───── **Original Article** ─────

Dendritic Spine Changes in Medial Prefrontal Cortex of Male Diabetic Rats Using Golgi-Impregnation Method

Mohammad-Taghi Joghataie PhD*· **, Mehrdad Roghani PhD•***, Mohammad-Reza Jalali MD‡, Tourandokht Baluchnejadmojarad PhD†, Maryam Sharayeli MD‡

Background: Neuropathy is one of the major complications contributing to morbidity in patients with diabetes mellitus. The effect of diabetes on brain has not been studied so much and no gross abnormality has been found in the central nervous system of patients with diabetic neuropathy.

This study was conducted to evaluate the time-dependent structural changes in medial prefrontal cortex of male diabetic rats using Golgi-impregnation method.

Methods: Male Wistar rats were randomly divided into the control and diabetic groups. For induction of diabetes, a single dose of streptozotocin (60 mg/kg) was injected intraperitoneally. At the end of the first and second months, the rats were transcardially perfused with a solution of phosphate buffer containing paraformaldehyde and Golgi-impregnated method was used to evaluate the changes of dendritic spines in medial prefrontal cortex.

Results: There was a significant reduction in the mean density of pyramidal neuron dendritic spines in the layers II and III of medial prefrontal cortex only after 2 months in the diabetic group compared to age-matched controls (*P* < 0.05).

Conclusion: Diabetes induces a reduction in the spine density of apical dendrites of medial prefrontal cortex only in two-month diabetic rats.

Introduction

Neuropathy is one of the major complications contributing to morbidity in patients with diabetes mellitus. Diabetes leads to a wide range of peripheral neuronal deficits such as reduced motor nerve conduction velocity, impaired sciatic nerve regeneration, axonal shrinkage in association with reduced neurofilament delivery, and deficient anterograde axonal transport.[1 – 2] In rats with

Authors' affiliations: Department of Anatomy, *Iran University of Medical Sciences, **University of Social Welfare and Rehabilitation, Department of Physiology, ***School of Medicine, Shahed University, †Iran University of Medical Sciences, ‡Department of Pathology, School of Medicine, Shahed University, Tehran, Iran.
•**Corresponding author and reprints:** Mehrdad Roghani PhD, Department of Physiology, Shahed University, Dehkadeh St., Keshavarz Blvd., Tehran, Iran.
Tel: +98-218-8964792, Fax: +98-218-8966310,
E-mail: mehjour@yahoo.com.

experimentally-induced diabetes by streptozotocin (STZ), the nerve damage is similar in many ways to the nerve degeneration seen in human diabetic neuropathy.[3] In addition, pathologic studies have suggested that diabetes is one of the risk factors for senile dementia of Alzheimer type.[4] Many studies of the relationship between diabetes and peripheral neuropathy have been done to date, but the effects of diabetes on brain have not been studied and no gross abnormality has been found in the central nervous system of patients with diabetic neuropathy.[5] On the other hand, diabetes mellitus is accompanied by disturbances in learning, memory, and cognitive skills in the human and experimental animals.[6] Medial prefrontal cortex (MPC) has been traditionally implicated in attentional processes, working memory, and behavioral flexibility.[7] Recently, some noticeable morphologic changes in dendritic spines of cerebral cortex of 4-week diabetic rats have been reported.[5] Therefore, this study was conducted to

evaluate time-dependent structural changes in MPC of male diabetic rats using Golgi-impregnation method.

Materials and Methods

Thirty-two male albino Wistar rats (Pasteur Institute, Tehran, Iran) weighing 290 – 320 g (10 – 12 weeks old) were housed in an air-conditioned colony room on a light/dark cycle (20 – 22°C and 30 – 40% humidity) and supplied with standard pelleted diet and tap water *ad libitum*.

The animals were randomly divided into four groups including 1 and 2 months controls and diabetics, each one containing at least 8 animals. Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg) dissolved in cold 0.9% saline solution immediately before use. Diabetes was verified by a serum glucose level higher than 250 mg/dL using glucose oxidation method (glucose oxidase kit, Zistchimie, Tehran).

Golgi-impregnation method

At the end of the first and second months, Golgi-impregnated method was used to evaluate the changes of dendritic spines in MPC region of the control and age-matched diabetic rats.[8] Briefly, the animals were perfused transcardially with a solution of 0.1 M phosphate buffer containing 4% paraformaldehyde (pH 7.4). After removing the forebrains, they were incubated in 1% potassium dichromate, 1% mercury chloride, 0.8% potassium chromate, and 0.5% potassium tungstate in distilled water at 20ºC for 14 – 16 days. After rinsing the brains with distilled water, they were incubated in 1% lithium hydroxide and 15% potassium nitrate in distilled water at 20ºC for two other days. The forebrains were cut on a freezing microtome (Leica, Germany) at -10ºC at a thickness of 50 µm (the blocks were soaked in gradient concentrations of 10% and 30% sucrose in phosphate buffer (0.1 M) for an overnight and at least 2 days, respectively). The sections were rinsed free floating in double distilled water, dehydrated in a series of ethanol, cleared with xylene, mounted onto gelatinized slides, and then coverslipped under Entelane.

Data analysis

Spines were counted on pyramidal neurons in layer II–III of MPC. In this respect, spines were readily identifiable at a magnification of 800 using an Olympus light microscope with digitalized photography facility. For a blinded assessment, all of the slides were coded before quantitative analysis, and the code was not broken until the analysis was completed. Pyramidal neurons were defined by the presence of a basilar dendritic tree, a distinct single apical dendrite, and dendritic spines. The spines were identified based on the morphologic criteria for mushroom and thin spines. Only protrusions perpendicular to the dendritic shaft that possessed a clear neck and bulbous head were counted. These spine types made up approximately 80 – 85% of the spine population. For each animal, spines were counted on 10 – 15 neurons. For each animal, those cells that exhibited dark and consistent impregnation throughout the cell body and dendritic tree were evaluated. Meanwhile, their relative isolation from neighboring impregnated neurons was an inclusion criterion. For each selected cell, the number of spines on at least four segments of the apical dendritic tree was determined. No primary dendrites were analyzed, and all of the segments selected for analysis were located 100 – 150 µm away from the cell body and not located at the terminal of a dendrite.

Computer-assisted tracings were done for each dendritic segment, and the length was determined using *Image tool* analysis software. The data were expressed as mean values of spine densities (number of spines per 100 µm) for each animal.

For statistical analysis, unpaired Student's *t*-test was used for comparing the control and diabetic groups. All data were expressed as mean ± SEM and $P < 0.05$ was considered significant.

Results

The body weight of the diabetic and normal rats was not different at the beginning of the study. After induction of diabetes, there was a significant reduction in this parameter in the diabetic group after 1 ($P < 0.005$) and 2 months ($P < 0.001$) (Figure 1). There was no significant difference in serum glucose level between the control and diabetic groups before the study. But the diabetic group showed a significant increase in serum glucose level at the end of the first and second months ($P < 0.001$) (Figure 2). !

Light microscopic examination of Golgi-impregnated tissue of MPC revealed reliable and consistent staining throughout the layers II and III.
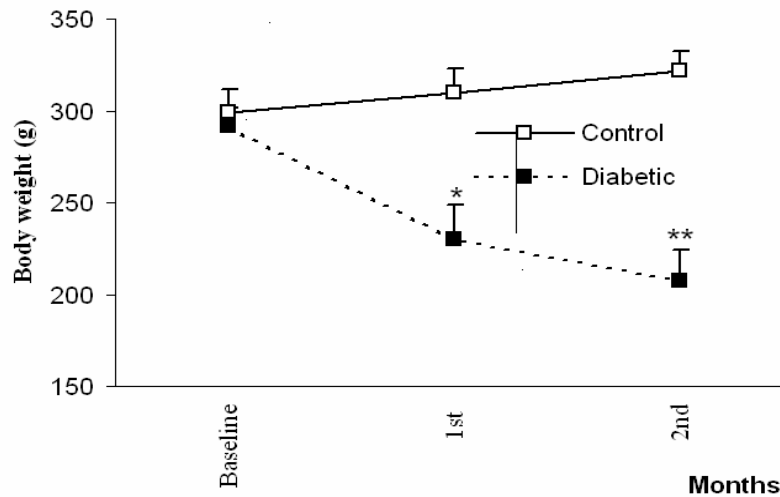
Figure 1. Body weight of the control and diabetic rats at different months (*$P < 0.005$, **$P < 0.001$).
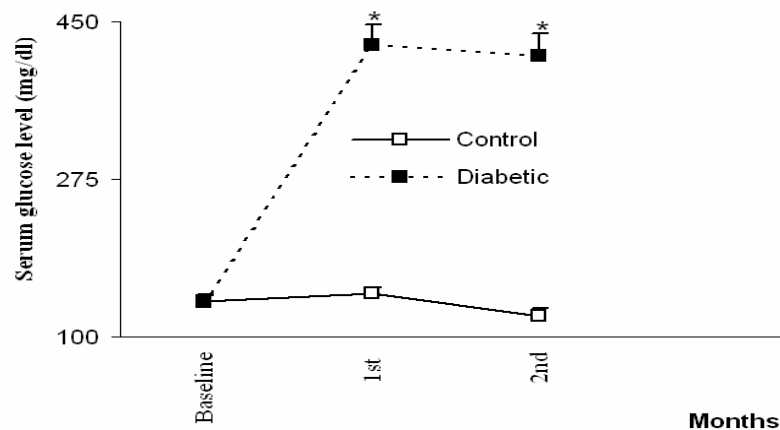

Figure 2. Serum glucose level of control and diabetic rats at different months (*$P < 0.001$).

Tracing of the apical dendrites of pyramidal neurons of MPC revealed significant changes in the number of dendritic spines in the diabetic rats compared to age-matched control group after 2 months (Figure 3). Quantitative analysis of the spine density of these neurons clearly revealed a significant decrease in the number of these spines (Figure 4) suggesting that spine and dendritic morphology may be sensitive to such stressful conditions like diabetes. In addition to the changes in the number of spines per selected length of dendrites, the shape of the remaining spines appeared to be different between the diabetic and control rats. Dendritic spines of the diabetic rats showed fewer and less pronounced protrusions compared to controls (Figure 3). On the contrary, observation of cell body area showed no

significant differences between the diabetic and control rats. !

Discussion

Although severe peripheral neuropathy has been reported in diabetic patients, the diabetic brain has not been studied so much and its possible dysfunctions have remained to be clarified. According to existing data, patients who diagnosed as having Alzheimer's disease have a relatively high frequency of diabetes mellitus.[9] However, no significant differences in the severity of Alzheimer-type pathologies such as senile plaques or neurofibrillary tangles between the diabetic and control subjects have been observed.[9] In addition, diabetics show impaired cognitive performance
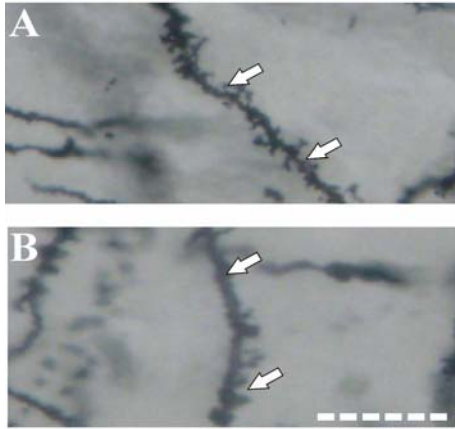
Figure 3. Photomicrograph of Golgi-stained dendritic spines from pyramidal neurons in the layers II–III of MPC in the control (A) and age-matched diabetic (B) rats two months after the study (scale bar = 25 μm).

compared to age-matched control subjects.[6] It seems that diabetes induces impairment of cognitive performance. The decrease of learning and cognitive abilities may not be restricted to disorders like Alzheimer's disease[2] and diabetics also show impaired cognitive performance compared to age-matched control subjects.[6]

In this study, body weight, intake of food, or water of the diabetic rats were significantly different from those of the control rats. Cell number and possible morphologic changes in the brains of the diabetic rats were also studied.

Previous studies failed to find any changes in neuronal cell number using Nissl staining.[5] Using Golgi staining in our study, morphologic changes were evident in the MPC area of the cortex in the diabetic rats after two months. Similar morphologic changes had been observed in the hippocampus at the time of delayed neuronal death induced by the transient global cerebral ischemia in rodents.[10] The initiating event that leads to delayed neuronal cell death is possibly the neuronal excitation caused by glutamate and subsequent calcium influx into the cell. Abnormal influxes of glutamate and/or calcium might also occur in diabetic brain, because the expressions of calbindin, synaptophysin, and syntaxin, which are the proteins related to a calcium binding protein, or synaptic secretions are reduced in such brains.[6] Their level indicates the degree of expression of glutamate, which initiates the release of other neuronal transmitters. Unusual influxes of glutamate and/or calcium might cause the morphologic changes in the diabetic brain,[11] as observed in this study. The neuronal cell death induced by ischemia is due to a failure of recovery process following excitatory damages to this particular neuronal circuitry.[12] Lack of several neurotrophic factors such as brain-derived neurotrophic factor (BDNF) or nerve growth factor (NGF) is also considered to cause the neuronal cell death after ischemia.[13] The reduced level of BDNF in diabetic hippocampus may induce some neuronal dysfunctions and morphologic changes.[14]

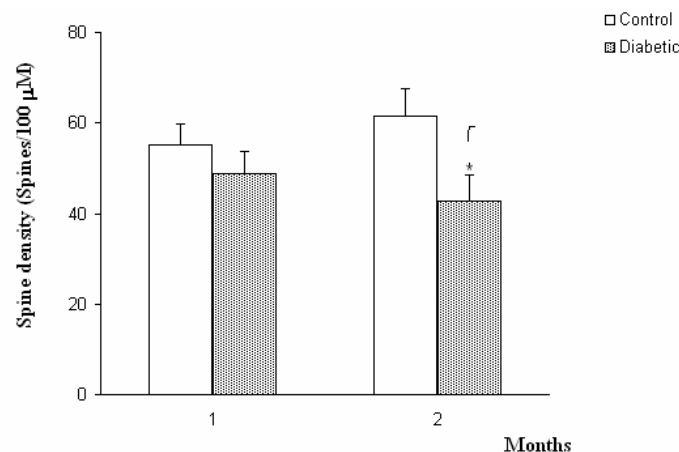In conclusion, to the best of our knowledge the

Figure 4. The mean spine density per selected length on the apical dendrites from pyramidal neurons of medial forebrain cortex of the intact and age-matched diabetic rats after one and two months. Vertical bars represent SEM values. Asterisk indicates significant difference relative to untreated controls ($P < 0.05$).

present report provides the first evidence that diabetes induces a reduction in the spine density of apical dendrites of MPC in two-month diabetic rats. Further studies are warranted to investigate the detailed mechanisms that lead to these abnormalities.

Acknowledgment

References

1   Baydas G, Nedzvetskii VS, Nerush PA, Kirichenko SV, Yoldas T. Altered expression of NCAM in hippocampus and cortex may underlie memory and learning deficits in rats with streptozotocin-induced diabetes mellitus. *Life Sci*. 2003; **73:** 1907 – 1916.

2   Sima AA, Li ZG. The effect of C-peptide on cognitive dysfunction and hippocampal apoptosis in type 1 diabetic rats. *Diabetes*. 2005; **54:** 1497 – 1505.

3   Reagan LP, McEwen BS. Diabetes, but not stress, reduces neuronal nitric oxide synthase expression in rat hippocampus: implications for hippocampal synaptic plasticity. *Neuroreport*. 2002; **13:** 1801 – 1804.

4   Popovic M, Biessels GJ, Isaacson RL, Gispen WH. Learning and memory in streptozotocin-induced diabetic rats in a novel spatial/object discrimination task. *Behav Brain Res*. 2001; **122:** 201 – 207.

5   Nitta A, Murai R, Suzuki N, Ito H, Nomoto H, Katoh G, et al. Diabetic neuropathies in brain are induced by deficiency of BDNF. *Neurotoxicol Teratol*. 2002; **24:** 695 – 701.

6   Parihar MS, Chaudhary M, Shetty R, Hemnani T. Susceptibility of hippocampus and cerebral cortex to oxidative damage in streptozotocin-treated mice: prevention by extracts of *Withania somnifera* and *Aloe vera*. *J Clin Neurosci*. 2004; **11:** 397 – 402.

7   Heidbredera CA, Groenewegen HJ. The medial prefrontal cortex in the rat: evidence for a dorso-ventral distinction based upon functional and anatomical characteristics. *Neurosci Biobehav Rev*. 2003; **27:** 555 –579.

8   Fujioka T, Sakata Y, Yamaguchi K, Shibasaki T, Katok H, Nakamura S. The effects of prenatal stress on the development of hypothalamic paraventricular neurons in fetal rats. *Neuroscience*. 1999; **92:** 1079 – 1088.

9   Harris Y, Gorelick PB, Freels S, Billingsley M, Brown N, Robinson D. Neuroepidemiology of vascular and Alzheimer's dementia among African-American women. *J Natl Med Assoc*. 1995; **87:** 741 – 745.

10 Shigeno T, Mima T, Takakura K, Graham DI, Kato G, Hashimoto Y. Amelioration of delayed neuronal death in the hippocampus by nerve growth factor. *J Neurosci*. 1991; **11:** 2914 – 2919.

11 Wisniewski K, Fedosiewicz-Wasiluk M, Holy ZZ, Car H, Grzeda E. Influence of NMDA, a potent agonist of glutamate receptors, on behavioral activity in 4-week streptozotocin-induced diabetic rats. *Pol J Pharmacol*. 2003; **55:** 345 – 351 .

12 Artola A, Kamal A, Ramakers GM, Biessels GJ, Gispen WH. Diabetes mellitus concomitantly facilitates the induction of long-term depression and inhibits that of long-term potentiation in hippocampus. *Eur J Neurosci*. 2005; **22:** 169 – 178 .

13 Lupien SB, Bluhm EJ, Ishii DN. Systemic insulin-like growth factor-I administration prevents cognitive impairment in diabetic rats, and brain IGF regulates learning/memory in normal adult rats. *J Neurosci Res*. 2003; **74:** 512 – 523.

14 Izumi Y, Yamada KA, Matsukawa M, Zorumski CF. Effects of insulin on long-term potentiation in hippocampal slices from diabetic rats. *Diabetologia*. 2003; **46:** 1007 – 1012.