RESEARCH ARTICLE

Zinc oxide nanoparticles modulate the gene expression of ZnT\textsubscript{1} and ZIP\textsubscript{8} to manipulate zinc homeostasis and stress-induced cytotoxicity in human neuroblastoma SH-SY5Y cells

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Abstract

Zinc ions (Zn\textsuperscript{2+}) are important messenger molecules involved in various physiological functions. To maintain the homeostasis of cytosolic Zn\textsuperscript{2+} concentration ([Zn\textsuperscript{2+}]\textsubscript{c}), Zrt/Irt-related proteins (ZIPs) and Zn\textsuperscript{2+} transporters (ZnTs) are the two families of proteins responsible for decreasing and increasing the [Zn\textsuperscript{2+}]\textsubscript{c}, respectively, by fluxing Zn\textsuperscript{2+} across the membranes of the cell and intracellular compartments in opposite directions. Most studies focus on the cytotoxicity incurred by a high concentration of [Zn\textsuperscript{2+}]\textsubscript{c} and less investigate the [Zn\textsuperscript{2+}]\textsubscript{c} at physiological levels. Zinc oxide-nanoparticle (ZnO-NP) is blood brain barrier-permeable and elevates the [Zn\textsuperscript{2+}]\textsubscript{c} to different levels according to the concentrations of ZnO-NP applied. In this study, we mildly elevated the [Zn\textsuperscript{2+}]\textsubscript{c} by ZnO-NP at concentrations below 1 \textmu g/ml, which had little cytotoxicity, in cultured human neuroblastoma SH-SY5Y cells and characterized the importance of Zn\textsuperscript{2+} transporters in 6-hydroxy dopamine (6-OHDA)-induced cell death. The results show that ZnO-NP treatment reduced the basal levels of reactive oxygen species and Bax/Bcl-2 mRNA ratios; in addition, ZnO-NP decreased the 6-OHDA-induced ROS production, p53 expression, and cell death. These results show that ZnO-NP-induced mild elevation in [Zn\textsuperscript{2+}]\textsubscript{c} activates beneficial effects in reducing the 6-OHDA-induced cytotoxic effects. Therefore, brain-delivery of ZnO-NP can be regarded as a potential therapy for neurodegenerative diseases.
Introduction

Zinc ion (Zn$^{2+}$) is essential for all living organisms and is the second most abundant transition element in human. It is a cofactor in many proteins regulating their catalytic activities and structure. In addition, recent emerging evidence has shown that Zn$^{2+}$ is a messenger in regulation of many cellular activities such as cell cycle, cell proliferation, differentiation and death via different signaling pathways [1, 2]. Cytosolic Zn$^{2+}$ concentration ([Zn$^{2+}$]$_{c}$) changes during cell cycle, differentiation and cell death [3]. During cell proliferation, the tyrosine phosphatases are suppressed by a small elevation of [Zn$^{2+}$]$_{c}$, to activate ERK pathway [4]. A number of transcription factors, such as p53, contain Zn$^{2+}$ binding motifs affecting cell cycle and survival [5].

The paradoxical, but vital, roles of Zn$^{2+}$ in nervous system have gained recognition recently [6, 7]. Zn$^{2+}$ is essential for neurogenesis, neuronal differentiation and synaptic transmission. The inhibition of synaptic Zn$^{2+}$ signaling in hippocampus and amygdala by Zn$^{2+}$ chelators affects cognition [8]. Zn$^{2+}$ deficiency reduces neurogenesis and associates with neuronal dysfunction. A correlation between Zn$^{2+}$ deficiency and depression has been demonstrated in both clinical studies and animal models [9, 10]. In contrast, high Zn$^{2+}$ levels block mitochondrial function and induce apoptosis in the development of pathophysiology of CNS disorders including epilepsy, schizophrenia and Alzheimer’s Disease [11]. At cellular level, high dose of Zn$^{2+}$ is neurotoxic causing cell death [12–14] and Zn$^{2+}$ deficiency causes caspase-dependent apoptosis in human neuronal precursor cells [15, 16]. Zn$^{2+}$ supplementation significantly reduces spinal cord ischemia-reperfusion injury in rats [17]. However, dietary Zn$^{2+}$ supplementation has restrictions and limitations in crossing brain-blood barrier (BBB), which has limited permeability for Zn$^{2+}$, especially when the desired final Zn$^{2+}$ level is higher than physiological levels [18]. Thus, controlled and targeted delivery of Zn$^{2+}$ is highly desirable.

Nanoparticles (NP) technologies have been used for the targeted delivery of chemicals [19]. In nervous system, polylactide-co-glycolide or BBB ligand specific-modified polylactide polymers are used to carry Zn$^{2+}$ across BBB [18, 19]. However, the rate is slow, the cellular or brain entrance are evidenced after several days [19]. We have previously demonstrated the entrance of zinc oxide-NP (ZnO-NP) into brain via olfactory bulb in rat and elevates the [Zn$^{2+}$]$_{c}$ in cultured cells [20]. Therefore, ZnO-NP has the potential to be a potent means for Zn$^{2+}$ delivery to regulate [Zn$^{2+}$]$_{c}$, homeostasis in the central nervous system.

The cellular uptake of ZnO-NP into intracellular compartments is via endocytosis followed by dissolution that occurs in acidic compartments to convert ZnO-NP to Zn$^{2+}$ [20]. Two classes of proteins are implicated in Zn$^{2+}$ transport for [Zn$^{2+}$]$_{c}$ homeostasis: solute-linked carrier 30 (SLC30, Zn transporter (ZnT)) and SLC39 (Zrt/Irt-related proteins (ZIP)) decrease and increase the [Zn$^{2+}$]$_{c}$, respectively, by fluxing Zn$^{2+}$ across the membranes of cell and intracellular organelles in opposite directions. The ZIP proteins then transport the accumulated Zn$^{2+}$ in these acidic compartments to the cytosol and ZnT proteins work corporately to flux Zn$^{2+}$ out of the cytosol. Therefore, ZnO-NP may be different from direct Zn$^{2+}$ application in regulating expression levels of Zn$^{2+}$ transporters to control Zn$^{2+}$ homeostasis.

ZnO-NP at high dosage causes apoptosis in lung [21] and neural stem cells [13] and interferes with the ion channel activities in primary cultured rat hippocampal neurons [22]. However, toxicity is not seen under exposure to ZnO-NP at low doses, such as 6 ppm (70 μM) [13], or 10 μM [20]. The importance of Zn$^{2+}$ to normal functioning of the central nervous system is increasingly appreciated [9, 15]. In this report, we mildly elevated the [Zn$^{2+}$]$_{c}$ in human neuroblastoma cells, SH-SY5Y, by ZnO-NP at concentrations below 1 μg/ml. ZnO-NP treatment greatly enhanced the expression level of ZnT, and less affected the expression of ZIP$_{4}$.

ZnO-NP treatment decreased the basal level of reactive oxygen species (ROS) and the expression ratio of Bax/Bcl-2. In addition, ZnO-NP treatment recued the cell death caused by the
6-hydroxy dopamine (6-OHDA). Therefore, BBB-permeable ZnO-NP provides a therapeutic strategy to treat neurodegeneration disorders by fin-tuning the \([Zn^{2+}]_c\).

**Materials and methods**

**Chemicals**

ZnO-NPs were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Their preparation protocols were described in our previous work [21]. The size range of ZnO-NP in solution was from 20 to 80 nm with an average of 45 nm. SH-SY5Y neuroblastoma cells were purchased from the American Type Culture Centre CRL2266 (Manassas, VA, USA). FluoZin-3-AM, Lipofectamine 2000®, reverse transcriptase III and TRIzol® reagent were purchased from Invitrogen Co. (Carlsbad, CA, USA). RNase-free DNAse I and RNeasy purification columns were purchased from Qiagen Inc. (Valencia, CA, USA). Random hexamer primers were obtained from Fermentas Inc. (Burlington, Canada). iQ SYBR Green Supermix was obtained from Bio-Rad Inc. (Hercules, CA, USA). Other chemicals were obtained from Merck KGaA (Darmstadt, Germany) otherwise indicated.

**Cell culture**

Human neuroblastoma SH-SY5Y cells were cultured in minimal essential medium (Gibico 41500–034) supplemented with F12 nutrient mixture (Gibico 21700–075) and 10% fetal bovine serum. The cells were kept in a humidified 5%-CO\(_2\) incubator at 37 °C [20].

\([Zn^{2+}]_c\) Measurements

Suspended cells were incubated in a Loading buffer (in mM, NaCl 150, glucose 5, Hepes 10, MgCl\(_2\) 1, KCl 5, CaCl\(_2\) 2.2, pH7.3) containing 10 \(\mu\)M of FluoZin-3-AM at 37˚C for 30 minutes. After washing out the FluoZin-3-AM by centrifugation and resuspending the cell in Loading buffer, the changes in the fluorescence intensity were recorded as described before [20].

**RT-PCR assay**

RNA extraction and reverse transcription were performed following the protocols suggested by the manufactures. The primers for the polymerase chain reactions (PCR, Q-Amp™ 2x Hot-Start PCR Master Mix) were listed in S1 Table in S1 File. The products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and photographed with ultraviolet trans-illumination. For quantitative PCR (qPCR), the kit used was IQ² Fast qPCR System and the instrument was from Illumina Inc. (Eco™ Real-time PCR system) [23].

**ROS measurements**

To quantify the production of ROS, we loaded the cells with 2',7'-dichlorodihydrofluorescein diacetate (H\(_2\)DCFDA, Molecular Probes®) and incubated at 378, 5% CO\(_2\) for 30 minutes. After replacing the medium, 6-OHDA or H\(_2\)O\(_2\) were added. The fluorescence intensities were measured by a microplate reader (Glomax-multidetection system, Promega, USA) with excitation at 485 nm and emission at 500–560 nm.

**ZIP\(_8\) and ZnT\(_1\) shRNA knockdown**

Plasmids expressing short hairpin RNAs (shRNA) against ZIP\(_8\) and ZnT\(_1\) were purchased from National RNAi Core Facility, Academia Sinica, Taiwan, and the target sequences of these shRNAs (4 for ZIP\(_8\) and 5 for ZnT\(_1\)) were listed in S2 Table in S1 File. Lipofectamine 2000®
was used to transfect these plasmids into SH-SY5Y cells [24]. An apoplasid was used as negative control.

**MTT assay**

The MTT assay, an index of cell viability and cell growth, is based on the ability of viable cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) [25]. All samples were assayed in triplicate from 5 batches of cells. The total time of ZnO-NP treatment was 24 hr and 6-OHDA was added at 18th hr. To enhance cell death caused by 6-OHDA, cells were incubated in a medium containing 0.5% of serum. For cells after shRNA transfection, the serum was 5% during all experiments.

**Statistical analysis**

Statistical analysis was performed using one-way analysis of variance and significant differences were assessed by Student’s t test. A p value less than 0.05 was regarded as statistically significant.

**Results**

**ZnO-NP elevates [Zn^{2+}]_c in cultured SH-SY5Y cells**

To examine ZnO-NP at low doses can elevate [Zn^{2+}]_c in cultured human neuroblastoma SH-SY5Y cells, we loaded the cells with FluoZin3, a Zn^{2+}-sensitive dye, and monitored the changes in fluorescence intensities (Fig 1). The addition of ZnO-NP (0.081 and 0.814 μg/ml, n = 3 each) increased the fluorescence intensity gradually during the 200-s recording period in a concentration-dependent manner. For 25-hr long-term treatment, the fluorescence intensities measured reached a maximum in 6 hr when treated with different concentrations of ZnO-NP (0.081, 0.814, and 8.14 μg/ml, n = 3 each and statistical symbols were shown in S1 Fig in S1 File). These results reveal that ZnO-NP apparently elevates the [Zn^{2+}]_c transiently in a concentration- and time-dependent mode even at low concentrations.

**ZnT1 and ZIP8 regulate the ZnO-NP-induced [Zn^{2+}] responses in SH-SY5Y cells**

ZIPs and ZnTs play important roles in maintaining the [Zn^{2+}]_c homeostasis. We first characterized the expression levels of ZnT and ZIP isoforms in cultured SH-SY5Y cells by RT-PCR and the results showed significant expressions of ZnT1, ZnT3, ZnT4, ZnT5, ZnT6, ZnT7, ZnT9 and ZnT10 (S2A Fig in S1 File) and ZIP1, ZIP3, ZIP4, ZIP6, ZIP7, ZIP8, ZIP9, ZIP10 ZIP11, ZIP13 and ZIP14 (S2B Fig in S1 File). ZnT1 is the main transporter at the plasma membrane to efflux Zn^{2+} out of cells and lowers the [Zn^{2+}]_c [26]; ZIP8 presents in the synaptic vesicles and lysosomes to transport Zn^{2+} from intracellular compartments to the cytosol [27, 28]. Since endocytosis is the main route for ZnO-NP entrance into the cell and dissolution into Zn^{2+} occurs in an acidic compartment [20], we focused on characterizing the involvement of ZnT1 and ZIP8 in modulating the ZnO-NP-induced [Zn^{2+}]_c response in SH-SY5Y cells (Fig 2). We adopted qPCR to investigate the mRNA levels of ZnT1 and ZIP8 in SH-SY5Y cells after the addition of ZnO-NP of different concentrations (Fig 2A & 2B, n = 3 for each concentration). The average results show that a low-dose of ZnO-NP (0.081 μg/ml) elevated the expression levels of ZnT1 and ZIP8 transiently in 6 hr (p < 0.05) and then declined to a basal level after 24 hrs. High doses of ZnO-NP (0.814 and 8.14 μg/ml) treatment maintained the expression of ZnT1 at a level 4–8 fold higher than the control group (p < 0.05) during the 24-hour exposure period. ZnO-NP at 0.814 μg/ml elevated and maintained the expression of ZIP8 at a level 2–3 fold
higher than the control group ($p < 0.05$), however, at 8.14 μg/ml, ZnO-NP had little effect on the expression of ZIP8. These results reveal that ZnO-NP exposure differentially enhances the expression of ZnT1 and ZIP8.

To verify the contributions of these transporters in regulating the $[\text{Zn}^{2+}]_c$ responses induced by ZnO-NP, we delivered specific shRNAs into the cells to reduce the translation of...
and ZIP8 (Fig 2C & 2D, respectively) (The original images of the Western blot were shown in S3 Fig in S1 File). The results of the Western blots revealed that most of these shRNAs decreased the protein levels of ZnT1 (H1-5) and ZIP8 (H6-9); among them, H5 and H9 were the most effective shRNAs in reducing the protein levels of ZnT1, by 88%, and ZIP8, by 70%, respectively. Treating transfected SH-SY5Y cells with ZnO-NP (0.814 μg/ml, Fig 2E, n = 3), the averaged changes in [Zn$^{2+}$]$_c$, comparing to the control group, was about 4-fold higher in cells expressing H5 ($p < 0.001$) and mostly abolished in cells expressing H9 ($p < 0.001$). It is likely that cells change the expression levels of these transporters to regulate the [Zn$^{2+}$]$_c$ in response to different stimulations.

ZnO-NP at a low dose increases the Bax/Bcl-2 expression level

To characterize the toxicity of ZnO-NP on SH-SY5Y cells, we treated the cells with different concentrations of ZnO-NP for 24 hr and monitored the viability by MTT assay (Fig 3A). The results show that ZnO-NP exposure reduced the viability in a dose dependent manner with an EC$_{50}$ of 6.8 ± 0.2 μg/ml (n = 15). Under 2 μg/ml, ZnO-NP had little effect on cell viability. We then examined the expression levels of Bax and Bcl-2 by qPCR in SH-SY5Y cells treated with

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ZnO-NP at 0.081 and 0.814 μg/ml for 24 hr (Fig 3B). The amounts of the PCR products expressed from Bax and Bcl-2 decreased and increased, respectively, as the concentrations of ZnO-NP increased (the original images of the agarose gel analysis of the PCR products were shown in the S4 Fig in S1 File). After normalization, the Bax/Bcl-2 expression ratio were significantly decreased to 0.69 ± 0.12 (p < 0.01) and 0.34 ± 0.08 (p < 0.001), respectively. In contrast, ZnO-NP at 8.14 μg/ml significantly increased the ratio to 1.49 ± 0.2 (n = 3, p < 0.01, not shown). Therefore, that ZnO-NP at low non-lethal doses decreases the Bax/Bcl-2 ratio indicating the blockage of apoptosis pathway.

ROS accumulation can trigger the expression of apoptosis-related genes. We then examined the intracellular ROS levels by loading the cells with H2DCFDA and monitored the changes in the fluorescence intensities in 2 hr (S5 Fig in S1 File). For control cells without ZnO-NP treatment, the ROS level increased over the recording period; in the presence of ZnO-NP (0.081 and 0.814 μg/ml), the ROS levels at the same duration were lower than that of the control group. These findings suggest that a low-dose exposure of ZnO-NP elicits beneficial effects in cells to reduce the oxidation stress and protect cells from death.

ZnO-NP counteracts stress-induced ROS generation and cell death in SH-SY5Y cells

The uptake of 6-OHDA, an analog of dopamine, into cells through dopamine transporters triggers the production of ROS and causes cell death. To verify ZnO-NP has a protective effect on the 6-OHDA-induced cell death, we pretreated the SH-SY5Y cells with a low dose of ZnO-NP (0.081 and 0.814 μg/ml) for 18 hr then 6-OHDA of different concentrations were added for another 6 hr (Fig 4A, n = 5). The results show that 6-OHDA at 50 and 100 μM significantly decreased the cell viability from 99.9 ± 7.0% of control group to 67.3 ± 10.3 (p < 0.01) and 42.1 ± 4.3 (p < 0.01), respectively. ZnO-NP pretreatment counteracted the 6-OHDA-induced cell death but not significant when 6-OHDA applied was 50 μM; in contrast, for 6-OHDA at 100 μM, the viabilities were significantly enhanced to 64.2 ± 12.9 (p < 0.01) and 53.4 ± 12.7% (p < 0.05) by ZnO-NP at 0.081 and 0.814 μg/ml, respectively. In addition, ZnO-NP (0.081 μg/ml) pretreatment significantly reduced the ROS level to 80 ± 6% of the Control group (n = 15, p < 0.05) and 6-OHDA treatment greatly elevated the ROS level to 135 ± 10% (n = 15, p < 0.05) (Fig 4B). ZnO-NP pretreatment could significantly reduce this increment to 98 ± 11% (n = 15, p < 0.05) (Fig 4B). Furthermore, ZnO-NP pretreatment reversed the effects of H2O2 in cell survival and ROS production (S6 Fig in S1 File). We then used RT-PCR to examine the expression level of p53, a transcription factor involved in the ROS-activated apoptosis pathway [29], in SH-SY5Y cells (Fig 4C and the original images were shown in the S7 Fig in S1 File). After normalization to that of the control group, 6-OHDA treatment significantly increased the expression level of p53 to 1.5 ± 0.3 (n = 3, p < 0.01) and this increment could be reduced by the pretreatment of ZnO-NP (0.081 μg/ml) to 0.9 ± 0.0 (n = 3, p < 0.05). These results suggest that ZnO-NP at a concentration below 1 μg/ml suppresses the production of ROS and reduced the expression of p53 to facilitate cell survival.

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ZnT1 and ZIP8 knockdown affected 6-OHDA-induced cytotoxicity

To verify the importance of ZnO-NP-induced elevation of $[\text{Zn}^{2+}]_c$ in protecting cells from death, we transfected the SH-SY5Y with shRNAs against ZnT1 and ZIP8, then examined the cell viability under 6-OHDA treatment with MTT assay (Fig 5, n = 15). Because of the damages caused by the transfection reagents, the culture medium contained 5% of serum during the experiment. The results show that 6-OHDA (50 μM) treatment decreased the viability from $100.8 \pm 4.7$ to $87.6 \pm 5.1$% ($p < 0.01$). Knockdown the expression of ZnT1 rescued the cell death caused by 6-OHDA to a level similar to that of the control group and the addition of ZnO-NP did not enhance the viability. In contrast, ZIP8 knockdown did not have such a protective effect in 6-OHDA-induced cell death ($100.0 \pm 1.9$ vs. $94.1 \pm 3.0$%) and the addition of ZnO-NP did not reverse the toxic effect of 6-OHDA. As shown in Fig 2E, knockdown the expression of ZnT1 and ZIP8 enhanced and suppressed the ZnO-NP-induced elevations of $[\text{Zn}^{2+}]_c$, respectively. Therefore, the release of Zn$^{2+}$ from the acidic compartments by ZIP8 and the elevation of $[\text{Zn}^{2+}]_c$ facilitated by ZnT1 are important in enhancing the viability of cells under different challenges.

Fig 4. ZnO-NP suppresses 6-OHDA-induced cytotoxicity in SH-SY5Y cells. Cells were incubated in a medium containing ZnO-NP (0, 0.081, 0.814 μg/ml) for 24 hr and 6-OHDA (50 or 100 μM) was added at 18th hr. A. Cell viability. The viability was measured by an MTT assay from 5 batches of cells. B. Normalized ROS production. Data presented were Mean ± SEM (n = 15). C. p53 mRNA levels. The ZnO-NP used was 0.081 μg/ml and cells were harvested for RT-PCR. The density of p53 products were normalized to that of β-actin and Control group (n = 3). The significance was analyzed by Student’s t-test; *: $p < 0.05$ and 0.01, respectively, when compared to the control group without 6-OHDA treatment or as indicated.

ZnT1 and ZIP8 knockdown affected 6-OHDA-induced cytotoxicity

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Discussion

This study finds that ZnO-NP potently induced the expressions of ZnT1 and ZIP8 to modulate [Zn$^{2+}$]$_c$, a crucial parameter for cytoviability in human neuroblastoma SH-SY5Y cells. Below lethal dosage under 1 μg/ml, ZnO-NP transiently elevated the [Zn$^{2+}$]$_c$ and decreased the Bax/Bcl-2 expression ratio. In addition, ZnO-NP suppressed the cytotoxicity, ROS production and p53 gene expression induced by 6-OHDA or H$_2$O$_2$. These results suggest the cell-protective function of ZnO-NP at low dosages against oxidative stresses and support a therapeutic strategy by delivering ZnO-NP into the CNS to suppress the development of neuropathological disorders.

Zn$^{2+}$ trafficking was investigated in these experiments. ZnO-NP-induced changes of [Zn$^{2+}$]$_c$ were studied in cells transfected with shRNA against ZnT1 to illustrate the role of ZnT1 for the efflux of Zn$^{2+}$. [Zn$^{2+}$]$_c$ and the expression of ZnT1 were coupled; both showed increases under exposure to low doses of ZnO-NP and returned to the basal levels after 24 hr. At high dosage (8.14 μg/ml), ZnO-NP induced a large increase in [Zn$^{2+}$]$_c$, coupled with an 8-fold increase in ZnT1 mRNA (at 6 hr). In this case, both the expression level of ZnT1 and [Zn$^{2+}$]$_c$
remained high throughout the observation period. Moreover, neurotoxicity induced by 6-OHDA was suppressed in the ZnT1-knockdowned cells. Our data show that $[\text{Zn}^{2+}]_c$ changes are coupled with the ZnT1 expression levels which are closely related to the neuron-protection activity of Zn$^{2+}$. ZnT1 is known to be a plasma membrane protein that is enriched in postsynaptic dendritic spines and plays a role in Zn$^{2+}$ homeostasis in synaptic neuron functions and diseases [30]. Su et al. reported a positive correlation between ZnT1 and Zn$^{2+}$ content in the spinal cord [31], and ZnT1 is shown to increase significantly with progression of Alzheimer’s disease [32].

Our data suggest that changes in ZnT1 expression can become a marker for $[\text{Zn}^{2+}]_c$ disturbance associated with neuroviability. Other ZnTs such as ZnT10, at Golgi, is down-regulated by an elevation of extracellular Zn$^{2+}$ in SH-SYSY cells [33]. IL-6 induces a down-regulation of ZnT10 and enhances the accumulation of Mn$^{2+}$ that might be correlated with Parkinson’s disease [34]. Further studies on ZnTs, ZIPs, and metallothioneins (MTs), are required to understand their roles in modulating the Zn$^{2+}$ homeostasis.

We have previously demonstrated the internalization of ZnO-NP by PC12 cells upon exposure to the ZnO-NPs for 10 min. Furthermore, after nasal exposure to airborne ZnO-NP, the NPs are found in rat brain under a transmission electron microscope [20]. We also verify that ZnO-NP elevates $[\text{Zn}^{2+}]_c$ in both cultured cells and rat white blood cells through endocytosis and subsequent dissolution in acidic compartments such as endosomes [21]. Conversion of ZnO to ions following entrance into lysosomes has also been shown in the studies of Xia et al. in which the labeled ZnO is traced in BEAS-2B cells [35]. Muller et al. also have demonstrated that ZnO dissolves rapidly in a lysosomal fluid at a pH of 5.2 [36].

ZIP8 has been shown to be localized in the lysosomal membrane and synaptosomes [27, 28]. Our data show that ZnO-NP-induced $[\text{Zn}^{2+}]_c$ changes are greatly suppressed in ZIP8 knockdowned cells, illustrating that ZIP8 is required for intracellular Zn$^{2+}$ release from those organelles after ZnO-NP was engulfed, which may be the main route for ZnO in elevating $[\text{Zn}^{2+}]_c$. The mRNA levels of ZIP8 and ZnT1 were positively correlated with the changes in $[\text{Zn}^{2+}]_c$ under exposure to ZnO-NP below 1 μg/ml. At a high dose of ZnO-NP (8.14 μg/ml), the expression of ZIP8 was small in contrast to $[\text{Zn}^{2+}]_c$ response and ZnT1 expression. The low level of ZIP8 prevents additional Zn$^{2+}$ fluxing to the cytosol and further cellular damage. These results suggest that there is a negative feedback between elevation of $[\text{Zn}^{2+}]_c$ and the expression of ZIP8.

ROS is known to cause DNA damage that activates the p53-linked apoptosis pathway through phosphorylation by ATM. Bcl-2 has been shown to be coupled with the pro-survival pathway to counteract the effects of mitochondrial damages induced by Bax. In addition, silencing the expression of ZnT1, but not ZIP8, can not only enhance the ZnO-NP-induced $[\text{Zn}^{2+}]_c$ elevation but rescue the 6-OHDA-induced cell death. It is likely that $[\text{Zn}^{2+}]_c$ response is a perquisite for ZnO-NP to reduce stress-induced cytotoxicity by suppressing ROS generation and augmenting expression of bcl-2.

Zn$^{2+}$ has been widely shown as a potential antioxidant for suppression of apoptosis [37–44]. In animal brain studies, Zn$^{2+}$ treatment decreases the Bax/Bcl-2 protein ratio [44]; treating SH-SYSY cells with a low dose of Zn$^{2+}$ can reverse a stress-induced increment of DNA fragmentation [12]. Zn$^{2+}$ supplementation can reduce the levels of ROS to prevent cardiomyocyte apoptosis and congenital heart defects [40]; it also promotes the recovery of spinal cord function [17, 45]. Zn$^{2+}$ has a protective effect on renal ischemia-reperfusion injury by augmenting superoxide dismutase activity and lowering the Bax/Bcl-2 expression ratio to reduce apoptosis [37]. Therefore, our results support that ZnO-NP, at sub-lethal dosage, causes a mild elevation of $[\text{Zn}^{2+}]_c$, which has been shown to enhance the expression of metallothioneins [46] and the activity of Zn$^{2+}$-related superoxide dismutase [47]. Both metallothioneins and dismutase can
lower the ROS level leading to the down-regulation of the expression of p53 and Bax/Bcl-2 ratio [47, 48]. In the future, we will further characterize how [Zn$^{2+}$], when elevated to different levels, regulates the ROS production.

In this and previous studies, we show that ZnO-NP dose-dependently exert paradoxical protective and cytotoxic functions through their ability to alter [Zn$^{2+}$], and modulate the expression of Zn$T_1$ and Zn$T_2$. Delivering ZnO-NP at a low dose into the central nervous system may provide a practical strategy to elevate the [Zn$^{2+}$] for potent neuroprotection. Further studies, both in vivo and in vitro, will be required using more sensitive and selective techniques to measure the homeostasis of [Zn$^{2+}$] and to assess the feasibility of using ZnO-NP for clinical application.

Supporting information
S1 File.
(DOCX)

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