

Effect of Gestational Diabetes on Purkinje and Granule Cells Distribution of the Rat Cerebellum in 21 and 28 days of Postnatal Life

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ABSTRACT

Introduction: Diabetes mellitus is associated with nervous system alterations in both human and animal models. This study was done to determine the effect of gestational diabetes on the Purkinje and granular cells in the cerebellum of rat offspring.

Methods: 10 Wistar rats Dams were randomly allocated in control and diabetic group. The experimental group received 40 mg/kg/body weight of streptozotocin (STZ) at the first day of gestation and control groups received saline injection intraperitoneally (IP). Six male offsprings of gestational diabetic mothers and control dams, at the 21, 28 postnatal days were randomly scarified and coronal sections of cerebellum (6 micrometer) serially collected. The neurons were stained with cresyl violet.

Results: The Purkinje cells density in the apex and depth of cerebellum in P21, in the experimental group was reduced 23% and 15% in comparison with the control group ($P<0.001$). The granular cells density in the experimental group was reduced 19.58% and 18.3% in comparison with the controls ($P<0.001$). The Purkinje cells density of cerebellum in P28, in the diabetic group reduced to 22.12% and 12.62% in comparison with the control group ($P<0.001$). The granular cells density in the diabetic group reduced 17.14% and 16.12% in comparison with the control group ($P<0.001$).

Discussion: The Purkinje and granular cells significantly reduced in gestational diabetes rat offspring.

Key Words:

Gestational diabetes,
Cerebellum, Purkinje
cell, Granular cell, Rat.

1. Introduction

Diabetes mellitus characterized by hyperglycemia, altered metabolism of lipids, carbohydrates and proteins (Yurii, Lebeda, Orlovskya, & Nikonenkoa, 2008).

Type I or insulin dependent, type II or insulin independent and Gestational diabetes are three general classifications of diabetes mellitus (Persaud, 2007). Diabetes mellitus is associated with peripheral neuropathy, and central

nervous system alterations in both human and animal models of the disease (Biessels, Heide, Kamal, Bleys & Gispen, 2002).

Dysfunctions of the central nervous system include abnormal expression of hypothalamic neuropeptides, hippocampal astrogliosis (Saravia et al., 2002), decreased hippocampal synaptic plasticity, neurotoxicity and changes in glutamate neurotransmission (Gardoniet al., 2002). Diabetic patients are prone to moderate alterations in memory and learning.

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Gestational diabetes mellitus (GDM) defined as impaired glucose tolerance affects approximately 4% of all pregnant women who have never before had diabetes, but who do have high blood glucose levels during pregnancy (Persaud, 2007).

The cerebellum has long been recognized as the primary center of motor coordination in the central nervous system (Gardoni et al., 2002; Ahmadpour & Haghiri, 2011). Recent studies in humans have also implicated the cerebellum in cognitive processing and sensory discrimination in medical conditions as diverse as pervasive developmental disorders, autism, and cerebellar vascular injuries (Allen, Yaqoob, & Harwood, 2005).

Disorder and disagreement in cerebellar structure is reported due to type 1 diabetes mellitus (Hernandez-Fonseca et al., 2009). Also, Min et al reported a decrease of neuron and thickness of cortex and white matter of cerebellum due to maternal diabetes in neonatal rats (Min et al., 2005).

Follow-up studies concerning the adverse effects of diabetic pregnancy on the developing brain have revealed neurobehavioral deficits in both sensory-cognitive and psychomotor functions, including altered auditory recognition memory processing at birth (Siddappa et al., 2004), reduce visual and memory performance at 8 and 12 months (DeBoer, Wewerka, Bauer, Georgieff, & Nelson, 2005), poorer performance on tests of general development in infants and toddlers and inferior performance in elementary school children (Ornoy, 2005).

While motor delay may be a sign of mild, nonspecific brain damage, the abnormalities in memory processing suggest alterations in hippocampal development and function (Nelson et al., 2000).

Although previous studies have shown the adverse effects of pre-gestational maternal diabetes on CNS including hippocampus, hypothalamus, cerebellum and cerebrum (Beauquis, Roig, Homo-Delarche, De Nicola, & Saravia, 2006; Ahmadpour & Haghiri, 2011) but there is no study about the effect of gestational diabetes on neuronal development of cerebellum. Therefore, this experimental study was design to assess the effect of gestational diabetes on the cerebellum at postnatal 21 and 28 day of Wistar rats.

2. Methods

2.1. Animals

This experimental study was performed at the Gorgan faculty of Medicine, Golestan University of medical sciences, Gorgan, Iran. Guidelines on the care and use of laboratory animals and approval of the ethic committee of Golestan University of medical sciences were obtained before study.

Experimental animals. Wistar rats, weighing 180-220 grams (12 weeks old) were used in this study. The animals were maintained in a climate-controlled room under a 12-hour alternating light/dark cycle, 20 °C to 25°C temperature, and 50% to 55% relative humidity. Dry food pellets and water were provided ad libitum.

2.2 Drugs

Streptozotocin (STZ) (Sigma, St. Louis, MO, USA) was dissolved in sterile saline solution (0.85%) to give 40 mg/kg dose intraperitoneally inject to female rats.

Histology

Animal groups and treatment: After 2 weeks of acclimation to the diet and the environment, female Wistar rats were placed with a proven breeder male overnight for breeding. Vaginal smears were done the next morning to check for the presence of sperm. Once sperm was detected that day was assigned as gestational day 1(GD). On day 1 of gestation, pregnant females were randomly divided into two control and diabetic groups.

Five female rats in diabetic group receiving 40 mg/kg/body weight of streptozotocin (STZ) and control groups (five rats) receiving an equivalent volume normal saline injection intraperitoneally (IP). Blood was sampled from the tail at 1 week after STZ injection. The dams with blood glucose level 120-250 mg/dl were considered as gestational diabetes (GDM). The pregnancy of dams was terminated physiologically.

Totally, six male offspring of gestational diabetic mothers and control mothers at postnatal 21 and 28 day (P21, P28) were randomly selected and were scarified. For light microscope preparations, cerebellum was fixed in 10% neutral-buffered formalin for histological procedure. The coronal sections (6 micrometer) were serially collected from bregma -9.96 mm to -11.88 mm of cerebellum (Paxinos & Watson, 1998). The sections were stained with cresyl violet.

Table 1. Maternal blood glucose level (mg/dl; Mean \pm SEM) on the insemination day, 21 and 28 day after delivery in control and streptozotocin-exposed groups.

Insemination Day		Day 21		Day 28	
Control	GDM	Control	GDM	Control	GDM
97.7 \pm 2.3	97.35 \pm 2.2	97.5 \pm 2.5	141.2 \pm 3*	97.8 \pm 2.0	155.5 \pm 3.5*

Results are expressed as Mean \pm SEM of the mean (*P < 0.001, n=5)

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Blood glucose measurements: Blood glucose level of mothers (before mating and after STZ injection) and offspring was obtained via tail vein and was estimated with a glucometer (ACCU-CHEK® Active Glucometer, Roche Diagnostics, Mann-heim, Germany).

Morphometric analysis:

At postnatal 21 and 28 day, the size (transvers diameter) of cerebellum was measured using the digital vernier caliper in the experimental and control groups.

In each sample, ten similar sections of anterior lobes of cerebellum were selected and images of five separate fields in the apex of cerebellar lobules and five separate fields in the depth of cerebellar lobules were captured by Olympus BX 51 microscope and DP12 digital camera attached to OLYSIA autobio report software (Olympus Optical, Co. LTD, Tokyo, Japan).

The morphometric analysis of cerebellum including densities of Purkinje cell, the number of Purkinje cells per 1000 μ m length of Purkinje cell line, diameter and area of the Purkinje cells were measured from high magnification (1000x). Also densities of granular cells (the number of the granular cells/ 10000 μ m² area of granular cell layer) and the thickness of cerebellar cortex including thickness of molecular layer (ML), Purkinje cell layer (PCL) and granular cell layer (GL) were measured from low magnification (20x objective) (Fig 1A and 1B).

Statistical analysis

Morphometric data is expressed as the mean \pm SEM and analyzed by the Student's "t" test using SPSS 16.5 software. P<0.05 was considered significant.

Table 2. The quantitative characteristics of the Purkinje cells of cerebellum in postnatal day 21 and 28 of gestational diabetes mellitus (GDM) and control mothers in Wistar rat.

Characteristics		Control (P21)	GD (P21)	Control (P28)	GD (P28)
Apex	PC density (PC Number /10000 μ m length of PCL)	10.5 \pm 0.6	8.08 \pm 0.5	10.8 \pm 0.8	8.41 \pm 0.7
	area of PCs (μ m ²)	155.43 \pm 7.2	219.75 \pm 9.1	145.54 \pm 7.7	175.8 \pm 8.7
	diameter of PCs (μ m)	12.44 \pm 0.3	15.61 \pm 0.5	12.63 \pm 0.4	14.18 \pm 0.5
	GC density at apex (GC Number /10000 μ m ² area of GCL)	26.56 \pm 0.6	22.21 \pm 0.6	25.72 \pm 0.7	21.31 \pm 0.6
	Depth	PC density (PC Number /10000 μ m length of PCL)	11.62 \pm 0.7	9.86 \pm 0.2	12.92 \pm 0.2
area of PCs (μ m ²)		119.85 \pm 5	186.2 \pm 6.6	119.48 \pm 9.4	148.62 \pm 7
diameter of PCs (μ m)		12.75 \pm 0.3	16.19 \pm 0.6	12.74 \pm 0.3	14.46 \pm 0.3
GC density (GC Number /10000 μ m ² area of GCL)		29.16 \pm 0.6	24.65 \pm 0.5	30.08 \pm 0.8	25.23 \pm 1.3

Results are expressed as Mean \pm SE of the mean, P<0.05 versus control, n=6

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3. Results

Blood glucose concentrations

The mean \pm SEM of blood glucose concentrations before mating and in day 21 and 28 after delivery of gestational diabetic and control mothers is depicted in Table 1. Blood glucose level significantly increased in gestational diabetic dams after STZ injection compared to controls ($P < 0.05$).

Morphometric results

The morphometric findings are depicted in Table 2, 3 and Fig 2 (apex and depth).

Purkinje cells density:

The Purkinje cells density of apex and depth(PC number /10000 μm length of PCL)of cerebellum in P21, in the experimental group reduced 23% and 15% in comparison with the control group (apex: 8.08 ± 0.5 vs 10.5 ± 0.6 , depth: 9.86 ± 0.61 vs 11.62 ± 0.72 , $P < 0.007$).

The Purkinje cells density of apex and depth of cerebellum in P28, in the experimental group reduced 22.12% and 12.62% in comparison with the control group (apex: 8.41 ± 0.72 vs 10.80 ± 0.87 , depth: 11.29 ± 0.27 vs 12.92 ± 0.29 , $P < 0.001$).

The mean area and diameter of the Purkinje cells in the treated group was significantly larger than the control group (Table 1).

Granular cells density:

In P21, the granular cells density of apex and depth(GC number /10000 μm^2 area of GCL) in the experimental group reduced 19.58% and 18.3% in comparison with the control group (apex: 22.21 ± 0.6 vs 26.56 ± 0.6 , depth: 24.65 ± 0.5 vs 29.16 ± 0.6 , $P < 0.001$). In P28, The granular cells density of apex and depth in the experimental group reduced 17.14% and 16.12% in comparison with the control group (apex: 21.31 ± 0.6 vs 25.72 ± 0.7 , depth: 25.23 ± 1.37 vs 30.08 ± 0.82 , $P < 0.001$).

The thickness of the cerebellar cortex layer:

The thickness of molecular, Purkinje and granular layers at the apex of cerebellar lobules of the experimental group significantly reduced in comparison with the control group ($P < 0.05$).

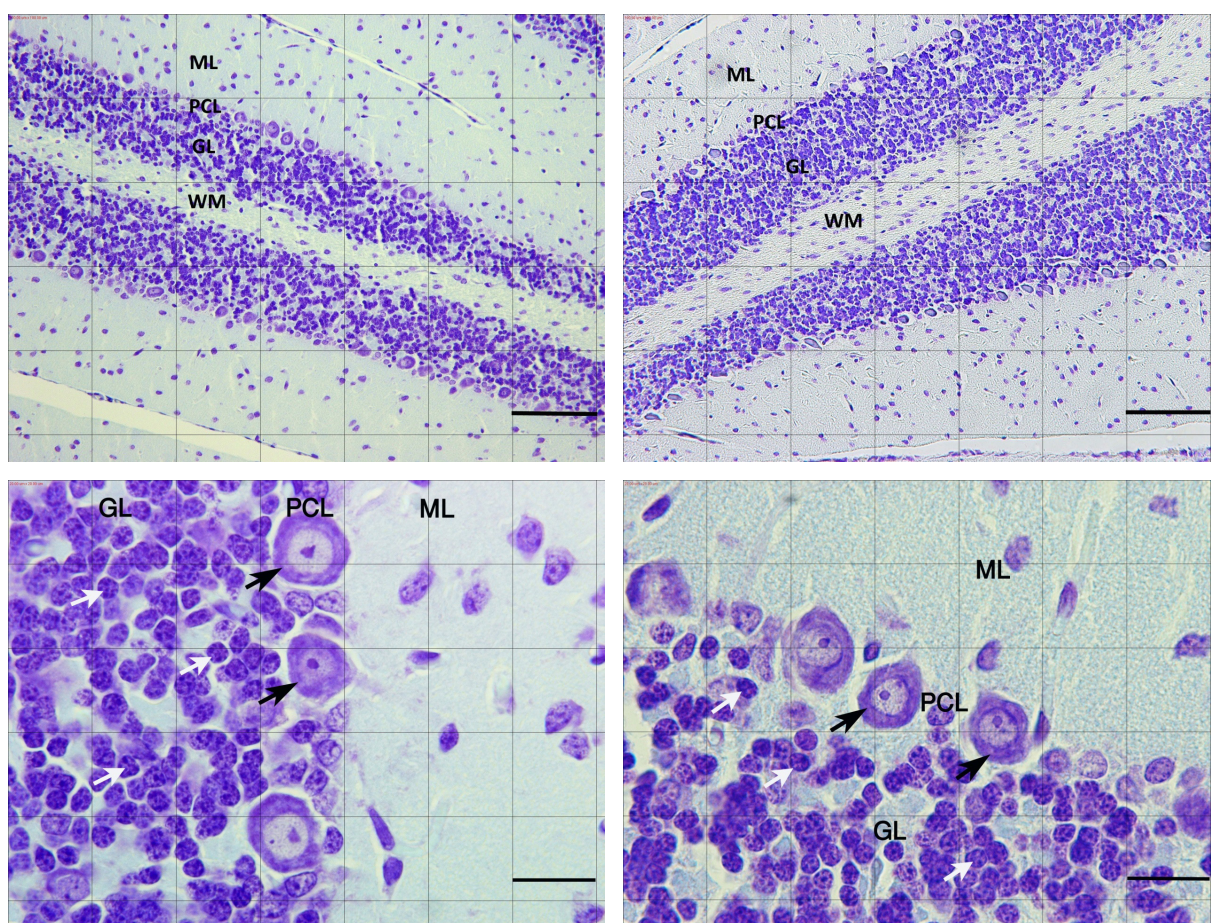
The thickness of molecular layer at the depth of cerebellar lobule in experimental group (P21) reduced in comparison with the controls (157.94 ± 6.10 vs 185.42 ± 9.44), the Purkinje layer in experimental group reduced compared to the control group (20.80 ± 0.32 vs 23.33 ± 0.39), and granular layer significantly reduced in comparison with the control group (114.18 ± 2.53 , 135.11 ± 11.42).

The thickness of molecular layer at the depth of cerebellum in experimental group (P28) reduced in comparison with the control group (166.85 ± 5.79 vs 194.49 ± 4.21), the Purkinje layer in experimental group reduced in comparison with the control group (21.32 ± 0.55 vs 23.77 ± 0.62), and granular layer significantly reduced in comparison with the control group (118.46 ± 4.13 , 138.39 ± 7.98 , $P < 0.05$).

Table 3. The thickness of the various layers of cerebellar cortex (μm) in postnatal day 21 and 28 (P21, P28) of gestational diabetes (GDM) mothers and control mothers in Wistar rat

Layer Control	P21			P28			
	GD	P	Control	GD	P	Control	
apex	ML	99.77 ± 4.6	89.37 ± 2.8	.05	117.73 ± 6.3	110.9 ± 3.4	.05
	PCL	22.25 ± 1.1	19.56 ± 0.5	.02	22.61 ± 0.9	19.97 ± 0.6	.03
	GL	215.86 ± 8.8	196.6 ± 2.9	.01	210.11 ± 4.4	176.15 ± 6.0	.01
depth	ML	185.41 ± 9.4	157.94 ± 6.1	.01	194.48 ± 4.2	166.84 ± 5.7	.003
	PCL	23.33 ± 0.3	20.8 ± 0.3	.001	23.77 ± 0.6	21.32 ± 0.5	.01
	GL	135.10 ± 11.4	114.17 ± 2.5	.02	138.38 ± 7.9	118.45 ± 4.1	.02

ML: molecular layer, PCL: Purkinje cell layer, GL: granule cell layer. Results are expressed as Mean \pm SE of the mean, $P < 0.05$ versus control, n=6



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Figure 1. Histological section of cerebellum cortex postnatal day 28 (P28) Wistar rat in A: control and B: case group (20× magnification, scale bar: 100 μm). C: control and D: case group (1000× magnification, scale bar: 20 μm). Section stained with cresyl violet. ML: molecular layer, PCL: Purkinje cell layer, GL: granule cell layer, WM: white matter, black arrow: Purkinje cell, white arrow: granular cell.

The transvers diameter of cerebellum in the experimental group (P21) significantly reduced in comparison with the control group (12.53 ± 0.43 vs 11.10 ± 0.05 mm) $P < 0.05$.

The transvers diameter of cerebellum in the experimental group (P28) significantly reduced in comparison with the control group (12.82 ± 0.67 vs 11.23 ± 0.03 mm) $P < 0.05$.

4. Discussion

The present study demonstrated that gestational diabetes produces a significant reduction in the density of the Purkinje and Granular cells and several layers of cerebellum in the postnatal day 21 and 28 of Wistar rats.

Previous studies have shown reducing neuronal cells density in animals with type 1 diabetes mellitus (Beauquiset et al., 2006; Ahmadpour & Haghiri, 2011). Also Hernandez-Fonseca et al., study has shown that STZ-induced diabetes increased apoptosis in pyramidal neu-

rons in cortex and cerebellar Purkinje cells in adults' rats (Hernandez-Fonseca et al., 2009).

Indeed, Khaksar et al. study has shown the adverse effects of maternal diabetes on reduction of neuron and thickness of cortex and white matter of cerebellum in neonatal rats (Khaksar, Jelodar & Hematian, 2010).

In spite of several studies regarding the effects of diabetes I and II on CNS including cerebellum, there is no investigation about the effect of gestational diabetes on cerebellar neurons in 21 and 28 day offspring.

Our animal model study demonstrated that gestational diabetes similar to type I and II diabetes mellitus, established a significant reduction in the cerebellar Purkinje and granular cells in the postnatal 21 and 28 day of Wistar rats.

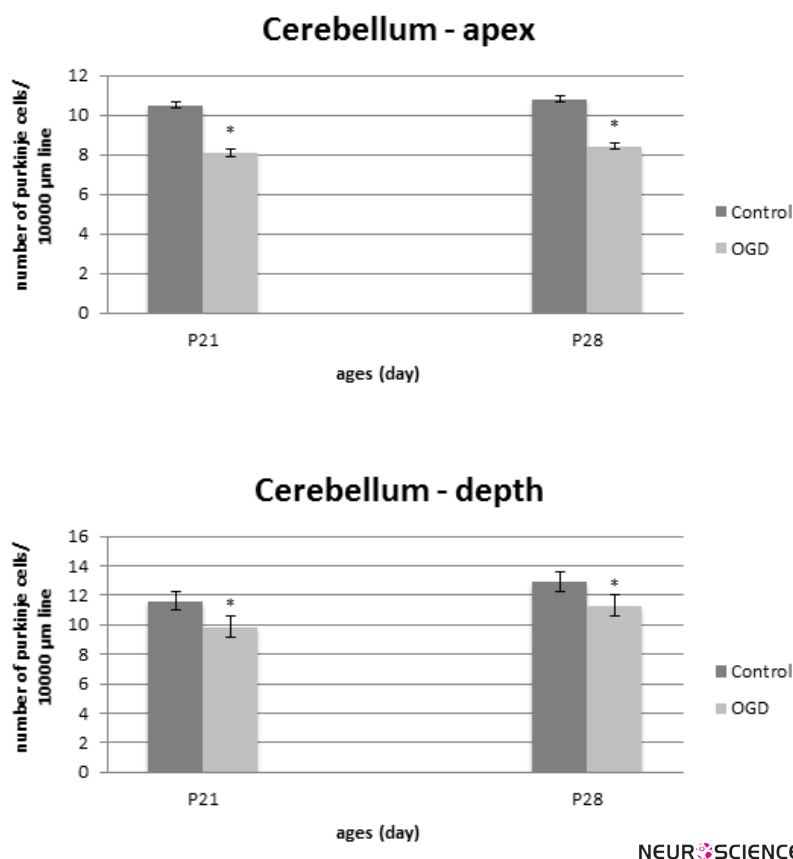


Figure 2. The mean number of Purkinje cells in apex and depth of cerebellum of postnatal day 21 and 28 of offspring in gestational diabetes mellitus (GDM) and control dams in Wistar rats. The cells were expressed as the number of Purkinje cells per 10000 μm^2 , (results are means \pm SEM, *Compared with controls $P < 0.05$, $n = 6$).

The reduction of Purkinje cell density of cerebellum can be due to program cell death or block of neurogenesis in CNS, including cerebellum (Hernandez-Fonseca et al., 2009). Hyperglycemia could induce cellular death by enhancing tissue acidosis (DeBoeret al., 2005).

Diabetes mellitus, regardless of its type, is associated with hyperglycemia. Several possible mechanisms are explained about cerebral alterations including neuronal loss of cerebellum due to hyperglycemia.

Diabetes mellitus is associated with increased oxidative stress in central nervous system (Grilloet al., 2003). The polyol pathway is activated during hyperglycemia and leads to consumption of NADPH and depletion of glutathione, which in turn lowers the threshold for intracellular oxidative injury (Klein & Waxman, 2003).

CNS complications of diabetes mellitus could be mediated through excessive free radicals generation (Okouchi, Okayama, & Aw, 2005; Ahmadpour & Haghiri, 2011). These radicals contribute to increase neuronal death by

oxidizing proteins, damaging DNA, and inducing the lipoperoxidation of cellular membranes (Hawkins & Davies, 2001).

Hyperglycemia may cause brain acidosis and dehydration, both involved in diminished cerebral blood flow and ischemia (Chen & Goeddel, 2002; DeBoer et al., 2005). Ischemia-related edema involves stimulation of brain Na-K-Cl cotransporter system facilitating edema formation and swelling of endothelial cells (Gardoni et al., 2002).

Also, in other cellular responses increase formation of advanced glycosylation end-products damages endothelial cells, therefore it is contributed to vascular damage indeed, during hyperglycemia. Diacylglycerol activation of protein kinase C has negative effects on cerebral blood flow and vascular permeability (Klein & Waxman, 2003).

Indeed, Several studies have shown that offspring of diabetic mothers have lower arachidonic acid (AA:20:4n-6)

and docosahexaenoic acid (DHA:22:6n-3) in cord blood (Min et al., 2005).

Arachidonic Acid metabolite and prostaglandin E2 plays an important role in neurogenesis. Zhao et al (2009) have reported that maternal arachidonic acid supplementation improves neurodevelopment in young adult offspring from rat dams with and without diabetes (Zhao, Del Bigio & Weiler, 2009).

Also, other possible mechanism regarding program cell deaths in diabetes mellitus (Allen et al., 2005; Lechuga-Sancho et al., 2006; Hernandez-Fonseca et al., 2009) can be due to decrease insulin or insulin-like growth factor signaling or an increase in cytokines such as TNF α (Chen & Goeddel, 2002).

Moreover, insulin-like growth factor has a neuroprotective and anti-apoptotic effect and down regulation of expression of insulin-like growth factor and its receptor in diabetes might also lead to neuronal loss (Romero, Liu, Asnaghi, Kern, & Lorenzi, 2002).

Furthermore, several studies have shown that the damage to both presynaptic and postsynaptic structures in the hippocampus in diabetes from hyperglycaemia induced alterations in the handling and homeostasis of intracellular calcium concentrations (Magarinos & McEwen, 2000).

Down regulation of nitric oxide synthase (NOS), mRNA and protein concentrations are the main factors in active response of cells due to hyperglycemia. These changes are shown in hippocampal neurons (Reagan & McEwen, 2002).

The increasing size of cerebellum in offsprings in experimental group compared to controls can indicate the edema in cerebellum due to gestational diabetes in dams.

This study showed the uncontrolled gestational diabetes induces reduction of the Purkinje neurons in offspring. Further studies are required for exploring the exact mechanism of CNS complications of gestational diabetes mellitus.

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