
Thigh muscle				
Zn (mg/kg)	28.49 ± 2.30	33.51 ± 2.77	29.68 ± 2.87	0.39
Fe (mg/kg)	141.71 ± 9.86	144.00 ± 9.75	144.16 ± 10.20	0.98
Cu (mg/kg)	1.68 ± 0.19	1.58 ± 0.14	1.30 ± 0.09	0.20
Mn (mg/kg)	0.67 ± 0.06	0.62 ± 0.07	0.46 ± 0.07	0.10

Means in a row with different superscripts were significantly different ($P < 0.05$). Data were expressed as mean values ± SE ($n = 8$)

Table 5 Effects of nano-ZnOs and zinc sulfate on the cecal microbial ecosystem

Item	Control	Nano-ZnO	$ZnSO_4$	*P*
Enteric group (%)	0.46 ± 0.03^a	0.72 ± 0.10^a	1.39 ± 0.21^b	<0.01
Bifidobacterium (%)	0.87 ± 0.07	0.71 ± 0.10	0.88 ± 0.12	0.44

Means in a row with different superscripts were significantly different ($P < 0.05$). Data were expressed as mean values ± SE ($n = 6$)

250 mg/kg nano-ZnOs and zinc sulfate for 7 weeks increased the zinc concentrations in the serum, liver, and kidney, which means that oral nano-ZnOs and zinc sulfate enhanced zinc absorption, which is in line with our previous study [18]. Wang et al. [7] also reported that oral exposure to 20- or 120-nm ZnO increased the kidney zinc level in mice. However, the zinc concentration in the thigh muscle was not affected by nano-ZnOs or zinc sulfate in our present study, which was in line with previous reports. They verified that the variation of muscle zinc was not large and the zinc concentration of muscle might not be a sensitive indicator for zinc status [40, 42, 43]. In addition, our results indicated that other mineral concentrations (Fe, Cu, and Mn) in the liver, kidney, and thigh muscle were not affected by oral nano-ZnOs or zinc sulfate, which partially explained our results that no anemia occurred in this study.

Intestinal microbiota is very important to human and animals via affecting metabolism and intestinal immunity [44, 45]. In this study, intestinal bacterial population was determined with flow cytometry analysis which is a rapid and reliable solution compared with methods based on cultivation or amplification of the nucleic acids [20, 21, 46]. Bacteria belonging to the enteric group are commonly considered as potential or conditioned gastrointestinal pathogens, while bifidobacterium was beneficial to the host [20]. Our results indicated that oral zinc sulfate increased the proportion of the enteric group, while nano-ZnOs did not affect the intestinal bacterial population (bifidobacterium and enteric groups), which suggested that oral 250 mg/kg zinc sulfate altered the intestinal microbiota and might be harmful to the intestinal immune system.

Conclusions

In summary, although intestinal microbiota and tissue concentrations of Fe, Cu, and Mn were not affected, gastrointestinal administration of 250 mg/kg nano-ZnOs for 7 weeks exhibited toxic effects on mice, including decreased body weight, liver damage (elevated serum GPT activity), and increased zinc accumulation in the liver and kidney. According to the literature and our findings, exposure to nano-ZnOs may lead to liver damage, which might be due to the accumulation of zinc. More researches are needed to investigate the forms of accumulated liver zinc (nano-ZnO or other zinc forms) after oral administration with nano-ZnOs. Compared to nano-ZnOs, oral 250 mg/kg zinc sulfate seemed to show similar or much more severe and acute toxicity in mice via acute decrease in body weight, increased serum GOT activity, and altered intestinal microbiota. In the future, it is better to investigate the long-term effects of nano-ZnOs and zinc sulfate with more suitable animal models, such as the model of pigs.

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Compliance with Ethical Standards Experiments were approved and conducted under the supervision of the Institutional Animal Care and Use Committee of Nanjing Agriculture University, China.

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