Increased Apoptosis in Subcortical Regions of The Visual Pathway in Offspring Born to Diabetic Rats

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Abstract

Objective: Diabetes in pregnancy is a prevalent disease that can affect the central nervous system of the fetus by hyperglycemia. This study aimed to investigate the impact of maternal diabetes on neuronal apoptosis in the superior colliculus (SC) and the lateral geniculate nucleus (LGN) in male neonates born to diabetic mothers.

Materials and Methods: In this experimental study, female adult rats were separated into three groups: control, diabetic (induced using an intraperitoneal injection of streptozotocin), and insulin-treated diabetic [diabetes controlled by subcutaneous neutral protamine hagedorn (NPH)-insulin injection]. Male neonates from each group were euthanized on 0, 7, and 14 postnatal days (P0, P7, and P14, respectively), and apoptotic cells were identified using TUNEL staining.

Results: The numerical density per unit area (NA) of apoptotic cells was significantly higher in SC and the dorsal LGN (dLGN) in neonates born to the diabetic rats compared to the control group at P0, P7, and P14. However, insulin treatment normalized the number of apoptotic cells.

Conclusion: This study demonstrated that maternal diabetes increased apoptosis in dLGN and SC of male neonates at P0, P7, and P14.

Keywords: Apoptosis, Lateral Geniculate Nucleus, Maternal Diabetes, Rat Brain, Superior Colliculus

Introduction

During pregnancy, diabetes may induce detrimental effects on fetal development (1). Gestational diabetes, a prevalent metabolic complication affecting about 15% of pregnancies, can lead to maternal and neonatal complications, including neurodevelopmental anomalies in infants (2, 3). Maternal hyperglycemia is associated with mitochondrial swelling in the neural tubes of offspring, which ultimately culminates in mitochondrial degeneration and subsequent cell death (4).

Because glucose readily crosses the placenta, maternal diabetes may have various adverse effects on the developing fetus, including increased oxidative stress, hypoxia, and apoptosis (5). Studies have indicated that a rise in oxidative stress levels are observed in the cord blood of neonates of diabetic mothers. This phenomenon has been linked to an elevated susceptibility to neurodevelopmental disorders in these infants (6).

In animal models it has been demonstrated that free radicals play a crucial role in regulating the timing and progression of neuronal development, differentiation, and synaptic plasticity. Imbalances in these signaling pathways can disrupt critical neurodevelopmental processes (7). Additionally, due to the high oxygen consumption of the brain and its limited antioxidant defenses, this organ is particularly vulnerable to oxidative damage (8). Free radicals can oxidize lipids, DNA, and proteins, thereby rendering these molecules biologically inactive and ultimately leading to cell death (7).

Vision loss and visual abnormalities are among the potential complications associated with diabetes (9). The subcortical visual nuclei of the diencephalon and mesencephalon are composed of numerous retinorecipient nuclei, including the superior colliculus (SC) nuclei and the lateral geniculate complex (10). The lateral geniculate nucleus (LGN) is divided into two parts: the dorsal part (dLGN), which transmits visual signals to the primary visual cortex, and the ventral part (vLGN), which sends

inhibitory signals to sensory-motor structures, including the superior colliculus. The vLGN also projects to areas associated with specific behavioral states, such as fear (11-13). The superior colliculus, a nucleus located on the dorsal surface of the mesencephalon, receives input from ganglion cells in the visual pathway in mice (12).

Considering the high prevalence of diabetes in pregnancy, we decided to investigate the surface density of apoptotic cells in the SC and dLGN, two nuclei of the visual pathway, in male rat neonates born to diabetic mothers.

Materials and Methods

All experiments were completed by the guidelines of the National Institutes of Health (NIH) supported by the Committee for the Care and Use of Laboratory Animals at Mashhad University of Medical Sciences (MUMS), Mashhad, Iran (IR.MUMS.MEDICAL.REC.1398.794).

Animals

Female Wistar rats (body weight: 200-250 g; age range: 6-8 weeks) were purchased from the animal house of MUMS, Mashhad, Iran. Animals were kept in a room at $23 \pm 2°C$ with 12-hour light/dark cycles and free access to food and water. Two female rats in each group were kept in the cages.

The animals (n=15) were randomly divided into three groups as follows:

i. Control group (Con.; n=5),
ii. Diabetic group (Dia.; n=5),
iii. Insulin-treated diabetic group (Ins.; n=5).

Induction of Diabetes

In the Dia. and Ins. groups, diabetes induction was performed with a single dose (65 mg/kg) intraperitoneal injection of Streptozotocin (STZ, Sigma Aldrich: S0130-1G, Germany) diluted in normal saline (14-16). A commercial digital glucometer (Accu-chek®, Germany) was used to measure fasting blood glucose 72 hours after STZ injection. Animals with fasting blood glucose levels of more than 150 mg/dL were considered diabetic and were divided into two groups, including Dia. and Ins. (16). In the Dia. group, the blood glucose level was measured by collecting blood from the end of the caudal vein daily. The mean blood glucose level in the Dia. group was 358.3 ± 84.24 mg/dL. Rats in the Ins. group received 2 to 4 units of protamine-zinc insulin (NPH) (Exir Pharmaceutical Company, Iran) two times a day percutaneously, after becoming diabetic (17). Injectable insulin levels were determined by measuring daily blood glucose to make sure that the rats' blood glucose levels were always within the normal range. In the Ins. group, the blood glucose level was measured by collecting blood from the end of the caudal vein daily until parturition. The mean blood glucose level in the Ins. group was 105.3 ± 10.14 mg/dL. The Con. group rats only received an intraperitoneal injection of normal saline. Female rats in all three groups were caged with healthy male rats (body weight: 330 ± 20 g; age

range: 12-13 weeks), one week after the establishment of diabetes in the Dia. group and after diabetes control in the Ins. group. Animals were allowed to give birth naturally; the birthday was considered P0 in neonates (18). The rat pups from diabetic and insulin-treated mothers were fed by healthy mothers to eliminate the possible effects of milk from diabetic rats, and thus focus only on the fetal period events (19). Fifteen male neonates from each group were randomly separated into three age subgroups: P0, P7, and P14 (5 animals per age subgroup). Since gender could interfere with the results, as a contextual variable, we decided to remove one of two genders (female) from the study.

Tissue preparation

Male neonates were sacrificed using CO_2 inhalation at P0, P7, and P14, and their brains were fixed in 10% formalin solution (10 of 37% formaldehyde solution (104003-Sigma-Aldrich, Germany) with 90% normal saline) for 72 hours after removal from their skull. After fixation, they were dehydrated by ascending concentrations of ethanol (Alcohol Pars Company, Iran) from 70 to 100%, then the tissues were immersed in paraffin. Coronal brain sections including the SC and the dLGB were prepared with a thickness of five micrometers. Five histological sections from the beginning to the end of SC and five histological sections from the beginning to the end of dLGB were selected in the brain of each neonate rat.

TUNEL staining

The investigation of apoptotic cell death in neurons and other cells was carried out using the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay. The slides were briefly deparaffinized, rehydrated, and incubated with hydrogen peroxide 3% (Sigma-7722-84-1, Germany) in methanol for 10 minutes, and then the slides were washed three times with phosphate-buffered saline (PBS). The sections were incubated with Proteinase K (Sigma-21627M, Germany) for 30 minutes at 37°C. After washing three times with PBS, the sections were incubated with Triton 0.3% for 10 minutes. The sections were incubated with TdT enzyme for two hours at 37°C, followed by three PBS washes. The following step involved incubating sections with 50 μl of peroxidase (POD) in the dark for 30 minutes, washing the sections incubated with 100 μl from the solution (ScyTek-ACV999) for 5 minutes and washing them with water. Finally, the sections were counterstained with Harris hematoxylin for 10 seconds. The slides were washed with water, dehydrated using ascending ethanol, clarified with Xylene, and mounted with coverslips (20).

Quantitative analysis

Tissue sections were examined under an 100× objective lens in a light microscope (Olympus BX51, Japan) connected to a camera (Olympus DP12, Japan) by ND25 and OP filter. The dLGB and the SC boundaries in rat neonates were delineated by cross-referencing with the classical atlases of the rat brain (21, 22). The surface density of apoptotic cells on tissue sections was estimated

using the counting frame of stereology (23).

Statistical analysis

GraphPad Prism 9 software (GraphPad, USA) was used to compare data using one- and two-way ANOVA, as well as the post hoc Tukey's test. All the data existed as mean ± SEM. The $P<0.05$ and was considered statistically significant.

Result

The distribution of apoptotic cells in the dLGN

The main effect of the numerical density of apoptotic cells in treatment groups showed a noteworthy difference [$F_{(2, 36)}$=114.9, $P\leq0.0001$]. In the Dia. group, the numerical density of apoptotic cells was remarkably increased at P0, P7, and P14 compared to the rat neonates in Con. group (each, $P\leq0.0001$). The numerical density of apoptotic cells decreased in the dLGN of neonate rats in the Ins. group compared to the Dia. group at P0, P7, and P14 (each, $P\leq0.0001$). A remarkable difference was not detected in the numerical density of apoptotic cells between neonate rats of the Ins. and Con. groups at P0, P7, and P14 ($P=0.0783$, $P=0.3285$, and $P=0.3581$ for P0, P7, and p14, respectively). The main effect was not meaningfully different in postnatal days [$F_{(2, 36)}$=4.201, $P=0.0229$]. The highest numerical density of apoptotic cells was for the Dia. group at P0 0.00005267( )0.000003839 ±, and the lowest numerical density of apoptotic cells was for the Con. group at P14 0.00001005(0.0000007547 ±). A significant difference was not seen in the main interaction effect of treatment groups×postnatal days [$F_{(4, 36)}$=0.8108, $P=0.5266$] (Fig.1A, B).

The distribution of apoptotic cells in the Superior Colliculus

TUNEL staining was used to estimate the numerical density of apoptotic neurons in the SC area. No significant difference was detected in the appearance of apoptotic cells on days 0, 7, and 14 in the Con. group. The main effect of the numerical density of apoptotic cells in treatment groups showed a significant difference [$F_{(2, 36)}$=319.6, $P\leq0.0001$]. In the Dia. group, the numerical density of apoptotic cells was remarkably increased at P0, P7, and P14 compared to the Con. group (each, $P\leq0.0001$). The numerical density of apoptotic cells decreased in the SC of rat neonates in the Ins. group compared to the Dia. group at P0, P7, and P14 (each, $P\leq0.0001$). A remarkable difference was not detected in the Numerical density of apoptotic cells between rat neonates of the Ins. and Con. groups at P0, P7, and P14 ($P=0.6535$, $P=0.6192$, and $P=0.6363$ for P0, P7, and p14 respectively). The main effect was not significantly different in postnatal days [$F_{(2, 36)}$=0.9822, $P=0.3843$]. The highest Numerical density of apoptotic cells was for the Dia. group at P0 0.00002647( ) 0.0000002667 ±, and the lowest Numerical density of apoptotic cells was for the Con. group at P14 0.000008067(0.000003811 ±). A significant difference was not seen in the main interaction effect of treatment groups×postnatal days [$F_{(4, 36)}$=0.03971, $P=0.9969$] (Fig.2A, B).
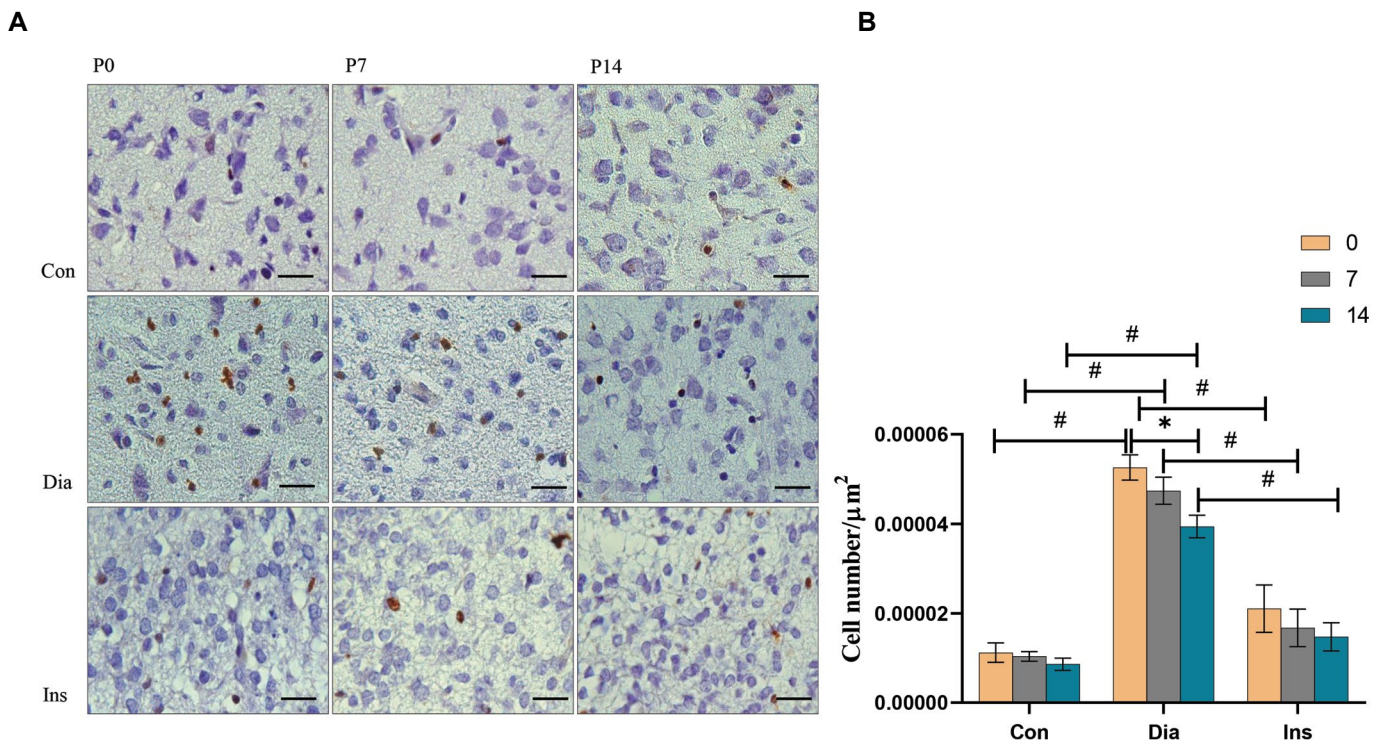

Fig.1: Immunoreactivity of the apoptotic cells in the dLGB at P0, P7, and P14 in different groups. **A.** Immunoreaction visualization with DAB, counterstaining with Harris hematoxylin (scale bars: 100 μm). **B.** Values represent the mean ± SEM. The level of significance between groups showed by # (#; P≤0.0001) and data compared with two-way ANOVA. The level of significance between postnatal days showed by * (*; P≤0.05), data compared with one-way ANOVA. Con; Neonates of control rats (n=5), Dia; Neonates of diabetic rats (n=5), Ins; Neonates of diabetic rats treated with insulin (n=5), and dLGB; Dorsal lateral geniculate body.
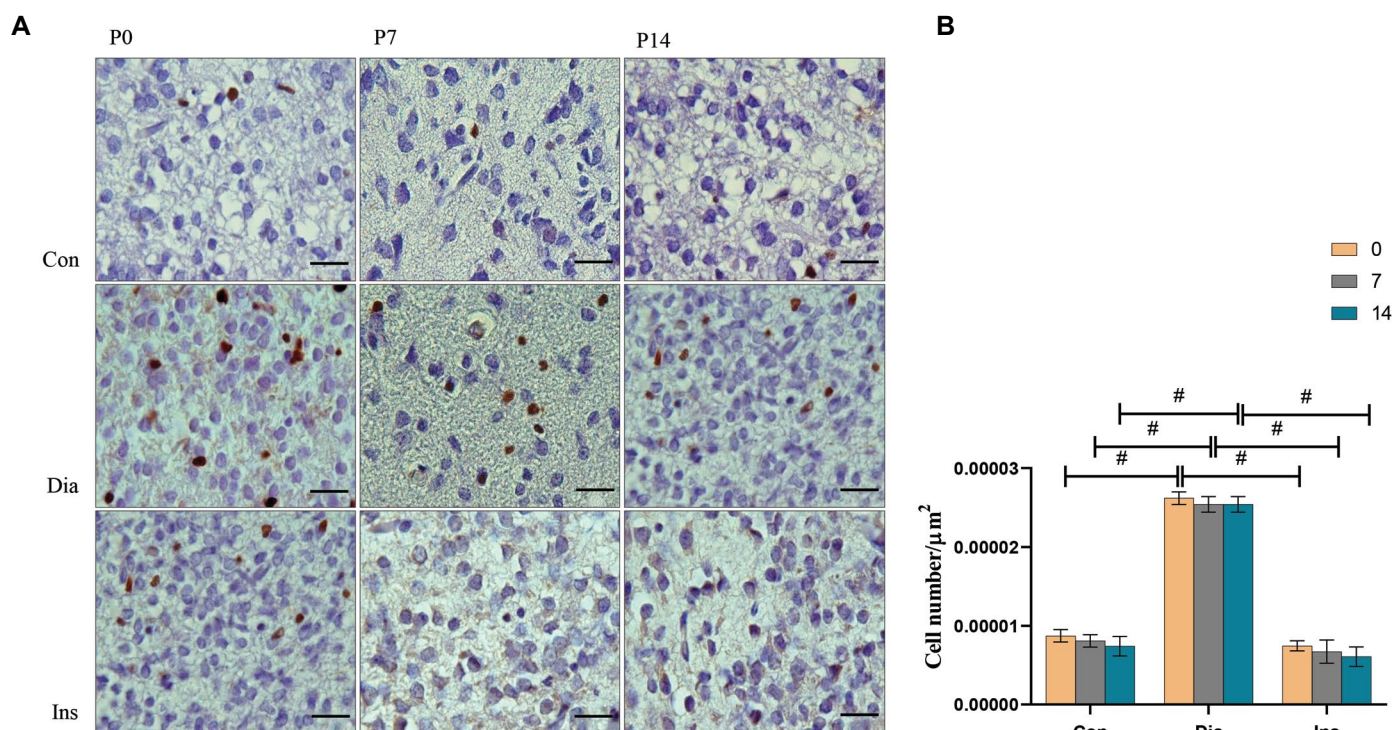
A

B

Fig.2: Immunoreactivity of the apoptotic cells in the SC at P0, P7, and P14 in different groups. A. Immunoreaction visualization with DAB, counterstaining with Harris hematoxylin (scale bars: 100 μm). B. Values represent the mean ± SEM. The level of significance between groups showed by # (#; P≤0.0001) and data compared with two-way ANOVA. SC; Superior colliculus, Con; Neonates of control rats (n=5), Dia; Neonates of diabetic rats (n=5), and Ins; Neonates of diabetic rats treated with insulin (n=5).

Discussion

This experimental study was aimed to determine the distribution of apoptotic cells in the SC and the dLGN of male neonate rats born to diabetic mothers. The deterioration in glucose homeostasis resulting from diabetes triggers neuronal damage. At this point, the molecular fundamentals of this neuronal susceptibility is only partially elucidated (24). It has been found previously that diabetic pregnancies cause several mechanisms, such as fetal hyperglycemia, neonatal hypoglycemia, hyperinsulinemia, oxidative stress, and hypoxia, to cause loss of neurons in the rat neonates (25).

The hyperglycemic state in diabetic mothers is transmitted to the fetus in utero. This condition negatively impacts the central nervous system development, with consequences that persist into adulthood. For instance, cortical visual evoked potential (cVEP), a marker of both visual acuity and neuronal myelination, is impaired by maternal metabolic disturbance (26). However, the precise mechanism, by which gestational diabetes affects brain development, remains unclear.

A study conducted on neonatal rats' hippocampus showed that maternal hyperglycemia during pregnancy alters the expression of genes that regulate neuronal cell apoptosis in the hippocampal area. As a result, there was an increase in the density of dark neurons (DNs), which are degenerating neurons in the hippocampus of newborn rats (25).

As the results of our study indicated, neuronal apoptosis in the dLGN and SC was found to increase significantly at all three time points under investigation in neonates born to diabetic mothers. Considering that diabetes during pregnancy increases blood glucose in the fetus, it may potentially lead to the loss of neurons in different areas of the brain of the fetus.

In the present study, the induction of apoptosis and neuronal death in the infant's SC and dLGN born to diabetic mothers may have occurred due to the following reasons: during gestation, maternal hyperglycemia may bring about alterations in the expression of two critical genes in the regulation of neuronal apoptosis: *Bcl-2* and *Bax*. Neuronal overexpression of *Bcl-2* prevents cell death and increases the number of neurons in various brain regions. Conversely, gene disruption studies targeting the Bax protein have demonstrated its significant role in initiating programmed cell death in neurons (27).

Microglial stimulation in neonates of diabetic mothers is associated with one of the complications they face, as it may lead to the activation of neuronal caspase 3 via TNFR1 (also known as p55) and Fas receptor. The release of TNF, TNFα, and FasL (Fas ligand) by microglial stimulation cause neurons to undergo apoptosis (28).

A potential pathway to apoptosis in infants of diabetic mothers could be the reduction of insulin growth factor 1 (IGF1) (29). IGF1 signaling through the IGF type 1 receptor (IGF1R) is critical for brain development. During embryonic development, IGF1 promotes neuronal

progenitor proliferation, while later on, it supports neuronal survival, growth, and synaptogenesis. IGF1 also stimulates the proliferation of oligodendrocyte progenitors, inhibits apoptosis, and promotes myelin production (30). Therefore, the decrease in IGF1 levels in infants of diabetic mothers may lead to apoptosis and neuronal death.

Diabetes also decreases antioxidant capacity, leading to increased oxidative stress. This rise in oxidative stress causes mitochondrial dysfunction, which may cause damage in neurons (31). On the other hand, hyperglycemia increases reactive oxygen species (ROS) production, which activates cytochrome C release from mitochondria into the cytoplasm. Cytochrome C promotes caspase-3 expression, stimulating endonuclease activity that cleaves DNA and condenses chromatin. Excess ROS also damages unsaturated fatty acid membranes, making cell and organelle membranes more permeable. The resulting water influx causes organelle and neuron swelling within cells. Degenerative characteristics of SC neurons in diabetic rats include condensed chromatin, cytoplasmic condensation with disorganized organelles, and cell shrinkage (32).

Currently, the precise mechanism by which diabetes increases glutamate accumulation in the extracellular matrix remains unknown. Nevertheless, this elevated glutamate level triggers an increase in the flow of intracellular Ca^{2+} and nitric oxide (NO) concentration. Consequently, the synthesis of various proteases, endonucleases, and phospholipases, such as Calpain and Caspase-3, is activated (33, 34).

Multiple studies have demonstrated that diabetes mellitus can hinder the brain's ability to regulate Ca^{2+}. This impairment can be particularly detrimental since numerous critical brain functions depend on the regulation of calcium signaling. Even a minor disruption of calcium signaling or calcium homeostasis may have adverse effects, such as neuronal demise (9, 35). In addition to the activation of proteases, endonucleases, and phospholipases, upregulation of intracellular Ca^{2+} also stimulates the expression of these enzymes. This leads to the destruction of various neuronal structures, including chromatin, the rough endoplasmic reticulum (rER), the Golgi complex, the cell membrane, and the cytoskeleton. This process is commonly referred to as chromatolysis, which causes the condensation of both chromatin and cytoplasm due to the disintegration of cell organelles (32). Type 1 diabetes mellitus (T1DM) has been shown to trigger structural brain degeneration, resulting in a loss of gray matter. Diabetes mellitus can also induce cortical and subcortical atrophy, further exacerbating the damage to the brain's structure (36). Insufficient research on the frequency of apoptosis in the LGB and SC regions in neonates of diabetic mothers poses challenges in interpreting these findings accurately.

Individuals who suffer from diabetes often experience visual impairments (37). This is due to the fact that diabetes can affect the visual pathway or visual cortex, which may cause problems with vision (38). Studies have shown that in diabetic rats, the superior colliculus (SC) undergoes neurodegenerative changes, which may also lead to visual disturbances (32, 39).

In the present study, it is suggested that maternal diabetes may have an impact on the production of brain glutamate or oxidative stress, or the overexpression of Bax, resulting in apoptosis and death in the SC and dLGB neurons of neonates born from diabetic mothers. In addition to its role in regulating blood glucose levels, insulin also has neuroprotective properties (34). The current research found that insulin treatment of diabetic mothers reduces the expression of apoptotic cells in the SC and dLGB of their neonates by regulating blood glucose levels in the mothers. Recent studies have shown that insulin has significant neuroprotective benefits. Experimental studies and clinical trials have demonstrated that insulin administration improves brain function in patients (40). Maternal diabetes has been suggested to significantly impact the regulation of insulin receptor and IGF-1R in the developing cerebellum. Nevertheless, maintaining optimal control of maternal hyperglycemia with insulin can help normalize these effects. Future research endeavors should thoroughly explore apoptosis and its visual function during diverse developmental stages. This would aid in developing a more comprehensive understanding of the effects of maternal diabetes on visual complications, thereby facilitating the development of effective prevention and treatment strategies.

Conclusion

The present study showed that diabetes in mothers at the time of pregnancy may increase the surface density of apoptotic cells in the SC and dLGN nuclei in the offspring. However, treating the mother with insulin helps reduce the number of apoptotic cells. These findings emphasize the importance of early intervention and prevention strategies to minimize the impact of maternal diabetes on the central nervous system of their newborns.

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Authors' Contributions

H.H., S.F.; Designed the study. N.A., F.T., J.B.; Have made substantial contributions to the achievement of data and/or analysis and interpretation of data. H.H., N.A., Gh.S., H.B.; Drafted the manuscript and revised it critically for important intellectual content. All authors have read and approved the final draft of the manuscript.

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