LOW HYPERGLYCEMIA INDUCES CA3 NEUROGENESIS IN MALE RATS

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Summary

The hippocampus is one of the most morphologically and synaptically plastic areas in the brain. Hyperglycemia can lead to functional and structural deficits in central nervous system. In the present study the effects of various rates of hyperglycemia on CA3 area of hippocampus neuronal density in male Wistar rats was vestigated.

At first forty male rats divided to four groups randomly (control and experimental 1, 2, 3). Hyperglycemia was induced by a single injection (i.p.) of streptozotocyn (45, 50,55mg/kg). Control animals were given an equal volume of citrate buffer. After one month, Animal was decapitated and their brain dissected, fixed in 10% formalin, sectioned in 7µm thickness and stained by toluidin blue. By applying systematic random sampling scheme the neuronal density of CA3 area of hippocampus was estimated. Statistical analyses showed significant increase ($p<0/05$) in the CA3 area of hippocampus neuronal density in experimental 1,2 in compare with control group. This increase is a kind of neurogenesis that induces with glucose increasing. Also there is a meaningful decrease in neuronal density in experimental 3 ($p<0/001$). Hyperglycemia induces elevated metabolism and hypoxia that is one of the reasons of neuronal degeneration because of increasing glucose.

Key words*:* Hyperglycemia, CA3, Neuronal Density

.**Running title**: CA3 neuronal density in various hyperglycemias

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Introduction

The hippocampus has been implicated in certain short-term memory. Indeed hippocampus lesions often produce short-term memory deficits. The hippocampus is preferentially susceptible to a wide variety of toxic insults and disease processes, including hypoxia-ischemia and hypoglycemia (1).Metabolic diseases such as diabetes and obesity have been associated with increased vulnerability to stress (2) and cognitive dysfunction (3).Diabetes mellitus can lead to functional and structural deficits in both the peripheral and central nervous system.

Neurogenesis occurs in the hippocampus of all mammals, including humans. Recent data show that newly born cells become functionally integrated into the dentate gyrus (DG) and have passive membrane properties, action potentials, and functional synaptic inputs similar to those found in mature dentate granule cells (4). Most important are findings that newly generated neurons play a significant role in synaptic plasticity and that a reduction in the number of these cells impairs learning and memory. Neurogenesis after brain injury not only leads to the replacement of damaged cells but might also contribute to functional recovery, suggesting the possibility of endogenous neural repair (5).

The pathogenesis of these deficits is multifactor and may involve microvascular dysfunction and oxidative stress (6).Cognitive deficits are also reported to occur in animal models of diabetes (Stroptozotocin induced) which can be prevented, but not fully reversed by insulin treatment (7). Diabetes also induced morphological changes in the presynaptic mossy fiber terminals (MFT) that form excitatory synaptic contacts with the proximal CA3 apical dendrites (8).

Oxidative stress induced by chronic hyperglycemia contributes to cerebrovascular complication in diabetes (9).Also diabetes mellitus is associated with an increased risk for cerebrovascular disease (10).Accumulating data support the conclusion that oxidative stress induced by chronic hyperglycemia plays a key role in both microvascular and macrovascular complications of diabetes, including Stroke (11).Many deleterious events contribute to oxidative damage to neurons in diabetes: because of high levels of polyunsaturated lipids in the brain, direct lipperoxidation frequently occurs causing lipid membrane disruption and consequent neurodegeneration (12).

Moreover, oxidative stress increase tissue levels of highly reactive and toxic substances and effects signal transduction pathways involved in neuronal and endothelial cell function. Primary diabetic encephalopathy is recognized as a late complication of both type 1 and type 2 diabetes (13). Impairments in learning, memory, problem salving and mental and motor speed are more common in

type1 diabetic patients than in the general population (14).A diabetic duration dependent decline in cognitive function occurs independently of hypoglycemic episodes(15) and impaired intellectual and cognitive developments in type 1 diabetic children correlate with diagnosis at young age, male sex and metabolic status. Cognitive deficits (16) and poor performances in abstract reasoning and complex psychomotor functioning occur in type 2 diabetes. Learning and memory dysfunctions are more prominent in elderly type 2 diabetic patients (17).It has not been determined whether this is because of potentiation of the normal aging process, a function of diabetes duration, or both. Notably Alzheimer disease is twice as prevalent in the diabetic population as in nondiabeti subjects (18).Several recent studies have implicated abnormal function of the insulin/IGF axis in the early pathogenesis of Alzheimer's disease. Insulin and IGF- 1 are believed to regulate s-amyloid levels and tau phosphorylation (19). Impaired spatial learning and memory occur in animal models of both type1 and type2 diabetes. In the hippocampus of STZ-induced rats, long –term potentiation is impaired, whereas long-term depression is enhanced indicating altered hippocampal synaptic plasticity, which are corrected by insulin treatment (20).

The aim of present experimental design was to induce various hyperglycemias and to assess the effects of that on CA3 neuronal density.

Materials and Methods

All experiment was conducted in faculty of science, Islamic Azad University of Mashhad, Iran (2011-2011).

Animal subjects:

Forty male, Wistar rats weighting between 200-250 g served as subjects for these experiments. All animals were housed individually and maintained on a 12/12 light/dark cycle, with lights on at 6.00h. Ambient temperature in the animal facility was kept at 22±2C$^{\circ}$ .Food and water was given ad libitum.

Groups:

Group 1- Normal- rats were not subjected to any procedures.

Group 2- Hyperglycemic- under STZ injection (45mgkg-1). (glucose level<200mgdl-1)

Group 3- Hyperglycemic- under STZ injection (50mgkg-1). (glucose level<300mgdl-1)

Group 4- Hyperglycemic- under STZ injection (55mgkg-1). (glucose level>400mgdl-1)

All the experimental protocols were conducted in faculty of science, Islamic Azad University of Mashhad, Iran (2011).All chemical used in this study were purchased from Sigma (UK).

Induction of hyperglycemia:

Hyperglycemia was induced in rats by a single injection of STZ (45, 50, 55mg kg-1) freshly dissolved in citrate buffer (PH 4.5).Control animals were injected with citrate buffer. Hyperglycemia was confirmed after 4 weeks.

The body weight was measured at the beginning and the end of the experiment. All animals were checked for glucose blood concentration at the beginning of the experiment. After 2 weeks from STZ-injection, as well as on the day before of experiment (21).

Tissue collection:

Animals were anesthetized with sodium pentobarbital (64mg/kg) and decapitated. The whole brain was removed and fixed in 10% paraformaldehyde. NaCl was added to the fixative to make the tissue float in order to overcome deformities during the fixation period. Paraffin embedded tissue blocks were sectioned at 7mμ thickness coronaly and stained with haematoxylin-eosin (22).

Measurement of neuronal density in CA3*:*

Hemotoxylin-eosin-stained serial paraffin sections were prepared from 10 hippocampus from individual animals in each group. Regions of hippocampus (CA3- CA3- CA3) were identified according to paxinos and Watson (23). Tissue blocks containing samples (brains) were serially cut throughout. Form several hundred sections per block; of each 20 section 3 serial sections were obtained. For example for the first series: 24st, 25st, 26st section and for the second series: 46 st ,47 st ,48st section and so on. Therefore we mounted every 3 section on a slide. At a practical level, Stereological methods are precise tools for obtaining quantitative information about three-dimensional structures based mainly on observations made on sections (24). All experiments were performed a minimum of two times.

The dissector principle was used to determine the numbers of neurons in each section. Form each section and it's adjacent neighbor two photos were taken, one from each section with a final magnification of 100. A two-dimensional unbiased counting frame was overlaid in a uniform, random manner on to regions of any two photos taken of both sections. Those cell nuclei selected by the frame

on the reference plane but not appearing on the adjacent look-up frame section were deemed to have their tops in the volume described by the product of the area of the counting frame and the distance between sections. These nuclei were counted (Q) to provide the numerical density of cells (NV) in the ventral horns of 100-spinal cord according to the equation:

$$NV = \frac{\sum a}{\sum frame \times V_{di\,\mathrm{sec}\,tor}}$$

Where $\sum a$ is the sum of counted neurons, h is the depth of the dissector equal to the section thickness (7 micron) and a (frame) is the scaled area of the dissector frame (24).

Statistical analyze:

The ratio of numerical density of neurons in each section of hippocampus was used as an index. Student's t test was used for comparison when only 2 groups were analyzed Statistical significance was chosen as ($p<0.05$).All results are reported as mean ±SEM.

Results

1-Hyperglycemia was assessed in this study by monitoring the blood glucose levels in both PBS and STZ injected rats. There was a significant increase ($p<0.001$) in blood glucose levels from 100±5 mg/dL in control to 470±18 mg/ml in diabetic rats. In Figur 1 was showed all part of hippocampus.

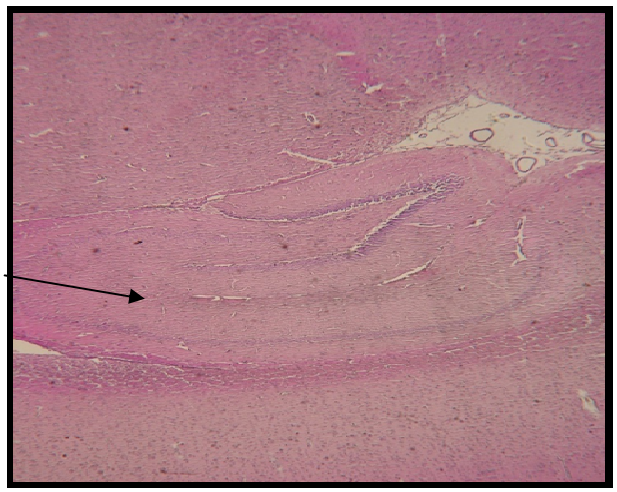

Fig.1: Photomicrograph illustrates neurons of the CA3 region of hippocampus at magnification of (20×). spik show the CA3.

2-Hyperglycemia produced evoked significant CA3 neuronal increase in Experimental 2 and 1. (Fig.2).This index was (1597±113) in control to (2357±53) and (2179±77) in hyperglycemic rats (p<o.o5).

3- In experimental 3, there is a meaningful decrease in CA3 neuronal density in compare to control group (914±18) (Fig.2)**.**

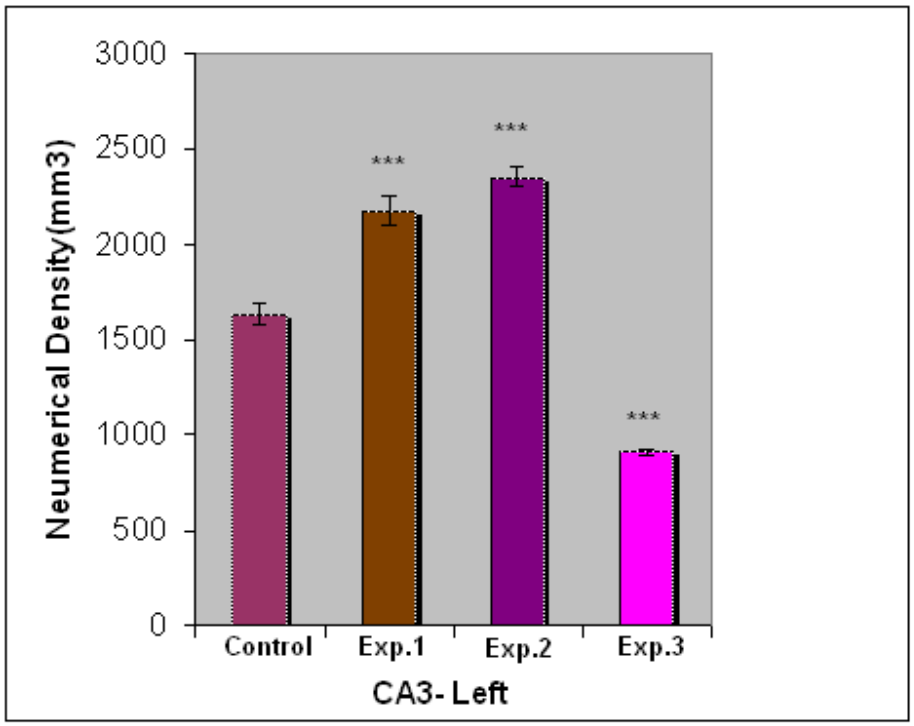

Fig.2: CA3 Neuronal density in hyperglycemic rats compare to control. The values are presented as means± SEM. n=10. *P<0.05 Student s t test compare hyperglycemic to controls.

4- All changes in different groups were obvious in left hippocampus in compare with right (Fig.3).

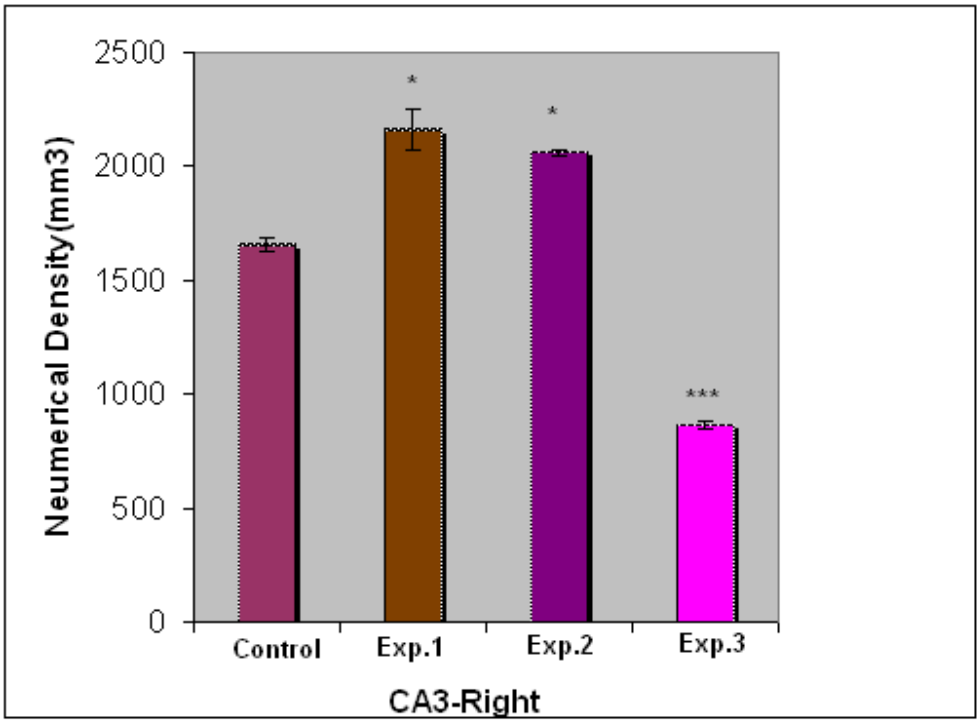

Fig.3: CA3 Neuronal density in hyperglycemic rats in right hippocampus. The values are presented as means± SEM. n=10. *P<0.05 Student s t test compare hyperglycemic to controls.

Discussion

Uncontrolled experimental hyperglycemia induced by (STZ) in rats is an endogenous chronic stressor that produces retraction and simplification of apical dendrites of hippocampus CA3 pyramidal neurons (9).One effect, synaptic vesicle depletion and dendrite atrophy occurs in hyperglycemia as well as after repeated stress and cort treatment. These changes occurred in concert with adrenal hypertrophy and elevated basal cort release as well as hypersensitivity and defective shut off of cort secretion after stress. Thus as an endogenous stressor STZ hyperglycemia not only accelerates the effects of exogenous stress to alter hippocampus morphology: it also produces structural changes that overlap only partially with those produced by stress and cort in the nondiabetic state (8).

That differences in the neuronal death pathomechanism among hippocampus sectors in hyperglycemia and ischemia may be related to the different duration and intensity of the oxidative stress in both applied models(acute in ischemia and chronics in STZ induced diabetes).It has been postulated that in cerebral ischemia neuronal apoptosis may be followed by necrotic phase(6)

In our morph metric studies on pyramidal cells in CA3, it has been shown that in high hyperglycemia there is neuronal loss and damage. As you see in Fig.2, there is a remarkable decrease in neuronal density in experimental 3 ($p<0.001$).

In low hyperglycemia (Experimental 1,2) neuronal density was increase meaningfully ($p<0.05$). The mechanism underlying the rise and decline in the hippocampus progenitor cell proliferation is unclear.

Our results suggest that the high amount of blood glucose could degenerate the neuron and lead them to death, but low glucose increase could induce the neurogeneses phenomena.

If left and right hippocampus was compared. It was clear that in left hippocampus the changes are very obvious but in right side in Experimental 2 there is not meaningful increase in compare with control group. It was postulated that left hippocampus has sensitive neuron that various rate of hyperglycemia effect on it (Fig.3).

The hippocampus is necessary for normal cognitive function, especially for processing recognition memory and transferring short-term memory items into long term storage (25).

This observation has allowed us to postulate that the neuronal death in the infant from diabetic mothers proceeds on an apoptotic pathway (26).Diabetes mellitus can result in a 30-40% reduction in brain iron (9).Iron in the form of cytochoromes is a required component of cellular oxidative metabolism in the brain and is thus essential for normal neuronal function. Iron deficiency of the heart, liver and skeletal muscle has been shown to affect cellular energy production and organ performance (27).

The hippocampus, especially the DG, is one of the iron rich areas of the rat brain, Finding suggest that the integrity of the prenatal hippocampus and its cholinergic input are important for normal development of memory and learning (16). In other hand, this area of the brain (hippocampus) particularly the dentate region is also vulnerable to damage when glucocorticoids are elevated as occurs, for example, when an organism is stressed (20).

The hippocampal morphological changes induced by stress are mediated by interactions between Gc secretion, excitatory amino acid, and are also correlated with deficits in hippocampus dependent memory (15).These results and our results confirm that an oxidative imbalance occurs in the hippocampus of hyperglycemic rats as we have previously shown. Several studies have pointed out that NF-kB(nuclar factor) activation is inhibited by a variety of antioxidants, such as N-acetyl-cystein,

butylanted hydroxyl anisole, vitamin E, and lipoic acid (10): these data suggest that antioxidants effect some steps of signaling events leading to phosphorylation, ubiquination and degradation (6).The role of oxidative stress and Nk-kB activation on hyperglycemia complications is well documented, moreover antioxidant treatment exerts a beneficial effect in experimental models of chronic injury in hyperglycemia and treatment with antioxidants can significantly reduce hyperglycemia complications (8).Reactive oxygen species activates a variety of target genes linked to the development of diabetic complications (28).

In addition, the loss of arachidonic acid content of the synaptosomal membrane, induced by hyperglycemia and by transient cerebral ischemia, making the membrane more resistant to oxidative stress. Oxidative stress induced by chronic hyperglycemia directly can damage ionic homeostasis and membrane transport systems in the brain (10) and may be this is one of the reasons for hippocampal neurons death. Apoptosis in hyperglycemia has been ascribed to oxidative stress (2).How ever, other experimental studies on streptozotocin induced rat diabetic, showed pathological changes, such as so-called dark neurons and neuronal loss, in different cerebral regions, especially in the hippocampus. A dominant opinion is that hyperglycemia aggravates ischaemic brain damage in experimental STZ-diabetes with transient cerebral ischemia in rats (3).It has been suggested that hyperglycemia and ischaemia evoke the oxidative stress following an impairment of the respiratory chain in mitochondria and an overproduction of the reactive oxygen species (ROS). ROS are considered as a main factor in the pathogenesis on neuronal death (3).The other reason for neuronal death in hyperglycemia is Iscemia. Some studies shown that there is differences in the neural death between hyperglycemia and Ischaemia (3).Pathomechanism of degenerative changes and neuronal loss through apoptosis or necrosis is not clear until now. It has been suggested that changes in intracellular calcium concentrations in oxidative stress may indicate the pathway of cell death .It is suggested that more sever injury with high intracellular calcium concentration (Ca+2) promotes necrotic cell death, where low (Ca+2) and milder injury promotes cell death through apoptosis(29). Studies on antioxidative treatment would deliver further data important in the exploration of neuronal death in diabetes and ischemia.

In total, it is concluded that hyperglycemia induces some changes in hippocampus neuronal structure and density. Statistical analysis show significant decrease in neuronal density (ND) in high

hyperglycemic rats compare to control but in low hyperglycemic groups1,2 neuronal density was increased..

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