The Zinc Ion Chelating Agent TPEN Attenuates Neuronal Death/apoptosis Caused by Hypoxia/ischemia Via Mediating the Pathophysiological Cascade Including Excitotoxicity, Oxidative Stress, and Inflammation

Wei-Ming Wang,^{1,2} Zhao Liu,¹ Ai-Jun Liu,² Yu-Xiang Wang,¹ Hong-Gang Wang,¹ Di An,¹ Bin Heng,¹ Lai-Hua Xie,³ Jun-Li Duan⁴ & Yan-Qiang Liu¹

¹ College of Life Sciences, Nankai University, Tianjin, China

² Department of Pharmacology, Second Military Medical University, Shanghai, China

³ Department of Cell Biology and Molecular Medicine, New Jersey Medical School, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA

⁴ Department of Gerontology, Xin Hua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Keywords

Excitotoxicity; Free Zn²⁺; Hypoxia/ischemia; Inflammation; Oxidative stress; TPEN.

Correspondence

Yan-Qiang Liu, PhD & Professor, College of Life Sciences, Nankai University, Tianjin 300071, China.

Tel.: +86-22-23508378;

Fax: +86-22-23508378;

E-mails: liuyanq@nankai.edu.cn and liuyanq2@126.com

and

Jun-Li Duan, MD & Professor, Department of Gerontology, Xin Hua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China.

Tel.: +86-21-25078999;

Fax: +86-21-65517520;

E-mail: duanjunli@163.com

Received 2 May 2015; revision 2 June 2015; accepted 3 June 2015

SUMMARY

Aims: We aim to determine the significant effect of TPEN, a Zn²⁺ chelator, in mediating the pathophysiological cascade in neuron death/apoptosis induced by hypoxia/ischemia.

Methods: We conducted both *in vivo* and *in vitro* experiments in this study. PC12 cells were used to establish hypoxia/ischemia model by applying oxygen–glucose deprivation (OGD). SHR-SP rats were used to establish an acute ischemic model by electrocoagulating middle cerebral artery occlusion. The effect of TPEN on neuron death/apoptosis was evaluated. In addition, the relative biomarkers of excitotoxicity, oxidative stress, and inflammation reactions in hypoxia/ischemia PC12 cell model as well as in SHR-SP rat hypoxia/ischemia model were also assessed. **Results:** TPEN significantly attenuates the neurological deficit, reduced the cerebral infarction area and the ratio of apoptotic neurons, and increased the expression of GluR2 in the rat hypoxia/ischemia brain. TPEN also increased blood SOD activity, decreased blood NOS activity and blood MDA and IL-6 contents in rats under hypoxia/ischemia. In addition, TPEN significantly inhibited the death and apoptosis of cells and attenuated the alteration of GluR2 and NR2 expression caused by OGD or OGD plus high Zn²⁺ treatments. **Conclusions:** Zn²⁺ is involved in neural cell apoptosis and/or death caused by hypoxia/ischemia via mediating excitotoxicity, oxidative stress, and inflammation.

doi: 10.1111/cns.12428

Introduction

Stroke is one of the age-related diseases and the second most common cause of death [1–3]. Ischemic stroke is the largest subtype, with an estimated incidence of > 80% of all strokes, and with high rate of morbidity, mortality, and disability [4–6]. Hypoxia is an important pathological condition of stroke [7]. Functional and behavioral deficits associated with nervous system damage from hypoxia/stroke are associated with neuronal death in brain tissue including hippocampus and cortex [7]. Neuronal death in brain

tissue following cerebral ischemia is represented as a mixed type of apoptosis and necrosis [8]. Previous studies have suggested that the ischemia-induced neuronal damage may involve in pathophysiological cascade reactions including excitotoxicity [7,9–11], oxidative stress [6], and inflammation [12,13]. However, detailed underlying mechanisms related to the neuronal death have not been well elucidated yet.

Zn²⁺ is the most abundant trace metal ion in the brain [14]. It is a constituent of about 3000 human proteins and required for over 300 cellular processes from DNA transcription to protein

synthesis, assisting in enzyme structural and catalytic activity, neurotransmission, intracellular signaling, and antibiotic activities [15–17]. The importance of Zn^{2+} in the whole organism is indicated by the wide variety of consequences of Zn^{2+} deficiency, including global disorders of development, such as growth retardation, and acute effects on peripheral tissues such as alopecia, parakeratosis, esophageal lesions, impaired wound healing, etc. [15]. It has been determined that, during neurotransmission in glutamatergic neurons, zinc and glutamate are co-released into the synaptic cleft [14]. It is now well recognized that Zn^{2+} influx from the extracellular space and its mobilization from intracellular pools such as mitochondria, cytosolic Zn^{2+} binding proteins, and lysosomes lead to neuronal damage because of hypoxia/ischemia [18–22]. On the other hand, excessive cytosolic Zn^{2+} depletion can also be deleterious to neurons [18,20,23,24], suggesting that tight regulation of Zn^{2+} homeostasis is crucial for neuronal survival. Although there are some experimental data elucidating the mechanism of free Zn^{2+} on neuronal damage by hypoxia/ischemia [24–26], it still needs more experimental evidences to clarify whether and how free Zn^{2+} is involved in the neuronal damage caused by hypoxia/ischemia.

Here we investigated the effect of N, N, N', N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), a Zn^{2+} chelator on neuron death/apoptosis and on some pathophysiological cascade reactions such as excitotoxicity, oxidative stress, and inflammation in both *in vitro* hypoxia/ischemia model [PC12 cell line treated by oxygen-glucose deprivation (OGD)] and *in vivo* hypoxia/ischemia rat model [by electrocoagulating middle cerebral artery occlusion (MCAO)]. We aim to provide more evidence demonstrating the significance of free Zn^{2+} in mediating pathophysiological cascade reactions such as excitotoxicity, oxidative stress, and inflammation in neuron death/apoptosis caused by hypoxia/ischemia.

Materials and Methods

Reagents and Relative Drugs

Anhydrous alcohol, calcium chloride, formaldehyde, glacial acetic acid, hydrochloric acid, magnesium chloride, methanol, potassium chlorate, sodium chloride, sodium hydroxide, and TTC stain were all purchased from China National Medicines Corporation (Shanghai, China). Ammonium persulfate, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), glycine, and ponceau were from Shanghai Boguang Biology Technology Co., Ltd (Shanghai, China). Glycerin, N, N, N', N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), and mouse anti-GluR2 monoclonal antibody were purchased from Sigma (St Louis, MO, USA). Tween-20, tris base and tetramethylethylenediamine (TEMED) were obtained from AMRESCO (Solon, OH, USA). Sodium dodecyl sulfate (SDS) was from Calbiochem (Darmstadt, Germany). Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining kit was purchased from Wuhan Boster Biological Technology, LTD (Wuhan, China). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, and fetal bovine serum (FBS) were purchased from Gibco. IL-6 ELISA assay kit was from R&D Systems, Inc. (Minneapolis, MN, USA). MDA, SOD, and NOS assay kits were from Nanjing Jiancheng Bioengineering Inc. (Nanjing, China). Coomassie Brilliant Blue was from Bio-Rad

(Richmond, CA, USA). PVDF membrane was from Amersham (Piscataway, NJ, USA). Develop and fixation solutions were obtained from the Eastman Kodak Company (Rochester, NY, USA). HRP-conjugated goat anti-mouse secondary antibody was from Beyotime Institute of Biotechnology (Shanghai, China). Mouse anti-GAPDH monoclonal antibody and total protein extraction reagent were purchased from Shanghai Kangcheng Biotechnology Co. (Shanghai, China).

The Experimented Animals and Management

Female stroke-prone spontaneously hypertensive rats (SHR-SP), aged 12 weeks, weight about 200 g were provided by the Animal Center of the Second Military Medical University (Shanghai, China). The rats were housed under controlled temperatures (23–25°C), relative humidity (70%), and lighting (8:00–20:00 light, 20:00–8:00 dark) with free access to food and tap water. All the animals used in the experiment received human care in compliance with institutional guidelines for health and care of experimental animals.

Intracerebroventricular Administration and MCAO Operation

Twenty SHR-SP rats were allotted to two groups (10/group) which the animals in one group were injected into 5 μL aCSF containing DMSO, whereas the animals in another group were injected into 5 μL 0.1 mol/L TPEN dissolved in aCSF containing DMSO by intracerebroventricular administration applying stereotaxic apparatus. After above intracerebroventricular administration for 30 min, all animals were underwent middle cerebral artery occlusion (MCAO) by electrocoagulation under being euthanized by i.p. 15% of chloral hydrate.

Determination of Neurological Deficit Score

After MCAO for 24 h, the neurological deficit score of all animals was determined according to the method [27]. The neurological deficit score was graded from 0 to 15 (normal score, 0; maximal deficit score, 15) according to 4 tests including balance beam test, wire suspension test, tail walking test, and the tail suspension test. In balance beam test, the rats were trained to traverse a square beam measuring 2.4 cm $\times$ 2.4 cm $\times$ 84 cm. According to the slipped status of foot and status of crossing the beam, the tested animals were, respectively, divided into 0–6 scores (normal score, 0; maximal deficit score, 6). In wire suspension test, to test grip strength and endurance, the front paws were positioned on a horizontal steel wire (2 mm diameter). Test parameters were latency to the first fall and the number of falls during a 2-min assessment period, and the tested animals were, respectively, divided into 0–3 scores (normal score, 0; maximal deficit score, 3). In tail walking test, the rats were trained to walk with the hind legs from the ground by lifting tail. Walking coordination was tested, and the tested animals were also, respectively, divided into 0–3 scores (normal score, 0; maximal deficit score, 3). In tail suspension test, the animal was suspended by the tip of their tail, stretched status of animals was assessed, and the tested animals were also, respectively, divided into 0–3 scores (normal score, 0; maximal deficit score, 3).

Assessment of Cerebral Infarct Size

After MCAO operation for 24 h and behavior test, the animals were euthanized. And the brain was removed and placed into cold 0.1 mol/L phosphate buffer (PBS, pH 7.4, -20°C) for a 2 min, sliced transversely into six 2 mm sections from the anterior to the posterior extremity, stained with 2% 2, 3, 5-triphenyl tetrazolium chloride (TTC) for 30 min in the dark, and fixed in 4% paraformaldehyde for more than 1 h. The infarct area of each section was measured by a computerized image analysis system (Leica, Germany), and the total infarct area of one brain was estimated by adding the infarct area of each section and corrected for brain edema.

Measurement of Apoptotic Cells in Brains by TUNEL Assay

The brains were carefully removed and fixed in 4% paraformaldehyde at room temperature for 4 h at 24 h after MCAO. Coronal section ($4\ \mu\text{m}$) was cut on a cryostat. TUNEL staining was performed using *In situ* Cell Death Detection Kit (Wuhan Boster Biological Engineering Co., Ltd, Wuhan, China), according to the protocol provided by manufacturer. Briefly, sections were first washed twice in PBS for 5 min and permeabilized for 2 min with 200 mL permeabilization solution (0.01 mol/L, pH 6.0 sodium citrate in PBS) at 95°C . After washing, the sections were incubated with TUNEL Reaction Mix for 1.5 h at 37°C in the dark. Then, the sections were incubated with POD and DAB/ H_2O_2 , respectively. The number of TUNEL-positive cells per square millimeter of tissue at the margin of the infarct area was counted under high-power magnification ($\times 400$). TUNEL-positive cells were expressed as percent of total cell count.

Determination of Serum SOD, MDA, NOS, and IL-6

The serum was obtained after the rat was deeply anesthetized. Serum superoxide dismutase (SOD), malondialdehyde (MDA), and NOS were assayed according to the instructions of the SOD, MDA, and NOS assay kits from the manufacturer (Nanjing Jiancheng Biotech, Nanjing, China). And serum interleukin-6 (IL-6) was examined with ELISA kits (R&D Systems Inc., Minneapolis, MN, USA) according to the protocol provided by manufacturer.

Western Blotting Assay for Detecting GluR2 in Brain Tissue

GluR2 in brain tissue was determined applying Western blotting method based on the instructions of the manufacturer. Tissues were homogenized by protein extraction reagent (Beyotime, Nanjing, China) supplemented with protease inhibitor cocktail (Pierce, Rockford, IL, USA) and phosphatase inhibitor cocktail (Pierce). About 50 μg of samples was run on 10% SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). The proteins were electrotransferred to nitrocellulose membranes. The nitrocellulose membranes were incubated with primary antibody (anti-GluR2 monoclonal antibody from mouse; Sigma Co) for 2 h at 25°C and then incubated with secondary antibody for 2 h. The

images were captured and analyzed by X-ray film-based technologies. GAPDH (anti-GAPDH monoclonal antibody from mouse; Sigma Co.) was used as a loading control. The optical density value of the target band was analyzed with gel image processing system.

PC12 Cells Culture

PC12 cell line was cultured in 1 g/L glucose Dulbecco's modified Eagle's medium (DMEM) with L-glutamine, 10% fetal bovine serum (FBS), 5% horse serum (HS), 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were incubated in humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C . DMEM, L-glutamine, FBS, HS, penicillin, and streptomycin were from DingGuo ChangSheng Biotechnology Co., Ltd., Beijing, China.

Oxygen–glucose Deprivation (OGD) Treatment in the Experiment

We used chemical methods to establish hypoxic-ischemic PC12 model (oxygen–glucose deprivation, OGD). The cells were cultured in the DMEM culture medium without glucose (Gibco, USA), and adding 2 mM sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) (AR, Tianjin, China) to remove the oxygen [28].

Measurement of PC12 Cell Viability by AO/EB Staining Assay

We applied acridine orange (AO)/ethidium bromide (EB) staining assay (AO and EB, respectively, combine with DNA of vital and death cells) to determine cell viability of every treatment. Briefly, PC12 cells were diluted to 1×10^5 cells/mL and plated at 100 μL per well in cover glass of a 96-well microplate with DMEM culture medium containing 10% fetal bovine serum and 5% horse serum for 24 h. Then, PC12 cells were treated with Control (normal culture medium), OGD (culture medium without glucose and adding 2 mM sodium dithionite), OGD plus TPEN (50 μM), OGD plus TPEN (80 μM), OGD plus Zn^{2+} (100 μM), OGD plus TPEN (50 μM), and Zn^{2+} (100 μM) for 3 h or 6 h. After washing with PBS, 20 μL of staining solution containing, respectively, 100 $\mu\text{g/L}$ AO and 100 $\mu\text{g/L}$ EB was added to cover glass. Then, number of AO staining (green) and EB staining (red) cells were counted under an immunofluorescence microscope (Olympus, BX51; Olympus Optical Co., Ltd, Tokyo, Japan). Cell viability was expressed as AO staining cells percent of total cells.

Measurement of Apoptotic Cells in PC12 by TUNEL Assay

TUNEL assay was performed on the PC12 cells of Control (normal culture medium), OGD (culture medium without glucose and adding 2 mM sodium dithionite), OGD plus TPEN (50 μM), OGD plus Zn^{2+} (100 μM), OGD with TPEN (50 μM), and Zn^{2+} (100 μM) for 3 h or 6 h with an *In Situ* Cell Death Detection Kit (Biyuntian, China) according to the manufacturer's instructions. Briefly, the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 and washed with PBS, and then were

permeabilized with 0.2% Triton-X 100 in methanol for 2 min at 4°C. The cells were incubated with TUNEL assay solution at 37°C for 60 min. Finally, the cells were washed with PBS and mounted with fluorescent mounting medium. The number of TUNEL-positive PC12 was obtained by counting cells in six randomly selected microscopic fields obtained from each coverslip at $\times 20$ objectives. The percentage of TUNEL-positive PC12 against the total number of PC12 was calculated and averaged.

Observation of PC12 Cells GluR2 and NR2 Expression by Immunofluorescence Method

Purified PC12 of Control (normal culture medium), OGD (culture medium without glucose and adding 2 mM sodium dithionite), OGD plus TPEN (50 μ M), OGD plus Zn^{2+} (100 μ M), OGD plus TPEN (50 μ M), and Zn^{2+} (100 μ M) for 3 h or 6 h were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 20 min, and blocked with 3% normal goat serum and 1% BSA for 30 min. The cells were then incubated at room temperature with primary antibodies: polyclonal rabbit anti-GluR2 or polyclonal goat anti-NR2 (1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequent antibody detection was carried out with secondary antibodies: TR-conjugated donkey anti-goat IgG or FITC-conjugated donkey anti-rabbit IgG (1:100; Santa Cruz Biotechnology, Inc). After rinsing three times with PBS, the cells

were mounted with a fluorescent mounting medium (Biyuntian, China). Expression of GluR2 and NR2 was observed under an immunofluorescence microscope (Olympus, BX51, Olympus Optical Co., Ltd).

Image-Pro Plus 6.0 software was used to determine the density of their expression.

Data Analysis

Data were presented as mean $\pm$ SD. Statistical significance was assessed using one-way ANOVA analysis and LSD test (*in vitro* experiment) or Student's *t*-test (*in vivo* experiment) with $P < 0.05$ considered significant. Origin 5.1 and SPSS 19.1 were applied to analyze the data.

Results

TPEN Alleviated Neurological Deficits and Reduced Brain Infarction Area in Acute Ischemic Rat Brains

The effects of TPEN on neurological deficit and brain infarction area in acute ischemic rats were shown in Figure 1. Figure 1A illustrates the location of MCAO operation. TPEN significantly decreased the neurological deficit score compared to the control

Figure 1 The effect of TPEN on neurological deficits and brain infarction area in acutely ischemic rat brain. **(A)** The coagulated points of middle cerebral artery. **(B)** Comparison of the neurological deficit scores ($n = 10$), ** $P < 0.01$, compared with control group (no TPEN treatment for acute ischemic rat). **(C)** Typical TTC staining map every coronal section, Scale Bar = 10 mm. **(D)** Infarction percent of every coronal section ($n = 10$), * $P < 0.05$ ** $P < 0.01$, compared with control group. **(E)** Average infarction percent of each treatment ($n = 10$), * $P < 0.05$, compared with control group.

group (Figure 1B, $P < 0.01$). TPEN group exhibited significant decrease in infarction area, compared to the control group (Figure 1C–E, $P < 0.05$ or $P < 0.01$).

TPEN Inhibited Neuron Apoptosis in Acutely Ischemic Rat Brain

As shown in representative images (Figure 2A) and summarized quantitative results (Figure 2B), the number of apoptotic neurons in TPEN group was significantly lower than in control group ($P < 0.05$).

TPEN Upregulated GluR2 Expression in Acutely Ischemic Rat Brain

As shown in Figure 3, the expression of GluR2 was upregulated (Figure 3A) in TPEN-treated group compared to the control group (Figure 3B, $P < 0.05$).

TPEN Reversed the Changes in Blood MDA, SOD, NOS, and IL-6 in Acutely Ischemic Rats

Next, we evaluated the levels of blood MDA, SOD, NOS, and IL-6 in acutely ischemic rats (Figure 4). Compared to the control group, TPEN treatment significantly decreased blood MDA concentration (Figure 4A, $P < 0.05$), total NOS (TNOS) (Figure 4C, $P < 0.05$), and IL-6 concentration (Figure 4E, $P < 0.05$), but

increased blood SOD activity (Figure 4B, $P < 0.05$). The level of iNOS activity remained unchanged (Figure 4D, $P > 0.05$).

TPEN Reversed Death and Apoptosis Caused by Hypoxia/ischemia in PC12 Cells

The effect of hypoxia/ischemia, Zn^{2+} , and TPEN on death or apoptosis in PC12 cells was shown in Figure 5. Immunofluorescence microscopic images clearly showed that OGD-induced hypoxia/ischemia increased the death rate in PC12 cells with a time-dependent manner (at 3 and 6 h) and was exacerbated by adding Zn^{2+} (50 μ M). TPEN partially attenuated the death rate enhanced by OGD or OGD plus high Zn^{2+} at both 3 h and 6 h (Figure 5A and B). Particularly, at 3 h, compared with OGD, OGD+TPEN50 and OGD+TPEN80 both significantly increased relative cell survival ratio ($P < 0.01$), whereas OGD+Zn significantly decreased relative cell survival ratio ($P < 0.01$), OGD+Zn+TPEN50 did not show significant difference ($P > 0.05$). Compared with OGD+Zn, OGD+Zn+TPEN50 also significantly increased relative cell survival ratio ($P < 0.01$). And at 6 h, compared with OGD, OGD+TPEN50 and OGD+TPEN80, respectively, increased relative cell survival ratio ($P < 0.01$ and $P < 0.05$), whereas OGD+Zn and OGD+Zn+TPEN50 decreased relative cell survival ratio ($P < 0.01$). TPEN did not decrease cell death rate induced synergistically by OGD plus Zn^{2+} after long-time treatment (6 h). In addition, the apoptotic rate in PC12 (green nuclei) was evaluated by TUNEL assay in different treatment group (Figure 5C). These results indicate that OGD time-dependently enhanced the apoptotic rate in PC12 cells,

Figure 2 TPEN inhibiting neuron apoptosis of acute ischemic rat. (A) Representative TUNEL staining map of each treatment, Scale Bar = 50 μ m. (B) Average percent of TUNEL-positive neuron in each treated rat ($n = 10$), * $P < 0.05$, compared with the control group.

which were further aggravated by adding Zn^{2+} . TPEN partially attenuated the apoptotic rate enhanced by OGD (with or without Zn^{2+}).

Figure 3 The effect of TPEN on GluR2 expression in acutely ischemic rat brains. (A) Representative Western blotting bands from the two groups. (B) Average relative expression levels of brain GluR2 ($n = 10$), $*P < 0.05$, compared to the control group.

TPEN Reversed the Changes of Intracellular GluR2 and NR2 Expression in PC12 Cells Induced by Hypoxia/ischemia

As shown in Figure 6, TPEN reversed changes of intracellular GluR2 and NR2 expression in PC12 cells induced by hypoxia/ischemia. GluR2 expression (Figure 6A & B) was decreased by hypoxia/ischemia (at both 3 h and 6 h, OGD vs. Control, $P < 0.05$), Zn^{2+} exacerbated the effect of OGD (at 3 h, OGD+Zn vs. OGD, $P < 0.05$), while TPEN reversed the decrease induced by OGD (at 3 h and 6 h, OGD+TPEN vs. OGD, $P < 0.05$, OGD+Zn+TPEN vs. OGD+Zn, $P < 0.01$). Figure 6C and D showed intracellular NR2 expression status of every treatment for 3 h and 6 h. NR2 expression (Figure 6C & D) was increased by hypoxia/ischemia (at 3 h, OGD vs. Control, $P < 0.05$; at 6 h, OGD vs. Control, $P < 0.01$). Zn^{2+} exacerbated the effect of OGD (at 3 h, OGD+Zn vs. OGD, $P < 0.05$; at 6 h, OGD+Zn vs. OGD, $P < 0.01$), whereas TPEN reversed the increase induced by OGD (at 3 h, OGD+TPEN vs. OGD, $P < 0.05$, at 6 h, OGD+TPEN vs. OGD, $P < 0.01$), but did not reverse the increase induced by synergistic effect of OGD plus Zn^{2+} (at 3 h and 6 h, OGD+Zn+TPEN vs. OGD+Zn, $P > 0.05$).

Discussion

Zn^{2+} is the most abundant trace metal ion in the brain [14]. It is stored in presynaptic vesicles together with glutamate and released into the extracellular space in an activity-dependent manner [29]. Released Zn^{2+} functions as a neurotransmitter or a neuromodulator [30,31] and plays important and wide-spectrum

Figure 4 The effect of TPEN on blood MDA, SOD, NOS, and IL-6 levels in acutely ischemic rats. (A) MDA concentration ($n = 10$). (B) SOD activity ($n = 10$). (C) Total NOS activity ($n = 10$). (D) Inducible NOS activity ($n = 10$). (E) IL-6 concentration ($n = 10$), $*P < 0.05$, compared to the control group in each panel.

physiological roles in cognition [32–34], brain development [35], sensory [36], behavior, and emotion [21,37]. On the other hand, Zn^{2+} is implicated directly or indirectly in the pathogenesis of numerous neurological diseases including Alzheimer's disease, amyotrophic lateral sclerosis, depression, epilepsy, ischemia, and schizophrenia [21]. As Koh et al. found that extracellular free Zn^{2+} may be involved in neuronal death in brain after transient global cerebral ischemia [38], there has accumulated a large amount of data showing the significance of Zn^{2+} in generating ischemic degenerative events following cerebral stroke [18,20–22]. In present study, we have observed that TPEN, a Zn^{2+} chelator significantly attenuates the neurological deficit and brain infarction area in acute ischemic rat brains. The results were in good agreement with those recently reported by Zhao et al. [39]

It has been determined that, during neurotransmission in glutamatergic neurons, Zn^{2+} and glutamate are co-released into the synaptic cleft [14]. It is also recognized that Zn^{2+} influx from the extracellular space and its mobilization from intracellular pools such as mitochondria, cytosolic Zn^{2+} binding proteins, and lysosomes lead to neuronal damage because of hypoxia/ischemia [18–22]. Excessive cytosolic Zn^{2+} can also be deleterious to neurons [18,20,23,24], suggesting that tight regulation of Zn^{2+} homeostasis

is crucial for neuronal survival. Some experimental data have indicated that neuronal damage by hypoxia/ischemia might be mediated by high free Zn^{2+} -induced neuronal apoptosis [24–26]. The results of our present experiments provide further evidence supporting this notion. Our *in vivo* experiments demonstrated that TPEN treatment decreases the neuronal apoptosis in acutely ischemic rat brains. Furthermore, our *in vitro* experiments indicated that OGD resulted in asynchronous increase of death and apoptosis in PC12 cells, which are further exacerbated by adding Zn^{2+} , whereas TPEN (a chelator of Zn^{2+}) treatment significantly reduced death and apoptosis rate of cells caused by OGD or OGD plus Zn^{2+} treatment (in a time-dependent manner). Based on previous reports on TPEN's effect on Zn^{2+} chelation in neuronal system [19], we selected the dosage of 50 and 80 μM for TPEN in our current study. As no difference was found in the death rate of cells between TPEN 50 μM group and 80 μM group, it was likely that TPEN at 50 μM could produce the maximal protective action.

The mechanisms for the ischemia-induced neuronal damage have been accounted for by complex factors such as oxidative stress [6,40], excitotoxicity [11,41,42], inflammation [12,13], etc. In the present study, our *in vivo* experiments demonstrated that TPEN treatment enhanced brain GluR2 expression, decreased

Figure 5 Hypoxia/ischemia induces death or apoptosis in PC12 cells, and TPEN reverses death or apoptosis induced by hypoxia. **(A)** Results of cell survival status obtained applying AO/EB staining method (green cells were alive, and red cells were death), Scale Bar = 50 μm . **(B)** Summarized results of relative cell survival ratio obtained applying AO/EB staining method ($n = 5$, $*P < 0.05$, $**P < 0.01$ compared with OGD; $***P < 0.001$ compared with OGD+Zn). **(C)** Immunofluorescence images showed apoptotic PC12 (green nuclei) as detected by terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick end labeling (TUNEL) in different treatment groups (the experiment was repeated three times), Scale Bar = 50 μm .

Figure 6 The level of intracellular GluR2 and NR2 expression. **(A)** Immunofluorescence images showing GluR2 expression, Scale Bar = 100 μ m. **(B)** Histogram of immunofluorescence density of GluR2 expression ($n = 3$) and NR2 expression. **(C)** Immunofluorescence images showing NR2 expression, Scale Bar = 100 μ m. **(D)** Histogram of immunofluorescence density of NR2 expression ($n = 3$) in PC12 cells under hypoxia/ischemia induced by OGD treatment, high Zn^{2+} , and TPEN separately or combined treatments for 3 and 6 h. * $P < 0.05$, ** $P < 0.01$, compared with Control, # $P < 0.05$, ## $P < 0.01$ compared with OGD, ^ $P < 0.01$ compared with OGD+Zn ($n = 6$).

blood MDA and IL-6 contents and NOS activity, increased blood SOD activity in acute ischemic rat. Our *in vitro* experiments indicated that OGD resulted in decrease of GluR2 expression and increase of NR2 expression in PC12 cells. Plus Zn^{2+} further exacerbated the decrease of GluR2 expression and increase of NR2 expression in PC12 cells, whereas TPTN treatment significantly attenuated the decrease of GluR2 expression and increase of NR2 expression caused by OGD and OGD+ Zn^{2+} in the cells. GluR2 and NR2 are subunits of AMPA receptor and NMDA receptor, respectively, and both AMPA receptor and NMDA receptor are ionotropic glutamate receptors which mediate signal transduction of glutamate. Glutamate produces its excitotoxicity by inducing influx of Ca^{2+} and Na^+ via AMPA receptor and NMDA receptor [43,44]. Whereas GluR2 might decrease the excitotoxicity of glutamate by inhibiting Ca^{2+} permeability of AMPA receptor, NR2 increases the excitotoxicity of glutamate by facilitating Na^+ permeability of NMDA receptor [45–47]. GluR1 is also important subunits of AMPA receptor and is involved in its open probability. It has been reported that GluR1 may correlate with the development of early global ischemia in rat [48]. It remains to be studied whether TPEN treatment affects GluR1 expression in our current model.

MDA, SOD, and NOS are all biomarkers of oxidative stress [49–51], whereas IL-6 is a cytokine and plays an important role in

immune modulation and inflammation reaction [52]. In fact, previous studies were, respectively, correlated to free Zn^{2+} with oxidative stress and excitotoxicity during hypoxia/ischemia [20,53–58]. Our present study provides potential links between free Zn^{2+} , oxidative stress, excitotoxicity, and inflammation reaction in neuronal damages during hypoxia/ischemia.

During the preparation of our present article, an excellent study has recently published by Zhao *et al.*, who reported that reducing zinc accumulation by TPEN rescued neurons from ischemia-induced apoptotic death by reducing poly (ADP-ribose) polymerase-1 activation in a *in vivo* model [39]. Our present study in both *in vitro* and *in vivo* models has further provided supportive evidence for the hypothesis that TPEN protects neurons from ischemia-induced injuries by reducing free Zn^{2+} and consequently preventing cell death mediated by excitotoxicity, oxidative stress, inflammation, and poly (ADP-ribose) polymerase-1 activation [39]. We understand this is still a hot research topic, and many controversial issues remain. Future studies on cellular and ionic mechanisms, such as specific effects of Zn^{2+} on ion transportation, are needed. In addition, some results obtained from *in vitro* experiments have provided supportive pathophysiological evidence (such as cell apoptosis and GluR2 expression influenced by TPEN) for *in vivo* experiment results at cellular level, and some results

from *in vitro* experiment (such as cell death ratio and NR2 expression influenced by TPEN) need to be confirmed by *in vivo* experiment. However, comprehensive *in vivo* and *in vitro* experimental evidence might be helpful to clarify that TPEN, the zinc ion chelating agent can mediate the pathophysiological cascade caused by hypoxia/ischemia.

It has been known that gender differences exist in cardiovascular and renal diseases in human and animal models, including in spontaneously hypertensive rats (SHR). Male SHR have higher blood pressures than females. This difference is likely mediated via a mechanism involving the androgen receptor and the renin-angiotensin system [59–61]. In addition, gender difference has also been found in brain damage during experimental focal ischemia in rats [62]. Therefore, to avoid any potential interference caused by above-mentioned gender difference, we used only female rats in our current study.

Based on comprehensive results obtained from our present study including *in vivo* and *in vitro* experiments, we conclude that extra free Zn^{2+} may trigger pathophysiological cascade reactions including oxidative stress, excitotoxicity, and inflammation reaction during hypoxia/ischemia and consequently induce the cerebral infarction and the neurological deficit in animals and human. Meanwhile, we understand our results were somehow descriptive and further mechanistic studies are warranted in the further study.

References

- Donnan GA, Fisher M, Macleod M, Davis SM. Stroke. *Lancet* 2008;**371**:1612–1623.
- Donnan GA, Fisher M, Macleod M, Davis SM. Secondary prevention of stroke - Reply. *Lancet* 2008;**372**:1036.
- Seshadri S, Beiser A, Kelly-Hayes M, et al. The lifetime risk of stroke - Estimates from the Framingham Study. *Stroke* 2006;**37**:345–350.
- Jauch EC, Cucchiara B, Adegoye O, et al. Part 11: adult Stroke 2010 American Heart Association Guidelines for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care. *Circulation* 2010;**122**:S818–S828.
- Rosamond W, Flegal K, Furie K, et al. Heart disease and stroke statistics - 2008 update - A report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2008;**117**:E25–E146.
- Guo JM, Liu AJ, Zang P, et al. ALDH2 protects against stroke by clearing 4-HNE. *Cell Res* 2013;**23**:915–930.
- Brose SA, Marquardt AL, Golovko MY. Fatty acid biosynthesis from glutamate and glutamine is specifically induced in neuronal cells under hypoxia. *J Neurochem* 2014;**129**:400–412.
- Northington FJ, Ferriero DM, Graham EM, Traystman RJ, Martin LJ. Early neurodegeneration after hypoxia-ischemia in neonatal rat is necrosis while delayed neuronal death is apoptosis. *Neurobiol Dis* 2001;**8**:207–219.
- Dallas M, Boycott HE, Atkinson L, et al. Hypoxia suppresses glutamate transport in astrocytes. *J Neurosci* 2007;**27**:3946–3955.
- Johnston MV. Excitotoxicity in neonatal hypoxia. Johnston Mental Retardation and Developmental Disabilities. *Res Rev* 2001;**7**:229–234.
- Lai TW, Zhang S, Wang YT. Excitotoxicity and stroke: identifying novel targets for neuroprotection. *Prog Neurobiol* 2014;**115**:157–188.
- Fann DYW, Lee SY, Manzanero S, Chunduri P, Sobey CG, Arumugam TV. Pathogenesis of acute stroke and the role of inflammasomes. *Ageing Res Rev* 2013;**12**:941–966.
- Park JH, Park OK, Cho JH, et al. Anti-inflammatory effect of Tanshinone I in neuroprotection against cerebral ischemia-reperfusion injury in the Gerbil Hippocampus. *Neurochem Res* 2014;**39**:1300–1312.
- Torres-Vega A, Pliego-Rivero BF, Otero-Ojeda GA, Gomez-Oliván LM, Veyra-Reyes P. Limbic system pathologies associated with deficiencies and excesses of the trace elements iron, zinc, copper, and selenium. *Nutr Rev* 2012;**70**:679–692.
- Samman S. Zinc. *Nutr Diet* 2007;**64**:S131–S134.
- Kelleher SL, McCormick NH, Velasquez V, Lopez V. Zinc in specialized secretory tissues: roles in the pancreas, prostate, and mammary gland. *Adv Nutr* 2011;**2**:101–111.
- De Leon-Rodriguez L, Lubag AJM, Sherry AD. Imaging free zinc levels in vivo - What can be learned? *Inorg Chim Acta* 2012;**393**:12–23.
- Sensi SL, Paoletti P, Koh JY, Aizenman E, Bush AI, Hershfinkel M. The neurophysiology and pathology of brain zinc. *J Neurosci* 2011;**31**:16076–16085.
- Medvedeva YV, Lin B, Shuttleworth CW, Weiss JH. Intracellular Zn^{2+} accumulation contributes to synaptic failure, mitochondrial depolarization, and cell death in an acute slice oxygen-glucose deprivation model of ischemia. *J Neurosci* 2009;**29**:1105–1114.
- Sensi SL, Paoletti P, Bush AI, Sekler I. Zinc in the physiology and pathology of the CNS. *Nat Rev Neurosci* 2009;**10**:780–U38.
- Bitanirwe BKY, Cunningham MG. Zinc: the brain's dark horse. *Synapse* 2009;**63**:1029–1049.
- Galasso SL, Dyck RH. The role of zinc in cerebral ischemia. *Mol Med* 2007;**13**:380–387.
- BarKalifa R, Hershfinkel M, Friedman JE, Kozak A, Sekler I. The lipophilic zinc chelator DP-b99 prevents zinc induced neuronal death. *Eur J Pharmacol* 2009;**618**:15–21.
- Calderone A, Jover T, Mashiko T, et al. Late calcium EDTA rescues hippocampal CA1 neurons from global ischemia-induced death. *J Neurosci* 2004;**24**:9903–9913.
- Noh KM, Yokota H, Mashiko T, Castillo PE, Zukin RS, Bennett MVL. Blockade of calcium-permeable AMPA receptors protects hippocampal neurons against global ischemia-induced death. *Proc Natl Acad Sci USA* 2005;**102**:12230–12235.
- Bonanni L, Chachar M, Jover-Mengual T, et al. Zinc-dependent multi-conductance channel activity in mitochondria isolated from ischemic brain. *J Neurosci* 2006;**26**:6851–6862.
- Hagiwara M, Pantoni L, Gutierrez JA, et al. Clinical and pathological responses to permanent middle cerebral artery occlusion in the Wistar rat. *Brain Pathol* 2000;**10**:561.
- Abudara V, Jiang RG, Eyzaguirre C. Behavior of junction channels between rat glomus cells during normoxia and hypoxia. *J Neurophysiol* 2002;**88**:639–649.
- Colvin RA, Fontaine CP, Laskowski M, Thomas D. Zn^{2+} transporters and Zn^{2+} homeostasis in neurons. *Eur J Pharmacol* 2003;**479**:171–185.
- Harrison NL, Gibbons SJ. Zn^{2+} : an endogenous modulator of ligand- and voltage-gated ion channels. *Neuropharmacology* 1994;**33**:935–952.
- Peters S, Koh J, Choi DW. Zinc selectively blocks the action of N-methyl-D-aspartate on cortical neurons. *Science* 1987;**236**:589–593.
- Takeda A, Fujii H, Minamino T, Tamano H. Intracellular Zn^{2+} signaling in cognition. *J Neurosci Res* 2014;**92**:819–824.
- Takeda A, Tamano H. Cognitive decline due to excess synaptic Zn^{2+} signaling in the hippocampus. *Front Aging Neurosci* 2014;**6**:26.
- Takeda A, Itagaki K, Ando M, Oku N. Involvement of N-methyl-D-aspartate receptor subunits in zinc-mediated modification of CA1 long-term potentiation in the developing hippocampus. *J Neurosci Res* 2012;**90**:551–558.
- Sawashita J, Takeda A, Okada S. Change of zinc distribution in rat brain with increasing age. *Brain Res Dev Brain Res* 1997;**102**:295–298.
- Takeda A, Ohnuma M, Sawashita J, Okada S. Zinc transport in the rat olfactory system. *Neurosci Lett* 1997;**225**:69–71.
- Takeda A. Zinc signaling in the hippocampus and its relation to pathogenesis of depression. *J Trace Elem Med Biol* 2012;**26**:80–84.
- Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW. The role of zinc in selective neuronal death after transient global cerebral ischemia. *Science* 1996;**272**:1013–1016.

Limitation

Please note that we injected TPEN directly via intracerebroventricular administration by applying stereotaxic apparatus. TPEN has been well recognized as specific Zn^{2+} chelator by previous works [63,64]. Although we did not measure the zinc concentrations in the blood, brain tissue, and its supernatant, we believe TPEN should reach the stroke lesion and produce its effect, as evidenced by the biochemical/functional results obtained. In addition, although we have provided the change of the cerebral infarct size which is one of brain pathological changes, more studies on brain pathological changes such as brain tissue histology might be helpful to clarify the effect of locally applied Zn^{2+} chelating agent TPEN on brain protection.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31272317), the National Basic Research Program of China (2009CB521901) and 111 Project (B08011).

Conflict of Interest

The authors declare no conflict of interest.

39. Zhao YM, Pan R, Li S, et al. Chelating intracellularly accumulated zinc decreased ischemic brain injury through reducing neuronal apoptotic death. *Stroke* 2014;**45**:1139–1147.
40. Speer RE, Karuppagounder SS, Basso M, et al. Hypoxia-inducible factor prolyl hydroxylases as targets for neuroprotection by “antioxidant” metal chelators: from ferroptosis to stroke. *Free Radical Bio Med* 2013;**62**:26–36.
41. Nunez-Figueroa Y, Ramirez-Sanchez J, Hansel G, et al. A novel multi-target ligand (JM-20) protects mitochondrial integrity, inhibits brain excitatory amino acid release and reduces cerebral ischemia injury in vitro and in vivo. *Neuropharmacology* 2014;**85**:517–527.
42. Bartlett TE, Wang YT. The intersections of NMDAR-dependent synaptic plasticity and cell survival. *Neuropharmacology* 2013;**74**:59–68.
43. Deng W, Rosenberg PA, Volpe JJ, Jensen FE. Calcium-permeable AMPA/kainate receptors mediate toxicity and preconditioning by oxygen-glucose deprivation in oligodendrocyte precursors. *Proc Natl Acad Sci USA* 2003;**100**:6801–6806.
44. Kosenko E, Llansola M, Montoliu C, et al. Glutamine synthetase activity and glutamine content in brain: modulation by NMDA receptors and nitric oxide. *Neurochem Int* 2003;**43**:493–499.
45. Zielinska M, Stafiej A, Law RO, Albrecht J. Effects of methionine sulfoximine on the glutamine and glutamate content and cell volume in rat cerebral cortical slices: involvement of mechanisms not related to inhibition of glutamine synthesis. *Neurotoxicology* 2004;**25**:443–449.
46. Kosenko E, Venediktova N, Kaminsky Y, Montoliu C, Felipe V. Sources of oxygen radicals in brain in acute ammonia intoxication in vivo. *Brain Res* 2003;**981**:193–200.
47. Radenovic L, Selakovic V, Janac B, Andjus PR. Neuroprotective efficiency of NMDA receptor blockade in the striatum and CA3 hippocampus after various durations of cerebral ischemia in gerbils. *Acta Physiol Hung* 2011;**98**:32–44.
48. Fu XZ, Zhang QG, Meng FJ, Zhang GY. NMDA receptor-mediated immediate Ser831 phosphorylation of GluR1 through CaMKII alpha in rat hippocampus during early global ischemia. *Neurosci Res* 2004;**48**:85–91.
49. He L, Liu B, Dai Z, et al. Alpha lipoic acid protects heart against myocardial ischemia-reperfusion injury through a mechanism involving aldehyde dehydrogenase 2 activation. *Eur J Pharmacol* 2012;**678**:32–38.
50. Selakovic V, Korenic A, Radenovic L. Spatial and temporal patterns of oxidative stress in the brain of gerbils submitted to different duration of global cerebral ischemia. *Int J Dev Neurosci* 2011;**29**:645–654.
51. Jiang Z, Li C, Arrick DM, Yang S, Baluna AE, Sun H. Role of nitric oxide synthases in early blood-brain barrier disruption following transient focal cerebral ischemia. *PLoS ONE* 2014;**9**:e93134.
52. Bustamante A, Sobrino T, Giralto D, et al. Prognostic value of blood interleukin-6 in the prediction of functional outcome after stroke: a systematic review and meta-analysis. *J Neuroimmunol* 2014;**274**:215–224.
53. Oteiza PI. Zinc and the modulation of redox homeostasis. *Free Radical Bio Med* 2012;**53**:1748–1759.
54. Jang BG, Won SJ, Kim JH, et al. EAAC1 gene deletion alters zinc homeostasis and enhances cortical neuronal injury after transient cerebral ischemia in mice. *J Trace Elem Med Biol* 2012;**26**:85–88.
55. Chen CJ, Liao SL. Neurotrophic and neurotoxic effects of zinc on neonatal cortical neurons. *Neurochem Int* 2003;**42**:471–479.
56. Canzoniero LMT, Manzerra P, Sheline CT, Choi DW. Membrane-permeant chelators can attenuate Zn²⁺-induced cortical neuronal death. *Neuropharmacology* 2003;**45**:420–428.
57. Domercq M, Mato S, Soria FN, Sanchez-Gomez MV, Alberdi E, Matute C. Zn²⁺-induced ERK activation mediates PARP-1-dependent ischemic-reoxygenation damage to oligodendrocytes. *Glia* 2013;**61**:383–393.
58. Frederickson CJ, Giblin LJ, Krezel A, et al. Concentrations of extracellular free zinc (pZn)(e) in the central nervous system of man, rat and rabbit during anesthesia, ischemia and reperfusion. *Exp Neurol* 2006;**202**:523.
59. Doumas M, Faselis C, Kokkinos P. Gender differences in hypertension: myths and reality. *Curr Hypertens Rep* 2013;**15**:321–330.
60. Reckelhoff JF, Zhang H, Srivastava K, et al. Gender differences in hypertension in spontaneously hypertensive rats: role of androgens and androgen receptor. *Hypertension* 1999;**34**:920–923.
61. Reckelhoff JF, Zhang H, Srivastava K. Gender differences in development of hypertension in spontaneously hypertensive rats: role of the renin-angiotensin system. *Hypertension* 2000;**35**:S480–S483.
62. Alkayed NJ, Harukuni I, Kimes AS, et al. Gender-Linked Brain Injury in Experimental Stroke. *Stroke* 1998;**29**:159–166.
63. Radford RJ, Lippard SJ. Chelators for investigating zinc metalloneurochemistry. *Curr Opin Chem Biol* 2013;**17**:129–136.
64. Dietz RM. Zn²⁺ Influx is critical for some forms of spreading depression in brain slices. *J Neurosci* 2008;**28**:8014–8024.