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## Effect of Maternal Diabetes on Hippocampus Neuronal Density in Neonatal Rats

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**Abstract:** The present study has been undertaken the effects of maternal diabetes on Hippocampus structure 1 day neonate individual's rats from diabetic mothers in both control and diabetic groups. Diabetes was induced by streptozotocin (60 mg kg<sup>-1</sup>) given by a single intraperitoneal injection to female Wistar rats. Control rats were injected with phosphate buffered saline. In neonates brains rapidly were removed and in all sample the number of neurons in CA1, CA2, CA3 was measured via stereological method in both control and experimental groups. Statistical analysis determines that there is a meaningful reduction in number of neurons in CA3 in neonate of diabetic mothers (p<0.05). That may be the reason for memory problem in these neonates.

**Key words:** Maternal diabetes, hippocampus, hyperglycemia, stereological methods

### INTRODUCTION

Pathological changes in the central nervous system of diabetic animals and human are usually subtle in the brain (Polanco *et al.*, 2005) selective vulnerability was reported especially in the cerebral cortex and in the hypothalamus (the preoptic-suprachiasmatic nuclei) in diabetic rats (Luo *et al.*, 2002).

The hippocampus is a important structure for memory processing. It is a particularly vulnerable and sensitive region of the brain that is also very important for declarative and spatial learning and memory. Hippocampal neurons are vulnerable to seizures, strokes and head trauma, as well as responding to stressful experiences. At the same time they show remarkable plasticity, involving long-term synaptic potentiation and depression, dendrite remodeling (synaptic turnover and neurogenesis in the case of the dentate gyrus (McEwen, 1999). The hippocampus has been implicated in certain short-term memory. Indeed hippocampal lesions often produce short-term memory deficits (Mumby *et al.*, 2002). The hippocampus is preferentially susceptible to a wide variety of toxic insults and disease processes, including hypoxia-ischemia and hypoglycemia (Piotrowski *et al.*, 2001). Metabolic diseases such as diabetes and obesity have been associated with increased vulnerability to stress (Damasceno *et al.*, 2002) and cognitive dysfunction (Nazer and Ramirez, 2000). Diabetes mellitus can lead to functional and structural deficits in both the peripheral and central nervous system. The pathogenesis of these deficits is multifactor and may involve microvascular dysfunction and oxidative stress

(Patil *et al.*, 2006). Cognitive deficits are also reported to occur in animal models of diabetes (Streptozotocin induced) which can be prevented, but not fully reversed by insulin treatment (Kuhad *et al.*, 2008). Diabetes also induced morphological changes in the presynaptic mossy fiber terminals (MFT) that form excitatory synaptic contacts with the proximal CA<sub>3</sub> apical dendrites (Magariños and McEwen, 2000).

Oxidative stress induced by chronic hyperglycemia contributes to cerebrovascular complication in diabetes (Damasceno *et al.*, 2002). Also diabetes mellitus is associated with an increased risk for cerebrovascular disease (Aragno *et al.*, 2002). 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 (Artola, 2008). Many deleterious events contribute to oxidative damage to neurons in diabetes: because of high levels of polyunsaturated lipids in the brain, direct lipid peroxidation frequently occurs causing lipid membrane disruption and consequent neurodegeneration (Ristow, 2004).

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 (Kinney *et al.*, 2003). Impairments in learning, memory, problem solving and mental and motor speed are more common in type 1 diabetic patients than in the general population (Deregner *et al.*, 2000). A diabetic duration dependent decline in cognitive function occurs

independently of hypoglycemic episodes (DeBoer *et al.*, 2005) and impaired intellectual and cognitive developments in type 1 diabetic children correlate with diagnosis at young age, male sex and metabolic status. Cognitive deficits (Ryan and Geckle, 2000) 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 (Sinclair *et al.*, 2000). 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 nondiabetic subjects (Arvanitakis *et al.*, 2005). 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  $\beta$ -amyloid levels and tau phosphorylation (Gasparini and Xu, 2003).

Impaired spatial learning and memory occur in animal models of both type 1 and type 2 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 (Gispén and Biessels, 2000).

Pregnant women who suffer from diabetes are more likely to have a child with memory problems; according to a new study. Fetal brain iron deficiency occurs in human pregnancies complicated by diabetes mellitus or more common in the offspring of these pregnancies. The aim of present research was to induce maternal diabetes mellitus and to assess the effects of that on Hippocampus structure (CA1, CA2, CA3) and measuring the neuronal density.

## MATERIALS AND METHODS

Female Wistar rats, weighting 200-250 g, were housed under standard laboratory conditions and kept under natural 12 h light: 12 h dark cycle. The animals procured from Razi Animal House, were housed 2 per cage with free access to standard food and water. Rats were acclimatized

to laboratory conditions before testing. Animal were divided into 2 different groups for estimat the effect of maternal hyperglycemia in neonates.

**Group 1:** Normal-rats were not subjected to any procedures

**Group 2:** Diabetes-under STZ injection

All the experimental protocols were conducted in Faculty of Science, Islamic Azad University of Mashhad, Iran (2008). All chemical used in this study were purchased from Sigma (UK).

**Induction of diabetes:** Diabetes was induced in rats by a single injection of STZ (60 mg kg<sup>-1</sup>) freshly dissolved in citrate buffer (pH 4.5). Age-matched control animals were injected with citrate buffer. Diabetes was confirmed after 8 weeks; only the animals with blood glucose level above 400 mg dL<sup>-1</sup> were included in the study.

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 the of experiment.

**Tissue collection:** Animals were anesthetized with sodium pentobarbital (64 mg kg<sup>-1</sup>) 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 7  $\mu$ m thickness coronally and stained with haematoxylin-eosin.

**Measurement of neuronal density in hippocampus:** Hemotoxylin-eosin-stained serial paraffin sections were prepared from 8 hippocampi from individual animals in each group. Regions of hippocampus (CA1 CA3) were identified according to Paxinos and Watson. Tissue blocks containing samples (brains) were serially cut throughout. From several hundred sections per block, of each 20 section 3 serial sections were obtained. For example for the first series: 24, 25, 26th section and for the second series: 46, 47, 48th 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 (Gundersen *et al.*, 1988). All experiments were performed a minimum of two times. 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  $\pm$  SEM.

## RESULTS AND DISCUSSION

Two weeks after STZ-injection the rat's blood glucose level was higher than 400 mg dL<sup>-1</sup>. At the end of the 10th week, the diabetic rats showed an increase in blood glucose level (470  $\pm$  18 mg dL<sup>-1</sup>).

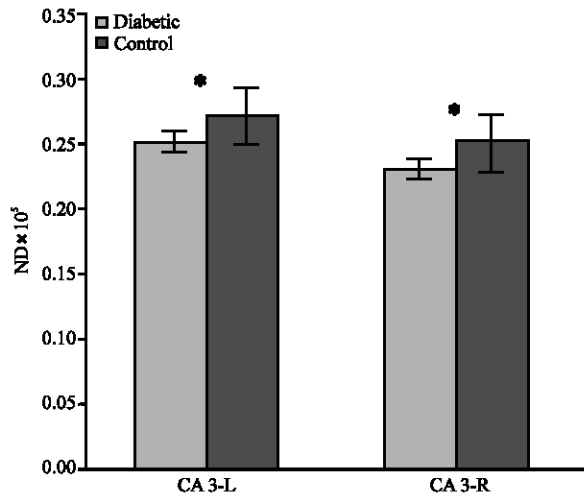


Fig. 1: Neuronal density CA<sub>3</sub> in neonate from diabetic mothers compare to control. The values are presented as Means±SEM n = 8 \*p<0.05. Student's t-test compare pups from Diabetic dams with pups from controls. CA3-L: CA3 in left hemisphere. CA3-R: CA3 in right hemisphere

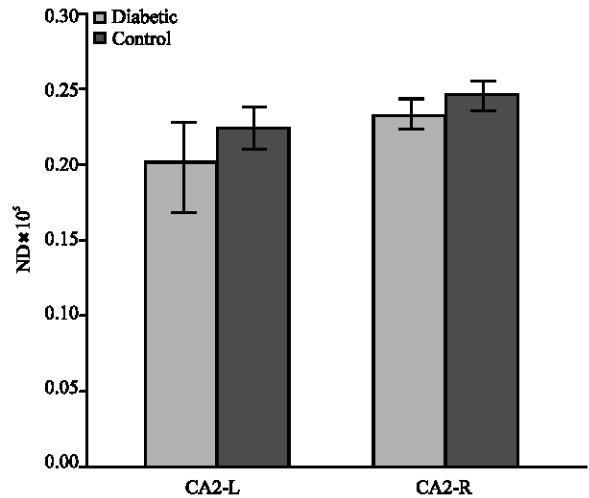


Fig. 3: Neuronal density CA<sub>2</sub> in neonate from diabetic mothers compare to control. The values are presented as means± SEM. n = 8. \*p<0.05 Student's t-test compare pups from Diabetic dams with pups from controls. CA2-L: CA2 in left hemisphere. CA2-R: CA2 in Right hemisphere.

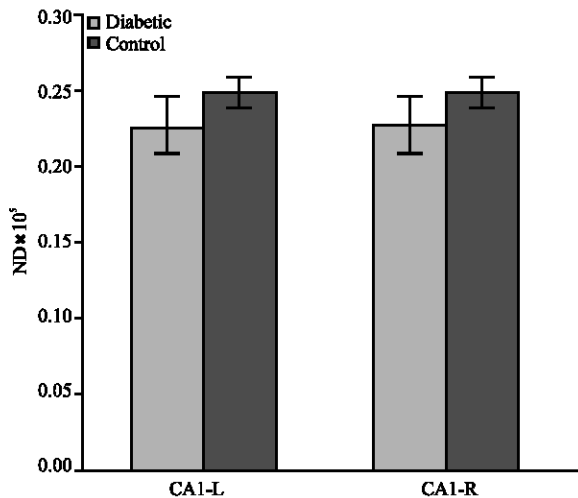


Fig. 2: Neuronal density CA<sub>1</sub> in neonate from diabetic mothers compare to control. The values are presented as means± SEM. n = 8. \*p<0.05 Student's t-test compare pups from Diabetic dams with pups from controls. CA1-L: CA1 in left hemisphere. CA1-R: CA1 in Right hemisphere

The body weight of diabetic animals increased to (230-260 g) during the experimental time, whole in the normal to (270-340 g). In the urine, all diabetic rats

demonstrated abnormal results: glucosuria (+++), ketone bodies (trace) and protein (trace). In the plasma all diabetic rats had remarkable increase in Creatinine, Uric acid, Urea, Triglycerides, Cholesterol. [P<sup>3+</sup>], [Ca<sup>2+</sup>] (p<0.05).

Maternal hyperglycemia produced evoked significant neuronal loss in CA<sub>3</sub> of neonate brains (Fig. 1). This decrease was 0.25±0.01 in control to 0.23±0.00 in neonates from diabetic mothers (p<0.05).

We estimat left and right regions of hippocampus and the results were the same. Although there was remarkable decreased in CA<sub>1</sub> and CA<sub>2</sub> but they were not meaningful (Fig. 2, 3).

In CA<sub>1</sub>, the mean of neuronal density was 0.24±0.01 in control to 0.22±0.02 in neonates from diabetic mothers (p = 0.05).

ND in CA<sub>2</sub> was 0.24±0.00 in control to 0.23±0.00 in experimental groups (p<0.08).

In morphometric study, there was more changes in neuronal shape in hippocampus regions of neonates from diabetic mothers camper to controls (Fig. 4).

In our morphometric studies on pyramidal cells in CA<sub>1</sub>, CA<sub>2</sub> and CA<sub>3</sub>, it has been shown that the experimental maternal hyperglycemia in rats results neuronal loss and damage expressed maximally in CA<sub>3</sub>. Although in experimental groups there is a remarkable neuronal density decrease in all hippocampus sectors (Fig. 1-3), but it is meaningful in CA<sub>3</sub> (Fig. 1).

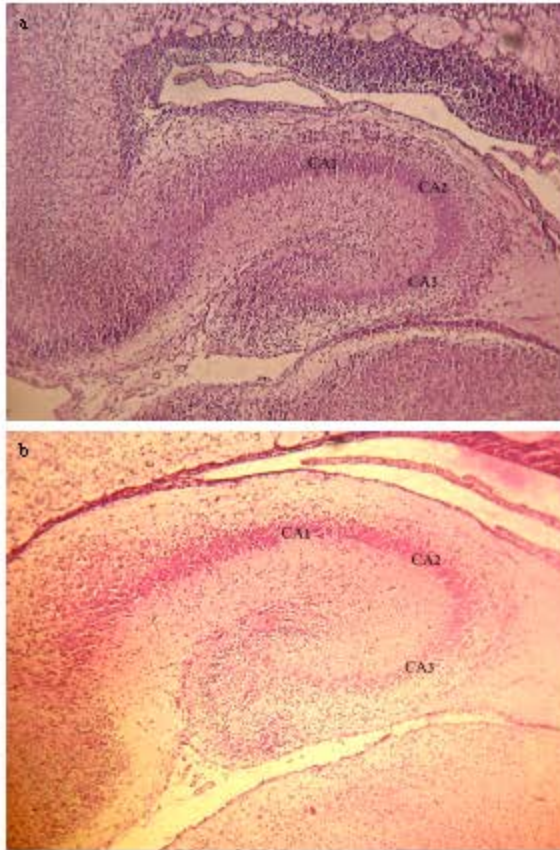


Fig. 4: Photomicrograph of the brain section of neonate at the region of the midhippocampus. (a) hippocampus of neonate from diabetic mother and (b) Control. (X40)

Diabetes causes morphometric neuronal changes (Sinclair *et al.*, 2000). This observation has allowed us to postulate that the neuronal death in the infant from diabetic mothers proceeds on an apoptotic pathway (Sima and Li, 2005). Gestational conditions increase fetal iron demand for erythropoiesis beyond placental iron transport capacity. Diabetes mellitus can result in a 30-40% reduction in neonatal brain iron (Damasceno *et al.*, 2002). Iron in the form of cytochromes is a required component of cellular oxidative metabolism in the brain and is thus essential for normal neuronal function (Omoy, 2007).

A higher prevalence of cognitive impairment has been documented in infants born to mothers with hyperglycemia gestational conditions (Gao and Gao, 2007). Iron deficiency could have a direct effect on brain development or could potentiate the effects of other adverse prenatal events. The cognitive nature of the

long-term impairments of infants of diabetic mothers (Nold and Georgieff, 2004) and growth-retarded infants suggests that factors targeting the developing hippocampus may be particularly important. The hippocampus is necessary for normal cognitive function, especially for processing recognition memory and transferring short-term memory items into long-term storage (McEwen, 1999).

In humans and animal models, the hippocampus appears to be particularly vulnerable to prenatal hypoxia-ischemia an event that occurs more commonly in gestations complicated by maternal diabetes mellitus or fetal growth retardation. We hypothesized that prenatal brain iron deficiency that occur in diabetic pregnancy increases the vulnerability of the hippocampus to hypoxic-ischemic injury (Barnes-Powell, 2007).

Iron deficiency of the heart, liver and skeletal muscle has been shown to affect cellular energy production and organ performance. Arguably, severe iron deficiency may lead to similar deficits in cellular energy metabolism and organ performance in the brain, resulting in a reduced ability to respond to restriction of oxygen and perfusion and in greater hippocampus damage. 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 (Ryan and Geckle, 2000). 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 (Gispens and Biessels, 2000).

Uncontrolled experimental diabetes induced by (STZ) in rats is an endogenous chronic stressor that produces retraction and simplification of apical dendrites of hippocampal CA<sub>1</sub> pyramidal neurons (Damasceno *et al.*, 2002).

One effect, synaptic vesicle depletion and dendrite atrophy occurs in diabetes 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 diabetes not only accelerates the effects of exogenous stress to alter hippocampal morphology; it also produce structural changes that overlap only partially with those produced by stress and cort in the nondiabetic state (Magariños and McEwen, 2000).

The hippocampal morphological changes induced by stress are mediated by interactions between Gc secretion, excitatory amino acid and are also correlated with deficits in hippocampal dependent memory (DeBoer *et al.*, 2005).

These results and present results confirm that an oxidative imbalance occurs in the hippocampus of neonates born from diabetic rats as we have earlier shown in diabetic rats. Several studies have pointed out that NF- $\kappa$ B (nuclear factor) activation is inhibited by a variety of antioxidants, such as N-acetyl-cysteine, butylated hydroxytoluene, vitamin E and lipoic acid (Aragno *et al.*, 2000). These data suggest that antioxidants effect some steps of signaling events leading to phosphorylation, ubiquitination and degradation (Patil *et al.*, 2006). The role of oxidative stress and Nf- $\kappa$ B activation on diabetic complications is well documented, moreover antioxidant treatment exerts a beneficial effect in experimental models of chronic injury in diabetes and treatment with antioxidants can significantly reduce diabetic complications (Magariños and McEwen, 2000). Reactive oxygen species activates a variety of target genes linked to the development of diabetic complications (Rakeshwar *et al.*, 2006).

In addition, the loss of arachidonic acid content of the synaptosomal membrane, induced by diabetes 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 (Aragno *et al.*, 2000) and may be this is one of the reasons for hippocampal neurons death. Apoptosis in diabetes has been ascribed to hyperglycemia and oxidative stress (Mumby *et al.*, 2002).

However, 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 (Piotrowski *et al.*, 2001). It has been suggested that diabetes 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 (Piotrowski *et al.*, 2001).

The other reason for neuronal death in diabetic pregnancy is Ischemia. It has been postulated that neurons with higher oxygen consumption in normal conditions are more susceptible to ischemia insult (Ristow, 2004).

Some studies shown that there is differences in the neural death between diabetic and Ischaemia (Piotrowski *et al.*, 2001) that differences in the neuronal death pathomechanism among hippocampal sectors in diabetes and ischaemia may be related to the different

duration and intensity of the oxidative stress in both applied models (acute in ischaemia and chronic in STZ-induced diabetes). It has been postulated that in cerebral ischaemia neuronal apoptosis may be followed by necrotic phase (Patil *et al.*, 2006). 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 severe injury with high intracellular calcium concentration ( $Ca^{+2}$ ) promotes necrotic cell death, where low ( $Ca^{+2}$ ) and milder injury promotes cell death through apoptosis (Misumi *et al.*, 2008). 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 maternal diabetes induces some changes in hippocampus neuronal structure and density. Statistical analysis show significant decrease in neuronal density (ND) of CA<sub>3</sub> in neonates from diabetic mothers compare to control. Although ND in CA1, CA2 have showed decrease but it is not meaningful. Maybe neurogenic process in hippocampus is responsible for these changes. Therefore, it is better in diabetic pregnant mother the level of glucose was maintained under normal condition.

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