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Streptozotocin-induced Hyperglycemia Produces Dark Neuron in CA3 Region of Hippocampus in Rats

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Abstract: Dark neurons have been reported in many pathological conditions, such as epilepsy, ischemia and hypoglycemia. All these pathological conditions cause increased extracellular excitatory neurotransmitters like glutamate. Glutamate has been involved in dark neuron formation. Increased extracellular glutamate has been reported in hippocampus of diabetic state. We aimed to study the effect of streptozotocin-induced diabetes on dark neuron formation in CA3 region of diabetic rats. 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 injected with saline only. In the end of 8 weeks, the brains were removed and hippocampi studied by gallyas method and transmission electron microscopy. Dark neurons in CA3 region of STZ -induced diabetic group showed high darkly Stained somata and axons. Dark neurons also showed shrinkage and detachment from surrounding tissues. Ultastructurally injured neurons in CA3 region of diabetic rats showed dark and electron dense appearance, chromatin condensation, margination and clumping. Present results showed that STZ-induced diabetes produces dark neuron in CA3 pyramidal layer of diabetic rats after 8 weeks. The mode of death in dark neurons is apoptosis.

Key words: CA3, dark neuron, diabetes, rat and stereptozotocin

INTRODUCTION

A century old enigma in neuropathology is the existence of dark neurons (Commermeyer *et al*., 1961). Dark neurons were noticed first to occur in neurosurgical biopsy because of their appearance (Ooigawa *et al*., 2006). Dark neurons have been reported in ischemia, epilepsy, spreading depression phenomena (SD) and hypoglycemia (Catarzi *et al*., 2007) all these pathological conditions cause disturbance in ion gradient and increased excitatory neurotransmitters like glycine and glutamate. Glutamate has an important role in dark neurons formation, and using glutamate antagonists prevent from dark neurons (Kherani *et al*., 2008). CA3 region of hippocampus has an important role in memory and receive glutamergic afferent from dentate gyrus (Ahmadpour *et al*., 2008; Zeng *et al*., 2000). Increased extracellular glutamate and subsequent neuronal death has been reported in hippocampus of type 1 diabetic rats (Grillo *et al*., 2005; Magarinos *et al*., 2000). We showed that diabetes type 1 leads to neuronal apoptosis in pyramidal layer of CA3 region (under press).It is believed increased extracellular glutamate act through disturbance in Na/K ATP ase pump and leads to neuronal death (Magarinos *et al*., 2000). In spite of the large bulk of studies on pathological conditions leading to dark neurons, there is little information about the effect(s) of hyperglycemia on dark neuron formation and the mode of death in dark neurons .in other hand the kind of death in dark neurons has not been fully revealed, for example ,in one study showed that the mode of death in dark neuron (in ischemic and epileptic paradigms)is neither necrosis nor apoptosis (Kovesdi *et al*., 2007; Gallyas *et al*., 2008). We hypothesized first: regarding to increased levels of glutamate in CA3 region of diabetic animals ,hyperglycemia may result to dark neurons formation, and second: the mode of dark neurons death is apoptosis. Thus we aimed to study effect of streptozotocin- induced diabetes on CA3 region of hippocampus in rats by use of modified gallyas method to identify dark neurons and transmission electron microscopy (TEM) to reveal the mode of neuronal death.

MATERIALS AND METHODS

All the experiments in this study were conducted in neuroscience unit of anatomy department (Mashhad).The study was carried out on male wistar rats (age: 8 weeks, body weight 240-260g, N=12 per group). All rats were maintained in animal house and were allowed free access to drinking Water and standard rodent diet. Experiments were performed during the light period of cycle and were conducted in accordance with Mashhad University of Medical Sciences (MUMS) animal ethic committee.

Induction of experimental diabetes: Diabetes 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 (Ates *et al*., 2007) control Animals were injected with saline only). Four days after the STZ

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injection, Fasting blood glucose was determined in blood samples, obtained by tail prick, by a Strip operated glucometer (BIONIME, Swiss). Rats were considered diabetic and included in the study if they had fasting plasma glucose levels>250. In the end of 8 weeks, the animals were anaesthetized by chloroform. Then the animals were transcardially perfused with 100 ml of saline followed by 200 ml of fixative containing 2% glutaraldehyde and 2%parafomaldehyde in 0.1 Phosphate buffer (pH, 7.4). The brains were removed and post fixed in the same fixative for 2 weeks. Serial coronal sections (Thickness = 5μm) were cut through the entire rostrocaudal extent of hippocampus in left and right hemispheres using a microtome.

Transmission electron microscopy (TEM): Two brains from each group were used for TEM study. The hippocampi were removed and processed as briefly follow: washing in phosphate buffer 0.1 M (pH = 7.3), fixation in osmium tetraoxide1%, dehydration by graded acetones,infiltration,embedding,primary trimming, thick section, thin sections (60-90nm) and staining with uranyl acetate and pb citrate. Electron micrographs were taken by EM900 (zeiss, Germany).

Gallyas staining method (Dark neurons staining): Demonstration of traumatized "dark neurons" was carried out using a developed silver impregnation method for demonstrating cytoskeletal damage (Gallyas *et al*., 1993) randomly selected Sections were dehydrated in a graded 1-propanol series and incubated at 56ºC for I6hr in an esterifying solution consisting of 1.2% H_2SO_4 and 98% I-propanol. After a IO min treatment in 8% acetic acid, sections were developed in a silicotungstate physical developer. Development was terminated by washing in 1% acetic acid for 30 min. Sections were then dehydrated, mounted and cover slipped with DPX and pictures were taken by Olympus microscope (BX 51, japan).

RESULTS

Light microscopic findings: Dark neurons in CA3 region of STZ -induced diabetic group showed high darkly Stained somata and axons, while in control group did not (Fig. 1 and 2). Dark neurons also showed shrinkage and detachment from surrounding tissues (Fig. 1 and 3). In some sections, darkly stained degenerated axons near the CA3 pyramidal neurons were observed (Fig. 3).

Transmission electron microscopy: Injured neurons showed the criteria like apoptotic neurons.Ultastructurally injured neurons in CA3 region of diabetic rats showed dark and electron dense apprerance.chromatin showed condensation, margination and clumping. Nuclear and cell membrane preserved. In some neurons swelled mitochondria and ribosomal rosettes were observed. Neuronal membrane showed irregularities. Apoptotic

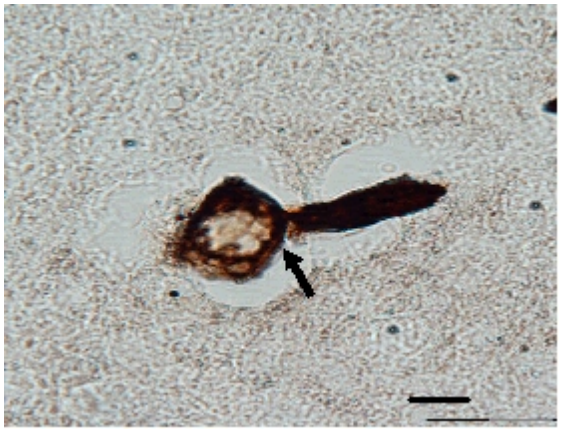

Fig. 1: A dark neuron stained by gallyas's method.somata and axon stained and neuron is detached from surrounding tissues. Scale bar 5μm

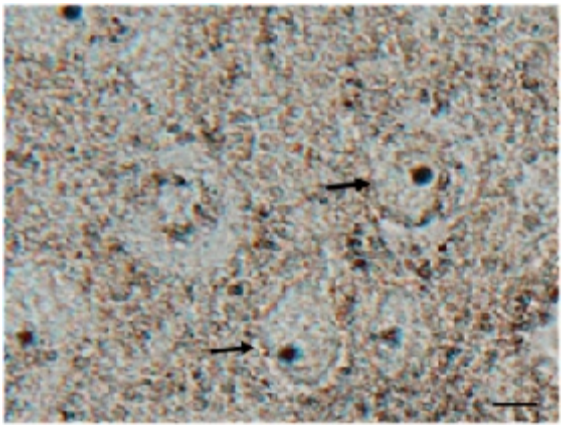

Fig. 2: Healthy neurons in control group. Scale bar 5μm

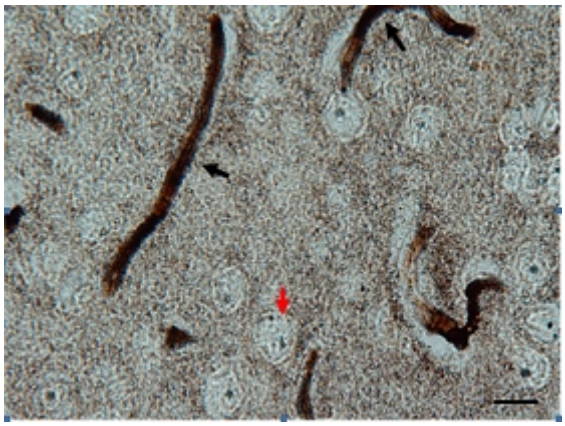

Fig. 3: Some degenerated axons can be seen (arrows). healthy neuron(red arrow). Scale bar 10 μm

bodies also observed (Fig. 4-6). Swelled mitochondria in some neurons and also in extracellular spaces were seen as mitochondrial cemetery! (Fig. 4) in some neurons chromatin margination and clumping were the major

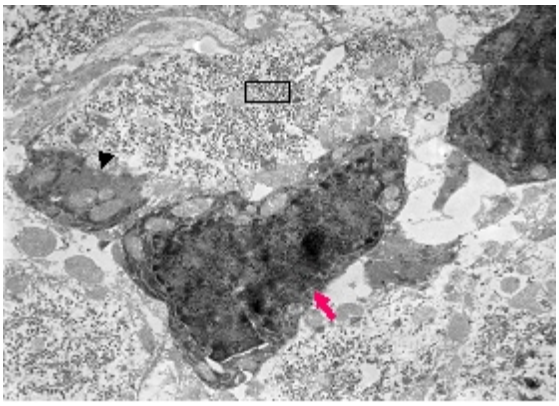

Fig. 4: Dark neuron (arrow) and apoptotic body (arrow head). Dark neurons in diabetic rats showed electro dense appearance and shrinkage. Rosette bodies (rectangle) are seen around the dark neuron. Scale bar 2 µm

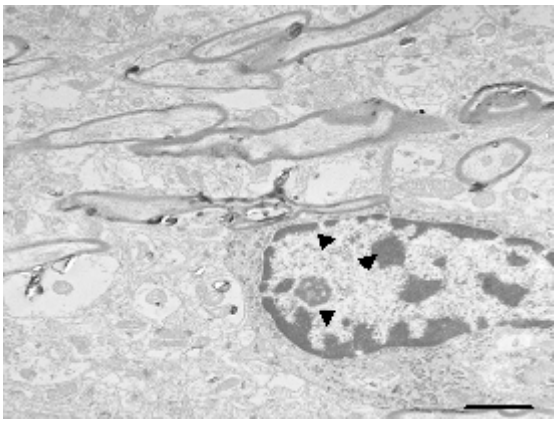

Fig. 5: Chromatin clumpig and margination (arrows heads) in pyramidal neuron of CA3 region in diabetic rats. Scale bar 2 µm

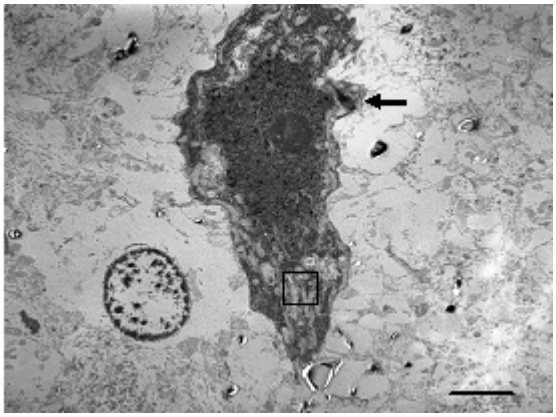

Fig. 6: Dark neuron in pyramidal layer of CA3 region in diabetic group. Dark neurons showed dark appearance, shrinkage and ruffled border (arrow). Dark neuron detached from surrounding tissue. Scale bar 4µm

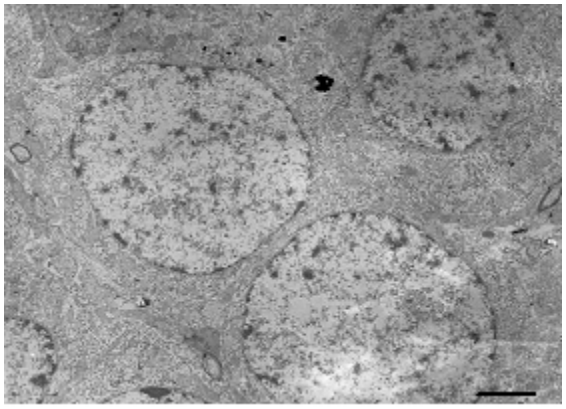

Fig. 7: Healthy neurons in control group. CA3 pyramidal neurons present Homogen and dispersed chromatin. Scale bar 2 µm

feature (Fig. 5and 6) in control animals, CA3 pyramidal neurons showed light appearance, with dispersed homogenous chromatin ,nucleolus and preserved cell membrane in contact with surrounding tissues (Fig. 7).

DISCUSSION

Our results showed that STZ-induced diabetes produces dark neuron in CA3 pyramidal layer of diabetic rats after 8 weeks.ultastructuraly a wide range of morphological changes were observed like apoptotic death. In this study we took advantage of tow methods, first: gallyas' method, as a selective method to detect dark neurons and second: TEM to approve the ultra structures of dark neurons. TEM study provide clear cut evidences to differentiate apoptotic neurons from other kinds of neuronal death like necrosis (Zeng *et al.,* 2000). At least four morphological subtypes of" dark" neurons are currently accepted (Graeber *et al.*, 2002): the Huntington type (observed in a mouse model of experimental Huntington disease), the artefactual type (produced by unintentional post-mortem mechanical injuries of various kinds), the reversible type (early stages of hypoglycemic, epileptic or ischemic injury) and the irreversible type (late stages of hypoglycemic, epileptic or ischemic injury). Perfusion of animals prevents artefactal type, as we did in preparing the brains (Kherani *et al.*, 2008). In this study, we reported that STZ-induced diabetes also produces dark neuron which has not been reported before. Ultrastructurally these neurons showed criteria like late phase of apoptosis (type II) namely: chromatin condensation, margination, preserved nuclear membrane, apoptotic bodies, shrinkage and electro dense appearance. These morphological findings are indicative of progressive and irreversible nature of these neuronal changes. The same morphological changes have been reported in CA3 apoptotic neurons after ischemia by Zeng *et al*. (2000). He also reported necrotic neurons in CA3

region. Gallyas *et al.* (2008) reported that ischemia and epilepsy induce a kind of dark neuron which is neither necrotic nor apoptotic. Ultrastructural changes reported by gallyas are the same as the apoptotic neurons criteria, like chromatin clumping,margination and electro dense appearance .we know that the most reliable criteria of apoptosis is chromatin changes and cell shrinkage (Zeng *et al.* 2000) although dark neurons can be produced by various pathological origins, but the reported morphological changes are almost the same. The mechanism of dark neurons production which is proposed by Gallyas *et al*. (2004) is gel-gel transition in an excitotoxic environment. The gel-gel phase transition is associated with morphological changes in neuron such as shrinkage which is not seen in necrosis. After completion of caspase cascade and segregation of nuclear chromatin, the apoptotic neurons also undergo a rapid shrinkage (Kerr and Harmon, 1991). Thus the mechanism of compaction in apoptotic neurons might involve the gel-gel phase transition (Pollack, 2001). Diabetes mellitus is an endocrine disease which is associated with neurochemical and neuropathological changes in brain tissue in particular hippocampus (Sima *et al.*, 2004). Previous studies have shown that hyperglycemia lead to excessive extracellular content of glutamate in CA3 region, and apoptosis in hippocampus of diabetic rats (Magarinos *et al*., 2000; Li *et al*., 2002; Grillo *et al*., 2005). Kherani (2008) showed that infusion of the selective agonist of glutamate-methyl-D-aspartate into brain tissue can produce dark neurons while glutamate antagonists prevent dark neuron formation. As mentioned above, increased glutamate in CA3 region triggers excitotoxity and subsequent neuronal death with major apoptotic features. Glutamate can trigger apoptosis or necrosis but, it depends on its receptors distribution and severity of insult (Portera-Cailliau *et al*., 1997). We believe that pathological paradigms are of great importance, for example ischemia or epilepsy release a large of glutamate into extracellular space which triggers necrosis. It seems be different in chronic diseases like diabetes mellitus which is associated with neurobiochemical and free radicals over production (Okouchi *et al*., 2005; Johansen *et al*., 2005). Excitotoxic and neurotoxic conditions in diabetic state are of less severity than of ischemic or epileptic, thus it gives the chance to neuron to choose the mode of death. Before we reported that the molecular pathway of neuronal death in diabetic state is apoptosis (Jafari *et al*., 2008). In this study we could also show that dark neurons produced by hyperglycemia are of apoptotic nature. We recommend doing more study on dark neuron formation in diabetes mellitus type1 chronologically.

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