European Review for Medical and Pharmacological Sciences 2015; 19: 4920-4926

Pro-inflammatory responses and oxidative stress induced by ZnO nanoparticles *in vivo* following intravenous injection

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Abstract. – OBJECTIVE: To determine the toxicological effect of ZnO nanoparticles (NPs), inflammatory responses, serum biological parameters and oxidative stress markers of Superoxide dismutase (SOD) were evaluated followed by intravenous treatment of ZnO NPs in mice.

MATERIALS AND METHODS: Inflammatory responses induced by a dose of 0.2 mg/kg ZnO NPs, followed by a single intravenous treatment were examined in mice. In addition, the serum biological parameters and oxidative stress markers were evaluated. Blood and spleen were collected following treatment. The mRNA transcript levels of inflammatory-related genes (TNF-α and IL1-β) were elevated in the spleen cells of mice treated with ZnO NPs at 12h.

RESULTS: The elevated levels of TNF-α and IL1-β in supernatants of spleen cell cultures of mice treated with ZnO NPs were also observed at 24h. The serum aspartate aminotransferase, glutamate pyruvate alanine aminotransferase, and lactate dehydrogenase levels significantly increased at 6h and 12h in ZnO NPs treated group, indicating liver cell injury and tissue damage. On the other hand, no elevation was observed in BUN and Cre, biochemical markers of kidney damage. SOD activities were significantly elevated at 24 h and 48 h.

CONCLUSIONS: This study shows the ZnO induced pro-inflammatory response *in vivo*, that this response may be related to oxidative stress, and to show hepatic damage at an early stage.

Key Words:
ZnO nanoparticles, Intravenous injection, Mice, Cytokines, Superoxide dismutase.

Introduction

Zinc oxide (ZnO) is a semiconductor material and is applied in light-emitting devices and electron emitters[1,2]. 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 high 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. Senthilkumar et al[5] have reported good quality ZnO NPs that disperse in water and organic solvents for biomedical applications. Sato et al[6,7] have successfully prepared non-cytotoxic and visible light-emitting ZnO NP fluorophores.

Recently, with the increasing attention that has been paid to nanotoxicity. Several reports have reported[8] the ZnO NPs toxicity *in vivo*: intranasal instillation, intratracheal instillation[9], oral administration[10] and dermal exposure[11]. In order to apply to bio-imaging, toxicity evaluation following intravenous administration needs to be performed. To our knowledge, little information is available on the toxic mechanisms of ZnO NPs following intravenous administration[12,13].

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Previous studies have evaluated the pro-inflammatory responses induced by silica NPs[14], TiO_2 NPs[15], carbon black NPs[16] and by gold NPs *in vivo*[17]. Several studies *in vitro*[18-21] have investigated the involvement of ZnO NPs in pro-inflammatory responses. However, to our knowledge, little information is available on pro-inflammatory responses of ZnO NPs *in vivo*[22]. Therefore, in the present study, we investigated inflammatory responses induced by ZnO NPs in mice followed by intravenous treatment. In addition, serum biological parameters and oxidative stress markers of superoxide dismutase (SOD) were evaluated to determine the toxicological effect of ZnO NPs.

Materials and Methods

Materials

ZnO dispersion (Modification type: cationic - 3-aminopropyl triethoxysilane) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Before intravenous administration, the dispersion was diluted with distilled water. The shape and average size of the particles were determined by transmission electron microscope (TEM) (JEM-2010, JEOL, Tokyo, Japan). The average size and the size distribution were analyzed by a dynamic light scattering particle size analyzer LB-550 (HORIBA, Kyoto, Japan).

Animal Experiment

Eight-week-old female ICR mice (B.W. 27.0-34.2 g) were purchased from CLEA Japan, Inc., (Tokyo, Japan). The mice were housed two to a cage in a 12h light/dark cycle with unlimited access to mouse chow and water and they were offered commercial diet. They were allowed to acclimate to their environment (20-22 °C, 45-55% humidity) for 1 week before treatment. The experimental procedure was approved by the Shimane University Animal Experimental Committee.

Based on LD_{50} (0.3 mg/kg) which obtained from our previous study[13], the lower dose (0.2 mg/kg) of ZnO NPs was used in this study in order to investigate biogenic effects. Six mice were used for each time point groups (6, 12, 24, 48, 72, and 96 h), and control group (n = 6) received vehicle alone. These mice received a single intravenous dose (0.2 mg/kg) via tail vein at 1 ml/kg body weight (0.1%), were sacrificed at the correct time point, and their blood and spleens were collected.

Biochemical Analysis

To obtain serum sample, one milliliter of fresh mouse blood was centrifuged immediately after sampling. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatinine (Cre), and blood urea nitrogen (BUN) in the serum were measured by an auto dry-chemistry analyzer SPOTCHEM™ SP4410 (Arkray Inc., Kyoto, Japan).

Cell Culture

The spleens were dissected out, and single-cell suspensions were prepared by mechanical dissociation in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS), 5 μM 2 ME, and 10 U/ml of penicillin. Spleen cells were filtered through a 40-μm nylon mesh and washed twice with culture medium. Spleen cells (5×10^5/well) were cultured for 40 h. After 40h, culture supernatants were collected and reserved at -20°C until cytokine (TNF-α and IL1-β) analysis; and cells were then immediately subjected to total RNA isolation according to previous study[23].

Real-time PCR

The mRNA transcript levels were measured by real-time RT-PCR. Using spleen cells, the total RNA was isolated using NucleoSpin® RNA II (MACHEREY-NAGEL Inc., Bethlehem, PA, USA). cDNA was obtained by reverse transcription using PrimeScript® RT Master Mix (Takara Bio, Inc., Otsu, Shiga, Japan). The RT reaction was conducted at 37°C for 15 min and ended by heating at 85°C for 5 sec followed by cooling at 4°C.

Real-time PCR was performed with a Thermal Cycler Dice Real Time System (Takara Bio, Inc., Otsu, Shiga, Japan) and SYBR® Premix Ex Taq™ II (Takara Bio, Inc., Otsu, Shiga, Japan). Relative mRNA transcript levels of cytokines (TNF-α and IL1-β) as compared with β-actin were calculated. PCR was performed at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Dissociation was started at 95°C for 15 sec, followed by 60°C for 30 sec and 95°C for 15 sec.

The following primers (sense and anti-sense, respectively) were used for INF-α: 5′-CATCTTCTCAAAATTCGAGTGACAA-3′ and 5′-TGGGAGTAGACAAGGTACAACCC-3′; for IL1-β: 5′-TGAACTCAAATGTGAAATGC -3′ and 5′-TGTCCATTGAGGTGGAGAGC-3′; and for β-actin: 5′-AGAGGGAAATCGTGCGTGAC-3′ and 5′-CAATAGTGATGACCTGGCCGT-3′.

Measurement of TNF-α and IL1-β

Spleen culture supernatants were collected and the production of cytokines (TNF-α and IL1-β) was evaluated using cytokine ELISA kits (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Absorbance at 450 nm was measured using microtiter-plate reader (Sunrise Rainbow Thermo RC-R, Tecan Japan Co., Ltd., Japan).

SOD Assay

SOD in serum was assayed using a SOD assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). Absorbance at 450 nm was measured using microtiter-plate reader.

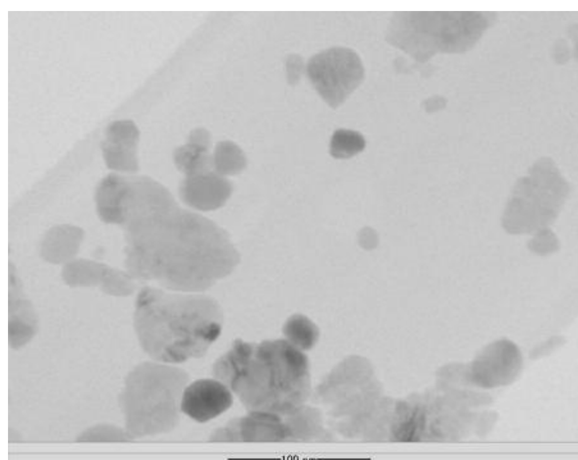

Figure 1. TEM image of ZnO nanoparticles in distilled water.

Statistical Analysis

The data were shown as the mean ± standard deviation. A factorial measure ANOVA was used with SPSS IBM 19 software (IBM, Armonk, NY, USA). Differences in groups were analyzed using the Dunnett test $p < 0.05$ was considered statistically significant.

Results

Characterization of ZnO NPs

The ZnO NPs employed in this study were observed by TEM (Figure 1), and size distribution was measured (Figure 2). The ZnO NPs were pebbly shape, and the average size was 58.5 nm. It was confirmed that the ZnO NPs is suitable for intravenous injection, because it showed pebbly shape, not the needle-like shape which is harmful for blood capillary, and the particle size is sufficiently-small compared to the diameter of blood capillary of mice.

Blood Biochemical Analysis

Blood biochemical analysis was performed following intravenous injection of 0.2 mg /kg of ZnO NP at 6 time points (Figure 3). The AST, ALT, and LDH levels were significantly higher than those in control groups 6h and 12h after treatment ($p < 0.01$), indicating liver cell and tissue damage. On the other hand, no elevation was observed in BUN and Cre, biochemical markers of kidney damage.

Expression of TNF-α and IL1-β genes

The mRNA transcript levels of inflammatory-related genes (TNF-α and IL1-β) in the spleen cells of mice treated with ZnO NPs were investigated. At 12h, the mRNA expressions of

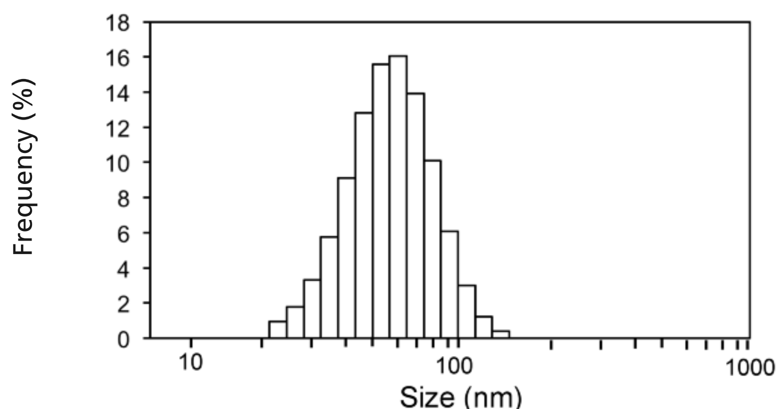

Figure 2. Size distribution of ZnO NPs.

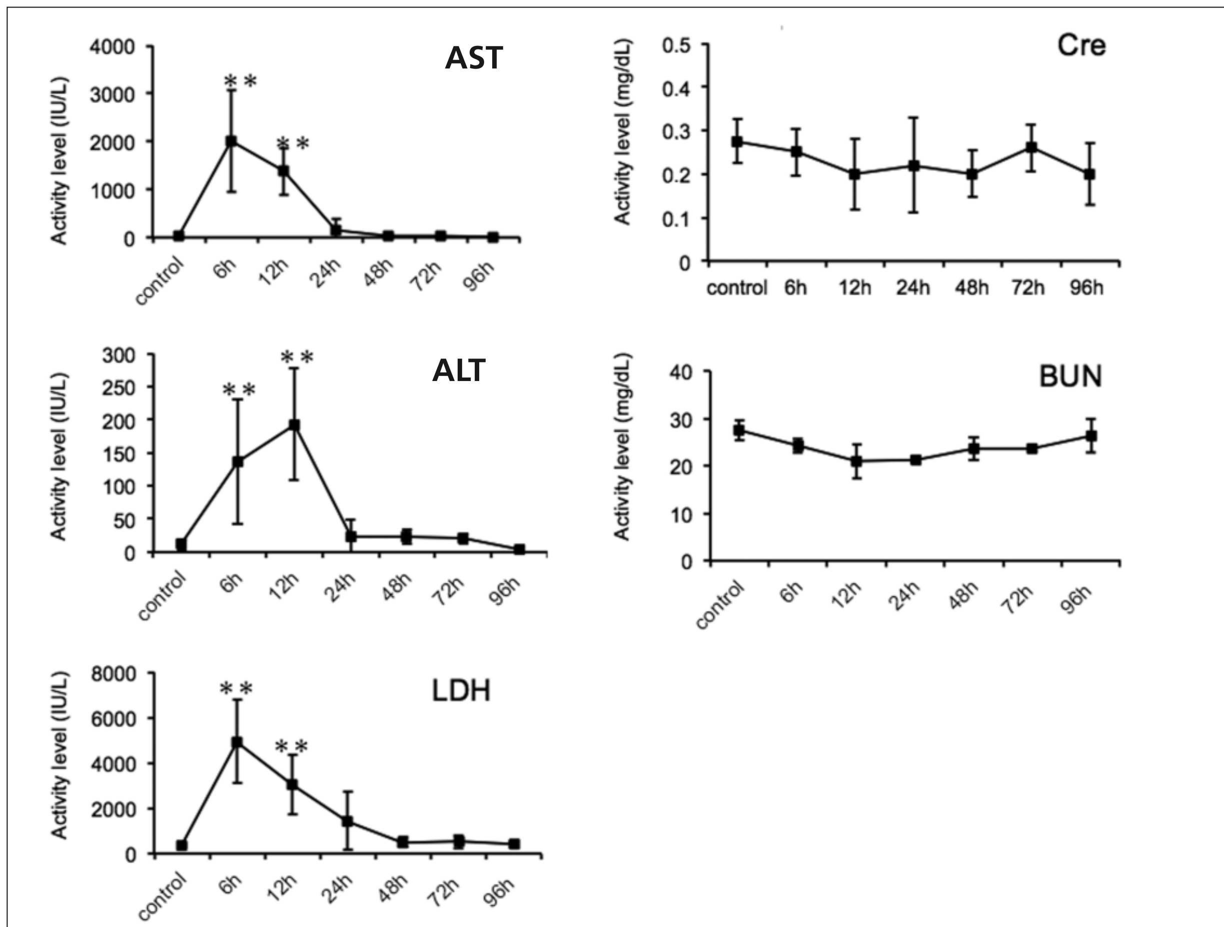

Figure 3. Biochemical analysis of the serum of the mice treated with ZnO NPs at different time points. ZnO NPs were intravenously administrated to mice at 0.2 mg/kg. The serum levels of AST, ALT, LDH, Cre, and BUN were measured. Data express the mean ± SD (n = 5). *, $p < 0.05$; **, $p < 0.01$ when compared to control groups (ANOVA, Dunnett test).

TNF-α (Figure 4 A) and IL1-β (Figure 4 B) were significantly elevated ($p < 0.01$).

Levels of TNF-α and IL1-β in mice

The levels of TNF-α and IL1-β in supernatants of spleen cell cultures of mice treated with ZnO NPs were also investigated. At 24h, the levels of TNF-α (Figure 4 C) and IL1-β (Figure 4 D) were significantly elevated.

SOD Levels

Figure 5 shows the serum SOD levels following intravenous injection of 0.2 mg/kg ZnO NPs at 6 time points. Serum SOD levels were significantly elevated at 24h and 48h ($p < 0.05$).

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. It is important to evaluate the toxicity before medical application.

In the present study, serum biochemical analysis was carried out (Figure 3). As a result, significant increase in AST, ALT, and LDH levels compared to the control groups at 6h and 12h after treatment. The liver is the largest detoxification organ. This indicates liver cell injury and tissue damage at an early stage. This is consistent with our previous study: hepatic sinusoid was partly dilated in mice at 1 day following intravenously administered 0.2 mg/kg dose of ZnO NPs and no pathological changes were observed in liver at 3 and 6 days following treatment[13]. The levels of Cre and BUN in the ZnO NPs exposed groups remained normal, indicating that ZnO NPs have no effect on kidney function. Also, this is in agreement with

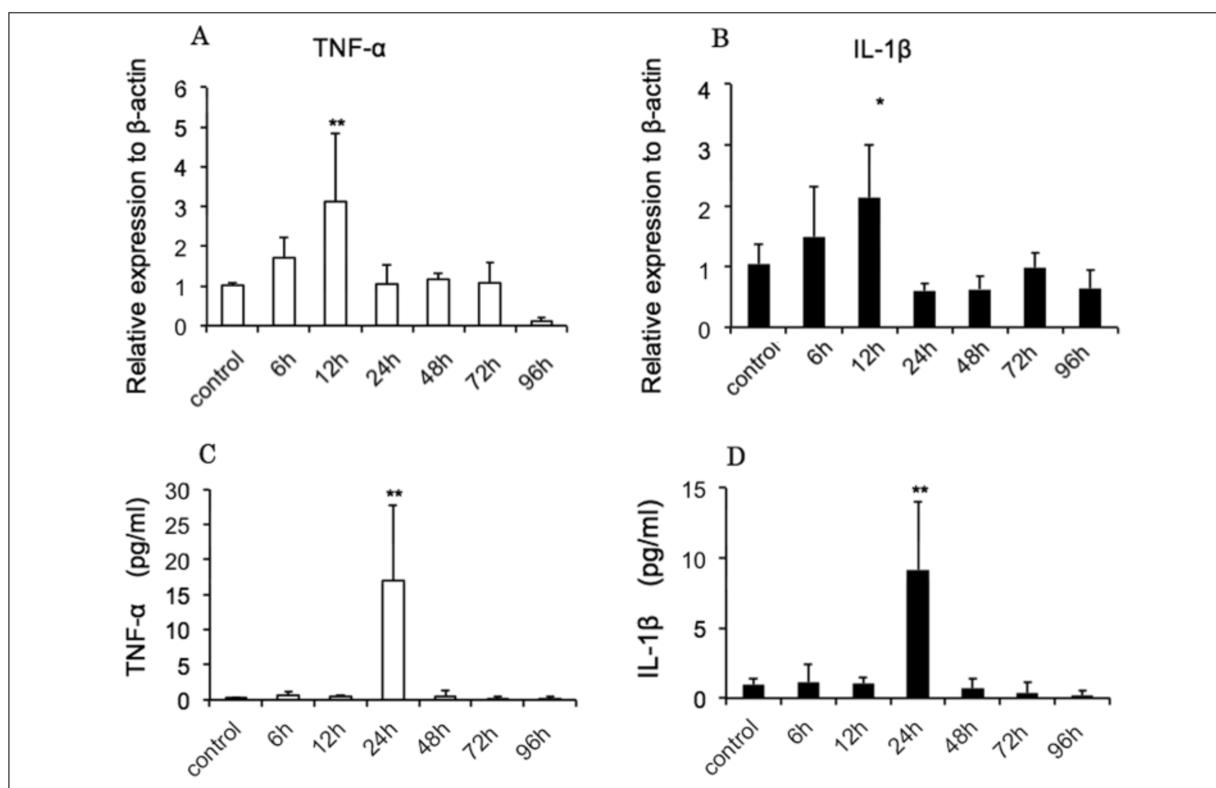

Figure 4. Changes in mRNA cytokine expression levels and cytokine levels in supernatants of spleen cell cultures at different time points following intravenous injection of ZnO NPs. mRNA levels of (A) TNF-α and (B) IL1-β. Levels (C) TNF-α and (D) IL1-β in spleen culture supernatants. Data express the mean ± SD (n = 5). *, $p < 0.05$; **, $p < 0.01$ when compared with control groups (ANOVA, Dunnett test).

our previous study showing no obvious pathological change at kidney following 0.2 mg/kg dose of ZnO NPs[13].

There are few *in vivo* studies regarding the effect of ZnO NPs on the pro-inflammatory response and the toxicological effects. Niwa et al[16] have shown that monocyte chemoattractant protein-1, interleukin-6, and C-reactive protein, were higher in the carbon black NPs-inhaled group than in the controls that followed. Park et al[14] have shown the increased mouse blood levels of TNF-α and IL1-β following a single treatment of silica NPs (50 mg/kg, i.p.). Chen et al[17] have investigated pro-inflammatory cytokine expression following a single i.p. injection of gold NPs in mice; a reduction in TNF-α and IL6-β mRNA levels in the fat were observed. Previous studies *in vitro* have investigated the involvement of ZnO NPs in pro-inflammatory responses. Prach et al[19] have shown that TNF-α was not induced by the ZnO particles in THP-1 cells. On the other hand, Cho et al[21] have reported increased IL-1β expression in THP-1 cells and elevated TNF-α and IL-1β expression in peripheral blood monocyte-derived macrophages by ZnO NPs. Increased levels of IL-8 were reported in colon carcinoma cells from human[18] and A 549 cells with ZnO NPs treatment[20].

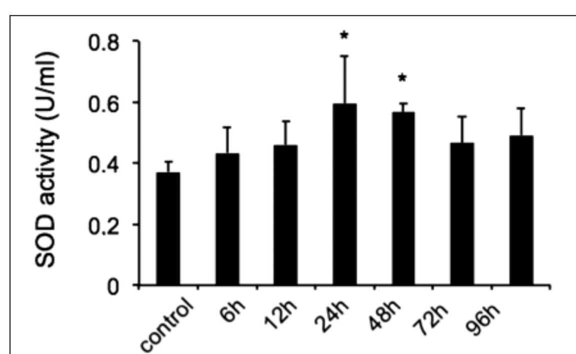

Figure 5. Serum SOD levels at different time points. ZnO NPs were intravenously administrated to mice at 0.2 mg/kg. Data express the mean ± SD (n = 5). *, $p < 0.05$; **, $p < 0.01$ when compared to control groups (ANOVA, Dunnett test).

In this study, the pro-inflammatory influences of ZnO NPs were revealed following intravenous injection in mice. As shown in Figure 4, increased mRNA expression and levels were observed. The mRNA expressions of TNF-α and IL1-β in the macrophages of mice reached their maximal level at 12 h (Figure 4 A and B); the levels of TNF-α and IL1-β in spleen culture supernatants of mice reached maximal levels at 24 h (Figure 4 C and D). These results indicate that ZnO NPs are eliminated quite rapidly.

Many studies have been reported on the association between oxidative stress and inflammation. In our study, significantly elevated serum SOD levels were observed at 24 h and 48 h (Figure 5). Although the pro-inflammatory effects were observed earlier, the relevance of oxidative stress in inflammation may be considered. Previously, we have reported that 8-hydroxy-2'-deoxyguanosine was evaluated following intravenous injection of 0.2 mg /kg ZnO NP in mice at 1day[13].

Conclusions

This study is the first to show the ZnO induced pro-inflammatory response *in vivo*, that this response may be related to oxidative stress, and to show hepatic damage at an early stage.

Acknowledgements

This work was supported by the Foundation for Priority Research Project (S-Green & Life Nanomaterials Project) of Shimane University and in part by JSPS KAKENHI Grant Number 26713025 (Grants-in-Aid for Young Scientists A) to J.F. The authors acknowledge the cooperation of Dr. Taisuke Hayashi of the Center for the Integrated Research in Science, Shimane University for providing the TEM facilities.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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