

Effect of maternal diabetes on gliogenesis in neonatal rat hippocampus

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Abstract

Background: Diabetes in pregnancy is a common metabolic disorder associated with various adverse outcomes in the offspring including impairments in attention and memory and alterations in social behavior. Glial cells are proven to have a critical role in normal function of neurons, and alteration in their activity could contribute to disturbance in the brain function. The aim of this study was to investigate the effect of maternal diabetes on hippocampal mRNA expression and distribution pattern of glial fibrillary acidic protein (GFAP) immunoreactive glial cells in the dentate gyrus (DG) of rat neonate at postnatal day 14 (P14). **Materials and Methods:** Wistar female rats were randomly allocated in control, diabetic, and insulin-treated diabetic groups. Diabetes was induced by injection of streptozotocin from 4 weeks before gestation until parturition. After delivery, the male offspring was euthanized at P14.

Results: Our results showed a significant higher level of hippocampal GFAP expression and an increase in the mean number of GFAP positive cells in the DG of diabetic group offspring ($P < 0.05$). We also found an insignificant up-regulation in the expression of GFAP and the mean number of positive cells in the insulin-treated diabetic group neonates as compared to control group ($P > 0.05$).

Conclusion: The present study revealed that diabetes during pregnancy strongly increased the glial cells production in the developing rat hippocampus.

Key Words: Glial cell, hippocampus, maternal diabetes, neonatal rat

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INTRODUCTION

Diabetes is a common metabolic disorder in pregnancy that is associated with structural and functional alterations of various organs including the central nervous system (CNS).^[1-3] Earlier studies clearly confirmed the negative effects of intrauterine hyperglycemia on the development of the CNS although the precise mechanism of maternal diabetes

effects on the CNS during fetal development remains unknown.^[4-6]

Maternal diabetes is characterized by an increase placental transport of glucose and other nutrients from the mother to the fetus.^[7] In addition, hypertrophy and hyperplasia of the pancreatic insulin-producing β cells have been recognized for many years as typical features in fetuses and newborn babies of diabetic mothers.^[8-10]

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Several lines of investigations provide evidence of the deleterious effects of maternal diabetes on long-lasting neurological impairment in their children that is manifest as impairment in attention and memory,^[4] deficits in balance and motor coordination,^[11] hyperactivity, and alter social behavior.^[12] Many researchers also reported other types of neurodevelopment disorders observed in offspring born to diabetic mothers including short attention span,^[13,14] delay in speech and language,^[4,12] lower cognitive scores,^[4] and learning difficulties at school age.^[13] Churchill *et al.* found that offspring of diabetic mothers had significantly lower mean intelligence quotient scores than control infants.^[4]

The hippocampus is a portion of the brain that has a crucial role in spatial learning and memory.^[15-17] It is demonstrated that hippocampal neurons are vulnerable to changes in glucose concentration, especially during CNS development.^[18] During postnatal development in rodents, first 2 weeks after birth is a period of continued active hippocampal dentate neurogenesis corresponding to the mid-second through the mid-third trimester of human gestation.^[19-21]

The importance of neurogenesis on CNS development comes from a wide variety of *in vitro* experimental data, the latter predominately derived from rodent studies.^[22] In mammals, the hippocampal dentate gyrus (DG) is one of the known brain regions where ongoing neurogenesis occurs throughout life.^[23-25] It has been documented that various environmental factors including toxins and diseases (such as diabetes) decrease the production of these new cells.^[26,27]

Glial cells are proving critical for normal CNS function. They have an important role in the blood-brain barrier maintenance, reactive oxygen species (ROS) protection, neuronal activity modulation, and synaptic transmission as well as control of extracellular pH, ion, and neurotransmitter concentrations.^[28-32] The previous studies demonstrated that CNS complications such as trauma, ischemia, tumors, neuroinflammation, and neurodegenerative disorders lead to astrocytic activation, also known as reactive gliosis or astrogliosis, increasing the production of intermediate filaments. In such conditions, reactive astrocytes became highly positive for glial fibrillary acidic protein (GFAP) and vimentin as intermediate filaments.^[30,32]

Because of glial cells have a critical role in normal function of neurons and alterations in their activity could contribute to disturbances in the brain function; in the present study, we investigated the effect of

diabetes during pregnancy on alterations in developing hippocampal astrocyte of rat neonates.

MATERIALS AND METHODS

Animals

Thirty young adult female Wistar rats (weighing 200–250 g) were used in this study. The dams were purchased from the animal house of Isfahan University of Medical Sciences. Animals were housed in a temperature and humidity controlled colony room under diurnal lighting conditions (12 h of darkness and 12 h of light) and were allowed to adapt to human handling. Food and water were provided *ad libitum*. All of the animal experiments were approved by the Ethics Committee for Animal Experiments at Isfahan University of Medical Sciences, Isfahan, Iran.

Treatment

Animals were subdivided into three groups as follows: Diabetic group (streptozotocin [STZ]-D; $n = 11$), diabetic group treated with insulin (STZ-INS; $n = 11$), and control group ($n = 8$).

Diabetes was induced by a single intraperitoneal injection of STZ (Sigma, 45 mg/kg body weight) which was freshly dissolved in normal saline.^[15] Glycemia was assessed by the puncture of tail vein using a digital glucometer (BIONIME, Switzerland). Only rats with glycemic values ≥ 350 mg/dL were considered as diabetics.^[18] Control animals were injected with normal saline only.

Treatment of diabetic animals was conducted after the verification of diabetes. Four to six units of protamine zinc insulin (NPH) (EXIR Pharmaceutical Company, Iran) were delivered subcutaneously. The dose of insulin was determined on the basis of a daily blood glucose test.

Animals were mated with nondiabetic males overnight starting a week after treatments. The presence of a vaginal plug the following morning was designated as day 1 of pregnancy (GD1). The pregnant rats confirmed as diabetic on the 1st day of pregnancy were separated and kept in individual cages until the birth of the offspring (20–22 days).

Histological preparation

At the end of pregnancy, animals were allowed to naturally deliver. The day of the birth was defined as postnatal day 0 (P0). Newborn rats born to diabetic and insulin-treated diabetic mothers were fostered onto control mothers to exclude other effects by the milk of diabetic rats, and thus enabled to focus only on the environment of the fetal period. Male offspring were

deeply anesthetized intraperitoneally with chloral hydrate (400 mg/kg body weight) and transcardially perfused at P14 with 0.9% saline followed by 4% buffered paraformaldehyde and brains were removed rapidly and then immersed in a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 48 h at 4°C. The brain tissues were processed by the routine histological method and embedded in paraffin blocks. For the stereological study, coronal serial sections (5 µm thickness) with 420 µm intervals were obtained through the entire hippocampus by a rotary microtome (Leica, Vienna, Austria) resulting a series of 12 coronal serial sections using systematic uniformly random sampling from each animal. Sections were considered for immunofluorescence staining, mounted on polylysine coated slides.

Immunofluorescence staining

To determine the amount and localization of GFAP in neonates hippocampus, we performed immunofluorescence technique as described previously.^[7] Briefly, the hippocampal sections were deparaffinized in xylene, rehydrated in a decreasing ethanol series, and rinsed in Tris-buffered saline (TBS; 0.1 M Tris-HCl, pH 7.4, and 0.9% NaCl). Antigen retrieval was performed with incubation of 10 mM citrate buffer solution for 15 min at 100°C. After several washes in TBS, sections were incubated in blocking solution (3% goat serum, 0.1% Triton X in TBS for 60 min at room temperature.^[33] Hippocampal sections were then incubated overnight with primary antibody; Rabbit polyclonal GFAP antibody (1:200; abcam; Cambridge, MA, USA) at 4°C. On the next day, after two washes with TBS, the sections were incubated in a secondary antibody; Goat Anti-Rabbit Alexa Fluor® 555 (IgG H and L); (1:1000; abcam; Cambridge, MA, USA) for 2 h at room temperature. Nuclear counterstaining was performed with 4', 6-diamidino-2-phenylindole dihydrochloride hydrate for 3 min. After two washes with TBS, slides mounted with glycerol buffer, coverslipped, and then visualized with a fluorescence microscope and digitally photographed (Zeiss, Axiophot, Germany). For negative control, the same protocol was used, but primary antibody was omitted.

The numerical density and number of GFAP-immunoreactive cells within the hippocampal dentate DG estimated by an investigator blinded to the protocol treatment, using the optical dissector technique according to our previous study.^[34] The optical dissector technique eliminates bias in counting because of cell size and shape. Briefly, for each section, 8–10 unbiased counting frames were

sampled in a systematically random fashion inside the area of DG. The preparations were examined under an Eclipse microscope (E200, Nikon, Tokyo, Japan), with a high numerical aperture (1.25) ×60 oil-immersion objective. Images were transferred to computer and an electronic microcator with digital readout (MT12, Heidenhain, Traunreut, Germany) using a high-resolution camera (BX51, Japan).

Real-time reverse transcription-polymerase chain reaction technique

The hippocampi were dissected immediately and stored at –80°C in RNA later stabilization reagent (Qiagen, Valencia, CA, USA) until use total RNA was isolated by using the RNeasy Mini Kit with RNase-Free DNase Set (Qiagen, Valencia, CA, USA) to ensure maximal removal of DNA during RNA purification. The RNA samples from three individual animals per group were used for the synthesis of cDNA with a RevertAid™ first strand cDNA Synthesis Kit (Fermentas; K1621; St. Leon-Rot, Germany) according to the manufacturer's recommendations. Relative gene expression analysis was performed with a Maxima SYBR Green/ROX quantitative polymerase chain reaction (qPCR) Master Mix (×2) Kit (Fermentas; K0221; St. Leon-Rot, Germany) based on the manufacturer's instructions recommendations. In each PCR, 10 µl Power SYBR Green PCR Master Mix ×2 was mixed with 2 µl cDNA and 10 pM/µl of each (forward and reverse) specific primer in a total volume of 20 µl. Relative quantitative real-time was performed using the Gene Amp 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). With the following cycling parameters: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, followed by amplicon dissociation (95°C for 15 s, 60°C for 1 min, increasing at 0.3°C/cycle until 95°C was reached). The endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the quantification of the mRNA target, and nonspecific amplifications were verified by a dissociation curve. All the reactions were performed in triplicate. Gene expression results were calculated using the delta cycle threshold ($2^{-\Delta\Delta Ct}$) method.^[35]

The primer sets were designed based on sequences from the National Center for Biotechnology Information (NCBI) database and checked for specificity using the NCBI BLAST tool (www.ncbi.nlm.nih.gov/BLAST); primers with no significant similarity to other loci were selected. The following primers were used: Sense: 5'-TTACCAGGCAGAACTTCGG-3'-anti sense: 5'-TCATCTTGAGCTTCTGCC-3 for GFAP;

sense: 5'-CTCCATTCTTCCACCTTTG-3', anti-sense: 5'-AGCCATATTCATTGTCATACCAG-3' for GAPDH.

Statistical analysis

Statistical analysis was performed using the SPSS statistical package (version 20; SPSS Inc., Chicago, IL, USA). Differences between groups were determined using independent sample *t*-test and one-way analysis of variance followed by Tukey's tests. The results are expressed as a mean \pm standard error of the mean and considered significant at $P < 0.05$.

RESULTS

The level of blood glucose concentration at before pregnancy was 102 ± 7.0 , 440 ± 45.3 , and 110 ± 8.8 in control, STZ-D, and STZ-INS groups, respectively. Whereas the level of blood glucose at the end of pregnancy was 105 ± 9.9 , 450 ± 56.4 , and 105 ± 6.1 in control, STZ-D, and STZ-INS groups, respectively. It was tried to maintain the level of blood glucose concentration during pregnancy period.

Immunofluorescence analysis

Figure 1 indicates the distribution of GFAP immunoreactive cells in hippocampal DG of control (a), STZ-D (b), and STZ-INS (c) groups' rat at P14. The statistical differences between the mean numbers of GFAP positive cells in DG of various studied groups pups at P14 also shows in Figure 2. As noted, there was a significant higher number of GFAP-immunoreactive cells in STZ-D group neonates in comparison to controls ($P < 0.05$). Nevertheless, no statistical differences were found in the mean number of GFAP positive cells in the DG between STZ-INS and control neonates [Figure 2].

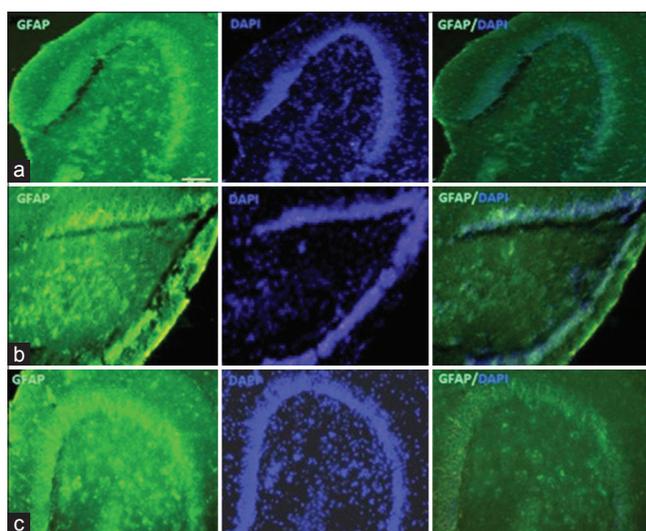


Figure 1: Photomicrographs showing distribution of glial fibrillary acidic protein-positive cells in hippocampal dentate gyrus of control (a), streptozotocin-D (b), streptozotocin-insulin and (c), rat offspring at postnatal days 14. Scale bar = 40 μ m

Real-time reverse transcription polymerase chain reaction

qRT-PCR was used to assay the effect of maternal diabetes and insulin therapy on the mRNA expression of GFAP marker in the hippocampus of rat newborns at P14. The expression levels of this marker in three studied groups are separately indicated in Figure 3.

Compared to control newborns, the expression of GFAP marker markedly up-regulated in offspring born to diabetic dams ($P < 0.05$). Regarding the expression of GFAP marker, we did not find any significant changes in their hippocampal expression between insulin-treated diabetic group neonates and controls at P14 ($P \geq 0.05$).

DISCUSSION

To our knowledge, this is the first study to examine the effect of diabetes during pregnancy period on hippocampal expression of GFAP gene and also numerical density of GFAP immunoreactive cells in DG of rat neonates using real-time qPCR and immunofluorescence staining methods. To induce diabetes in the current study we used a moderate dose of STZ (45 mg/kg) in agreement with the other works,^[36,37] this dose induced moderate diabetes with maternal and fetal hyperglycemia, maternal insulinopenia, and neonatal hyperinsulinemia, and also had a marginal effect on litter size.^[36-39]

The existing evidence clearly shows that diabetes during pregnancy is associated with higher risk of CNS abnormalities in offspring, including long-lasting neurological impairment that is manifested as impairments in attention and memory, hyperactivity, and altered social behavior.^[5,36,40] Various studies have

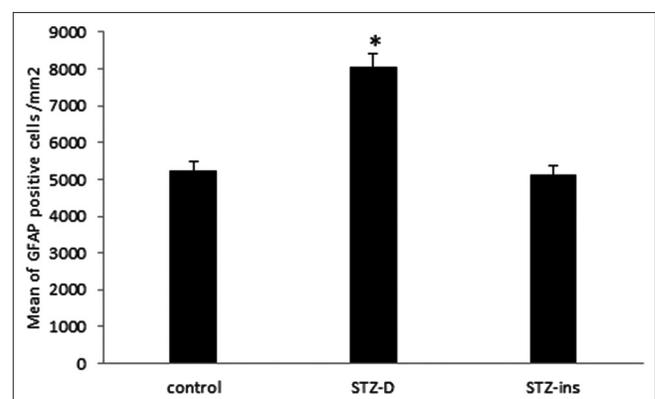


Figure 2: Comparison in the mean number of glial fibrillary acidic protein-positive cells in control, streptozotocin-D, and streptozotocin-insulin groups. The mean number of glial fibrillary acidic protein-positive cells showed a significantly increase in diabetic group as compared to control dams ($P < 0.05$). Values are mean \pm standard error of the mean; $n = 10$. * $P < 0.05$ compared to controls

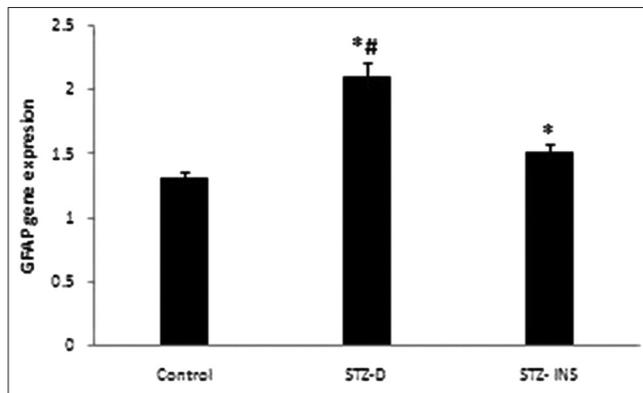


Figure 3: Comparison in glial fibrillary acidic protein expression in streptozotocin-D, streptozotocin-insulin and control groups. The glial fibrillary acidic protein expression showed a significantly increase in diabetic group pups as compared to control dams ($P < 0.05$). Values are mean \pm standard error of the mean; $n = 10$. * $P < 0.05$ compared to controls, # $P < 0.05$ compared to streptozotocin-insulin

addressed the question of possible CNS development disturbances induced by maternal diabetes, which may result in the alteration of many developmental events such as neurogenesis, migration, differentiation, and cell survival suggesting that maternal diabetes causes neuropathology via a number of mechanisms.^[41-43] Therefore, the effect of diabetes during pregnancy on the expression of genes that regulate brain growth/development is of considerable interest.

The present study results showed a significant higher number of GFAP-immunoreactive cells in DG of pups born to diabetic group as compared to others. These results show an increase in production of newly generated neurons in DG of neonates born to diabetic mothers. GFAP is an intermediate filament that is routinely known as a marker for astrocytes and other glial cells, but a large amount of newly generated neural cells also express GFAP in CNS.^[28-32] In contrast to microtubules and actin filaments, the composition of intermediate filaments changes among cell types, their developmental stages, and functional status.^[44] Astrocyte precursors and immature astrocytes present principally nestin and vimentin and, as astrocytes mature, GFAP becomes increasingly expressed.^[45]

Previous works reported that GFAP expression in freshly generated hippocampal neuron originates from precursor cells (type 1) which have a proliferative capacity. To date, many studies have demonstrated the existence of neural stem/progenitor cells in the CNSs of rodents and humans, restricted in two specific zone, subventricular zone of the lateral ventricles, and subgranular zone in the DG of the hippocampus.^[46,47]

Astrocytes are vulnerable to hypoxia *in vitro* and may be targets of hyperglycemic conditions.^[31] In a study

by Muranyi *et al.*, hyperglycemia caused astrocyte activation in early stage and astrocyte death in late stage.^[30] Further studies revealed that increased astrocyte damage coincided with enhanced production of free radicals.^[31] The other study indicated that diabetes or hyperglycemia caused early damage to astrocytes and increased production of reactive nitrogen species and ROS.^[30]

Saravia *et al.* demonstrated that astrocyte number was higher in early life in prediabetic mice than in control when transient hyperinsulinemia and lower glycemia were found.^[48] The number of GFAP positive cells further increased after the onset of diabetes in mice. Similarly, in STZ-treated diabetic mice, the number of GFAP immunoreactive cells was higher than in vehicle-treated mice.^[48] The researchers have also demonstrated that diabetic mice also showed abnormal expression of astrocyte markers in hippocampus. Thus, increased number of GFAP positive cells, indicative of astrogliosis, was found in stratum radiatum below the CA1 hippocampal subfield of diabetic mice.^[49]

In addition, in experimental models of type 1 diabetes mellitus a vast spectrum of neuronal changes have been reported.^[11,13,50] These pathological abnormalities include synaptic and neuronal alterations, degeneration, and neuronal loss, which collectively can lead to cognitive impairment and higher risk of development dementia.^[4,12,15,36]

CONCLUSION

Taken together, the present study indicated that maternal hyperglycemia may result in developmentally induced increase in the hippocampal GFAP expression and numerical density of GFAP positive cells in the DG of the offspring. These alterations may result in a delay in normal hippocampal development, and could be a reason for the structural, behavioral, and cognitive abnormalities observed in offspring of diabetic mothers. Future studies will be need to determine the effects of diabetes during pregnancy on the expression of other key molecules involved in the development of CNS.

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Conflicts of interest

There are no conflicts of interest.

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