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1. Introduction

Diabetes mellitus type 1 (T1D) or insulin dependent diabetes mellitus (IDDM) is an endocrine metabolic disorder which is defined by absolute or partial lack of insulin and hyperglycemia (1). Traditionally the complications of diabetes were classified as acute complications like diabetic keto acidosis (DKA) and chronic complications. Chronic complications comprise vascular and nonvascular complications. The vascular complications are further subdivided into microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular complications (coronary artery disease, CAD, and cerebrovascular disease) (2). Despite the first record of diabetes-related cognitive dysfunctions in 1922 (3), for a long period diabetic nephropathy, peripheral neuropathy, and retinopathy were considered as late diabetes microvascular complications and it was believed that central nervous system (CNS) as an insulin independent organ, spares from diabetic complications. However in recent decades studies have provided evidence that indicate the deleterious effects of T1DM on structure and functions of the brain (4-6). Duration related or chronic effects of T1DM on the brain, T1DM encephalopathy, are manifested at the all levels of CNS from microscopic to macroscopic level. Macroscopically neuroimaging studies have demonstrated a high incidence of abnormalities like temporal lobe sclerosis, decreases in white matter volume in parahippocampus, temporal and frontal lobes as well as decreased gray matter volumes of the thalami, hippocampi, and insular cortex, decreased gray matter densities of superior and middle temporal gyri and frontal gyri (7, 8). In experimental models of T1DM a vast spectrum of neuronal changes have been reported. These pathological abnormalities include synaptic and neuronal alterations, degeneration, increased cerebral microvascular permeability, and neuronal loss which collectively can lead to cognitive impairment and higher risk of development dementia (9-11). Although the mechanisms through which hyperglycemia might mediate these effects are not completely understood it seems hyperglycemia increases oxidative stress in
mitochondria and subsequent free radicals generation. Increased free radicals damage cellular membrane (lipid per oxidation) and initiate death signaling pathways (12-14). One of the most sensitive regions of the brain to the metabolic disorders and oxidative stress is hippocampus (15). The hippocampus itself is divided into two interlocking sectors, the dentate gyrus and the hippocampus proper (cornu ammonis). The dentate gyrus has three layers: (1) the granular layer containing the densely packed cell bodies of the granule cells; (2) the molecular layer formed by the intertwining apical dendrites of the granule cells and their afferents; (3) the polymorph layer in the hilus of the dentate gyrus containing the initial segments of the granule-cell axons as they gather to form the glutamergic mossy fiber bundle. Hippocampus proper as an archeocortical structure has been divided into seven layers as follows: (1) The alveus; containing the axons of the pyramidal cells (2) the stratum oriens, a layer between the alveus and the pyramidal cell bodies which contains the basal dendrites of the pyramidal cells (3) the stratum pyramidal (4) the stratum radiatum and (5) the stratum lacunosum/molecular which are, respectively, the proximal and distal segments of the apical dendritic tree. In the CA3 field an additional layer is recognized: the stratum lucidum, interposed between the pyramidal cell bodies and the stratum radiatum, receiving the mossy-fibers input from the dentate granule cells. Each CA3 giant pyramidal neuron with large dendritic spines receive as many as 10-50 mossy fibers from dentate gyrus, and send their axons into the fimbria. New memory formation and consolidation process of events by hippocampus depend on the integrity of hippocampus internal circuits (16, 17) (fig1).

**Figure 1.** Functional circuits of hippocampus. Inputs from extensive cortical and subcortical areas reach dentate gyrus. Mossy fibers, axons of granular cells, synapse with CA3 pyramidal neurons. CA3 pyramidal neurons send collateral to CA1. Axons from these two regions reach limbic related regions.
Hippocampus structural complexity has made it vulnerable to the many pathological conditions such as diabetes mellitus type 1 (18). It is a crucial part of the limbic system, which plays a pivotal role in memory formation, emotional, adaptive and reproductive behaviors (16 17 and19). Studies have shown that cell proliferation continues in granular layer of DG constantly. This unique neuronal renew is necessary for memory formation (20, 21). Any factor disturbing the balance between neuronal proliferations /death may result to memory and learning impairment (22). Studies have demonstrated that experimental diabetes causes decreased granular cells proliferation and neuronal death (necrosis / apoptosis) in CA3 and DG regions (23). Although neuronal death has been considered as the main leading cause of diabetic CNS and peripheral neuropathies the mode of neuronal death in T1DM has remained as a matter of controversy (24, 25, and 26). Neuronal death has been known as a common feature of neurodegenerative diseases like Alzheimer and diabetes (27). Studies have suggested free radicals and glutamate excitotoxicity as the main driving causes of neuronal death in diabetic paradigm (27-28). Interestingly these factors have been implicated in another mysterious and different type of neuronal death which is called “Dark” neuron. This kind of neuron has been reported in various pathological conditions like stroke, epilepsy, hypoglycemia, aging and spreading depression phenomena (SD). On the other hand, dark neuron formation has been reported in stress full conditions such as acute physical stress, normal ageing process in cerebellum and postmortem (nonenzymatic). All of these pathologic conditions cause disturbance in ion gradient (Na/K ATPase pump), and increases excitatory neurotransmitters like glutamate (27, 28). Despite the role of hyperglycemia in increasing oxidative stress and extracellular level of glutamate in hippocampus, 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. In spite of new therapies like intranasal insulin, C peptide and antioxidants (9) diabetic central neuropathy and its underlying mechanisms have remained far from fully understood.

**Purpose:** Obviously understanding the neuronal death mechanisms as a common feature of neurodegenerative diseases like Alzheimer and diabetes would contribute to better understanding of its pathophysiology and new treatment approaches. As stated before dark neurons can form in enzyme-independent condition. Therefore, there may be a need to revise the cell death concept and types. This study was conducted to clarify the following questions:(1) Does hyperglycemia lead to dark neurons formation in granule layer of DG?(2) What is the nature and entity of the ultrastructural changes?

## 2. Materials and method

**Experimental diabetes mellitus induction**

Streptozotocin is a glucosamine–nitrosourea compound isolated from Streptomyces achromogenes. As an alkylating agent it interferes with glucose transport. It is taken up into beta cells of pancreas via the specific transporter, GLU-2, inducing multiple DNA strands breaks. Because of the absence of The GLUT-2 glucose, STZ direct effects on the brain tissue is eliminated following systemic administration (29).
Induction of experimental diabetes

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 complied with the regulations of the European Convention on Vertebrate Animals Protection (2005). 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) (30). 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 follows briefly: 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, thick sectioning, thin sectioning (60–90 nm), staining with uranyl acetate and lead citrate. To identify DG region, the semi thin were stained by 1% Toluidine Blue. Finally, electron micrographs were taken by EM900 (Zeiss, Germany) equipped to TFPO camera.

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 (28). Four sections from each animal (16 sections per group) were selected by uniform random sampling. Dark neurons staining was done as our previous study (27) and follows as briefly: (a) random systematically selection of paraffin embedded sections, (b) dehydration in a graded 1-propanol series, (c) incubation at 560C for 16 hours in an esterifying solution consisting of 1.2% sulphuric acid, (d) 1-propanol(98%), (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 and therefore only cell bodies were counted (26).
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.

3. 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) (fig2). Diabetic rats also exhibited obvious signs of diabetes namely: polyuria and polydipsia.

Counting the DNs

The numbers of DNs in diabetic animals were counted 223±25 and those of normal group counted5.75±4.34. The comparison between the numbers of DNs in two groups showed significant level of difference (p<0.05) (Figure 2)

Light microscopy findings

Dark neurons (DNs) in DG granular layer of STZ-induced diabetic group showed preserved cell integrity, detached from surrounding tissues, high darkly brown stained somata and degenerated axons (Figure 3-6). Filamentous (thread like) structures were noticed in soma and neuritis (Figure 4). Some granular cells showed small mitochondrion size brown grain in their perikarya (Figure 5). In control animals, some scattered DNs were also found in DG granular layer, while surrounding normal neurons were not stained (Figure 7). Staining by toluidine blue showed some neurons were deeply stained (hyperbasophilia) (figure8,9).

TEM findings

Characterization of neuronal death was according to our previous study, hence chromatin changes like clumping, margination and condensation was considered the most important evidence of non-necrotic death. Of course, other morphological characters such as cell shrinkage and dark appearance were considered. Integrity of neuronal membrane preserved in most of cases (Figs 10–14). Chromatin clumping, condensation and margination were noticed in diabetic group. The pattern of chromatin changes showed some differences. Tiny and dispersed chromatin clump in electron dense nucleus and nucleolus without chromatin adherence were seen in some dark appearance neuron(figs10,12,13) while in some chromatin clumping was more conspicuous and nucleus appearance was lighter (fig14). Other morphological changes included: reduced inter-organelles spaces, electron dense appearance, shrinkage, detachment from surrounding tissues, degenerating axon(figs11,12) and apoptotic-body (14). Swelled mitochondria were observed in cytoplasm of shrunken dark neurons (fig10). In control animals some healthy looking neurons with increased electrondensity and apoptotic bodies were observed (14). The normal healthy neuron showed normal dispersed and light chromatin (fig 14).
Figure 2. Counting of DNs in diabetic animals (Dia) showed significant level of difference to control group (Con). *p<0.05.

Figure 3. Reversible type of dark neurons are scattered between some dark neuron. These neurons are characterized with light brown color that is indicative of recovering phase (arrowheads). Scale bar 5 μm
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Figure 4. A DN in the granular layer of diabetic group stained darkly brown (center). Soma of this DN shows some thread like structures (white arrow). An axosomatic synapse is also seen (right arrow). Scale bar 5 µm.

Figure 5. Dark neuron. Highly dark stained degenerated neurons. In center a dark neuron (red arrowhead) and numerous degenerated neuronal particles are seen. Diabetic group. Scale bar 5 µm.
Figure 6. 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 7. DG granule cells in control group. DNs (arrow) dispersed in the granular layer. Scale bar 25 μm.
Figure 8. Semi thin sections (1μm) stained by toluidine blue. Arrows indicate dark neuron among the healthy granular layer cells of DG (control). Scale bar 25μm

Figure 9. Semi thin sections (1μm) stained by toluidine blue. Arrow indicates normal neuron among the dark, hyperbasophilic neurons of DG. Scale bar 25μm
Figure 10. A DN in diabetic rats. Chromatin condensation, margination and clumping (white arrow), swollen mitochondria (arrows, right and left) are seen around the nucleus. Scale bar 2 μm.

Figure 11. 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 12.** Normal neuron (center) and its nucleolus (N). Two dark neurons (D) with chromatin clumping. A large mass of chromatin is attached to nucleolus. Scale bar 2μm

**Figure 13.** A dark neuron (white arrow). The pattern of chromatin clumping and nucleolus is different. Scale bar 4μm
Figure 14. Control group: apoptic neurons (AP) are seen with chromatin margination and clumping. Apoptotic like bodies (arrowheads). Right of photograph (star) shows normal neuron. Scale bar 4 μm.
4. Discussion

Dark neurons have been reported in the brain of experimental animals exposed to various pathological conditions. Morphologically DNs are characterized by at least six features namely: hyperbasophilia, argyrophilia, disappearance of antigenicity, ultrastructural compaction, volume reduction and increased electron density (31). On the basis of ultrastructural differences four types of dark neurons are described: the Huntington type, the artefactual, the reversible, and the irreversible (32). They have been reported in Huntington, epilepsy, SD, hypoglycemia, and also in aging process (28). The result of our study showed that uncontrolled TIDM accelerates the rate of DNs formation in granular later of DG. We could also show that DNs occur in normal condition that implicates the common nature of dark neuron (31, 32). For demonstration of DNs, we used the selective type-III argyrophilia (method of Gallyas). Gallyas’ 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 (31). DNs of both groups have common features like deep hyperbasophilia, dark staining, and neuronal shrinkage. So the reaction of neurons to different paradigms has resulted to a common morphology. DNs are the final product of a Series of physico-chemical reactions initiated from extracellular milieu and propagate into the neuron (33). At present the only proposed explanation for mechanism of formation of dark neurons is the gel concept. In this concept intra neuronal gel constitute a trabecular network surrounded by fluid. Various noxae e.g. free radicals induce release of noncovalent stored energy from gel state and as a results of gel contracture a large volume of cytoplasm contents is pressed out and lead to neuronal compaction and electron density of dark neurons. It seems cytoskeletal network would be essential in these phenomena (33-35). However, it has not been defined as some different aspects of neuronal reactions. For instance some neurons with small mitochondrion size brown grain in their perikarya were noticed. It is believed these types of neurons are in recovering phase (reversible type) in contrast to real dark neuron (dead or irreversible) (36).Interestingly reversible dark neurons were only seen in diabetic group. At present we can’t explain why reversible neurons were seen only in diabetic group but the severity of initiating insult, not its nature, may be a determinant. In diabetes more neurons were probably exposed to noxa e.g. free radicals but the response of neurons would be selective (36). Studies have documented evidence that imply the role of hyperglycemia and increased oxidative stress in neuronal death (26, 37). Based on our results it can be inferred that neurodegeneration or aging process progresses more quickly in diabetes type1 (39). Although the rate of DNs was not significant in control animals, it may raise traumatic origin of DNs. Perfusion of animals before brains harvesting eliminates traumatic origin of DNs (38) as we did in this study. To reveal the ultrastructural changes, we took advantage of TEM study. TEM study provides clear-cut evidences to differentiate the mode of cell death (40). Morphological study of DNs by TEM showed chromatin changes, darkness, and shrinkage and swelled mitochondria.
The pattern of chromatin in DNs showed some differences as follows: (1) chromatin clumping with electron-dense appearance and normal shape of nucleus boundaries (most seen in control animals) (2) dispersed tiny clumped chromatin with relatively dark appearance and crenated outlines of nucleus (3) large clumped irregular chromatin with irregular outlines of nucleus. The last two patterns were only seen in diabetic animals. To the best of our knowledge, this diversity in chromatin and nucleus morphology was not discussed in other related researches. Another characteristic of dark neuron was swelled mitochondria. In line with our findings, the same characteristics have been reported in dark neurons (41). The same Chromatin changes (condensation and margination), neuronal darkness and shrinkage are considered as the hallmarks of apoptotic death. Although the apoptotic nature of death in DNs has been discounted and reasoned to TUNEL assay, it should be emphasized that TUNEL assay is based on caspase activity which is not always sole determinant of apoptotic death (40, 42, and 43). Based on our results in TEM, the different nuclear chromatin patterns can be explained in two ways: diverse patterns of chromatin clumping/condensation as a continuum or response of neuronal subtypes e.g. basket cells in granular layer. It seems apoptotic neurons or DNs represent 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) and necrosis (40). The mechanism of DNs production that is proposed 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. Thus, the mechanism of compaction in apoptotic neurons might involve the gel–gel phase transition (44-46). In conclusion; dark neurons occur naturally in CNS and diabetes mellitus as a metabolic disorder (common nature of dark neurons formation) accelerates dark neurons formation and consequently brain aging. We propose the future studies focus more on the preventive mechanisms of DNs formation in TIDM.

Author details
Shahriar Ahmadpour
Advanced Medical Technology Department, Iranian Applied Research Center for Public Health and Sustainable Development (IRCPHD), North Khorasan University of Medical Sciences, Bojnurd, Iran

5. References


