Acute Effects of Pulmonary Exposure to Zinc Oxide Nanoparticles on the Brain *in vivo*

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ABSTRACT

Although the applications for zinc oxide nanoparticles (ZnONP) are continually increasing, the neurotoxicity of this material remains unclear. This study investigated the acute effects of pulmonary exposure to ZnONP on the brain in terms of behavioral changes, oxidative stress, inflammation, and tau and autophagy expressions using rat subjects. Based on the test subjects' performance in a Morris water maze and an elevated-plus maze, respectively, no major alterations occurred in spatial cognition or learning ability, or anxiety. Following exposure to 10 mg kg⁻¹ of ZnONP, we observed that the level of 8-hydroxy-2'-deoxyguanosine (8-OHdG)/dG significantly increased in the hippocampus, whereas those of interleukin (IL)-1 β and IL-6 significantly decreased in the cerebellum and the cortex. Additionally, microglia were activated in the hippocampus. Tau protein expression was strong in the cerebellum and the hippocampus, but no significant expression of Beclin-1, light chain 3 (LC3) II/I, or ubiquitin was detected. Our results suggest that acute exposure to ZnONP induces oxidative stress, microglia activation, and tau protein expression in the brain, leading to neurotoxicity.

Keywords: Autophagy; Central nervous system; Elevated-plus maze; Morris water maze; Nanoparticle; Ubiquitin.

INTRODUCTION

Zinc oxide nanoparticles (ZnONP) are used in many commercial nanomaterials and food additives, and have been detected in ambient air and occupational environments (Fine *et al.*, 1997; Grommes and Soehnlein, 2011). In the United States, the permissible exposure limit (PEL) for zinc oxide (ZnO) in occupational environment is set to 5 mg m⁻³

of respirable particles (Gordon and Fine, 1993). Notably, pulmonary exposure of ZnO has been linked to adverse human health effects. For example, metal fume fever occurs after exposure to ZnO for a few hours (Kwon *et al.*, 2014), and acquired tolerance occurs after repeated exposures (Fine *et al.*, 2000). A study showed that zinc-hexachloroethane smoke caused lung fibrosis after exposure (Richards *et al.*, 1989). Our previous reports showed cardiopulmonary effects caused by ZnONP exposure *in vivo* (Chuang *et al.*, 2014; Pan *et al.*, 2015; Chuang *et al.*, 2017) and *in vitro* (Pan *et al.*, 2014). However, neurotoxicity that occurs with pulmonary exposure of ZnONP remains unclear.

A clinical study observed that metal fume exposure contributes to neuropsychological disorders in welders,

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including a loss of balance, cognitive disorders, and Parkinsonism (Bowler *et al.*, 2007). Clinical observations suggest that chronic exposure to metal fumes may be associated with the development of neurodegenerative diseases. Increasing numbers of studies have highlighted the possibility of neurotoxicity caused by nanoparticles (Sharma and Sharma, 2012; Mushtaq *et al.*, 2015). ZnONP, for example, were indicated to regulate synaptic transmission *in vitro* (Zhao *et al.*, 2009) and to disturb spatial cognition *in vivo* (Han *et al.*, 2011). A recent study indicated that anxiogenic behavior of male Swiss mice could be associated with exposure of ZnONP by intraperitoneal injection (de Souza *et al.*, 2018). Currently, three possible pathways are potentially involved in the influence of nanoparticles on the brain: (1) direct penetration of the blood-brain barrier (BBB), (2) translocation along the olfactory nerve, and (3) indirect effects from oxidative stress and inflammation. Due to the unique physicochemistry of ZnONP, their neurotoxicity might not result from a single pathway. It was reported that ZnONP can reach the brain via the olfactory bulb-brain translocation pathway after exposure (Garcia and Kimbell, 2009; Kao *et al.*, 2012). Also, ZnONP is able to interact with plasma and brain proteins and regulate rat blood and brain homogenates (Shim *et al.*, 2014). Therefore, it is important to understand the neurotoxicity of the central nervous system (CNS) after pulmonary exposure of ZnONP.

Notably, inhaled nanoparticles are associated with oxidative-inflammatory reactions at sites of deposition (Oberdorster *et al.*, 2009). Combustion-derived Zn has been linked to increase of oxidative DNA damage (Xiao *et al.*, 2013; Shao *et al.*, 2016; Shao *et al.*, 2017). Oxidative stress is recognized to initiate neurodegenerative diseases, leading to loss of cognitive function (Calderon-Garciduenas *et al.*, 2004). Inflammation is a critical response in the development of CNS abnormalities. For example, proinflammatory cytokines, and excitatory amino acids and nitroxidative species may be associated with cognitive deficits and neurodegenerative diseases (Uttara *et al.*, 2009). Activation of microglia was implicated in neuronal injury, which is associated with the occurrence of many neurodegenerative diseases and conditions (Carnevale *et al.*, 2007; Dheen *et al.*, 2007). The microglial response supports the potential of a common underlying neuroinflammatory mechanism, which is reprogrammed to a heightened proinflammatory phenotype (priming) to become deleterious in neurodegenerative diseases. Previous studies have reported that ZnONP caused microglia activation and neuron death (Sruthi and Mohanan, 2015; Sharma *et al.*, 2017).

Tau proteins accumulated intraneuronally is believed as an important hallmark in the development of neurodegenerative diseases (Serrano-Pozo *et al.*, 2011). Additionally, autophagy is able to play an essential degradative mechanism that remove tau from cells in brain. Together, oxidative-inflammatory responses and tau-cleaning mechanisms could be vital to regulating neurodegenerative diseases. The neurotoxic potential of ZnONP was recently investigated; however, their impacts on the CNS remain unclear. We hypothesized that inhaled ZnONP cause neurotoxicity on CNS. The objective of the present study was to investigate the effects

of ZnONP on brain. We investigated behavioral alterations, oxidative stress and inflammation as well as tau, autophagy and ubiquitin expressions after acute pulmonary exposure.

METHODS

Particles and Reagent Sources

ZnONP at 50 nm in average diameter and $10.8 \text{ m}^2 \text{ g}^{-1}$ in surface area were obtained from Sigma-Aldrich (CAS No. 1314-13-2; St. Louis, MO, USA). Their physicochemical characteristics were previously reported (Chuang *et al.*, 2014). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless explicitly stated otherwise.

Physical Characterization of ZnONP

We dispersed ZnONP in phosphate-buffered saline (PBS) supplemented with 5% bovine serum albumin (BSA) at final concentrations of 5 and 10 mg mL^{-1} with 30 min of sonication. Serum protein was reported to well disperse nanoparticles when suspended in solution (Chuang *et al.*, 2014). Hydrodynamic diameters were determined using a Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK). ZnONP solutions in PBS with 5% BSA were then air-dried on grids followed by adherence to carbon adhesive tabs on aluminum scanning electron microscopic (SEM) stubs. An FEI Ultra High Resolution Field Emission-SEM (FE-SEM; Nova NanoSEM 230; Hillsboro, OR, USA) was used to investigate the morphology of ZnONP at 5 and 10 mg mL^{-1} .

Animals

Seven-week-old male Sprague Dawley (SD) rats were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Rats were housed in plastic cages under a $22 \pm 2^\circ\text{C}$ temperature and $55 \pm 10\%$ relative humidity in a 12/12-h light/dark cycle. All animal experiments complied with protocols of the Institutional Animal Care and Use Committee (IACUC) at National Taiwan University (Taipei, Taiwan; IACUC approval No. 20150233).

Experimental Design

The experimental design is shown in Fig. 1. On Day 0, rats were given intratracheal (IT) instillation of 0 (vehicle control), 5, and 10 mg kg^{-1} body weight of ZnONP in $200 \mu\text{L}$ PBS (with 5% BSA) per rat under light anesthesia (2% isoflurane) using a rodent anesthesia machine ($n = 15$ per treatment; Northern Vaporisers Ltd., Skipton, UK). On Day 1, 5 rats each treatment were randomly selected for elevated-plus maze followed by euthanasia (by CO_2) after the experiment. The samples were collected for biochemical analyses. Rats from the biochemical analyses were immediately decapitated. The cerebellum, cortex, and hippocampus of each rat were obtained, frozen in liquid nitrogen, and stored at -80°C for biochemical and metal analyses. The rest of 10 rats were performed a Morris water maze (MWM; $n = 10$ per treatment) on Days 8–13. On Day 13, the elevated-plus maze was conducted followed by euthanasia after the experiment. The behavior examination was conducted in an animal behavior room, which is a soundproof room with controlled environment (e.g., light, temperature and humidity). Rats for histology

Fig. 1. Overview of the experimental design for zinc oxide nanoparticle (ZnONP) exposure in SD rats. Rats were exposed to 0 (PBS vehicle control with 5% BSA), 5, and 10 mg kg⁻¹ ZnONP via intratracheal (IT) instillation. Elevated-plus maze and Morris water maze was conducted to observe behavior changes by ZnONP. Cerebellum, cortex and hippocampus were collected for biochemical analyses and histology.

were transcardially perfused with 30 mL saline plus a 4% paraformaldehyde (PFA) solution in 0.1 M phosphate buffer (pH 7.4) after the experiment. Brains were quickly removed from the skull and immersed overnight in 10% formaldehyde at 4°C for pathological observation. The concentrations used for ZnONP administration was selected to induce lung inflammation after exposure (Juan, 2011). The ZnONP doses were relevant for human exposure for occupational exposure scenarios according to a previous method (Vietti *et al.*, 2013; Rovira *et al.*, 2014): the alveolar surface area, breathing frequency (30 times min⁻¹), lung deposition rate (30%), minute ventilation of 20 L min⁻¹, and the recommended ZnO standard of 5 mg m⁻³ (Fine *et al.*, 1997). The human-equivalent mass concentration (7.2 mg day⁻¹) would be achieved in 36 days with 5 mg kg⁻¹ and in 71 days with 10 mg kg⁻¹.

Morris Water Maze (MWM)

The MWM was used to examine spatial learning, according to our previous report (Chen *et al.*, 2010). Briefly, the MWM consisted of a circular pool with a diameter of 120 cm and a height of 60 cm. A white platform (9 cm with rough surface) was submerged 1 cm below the water surface. The pool was filled with water containing milk and the water temperature was kept at 25°C. The pool was divided into four equal quadrants, and each quadrant was marked with a different visual cue. There were two phases during the study period: an acquisition phase (Days 1–4) and a probe trial phase (Day 5) for examining spatial learning and memory ability, respectively. The reference for the MWM has been described previously (da Silva *et al.*, 2018). The endogenous reference such as recording camera apparatus was fixed in the experimental room during the study. The researcher was stood on the same place throughout the training and experiment. All data were recorded using a video camera-based system (EthoVision 3.1; Noldus, Wageningen, Netherlands).

Elevated-plus Maze

An elevated-plus maze was used to evaluate anxiety, according to a previous report (Walf and Frye, 2007). The elevated-plus maze consisted of two open arms, two enclosed

arms, and a central platform made of black Plexiglas. Times spent in the open and closed arms and the number of entries made into each arm were recorded with a video camera (EthoVision 3.1; Noldus). The level of anxiety was measured by the variables of the time spent and the percentages of entries in the open and closed arms. Anxiety index (Anxiety index = 1 – [(open arm time/test duration) + (open arm entries/total number of entries)]/2) was calculated according to a previous report (da Costa Estrela *et al.*, 2015).

Oxidative Stress

WelPre DNA kit (Wegene Biotech, Taipei, Taiwan) was used to extract DNA from brain tissue, according to the manufacturer's instructions. A NanoVue Spectrophotometer (Biochrom, Holliston, MA, USA) was used to determine DNA concentrations and purity. Samples were spiked with 20 µL of ¹⁵N₅-8-hydroxy-2'-deoxyguanosine (¹⁵N₅-8-OHdG; 25 ng mL⁻¹, 99% purity; Cambridge Isotope Laboratories, Andover, MA, USA) and ¹³C₃-8-NG (25 ng mL⁻¹), and these served as the internal standards. Samples were vortexed for solid-phase extraction (SPE; Sep-Pak C₁₈ cartridge; Waters, Milford, MA, USA). Details of DNA isolation and preparation were previously reported (Li *et al.*, 2005; Wu *et al.*, 2016). Levels of 8-NO₂Gua, 8-OHdG, ¹⁵N₅-8-OHdG, and ¹³C₃-8-NG were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS; TSQ Quantum™ Access MAX; Thermo Scientific, MA, USA). Levels of deoxyguanosine (dG) and guanine (Gua) were determined using high-performance liquid chromatography (HPLC) with UV detection (HPLC-UV; AS950 and PU980; Jasco, Easton, MD, USA). LC-MS/MS and HPLC-UV data were analyzed by Xcalibur 2.2 (Thermo Fisher Scientific) and the Scientific Information Service Corporation chromatography data system (Scientific Information Service Corporation, Taipei, Taiwan), respectively. Spectra of the LC-MS/MS and HPLC-UV analyses are given in Supplementary Information Figs. S1 and S2.

Brain Inflammation

A Bio-Plex rat cytokine assay (Bio-Rad, Hercules, CA, USA) was used in this study. Levels of interleukin (IL)-1α,

IL-1 β , IL-6, and tumor necrosis factor (TNF)- α in brain tissues were acquired with a Bio-Plex 200 system with Luminex (Bio-Rad) and analyzed using Bio-Plex Manager (Bio-Rad).

Immunohistochemistry

For immunohistochemistry, the brain tissues were embedded in paraffin and sliced at a thickness of 5 μ m after deparaffinization and antigen retrieval for the section. Tissue was stained with activated microglial marker Iba-1 (GeneTex, San Antonio, TX, USA). DAPI was used for nuclear staining. For neuron detection, Nissl staining buffer (1% Cresyl Violet, 1% Glacial Acetic Acid in ddH₂O) was used to measure Nissl bodies in the cytoplasm of neurons. To detect brain lesions, tissue sections were visualized by a fluorescent microscope (EVOS FL imaging system; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Images were taken and stitched into large image by automated microscopy (TissueFAXS; TissueGnostics GmbH, Vienna, Austria).

Western Blot Analysis

Tissue lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto polyvinylidene difluoride (PVDF) membranes (Millipore, Darmstadt, Germany). The primary antibodies for Beclin 1 (1:1000), light chain 3 (LC3; 1:1000), tau (1:1000), ubiquitin (1:1000), and β -actin (1:1000) were obtained from Cell Signaling (Danvers, MA, USA). Anti-rabbit (HRP)-conjugated secondary antibodies were obtained from Chemicon International (MA, USA) and Merck Millipore (MA, USA), respectively. After blocking with 5% skim milk in PBST, an HRP-labeled secondary antibody was incubated and washed with TBST. Enhanced chemiluminescence (ECL) Western blotting reagents were used. Proteins were visualized through incubation with the colorimetric substrates NBT and BCIP. Quantitative data were obtained using Image-Pro vers. 4 (Media Cybernetics, MD, USA) for Windows. All data were adjusted to the control (multiples of change of the control). Details of the Western blot analysis were previously reported (Chen *et al.*, 2004).

Zn in Brain Tissues

Inductively coupled plasma mass spectrometry (ICP-MS; Model 7500c; Agilent, USA) was used to determine Zn concentrations in cerebellum and cortex. Samples were digested using concentrated nitric acid (Fisher Scientific, USA) in a MARS 5 microwave system (CEM, USA) in CEM advanced Teflon-lined composite vessels (Jones *et al.*, 2006). Nitric acid and deionized water (> 18 M Ω) were added to the samples to a final concentration of 5% nitric acid. Nitric acid and deionized water blanks were used to detect contamination during the analytical process.

Histology

Brains were excised and then fixed with 10% neutral buffered formalin. Samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Histology was blindly examined under light microscopy.

Statistical Analysis

One-way analysis of variance (ANOVA) with Fisher's least significant difference *post hoc* test was used for comparisons among multiple values. Statistical analyses were performed using GraphPad vers. 5 for Microsoft Windows (La Jolla, CA, USA). The level of significance was set to $p < 0.05$.

RESULTS AND DISCUSSION

Physical Characterization of ZnONP

To study the effects of ZnONP on the CNS *in vivo*, we first investigated the physical modification of ZnONP when suspended in the BSA-containing PBS vehicle for pulmonary exposure. Fig. 2 shows the physical characterization of ZnONP at 5 and 10 mg mL⁻¹ used in this study. There was no significant difference in the hydrodynamic diameters of ZnONP at 5 and 10 mg mL⁻¹, which were 271 ± 23 and 257 ± 14 nm, respectively. The results indicate that the ZnONP was well suspended with some spherical aggregation in the solution, even at the highest mass concentration. ZnONP suspended in PBS with 5% BSA were mainly spherical particles with some aggregation. BSA is commonly used in nanoparticle testing because it is recognized as a good surfactant to adequately disperse nanoparticles when suspended in biological solutions (Cho *et al.*, 2012).

Notably, although BSA is able to disperse ZnONP in PBS, size fractions remained between 257 (5 mg mL⁻¹) and 271 nm (10 mg mL⁻¹). On the other hand, the possibility of ZnONP bypassing the BBB is relatively low due to the large size fractions. The olfactory bulb is also considered a route for nanoparticle translocation into the brain. In the present study, IT instillation was used for *in vivo* exposure, thus ZnONP entering the brain via the olfactory bulb was insignificant. Lung-to-brain inflammation may have been one of the pathways of ZnONP-induced neurotoxicity in the present study. In the present study, the rats were received a single administration of ZnONP. The mass concentrations administrated to rats were relevant to occupational exposure scenarios in humans (Rovira *et al.*, 2014), considering the alveolar surface area, breathing frequency, and lung deposition rate. Furthermore, workplace conditions, such as working day ventilation and PEL for ZnO (5 mg m⁻³), were considered. The exposure concentration was chosen to induce significant oxidative stress and inflammation after pulmonary exposure. Additionally, sex hormones have been associated with neurodegenerative disease (Vegeto *et al.*, 2008; Villa *et al.*, 2016). In the present study, male rats were used to control the effects of estrogens on the development of neurodegenerative disease after ZnONP exposure. The acute effects of ZnONP were investigated after 1 day and 7 days of exposure.

Behavioral Observations

Behavioral alterations caused by ZnONP exposure were firstly evaluated, including spatial orientation and memory by the MWM and anxiety by the elevated-plus maze. Fig. 3 shows the acquisition phase (Days 1–4) and probe trial (Day 5) using the MWM to respectively examine spatial

Fig. 2. Physical analyses of zinc oxide nanoparticles (ZnONP) when dispersed in 5% BSA in PBS. The hydrodynamic diameters of ZnONP at 5 and 10 mg mL⁻¹ were 271 ± 23 and 257 ± 14 nm, respectively. The ZnONP mainly presented as spherical aggregates.

learning and memory ability. There were no significant differences in the escape latency or distance moved among the control, and 5 and 10 mg kg⁻¹ ZnONP exposure groups. For the probe trial, there were no significant differences in the number of times crossing the target quadrant, time in the target quadrant, or the swimming velocity among the control, and 5 and 10 mg kg⁻¹ ZnONP exposure groups. Fig. 4 shows the levels of anxiety determined by the elevated-plus maze on Days 1 and 5 after exposure in the control, and 5 and 10 mg kg⁻¹ ZnONP exposure groups. There were no significant differences in the anxiety index after ZnONP exposure on Days 1 and 5. Additionally, there were no significant differences in the open-arm time, open-arm entries (%), closed-arm time or closed-arm entries (%) after ZnONP exposure on Days 1 and 5 (Fig. S3).

We observed acute exposure of ZnONP did not occur significant alteration in behavior in the rats. Our observations are inconsistent with some previous studies of ZnONP exposure. For example, one reported showed that ZnONP impaired the spatial cognitive capability of Wistar rats after 8 weeks of exposure (Han *et al.*, 2011). Swiss mice exposed to 5.6 mg kg⁻¹ of ZnONP 8 times (every other day) damaged spatial cognition (Xie *et al.*, 2012). Previous studies showed that exposure to ZnONP induced anxiety-like behaviors in rats after an intraperitoneal (*i.p.*) injection of 5–20 mg ZnONP kg⁻¹ (Torabi *et al.*, 2013). Different observations in spatial cognition, memory ability, and anxiety in previous reports could have resulted from distinct exposure durations (single and repeated) and administration routes (*IT* and *i.p.*). A reported showed that Zn may have the anxiogenic effects

Fig. 3. Morris water maze for examination of spatial learning (acquisition phase, Days 1–4) and memory ability (probe trial, Day 5). There were no significant differences in the escape latency or distance, the number of times crossing the target quadrant, the time in the target quadrant, of the swimming velocity after 5 and 10 mg kg⁻¹ of ZnONP exposure.

Fig. 4. The elevated-plus maze for examination of anxiety on Days 1 and 5 after exposure to control, and 5 and 10 mg kg⁻¹ zinc oxide nanoparticles (ZnONP). There were no significant differences in the anxiety index after ZnONP exposure on Days 1 and 5.

after administration (Samardzic *et al.*, 2013; de Souza *et al.*, 2018), which could explain the results observed in the present study. The percent of time spent in the open arms decreased with exposure to 10 mg kg⁻¹ ZnONP, which is similar as a previous study (de Souza *et al.*, 2018). The insignificant results may be due to the small sample size used for behavior observation.

Brain Oxidative Stress and Inflammation

To study early-phase responses to acute ZnONP exposure,

the cerebellum, cortex, and hippocampus of rat brains were examined. Fig. 5 shows brain oxidative stress and inflammation levels in the cerebellum, cortex, and hippocampus after ZnONP exposure. Levels of 8-OHdG/dG were significant increased by 10 mg kg⁻¹ of ZnONP exposure in the hippocampus ($p < 0.05$; Fig. 5(a)). However, there were no significant differences in 8-NO₂Gua/Gua levels in the cerebellum, cortex, or hippocampus after ZnONP exposure. These results suggest that the hippocampus may be sensitive to reactive oxygen species (ROS) generated by exposure to high levels of ZnONP.

The hippocampus plays an important role in cognition and memory functions. It depends on synaptic transmission properties which mainly refer to neuronal network and synaptic functions (Min *et al.*, 2009). An imbalance of cytokines and ROS may directly cause neuronal injuries, leading to modifications of the volume of the hippocampus or disturbances in the transmitter system (Jayatissa *et al.*, 2008). Although we observed an increase in oxidative stress in the hippocampus, subsequent impairment of cognition and memory functions was not observed in the present study.

Brain inflammation caused by ZnONP in mice was previously observed (Tian *et al.*, 2015; Ansar *et al.*, 2017). However, responses of different areas of the brain to ZnONP exposure remain unclear. We observed that IL-1 β and IL-6 significantly decreased in the cerebellum and cortex after exposure to 10 mg kg⁻¹ ZnONP ($p < 0.05$; Fig. 5(b)). There

were no significant differences in IL-1 α and TNF- α in the cerebellum, cortex, and hippocampus after ZnONP exposure. The cerebellum is involved in motor control, cognitive function, and fear and pleasure responses (Wolf *et al.*, 2009), whereas the cortex plays roles in memory, attention, cognition, and consciousness (Cabeza *et al.*, 2008). Significant reductions in IL-1 β and IL-6 observed in the present study could have been due to anti-inflammatory responses in the cerebellum and cortex. The anti-inflammatory responses could associate with the behavioral results.

Neuron Loss and Iba-1 Stain

Fig. 6 shows results of Nissl and Iba-1 stain in the hippocampus after exposure to the 5 and 10 mg kg⁻¹ ZnONP. There were no significant changes of neuron lost (by Nissl stain) observed in hippocampus after ZnONP exposure. We

Fig. 5. (a) Levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG)/dG and 8-NO₂Gua/guanine (Gua) in the cerebellum, cortex, and hippocampus after zinc oxide nanoparticle (ZnONP) exposure. 8-OHdG/dG in the hippocampus significantly increased with exposure to 10 mg kg⁻¹ ZnONP ($p < 0.05$). (b) Interleukin (IL)-1 α , IL-1 β , IL-6, and tumor necrosis factor (TNF)- α in the cerebellum, cortex, and hippocampus after zinc oxide nanoparticle (ZnONP) exposure. IL-1 β and IL-6 significantly decreased in the cerebellum and cortex after exposure to 10 mg kg⁻¹ ZnONP ($p < 0.05$). * $p < 0.05$.

Fig. 6. Nissl stain and Iba-1 signals in the hippocampus of control, 5 and 10 mg kg⁻¹ exposure of ZnONP for 7 days (× 40). We observed that significant microglial activation in the hippocampus occurred after ZnONP exposure at 10 mg kg⁻¹.

observed significant expression of Iba-1 in hippocampus after exposure of 10 mg kg⁻¹ ZnONP when compared with the control. Our observation was in line with a previous report that ambient particles caused microglial activation, oxidative stress, neuroinflammation, and impaired neurogenesis in various brain regions such as the hippocampal subgranular zone and subventricular zone (Costa *et al.*, 2015). Microglia play an important role in maintaining brain functions, as they are resident innate immune cells that act as sentinels to survey the central nervous system environment (Hickman *et al.*, 2013). Notably, microglia activation plays dual roles in regulation of inflammatory responses (Tang and Le, 2016). Microglia respond to a vast repertoire of stimuli, including cellular damage, environmental stress, and pathogens (Block *et al.*, 2007). Exacerbated and unregulated microglial proinflammatory responses are related to neuropathology and CNS diseases (Block *et al.*, 2007), which are also linked to microglial priming (Perry and Holmes, 2014). A previous report showed that gold nanoparticle inhibited inflammatory reaction by microglia (Park *et al.*, 2019). Therefore, further investigation is required to confirm the observation.

Beclin-1, LC3, Tau, and Ubiquitin Expression in Brain

Alteration in inflammation in the cerebellum and cortex were found after exposure to 10 mg kg⁻¹ ZnONP. Therefore, rats exposed to 10 mg kg⁻¹ ZnONP were used for further investigation of Beclin-1, LC3 II (conjugated LC3), tau, and

ubiquitin in the hippocampus, cerebellum, and cortex (Fig. 7). There were no significant alterations in Beclin-1 or the LC3 II/I ratio in the cerebellum, cortex, and hippocampus with ZnONP exposure compared to the control. We observed that ZnONP at 10 mg kg⁻¹ significantly increased tau expressions in the cerebellum and hippocampus compared to the control ($p < 0.05$). There was no significant change in the expression of ubiquitin in the cerebellum, cortex, and hippocampus after control and 10 mg kg⁻¹ of ZnONP exposure.

Tau overexpression in neuroblastoma cells can lead to tau aggregation and the appearance of smaller proteolytic fragments (Chesser *et al.*, 2013). Tau overexpression in the brain may lead to tau accumulation, but there are some mechanisms that can clean tau from healthy brains. Degradative mechanisms, such as autophagy, remove tau aggregates from cells (Johansen and Lamark, 2011; Chesser *et al.*, 2013). Previous studies have indicated autophagy regulation by ZnONP *in vitro* (Yu *et al.*, 2013; Arakha *et al.*, 2017; Bai *et al.*, 2017). However, we observed no significant alterations in Beclin-1 or LC3 II expressions in brain sections. The results suggest that ZnONP exposure inactivated autophagy in the brain. There was no significant neuron loss of brain by ZnONP exposure. Autophagy inactivation and loss of basal autophagy could result in tau accumulation (Levine and Kroemer, 2008). A study showed that ZnONP could be an anti-glycation agent inhibiting AGE formation as well as protecting the protein structure from oxidation

Fig. 7. Expressions of Beclin-1, light chain 3 (LC3) II/I ratio, tau, and ubiquitin in the cerebellum, cortex, and hippocampus after control and 10 mg kg⁻¹ of zinc oxide nanoparticle (ZnONP) exposure. Levels of tau significantly increased after exposure to ZnONP in the cerebellum and hippocampus compared to the control ($p < 0.05$). * $p < 0.05$.

(Ashraf *et al.*, 2018). It suggests that low levels of ZnONP may have neuroprotective effects, inhibiting protein oxidative and activating autophagy. However, high concentrations of ZnONP could cause autophagy inactivation, leading to the formation of tau aggregates in the brain.

Zn in the Brain

Zn is considered a toxic element for lung toxicity (Adamson *et al.*, 2000); however, the effects of ZnONP in brain remain unclear. The CNS is a sensitive site to interact with ions in physiological and pathological conditions (Chu and Xiong, 2012). Zinc ions, for example, are known to deteriorate the CNS during development. Zinc ions are able to leach from ZnONP and then diffuse to cells or organs, whereas the remaining ZnONP are able to continually release zinc ions to the lung-to-systemic environment (Guerinot, 2000). The BBB is the interface between the systemic system and the brain, which is a critical barrier for regulating substance transport into the brain. Fig. 8 shows levels of Zn

in the cerebellum and cortex after exposure to the control, and 5 and 10 mg kg⁻¹ ZnONP ($n = 5$). The Zn level was higher in the cerebellum after 10 mg kg⁻¹ of ZnONP exposure, but the level did not reach statistical significance. Also, there were no significant differences in Zn levels in the cerebellum or cortex after ZnONP exposure. Our results are consistent with those of previous studies (Cho *et al.*, 2013; Adamcakova-Dodd *et al.*, 2014), which could be due to the time point we observed after ZnONP exposure. ZnONP can cause alterations in the integrity and permeability of the BBB due to inflammation (Giovanni *et al.*, 2015; Setyawati *et al.*, 2015). The observation could be explained by the decrease of inflammation in brain and behavior results.

Brain Histology

Fig. 9 shows results of brain histology in the cerebellum, cortex, and hippocampus after 7-day exposure to the control, and 5 and 10 mg kg⁻¹ ZnONP. No significant pathological changes were observed in the three regions of the brain after

Fig. 8. Levels of Zn in the cerebellum and cortex after exposure to the control, and 5 and 10 mg kg⁻¹ zinc oxide nanoparticles (ZnONP) ($n = 5$). There were no significant differences of the Zn level in the cerebellum or cortex after ZnONP exposure.

Fig. 9. H&E staining of the cerebellum, cortex, and hippocampus after exposure to the control, and 5 and 10 mg kg⁻¹ zinc oxide nanoparticles (ZnONP) (400×). No significant pathological changes were observed in the three regions of the brain after ZnONP exposure. Scale bars measure 50 μm.

ZnONP exposure. The outbred SD rat used in the present study could lead to different results of ZnONP neurotoxicity due to its genetic variation. Therefore, evaluation of ZnONP neurotoxicity by more strains may be required in future works.

CONCLUSIONS

In summary, our results indicate that acute pulmonary exposure to ZnONP caused neurotoxicity in rats. We observed

the occurrence of oxidative stress, microglia activation, and tau accumulation in different regions of the brain after exposure. Thus, acute exposure to ZnONP may also induce neurotoxicity in humans. Our findings provide clues to the effects of ZnONP metal fumes on neurodegenerative diseases and the underlying CNS responses.

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DISCLAIMER

The authors declare that they have no conflicts of interest.

SUPPLEMENTARY MATERIAL

Supplementary data associated with this article can be found in the online version at <http://www.aaqr.org>.

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