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The effect of the maternal induced diabetes on the postnatal development of the dorsomedial nucleus in hypothalamus of the albino rat

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Abstract
Maternal diabetes constitutes an unfavorable environment for embryonic and postnatal development. The aim of the present work was to detect the effect of maternal induced diabetes on the structural organization of the dorsomedial nucleus during postnatal development. Hypothalamic specimens of the offspring of diabetic mothers were studied at ages of newborn, 21 days and two months. Several techniques were used in this work including, gallocyanine-chrom-alum staining technique, Golgi –Cox method and immunohistochemistry. In addition, morphometric study was done to estimate the number of neurons in the dorsomedial nucleus. In the newborn, some neurons in the dorsomedial nucleus showed the presence of darkly stained nuclei. With the progress of age these degenerative changes became more prominent. Marked reduction in the extension and branching of dendrites were observed. In addition, an apparent increase in the number of astrocytes was found. Measuring the number of cells per area demonstrated the presence of a highly significant decrease in number at the ages of 21 days and two months in the offspring of diabetic mothers in comparison with the control group. In conclusion, this study indicated that maternal diabetes affect the development and structure of the dorsomedial nucleus. These changes suggest that there should be strict control of diabetes before and during pregnancy.

Keywords: The dorsomedial nucleus, maternal diabetes, astrocytes, hypothalamus

1. Introduction
The hypothalamus is a critical integrator of several homeostatic processes that are required for the survival of vertebrates. Disruption of the development of the hypothalamus has the potential of perturbing important physiological processes with lifelong consequences (Caqueret *et al*., 2005) [1]. Maternal diabetes constitutes an unfavorable environment for embryonic and fetoplacental development. Despite current treatments, pregnant women with either type 1 or type 2 diabetes are at increased risk of miscarriage, stillbirth, offspring congenital malformations, placental abnormalities, and intrauterine malprogramming (Vambergue and Fajardy 2011) [2]. Diabetes during pregnancy affects the health of both the mothers and their infants (Hami *et al*., 2015) [3]. Perinatal hyperinsulinism is pathognomonic in the offspring of the diabetic mothers (Plagemann, 2005) [4]. No sufficient studies were done on the effect of the maternal diabetes on the structure of the individual hypothalamic nuclei. The dorsomedial hypothalamic nucleus was found to has an important role in many processes that control both food intake and body weight regulation (Bellinger and Bernardis 2002) [5]. The present work was carried out to detect the effect of maternal induced diabetes on the structural organization of the dorsomedial hypothalamic nucleus in the offspring during postnatal development.

2. Material and methods
In this work, a total number of sixty adult female albino rats weighing 200-300 g and 3 months old were used. They were divided into two groups:

1. Group I (Control) is composed of 30 adult female rats. They received no treatment.
2. Group II (Experimental) is composed of 30 adult female rats in which diabetes was induced. Induction of diabetes was carried out by single intraperitoneal injection of alloxan monohydrate (Sigma St Louis, M.O.,USA) dissolved in 0.9% cold normal saline solution at a dose of 150 mg/kg body weight. The diabetes was assessed by determining the blood glucose concentration 72 h after injection of alloxan. The rats with blood glucose level above 200 mg/dl were then selected for the study. In both groups, mating was allowed between the female and male rats. Every morning the females were examined for the presence of the vaginal plug and vaginal smears were examined to detect the commence of pregnancy. Female rats were allowed to deliver spontaneously. Their offspring were studied at the ages of newborn, 21 days and two months.

In both offspring of the control mothers and those of the diabetic mothers groups, 6 pups were studied by gallocyanine chrom-alum staining technique to demonstrate the cytoarchitecture of the dorsomedial nucleus in the hypothalamus. The animals were anaesthetized by ether inhalation. The rats were perfused in the left ventricle by normal saline followed by Bouin's fluid. The brains were extracted from the skull. Two cuts were made to determine the site of hypothalamus. The first one cranial to optic chiasma and another one caudal to the mamillary bodies. The specimens then were put in Bouin's fixative solution for 12-24 hours according to the age of the specimen. Bouin's fixative was used as it penetrates rapidly the brain tissue and causes little shrinkage. Another 3 rats were processed to be studied by Golgi-Cox technique to show the development of the cell processes. The animals were anaesthetized by ether inhalation and perfused by normal saline. The brains once removed from the skulls were put in the following fluid, which was freshly prepared:

5% potassium dichromate 16.0 volumes

5% mercuric chloride	16.0 volumes
5% potassium chromate	8.0 volumes
Distilled water	20.0 volumes

The fluid was kept in a dark bottle that contains a cotton piece at its bottom to ensure complete bathing of the brains with the fluid. The fluid was changed after 48 hours. The specimens were left in this fixative in the dark for six weeks. Then they were processed according to steps described by Drury and Wallington (1980) [6]. The immune his to chemical study using anti-Glial fibrillary acidic protein was performed in the two months age group to demonstrate astrocytes according to steps described by (Cattoretti *et al*., 1993) [7]. Morphometric study was done on gallocyanine stained sections to estimate the number of neurons in the dorsomedial nucleus both in the offspring of control mothers and in those of diabetic mothers in all the studied age groups using an image analysis system (Leica Q500 Mc) that proposed counting the cells per specific area (12360 μ^2). Statistical analysis using t-test was performed to compare experimental with control groups (Peterson, 1985) [8].

3. Results

A- Control group

In the newborn rats, light microscopic examination reveals that the cells of the dorsomedial nucleus are rounded with vesicular nuclei and prominent nucleoli (Fig.1). In the twenty-one days, old rats, the cells of the dorsomedial nucleus are of variable size and shape with vesicular nuclei and prominent nucleoli. (Fig.2). Golgi – Cox stain shows the presence of multipolar neurons with extended branched dendrites carrying many spines (Fig.3). At the age of two months, the cells of the dorsomedial nucleus are of variable size. They are polyhedral or rounded in shape with increase in Nissl substance than in the previous ages (Fig.4). Golgi-Cox stain shows the presence of multipolar cells which have long extended and branching dendrites. These dendrites carry a lot of spines (Fig.5). Immuno his to chemical staining using anti GFAP demonstrates the presence of few astrocytes in the nucleus, which appear star shaped with branching processes between neurons (Fig. 6).

B- Experimental group

In the newborn, the cells in the dorsomedial nucleus appear to be dispersed. Most of the cells appear to be lightly stained. Some cells have darkly stained nuclei (Fig. 7). At twenty-one days old rat, cells of the dorsomedial nucleus have darkly stained nuclei. In addition, some cells with pyknotic nuclei and lysis of the cytoplasm can be observed (Fig. 8). Golgi-Cox stain shows a marked decrease in extension and branching of dendrites of the neurons in the dorsomedial nuclei (Fig. 9). At the age of two months, many cells in the dorsomedial nucleus have darkly stained nuclei with the presence of some cells that show lysis of the cytoplasm (Fig. 10). Golgi-Cox stain shows a decrease in extension and branching of the dendrites in the neurones the dorsomedial nucleus (Fig.11). Apparent increase in the astrocytes in comparison with the control can be observed indicating increase in the expression of GFAP within the dorsomedial nucleus (Fig.12).

3.1 Morphometric analysis

The mean number of cells in the dorsomedial nucleus per an area of 12360 μ^2 in the in the newly born rats offspring of control mothers is 164.8 ± 1.985. While the mean number in the offspring of diabetic mothers is 157 ± 1.897. It is obvious that there is a decrease in the mean number of cells in the offspring of diabetic mothers. This decrease in the mean number of cells is statisticaly significant ($p < 0.05$). The mean numbers of cells in the dorsomedial nucleus per an area of 12360 μ^2 21 days and two months old rats offspring of control mothers are 176.2 ± 2.337 and 153.2 ± 2.083 respectively. While the mean number in the offspring of diabetic mothers are 160.8 ± 2.956 and 113 ±2.569 respectively. It is clear that there is a decrease in the mean number of cells in the offspring of diabetic mothers. This decrease is highly significant ($P < 0.01$) (Table 1 and Fig 13).

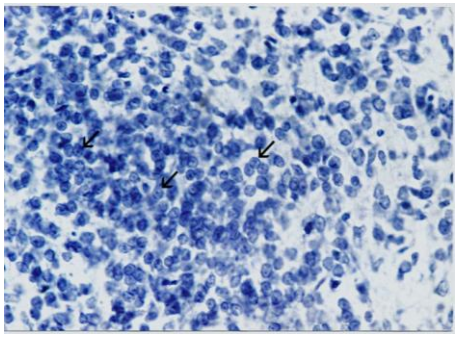

Fig 1: A photomicrograph showing the dorsomedial nucleus of the hypothalamus of newborn rat offspring of control mother. The cells are rounded and closely packed with prominent nucleoli (arrows). (Gallocyanin stain x 400)

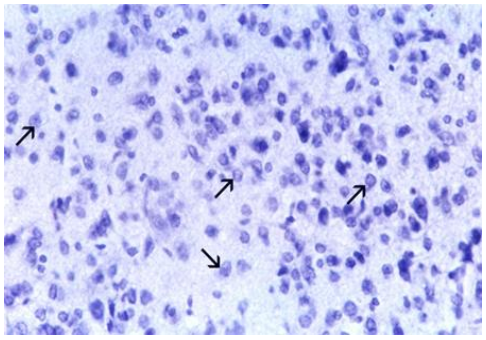

Fig 2: A photomicrograph showing the dorsomedial nucleus of the hypothalamus of twenty-one days old rat offspring of control mother. The cells are of variable shape and size with vesicular nuclei (arrows). (Gallocyanin stain x 400)

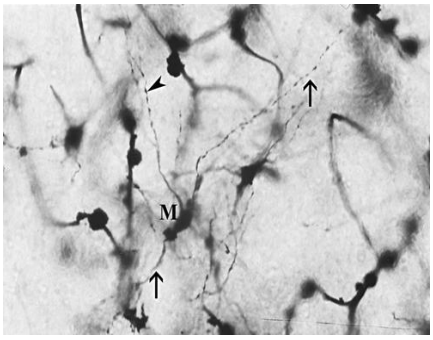

Fig 3: A photomicrograph showing the dorsomedial nucleus in the hypothalamus of twenty-one days old rat offspring of control mother. Note the presence of multipolar neuron (M) and the extension and branching of dendrites (arrows) with numerous spines (arrow head). (Golgi - Cox stain x 250

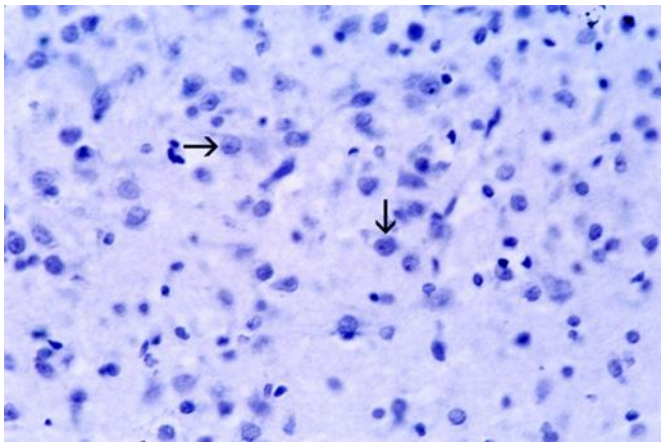

Fig 4: A photomicrograph showing the dorsomedial nucleus of the hypothalamus of of two months old rat offspring of control mother. The nucleus consists of cells that are of variable shape with vesicular nuclei (arrows). (Gallocyanin stain x 400)

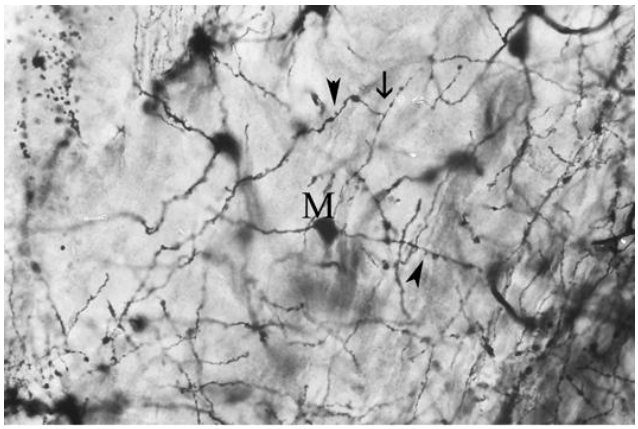

Fig 5: A photomicrograph showing the dorsomedial nucleus in the hypothalamus of two months old rat offspring of control mother Note the presence of multipolar neuron with extended and branched dendrites (arrow). The dendrites carry numerous spines (arrow heads). (Golgi-Cox stain x 250)

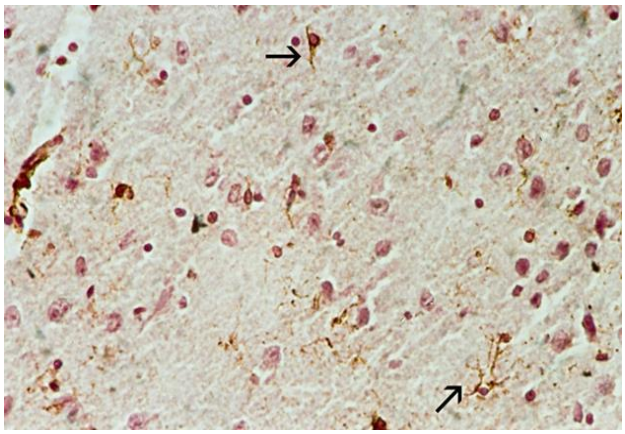

Fig 6: A photomicrograph showing the dorsomedial nucleus of the hypothalamus of two months old rat offspring of control mother. Note the presence of few star shaped astrocytes (arrows). (Anti GFAP immunostaining x 400)

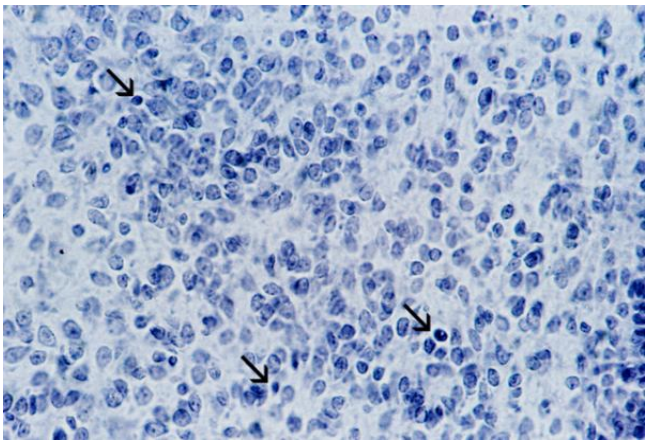

Fig 7: A photomicrograph showing the dorsomedial nucleus in the hypothalamus of newborn rat offspring of diabetic mother. Some cells with darkly stained nuclei can be observed (arrows). Note that most of cells are lightly stained. (Gallocyanin stain x 400)

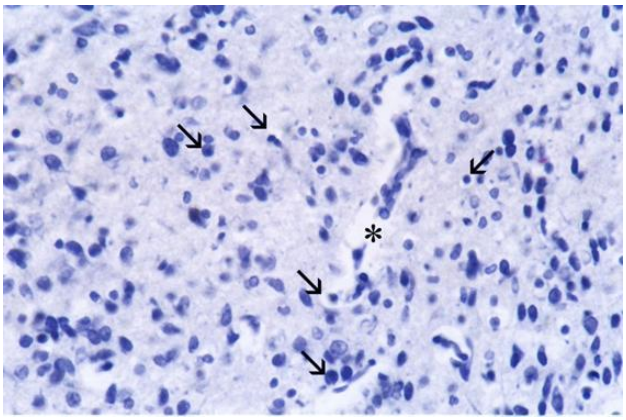

Fig 8: A photomicrograph showing the dorsomedial nucleus in the hypothalamus of twenty-one day rat offspring of diabetic mother. Many cells have darkly stained nuclei and cytoplasmic lysis (arrows).Note the presence of an area of cell loss (*). (Gallocyanin stain x 400)

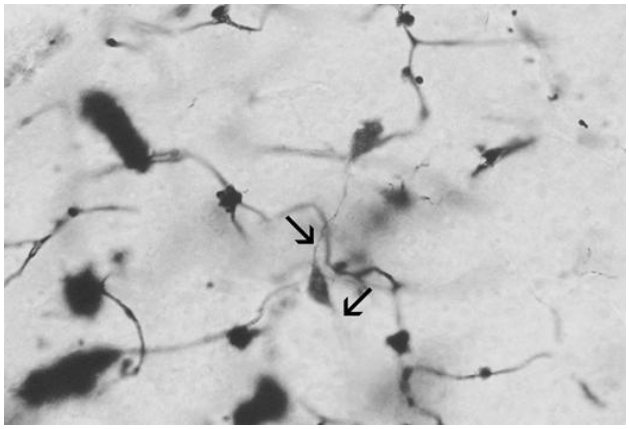

Fig 9: A photomicrograph showing the dorsomedial nucleus in the hypothalamus of twenty-one days old rat offspring of diabetic mother. Note the decrease in the extension and branching of dendrites (arrows). (Golgi - Cox stain x 250)

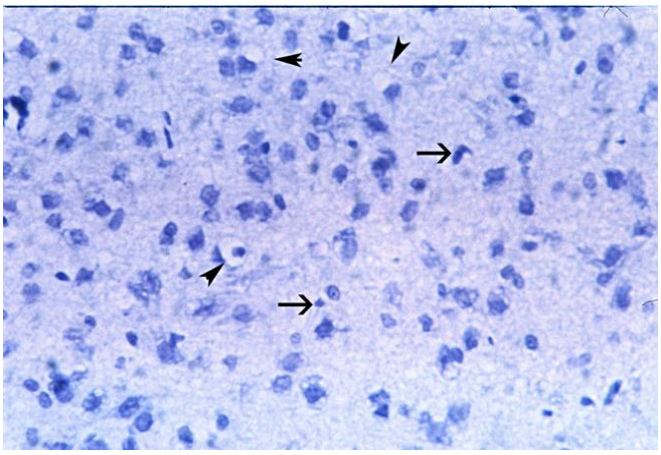

Fig 10: A photomicrograph showing the dorsomedial nucleus in the hypothalamus of two months old rat offspring of diabetic mother. Many cells have pyknotic nuclei (arrows). Some cells show lysis of cytoplasm (arrows heads). (Gallocyanin stain x 400)

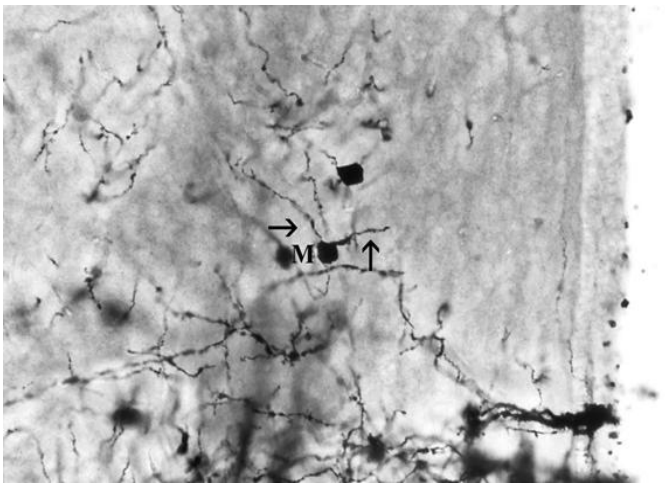

Fig 11: A photomicrograph showing the dorsomedial nucleus in the hypothalamus of two months old rat offspring of diabetic mother. Note the decrease in the extension and branching of the dendrites (arrows). (Golgi-Cox stain x 250)

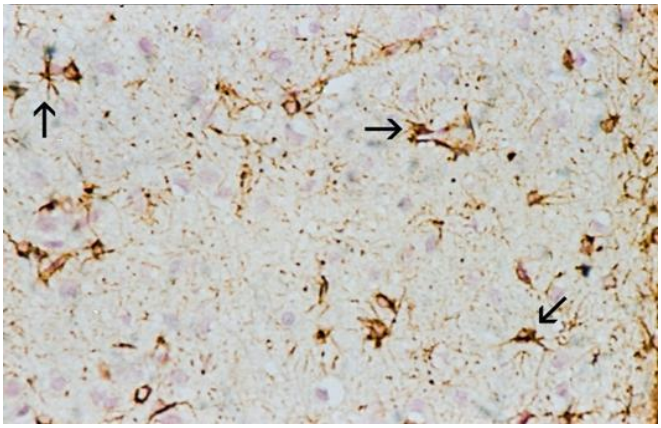

Fig 12: A photomicrograph showing the dorsomedial nucleus in the hypothalamus of two months old rat offspring of diabetic mother. Note the apparent increase in astrocytes (arrows). (Anti GFAP immunostaining x 400)

Table 1: The mean number of cells in the dorsomedial nucleus per an area of 12360 µ ² of the offspring of control mothers and that of diabetic mothers at different ages.

Ages	Offspring of control mothers			Offspring of diabetic mothers			t
	N	Mean	SE	N	Mean	SE	
Newborn	5	164.8	1.985	5	157	1.897	* 2.841
21 days old rat	5	176.2	2.337	5	160.80	2.956	** 4.468
Two months old rat	5	153.2	2.083	5	113.00	2.569	** 12.154

SE=Sandard Error, N = number
*Statistical significance difference (P<0.05)
**Highly Statistical significance difference (P<0.01)

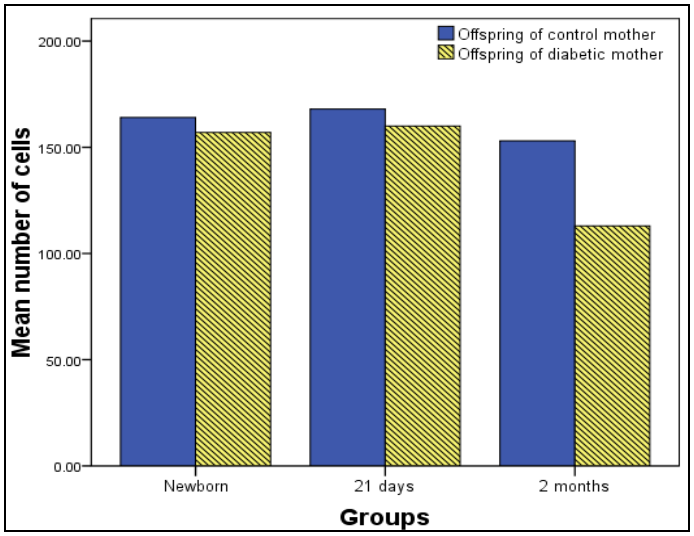

Fig 13: It shows the relation between the number of cells of the dorsomedial nucleus per an area of 12360 µ ² in offspring of control mothers and offspring of diabetic mother's rats among different age groups.

4. Discussion

The cells of the dorsomedial nucleus were found to be rounded in the newborn and closely packed in the newborn rats. They became of variable shape and size with vesicular nuclei and prominent nucleoli and appeared to be less closely packed in the twenty-one and two months old rats this was in agreement with Hyyppa (1969) [9] who reported that the cells early in development were rounded but later became become fusiform or triangular.

This study demonstrated that the dorsomedial nucleus in the newborn offspring of diabetic mothers the showed the presence of some cells with darkly stained nuclei but at the age of two months, many cells with lysis of cytoplasm and pyknotic nuclei had been demonstrated. These indicated apoptosis (Bredesen 2000) [10]. These changes could be explained by mechanisms such as hypoxia and oxidative stress, especially through the pathway of hexosamine biosynthesis that might be involved in diabetes embryopathy, by inducing apoptosis during critical phases of organ development (Simeoni and Barker 2009) [11]. The critical period for hypothalamic development in the rat is in the first 21 days of life (Pozzo Miller and Aoki 1992) [12]. Moreover, disturbance in catecholamines of hypothalamic nuclei has a role in explaining the effect of maternal diabetes on the development of hypothalamic nuclei (Plagmman *et al*., 1998)

[13]. This was inconsistent with Plagemann *et al*. (1999) [14] who showed that no alterations were observed in the dorsomedial nucleus in the hypothalamus of twenty-one days old offspring rats of diabetic mothers. They suggested that the dorsomedial nucleus serve as a functional interneuron between ventromedial nucleus and the lateral hypothalamic area.

In accordance with (Kitzmiller *et al*., 1996) [15] glucose passes from the maternal into the fetal circulation via the placental barrier, while maternal insulin does not. Witkop *et al*. (2009) [16] stated that when a fetus (over 12th week of gestation) is exposed to high levels of maternal glucose, it responds by secreting high levels of insulin in its circulation to control hyperglycemia. Therefore, maternal insulin concentrations do not have a direct effect on fetal glucose and insulin metabolism, but only an indirect one by influencing maternal glucose levels (Weiss, 1988) [17]. Franke *et al*. (2005) [18] found that induced maternal hypoinsulinemia as well as its normalization by islet transplantation was unlikely to have direct effects on the fetus. Leloup (2004) [19] reported that insulin binding was increased in hypothalamic and extrahypothalamic-related nuclei in the offspring of hyperglycemic mothers. This increase was specific to areas involved in the nervous control of metabolism and could be a factor in glucose intolerance and impairment of insulin secretion exhibited by young-adult rats from hyperglycemic mothers. It is documented that elevated insulin levels in fetal and perinatal life (hyperinsulinaemia) are pathognomonic in the children of mothers with diabetes during pregnancy [4].

Golgi – Cox technique showed a decrease in the extension and in the branching of the dendrites in cells of the dorsomedial nucleus in the offspring of diabetic mothers in comparison with the control groups. These observations were supported by Jing *et al*. (2014) [20] who suggested that the maternal hyperglycemia retard the dendritic development in the fetal brain and that these changes partially resulted from abnormal insulin/Insulin-like growth factor-I signaling in the brain. This abnormal signaling was discussed by Haghir *et al*. (2013) [21] who revealed that diabetes during pregnancy strongly influences the regulation of both insulin receptors (InsR) and insulin like growth factor 1(IGF-1R) in the developing cerebellum. They found that 2 weeks after birth, mRNA expression and protein levels of both InsR and IGF-1R in cerebellum in the offspring of diabetic mothers was significantly downregulated.

The morphometric study showed that the dorsomedial nucleus showed a highly significant decrease in number of cells per a fixed area at the ages of twenty-one days and two months in the offspring of diabetic mothers in comparison with the offspring of control mothers. This was in accordance with Tehranipour and Ghasemzade (2010) [22] who reported that there was a significant reduction in the number of neurons in neonate hypothalamus of diabetic mothers. The decrease in number of cells described in the present work can be explained by the study of Li *et al*. [23] who reported that insulin-like growth factor has a neuroprotective anti-apoptotic effect and down regulation of expression of (insulin-like growth factor) and its receptor in the brain of offspring of diabetic mothers (Ramsay *et al*, 1994) [24] could be expected to lead to neuronal loss.

Expression of glial-fibrillary acidic protein (GFAP), an intermediate filament protein, is commonly used as a marker of astrocytes (Buckman *et al*., 2013) [25]. Extensive studies of astrocyte–neuron interactions have been carried out in a number of brain areas Gerics *et al*. (2006) [26]. In the offspring of control mothers, this work revealed scanty number of astrocytes seen in the dorsomedial nucleus. These results were in accordance with Zilles *et al*. (1991) [27] who found that in the hypothalamus of adult rats, the number of GFAP-expressing astrocytes was highest in the periventricular region, falling with increasing distance from the third ventricle, with nearly no GFAP immunoreactivity in ventromedial nucleus, dorsomedial nucleus and lateral hypothalamic area. This study demonstrated an increase in the expression of GFAP in the dorsomedial nucleus in the adult offspring of diabetic rats. These findings can be explained by that reactive astrogliosis occurs in response to inflammation and injury to the central nervous system as reported by Buckman *et al*. (2013) [25] who defined astrogliosis as the hypertrophy and hyperplasia of astrocytes in response to acute or chronic insults in the brain. Chronic fetal hypoxia present in maternal diabetes mellitus may increase the inflammatory burden incurred by the fetus (Loukovaara *et al*., 2004) [28] and the produced inflammatory cytokines affect neuronal development (Zalcman *et al*., 1994) [29]. In support of the present results, Magrinos and Mac Ewen (2000) [30] reported that dysfunctional glucose regulation and/or insufficient insulin availability elicits neuronal synaptic reorganization in the experimental diabetic rodents. Astrocytes interact with hypothalamic neurons to modulate neuronal maturation and neuroendocrine activity Theodosius and MacVicar (1996) [31]. In addition, astrocytes are known to regulate synaptic plasticity in hypothalamo-neurohypophysial system (Panatier, 2009) [32]. Therefore, alterations in the number of astrocytes as well as a disturbed glia to neuron ratio and glia-neuron interaction might have permanent consequences for hypothalamic organization and neuroendocrine functions. It is documented that the dorsomedial hypothalamic nucleus is critical for the expression of circadian rhythms and receives input from systems that monitor food availability (Gooly *et al*. 2006) [33] and so its structural changes in the offspring of diabetic mothers as was shown by this study can disturb the normal mechanism s involved in the body weight control.

5. Conclusion

This study demonstrated that maternal diabetes can structurally affect the development of the dorsomedial nucleus. It is recommended that there should be strict control of the diabetes before pregnancy. Women with diabetes mellitus at reproductive age should be identified as members of a high-risk group and an intensive glycemic control before conception and throughout pregnancy is a necessity.

6. References

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