Folia Neuropathologica

# Apoptotic neuronal changes enhanced by zinc chelator - TPEN in organotypic rat hippocampal cultures exposed to anoxia

**Ewa Nagańska, Ewa Matyja**

Department of Clinical and Experimental Neuropathology, Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

*Folia Neuropathol 2006; 44 (2): 125-132*

**Abstract**

*Both, the neurotoxic and neuroprotective effects of zinc have been well established, but the exact mechanism of its dual abilities still remains unclear. It has been shown that zinc deficiency leads to progressive neuronal injury. Therefore a safe zinc concentration levels seem to be necessary in neuronal protection from different noxious factors.*
*This study was undertaken to determine the effect of zinc chelating agent – TPEN on neuronal morphological changes in organotypic hippocampal culture and its effect on post-anoxic changes in this model. The study evidenced that exposition to 15 μM of TPEN induced various stages of apoptotic changes in hippocampal pyramidal neurons and enhanced the anoxia-induced neuronal apoptosis in this model. These results confirmed the hypothesis that manipulations of intracellular pool of zinc by zinc-chelating agents may be a cause of both induction and prevention of apoptotic cell death in various pathological conditions.*

***Key words:*** *TPEN, apoptosis, hippocampus in vitro.*

## Introduction

Zinc is one of the well known neuromodulatory agents [39,40,44]. After exposition to different injuring factors zinc accumulates especially in degenerating neurones of CA1 hippocampal subfield. It has been documented that transient ischemia/hypoxia may induce the increase of extracellular zinc concentration accompanied by over-expression of zinc transporter ZnT-1 gene [19,43]. Zinc may play a casual role in various forms of apoptosis and its accumulation has been demonstrated in central neurons undergoing apoptosis during development [21]. Zinc chelating agents are thought to be responsible for decrease of neurotoxic properties of zinc [4,8].

Our previous ultrastructural studies showed the neuroprotective effect of zinc on apoptotic cell death in a model of anoxia *in vitro* [28].

The aim of this study was the evaluation of the effect of zinc-chelator - TPEN on the course of morphological changes in the model of organotypic hippocampal culture exposed to anoxia to answer the question if intracellular zinc deficiency could potentiate postanoxic neuronal injuries.

## Material and methods

The experiments were performed on organotypic hippocampal cultures prepared from 2- to 3-day-old Wistar rats. In sterile conditions the hippocampi were

**Communicating author:**
Ewa Naganska, MD PhD, Department of Clinical and Experimental Neuropathology Medical Research Centre, Polish Academy of Sciences, Pawinskiego 5 St, 02-105 Warsaw, Poland; tel. +48 22 668 53 69; fax +48 22 668 55 32, e-mail: enaganska@yahoo.com

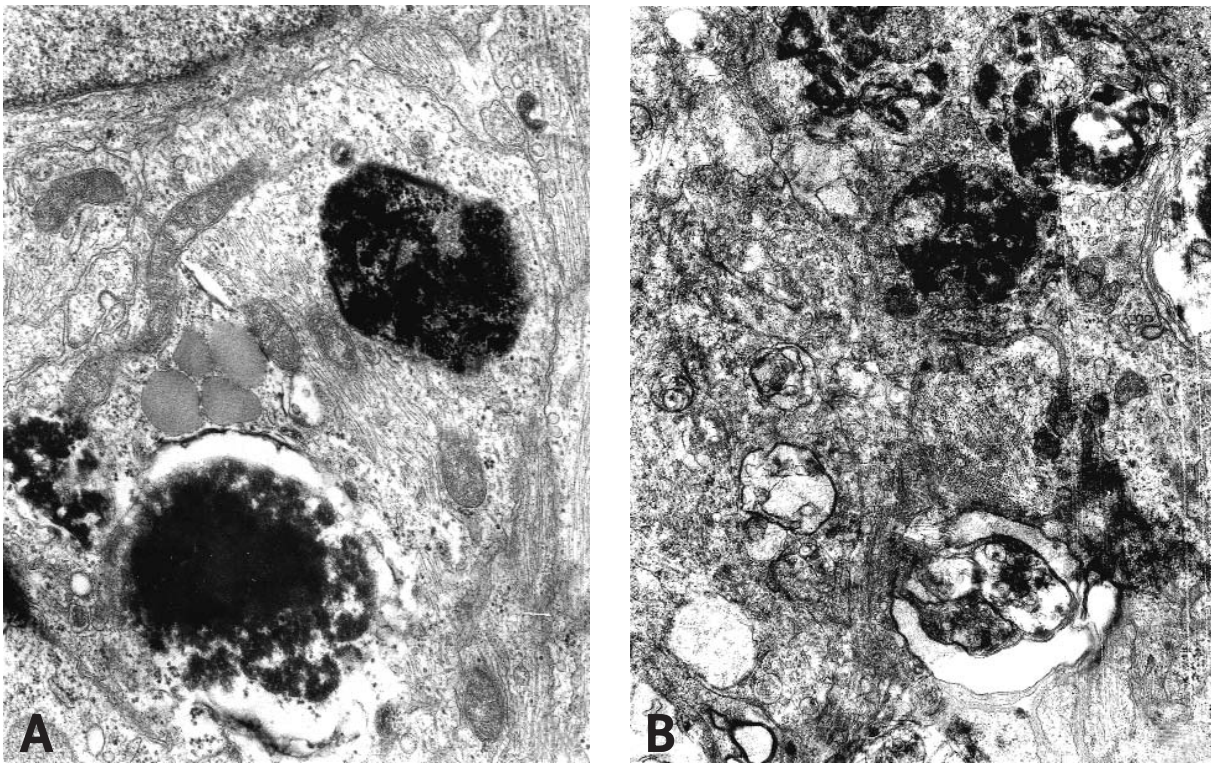
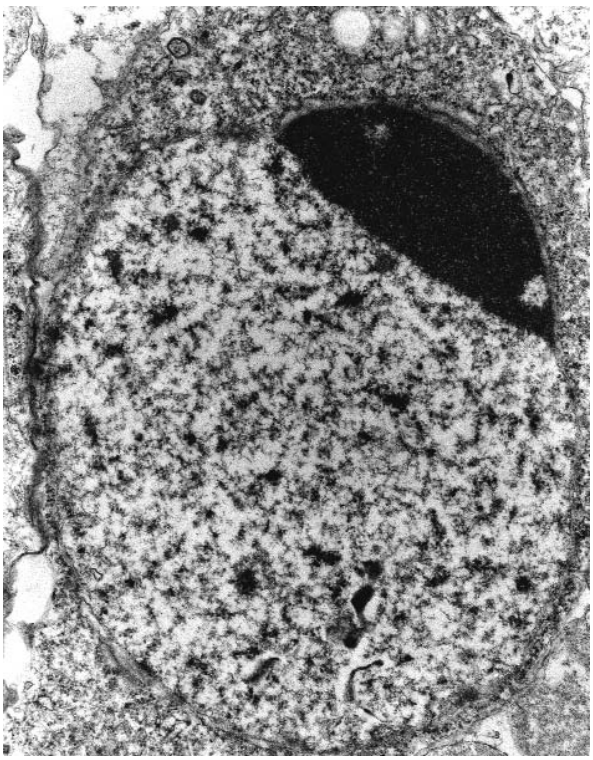



**Fig. 1.** Hippocampal culture, 24 hours after exposition to 15 µM TPEN. Apoptotic bodies containing fragments of condensed chromatin and cytoorganelles. x 18 750



dissected out from both cerebral hemispheres, placed in dishes containing Eagle Minimal Essential Medium (MEM) and cut coronally into thin slices. The explants were placed on collagen-coated cover glasses with 2 drops of nutrient medium and sealed into the Maximow chambers. The cultures were kept at 36.6°C in a medium consisting of 20% inactivated foetal bovine serum and 80% of MEM, supplemented with glucose to a final concentration of 600 mg%, with antibiotics. The medium was renewed twice a week. On the 14-18 day *in vitro* the well differentiated and sensitive to anoxic injury cultures were divided into the following experimental groups: 1. cultures exposed to TPEN (*N,N,N'N'*-tetrakis-(2-pyridylmethyl) ethylene-diamine) in concentration of 15 µM; 2. cultures exposed to 20-minutes anoxia in a pure nitrogen atmosphere in flasks adapted for permanent gas flow;

**Fig. 2.** Hippocampal culture, 5 days after exposition to 15 µM TPEN. Pyramidal neuron with characteristic apoptotic form of condensed chromatin in the close proximity to nuclear membrane, so-called "half--moon". x 18 750

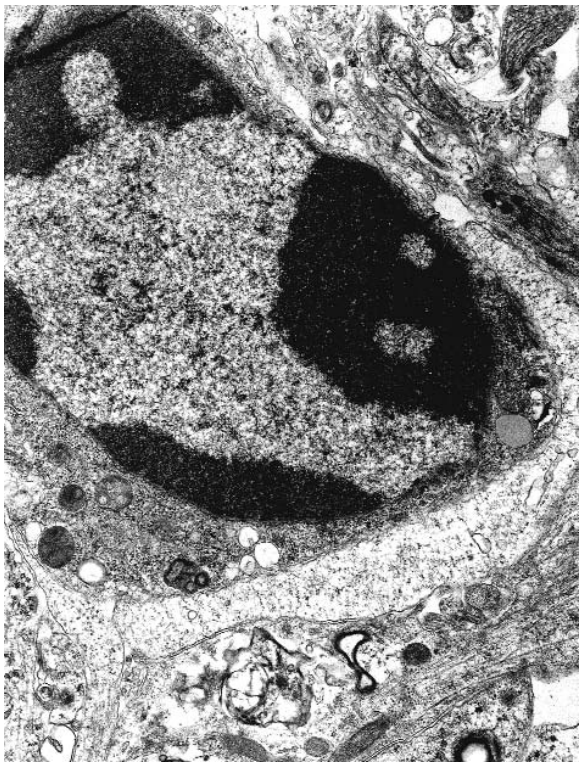


**Fig. 3.** Hippocampal culture, 5 days after exposition to 15 μM TPEN. Neuronal cell with nucleus containing aggregations of chromatin under nuclear membrane. x 25 000

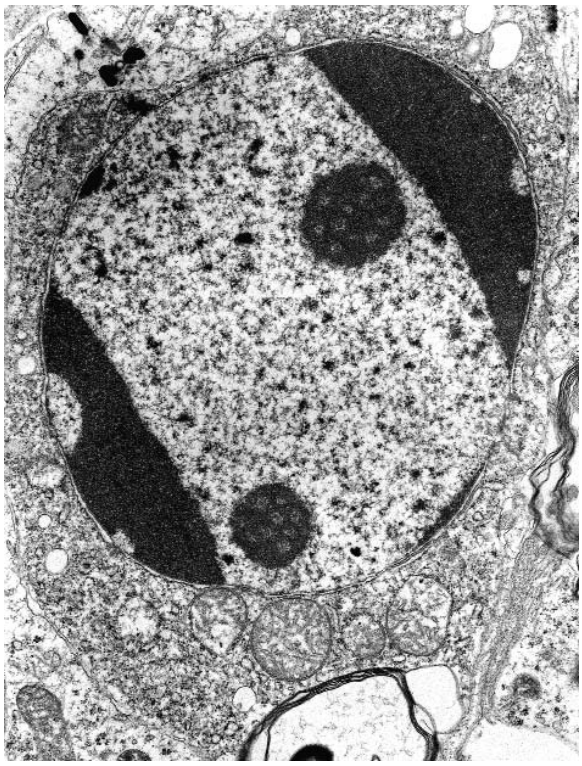


**Fig. 4.** Hippocampal culture, 5 days after exposition to 15 μM TPEN. Neuronal cell with nucleus containing aggregations of chromatin in the form of "cups" characteristic of apoptosis. x 18 750

3. cultures exposed to 20-minutes anoxia, pretreated with TPEN (15μM); 4. control cultures grown in standard conditions. After 30 minutes, 2 and 24 hours, 3 and 5 days the cultures from experimental and control groups were processed for electron microscopy. They were rinsed in cacodylate buffer, pH 7.2, fixed in a mixture containing 0.8% formaldehyde and 2.5% glutaraldehyde for 1 hour, postfixed in 1% osmium tetroxide, dehydrated in alcohols in graded concentrations, and embedded in Epon 812. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined under a JEOL XB 1500 electron microscope.

## Results

### The effect of TPEN on ultrastructural features in organotypic hippocampal cultures

Exposure to TPEN in 15 μM concentration led to progressive ultrastructural changes in the structure of both nucleus and cytoorganelles of piramidal neurones. After 30 minutes and 2 hours of the experiment a slight vacuolization of cytoplasm and swelling of mitochondria were observed. Numerous neurones showed extensive changes within the mitochondrial matrix with loss of mitochondrial cristae. After 24 hours following the exposure, the pyramidal neurones displayed dilatation of Golgi apparatus channels and extensive vacuolization of cytoplasm, whereas the nucleus maintained its normal appearance. Some massively damaged cells, presenting morphological criteria of necrosis and/or apoptosis were noticed. There were also neurones exhibiting typical apoptotic features with condensed cytoplasm containing numerous well preserved cytoorganelles. Numerous apoptotic bodies were seen (Fig. 1).

The most prominent apoptotic neuronal changes were observed after 5 days following the exposure to TPEN. A lot of pyramidal neurones showed the aggregation of chromatin close to the nuclear membrane, often in the form of so-called "half-moon" (Fig. 2). Frequently, the condensed chromatin formed numerous aggregations under the nuclear membrane

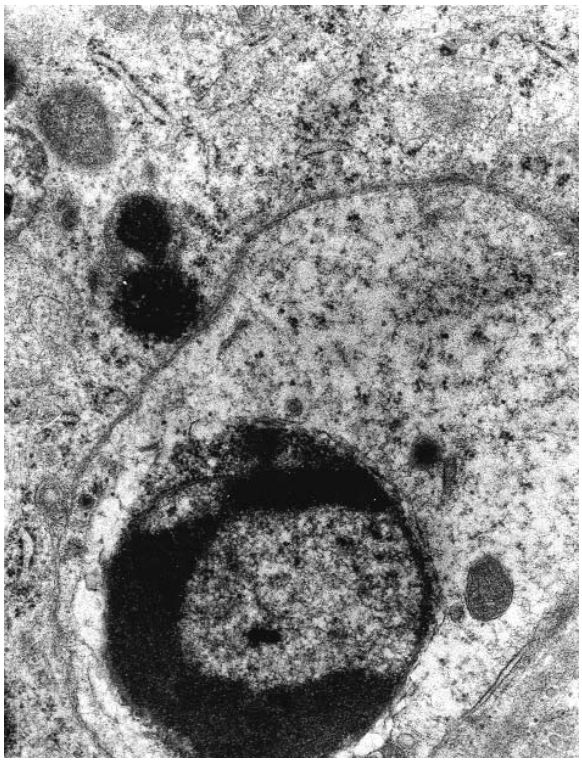


**Fig. 5.** Hippocampal culture, 5 days after exposition to 15 µM TPEN. Nucleus containing condensed chromatin and lack of the nuclear membrane integrity. x 18.750

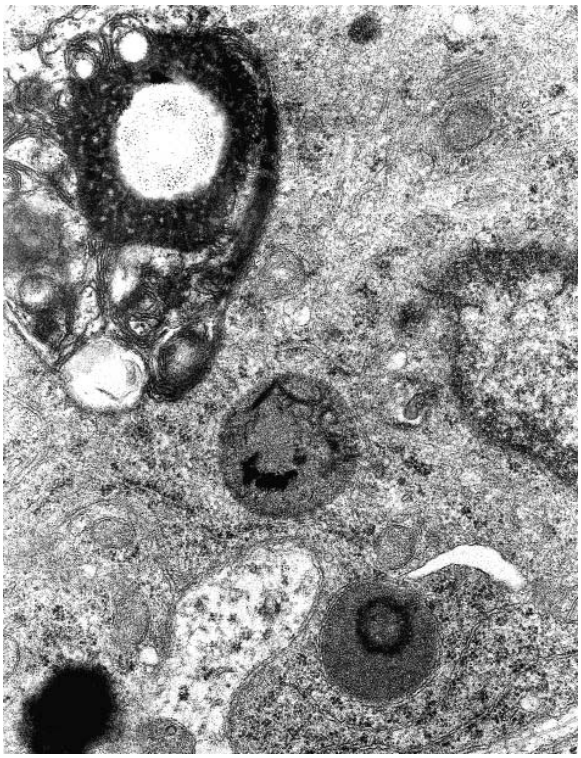
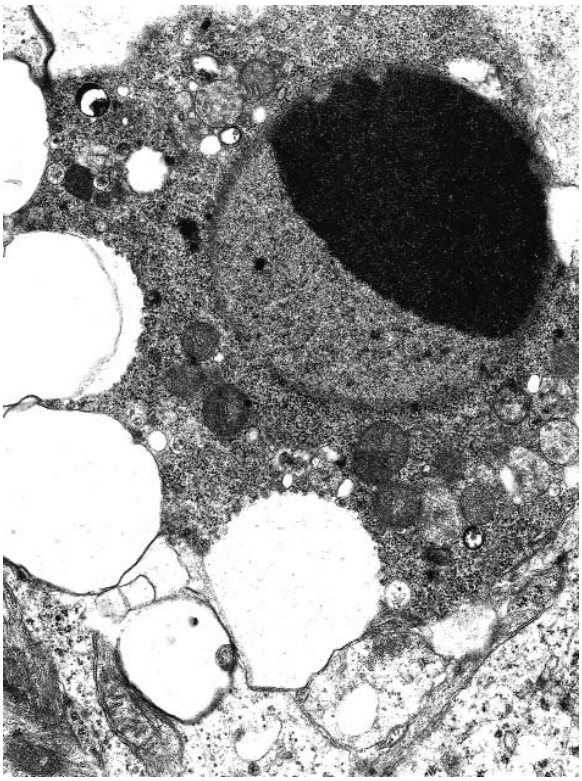


**Fig. 6.** Hippocampal culture, 5 days after exposition to 15 µM TPEN. Apoptotic body containing chromatin clumps and fragments of cytoplasm with destructed cytoorganelles. x 18 750



(Fig. 3) or "cups", typical of apoptosis (Fig. 4). Some cells, displaying marked condensation of cytoplasm and aggregation of nuclear chromatin, lacked the nuclear membrane integrity (Fig. 5). A large number of apoptotic bodies containing chromatin clumps and fragments of cytoplasm with destructed cytoorganelles were frequently observed (Fig. 6).

## The effect of TPEN on the development of post-anoxic morphological changes in organotypic rat hippocampal culture

Cultures exposed to 20-minutes anoxia but pretreated with TPEN in concentration of 15 µM showed a large number of cells with morphological features of both necrosis and apoptosis. A set of cells exhibited electron-dense cytoplasm with damaged organelles and disrupted cell membranes. A large

**Fig. 7.** Hippocampal culture, 24 hours after exposition to 15 µM TPEN and 20-minutes anoxia. Neuronal cell containing nucleus with characteristic apoptotic form of condensed chromatin. x 25 000

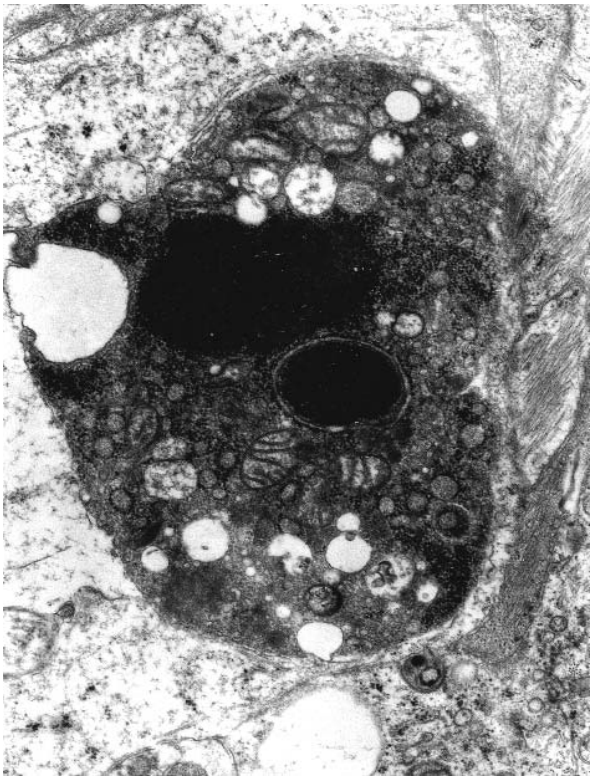


**Fig. 8.** Hippocampal culture, 24 hours after exposition to 15 μM TPEN and 20-minutes anoxia. Neuronal cell with electron-dense cytoplasm containing condensed masses of chromatin and more or less damaged cytoorganelles. x 30 000

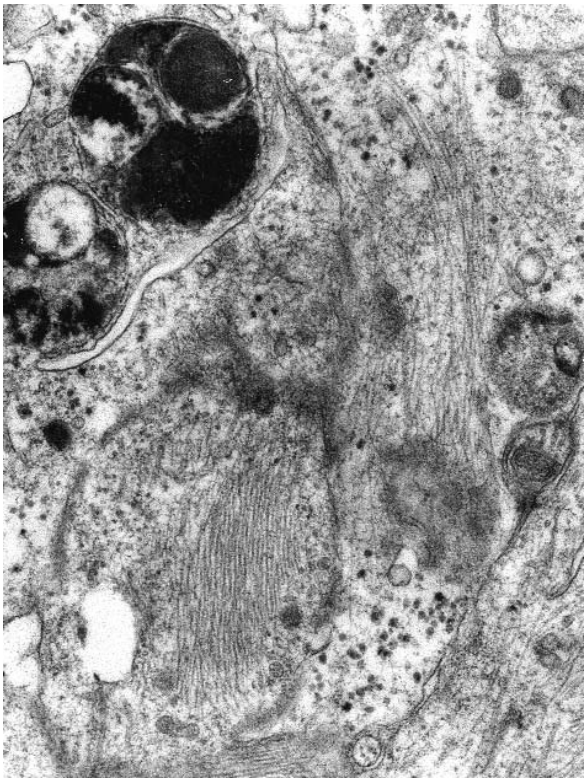


**Fig. 9.** Hippocampal culture, 24 hours after exposition to 15 μM TPEN and 20-minutes anoxia. Apoptotic bodies with fragments of condensed chromatin and damaged cytoorganelles. x 50 000

number of cells revealed typical apoptotic changes, especially characterisic condensation of nuclear chromatin (Fig. 7, 8). The ongoing apoptotic process was confirmed by the presence of numerous apoptotic bodies (Fig. 9). Some cells exhibited the ultrastructural features typical of both necrosis and apoptosis i.e. destruction of cytoorganelles and clumps of condensed nuclear chromatin, reflecting so-called "apoptotic-necrotic" continuum (Fig. 10). After 5 days of observation the hippocampal cultures displayed advanced morphological changes of neuronal cells including severe vacuolisation of cytoplasm, destruction of cytoorganelles and massive condensation of nuclear chromatin with only partial preservation of the nuclear membrane (Fig. 11).

## Discussion

Zinc is one of the trace elements playing an important role in the maintenance of structural and functional integrity of cells and tissues. Zinc in micromolar concentrations is necessary to maintain proper functioning of many enzymes, transcription factors and structural proteins [39,44]. The central nervous system, as well as other tissues, contains significant amounts of zinc [11]. The increasing evidence confirms the crucial role of zinc in many physiological processes but on the other hand, zinc seems to be a very important factor in the pathogenesis of different neurodegenerative diseases [20,37]. Neuroprotective and neurotoxic effects of zinc have been established in different experimental models [10,20,34]. Zinc is thought to be an endogenous modulator of synaptic activating transmitter – glutamate (GLU) through NMDA, AMPA and metabotropic glutamatergic receptors [7,13,18,31,47]. The complex effect of $Zn^{2+}$ on many metabolic processes suggests that zinc may play a modulating role in neurodegenerative processes [7,17,26,41,45,46,48,49]. Swelling of mitochondria is one of the most prominent ultrastructural changes resulting from

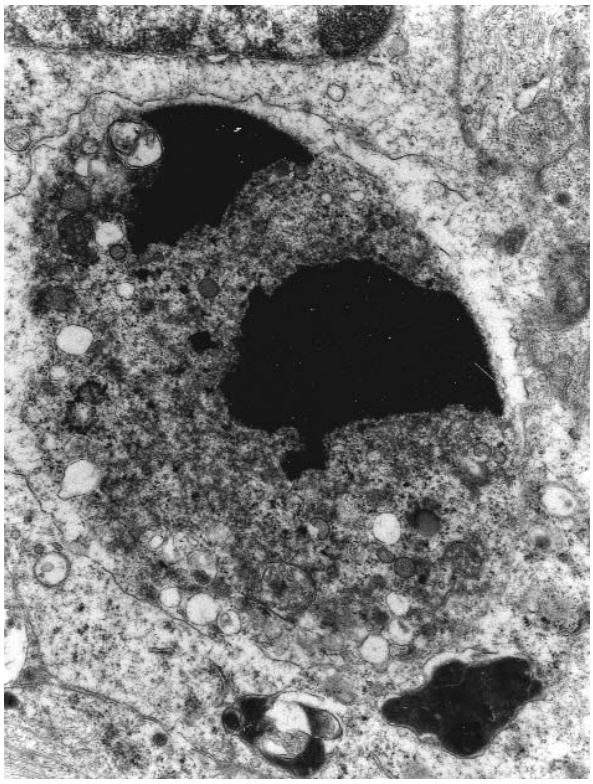


**Fig. 10.** Hippocampal culture, 24 hours after exposition to 15 μM TPEN and 20-minutes anoxia. Massively damaged neuronal cell with completely disrupted cytoorganelles and masses of condensed chromatin. x 25 000

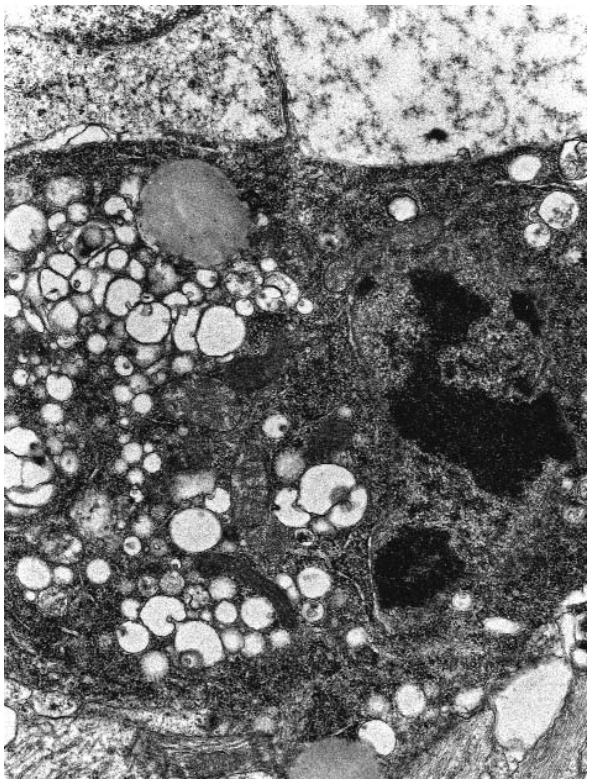


**Fig. 11.** Hippocampal culture, 5 days after exposition to 15 μM TPEN and 20-minutes anoxia. Fragment of neuronal cell with marked vacuolisation of cytoplasm, lipid drops and clumps of condensed nuclear chromatin. x 25 000

postanoxic overaccumulation of zinc in postsynaptic neurones [23]. It probably follows permeability transition pore in the mitochondrial membrane [15]. Recent data shows that zinc ions from synaptic vesicles, and also of intramitochondrial origin, play an important role in pathogenesis of these changes [35,36]. Some authors point out that cellular changes resulting from the neurotoxic effect of zinc exhibit both necrotic and apoptotic features [12,16].

The reduction of zinc pool by chelating agents in physiological conditions might lead to substantial disturbances in intracellular biochemical reactions. Depletion of zinc intracellular concentration turned out to be crucial in loss of cell defence against injuring factors [32].

The present ultrastructural study demonstrated the toxic effect of zinc-chelating agent - TPEN on the pyramidal rat hippocampal neurones *in vitro*. The pyramidal neurones showed characteristic sequence of morphological changes typical of apoptosis, especially after 5 days since exposition. Pyramidal neurones exhibited morphological features of both early apoptotic changes with a characteristic pattern of chromatin clumping and late stages of apoptosis with formation of typical apoptotic bodies. The toxic effect of TPEN was enhanced by exposition to 20-minutes anoxia. It is consistent with our previous ultrastructural studies based on a model of anoxia *in vitro* which had evidenced the protective effect of $ZnCl_2$ on development of late postanoxic changes connected with apoptosis [28]. The neuroprotective effect of zinc is probably connected with inhibition of NMDA receptors. The regulatory role of zinc in the process of apoptotic cell death was the subject of different experimental models [2,3,22,23,28,33,51]. In physiological conditions endogenous zinc plays an inhibiting role of apoptosis [30,50], probably by inhibition of the endonucleases activity responsible for DNA degradation and by interactions with transcription factors and kinases or by its antioxida-

tive properties [25]. Some authors emphasise the main inhibitive effect of zinc on caspase-3 [5,6,24,30,38]. On the other hand, zinc seems to have a modulatory effect on the apoptotic process by increasing the permeability of mitochondrial megachannels and causing the cascade of caspase reactions [15,42].

It has been previously documented that TPEN causes removal of zinc from zinc-dependent transcription factors. TPEN is thought to be a potentially efficient agent which prevents neuronal death due to a decrease of toxic concentrations of zinc [8,9]. However, the reduction of zinc pool by chelating agents in physiological conditions may lead to substantial disturbances in intracellular biochemical reactions.

Extensive decrease of zinc concentration leads to activation of apoptosis in different cells including neurones [1,27,29]. The exact mechanism of this effect remains unclear, but typical apoptotic changes have been observed in neurones in different experimental models, both *in vivo* [4,8] and *in vitro* [1,5,14,33]. The present study supports the opinion that the instability in intracellular zinc concentration may result in abnormality in cell death control in various pathological processes.

## References


1. Ahn YH, Kim YH, Hong SH, Koh JY. Depletion of intracellular zinc induces protein synthesis-dependent neuronal apoptosis in mouse cortical culture. Exp Neurol 1998; 154: 47-56.
2. Ahn YH, Koh JY, Hong SH. Protein synthesis-dependent but Bcl-2-independent cytochrome C release in zinc depletion-induced neuronal apoptosis. J Neurosci Res 2000; 61: 508-514.
3. Aizenman E, Stout AK, Hartnett KA, Dineley KE, McLaughlin BA, Reynolds IJ. Induction of neuronal apoptosis by thiol oxidation: putative role of intracellular zinc release. J Neurochem 2000; 75: 1878-1888.
4. Armstrong C, Leong W, Lees GJ. Comparative effects of metal chelating agents on the neuronal cytotoxicity induced by copper (Cu2+), iron (Fe3+) and zinc in the hippocampus. Brain Res 2001; 892: 51-62.
5. Chai F, Truong-Tran AQ, Ho LH, Zalewski PD. Regulation of caspase activation and apoptosis by cellular zinc fluxes and zinc deprivation: A review. Immunol Cell Biol 1999; 77:272-278.
6. Chimienti F, Seve M, Richard S, Mathieu J, Favier A. Role of cellular zinc in programmed cell death: temporal relationship between zinc depletion, activation of caspases, and cleavage of Sp family transcription factors. Biochem Pharmacol 2001; 62: 51-62.
7. Christine CW, Choi DW. Effect of zinc on NMDA receptor-mediated channel currents in cortical neurons. J Neurosci 1990; 10: 108-116.
8. Cuajungco MP, Lees GJ. Prevention of zinc neurotoxicity in vivo by N,N,N',N' - tetrakis (2-pyridylmethyl) ethylene-diamine (TPEN). Neuroreport 1996; 7: 1301-1304.
9. Cuajungco MP, Lees GJ. Diverse effects of metal chelating agents on the neuronal cytotoxicity of zinc in the hippocampus. Brain Res 1998; 13: 118-129.
10. Dineley KE, Votyakova TV, Reynolds IJ. Zinc inhibition of cellular energy production: implications for mitochondria and neurodegeneration. J Neurochem 2003; 85: 563-570.
11. Frederickson CJ, Moncrieff DW. Zinc-containing neurons. Biol Signals 1994; 3: 127-139.
12. Gwag BJ, Koh JY, DeMaro JA, Ying HS, Jacquin M, Choi DW. Slowly triggered excitotoxicity occurs by necrosis in cortical cultures. Neuroscience 1997; 77: 393-401.
13. Huang EP. Metal ions and synaptic transmission: think zinc. Proc Natl Acad Sci U S A 1997; 94: 13386-13387. Review.
14. Hyun HJ, Sohn JH, Ha DW, Ahn YH, Koh JY, Yoon YH. Depletion of intracellular zinc and copper with TPEN results in apoptosis of cultured human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2001; 42: 460-465.
15. Jiang D, Sullivan PG, Sensi SL, Steward O, Weiss JH. Zn(2+) induces permeability transition pore opening and release of pro-apoptotic peptides from neuronal mitochondria. J Biol Chem 2001; 14: 47524-47529.
16. Kim YH, Kim EY, Gwag BJ, Sohn S, Koh JY. Zinc-induced cortical neuronal death with features of apoptosis and necrosis: mediation by free radicals. Neuroscience 1999; 89: 175-182.
17. Koh JY, Choi DW. Zinc alters excitatory amino acid neurotoxicity on cortical neurons. J Neurosci 1988; 8: 2164-2171.
18. Koh JY, Choi DW. Zinc toxicity on cultured cortical neurons: involvement of N-methyl-D-aspartate receptors. Neuroscience 1994; 60: 1049-1057.
19. Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW. The role of zinc in selective euronal death after transient global cerebral ischemia. Science 1996; 272: 1013-1016.
20. Koh JY. Zinc and disease of the brain. Mol Neurobiol 2001; 24: 99-106.
21. Lee JY, Hwang JJ, Park MH, Koh JY. Cytosolic labile zinc: a marker for apoptosis in the developing rat brain. Eur J Neurosci 2006; 23: 435-442.
22. Lobner D, Canzoniero LM, Manzerra P. Zinc-induced neuronal death in cortical neurons. Cell Mol Biol 2000; 46: 797-806.
23. Manev H, Kharlamov E, Uz T, Mason RP, Cagnoli CM. Characterization of zinc-induced neuronal death in primary cultures of rat cerebellar granule cells. Exp Neurol 1997; 146: 171-178.
24. Marini M, Frabetti F, Canaider S, Dini L, Falcieri E, Poirier GG. Modulation of caspase-3 activity by zinc ions and by the cell redox state. Exp Cell Res 2001; 266: 323-332.
25. Marini M, Musiani D. Micromolar zinc affects endonucleolytic activity in hydrogen peroxide-mediated apoptosis. Exp Cell Res 1998; 239: 393-398.
26. Mayer ML, Vyklicky L Jr, Westbrook GL. Modulation of excitatory amino acid receptors by group IIB metal cations in cultured mouse hippocampal neurones. J Physiol 1989; 415: 329-350.
27. McCabe MJ Jr, Jiang SA, Orrenius S. Chelation of intracellular zinc triggers apoptosis in mature thymocytes. Lab Invest 1993; 69: 101-110.

28. Naganska E, Matyja E. The protective effect of ZnCl2 pretreatment on the development of postanoxic neuronal damage in organotypic rat hippocampal cultures. Ultrastruct Pathol 2002; 26: 383-391.
29. Nakatani T, Tawaramoto M, Opare Kennedy D, Kojima A, Matsui-Yuasa I. Apoptosis induced by chelation of intracellular zinc is associated with depletion of cellular reduced glutathione level in rat hepatocytes. Chem Biol Interact 2000; 125: 151-163.
30. Perry DK, Smyth MJ, Stennicke HR, Salvesen GS, Duriez P, Poirier GG, Hannun YA. Zinc is a potent inhibitor of the apoptotic protease, caspase-3. A novel target for zinc in the inhibition of apoptosis. J Biol Chem 1997; 272: 18530-18533.
31. Peters S, Koh J, Choi DW. Zinc selectively blocks the action of N-methyl-D-aspartate on cortical neurons. Science 1987; 236: 589-593.
32. Rudolf E, Cervinka M, Cerman J. Zinc has ambiguous effects on chromium (VI)-induced oxidative stress and apoptosis. J Trace Elem Med Biol 2005; 18: 251-260.
33. Sakabe I, Paul S, Dansithong W, Shinozawa T. Induction of apoptosis in Neuro-2A cells by Zn2+ chelating. Cell Struct Funct 1998; 23: 95-99.
34. Sensi SL, Yin HZ, Weiss JH. AMPA/kainate receptor-triggered Zn2+ entry into cortical neurons induces mitochondrial Zn2+ uptake and persistent mitochondrial dysfunction. Eur J Neurosci 2000; 12: 3813-3818.
35. Sensi SL. Zn2+, mitochondria and neuronal injury. J Neurochem 2003a; 85 Suppl 2: 10.
36. Sensi SL, Ton-That D, Sullivan PG, Jonas EA, Gee KR, Kaczmarek LK, Weiss JH. Modulation of mitochondrial function by endogenous Zn2+ pools. Proc Natl Acad Sci U S A 2003b; 100: 6157-6162.
37. Shaw CE, al-Chalabi A, Leigh N. Progress in the pathogenesis of amyotrophic lateral sclerosis. Curr Neurol Neurosci Rep 2001; 1: 69-76.
38. Sugawara T, Noshita N, Lewen A, Gasche Y, Ferrand-Drake M, Fujimura M, Morita-Fujimura Y, Chan PH. Overexpression of copper/zinc superoxide dismutase in transgenic rats protects vulnerable neurons against ischemic damage by blocking the mitochondrial pathway of caspase activation. J Neurosci 2002; 22: 209-217.
39. Takeda A. Movement of zinc and its functional significance in the brain. Brain Res Brain Res Rev 2000; 34: 137-148.
40. Takeda A. Zinc homeostasis and functions of zinc in the brain. Biometals 2001; 14: 343-351.
41. Terse PS, Komiskey HL. Modulation of a competitive N-methyl-D-aspartate receptor antagonist binding by zinc oxide. Brain Res 1997; 744: 347-350.
42. Truong-Tran AQ, Carter J, Ruffin RE, Zalewski PD. The role of zinc in caspase activation and apoptotic cell death. Biometals 2001; 14: 315-330.
43. Tsuda M, Imaizumi K, Katayama T, Kitagawa K, Wanaka A, Tohyama M, Takagi T. Expression of zinc transporter gene, ZnT-1, is induced after transient forebrain ischemia in the gerbil. J Neurosci 1997; 17: 6678-6684.
44. Vallee BL, Falchuk KH. The biochemical basis of zinc physiology. Physiol Rev 1993; 73: 79-118.
45. Vogt K, Mellor J, Tong G, Nicoll R. The actions of synaptically released zinc at hippocampal mossy fiber synapses. Neuron 2000; 26: 187-196.
46. Weiss JH, Hartley DM, Koh JY, Choi DW. AMPA receptor activation potentiates zinc neurotoxicity. Neuron 1993; 10: 43-49.
47. Xie X, Gerber U, Gähwiler BH, Smart TG. Interaction of zinc ionotropic and metabotropic glutamate receptors in rat hippocampal slices. Neurosci Lett 1993; 159: 46-50.
48. Yeh GC, Bonhaus DW, McNamara JO. Evidence that zinc inhibits N-methyl-D-aspartate receptor-gated ion channel activation by noncompetitive antagonism of glycine binding. Mol Pharmacol 1990; 38: 14-19.
49. Yokoyama M, Koh J, Choi DW. Brief exposure to zinc is toxic to cortical neurons. Neurosci Lett 1986; 71: 351-355.
50. Zalewski PD, Forbes IJ, Giannakis C. Physiological role for zinc in prevention of apoptosis (gene-directed death). Biochem Int 1991; 24: 1093-1101.
51. Zalewski PD, Forbes IJ, Betts WH. Correlation of apoptosis with change in intracellular labile Zn(II) using zinquin [(2-methyl-8-p-toluenesulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent probe for Zn(II). Biochem J 1993; 296: 403-408.