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# Neuronal death/apoptosis induced by intracellular zinc deficiency associated with changes in amino-acid neurotransmitters and glutamate receptor subtypes

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ABSTRACT

In the present study, a model of zinc deficiency was developed by exposing primary neurons to an N,N,N′,N′-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN)-containing medium. The cell survival rate, apoptosis rate, intracellular and extracellular concentrations of 4 amino acids, and the expression of 2 glutamate receptor subtypes α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (GluR2)and *N*-methyl-D-aspartate receptor subtype 2B (NR2B) were evaluated in zinc-deficient cells. The results revealed that zinc deficiency led to a decrease in cell viability and an increase in the apoptosis rate. Additionally, in cultured neurons, zinc deficiency led to an increase in the concentration of aspartic acid (Asp) and a decrease in the concentrations of glutamate (Glu), glycine (Gly), and gamma-aminobutyric acid (GABA). These changes were reversed by concurrent zinc supplementation. Furthermore, zinc deficiency led to an increase in the secreted amounts of Glu, Gly, and Asp but a decrease in secreted amounts of GABA, as measured using the concentrations of these amino acids in the cell-culture medium. These changes were partially reversed by zinc supplementation. Finally, zinc deficiency led to a significant decrease in GluR2 expression and an increase in NR2B expression in cultured neurons, whereas simultaneous treatment with zinc sulfate ($ZnSO_4$) prevented these changes. These results suggest that zinc deficiency-induced neuronal death/apoptosis involves changes in the concentrations of 4 amino acid neurotransmitters and the expression of 2 glutamate receptor subtypes.

## 1. Introduction

Zinc has long been regarded as an important nutrient, and is involved in several biochemical pathways [1]. Zinc is essential for the activity of multiple enzymes involved in cell survival pathways and metabolic homeostasis [2,3]. In addition, some studies have reported that zinc plays an important role in hippocampal neurogenesis in rats [4]. Under normal conditions, a dynamic balance of zinc is maintained throughout the body in multiple ways. Perturbations in zinc homeostasis can cause several physiological disorders and neurological diseases, and result in brain dysfunction. Previous studies have reported that intracellular zinc deficiency induces apoptosis and cell death in cultured neurons [5,6]. The exact mechanism underlying zinc deficiency-induced neuronal apoptosis remains to be elucidated.

Neurotransmitters play an important role in the transmission of signals across chemical synapses. They are released from pre-synaptic neurons and bind to receptors on post-synaptic neurons, resulting in a change in the excitatory state of these neurons [7]. Glutamate, an excitatory neurotransmitter, is released into the synapse [8,9]. Changes in glutamate metabolism can cause the release of large amounts of glutamate, resulting in cellular excitotoxicity and diseases of the nervous system [10]. Glutamate is released from neurons and binds to specific receptors, thus regulating cellular excitability [8]. Ionic glutamate receptors (iGluRs) are important glutamate receptors and include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), *N*-methyl-D-aspartate (NMDA), and kainite receptor subtypes [11,12]. *N*-methyl-D-aspartate (NMDA) receptor subtype 2B (NR2B) is the regulatory subunit of the functional NMDA receptor [13], and glutamate receptor 2 (GluR2) is an important component of the AMPA receptor. Previous studies have reported that impairment of signaling via both the AMPA and NMDA receptors is involved in multiple brain pathologies. Overactivation of NMDA receptors may cause calcium overload within a cell, and this may lead to cytotoxicity and subsequently, to neuropathy [14–16]. Glycine (Gly) and γ-aminobutyric acid (GABA) are the main inhibitory neurotransmitters in the central nervous system, and thus regulate neuronal excitation. Gly triggers the opening of the ionic channels coupled to Gly receptors, which induces hyperpolarization in the postsynaptic neuron and inhibits neuronal excitation

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[17,18]. GABA also inhibits neuronal firing, and has been implicated in the regulation of cognitive functions [19,20]. Reports suggest that in some diseases, cellular apoptosis is closely related to changes in the concentrations of amino acid neurotransmitters [21].

In the present study, we attempted to elucidate the mechanism underlying zinc deficiency-induced neuronal death/apoptosis by evaluating the effects of zinc deficiency and concomitant $Zn^{2+}$ supplementation on the concentration of amino acid neurotransmitters and the expression of glutamate receptors in cultured neurons.

## 2. Materials and methods

### 2.1. Chemicals and animals

4-(2-Hydroxyethyl)-1-Piperazine ethane sulfonic acid (HEPES), L-glutamic acid, and glycine (purity > 99%) were obtained from Genview Scientific Inc. (Tallahassee, USA). Antibiotic solution, poly-L-lysine, γ-aminobutyric acid (purity ≥ 99.0%), and N,N,N′,N′-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) were obtained from Sigma-Aldrich (St. Louis, MO, USA). L-Aspartic acid (purity = 99%) was obtained from KisanBiotech Co. Ltd. (Seoul. Korea). 2,4-Dinitrofluorobenzene(DNFB)was obtained from Yolne Chemical Co. Ltd. (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) was obtained from Amresco (Solon, OH, USA). Dulbecco's Modified Eagle's Medium (DMEM)/nutrient mixture F12 with Glutamax™-1 and B27 supplements were obtained from Gibco (Gran Island, NY, USA). Dimethyl sulfoxide was obtained from Dingguo Changsheng Biotechology Co. Ltd. (Beijing, China). The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit and the fixative used in immunohistochemistry were purchased from Biyuntian Co. (Beijing, China). The GluR2 immunohistochemical assay kit [polyclonal rabbit-anti GluR2 (N-19), donkey anti-rabbit IgG-TR, etc.] and the NR2B immunohistochemical assay kit [polyclonal goat anti-NMDA NR2B (C-20), donkey anti-goat IgG-FITC, etc.] were purchased from Santa Cruz Biotechnology, Inc. (CA, USA).

All animals used for experiments in the present study were obtained from the experimental animal center of the Academy of Military Medical Sciences, and were cared for in accordance with the institutional guidelines for the health and care of experimental animals. The experimental protocols were approved by the Committee on the Ethics of Animal Experiments of Nankai University.

### 2.2. Primary neuronal culture

Primary hippocampal neurons were collected from brains of newborn Wistar rats (approximately 1 day old). The hippocampus was isolated from the brain of each rat and treated with 0.125% trypsin for 20 min at 37 °C, triturated in a solution of DMEM/F12 with Glutamax™-1 and 15% fetal bovine serum, and centrifuged for 15 min. The cells were suspended in DMEM/F12 medium containing Glutamax™-1 and supplemented with 2% B27, and 1% antibiotic solution. The cells were plated onto cover glasses pre-coated with poly-L-lysine (0.1 mg/mL) in multiwall cell-culture plates at a density of 1.0–5.0 × $10^5$ cells/$cm^2$. Cells were cultured in a humidified incubator (Sanyo, Japan) at 37 °C with 5% $CO_2$ for 7 days, following which they subjected to treatment and further experiments. The medium was changed twice a week by replacing half the volume of culture medium with serum-free DMEM/F12 medium with Glutamax™-1.

### 2.3. Experimental groups and TPEN treatment

There were 3 experimental groups in the present study: the control group, the TPEN exposure group, and the TPEN exposure plus $ZnSO_4$ treatment (TPEN + $ZnSO_4$) group. Treatment lasted for 24 h. The model of zinc deficiency in primary neurons was established by adding 5 μM of TPEN to the culture medium. To assess whether $Zn^{2+}$ supplementation can rescue the effects of zinc deficiency, in the TPEN + $ZnSO_4$ condition, 5 μM of TPEN and 10 μM of $ZnSO_4$ were added to the culture medium.

### 2.4. Measurement of cell viability using the MTT assay

Viability of the cultured primary hippocampal neurons was determined using an MTT assay, which measures the activity of mitochondrial succinate dehydrogenase within viable cells by the reduction of exogenous MTT to insoluble purple formazan crystals. Neurons were plated in 96-well plates at a density of 1 × $10^5$ cells/mL. The volume of medium added to each well was 200 mL. For the assay, MTT solution was added to each well. The final concentration of the MTT solution was 1 mg/mL. Neurons were incubated in this solution at 37 °C for 4 h, following which the medium was discarded and the formazan crystals were dissolved in 150 μL of DMSO. The absorbance of this solution at 570 nm was measured using a Beauty Diagnostic Microplate Reader (Molecular Devices, Sunnyvale, California, USA). The results were expressed in terms of percentage of the absorbance value obtained from the control group.

### 2.5. Measurement of cell apoptosis rate using the TUNEL assay

Neurons, at a density of 1 × $10^5$ cells/mL, were grown on six-chamber well slides pre-coated with poly-L-lysine (PLL). After 24 h of treatment, cells were washed thrice with phosphate buffered saline (PBS), following which 2 mL of TUNEL assay solution was added to each well. The cells were then fixed for 30 min, and washed thrice with PBS, then permeabilized with 0.1% Triton X-100 PBS on an ice bath. According to the manufacturer's instructions, cells processed for TUNEL staining were co-stained with 4, 6-diamidino-2-phenylindole (DAPI) for 2 min. The number of TUNEL-positive neurons and the total number of neurons were counted. The percentage of TUNEL-positive cells was calculated and averaged. The results were expressed in terms of the percentage of TUNEL-positive cells.

### 2.6. Determination of amino acid concentrations using high-performance liquid chromatography (HPLC)

The concentrations of 4 free amino acid neurotransmitters (Glu, Asp, Gly, and GABA) were measured in primary neurons and the medium they were cultured in using a CoM6000 HPLC System consisting of two 6000 LDS pumps, a 6000 UV–Vis detector (360 nm), and a Comatex C18 (5 μm, 250 mm × 4.6 mm pore size) (CoMetro Technology, USA).

Neurons were cultured at a density of 1.12 × $10^5$ cells/$cm^2$. After treatment with the respective chemical agents, culture medium was separately collected from neurons of all three experimental groups and centrifuged for 10 min at 1000 rpm to remove any remaining cells. Subsequently, acetonitrile was added to the supernatant (1:1, v:v) in order to precipitate proteins, which were separated out after centrifugation at 14,000 rpm for 20 min at 4 °C. The same process was carried out using blank culture media (culture media that was not used to grow any cells). To obtain amino acids from cultured neurons, 100 μL RIPA lysis buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% NP-40) was added to fully lyse the cells. A 5-μL sample of the lysis solution was used for protein quantification using a bicinchoninic acid-protein quantification assay kit by following the manufacturer's instructions. Subsequently, 80 μL of acetonitrile was added to 80 μL of the lysis solution for protein precipitation, and the mixture was centrifuged at 14,000 rpm for 20 min at 4 °C. The sample solutions were derivatized by the addition of 0.1 M carbonate buffer and 2,4-dinitrochlorobenzene (DNCB)-acetonitrile (1:1000, v/v) followed by incubation in a 60 °C water-bath for 60 min in the dark. The derivatization reaction was stopped by the addition of mobile phase B. The mobile phase consisted