ORIGINAL

Distribution and toxicity evaluation of ZnO dispersion nanoparticles in single intravenously exposed mice

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Abstract : ZnO nanoparticles (NPs) have been widely used in various commercial products. Application of ZnO NPs is expected to apply to cancer diagnosis and therapy, used as drug delivery carriers. In the present study, the lethal dose 50 (LD_{50}) of intravenously administered ZnO NPs (0.3 mg/kg) was calculated in mice. Blood kinetics and tissue distribution of a toxic dose of ZnO NPs (0.2 mg/kg, 0.05 mg/kg) were investigated after intravenous exposure. In addition, 8-hydroxy-2'-deoxyguanosine (8-OHdG) was evaluated. Following the injection, ZnO NPs were rapidly removed from the blood and distributed to organs. Pulmonary emphysema was observed pathologically study in mice at 3 days after the 0.2 mg/kg dose and at 6 days after the 0.05 mg/kg dose. ZnO NPs were mainly accumulated in the lung and spleen within 60 min. From the long-term tissue distribution study, the liver showed peak concentration at 6 days, and spleen peaked at 1 day. The lungs kept high levels until 6 days. Tissue distribution and pathological study showed that the spleen, liver, and lungs are target organs for ZnO NPs. Accumulation in the liver and spleen may be due to the phagocytosis by macrophages. A dose-dependent increase in 8-OHdG was observed in mice treated with ZnO NPs. This study is the first to show information on kinetics and target organs following intravenous ZnO injection. J. Med. Invest. 62 : 45-50, February, 2015

Keywords **:** *Nanoparticles, Intravenous injection, ; ZnO, Acute toxicity, Tissue distribution*

INTRODUCTION

Zinc oxide (ZnO) is a semiconductor material and is applied in light-emitting devices (1) and electron emitters (2). ZnO nanoparticles (NPs) have been widely used in various commercial products such as sensors, electronics, antibacterial reagents, rubber additives, paints, pigments, cosmetics, and food additives (3). ZnO is suitable for bio-imaging applications because it has a wide band gap (Eg=3.37 eV) with a large excitation binding energy (60 eV) and ensures efficient UV-blue emission at room temperature (4). Zn is an essential element, and ZnO is less toxic than quantum dot, which contains Cd. Therefore, application of ZnO NPs are expected to apply to cancer diagnosis and therapy, used as drug delivery carriers. Senthilkumar *et al.* have reported good quality ZnO NPs that disperse in water and organic solvents by a different surface treatment procedure for biomedical applications (5). Sato *et al.* have successfully prepared non-cytotoxic and visible light-emitting ZnO NP fluorophores as probes with binding sites to biomolecules on the surface for biological imaging (6, 7). However, no data is available on the toxicity of these materials *in vivo*.

Recently, with the increasing attention that has been paid to nanotoxicity, several studies have reported the toxicity of ZnO NPs *in vivo* : intranasal instillation (8), intratracheal instillation (9), and oral administration (10), dermal exposure (11). In order to apply to bio-imaging, toxicity evaluation following intravenous administration needs to be performed. To our knowledge, no information is available on ZnO NP toxicity following intravenous administration. Moreover, the toxicological mechanism and tissue distribution of ZnO NPs have not yet been systematically studied following intravenous injection. In the present study, the blood kinetics and tissue distribution of a toxic dose of ZnO NPs were investigated up to 6 days after intravenous exposure. The toxicity of ZnO NPs have shown to be related to oxidative stress, lipid peroxidation, cell membrane leakage, and oxidative DNA damage *in vitro* (12, 13). In the present study, 8-hydroxy-2'-deoxyguanosine (8-OHdG), commonly used as a marker for oxidative stress (14), was evaluated following intravenous ZnO NPs injection.

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MATERIALS AND METHODS

Materials

ZnO dispersion NPs, < 100 nm particle size (DLS), < 35 nm avg. part. size (APS), 50 wt. % in H_2O (Modification type : cationic - 3-Aminopropyl triethoxysilane) was purchased from Sigma-Aldrich (St. Lous, MO, USA). Before intravenous administration, the dispersion was diluted with distilled water to prevent aggregation.

Animals

Eight-week-old female ICR mice (B.W. 27.0-34.2 g) were purchased from CLEA Japan, Incorporated (Tokyo, Japan). The mice were housed two to a cage in a 12 h light/dark cycle with unlimited access to mouse chow and water. They were allowed to acclimate to their environment for 1 week before treatment. In a preliminary experiment, gender difference was not observed regarding ZnO NPs. These mice received a single intravenous dose via the tail vein at 1 ml/kg body weight (0.027 ml-0.034 ml). The experimental

protocols were approved by the Shimane University Animal Experimental Committee.

LD_{50} estimation

LD_{50} estimation was carried out using the Litchfield-Wilcoxon method (15). A total of 65 mice were exposed to 500, 50, 5, 0.5, 0.4, 0.38, 0.36, 0.34, 0.33, 0.25, 0.20, 0.10, and 0.05 mg/kg of ZnO NPs via the tail vein. After injection, symptoms and mortality were observed.

Kinetic study

Based on LD_{50}, a dose of 0.2 mg/kg of ZnO NPs was used in this study. Six mice were used for each time point (5, 15, 30, and 60 min), and an additional group (n=24) for each time point (5, 15, 30, and 60 min) received an equivalent volume of distilled water as a control. These mice received a single intravenous dose via the tail vein and were sacrificed at the due time point. Blood and tissues (lung, liver, kidney, and spleen) were collected and stored at -20°C until the Zn assay.

Tissue accumulation study

For this study, 6 mice were used for each time point (1, 3, and 6 days), and an additional group (n=18) for each time point (1, 3, and 6 days)received an equivalent volume of distilled water as a control. These mice received a single intravenous dose (0.05, 0.2 mg/kg) via the tail vein and were sacrificed at the due time point. Blood and tissues (lung, liver, kidney, and spleen) were collected and stored at -20°C until the Zn assay.

Pathological examination

Tissue samples (liver, kidney, lung, and spleen) harvested at each time point (1 h, 1 day, 3 days, and 6 days) following a single intravenous dose (0.05, 0.2 mg/kg) were fixed in 10% formalin for routine histologic processing. Paraffin sections were stained with hematoxylin and eosin for pathological examination. A control mouse was also examined in the same manner.

8-OHdG analysis

The concentrations of 8-OHdG in urine samples were measured using a competitive immunochromatography automatic analyzer (ICR-001, Techno Medica Co., Ltd., Yokohama, Japan), which can measure both 8-OHdG and creatinine. The collected sample was diluted 9-fold with distilled water, and 100 µl of urine was placed into each inlet. The level of 8-OHdG in urine was expressed as ng/mg creatinine.

Zn assay

Zn in whole blood (200 µl) and homogenized tissue samples were extracted by 0.01 M nitric acid (10 ml) by shaking overnight. The samples were centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant was subjected to Zn analysis by Metallo Assay Zinc LS (AKJ Global Technology Co., Ltd., Chiba, Japan). Dissociated Zn ions give a red-colored complex with 5-Br-PAPS (as chelator). The intensity of the colored complex is proportional to the Zn concentration in the sample, and the concentration of Zn in the sample is in turn determined by observation of the absorbance at 560 nm using microtiter-plate reader (Sunrise Rainbow thermo RC-R, Tecan Japan Co., Ltd., Japan). Absorbance at 700 nm was used for correction. The coefficients of variation for intra- and inter-day precision were less than 5% and 9%, respectively.

Statistical analysis

The data were expressed as the mean ± standard deviation. The factorial measure ANOVA was used to compare the Zn concentration and 8-OHdG concentration among groups with the program SPSS IBM 19 (IBM, Armonk, New York, USA). Differences in groups were analyzed using the Dunnet test.

RESULTS

LD_{50}

To obtain the LD_{50} in ICR mice, 500, 50, 5, 0.5, 0.4, 0.38, 0.36, 0.34, 0.33, 0.25, 0.20, 0.10, and 0.05 mg/kg of ZnO were exposed to ICR mice via the tail vein. The LD_{50} in ICR mice was calculated to be 0.3 mg/kg.

Blood kinetics

Based on the LD_{50}, a dose of 0.2 mg/kg of ZnO NPs were used in this study. In the present study, the data of blood presented as increased Zn level after subtraction of the Zn level in the control group. The time-course change in the blood Zn levels following intravenous injection is shown in Fig. 1. The blood Zn concentration peaked at 5 min and rapidly decreased after 15 min, then changed moderately at the next time point. During the experiment, no mortality was observed and marked hemolysis was not observed in control in groups.

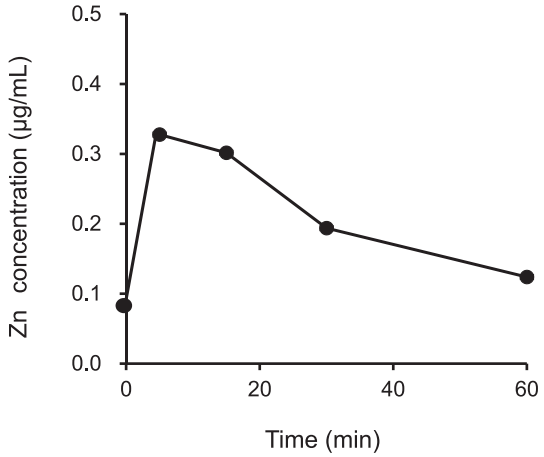

Fig. 1. Time-course change in blood Zn levels following intravenous administration (0.2 mg/kg) in mice. The data presented as increased Zn levels after subtraction of the Zn level in the control group. Data express mean ± S.D. (n=6) but the S.D. is within each symbol.

Short-term tissue distribution of ZnO

The short-term tissue distribution of ZnO was investigated at 4 time points (5 min, 15 min, 30 min, and 1 h) following intravenous injection of ZnO NPs (0.2 mg/kg) (Fig. 2). In general, an intravenously injected substance is first distributed in the lungs and then distributed to whole body via blood circulation. The level in the liver (Fig. 2A) and the lung (Fig. 2C) peaked at 5 min, and that in the kidney (Fig. 2B) and spleen (Fig. 2D) peaked at 15 min. The liver showed the lowest concentration among 4 tissues (Fig. 2A), and the kidney level rapidly decreased after 15 min to the same level as the control group (Fig. 2B).

Long-term tissue distribution of ZnO

The long-term tissue accumulation of ZnO NPs were evaluated at 3 time points (1 day, 3 days, and 6 days) following intravenous injection of ZnO NPs (0.05 mg/kg, 0.2 mg/kg) (Fig. 3). To investigate the dose-dependent effect of ZnO NPs, these two doses were investigated. At a dose of 0.05 mg/kg, only kidney levels at 1 day showed significantly higher levels than those of the control group (Fig. 3A). At a dose of 0.2 mg/kg, the spleen showed a significantly

higher Zn level at 1 day than that of the control group (Fig. 3B). Within 3 time points, Zn concentration in the spleen was gradually decreased. In contrast, the liver showed a significantly higher Zn level than that of the control group at 6 days after the 0.2 mg/kg dose. In the present study, dose-dependent distribution was not observed.

8-OHdG analysis

Fig. 4 shows the 8-OHdG concentration in urine at several time points (1 day, 3 days, and 6 days). The highest concentration was observed at 1 day and gradually decreased at 6 days. A dose-dependent effect was observed.

Pathological examination

Fig. 5 shows the time-course pathological findings for the lung, liver, kidney, and spleen of mice dosed with ZnO NPs. Hepatic sinusoid was partly dilated in mice at 1 day following intravenously administered 0.2 mg/kg dose of ZnO NPs. Destruction and dilation of the alveolar wall, which suggests pulmonary emphysema, was observed in mice at 3 days after the 0.2 mg/kg dose and at 6 days after the 0.05 mg/kg dose. No pathological change was observed in the kidney or spleen. In the present study, pathological findings were not correlated to Zn levels in tissue.

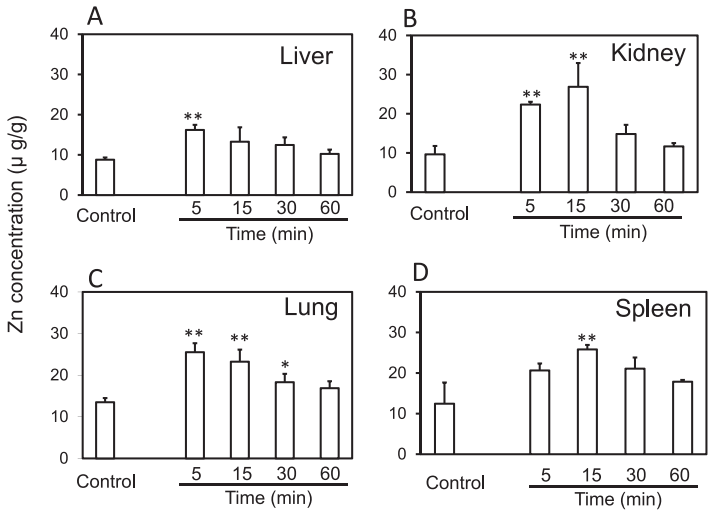

Fig. 2. Short-term tissue distribution of Zn following intravenous administration of a 0.2 mg/kg dose of ZnO NPs in mice : Liver (A), Kidney (B), Lung (C), and Spleen (D). Data express mean±S.D. (n=6). *, p< 0.05 ; **, p< 0.01 when compared between control groups (ANOVA, Dunnet test).

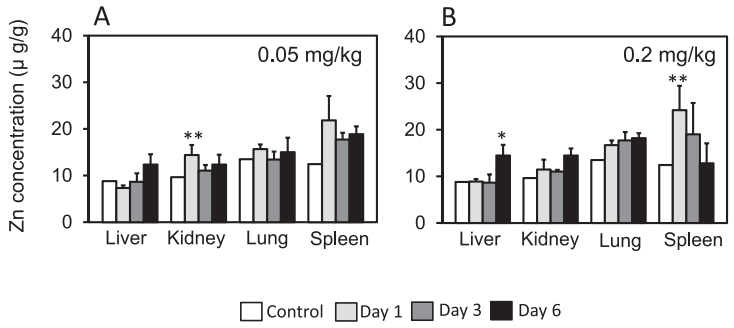

Fig. 3. Long-term tissue distribution of Zn following single intravenous administration of a 0.05 mg/kg dose (A) and a 0.2 mg/kg dose (B) of ZnO NPs in mice. Data express mean±S.D. (n=6). *, p< 0.05 ; ** p< 0.01 when compared between control groups (ANOVA, Dunnet test).

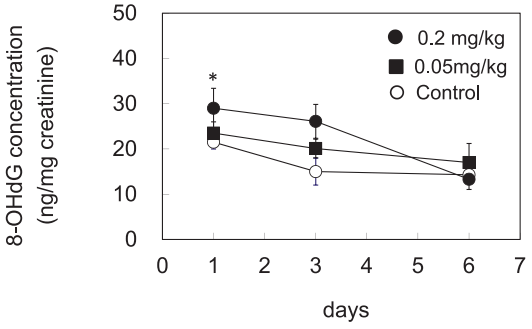

Fig. 4. Time-course variation in concentrations of 8-OHdG in the urine of mice after single intravenous administration of ZnO NPs (0.05 mg/kg 0.2 mg/kg). Data express mean±S.D. (n=6). *, p< 0.05 when compared between control groups (ANOVA, Dunnet test).

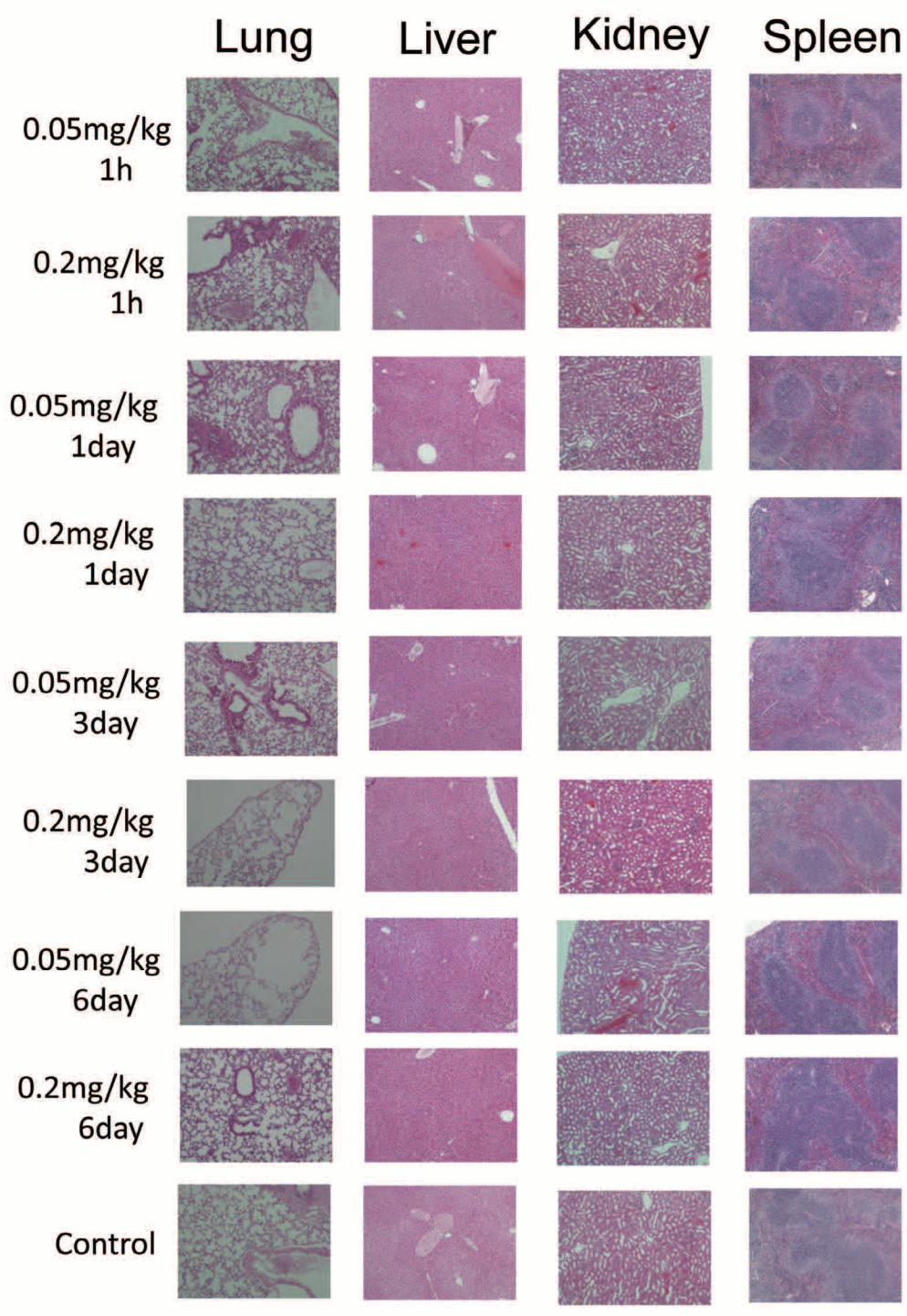

Fig. 5. The histopathological changes in the liver, kidney, lungs, and spleen (HE, 100x).

DISCUSSION

ZnO NPs are widely used in commercial products such as semiconductors, zinc oxide ointments, baby powder, and sunscreen. Because Zn is an essential element, biological and medical application is expected. We consider the medical application of ZnO for bio-imaging applications such as cancer diagnosis, and it is important to evaluate the toxicity before medical application. Several previous studies *in vivo* have shown the toxicity of ZnO NPs. The present study investigated the tissue distribution and accumulation of intravenously administered ZnO NPs. Following injection, a rapid decline in blood ZnO concentration was observed within 30 min following injection (Fig. 1) : ZnO NPs were rapidly distributed to organs. The present study indicated that ZnO NPs were mainly accumulated in the lungs and spleen within 60 min after injection (Fig. 2). The Zn level in the kidney peaked at 15 min and rapidly decreased to a normal level at 30 min.

In the long-term tissue distribution study, the liver showed a peak concentration at 6 days (Fig. 3B), and the spleen peaked at 1 day (Fig. 3B). Accumulation in the liver and spleen following intravenous injection of NPs such as silica, titanium dioxide, quantum dots, gold, and silver, have been reported by previous studies

(16-19). Xie *et al.* have revealed that the TiO_2 NPs were taken up mainly by liver and spleen endocytosis, and they were also detected in phagosomes (18). Macrophages, involved in the uptake and metabolism of foreign molecules and particles, are located primarily in the liver, spleen, lungs, and bone marrow (20). When NPs are administered intravenously, various serum proteins bind to the surface of the NPs, which are recognized by the scavenger receptors on the macrophage cell surface and internalized, leading to a significant loss of NPs from circulation (21). In the present study, the Zn level in the liver was relatively low. This may be due to the difference in particle size, and the small-particle content may be low in the present study. Lankveld *et al.* have reported that the 20 nm Ag particles mainly distributed to the liver, whereas the larger particles mainly distributed to the spleen (17). In contrast, a previous study has shown that the kidney, liver, and lungs would be possible target organs following single oral ZnO NPs administration (22). The difference in tissue distribution is likely due to the ionization properties of ZnO : ZnO is soluble in acidic conditions and readily dissolves under gastric conditions. A pathological change was only observed in the lungs (pulmonary emphysema) and liver (hepatic sinusoid) dilation (Fig. 5). This may be due to the higher retention of ZnO in the lungs (Figs. 2 and 3).

The toxicity of ZnO NPs have been shown to be related to oxidative stress, lipid peroxidation, cell membrane leakage, and oxidative DNA damage *in vitro* (13, 23). Previous studies have shown that ZnO NPs have cytotoxicity via Zn^{2+} release (24, 25). Generally, ZnO NPs are poorly water soluble but easily release Zn^{2+} into a culture medium. 8-OHdG is one of the major products of reactive oxygen species (ROS)-induced DNA damage (26). It is widely used as an important biomarker of oxidative DNA damage (27). In the present study, 8-OHdG in urine at 3 time points (1 day, 3 days, and 6 days) was evaluated (Fig. 4). 8-OHdG levels peaked at 1 day and decreased to a normal level, and the level was dose dependent. This indicates that ZnO NPs cause oxidative stress by generating ROS. However, the levels were not remarkably high when compared to the spleen and lungs : Zn^{2+} release might be smaller by intravenous injection when compared to oral administration because of phagocytosis. A previous study has shown that silica NPs generate ROS, and the generated ROS may trigger pro-inflammatory responses both *in vivo* and *in vitro* (28). This has not been confirmed with regard to ZnO NPs, and we are planning to investigate the pro-inflammatory response relevance in ZnO NPs toxicities. Further study is needed to evaluate the toxicological effect of ZnO NPs.

CONCLUSION

In this study, the blood kinetics and tissue distribution of a toxic dose of ZnO NPs were first investigated following intravenous exposure, and 8-OHdG was evaluated in the urine of ZnO NP-administered mice. Following injection, ZnO NPs were rapidly removed from the blood and distributed to various organs. Tissue distribution and pathological study showed that the spleen, liver, and lungs are target organs for ZnO NPs. A dose-dependent increase in 8-OHdG was observed in ZnO NP-treated mice. These results provide information on kinetic and target organs following intravenous ZnO injection.

CONFLICT OF INTERESTS

None of the authors have any conflicts of interest.

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