Iranian Journal of Basic Medical Sciences

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IJBMS

Gestational diabetes influences retinal Muller cells in rat's offspring

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ARTICLE INFO

Article type:
Short communication

Article history:
Received: Dec 4, 2016
Accepted: Oct 20, 2016

Keywords:
Gestational diabetes
GFAP
Mellitus
Muller cell
Retina

ABSTRACT

Objective(s): The Muller cell is the principal glial cell of the vertebrate retina. The expression of Glial fibrillary acidic protein (GFAP) in the Muller cells was used as a cellular marker for retinal damage. This study was done to evaluate the effect of gestational diabetes on retinal Muller cells in rat's offspring.

Materials and Methods: In this experimental study, 12 Wistar rat dams were randomly allocated in control and diabetic groups. Gestational diabetes was induced by 40 mg/kg/body weight of streptozotocin at the first day of gestation, intraperitoneally. Dams in control group received an equivalent volume normal saline. Eye of six offspring of each group were removed at postnatal day 28 (P28). The histopathological changes in retina were examined through H&E staining and ultrastructure transmission electron microscopy (TEM). The expression of GFAP was examined using Immunohisto-chemical staining of GFAP in Muller cells. Photographs of retina were taken using Olympus BX51 microscope and a digital camera DP12 and EM LEO906; Zeiss, Germany.

Results: In the control rat's offspring, GFAP expression was not significant in Muller cells. According to the optical microscope images, GFAP expression was observed in the processes of the Muller cell in the inner plexiform layer of retina in offspring of diabetic mothers. In TEM technique, nuclear fragmentation and apoptotic bodies were observed in Muller cell of diabetic offspring.

Conclusion: This study showed that the uncontrolled gestational diabetes can increase GFAP expression in Muller cells and retinal thickness of retinal layer in rat offspring's, therefore uncontrolled gestational can damage the Muller cells.

► *Please cite this article as:*
Tabasi A, Ghafari S, Mehdizadeh M, Asadi Shekari M, Golalipour MJ. Gestational diabetes influences retinal Muller cells in rat's offspring. Iran J Basic Med Sci 2017; 20:216-221; http://dx.doi.org/10.22038/ijbms.2017.8251

Introduction

Gestational Diabetes Mellitus (GDM) is the most common metabolic complications of pregnancy, and causes fetal mortality and morbidity (1, 2). GDM is a state of glucose intolerance with the onset or first recognition occurring during pregnancy (3) and approximately occurs in 2-5 % of all pregnancies (4). Offspring of mothers with GDM are at increased risk for diabetes and obesity (5, 6). The gestational diabetes prevalence is reported 1-3% in the United States, 10.9% in Asian countries, 5.2% in Europe (7). A meta-analysis study indicated the prevalence of GDM ranged between 1.3% to 8.9% in different regions of Iran (8).

Diabetes is associated with long-term complications that affect almost every part of the body, often leading to blindness, cardiovascular disease and kidney failure and nerve damage (9). Long-term hyperglycemia causes irreversible pathological changes in the retina and leading to an increase in diabetic retinopathy (10). The decrease of retina ganglionic cell layer thickness in diabetes type 1 indicates that the retinal layers are mostly influenced by the effects of diabetes (11). Diabetes-induced cell death has been observed in numerous retinal cell types such as endothelial cells and pericyte, neural retinal cells (ganglion cells) and retinal glial cells (Muller cells, astrocytes and microglia) (12).

The Muller cell is the principal glial cell of the vertebrate retina; in the avascular retinae of many vertebrates (including mammals) it constitutes the only type of macroglial cells. Muller cells are specialized radial glial cells which span the entire thickness of the retina and contact/ensheath all retinal neuronal somata and their processes. Muller cells constitute an anatomical link between the retinal neurons and the compartments with which these need to exchange molecules, i. e., the retinal blood vessels, the vitreous body and the subretinal

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space (which, together with the retinal pigment epithelium (RPE), constitutes the pathway to the choroidal blood vessels) (13). Retinal glial cells, primarily Muller glia change from quiescent to an injury-associated phenotype and express high levels of Glial fibrillary acidic protein (GFAP; a hallmark of glial cell activation) in the human retina during early diabetes (14).

It has been reported that diabetes induces damage in avascular retinal neurons and Muller glial cells (11, 12). Generally, the mammalian retina contains three types of glial cells. In addition to microglial cells, there are two forms of neuron-supporting macroglial cells, astrocytes and Muller (radial glial) cells (13).

To assess astrocyte change, labeling experiments were performed on control and diabetic retinas using antibodies to GFAP a marker that labels retinal astrocytes but not Muller cells (15,16). Muller cells in the mammalian retina normally express low levels of glial fibrillary acidic protein (GFAP); however its expression is unregulated in response to the loss of retinal neurons. The change in expression of GFAP is one of the earliest indicators of retinal damage and is correlated with the time course of disease (17).

GFAP expression in the retinas of the diabetic rats was also detected in the end feet of the Muller cells. In the retina of control rats, GFAP expression was limited to astrocytes and was not detected in Muller cells even at 40 weeks of follow-up. The expression of glial fibrillary acidic protein in Muller cells was used as a cellular marker for retinal damage (18).

Diabetes induces abnormalities in retinal Muller cells, including increased expression of glial fibrillary acidic protein, reduction of glutamine syntheses and decreased function of glutamate transporter (19).

Plasma cell membrane of retinal Muller glial cell has an important function in regulation volume through outward water transport. Recent studies indicated that retinal edema can be caused by swollen Muller glial cells following cell injury and upregulation of GFAP. Therefore, this study was done to determine the effect of induced gestational diabetes on the expression GFAP in Muller cells of retinal layer in rat's offspring (20).

Also, a study has shown that the uncontrolled gestational diabetes can reduces the number of ganglionic neurons and increase apoptotic ganglionic cells of retina layer in rat offspring (21).

Regarding the important role of Muller cell in supporting of neuronal retinal cell, this study was done to determine the effect of induced gestational diabetes on Muller cells of retinal layer in rat's offspring.

Materials and Methods

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 Ethics Committee of Golestan University of Medical Sciences were obtained before the study.

Wistar rats, weighing 180-220 g (12 weeks old) were used in this study. The animals were maintained in a climate-controlled room under a 12 hr 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*.

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 observed that day assigned as gestational day 0 (GD0). On day 1 of gestation, pregnant females randomly divided in two control and diabetic groups. Six female rats in diabetic group were received 40 mg/kg/body weight of streptozotocin (STZ) (Sigma, St Louis, MO, USA) dissolved in sterile saline solution (0.85%) and control group (six rats) were received an equivalent volume normal saline intraperitoneally (IP).

Blood glucose level of mothers (both before mating and 72 hr after STZ injection) was obtained via tail vein and was measured with a glucometer (ACCU-CHEK® Active Glucometer, Roche Diagnostics, Mannheim, Germany) (22). The dams with blood glucose level 120-250 mg/dl were considered as gestational diabetes (21, 23).

Six offspring of gestational diabetic mothers and control mothers in 28th day after birth (postnatal day 28) were randomly selected and were killed quickly with anesthesia. For light microscope preparations eyes were fixed in 10% neutral-buffered formalin and the tissue processing eyes sectioned at 6-micrometer thickness using a microtome (Microm HM 325, Germany).

Eye tissue sections were stained using hematoxylin & eosin. For morphometric evaluation, 10-20 sections were observed with digital light microscopy. A photograph of sections was produced using an Olympus BX51 microscope and a DP12 digital camera. The density of Muller cells evaluated in 60000 μm^2 inner nucleus layer of eye and the thickness of inner retinal layer using OLYSIA Autobioreport software.

Immunohistochemistry

Immunocytochemical labeling to detect the Muller cells was performed by monoclonal antibody anti GFAP (Millipore corporation Billerica, USA) on eye coronal sections with 6 μm thickness. In brief, deparaffinized sections were preincubated with citrate buffer and were washed for 9 min in 0.01 M phosphate-buffered saline (PBS, pH 7.4) and treated with 0.3% hydrogen peroxide in 0.01 M PBS including 10% methanol. The eye sections were preincubated with blocking reagent and washed in 0.01 M PBS. Then, eye sections were incubated with antiGFAP (1:600) in a humidified

Table 1. The mean±SEM of blood glucose level (mg/dl) in control and gestational diabetes dams in day 0 and day 3 after induction of diabetes

Day	Control	Gestational diabetes	*P*-value
GD0	99.60±6.2	100.42±2.1	NS
GD3	92.53±5.3	211.60±6.3	* 0.001

NS: Non significant, * *P*-value <0.05, n=6)

chamber for 1 hr at room temperature. After rinse in 0.01 M PBS, the sections were incubated with the biotinylated secondary for 10 min and then with Streptavidin HRP and rinsed in PBS. Immunoreactivity was visualized using 3,3' diaminobenzidine (DAB; chromogen reagent) for 30 min at room temperature. Subsequently, the tissue specimen was counterstained with Mayer's hematoxylin and mounted with Entellan (Merck, USA).

Electron microscopy technique

The eyes were removed and immersed in the fixative solution (250 ml of 4% paraformaldehyde, pH 7.4 at room temperature), overnight. A 400-µm block of area retina was dissected and fixed in buffered 2.5% glutaraldehyde for an additional 48 hr. Then the sections were washed in PBS solution and postfixed in 1% OSO_4 for 2 hr at room temperature. After dehydration in ascending graded ethanol, were embedded in Epon 812 resin. After that, they were put onto slices with resin and polymerized for 48 hr at 60 °C. Subsequently, 60 nm sections were cut and stained with 1% uranyl acetate and 2% lead citrate. Sections were examined with a Philips EM300 transmission electron microscope.

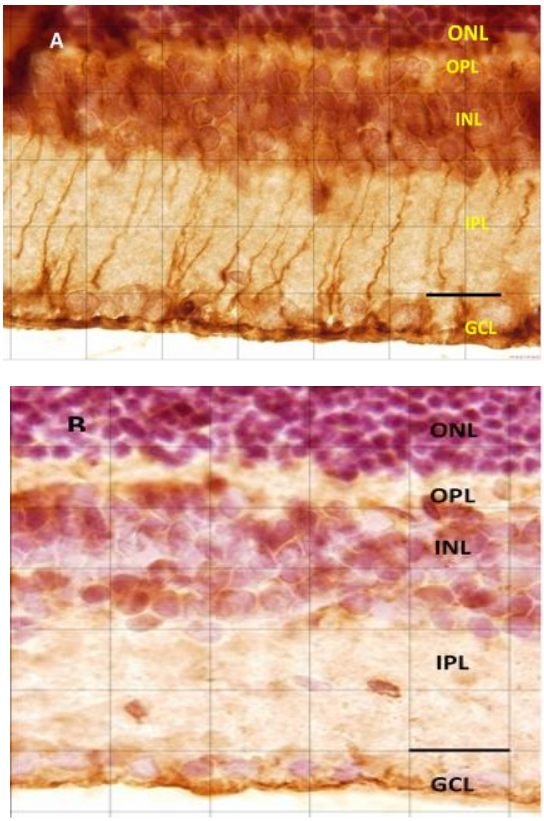
Statistical analysis

Statistical analysis was done by means of the statistical package SPSS 16. All data are given as mean ±standard error of the mean (SEM). Comparisons between pairs of groups were carried out using Student's t test. Values of $P<0.05$ were considered to be statistically significant.

Results

Blood glucose concentrations

The mean±SEM of blood glucose concentrations before mating and 72 hr after STZ injection were 100.42±2.1 and 211.60±6.30 mg/dl in diabetic dams. In control dams the mean±SEM of blood glucose concentrations before mating and 72 hr after STZ injection were 99.60±6.2 and 92.53±5.3 mg/dl, Table 1.



Figuer 1. Immunohistochemistry by monoclonal antibody anti GFAP of retinal layers in postnatal day 28 of Wistar rat. A) It is prominent in Muller cell inner processes in gestational diabetic retina, B) Control retina is GFAP negative (not shown). (ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglionic cell layer, 1000X, Scale bar: 20 µm)

Morphometric results

Number of Muller cell processes

The number of Muller cell processes per 6000 µm² area of inner nucleus cell layer of retina were significantly increased in offspring of gestational diabetic mothers in comparison to controls ($P<0.05$) Figure 1, Table 2.

Retinal layer thickness

The thickness of INL significantly increased from 26.36±0.93 µm in the control group to 29.89±0.46 µm in the experimental group ($P<0.001$) and the thickness of retinal layer significantly increased from 173.31±3.8 mm in the control group to 182.52±2.5 µm in the gestational diabetic group ($P<0.05$), Table 2.

Table 2. The number of Muller cell processes and the thickness of retinal layer in postnatal day 28 of gestational diabetic and controls rat offspring

Characteristics	Control	Gestational diabetes	*P*-value
Number of Muller cell processes per (6000 µm²)	-	46.09±2.3	<0.05*
Thickness of retinal layer (µm)	173.31±3.8	182.52±2.5	<0.037*
Thickness of INL (µm)	26.36±0.93	29.89±0.46	<0.001*

(Results were shown as mean±SEM, * *P*-value <0.05, n=6)

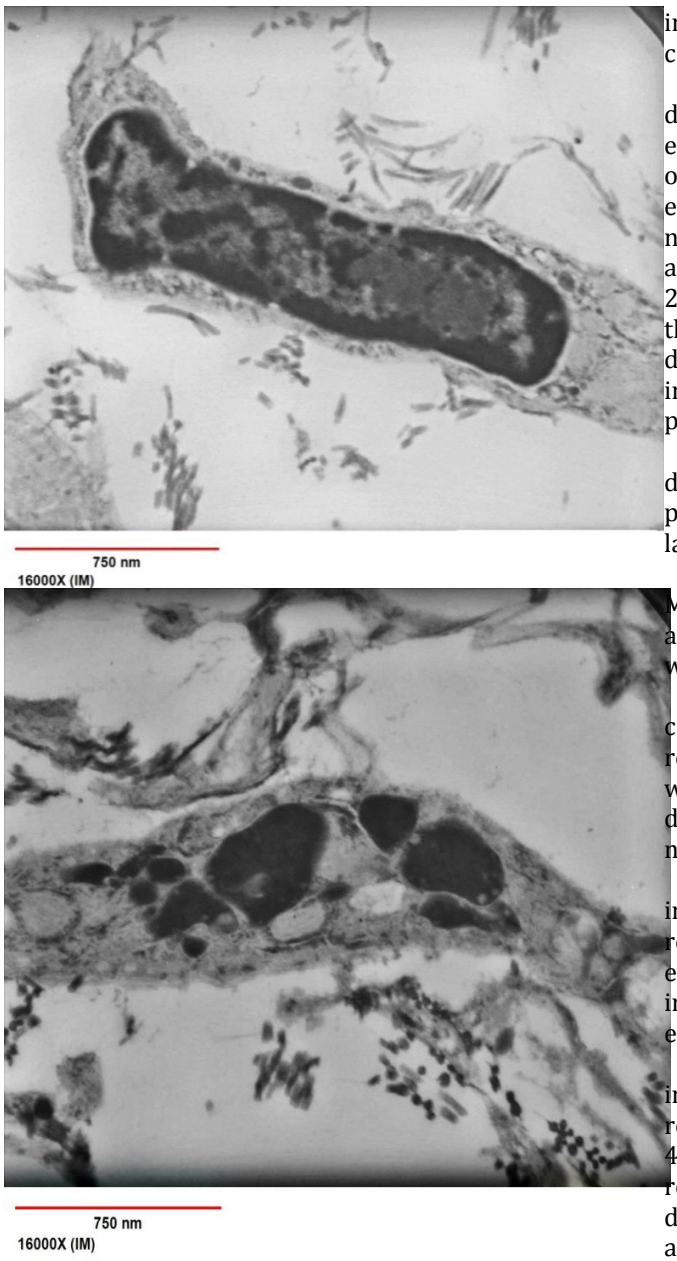


Figure 2. Electron micrograph, Muller cells in the retina in postnatal day 28 (P28) of Wistar rat

A) Gestational diabetes group showing nuclear fragmentation and apoptotic bodies in Muller cell. B) Normal structure in control group. 16000X (Scale bar: 750 nm)

Electron microscopy findings

Nuclear fragmentation and apoptotic bodies were observed in Muller cell of gestational diabetic offspring whereas control group showed normal structure by electron microscope, Figure 2.

Discussion

The focus of this article was the effect of gestational diabetes on retinal Muller cells. Muller cells are critically positioned between the vasculature and the neurons of the retina, has a important role in regulating the molecular composition of the retinal microenvironment (24).

The main clinical lesion that is caused by diabetes in the retina are those of blood vessels, but evidence is also mounting that neural and glial cells of the retina are affected early in both human and experimental diabetes (21, 25). The most prominent neuronal abnormality is apoptosis of cells whose size and location are consistent with ganglion cells (13, 21). The glial alterations explained to date relate to the pattern and level of expression of GFAP. In diabetes, Muller cells acquire prominent GFAP immunoreactivity throughout the extension of their processes (26).

This study showed that uncontrolled gestational diabetes increased expression of glial fibrillary acidic protein GFAP in the retinal Muller cells and retinal layer thickness of rat offspring.

Our finding is similar to several studies including Mizutani *et al* (1998), Ly *et al* (2011) , Li *et al* (2001) and Mancini et al (2013), although these studies were done on diabetes type 1.

Mizutani *et al* (1998) study on human eyes from certified eye banks through the national disease research interchange showed that the level of GFAP was increased in the diabetic retinas (161±106 densitometric units/μg protein vs 55±45 in the nondiabetic retinas, P= 0.03) (16).

Also, Li *et al* (2001) study in animal models using immunohistochemistry method by anti GFAP reported in the retinas from control rats, GFAP expression was limited to astrocyte. GFAP expression in the retinas of the diabetic rats was detected in the end feet of the Muller cells (27).

Indeed, Ly *et al* (2011) study on rats retina using immunohistochemistry method by anti GFAP reported Muller cell were labeled for the GFAP after 4 and 6 weeks of diabetes. Muller cells in central retina display increased GFAP from 10 weeks of diabetes, whereas retinal occurs in the peripheral after 6 weeks of diabetes (28).

Furthermore, Mancini *et al* (2013) study on two groups of Wistar rats injected with STZ two days after birth, reported in the two diabetic groups increased retinal immunoreactivity of GFAP in Muller cells but in the nondiabetic group GFAP expression was limited to astrocyte (29).

In our study, we observed increased GFAP expression in stalk of Muller cells in inner nuclear layer of retina. The cytological changes observed in Muller cells in response to injury are accompanied by significant alteration in gene expression. Whereas proteins such as GFAP, glutamate/aspartate transporter (GLAST) are upregulatrd under phatological conditions. Other proteins appear to be down-regulated.

Immunocytochemical and *in situ* hybridization studies have shown GFAP is not expressed by Muller

cells in embryonic or adult mouse retina, GFAP is integrated into Muller cell cytoskeleton and turns over extremely slowly or not at all. In contrast, GFAP mRNA is transcribed for a limited time and the gene is subsequently turned off. The increase in GFAP expression has been shown to be due to transcriptional activation of the GFAP gene in Muller cells. However, the cis and trans-activating factors that regulate GFAP gene expression in Muller cells have not been identified so far. Cell transfection and GFAP-lacZ transgenic mice studies indicate that cis elements that stimulate GFAP transcription in astrocytes and Muller cells are different. There is some evidence that growth factors and cytokines are the signaling molecules involved in GFAP induction.

Cellular mechanisms responsible for GFAP expression or mitotic activity in Muller cells: Molecules emanating from degenerating photoreceptors, and cytokines released from retinal compartments or secreted by activated macrophages, might act on Muller cells to induce GFAP expression or mitotic activity (30). Also in this study nuclear fragmentation and apoptotic bodies were observed in Muller cell of gestational diabetic offspring compared with normal structure control group in electron microscope images. This study is similar to Kumar *et al* study they reported degenerated and swollen Muller cell processes in TEM ultramicrograph in diabetic group (31).

Conclusion

We concluded that the uncontrolled gestational diabetes can increase GFAP expression and retinal layer thickness in Muller cells of retina layer in rat offspring. We suggested the babies born from mothers with gestational diabetes must be screened for retinal damage and dysfunction.

Acknowledgment

The study was supported by Gorgan Congenital Malformations Research Center and Deputy of Research of Golestan University of Medical Sciences, Gorgan, Iran (Grant number: 265727). The author's thanks from Neurosciences Research Center, Kerman University of Medical Sciences, Kerman, Iran.

This article is derived from thesis of Akramsadat Tabasi for MSc degree in field of anatomical sciences.

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