Zinc oxide nanoparticles modulate the gene expression of ZnT_1 and ZIP_8 to manipulate zinc homeostasis and stress-induced cytotoxicity in human neuroblastoma SH-SY5Y cells

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Abstract

Zinc ions (Zn^{2+}) are important messenger molecules involved in various physiological functions. To maintain the homeostasis of cytosolic Zn^{2+} concentration ($[Zn^{2+}]_c$), Zrt/Irt-related proteins (ZIPs) and Zn^{2+} transporters (ZnTs) are the two families of proteins responsible for decreasing and increasing the $[Zn^{2+}]_c$, respectively, by fluxing Zn^{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 $[Zn^{2+}]_c$ and less investigate the $[Zn^{2+}]_c$ at physiological levels. Zinc oxide-nanoparticle (ZnO-NP) is blood brain barrier-permeable and elevates the $[Zn^{2+}]_c$ to different levels according to the concentrations of ZnO-NP applied. In this study, we mildly elevated the $[Zn^{2+}]_c$ by zinc oxide-nanoparticles (ZnO-NP) at concentrations below 1 μg/ml, which had little cytotoxicity, in cultured human neuroblastoma SH-SY5Y cells and characterized the importance of Zn^{2+} transporters in 6-hydroxy dopamine (6-OHDA)-induced cell death. The results show that ZnO-NP at low concentrations elevated the $[Zn^{2+}]_c$ transiently in 6 hr, then declined gradually to a basal level in 24 hr. Knocking down the expression levels of *ZnT_1* (mostly at the plasma membrane) and *ZIP_8* (present in endosomes and lysosomes) increased and decreased the ZnO-NP-induced elevation of $[Zn^{2+}]_c$, respectively. 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. Therefore, mild elevations in $[Zn^{2+}]_c$ induced by ZnO-NP activate beneficial effects in reducing the 6-OHDA-induced cytotoxic effects. Therefore, brain-delivery of ZnO-NP can be regarded as a potential therapy for neurological disease.

Keywords: neuroviability, zinc homeostasis, zinc oxide-nanoparticle, *ZIP_8*, *ZnT_1*, neurodegeneration

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 were 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-realted 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 concentration of $[Zn^{2+}]_c$ 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_1 and less affected the expression of ZIP_8. 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-NP were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Their preparation followed protocols 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, 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, German) 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 ºC (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 μM 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 HotStart PCR Master Mix) were listed in Supplementary Table S1. The products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and photographed with ultraviolet trans-

117 illumination. For quantitative PCR (qPCR), the kit used was IQ^2 Fast qPCR System and the
118 instrument was from Illumina Inc. (Eco™ Real-time PCR system) (23).

119 **MTT assay**

120 The MTT assay, an index of cell viability and cell growth, is based on the ability of viable cells
121 to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (24). All samples
122 were assayed in triplicate, and the mean for each experiment was calculated. Five batches of cells
123 were used in this experiments.

124 **ROS measurements**

125 To quantify the production of ROS, we loaded the cells with 2',7'-dichlorodihydrofluorescein
126 diacetate (H_2DCFDA, Molecular Probes®) and incubated at 37℃, 5% CO_2 for 30 minutes. After
127 replacing the medium, H_2O_2 or 6-OHDA were added. The fluorescence intensities were measured by
128 a microplate reader (Glomax-multidetection system, Promega, USA) with excitation at 485 nm and
129 emission at 500 - 560 nm.

130 ***ZIP_8* and *ZnT_1* shRNA knockdown**

131 Plasmids expressing short hairpin RNAs (shRNA) against ZIP_8 and ZnT_1 were purchased from
132 National RNAi Core Facility, Academia Sinica, Taiwan, and the target sequences of these shRNAs
133 (4 for *ZIP_8* and 5 for *ZnT_1*) were listed in Supplementary Table S2. Lipofectamine 2000® (Invitrogen,
134 Carlsbad, CA) was used to transfect these plasmids into SH-SY5Y cells(25). An apoplasmid was
135 used as negative control.

136 **Statistical analysis**

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

140

141

Results:

1. 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) 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). These results reveal that ZnO-NP apparently elevates the $[Zn^{2+}]_c$ transiently in a concentration- and time-dependent mode even at low concentrations.

2. ZnT_1 and ZIP_8 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 ZnT_1, ZnT_3, ZnT_4, ZnT_5, ZnT_6, ZnT_7, ZnT_9 and ZnT_{10} (Supplementary Fig. S1A) and ZIP_1, ZIP_3, ZIP_4, ZIP_6, ZIP_7, ZIP_8, ZIP_9, ZIP_{10}, ZIP_{11}, ZIP_{13} and ZIP_{14} (Supplementary Fig. S1B). ZnT_1 is the main transporter at the plasma membrane to efflux Zn^{2+} out of cells and lowers the $[Zn^{2+}]_c$ (26); ZIP_8 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 ZnT_1 and ZIP_8 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 ZnT_1 and ZIP_8 in SH-SY5Y cells after the addition of ZnO-NP of different concentrations. The average results show that a low-dose of ZnO-NP (0.081 μg/ ml) elevated the expression levels of ZnT_1 and ZIP_8 transiently in 6 hr 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 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, 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 $[Zn^{2+}]_c$ responses induced by ZnO-NP, we delivered specific shRNAs into the cells to reduce the translation of *ZnT1* and *ZIP8* (Fig. 2C & D, respectively). 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), the averaged changes in $[Zn^{2+}]_c$, comparing to the control group, was about 4-fold higher in cells expressing H5 and mostly abolished in cells expressing H9 (Fig. 2E). It is likely that cells change the expression levels of these transporters to regulate the $[Zn^{2+}]_c$ in response to different stimulations.

3. 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. 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 ZnO-NP at 0.081 and 0.814 μg/ml for 6 hr (Fig. 3C). The amounts of the PCR products expressed from *Bax* and *Bcl-2* decreased and increased, respectively, as the concentrations of

ZnO-NP increased; in contrast, ZnO-NP at 8.14 μg/ml significantly increased the ratio to 1.49 ± 0.2. Therefore, that ZnO-NP at low non-lethal dose 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 H_2DCFDA and monitored the changes in the fluorescence intensities in 2 hr (Supplementary Figure S2). 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 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. b

4. ZnO-NP counteracted 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), which showed little effect on cell death in 24 hr (Fig. 3A). We then added 6-OHDA and monitored the survival rate at 6 hr later (Fig. 4). The results show that 6-OHDA significantly caused cell death with a dose-dependent manner at 50 and 100 μM. ZnO-NP pretreatment counteracted the 6-OHDA-induced cell death and became significant at 100 μM of 6-OHDA. In addition, ZnO-NP (0.081 μg/ml) pretreatment significantly suppresses the 6-OHDA-induced production of ROS. Similarly, ZnO-NP pretreatment reversed the effects of H_2O_2 in cell survival and ROS production (Supplementary Figure S3). We then used RT-PCR to examine the expression level of *p53*, a transcription factor involved in the activation of apoptosis pathway, in SH-SY5Y cells (Fig. 4C). It is apparently that ZnO-NP pretreatment reduced the expression of *p53* enhanced by 6-OHDA. These results suggest that ZnO-NP at a concentration

below 1 μg/ml suppresses the production of ROS and facilitates cell survival.

5. ZnT1 and ZIP8 knockdown affected 6-OHDA-induced cytotoxicity

To verify the importance of ZnO-NP-induced elevation of $[Zn^{2+}]_c$ 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). The results show that knockdown the expression of *ZnT1* recused 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 any more. In contrast, *ZIP8* knockdown did not have such a protective effect in 6-OHDA-induced cell death 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 $[Zn^{2+}]_c$, respectively. Therefore, the release of Zn^{2+} from the acidic compartments by ZIP_8 and the elevation of $[Zn^{2+}]_c$ facilitated by ZnT_1 are important in enhancing the viability of cells under different challenges.

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_2O_2. These results suggest the cell-protective function of ZnO-NP at lose 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 $[Zn^{2+}]_c$ changes were studied in cells transfected with snRNA against *ZnT1* to illustrate the role of ZnT_1 for the efflux of Zn^{2+}. $[Zn^{2+}]_c$ and the expression of *ZnT1* were coupled; both showed increases under exposure to low dose 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*-kockdowned cells. Our data show that $[Zn^{2+}]_c$ changes are coupled with the *ZnT1* expression levels which are closely related to the neuron-protection activity of Zn^{2+}. ZnT_1 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 (29). Su *et al.* reported a positive correlation between ZnT_1 and Zn^{2+} content in the spinal cord (30), and ZnT_1 is shown to increase significantly with progression of Alzheimer's disease (31).

Our data suggest that changes in *ZnT1* expression can become a marker for $[Zn^{2+}]_c$ disturbance associated with neuroviability. Other ZnTs such as ZnT_{10}, at Golgi, is down-regulated by an elevation of extracellular Zn^{2+} in SH-SY5Y cells (32). IL-6 induces a down-regulation of *ZnT10* and enhances the accumulation of Mn^{2+} that might be correlated with Parkinson's disease (33). Further

257 studies on ZnTs, ZIPs, and metallothioneins (MTs), are required to understand their roles in
258 modulating the Zn^{2+} homeostasis.

259 We have previously demonstrated the internalization of ZnO-NP by PC12 cells upon exposure
260 to the nanoparticles for 10 min. Furthermore, after nasal exposure to airborne ZnO-NP, the
261 nanoparticles are found in rat brain under a transmission electron microscope (20). We also verify
262 that ZnO-NP elevates $[Zn^{2+}]_c$ in both cultured cells and rat white blood cells through endocytosis and
263 subsequent dissolution in acidic compartments such as endosomes (21). Conversion of ZnO to ions
264 following entrance into lysosomes has also been shown in the studies of Xia *et al.* in which the
265 labeled ZnO was traced in BEAS-2B cells (34). Muller *et al.* also have demonstrated that ZnO
266 dissolves rapidly in a lysosomal fluid at a pH of 5.2 (35).

267 ZIP_8 has been shown to be localized in the lysosomal membrane or in synaptosomes (27, 28).
268 Our data show that ZnO-NP-induced $[Zn^{2+}]_c$ changes are greatly suppressed in *ZIP_8*-knockdowned
269 cells, illustrating that *ZIP_8* is required for intracellular Zn^{2+} release from those organelles after ZnO-
270 NP was engulfed, which may be the main route for ZnO in elevating $[Zn^{2+}]_c$. The mRNA levels of
271 *ZIP_8* and *ZnT_1* were positively correlated with the changes in $[Zn^{2+}]_c$ under exposure to ZnO-NP
272 below 1 μg/ml. At a high dose of ZnO-NP (8.14 μg/ml), the expression of *ZIP_8* was small in contrast
273 to $[Zn^{2+}]_c$ response and *ZnT_1* expression. The low level of ZIP_8 prevent additional Zn^{2+} fluxing to the
274 cytosol and further cellular damage. These results suggest that there is a negative feedback between
275 elevation of $[Zn^{2+}]_c$ and the expression of *ZIP_8*.

276 ROS is known to cause DNA damage that activates the *p53*-linked apoptosis pathway through
277 phosphorylation by ATM. *Bcl-2* has been shown to be coupled with the pro-survival pathway to
278 counteract effects of mitochondrial damage induced by *Bax*. In addition, silencing the expression of
279 *ZnT_1*, but not *ZIP_8*, can not only enhance the ZnO-NP-induced $[Zn^{2+}]_c$ elevation but rescue the 6-
280 OHDA-induced cell death. It is likely that $[Zn^{2+}]_c$ response is a perquisite for ZnO-NP to reduce
281 stress-induced cytotoxicity by suppressing ROS generation and augmenting expression of *bcl-2*.

282 Zn^{2+} has been widely shown as a potential antioxidant for suppression of apoptosis (36-43). In
283 animal brain studies, Zn^{2+} treatment decreases the *Bax*/*Bcl-2* protein ratio (43); treating SH-SY5Y
284 cells with a low dose of Zn^{2+} can reverse a stress-induced increment of DNA fragmentation (12).
285 Zn^{2+} deficiency has been shown to reduce stem cell proliferation, increase neuronal precursor
286 apoptosis and impair neuronal differentiation (15, 44) as well as associate with neuronal dysfunction,
287 such as attention-deficit hyperactivity disorder (9) and depression-like symptoms (45, 46). In
288 contrast, Zn^{2+} supplementation can reduce the levels of ROS to prevent cardiomyocyte apoptosis and
289 congenital heart defects (39); it also promotes the recovery of spinal cord function (17, 47). Zn^{2+} has
290 a protective effect on renal ischemia-reperfusion injury by augmenting superoxide dismutase activity
291 and lowering the *Bax/Bcl-2* expression ratio to reduce apoptosis (36). Our results support that ZnO-
292 NP, at sub-lethal dosage, plays a protective role by reducing ROS generation and the expression of
293 *Bax*/*Bcl-2*.

294 In this and previous studies, we show that ZnO-NP dose-dependently exert paradoxical
295 protective and cytotoxic functions through their ability to alter $[Zn^{2+}]_c$ and modulate the expression
296 of ZnT_1 and ZIP_8. Delivering ZnO-NP at a low dose into the central nervous system may provide a
297 practical strategy to elevate the $[Zn^{2+}]_c$ for potent neuroprotection. Further studies, both *in vivo* and
298 *in vitro,* will be required using more sensitive and selective techniques to measure the homeostasis of
299 $[Zn^{2+}]_c$ and to assess the feasibility of using ZnO-NP for clinical application.
300

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306 **Figure Legends**

307 **Figure 1** ZnO-NP exposure induces a transient elevation of $[Zn^{2+}]_c$ in SH-SY5Y cells. We loaded the
308 cells with FluoZin-3 and monitored the changes of the fluorescence intensities from a group of
309 suspended cells stimulated with different concentrations of ZnO-NPs. A. The short-term $[Zn^{2+}]_c$
310 responses. ZnO-NP (0, 0.081, and 0.814 μg/ml) were added at the beginning of the recording and the
311 fluorescence intensities were normalized to the value at the time zero (F/F_0). B. Long-term exposure
312 of ZnO-NP. The fluorescence intensities from ZnO-NP-treated suspension cells were normalized to
313 the control group without ZnO-NP treatment (Normalized $[Zn^{2+}]_c$) at different time after ZnO-NP
314 exposure. Data presented were Mean ± SEM from 3 batches of cells.
315
316 **Figure 2** Knockdown the expressions of specific Zn^{2+} transporters interfere $[Zn^{2+}]_c$ responses in SH-
317 SY5Y cells. A. and B. The expression levels of *ZnT1* and *ZIP8*, respectively. Cells were treated with
318 different concentrations of ZnO-NP for 0, 6 and 24 hr and the mRNA levels of ZnT_1 and ZIP_8 were
319 analyzed by RT-PCR. The expression levels were normalized to that of β-actin. C. and D. Expression
320 knockdown of *ZnT1* and *ZIP8*, respectively. Specific shRNAs against ZnT_1 (H1-5) and ZIP_8 (H6-9)
321 were delivered into the cells for 1 day and the protein levels were examined by Western blot (upper
322 panel). The intensities of each protein bands were normalized to that of β-actin (lower panel). E.
323 $[Zn^{2+}]_c$ responses in transfected cells. Cells were transfected with H5 and H9 shRNAs for 1 day and
324 then loaded with FluoZin3. The changes in the fluorescence intensities ($\Delta F/F_0$) induced by ZnO-NP
325 (0.814 μg/ml) were calculated. Data presented were Mean ± S.E.M from 3 bathes of cells. ***: $p <$
326 0.001 (Student's *t*-test) when compared to the control group.
327
328
329

Figure 3 Low-dose ZnO-NP exposure reduces basal apoptosis signal in SH-SY5Y cells. A. Dose-dependent cell viability. After a 24-hr ZnO-NP exposure at different concentrations, the cell viability was analyzed by an MTT assay. The dose-dependence were fitted by a Boltzmann equation with an EC_{50} of 6.8 ± 0.2 μg/ml. Data presented were Mean ± SEM from 15 batches of cells. B. The *Bax/Bcl-2* ratio. Cells were treated with ZnO-NP for 6 hr and then the mRNA were collected for RT-PCR to analyze the expression level (upper panel) of *Bax* and *Bcl-2*. The intensities of the PCR products were normalized to the level β-actin and then used to calculate the *Bax*/*Bcl-2* ratio (Lower panel). Data presented were Mean ± S.E.M from 3 bathes of cells. ** and ***: $p < 0.01$ and 0.001, respectively, by Student's *t*-test when compared to the control group.

Figure 4 ZnO-NP suppresses 6-OHDA-induced cytotoxicity in SH-SY5Y cells. A. Cell viability. Cells were pretreated with ZnO-NP (0, 0.081, and 0.814 μg/ml) for 18 hr and then incubated with 6-OHDA (50 or 100 μM) for another 6 hr. The viability was measured by an MTT assay. B. ROS production. Cells were pretreated with or without ZnO-NP (0.081 μg/ml) for 2 hr, then 6-OHDA were added for another 1 hr. Data presented were Mean ± SEM from 15 batches of cells. The significance were analyzed by Student's *t*-test; ** and ***: $p < 0.01$ and 0.001, respectively, when compared to the control group without 6-OHDA treatment or as indicated. C. *p53* mRNA levels. Cells were pretreated with ZnO-NP (0.081 μg/ml) for 18 hr and then incubated with 6-OHDA for 6 hr. Cells were then harvested for RT-PCR and the density of *p53* products were normalized with that of β-actin and control group.

Figure 5 ZnO-NP altered 6-OHDA-induced cytotoxicity in cells with transporter knockdown. H5 and H9 snRNAs were transfected into SH-SY5Y cells for 24 hr to knock down ZnT_1 and ZIP_8, respectively. Cells were pretreated with ZnO-NP of different concentrations for 16 hr and then 6-OHDA (50 μM) for another 6 hr. The cell viability was determined by MTT assay. Data presented

were Mean ± SEM (n = 15) and the significance were analyzed by Student's *t*-test; * and **: $p <$ 0.05 and 0.01, respectively, when compared to the group without 6-OHDA treatment.

360 **References**

361 1. Maret W. Zinc biochemistry: from a single zinc enzyme to a key element of life. Advances in
362 nutrition. 2013;4(1):82-91.
363 2. Maret W. New perspectives of zinc coordination environments in proteins. Journal of inorganic
364 biochemistry. 2012;111:110-6.
365 3. Li Y, Maret W. Transient fluctuations of intracellular zinc ions in cell proliferation. Experimental
366 cell research. 2009;315(14):2463-70.
367 4. Krezel A, Maret W. Zinc-buffering capacity of a eukaryotic cell at physiological pZn. Journal of
368 biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic
369 Chemistry. 2006;11(8):1049-62.
370 5. Cho Y, Gorina S, Jeffrey PD, Pavletich NP. Crystal structure of a p53 tumor suppressor-DNA
371 complex: understanding tumorigenic mutations. Science. 1994;265(5170):346-55.
372 6. Sindreu C, Storm DR. Modulation of neuronal signal transduction and memory formation by
373 synaptic zinc. Frontiers in behavioral neuroscience. 2011;5:68.
374 7. Kawada H, Blessing K, Kiyota T, Woolman T, Winchester L, Kador PF. Effects of multifunctional
375 antioxidants on mitochondrial dysfunction and amyloid-beta metal dyshomeostasis. Journal of
376 Alzheimer's disease : JAD. 2015;44(1):297-307.
377 8. Takeda A, Tamano H. Significance of the degree of synaptic Zn signaling in cognition.
378 Biometals : an international journal on the role of metal ions in biology, biochemistry, and

379 medicine. 2015.

380 9. Lepping P, Huber M. Role of zinc in the pathogenesis of attention-deficit hyperactivity disorder:
381 implications for research and treatment. CNS drugs. 2010;24(9):721-8.

382 10. Levenson CW, Morris D. Zinc and neurogenesis: making new neurons from development to
383 adulthood. Advances in nutrition. 2011;2(2):96-100.

384 11. Marger L, Schubert CR, Bertrand D. Zinc: an underappreciated modulatory factor of brain
385 function. Biochemical pharmacology. 2014;91(4):426-35.

386 12. An WL, Pei JJ, Nishimura T, Winblad B, Cowburn RF. Zinc-induced anti-apoptotic effects in SH-
387 SY5Y neuroblastoma cells via the extracellular signal-regulated kinase 1/2. Brain research
388 Molecular brain research. 2005;135(1-2):40-7.

389 13. Deng X, Luan Q, Chen W, Wang Y, Wu M, Zhang H, et al. Nanosized zinc oxide particles induce
390 neural stem cell apoptosis. Nanotechnology. 2009;20(11):115101.

391 14. Kim JH, Jeong MS, Kim DY, Her S, Wie MB. Zinc oxide nanoparticles induce lipoxygenase-
392 mediated apoptosis and necrosis in human neuroblastoma SH-SY5Y cells. Neurochemistry
393 international. 2015;90:204-14.

394 15. Szewczyk B. Zinc homeostasis and neurodegenerative disorders. Frontiers in aging
395 neuroscience. 2013;5:33.

396 16. Gower-Winter SD, Corniola RS, Morgan TJ, Jr., Levenson CW. Zinc deficiency regulates
397 hippocampal gene expression and impairs neuronal differentiation. Nutritional neuroscience.

2013;16(4):174-82.

17. Wang Y, Su R, Lv G, Cao Y, Fan Z, Wang Y, et al. Supplement zinc as an effective treatment for spinal cord ischemia/reperfusion injury in rats. Brain research. 2014;1545:45-53.

18. Grabrucker AM, Rowan M, Garner CC. Brain-Delivery of Zinc-Ions as Potential Treatment for Neurological Diseases: Mini Review. Drug delivery letters. 2011;1(1):13-23.

19. Grabrucker AM, Garner CC, Boeckers TM, Bondioli L, Ruozi B, Forni F, et al. Development of novel Zn2+ loaded nanoparticles designed for cell-type targeted drug release in CNS neurons: in vitro evidences. PloS one. 2011;6(3):e17851.

20. Kao YY, Cheng TJ, Yang DM, Wang CT, Chiung YM, Liu PS. Demonstration of an Olfactory Bulb-Brain Translocation Pathway for ZnO Nanoparticles in Rodent Cells In Vitro and In Vivo. Journal of molecular neuroscience : MN. 2012;48(2):464-71.

21. Kao YY, Chen YC, Cheng TJ, Chiung YM, Liu PS. Zinc oxide nanoparticles interfere with zinc ion homeostasis to cause cytotoxicity. Toxicol Sci. 2012;125(2):462-72.

22. Zhao J, Xu L, Zhang T, Ren G, Yang Z. Influences of nanoparticle zinc oxide on acutely isolated rat hippocampal CA3 pyramidal neurons. Neurotoxicology. 2009;30(2):220-30.

23. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry. 2009;55(4):611-22.

24. Gerlier D, Thomasset N. Use of MTT colorimetric assay to measure cell activation. Journal of

immunological methods. 1986;94(1-2):57-63.

25. Yuan SS, Hou MF, Hsieh YC, Huang CY, Lee YC, Chen YJ, et al. Role of MRE11 in cell proliferation, tumor invasion, and DNA repair in breast cancer. J Natl Cancer Inst. 2012;104(19):1485-502.

26. Zhong ML, Chi ZH, Shan ZY, Teng WP, Wang ZY. Widespread expression of zinc transporter ZnT (SLC30) family members in mouse endocrine cells. Histochemistry and cell biology. 2012;138(4):605-16.

27. Aydemir TB, Liuzzi JP, McClellan S, Cousins RJ. Zinc transporter ZIP8 (SLC39A8) and zinc influence IFN-gamma expression in activated human T cells. Journal of leukocyte biology. 2009;86(2):337-48.

28. Lichten LA, Cousins RJ. Mammalian zinc transporters: nutritional and physiologic regulation. Annual review of nutrition. 2009;29:153-76.

29. Sindreu C, Bayes A, Altafaj X, Perez-Clausell J. Zinc transporter-1 concentrates at the postsynaptic density of hippocampal synapses. Molecular brain. 2014;7:16.

30. Su R, Mei X, Wang Y, Zhang L. Regulation of zinc transporter 1 expression in dorsal horn of spinal cord after acute spinal cord injury of rats by dietary zinc. Biological trace element research. 2012;149(2):219-26.

31. Beyer N, Coulson DT, Heggarty S, Ravid R, Hellemans J, Irvine GB, et al. Zinc transporter mRNA levels in Alzheimer's disease postmortem brain. Journal of Alzheimer's disease : JAD. 2012;29(4):863-73.

436 32. Bosomworth HJ, Thornton JK, Coneyworth LJ, Ford D, Valentine RA. Efflux function, tissue-
437 specific expression and intracellular trafficking of the Zn transporter ZnT10 indicate roles in
438 adult Zn homeostasis. Metallomics : integrated biometal science. 2012;4(8):771-9.
439 33. Fujishiro H, Yoshida M, Nakano Y, Himeno S. Interleukin-6 enhances manganese accumulation
440 in SH-SY5Y cells: implications of the up-regulation of ZIP14 and the down-regulation of ZnT10.
441 Metallomics : integrated biometal science. 2014;6(4):944-9.
442 34. Xia T, Kovochich M, Liong M, Madler L, Gilbert B, Shi H, et al. Comparison of the mechanism of
443 toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress
444 properties. ACS nano. 2008;2(10):2121-34.
445 35. Muller KH, Kulkarni J, Motskin M, Goode A, Winship P, Skepper JN, et al. pH-dependent toxicity
446 of high aspect ratio ZnO nanowires in macrophages due to intracellular dissolution. ACS nano.
447 2010;4(11):6767-79.
448 36. Guo L, Li P, Meng C, Lu R, Yang Y, Zhou Y, et al. Protective effect of zinc on mouse renal
449 ischemia-reperfusion injury by anti-apoptosis and antioxidation. Current pharmaceutical
450 biotechnology. 2014;15(6):577-82.
451 37. Zheng J, Zhang Y, Xu W, Luo Y, Hao J, Shen XL, et al. Zinc protects HepG2 cells against the
452 oxidative damage and DNA damage induced by ochratoxin A. Toxicology and applied
453 pharmacology. 2013;268(2):123-31.
454 38. Li B, Tan Y, Sun W, Fu Y, Miao L, Cai L. The role of zinc in the prevention of diabetic

455 cardiomyopathy and nephropathy. Toxicology mechanisms and methods. 2013;23(1):27-33.

456 39. Kumar SD, Vijaya M, Samy RP, Dheen ST, Ren M, Watt F, et al. Zinc supplementation prevents
457 cardiomyocyte apoptosis and congenital heart defects in embryos of diabetic mice. Free radical
458 biology & medicine. 2012;53(8):1595-606.

459 40. Huang YZ, McNamara JO. Neuroprotective effects of reactive oxygen species mediated by
460 BDNF-independent activation of TrkB. The Journal of neuroscience : the official journal of the
461 Society for Neuroscience. 2012;32(44):15521-32.

462 41. Bosco MD, Mohanasundaram DM, Drogemuller CJ, Lang CJ, Zalewski PD, Coates PT. Zinc and
463 zinc transporter regulation in pancreatic islets and the potential role of zinc in islet
464 transplantation. The review of diabetic studies : RDS. 2010;7(4):263-74.

465 42. Ohly P, Dohle C, Abel J, Seissler J, Gleichmann H. Zinc sulphate induces metallothionein in
466 pancreatic islets of mice and protects against diabetes induced by multiple low doses of
467 streptozotocin. Diabetologia. 2000;43(8):1020-30.

468 43. Singla N, Dhawan DK. Zinc down regulates Apaf-1-dependent Bax/Bcl-2 mediated caspases
469 activation during aluminium induced neurotoxicity. Biometals : an international journal on the
470 role of metal ions in biology, biochemistry, and medicine. 2015;28(1):61-73.

471 44. Adamo AM, Oteiza PI. Zinc deficiency and neurodevelopment: the case of neurons. BioFactors.
472 2010;36(2):117-24.

473 45. Tassabehji NM, Corniola RS, Alshingiti A, Levenson CW. Zinc deficiency induces depression-like

474 symptoms in adult rats. Physiology & behavior. 2008;95(3):365-9.

475 46. Whittle N, Lubec G, Singewald N. Zinc deficiency induces enhanced depression-like behaviour

476 and altered limbic activation reversed by antidepressant treatment in mice. Amino acids.

477 2009;36(1):147-58.

478 47. Wang Y, Me X, Zhang L, Lv G. Supplement moderate zinc as an effective treatment for spinal

479 cord injury. Medical hypotheses. 2011;77(4):589-90.

480

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Figure 1

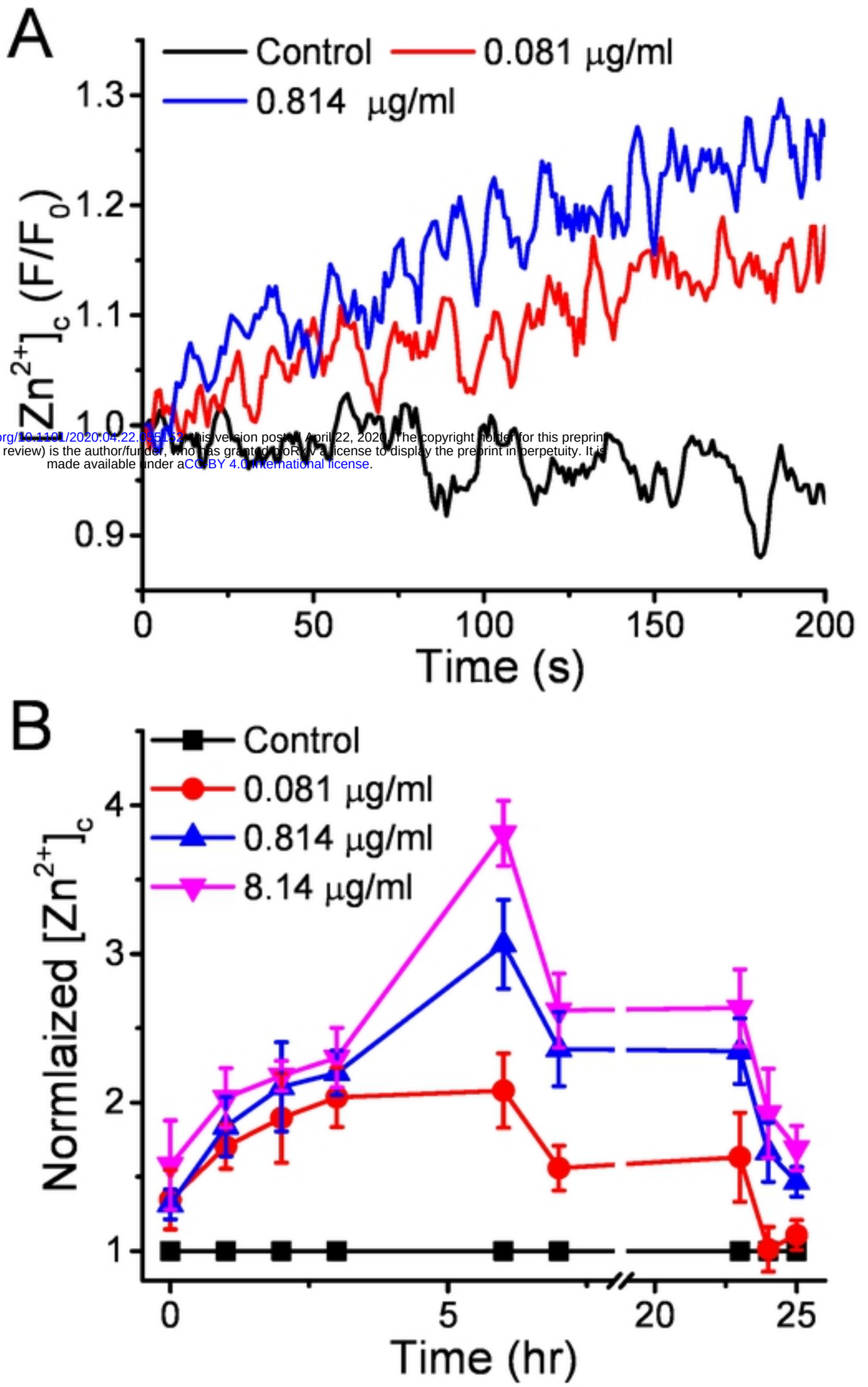


Figure 2

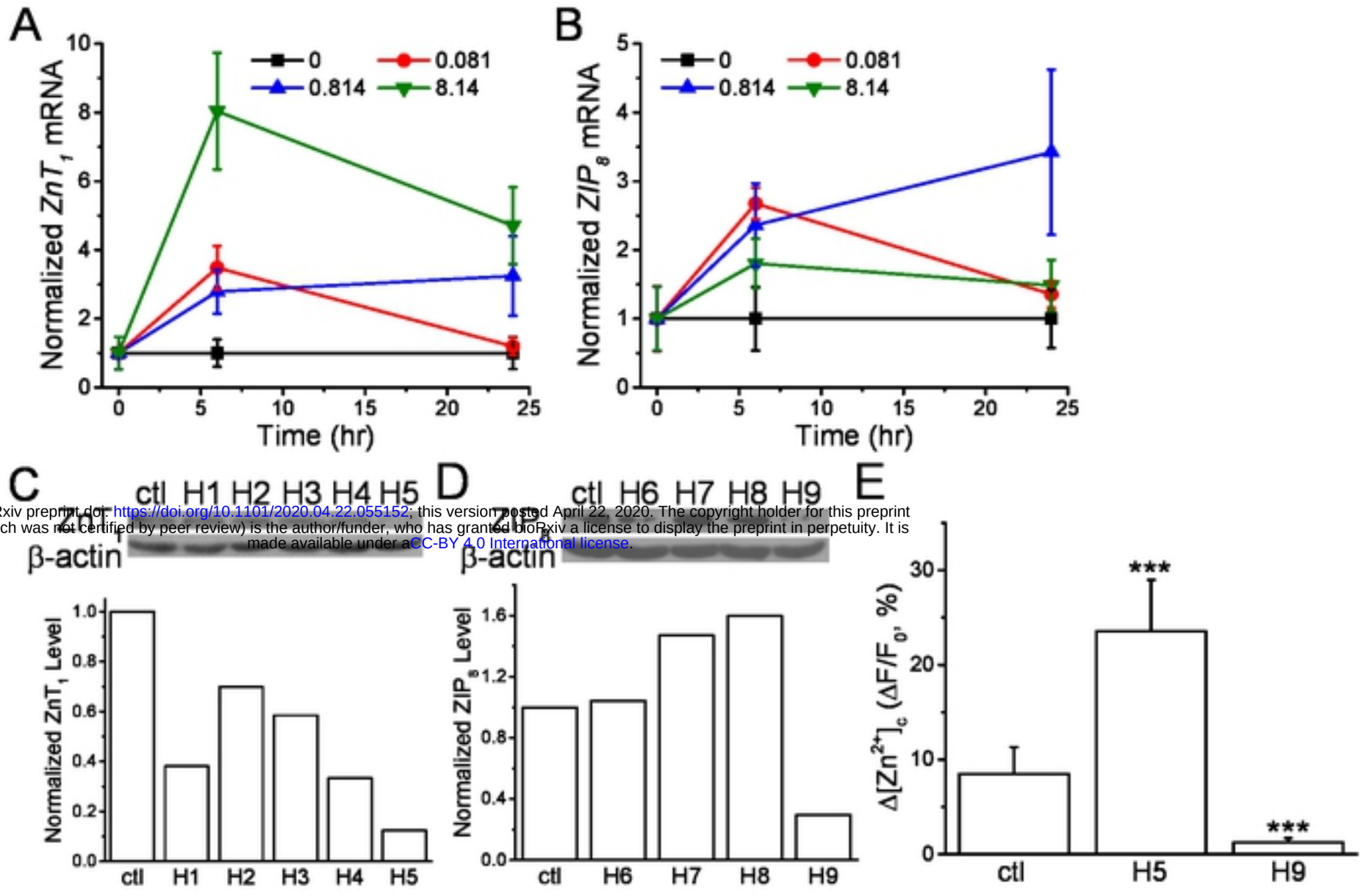
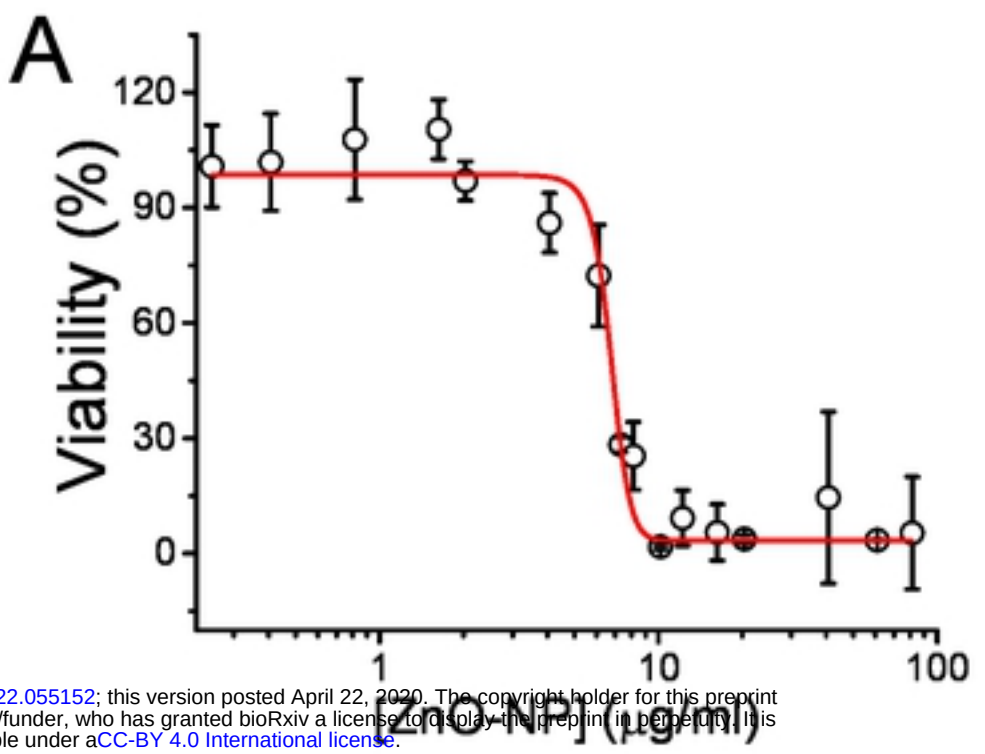


Figure 3



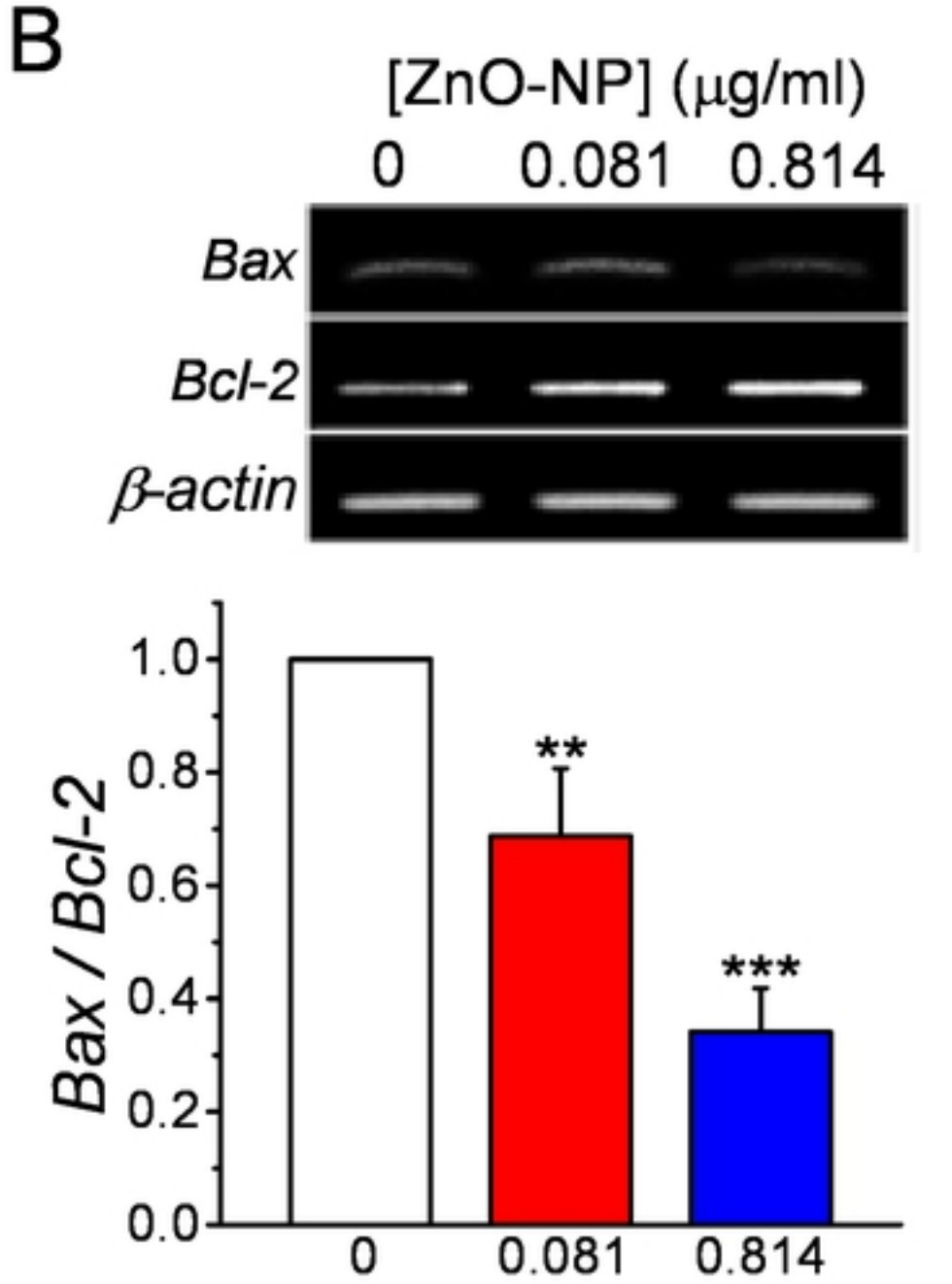


Figure 4

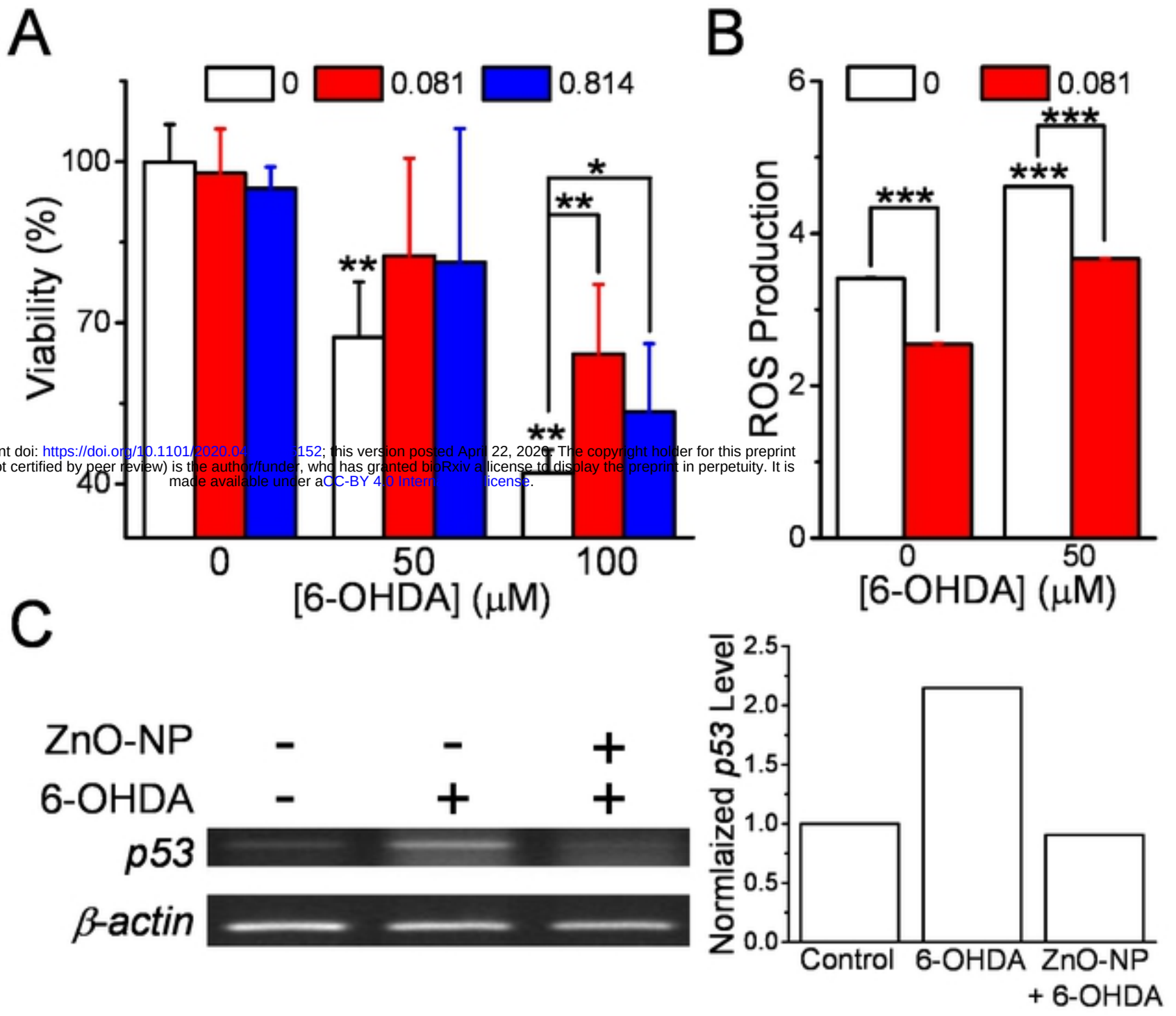


Figure 5