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Comparative study on the effects of micro- and nano-sized zinc oxide supplementation on zinc-deficient mice

Ja-Seon Yoon [1], **Sang Yoon Nam** [1], **Beom Jun Lee** [1,*], **Hyun Jik Lee** [1,2,*]

[1]College of Veterinary Medicine and Veterinary Medicine Center, Chungbuk National University, Cheongju 28644, Korea
[2]Institute for Stem Cell & Regenerative Medicine (ISCRM), Chungbuk National University, Cheongju 28644, Korea

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***Corresponding authors:**
Beom Jun Lee
College of Veterinary Medicine and Veterinary Medicine Center, Chungbuk National University, 1 Chungdae-ro, Seowon-gu, Cheongju 28644, Korea.
Email: beomjun@cbu.ac.kr
https://orcid.org/0000-0002-7013-8086

Hyun Jik Lee
College of Veterinary Medicine and Veterinary Medicine Center, Institute for Stem Cell & Regenerative Medicine (ISCRM), Chungbuk National University, 1 Chungdae-ro, Seowon-gu, Cheongju 28644, Korea.
Email: leehyunjik@chungbuk.ac.kr
https://orcid.org/0000-0002-2762-2649

ABSTRACT

Background: Zinc (Zn) is an essential cofactor for physiological homeostasis in the body. Zn oxide (ZnO), an inorganic compound that supplies Zn, exists in various sizes, and its bioavailability may vary depending on the size *in vivo*. However, comparative studies on the nutritional effects of micro-sized ZnO (M-ZnO) and nano-sized ZnO (N-ZnO) supplementation on Zn deficiency (ZnD) animal models have not been reported.
Objectives: This study investigated the nutritional bioavailability of N-ZnO and M-ZnO particles in dietary-induced ZnD mice.
Methods: Animals were divided into six experimental groups: normal group, ZnD control group, and four ZnO treatment groups (Nano-Low, Nano-High, Micro-Low, and Micro-High). After ZnD induction, N-ZnO or M-ZnO was administered orally every day for 4 weeks.
Results: ZnD-associated clinical signs almost disappeared 7 days after N-ZnO or M-ZnO administration. Serum Zn concentrations were higher in the Nano-High group than in the ZnD and M-ZnO groups on day 7 of ZnO treatment. In the liver and testis, Nano-Low and Nano-High groups showed significantly higher Zn concentrations than the other groups after 14-day treatment. ZnO supplementation increased *Mt-1* mRNA expression in the liver and testis and *Mt-2* mRNA expression in the liver. Based on hematoxylin-and-eosin staining results, N-ZnO supplementation alleviated histological damage induced by ZnD in the testis and liver.
Conclusions: This study suggested that N-ZnO can be utilized faster than M-ZnO for nutritional restoration at the early stage of ZnD condition and presented *Mt-1* as an indicator of Zn status in the serum, liver, and testis.

Keywords: Zinc oxide (ZnO); zinc deficiency; nanoparticles; metallothionein; bioavailability

INTRODUCTION

Zinc (Zn) is one of the essential minerals that act as a cofactor of physiological homeostasis in animals [1]. Zn is necessary for various biochemical processes, including DNA, RNA, and protein synthesis. In addition, Zn is crucial for reproduction in testicular function, especially in the formation and maturation of sperm and fertilization [2]. The deficiency of Zn causes

ORCID iDs
Ja-Seon Yoon
https://orcid.org/0000-0002-3084-2562
Sang Yoon Nam
https://orcid.org/0000-0001-7576-6543
Beom Jun Lee
https://orcid.org/0000-0002-7013-8086
Hyun Jik Lee
https://orcid.org/0000-0002-2762-2649

Author Contributions
Conceptualization: Yoon J, Lee BJ; Data curation: Yoon J, Lee HJ; Formal analysis: Yoon J, Nam SY, Lee HJ; Funding acquisition: Lee HJ; Investigation: Yoon J, Lee BJ, Lee HJ; Manuscript writing-original draft: Nam SY, Lee BJ, Lee HJ.

Conflict of Interest
The authors declare no conflicts of interest.

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health problems, including skin irritants, poor wound healing, cognitive impairment, and reproductive system dysfunction [3-5]. Zn deficiency (ZnD) in humans occurs due to a high phytate, fiber diet, general malabsorption-inducing diseases, and excessive specific mineral ingestion competing with Zn absorption [6, 7]. Therefore, developing an efficient Zn delivery strategy that maintains normal Zn serum levels can prevent ZnD-associated diseases.

Metallothionein (MT) is a major Zn importer that plays an important role in the transport, storage, and distribution of Zn [6-8]. It is a cystine-rich, low-molecular-weight protein with a high affinity for physiological and nonphysiological heavy metals. Each MT molecule exists in two separate clusters and can bind up to seven Zn atoms [9]. MT-1 and MT-2 isoforms are most widely expressed in many cell types in different tissues and organs. MT-3 is expressed in the brain, and MT-4 is expressed in differentiating stratified squamous epithelium [10,11]. It is also associated with the detoxification of heavy metals and the homeostasis of essential metal ions due to their high affinity for these metals [12,13]. In addition, a previous study reported that Zn intake upregulated *MT* gene expression, indicating that *MT* expression can be used as a biomarker of Zn bioavailability in the body [14].

Zn oxide (ZnO) is an inorganic compound that supplies Zn. Natural and synthetic ZnO exist in various sizes, and their bioavailability varies depending on the size *in vivo*. As nanotechnology quickly advances, nano-sized substances are being used in numerous fields. According to the International Organization for Standardization, nanoscale substances are substances with at least one external dimension in a size range of < 100 nm [15]. Due to their small size, large specific surface area, and high reactive surface sites, nanoscale Zn usually has unique physical and/or chemical characteristics [16,17]. Nano-sized ZnO (N-ZnO) is one of the most widely used nanoparticles in biomedical products [18]. Although several toxicological effects of a high dose of N-ZnO have been reported [19,20], the nutritional effects of N-ZnO supplementation on ZnD disease models are unknown. Besides of N-ZnO, many researchers also reported that the effect of micro-sized ZnO (M-ZnO). For example, a previous study reported that supplementation with M-ZnO or N-ZnO could be a less toxic potential alternative to natural Zn source [16,21]. However, another study demonstrated the cytotoxicity of M-ZnO aspiration, which causes inflammation and sedimentation of aspirated ZnO particle in human airway due to the size of the aspirated ZnO particles [22]. In addition, comparative studies on the effects of M-ZnO or N-ZnO supplementation on ZnD-associated clinical signs are not reported yet. Therefore, this study investigated the nutritional bioavailabilities and therapeutic effects of N-ZnO and M-ZnO on ZnD mice models.

MATERIALS AND METHODS

Materials

M-ZnO (powder; particle size < 5 μm; purity 99.9%) and N-ZnO (nanopowder; particle size < 50 nm; purity > 97%; surface area > 10.8 m^2/g) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). N-ZnO solubility was evaluated in acidic (pH 1.5) or neutral (pH 7.0) conditions. N-ZnO and M-ZnO (40 mg) were dissolved with 1 ml deionized water (DW), respectively. The acidic pH was adjusted to 1.5 using 2 N HCl. The N-ZnO and M-ZnO solutions were allowed to stand for 12 h at 37°C with a shaking water bath. M-ZnO was completely dissolved in the acidic (pH 1.5) condition. In contrast, N-ZnO in the acidic condition showed minimal dissolution for up to 12 h. In neutral (pH 7.0) condition, M-ZnO and N-ZnO were not dissolved completely (**Supplementary Fig. 1**). The AIN-93M

purified rodent diet contained 14% casein, 10% sucrose, 4% soybean oil, 0.0008% t-butyl hydroquinone, 46.5692% cornstarch, 15.5% dextrose, 5% cellulose, 3.5% salt mix, 1% vitamin mix, 0.25% choline bitartrate, and 0.18% L-cystine. Meanwhile, the ZnD diet contained 14% egg whites, 9.87% sucrose, 4% soybean oil, 0.0008% t-butyl hydroquinone, 46.7492% cornstarch, 15.5% dextrose, 5% cellulose, 3.5% mineral mix, 1% vitamin mix, 0.25% choline bitartrate, and 0.13% biotin/sucrose premix (1 mg/biotin/g). The composition of basal and ZnD diets is described in **Supplementary Table 1**.

Animals

Male ICR mice (4 weeks old) were obtained from Central Laboratory Animal, Inc. (SLC, Inc., Shizuoka, Japan). All animals were housed in polycarbonate cages (4–5 mice per cage). The temperature and relative humidity were maintained at 20°C ± 2°C and 50% ± 20%, respectively. Light and dark cycles were at 12/12 h. Mice were fed the AIN-93M purified rodent diet (Dyets, Inc., Bethlehem, PA, USA) and water *ad libitum*. The animal experiment was conducted in accordance with the "Guide for Care and Use of Laboratory Animals" of Chungbuk National University Institutional Animal Care and Use Committee (CBNUA-530-13-01). Mice were allowed to acclimatize to their environment for 1 week before experimentation. During the experimental period, the body weights and food intake of the animals were recorded weekly.

Experimental design

There was a total of 8 weeks, including the ZnD induction period for 4 weeks and the ZnO treatment period for 4 weeks. Animals were divided into six experimental groups: normal group, ZnD control group, and four ZnO treatment groups (Nano-Low [NL], Nano-High [NH], Micro-Low [ML], and Micro-High [MH]). To induce ZnD condition in mice, animals in the ZnD control and four ZnO treatment groups were fed a ZnD diet (2.6 mg Zn/kg diet) for 4 weeks. Mice in the normal group were fed a basal diet (50.65 mg Zn/kg diet) during the 8-week experimental period. Mice were treated orally with M-ZnO and N-ZnO (8 and 40 mg Zn/kg body weight) in a 1.0% sodium carboxymethyl cellulose solution daily for 4 weeks of the experimental period. Mice in the ZnD control group were treated with a 1.0% sodium carboxymethyl cellulose solution (vehicle).

Sample collection

Blood samples were collected via the abdominal vein and immediately transferred into serum separator tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA). The serum was obtained by centrifuging whole blood at 3,000 rpm for 15 min at 4°C. The liver and testis were obtained and washed with 0.9% saline. The organs were weighed, and half of the organs were frozen in liquid nitrogen. The other half of the remaining organs were fixed in 10% neutral buffered formalin.

Measurement of Zn concentration in the serum and tissues

After oral administration of M-ZnO and N-ZnO, the Zn content in the serum and tissues was analyzed. The serum and tissues were predigested with nitric acid (HNO_3) and heated at ~160°C using concentrated HNO_3 and H_2O_2 until the solution was colorless and clear. Completely digested samples were diluted with 5 ml DW and filtered at 0.45 μm before analysis. The total Zn concentration of serum and tissues was quantified by inductively coupled plasma-mass spectrometry (ICP-MS; 820-MS; Varian, Santa Clara, CA, USA) analysis.

Total RNA extraction and semiquantitative real-time polymerase chain reaction

Total RNA was extracted from mouse tissues using a Ribo EX (Geneall, Daejeon, Korea) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed to first-strand cDNA using a ReverTra Ace (Toyobo, Tokyo, Japan) and amplified with the Accu Power® PCR premix (Bioneer, Daejeon, Korea). PCRs were performed using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The following primers were used: *Mt-1* forward primer 5′-ATGGACCCCAACTGCTCCTG-3′ and reverse primer 5′-TCAGGCACAGCACGTGCACT-3′ (GenBank accession no. NM013602.3; product size, 186 bp); *Mt-2* forward primer 5′-CAAACCGATCTCTCGTCGAT-3′ and reverse primer 5′-AGGAGCAGCAGCTTTTCTTG-3′ (GenBank accession no. NM008630.2; product size, 150 bp); and *β-actin* forward primer 5′-ACCCACACTGTGCCCATCTA-3′ and reverse primer 5′-CACGCTCGGTCAGGATCTTC-3′ (GenBank accession no. NM007393.3; product size, 112 bp). *β-Actin* mRNA was used as an internal control to normalize the expression of the target transcripts. PCR products were separated on a 2.3% agarose gel in Tris-borate-EDTA buffer and imaged with a Gel Documentation System. The band intensity was quantified using ImageJ software (available at rsb.Info.nih.gov/ij).

Histopathological examination

Histopathological analysis was performed by examining the morphological changes in the organs. The sample tissues were fixed with 10% neutral buffered formalin and processed using routine histological techniques. After paraffin embedding, 3.5 μm cutting sections of tissues were obtained, stained with hematoxylin and eosin (H&E), and examined under a light microscope (Olympus BX-53, Tokyo, Japan).

Statistical analysis

Data were expressed as the mean ± standard deviation. Data were analyzed using a nonparametric one-way analysis of variance, and a significant difference among treatment groups was evaluated by the least-squares difference method for multiple comparisons to the controls. The difference at the $p < 0.05$ level was sequenced to be statistically significant.

RESULTS

Validation of the ZnD diet-induced ZnD mice model

To induce the ZnD mice model, mice were treated with a ZnD diet for 4 weeks. Analysis of the Zn content in basal and ZnD diets was performed by ICP-MS. The basal diet contained 51.06 mg Zn/kg diet, and the ZnD diet contained 5.36 mg Zn/kg diet. The Zn content in the ZnD diet was significantly lower than that in the basal diet (**Fig. 1A**). After ZnD induction for 4 weeks, the Zn content in the serum was analyzed during the ZnD induction period. On the fourth week of the experiment, the serum Zn concentration of mice of the ZnD control group decreased significantly compared to the normal group (**Fig. 1B**). The feeding of a ZnD diet to mice caused several clinical signs, such as alopecia, skin wounds, keratinization in the tail region, erect of fur, and eye lesions (**Supplementary Table 2** and **Fig. 1C**). Taken together, these findings indicated that ZnD diet-induced ZnD mice model was validated by serum Zn concentration and clinical sign data.

Effects of M-ZnO or N-ZnO treatment on serum concentrations in the ZnD mice model

To investigate the effects of M-ZnO or N-ZnO, ZnD mice were treated with M-ZnO or N-ZnO (8 and 40 mg Zn/kg body weight) for 4 weeks (**Fig. 2A**). The changes in body weights of mice were measured during the experimental periods (**Fig. 2B**). There were no significant changes

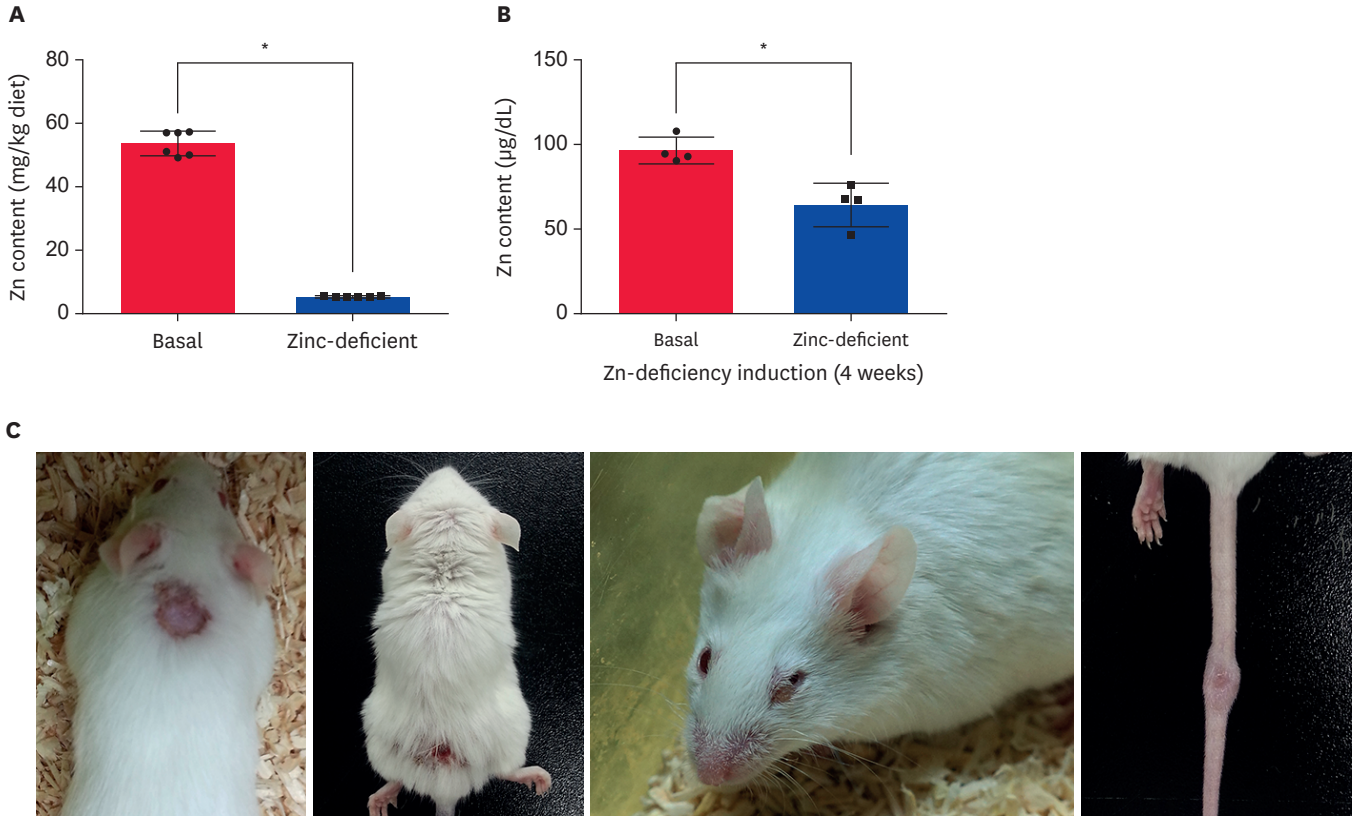

Fig. 1. Induction Zn-deficient mice model. (A) Zn content in diets as determined by ICP-MS. Bars represent the means ± SD (n = 6). (B) Zn concentration in the serum of mice during Zn deficiency induction period. The Zn concentration was determined by ICP-MS (n = 4). (C) Clinical signs (alopecia, erect of fur, eye lesion, and tail lesion) of mice with Zn deficiency.
*Significantly different from basal diet ($p < 0.05$).

in body weights of mice among all experimental groups. In addition, the changes in the food intake of mice were measured during the experimental periods. The food intake of mice in the ZnD control group significantly decreased compared to the normal control group at 7 and 8 weeks (**Fig. 2C**). ZnO treatments, regardless of nano- or micro-sized particles, recovered those clinical signs on day 7 (**Supplementary Table 3**). The recovery process of alopecia in ZnD mice with a high dose of N-ZnO (NH) is shown in **Fig. 2D**.

Furthermore, this study investigated the Zn concentration in the serum, liver, and testis samples using ICP-MS. The serum Zn concentration increased significantly in the NL, NH, and MH groups than ZnD group after 7-day treatments (**Fig. 3A**). The serum Zn level in the NL group was the higher than that in ML and ZnD groups during all experimental periods (**Fig. 3A**). The serum Zn level in the NH group was similar to that in the MH group during all experimental periods (**Fig. 3A**). In the liver tissue samples, there were no significant differences in Zn content levels of the liver in mice of the ZnO treatment groups compared to the ZnD control group after 7-day treatment (**Fig. 3B**). The MH, NL, and NH groups showed significantly higher Zn concentration than the ZnD control group after 14-day treatment (**Fig. 3B**). The Zn level in the NH group was significantly higher than that in the MH group after 28-day treatment (**Fig. 3B**). In the testis tissue samples, the Zn concentration of the testis in the NL and NH groups were significantly higher than that in the ML and MH groups, respectively (**Fig. 3C**). Collectively, this study indicated that N-ZnO treatment rather than M-ZnO can be an effective strategy for alleviating the Zn concentration and clinical signs in the early stage of ZnO treatment.

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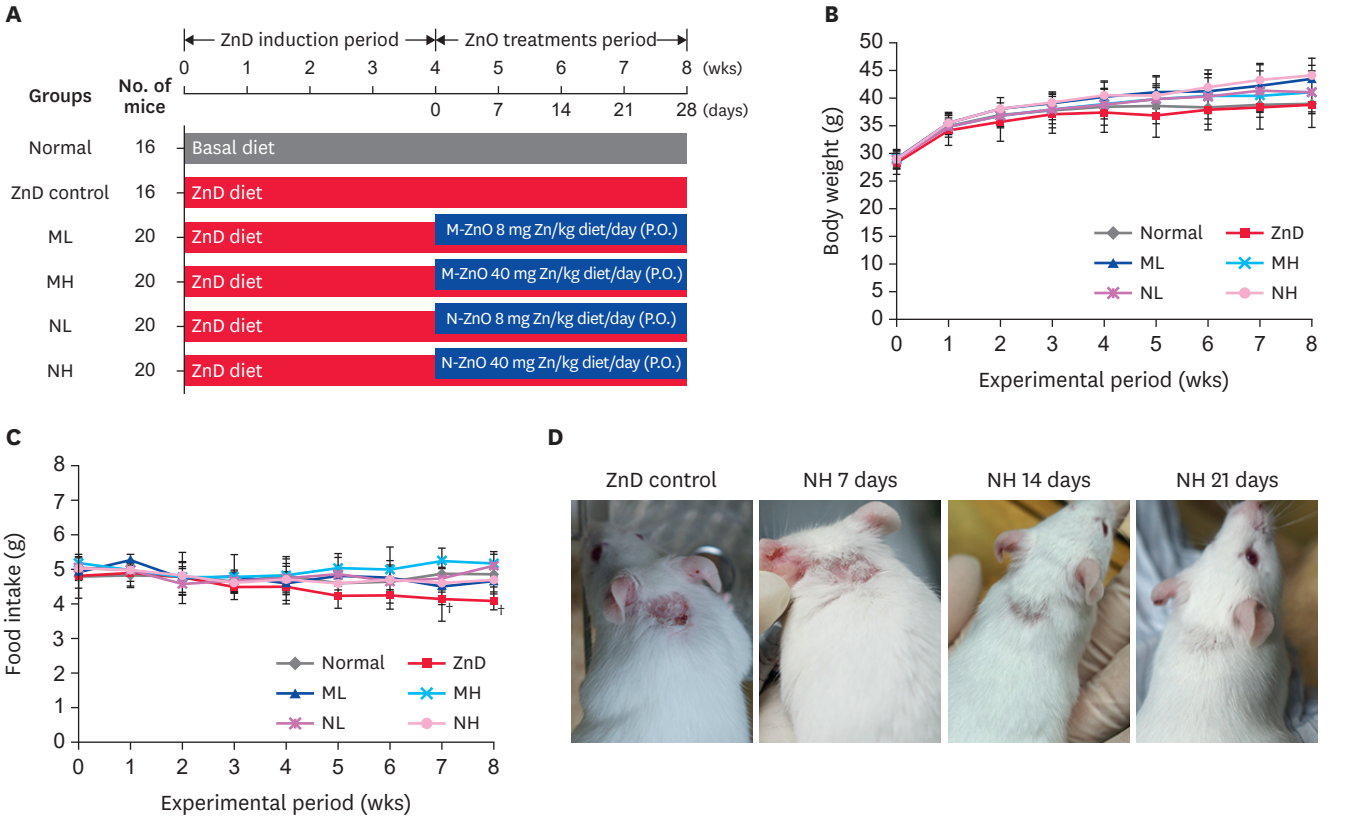

Fig. 2. Effect of M-ZnO and N-ZnO treatment on body weight and food intake of Zn-deficient mice model. (A) Experimental design for treatments of M-ZnO and N-ZnO in Zn-deficient mice. Zn-deficient mice were induced for 4 weeks by feeding a Zn-deficient diet and M-ZnO and N-ZnO oral treatments were performed daily for 4 weeks. (B and C) Changes in body weights and food intake of mice treated with M-ZnO and N-ZnO. Values represent the means ± SD (n = 16). (D) Clinical signs (alopecia, erect of fur, and eye lesion) of mice with ZnD and ZnD with high dose of N-ZnO on 0–21 days.
ZnD, Zn-deficient control group, ZnO, Zn oxide; ML, Micro-Low group (8 mg Zn/kg body weight); MH, Micro-High group (40 mg Zn/kg body weight); NL, Nano-Low group (8 mg Zn/kg body weight); NH, Nano-High group (40 mg Zn/kg body weight); M-ZnO, micro-sized ZnO; N-ZnO, nano-sized ZnO.

Histopathology of liver and testis tissue samples after ZnO treatment in the ZnD mice model

Histopathological observations were performed on the liver and testis section with H&E staining on day 7 after ZnO treatments (**Fig. 4A and C**) and day 28 after ZnO treatments (**Fig. 4B and D**). In the liver tissue samples, there were no apparent abnormal findings in all ZnO-treated groups on days 7 and 28 (**Fig. 4A and B**). In the testis, the ZnD control group at day 7 and 28 showed many histopathological changes, including degeneration of spermatids and spermatogenic cells, widened interstitial spaces and vacuolization of the seminiferous epithelium (**Fig. 4C**). However, the NH group at 7 days restored the degeneration of spermatids and spermatogenic cells and the vacuolization of seminiferous epithelium. On day 28 of ZnO administration, all ZnO treatment groups showed recovered histology similar to the normal group (**Fig. 4D**). Therefore, these findings indicated that ZnO-induced restoration of histological damage in the liver is faster than that in the testis.

Effects of ZnO treatment on MT mRNA expression in the liver and testis

Next, this study investigated the effects of ZnO treatment on *Mt-1* and *Mt-2* mRNA expression in the liver and testis. In the liver tissue samples, *Mt-1* and *Mt-2* mRNA expression was significantly increased in all treatment groups compared to the ZnD control group (**Fig. 5A and B**). In particular, the NH group showed high levels of *Mt-1* and *Mt-2* mRNA expression

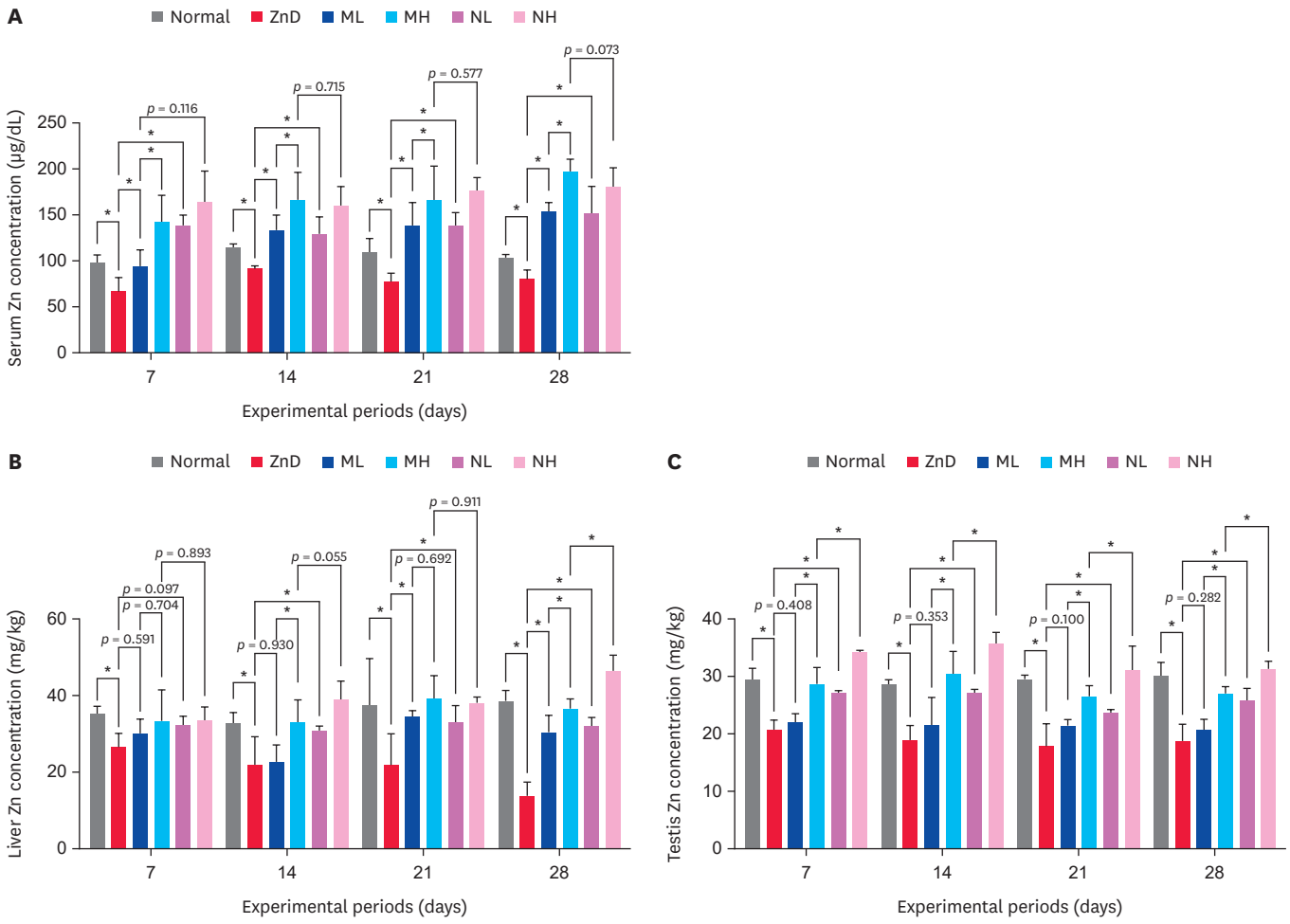

Fig. 3. Effect of M-ZnO and N-ZnO treatment on Zn concentration of mice. (A-C) After Zn deficiency induction, low or high doses of M-ZnO or N-ZnO treatments were performed daily for 4 weeks. (A) Zn concentration in the serum of mice after ZnO treatments for 7–28 days (n = 9). (B) Zn concentration in the liver of mice after ZnO treatments for 7–28 days (n = 9). (C) Zn concentration in the testis of mice after ZnO treatments for 7–28 days (n = 6). Values represent the means ± SD. ZnD, Zn-deficient control group; ML, Micro-Low group (8 mg Zn/kg body weight); MH, Micro-High group (40 mg Zn/kg body weight); NL, Nano-Low group (8 mg Zn/kg body weight); NH, Nano-High group (40 mg Zn/kg body weight); M-ZnO, micro-sized ZnO; N-ZnO, nano-sized ZnO; ZnO, Zn oxide.
* indicates statistically significant ($p < 0.05$).

compared to the ML, MH, and NL groups (**Fig. 5A and B**). ZnO-induced *Mt-1* and *Mt-2* mRNA expression was gradually decreased in a time-dependent manner (**Fig. 5A and B**).

The relative expression levels of *Mt-1* mRNA in the testis are shown in **Fig. 5C**. On day 7 of ZnO administration, *Mt-1* mRNA expression was significantly increased in the NL, NH, and MH groups compared to the ZnD control group (**Fig. 5C**). In addition, the NH group showed a high level of *Mt-1* mRNA expression compared to the ML or MH group (**Fig. 5C**). However, ZnD induction or ZnO treatment did not affect *Mt-2* mRNA expression levels during all experimental periods (**Fig. 5D**). Therefore, these findings showed that ZnD induction and ZnO treatment regulated *Mt-1* mRNA genes in the liver and testis samples, indicating that the *Mt-1* mRNA gene can be used to an indicator of Zn concentration in the liver and testis tissues.

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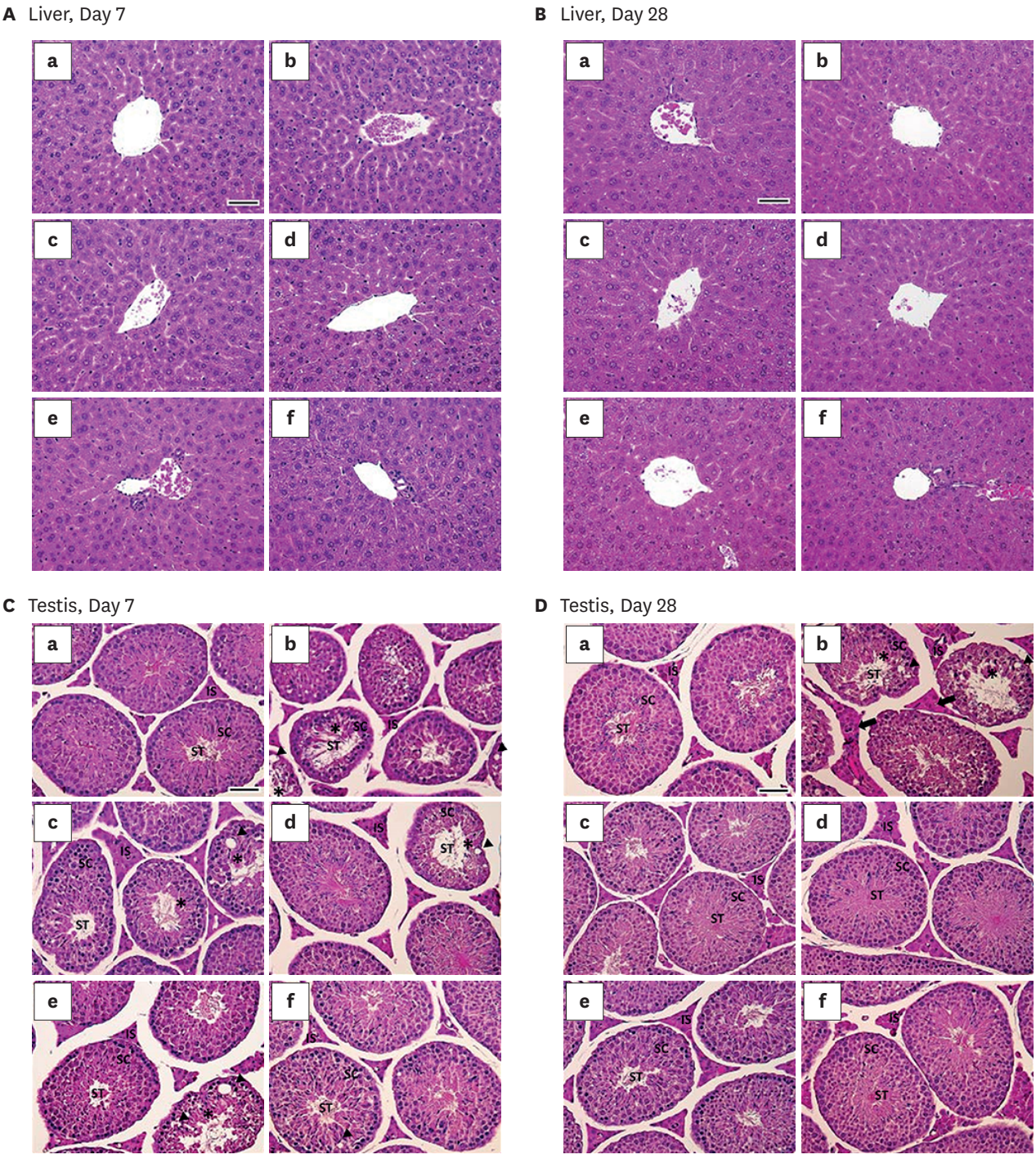

Fig. 4. Histopathology of liver and testis after Zn oxide treatments for 7 and 28 days. Liver and testis tissue samples are stained with hematoxylin and eosin staining. a: Normal group, b: Zn-deficient control group, c: Micro-Low group (8 mg Zn/kg body weight), d: Micro-High group (40 mg Zn/kg body weight), e: Nano-Low group (8 mg Zn/kg body weight), f: Nano-High group (40 mg Zn/kg body weight). Marked degeneration of spermatids and spermatogenic cells (asterisk), widened interstitial spaces (arrow) and vacuolization of seminiferous tubules with epithelial disorganization (arrow head) are noted. Bar size = 5 μm. ST, spermatids; SG, spermatogenic cells; IS, interstitial space.

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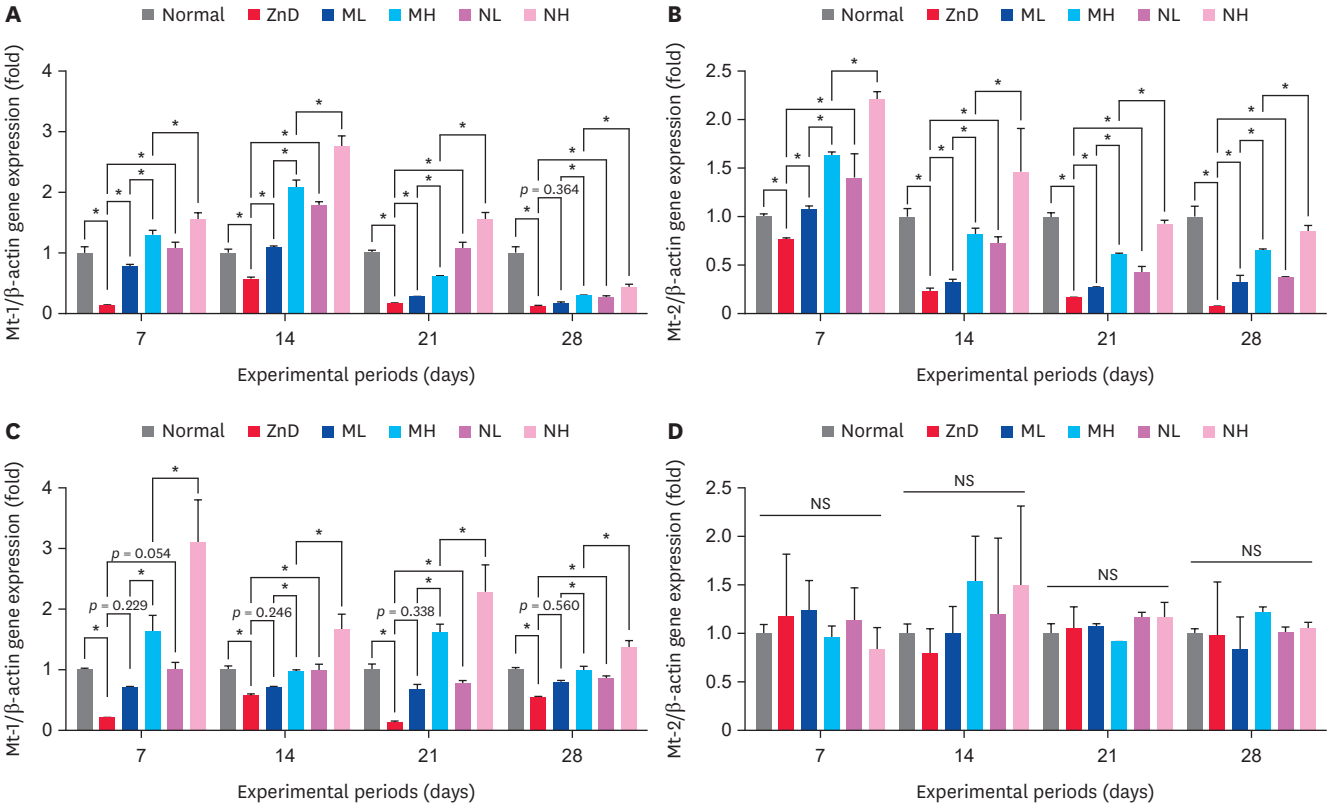

Fig. 5. Effect of M-ZnO and N-ZnO treatment on *Mt-1* and *Mt-2* mRNA expression of mice. (A-D) After Zn deficiency induction, low or high doses of M-ZnO or N-ZnO treatments were performed daily for 4 weeks. (A and B) Relative *Mt-1* and *Mt-2* mRNA expressions in the liver after ZnO treatments for 7–28 days (n = 3). (C and D) Relative *Mt-1* and *Mt-2* mRNA expressions in the testis after ZnO treatments for 7–28 days (n = 3). Values represent the means ± SD.
ZnD, Zn-deficient control group; ML, Micro-Low group (8 mg Zn/kg body weight); MH, Micro-High group (40 mg Zn/kg body weight); NL, Nano-Low group (8 mg Zn/kg body weight); NH, Nano-High group (40 mg Zn/kg body weight); M-ZnO, micro-sized ZnO; N-ZnO, nano-sized ZnO; ZnO, Zn oxide.
*indicates statistically significant ($p < 0.05$). NS indicates not statistically significant.

DISCUSSION

This study demonstrated that M-ZnO or N-ZnO supplementation recovered ZnD-induced clinical signs and histological injuries of the liver and testis. Despite the low dose of N-ZnO, its treatment efficiently restored Zn concentrations in the serum, liver, and testis and facilitated histological regeneration. In addition, *Mt-1* mRNA gene expression was closely associated with Zn levels in the liver and testis. Among the clinical signs observed in ZnD, alopecia is a typical symptom of ZnD, consistent with previous data [23]. Data showed that alopecia occurrence in all ZnO treatment groups on day 7 was lower than in ZnD mice. In addition, hair began to grow on the fourth day of administration and recovered hair loss completely on day 21. At 7 days after ZnO treatments, the recovery degree from clinical signs, such as skin wounds, keratinization, and fur erection, was confirmed in accordance with the ZnO doses and particle sizes. The keratinization in the tail region and erection of fur almost disappeared on day 7 of N-ZnO or M-ZnO administration. Furthermore, a previous study reported that ZnD in rats decreased feed intake and body weight [24,25]. ZnD induction for 8 weeks did not cause any significant difference in the body weight of mice, although the food intake in the ZnD control group was significantly decreased compared to the normal group from weeks 7 and 8. These findings indicated that ZnO supplementation for 7 days could be an effective strategy for ZnD treatment. A recent study also reported that prophylactic treatment of Zn improved the short-term neurological recovery in mice with mild head injury [26].

Zn absorption occurs mainly in the duodenum and proximal small intestine [27] and is estimated to account for approximately 20%–40% of ingested Zn. Absorbed Zn enters the portal circulation and binds to albumin at 84%, α_2-macroglobulin at 15%, and other amino acids at 1% and is carried to the liver [28]. The intracellular Zn concentration is tightly controlled by Zn importers (Zrt-Irt-like protein), Zn exporters (Zn transporters [ZnTs]), and Zn-binding proteins, such as MTs [10,12,13,29]. According to a previous study, the Zn status and the ZnO size determine Zn bioavailability [17,30]. Indeed, the Zn absorption of young men on a low Zn diet is higher than men on a high Zn diet [30]. In addition, N-ZnO, due to its small size, has a large surface and high physical characteristics; thus, N-ZnO is absorbed more efficiently than M-ZnO [17]. Consistently, our study demonstrated that Zn absorption in a low dose of N-ZnO treatment is higher than in a low dose of M-ZnO. However, the difference between Zn absorption in a high dose of M-ZnO and that in a high dose of N-ZnO is not statistically different. Therefore, present study indicates that the absorption of ZnO at low dose is particle size-dependent, but ZnO absorption at high dose is sufficient to increase blood Zn concentration in a particle size-independent manner.

The liver is the major organ controlling Zn metabolism, which is affected by many liver diseases, including liver cirrhosis and tumor [31]. This study showed no significant difference in the hepatic Zn concentration between the treatment groups and the ZnD control group on day 7. Also, the NL, MH, and NH groups showed a significant increase in the hepatic Zn concentration compared to other ZnO treatment groups on day 14, indicating that absorbed Zn might be used primarily for body function rather than storage in the ZnD state, N-ZnO can be utilized for the body more quickly, and eventually, excess Zn is stored in the liver. After 21 days of ZnO treatments, all four ZnO-treated groups recovered the hepatic Zn levels to the normal group. This is because the rest of Zn is not absorbed or stored after being restored to normal levels, and Zn is excreted by homeostatic effects. Indeed, unabsorbed dietary Zn and endogenous Zn excretion are eliminated mainly via fecal excretion [32]. ZnD can cause aberrant testicular development, reduced spermatogenesis, and male sterility. Thus, testis require a high Zn intake to maintain their optimal function [33]. In this study, the NL and NH groups restored ZnD to normal levels on day 7 of ZnO treatments. These findings indicated that the N-ZnO groups initially accumulated higher Zn levels than the M-ZnO group, and a low dose of N-ZnO supplementation is sufficient for ZnD treatment in the liver and testis. The previous study demonstrated that ZnD could cause severe damage to the testis, such as atrophy of testicular tubules and spermatid inhibition in rodents [34]. Consistently, ZnD groups after ZnD induction revealed atrophy of testicular tubules compared to the normal group. After ZnO treatments for 7 days, the damages in the NL, NH, and MH groups were almost restored compared to the ZnD control group. In addition, several studies reported that the cytotoxicity of ZnO treatment depends on the dose and exposure time through oxidative stress [35,36]. However, this study showed no significant clinical findings in the liver and testis in all ZnO treatment groups throughout the experimental periods, indicating that ZnO supplementation does not cause the cytotoxic effects in the ZnD model with low Zn status. Besides of our clinical findings in liver and testis tissues, previous researchers showed that ZnD stimulated pro-inflammatory cytokines, such as IL-1β, IL-2 and IL-6. Indeed, ZnD in human increases IL-1beta in LPS-stimulated peripheral blood monuclear cells (PBMC), but decreased IL-2 and IL-6 in phytohaemagglutin and LPS-induced PBMC [37]. In addition, zinc supplementation alleviated oxidative stress and chronic inflammation in patients with ZnD and age-related diseases [38].

In various tissues of growing mice, Zn status affected MT mRNA synthesis and protein expression levels [39]. These data showed that *Mt-1* mRNA is highly expressed in the liver and testis. In addition, *Mt-1* but not *Mt-2* mRNA expression was reduced by ZnD and recovered by ZnO treatment. Therefore, this study first suggested that *Mt-1* could be used as a reliable biomarker that can indirectly confirm the Zn level in the liver and testis. A previous study also reported that leukocyte MT expression was decreased by Zn depletion but recovered by Zn supplementation, presenting that MT among ZnTs in leukocytes is closely related to the plasma Zn status [8]. According to another study, there was a significant linear correlation between *Mt-1* mRNA expression and tissue distributions of Zn [40]. Therefore, previous and present findings suggested that cellular *Mt-1* mRNA expression can be used as a Zn status indicator in the serum and organ. This study further showed the small particle size of N-ZnO, despite the low dose, upregulated serum Zn concentration and *Mt-1* mRNA expression in the early stage of Zn recovery in the testis and liver, indicating that a low dose of N-ZnO supplementation is sufficient for the recovery of serum and intracellular Zn status.

In conclusion, this study demonstrated that ZnO supplementation restored Zn concentration and histological damage in the ZnD mice model and suggested that low-dose treatment of N-ZnO is a promising strategy for ZnD-associated diseases with minimal cytotoxicity.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

Composition of the basal and Zn-deficient diets

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Supplementary Table 2

The number of mice with clinical signs during of Zn deficiency induction

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Supplementary Table 3

The number of mice with clinical signs after ZnO administration for 7 days

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Supplementary Fig. 1

Solubility of nano- and micro-sized ZnO at pH 1.5 and 7.0. ZnO particles were dissolved with deionized water at pH 1.5 and pH 7.0, respectively. A: micro-sized ZnO and B: nano-sized ZnO.

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