ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Diabetes mellitus type 1 induces dark neuron formation in the dentate gyrus: a study by Gallyas' method and transmission electron microscopy

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Abstract

Background: Diabetes mellitus type 1 is a chronic endogenous stressor. We investigated the effects of a diabetes mellitus type 1 on dark neuron formation in granular layer of dentate gyrus. *Materials and Methods*: Diabetes was induced by a single intraperitoneal (IP) injection of streptozotocin (STZ) at a dose of 60 mg/kg dissolved in saline. Control animals were received only saline. In the end of eight weeks, the brains were removed and hippocampi studied by Gallyas' method and transmission electron microscopy. *Results*: The comparison between the rate of dark neurons in diabetic group (223±25) and of control (5.75±4.34) showed significant level of difference ($p<0.05$). Ultrastructurally dark neurons showed apoptotic death criteria namely: dark and electron dense appearance, chromatin condensation, margination and clumping. *Conclusions*: Present results suggest that STZ-induced diabetes accelerates dark neuron formation with apoptotic criteria in granule layer of dentate gyrus.

Keywords: granule cell, dentate gyrus, dark neuron, diabetes, rat, streptozotocin.

Introduction

The mode of death and the pathogenesis of "dark" neurons have not been fully understood, because they occurred in various pathological conditions and the causes of formation remain unresolved [1, 2]. Dark neurons have been reported in excitotoxic conditions like ischemia, epilepsy, spreading depression phenomena (SD) and hypoglycemia [3]. On the other hand, dark neuron formation has been reported in stress full condition such as acute physical stress [4] and normal ageing process in cerebellum [5]. All of these pathologic conditions cause disturbance in ion gradient, increased excitatory neurotransmitters like glutamate and free radical generation [5, 6]. Diabetes mellitus (DM) is a chronic endogenous stressor that is associated with increased oxidative stress in central nervous system in particular hippocampus [7, 8]. Dentate gyrus (DG) is a part of hippocampal formation that plays an important role in memory and learning in animals and human. Studies have shown DM suppresses neuronal proliferation and enhances neuronal death that collectively resulting to memory impairment [9, 10]. Although hyperglycemia causes increased oxidative stress and extracellular level of glutamate in hippocampus, but there is little information about the effect(s) of a chronic endogenous stressor like diabetes type 1 on dark neuron formation in DG granule cells. Thus, we aimed to study effect(s) of streptozotocin-induced diabetes, as the known experimental model of type 1 diabetes (T1D) on the DG of hippocampus in rats by use of Gallyas' method to identify dark neurons and transmission electron microscopy (TEM) to find the answer of these questions: (1) Does hyperglycemia lead to dark neurons formation in granule layer of DG? (2) What is the nature of the ultrastructural changes?

Materials and Methods

This study was carried out on male Wistar rats (age eight weeks, body weight 240–260 g, n=6 per group). All rats maintained in animal house and allowed free access to drinking water and standard rodent diet. Experiments performed during the light period of cycle and conducted in accordance with Regional Committee of Ethic of Mashhad University of Medical Sciences (MUMS) complied with the regulations of the European Convention on Vertebrate Animals Protection (2005).

Induction of experimental diabetes

We considered fasting blood glucose (FBG) >250 mg/dL as a diabetic. T1D was induced by a single intraperitoneal (IP) injection of STZ (Sigma Chemical, St. Louis, Mo) at a dose of 60 mg/kg dissolved in saline (control animals were injected with saline only) [11]. Four days after the STZ injection, FBG was determined in blood samples of tail veins by a digital glucometer (BIONIME, Swiss). In the end of eight weeks, the animals were anesthetized by chloroform. Then perfusion was done transcardially with 100 mL of saline followed by 200 mL of fixative containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate

buffer (pH 7.4). The harvested brains were post-fixed in the same fixative for two weeks. Then the brain further processed through graded ethanol followed by xylene and paraffin. Serial coronal sections (thickness 10 μm) were made through the entire extent of hippocampus in left and right hemispheres using a microtome.

Transmission electron microscopy (TEM)

The hippocampi (two for each group) were removed and processed as briefly follow: washing in phosphate buffer 0.1 M (pH 7.4), fixation in 1% osmium tetroxide, dehydration by graded acetones (50, 70, 80, 90 each 20 minutes, and 100 three changes ×30 minutes), infiltration by resin/acetone (1/3 overnight, 1/1 8 hours and 3/1 8 hours), resin (overnight) and embedding, then the blocks were sent to Shahid Beheshti University of Medical Sciences (Tehran, Iran) for primary trimming, thick sectioning, thin sectioning (60–90 nm), staining with uranyl acetate and lead citrate. To identify DG region, the semithin were stained by 1% Toluidine Blue. Finally, electron micrographs were taken by EM900 (Zeiss, Germany) equipped to TFPO camera and sent back for analysis.

Gallyas' method (dark neurons staining)

Gallyas' method is a useful method for detecting of DNs. This argyrophil staining is based on the damage in cytoskeleton and DNs show characteristic morphological features like shrunken dark somata and dendrites [12]. Four sections from each animal (16 sections per group) were selected by uniform random sampling. Dark neurons staining was done as our previous study (8) as briefly as follow: (a) random systematically selection of paraffin embedded sections, (b) dehydration in a graded 1-propanol series, (c) incubation at 56^0C for 16 hours in an esterifying solution consisting of 1.2% sulphuric acid, (d) 98% 1-propanol, (e) treatments in 8% acetic acid (10 minutes), (f) developing in a silicotungstate physical developer, (g) development termination by washing in 1% acetic acid (30 minutes), and (h) dehydration. The superior and inferior blades of the dentate gyrus were studied and pictures were taken by Olympus microscope (BX51, Japan) equipped with Motic Image Plus 2 software (Motic China Group, LTD). Counting of DNs was carried out according to the stereological bases so only cell bodies were counted [13].

Statistical analysis

All data are expressed as mean±SD. Statistical comparison for the number of DNs between two groups was made using Student *t*-test. Statistically significant difference was accepted at the $p<0.05$ level.

Results

The day 4 after STZ injection rats were severely diabetic as indicated by their elevated plasma glucose (567.92±45.20 mg/dL) while plasma glucose of control group showed normoglycemic range (101±6.310 mg/dL) ($p<0.001$). Diabetic rats also exhibited obvious signs of diabetes namely: polyuria and polydipsia.

Light microscopy findings

Dark neurons (DNs) in DG granular layer of STZ-induced diabetic group showed preserved cell integrity while detached from surrounding tissues, high darkly brown stained somata and degenerated axons (Figure 1). Filamentous (thread like) structures were noticed in soma and neuritis (Figure 3). Some granular cells showed small mitochondrion size brown grain in their perikarya (Figure 2). In control animals, some scattered DNs were also found in DG granular layer, while surrounding normal neurons did not stained (Figure 4).

Counting the DNs

The numbers of DNs in diabetic animals were counted 223±25 and those of normal group counted 5.75±4.34. The comparison between the numbers of DNs in two groups showed significant level of difference ($p<0.05$) (Figure 5).

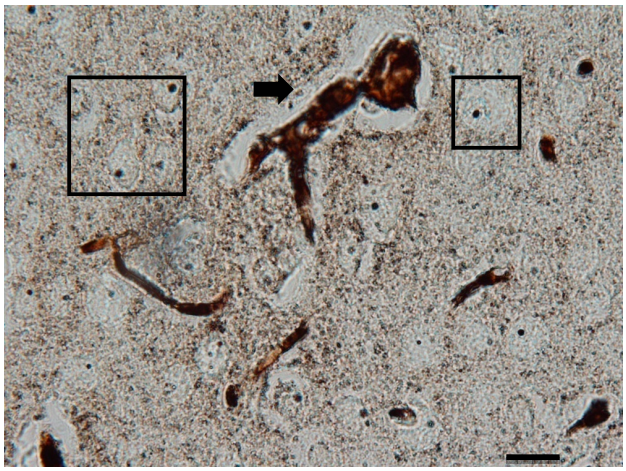

Figure 1 – *A DN stained by Gallyas' method. Somata and axon stained intensely (arrowhead). DN is detached from surrounding tissues and scattered among healthy neuron (windows). Scale bar 5 μm.*

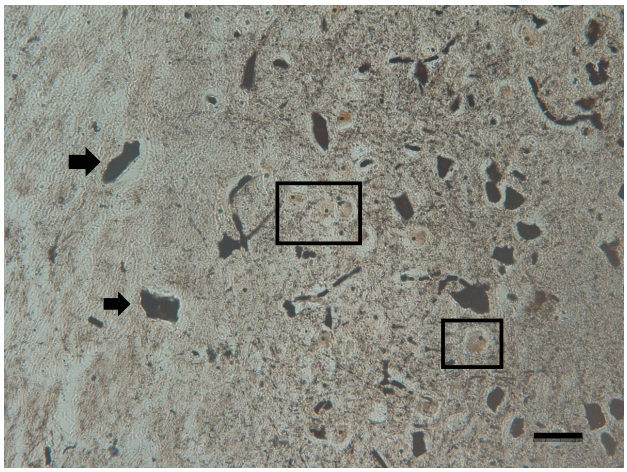

Figure 2 – *DNs in the granular layer of diabetic group, (arrowheads). The perikarya of dark neurons stained highly dark (arrowhead). Some scattered neurons stained lightly brown (in windows) which are indicative of recovery phase. Scale bar 25 μm.*

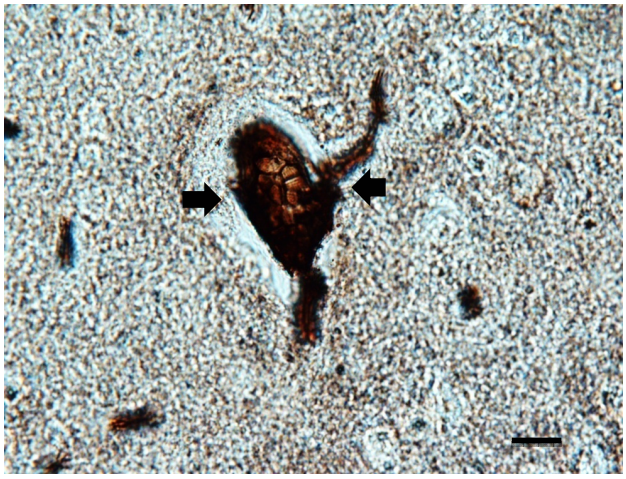

Figure 3 – *A DN in the granular layer of diabetic group stained darkly brown (arrow). Soma of this DN shows some thread like structures. An axosomatic synapse is also seen (right arrow). Scale bar 5 µm.*

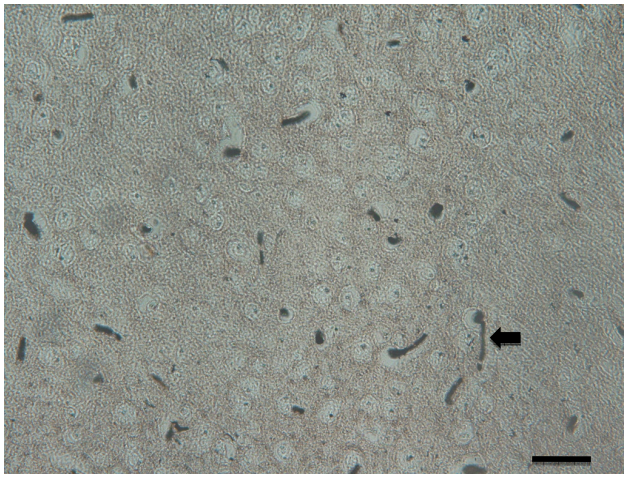

Figure 4 – *DG granule cells in control group. DNs (arrow) dispersed in the granular layer. Scale bar 25 µm.*

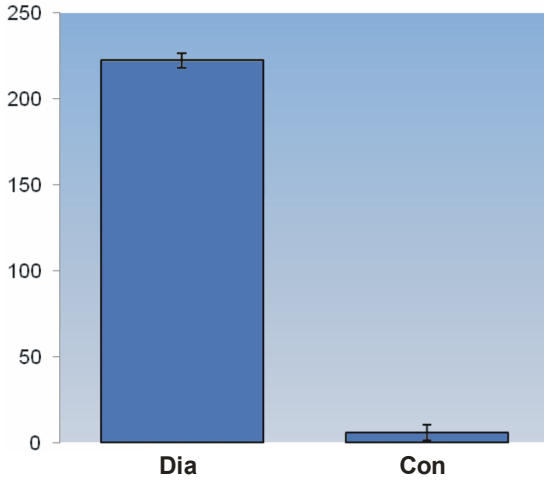

Figure 5 – *Counting of DNs in diabetic animals (Dia) showed significant level of difference to control group (Con). *p<0.05.*

TEM findings

Characterization of apoptotic death was according to our previous study, hence chromatin changes like clumping, margination and condensation were considered the most important evidence of apoptosis. Of course, other morphological characters such as cell shrinkage dark appearance were considered. Integrity of neuronal membrane preserved in most of cases (Figures 6–8).

Chromatin clumping, condensation and margination were noticed in diabetic group (Figures 7 and 8).

Other morphological changes included: electron dense appearance, shrinkage, detachment from surrounding tissues, degenerating axon and apoptotic body (Figure 6).

Swelled mitochondria were observed in cytoplasm of shrunken dark neurons (Figure 7).

Some chromatin changes like margination, clumping and condensation were also observed in control group. In contrast, the normal healthy neuron showed normal dispersed and light chromatin, rough endoplasmic reticulum and numerous synaptic terminals (Figure 9).

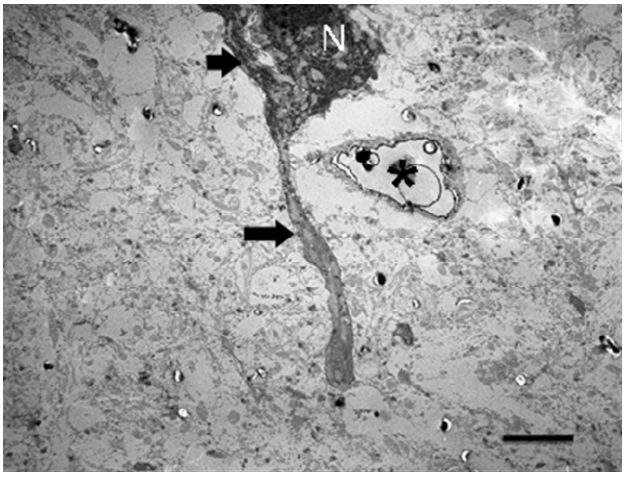

Figure 6 – *A DN in diabetic rats with degenerated axon (long arrow), dark perikarya (short arrow). Degenerative vacuolization has occurred around the DN and a vessel (star). Scale bar 5 µm.*

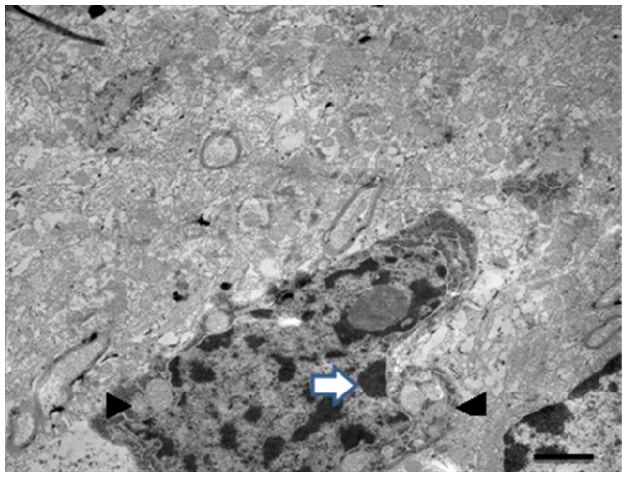

Figure 7 – *A DN in diabetic rats. Chromatin condensation, margination and clumping (white arrow), swollen mitochondria (arrowheads, right and left) are seen around the nucleus. Scale bar 2 µm.*

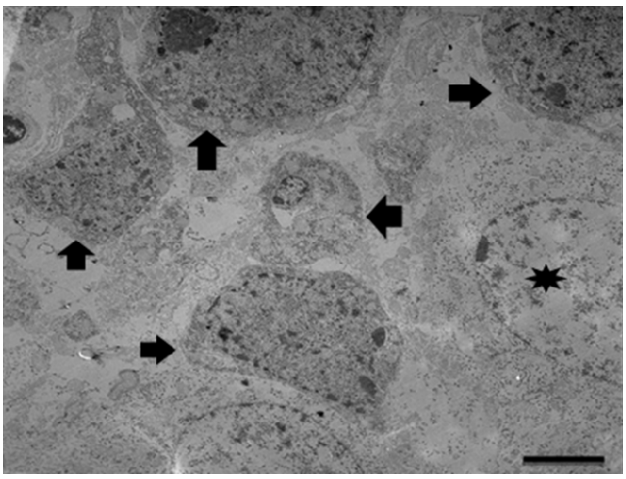

Figure 8 – ***Control group: DNs (arrows) with chromatin margination and clumping. In center, an apoptotic body is seen. Right of photograph (star) shows normal neuron. Scale bar 4 µm.***

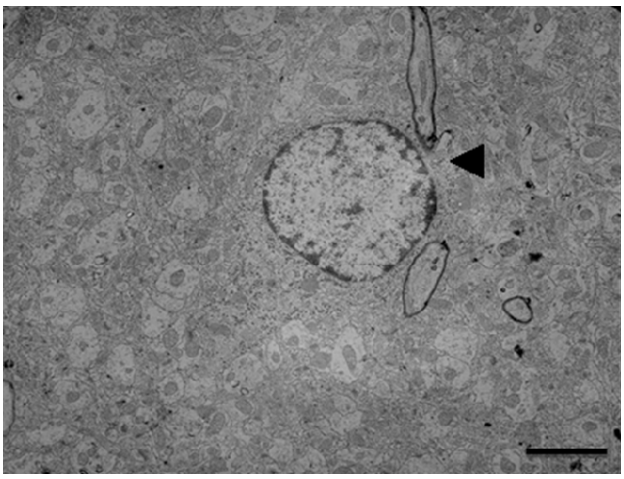

Figure 9 – ***A normal neuron in control group. An axosomatic synapses (arrow head). Scale bar 4 µm.***

Discussion

The results of this study showed that: (1) uncontrolled STZ-induced diabetes triggers DNs formation in DG granule cells of diabetic rats, (2) formation of DNs can occur in granular layer of healthy animals, (3) ultra-structurally, the DNs in DG granule layer showed morphological changes resemble to apoptotic death, namely: high electron density, shrinkage, irregularity and apoptosis like chromatin changes. Studies have shown cell proliferation continues in granular layer of DG constantly. This unique neuronal renew is necessary for memory formation. Any factor disturbs the balance between neuronal proliferation and neuronal lose may result to memory and learning impairment [10, 14]. We could show that hyperglycemia induces DN formation in DG granular layer, which has not been reported before. For demonstration of DNs, we used the selective method of Gallyas. This method is based on the reaction between the physical developer and few chemical groups in tissue. The final product of this chemical reaction would be formation of the crystallization nuclei whose enlargement produces the metallic silver grains constituting the microscopic image [12]. In this method, some neurons may show light brown appearance with mitochondrion-sized, silver-stained dot in their cytoplasm that is indicative of recovering phase [15]. In diabetic group, some scattered neurons were also observed with silver-stained dot in their perikarya in vicinity of DNs that is indicative of selective vulnerability of neurons to insult [16]. According to results of this study and our previous findings [17], it seems the main CNS complications of diabetes mellitus type 1 are mediated through excessive free radicals generation [18, 19]. Although the rate of DNs was not significant in control animals but it may raise a question, that is, why DNs occurred in granule cell of DG in healthy animals? Are they of traumatic origin or normal one? Perfusion of animals before brains harvesting eliminates traumatic origin of DNs [6], as we did in this study. The only explanation for presence of DNs in control group may be due to ageing process and free radicals generation [5]. It can conclude that diabetes mellitus type 1 may trigger DNs formation at least by acceleration of aging process by hyperglycemia and increased free radicals generation. To reveal ultra-structural changes, we took advantage of TEM study. TEM study provides clear-cut evidences to differentiate the mode of cell death [20]. Morphological study of DNs by TEM showed apoptotic like chromatin changes, darkness, and shrinkage and swelled mitochondria. The same morphological changes have been reported in DNs by Gallyas F *et al.* [2]. Although he discounted the apoptotic nature of death in DNs, and reasoned to TUNEL assay, but it should be emphasized TUNEL assay is based on caspase activity which is not always sole determinant of apoptotic death [21]. Studies have reported chromatin changes (condensation and margination), neuronal darkness and shrinkage as the hallmarks of apoptotic death [22, 23]. It seems apoptotic neurons or DNs represents a common way of death with some differences in intracellular pathways. Cell death can be classified into two major categories: apoptosis (with a variety of chromatin changes) necrosis [21]. The mechanism of DNs production that is proposed by Gallyas F *et al.* [24] is gel-gel transition. The gel–gel phase transition is associated with morphological changes in neuron such as shrinkage, which is not seen in necrosis. Apoptotic neurons also undergo a rapid shrinkage [25]. Thus, the mechanism of compaction in apoptotic neurons might involve the gel–gel phase transition [24, 26].

Conclusions

According to our morphological findings, we believe DNs can be classified as apoptotic type with some probable difference. In summary, the evidences of this study suggest first: diabetes mellitus type 1 (hyper-glycemia) produces DNs with apoptotic death criteria in granular layer of DG, and second: DNs formation in granular layer of DG accelerate in the hyperglycemic paradigm.

Acknowledgements

This work was supported financially by Vice Chancellorship of Mashhad University of Medical Sciences. We wish to thank Hamideh Bahrami for her honestly help in preparation of this manuscript and also Professor Shakibai from Germany for his generous helps.

References

[1] Kövesdi E, Pál J, Gallyas F, *The fate of "dark" neurons produced by transient focal cerebral ischemia in a non-necrotic and non-excitotoxic environment: neurobiological aspects*, Brain Res, 2007, 1147:272–283.

[2] Gallyas F, Kiglics V, Baracskay P, Juhász G, Czurkó A, *The mode of death of epilepsy-induced "dark" neurons is neither necrosis nor apoptosis: an electron-microscopic study*, Brain Res, 2008, 1239:207–215.

[3] Catarzi D, Colotta V, Varano F, *Competitive AMPA receptor antagonists*, Med Res Rev, 2007, 27(2):239–278.

[4] Ishida K, Ungusparkorn C, Hida H, Aihara N, Ida K, Nishino H, *Argyrophilic dark neurons distribute with different pattern in the brain after over hours treadmill running and swimming in the rat*, Neurosci Lett, 1999, 277(3):149–152.

[5] Vohra BP, James TJ, Sharma SP, Kansal VK, Chudhary A, Gupta SK, *Dark neurons in the ageing cerebellum: their mode of formation and effect of Maharishi Amrit Kalash*, Biogerontology, 2002, 3(6):347–354.

[6] Kherani SZ, Auer RN, *Pharmacological analysis of the mechanism of dark neuron production in cerebral cortex*, Acta Neuropathol, 2008, 116(4):447–452.

[7] Grillo CA, Piroli GG, Wood GE, Rezinkov LR, McEwen BS, Reagan LP, *Immunocytochemical analysis of synaptic proteins provides new insights into diabetes-mediated plasticity in the rat hippocampus*, Neuroscience, 2005, 136(2):477–486.

[8] Ahmadpour Sh, Sadeghi Y, Haghir H, *Streptozotocin-induced hyperglycemia produces dark neuron in CA3 region of hippocampus in rats*, Asian J Med Sci, 2010, 2(1):11–15.

[9] Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR, *Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus*, Proc Natl Acad Sci U S A, 2006, 103(46):17501–17506.

[10] Choi JH, Hwang IK, Yi SS, Yoo KS, Lee CH, Shin HC, Yoon YS, Won MH, *Effects of streptozotocin-induced type 1 diabetes on cell proliferation and neuronal differentiation in the dentate gyrus; correlation with memory impairment*, Korean J Anat, 2009, 42(1):41–48.

[11] Ates O, Cayli SR, Yucel N, Altinoz E, Kocak A, Durak MA, Turkoz Y, Yologlu S, *Central nervous system protection by resveratrol in streptozotocin-induced diabetic rats*, J Clin Neurosci, 2007, 14(3):256–260.

[12] Gallyas F, *Physico-chemical mechanism of the argyrophil III reaction*, Histochemistry, 1982, 74(3):409–421.

[13] Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sørensen FB, Vesterby A *et al.*, *Some new, simple and efficient stereological methods and their use in pathological research and diagnosis*, AMPIS, 1988, 96(5):379–394.

[14] Jackson-Guilford J, Leander JD, Nisenbaum LK, *The effect of streptozotocin-induced diabetes on cell proliferation in the rat dentate gyrus*, Neurosci Lett, 2000, 293(2):91–94.

[15] Csordás A, Mázló M, Gallyas F, *Recovery versus death of "dark" (compacted) neurons in non-impaired parenchymal environment: light and electron microscopic observations*, Acta Neuropathol, 2003, 106(1):37–49.

[16] Gallyas F, Zoltay G, Horváth Z, Dávid K, Kellényi L, *An immediate morphopathologic response of neurons to electroshock; a reliable model for producing "dark" neurons in experimental neuropathology*, Neurobiology (Bp), 1993, 1(2):133–146.

[17] Ahmadpour Sh, Sadeghi Y, Hami J, Haghir H, *Effect of insulin and ascorbic acid therapy on plasma Cu level in stereptozotocin-induced diabetic rats*, J Birjand Univ Med Sci, 2008, 15(3):26–32.

[18] Okouchi M, Okayama N, Aw YT, *Differential susceptibility of naïve and differentiated PC-12 cells to methylglyoxal-induced apoptosis: influence of cellular redox*, Curr Neurovasc Res, 2005, 2(1):13–22.

[19] Ziegler D, Sohr CG, Nourooz-Zadeh J, *Oxidative stress and antioxidant defense in relation to the severity of diabetic polyneuropathy and cardiovascular autonomic neuropathy*, Diabetes Care, 2004, 27(9):2178–2183.

[20] Zeng YS, Xu ZC, *Co-existence of necrosis and apoptosis in rat hippocampus following transient forebrain ischemia*, Neurosci Res, 2000, 37(2):113–125.

[21] Bröker LE, Kruyt FA, Giaccone G, *Cell death independent of caspases: a review*, Clin Cancer Res, 2005, 11(9):3155–3162.

[22] Nagańska E, Matyja E, *Ultrastructural characteristics of necrotic and apoptotic mode of neuronal cell death in a model of anoxia in vitro*, Folia Neuropathol, 2001, 39(3):129–139.

[23] Yardimoglu M, Ilbay G, Kokturk S, Onar FD, Sahin D, Alkan F, Dalcik H, *Light and electron microscopic examinations in the hippocampus of the rat brain following PTZ-induced epileptic seizures*, JABS Journal of Applied Biological Sciences, 2007, 1(3):97–106.

[24] Gallyas F, Csordás A, Schwarcz A, Mázló M, *"Dark" (compacted) neurons may not die through the necrotic pathway*, Exp Brain Res, 2005, 160(4):473–486.

[25] Rello S, Stockert JC, Moreno V, Gámez A, Pacheco M, Juarranz A, Cañete M, Villanueva A, *Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments*, Apoptosis, 2005, 10(1):201–208.

[26] Pollack GH, *Phase transitions and the molecular mechanism of contraction*, Biophys Chem, 1996, 59(3):315–328.

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Received: September 26th, 2010

Accepted: April 28th, 2011