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Morphometric study of the spinal cord in foetuses of diabetic pregnancies

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ABSTRACT

This study was conducted to evaluate the effects of maternal diabetes on the foetal spinal cord. Sixteen adult female rats were divided into two equal groups. Diabetes was induced in one group by alloxan. Both groups became pregnant by natural mating. At days 17, 18, 19 and 20 of pregnancy, the spinal cord was collected from the foetuses of all rats, and the body mass and number of foetuses were also measured. Various histological parameters were determined using routine histological techniques. The results revealed a decrease in the transverse diameter of the spinal cord, the transverse diameter of central canal, the number of cells in the white and gray matters and an increase in the vertical diameter of the spinal cord and the vertical diameter of the central canal in the foetuses of diabetic mothers (FDM) as compared to the control group. The body mass of FDM was significantly ($P<0.05$) higher than that of the control and the number of foetuses in FDM was significantly ($P<0.05$) lower than the control. Maternal hyperglycaemia exhibited deleterious effects on the spinal cord during foetal life, which affected the shape, structure and cell number of the spinal cord.

Key words: maternal diabetes, rat, foetus, alloxan, spinal cord

Introduction

The pancreas, by producing insulin, allows the body to use glucose efficiently. However, in diabetes, the pancreas insufficiently controls the insulin hormone, causing the blood sugar levels to rise (JONES, 2001). In diabetic mothers, the placental transport of glucose and other nutrients increases during pregnancy, resulting in foetal and neonatal Macrosomia (PERSSON and HANSON, 1998). Data indicate that maternal diabetes increases the incidence of major malformations (CUNINGHAM et al., 2005).

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One of the mammalian systems that is clearly impaired in diabetes is the nervous system (CECIL et al., 2003). An increased number of malformations occur in infants born from mothers with maternal diabetes involving the central nervous system (CNS) (ABERG et al., 2001) such as anencephaly, spina bifidia and hydrocephaly (CUNINGHAM et al., 2005). It has been shown that diabetes may lead to neurophysiological alterations, cognitive abnormalities, white matter hyperintensities and changes in the gray matter density (MUSEN et al., 2006**).** In addition, white matter microstructure deficits have been seen in type 1 diabetes (KODL et al., 2008). Studies have demonstrated that diabetes induces alterations in the dendritic morphology of cortical neurons (MARTINEZ-TELLEZ et al., 2005), changes in the hippocampal neuronal structure and density (TEHRANIPOUR and KHAKZAD, 2008), apoptosis in the neurons of the hippocampus (LI et al., 2002) and disturbs the proliferation and cell death of neural progenitors of the spinal cord (GAO and GAO, 2007). Diabetes leads to a decrease in nerve Na+,K(+)-ATPase activity, which is effective in the pathogenesis of diabetic neuropathy (SCARPINI et al., 1993). The purpose of this investigation is to evaluate the possibility of congenital spinal cord malformations in foetuses of diabetic rats, at days 17, 18, 19 and 20 of pregnancy.

Materials and methods

Sixteen adult female Sprague Dawley rats (200-230 g and 4-5 months old) were housed in an air-conditioned room (22 ± 2 °C) and supplied with standard pellet food with tap water *ad libitum.* The animals were divided into two groups: diabetics and normal (control). The animals were cared for and treated in accordance with the guidelines for laboratory animals, established by the National Institute of Health, as well as by the local ethical committee.

Diabetes was induced in thirty rats by a single intraperitoneal injection (150 mg/kg) of alloxan tetrahydrate (Sigma, St. Louis, MO) according to our previous experience (KHAKSAR et al., 2010; KHAKSAR et al., 2011). The animals were fasted 12 h before and after alloxan injection. Rats with blood glucose above 200 mg/dl, as well as with polydipsia, polyuria and polyphagia for at least 1 week, were considered to be diabetic and were selected for the experiment (SZKUDELSKI, 2001).

Female animals of both groups in oestrus stage were caged with male rats for mating. Mating was confirmed by vaginal plug observation (TURNER and BAGNARA, 1976). At days 17 to 20 of pregnancy, two rats from the the diabetic and control groups were anaesthetized (by using diethyl ether) and scarified daily. The foetuses were collected from both groups by surgery and the spinal cord was isolated from the foetuses of the rats. At the same time the body mass and number of foetuses were measured.

All tissue samples were fixed in 5% buffered formalin fixative for histopathological investigations and subsequently embedded in paraffin. Sections (5 microns thickness)

were stained with H&E and Green Masson's trichrome techniques, and observed with an Olympus BX51 microscope for evaluation of histomorphometrical parameters such as:

1) Transverse and vertical diameters of spinal cord (μm). 2) Transverse and vertical diameters of the central canal of spinal cord (μm). 3) The number of cells in the gray and white matter separately per unit (mm^2). 4) The ratio of gray matter to white matter.

The transverse and vertical diameters of the spinal cord and the central canal were measured by ocular micrometer and Olympus BX51 light microscope, using Olysia software. The number of cells per unit (mm^2) in both the white and gray matter and the ratio of gray matter to white matter were counted by ocular graticule and an Olympus BX51 light microscope, using Olysia software. Analysis of particularly morphometric data was carried out by Student's T test using the SPSS program.

Results

The foetal body mass changes of the diabetic and control groups are shown in Fig. 1. The mean body mass in the foetuses of diabetic mothers (FDM) was significantly ($P<0.05$) higher than that of the controls. Fig. 2 demonstrates the number of foetuses in the FDM and control groups on all days. The number of foetuses of diabetic mothers (FDM) was significantly ($P<0.05$) lower than that of the control group.

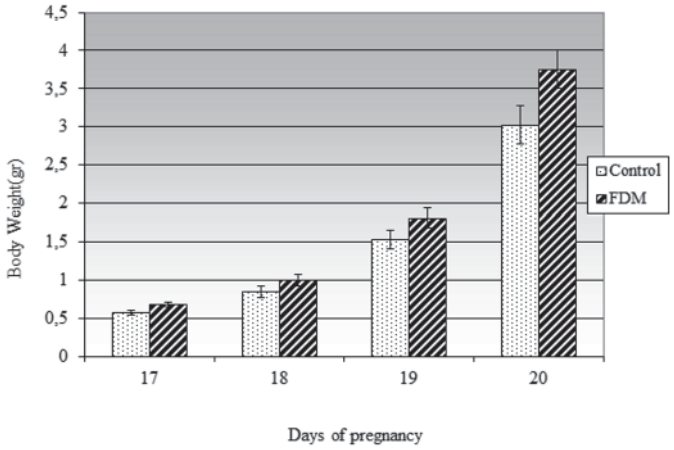

Fig. 1. Comparison of the body mass of foetuses of rats from normal (control) and diabetic mothers (FDM) at 17 to 20 days of pregnancy. The body mass of foetuses of diabetic mothers (FDM) increased significantly ($P<0.05$) compared to normal mothers (Control) at days 17, 18, 19 and 20 of pregnancy.

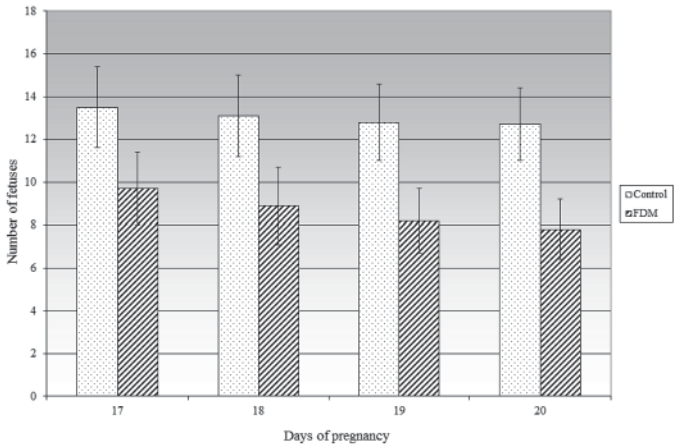

Fig. 2. Comparison of the number of foetuses of rats from normal (control) and diabetic mothers (FDM) at 17 to 20 days of pregnancy. The number of foetuses in diabetic mothers (FDM) decreased significantly ($P<0.05$) in comparison with normal mothers (control) at days 17, 18, 19 and 20 of pregnancy.

Table 1 presents the different parameters of the spinal cords of foetuses obtained from diabetic (FDM) and control mothers at days 17, 18, 19 and 20 of pregnancy.

The transverse spinal cord diameter was lower in FDMs compared to that of controls, which was significant ($P<0.05$) at days 18 and 19 of pregnancy. The percentage of reduction was 4.2%, 6.2%, 5.4% and 4.6% for days 17, 18, 19 and 20 of pregnancy, respectively. The vertical spinal cord diameter was higher in FDMs compared to control. This reduction was significant ($P<0.05$) at days 18, 19 and 20 of pregnancy. The difference at days 17, 18, 19 and 20 as percentages was 5.5%, 5.8%, 5.7% and 6%, respectively. The transverse diameter of the central canal was lower in FDMs compared to controls but this reduction was only significant ($P<0.05$) at day 20 of pregnancy. At day 20, this value was 33.6μm in FDMs but 38.4μm in controls. The vertical diameter of the central canal was insignificantly increased in FDMs compared to controls, which was 81.7 μm in FDMs and 75.6μm in controls at day 20 of pregnancy.

Table 1. Comparison of foetuses of diabetic mothers and control parameters of spinal cord at 17, 18, 19 and 20 days of pregnancy.

Age (day)	17		18	
Group	FDM	Control	FDM	Control
TDS (μm)	746.3 ± 48.4	779.4 ± 41.6	831.6 ± 33.3	887 ± 29.8*
VDS (μm)	664.7 ± 45.1	629.9 ± 48.5	763.2 ± 35. 8	721.2 ± 42.9*
TDC (μm)	26.2 ± 2.5	27.1 ± 2.3	29.6 ± 2.2	30.8 ± 1.9
VDC (μm)	168.5 ± 12.2	163.1 ± 14	103.1 ± 7.3	100.73 ± 6.2
GWR	3.1 ± 0.2	3.2 ± 0.2	2.7 ± 0.2	2.8 ± 0.2
NCG (n/mm^2)	13992.4 ± 777.4	14425.5 ± 768.2	10453 ± 567.1	10785.2 ± 546.3
NCW (n/mm^2)	247.5 ± 20.5	273.4 ± 19.1*	435.8 ± 31.4	450.4 ± 28.3
Age (day)	19		20	
Group	FDM	Control	FDM	Control
TDS (μm)	949.3 ± 59.2	1003.4 ± 58.3*	1309.1 ± 107.2	1371.8 ± 119.9
VDS (μm)	868.1 ± 30.5	820.4 ± 38.4*	981.3 ± 56.9	925.7 ± 54.1*
TDC (μm)	32.5 ± 5.3	33.9 ± 3.7	33.6 ± 4.2	38.4 ± 3.1*
VDC (μm)	95.3 ± 11.8	91.4 ± 11	81.7 ± 4.7	75.6 ± 5.2
GWR	2.2 ± 0.2	2.3 ± 0.2	1.9 ± 0.1	2 ± 0.1
NCG (n/mm^2)	11248.5 ± 630.4	11705.6 ± 621.1	11963.9 ± 892.9	12468.3 ± 864.3
NCW (n/mm^2)	831.8 ± 78.4	862.7 ± 75.6	1593.2 ± 121.9	1651.7 ± 110.8

FDM (Foetuses of diabetic mothers), TDS (Transverse diameter of spinal cord), VDS (Vertical diameter of spinal cord), TDC (Transverse diameter of Central canal), VDC (Vertical diameter of Central canal), GWR (Ratio of gray matter to white matter), NCG (Number of cells of gray matter), NCW (Number of cells of white matter), Values are demonstrated with mean ± SD. Asterisk (*) represents a significant difference at $P<0.05$.

The number of cells in the gray matter was insignificantly lower in FDMs compared to the control group. At day 20 of pregnancy, this value was 11963.9/mm^2 in the FDMs but 12468.3/mm^2 in the control group. The differences as percentages were 3%, 3%, 3.9% and 4% at days 17, 18, 19 and 20 of pregnancy, respectively. The number of cells in the white matter was lower in FDMs compared to those of the controls. At day 17 of pregnancy this reduction was significant ($P<0.05$), 247.5/mm^2 in FDMs but 273.4/mm^2 in the control group. The decreases in the number of cells in the white matter as percentages at days 17, 18, 19 and 20 were 9.4%, 3.2%, 3.6% and 3.5%, respectively. The ratio of gray matter to white matter was insignificantly lower ($P<0.05$) in FDMs compared to that of controls at days 17, 18, 19 and 20 of pregnancy.

Figs 3 and 4 show sections of the spinal cord in FDMs and the control groups at day 17 of pregnancy.

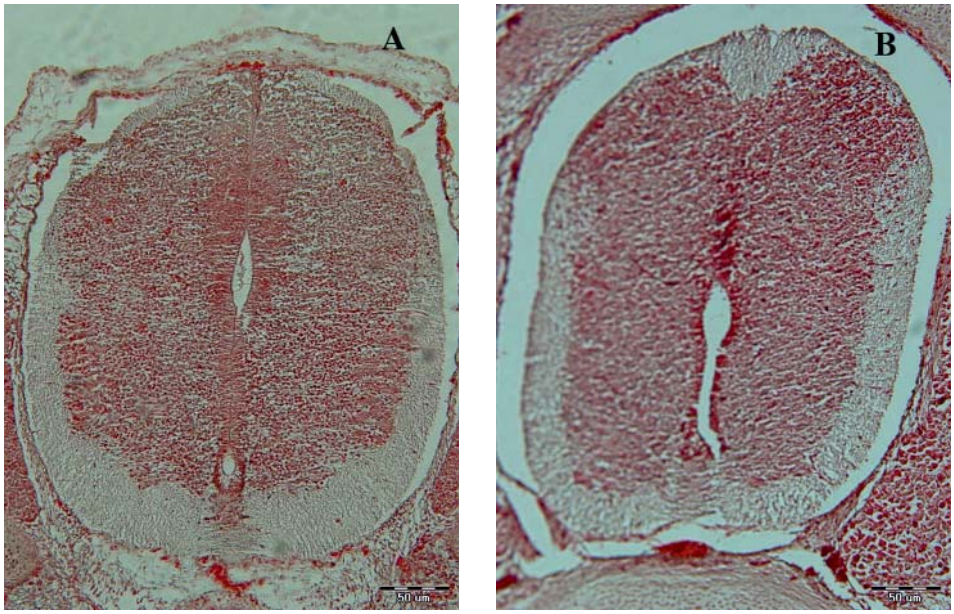

Fig. 3. Comparison of the transverse and vertical diameters of foetal spinal cord at day 17 of pregnancy in control (A) and FDM (B) groups (Staining: Hematoxilin&Eosin).

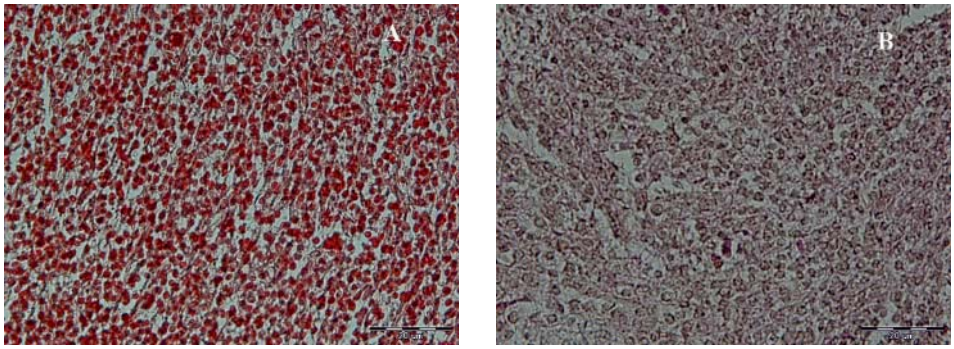

Fig. 4. Comparison of the cells number of gray matter in foetal spinal cord at day 17 of pregnancy in control (A) and FDM (B) groups (Staining: Hematoxilin&Eosin).

Discussion

The body mass of the foetuses of diabetic mothers was significantly higher than that of the control (Macrosomia), which is due to an increase in placental transport of glucose

and other nutrients (JONES, 2001). Our previous studies demonstrated that the body mass of neonates and foetuses of diabetic rats was significantly increased (KHAKSAR et al., 2010; KHAKSAR et al., 2011). Excessive gestational mass gain and hyperglycaemia may overstimulate the fetal pancreatic β cells and consequently bring about fetal hyperinsulinism. Insulin itself is a growth hormone for the fetus, resulting in higher birth mass and in impaired glucose tolerance and obesity in adolescence (WROTNIAK et al., 2008).

The number of foetuses of diabetic mothers was significantly fewer than that of the control group. MOLEY et al. (1998) reported that Hyperglycaemia induces apoptosis in pre-implantation embryos through cell death effector pathways; their results indicated that hyperglycaemic conditions, either *in vivo* or *in vitro*, modulate the expression of an apoptosis regulatory gene as early as the pre-implantation blastocyst stage in the mouse. Previous studies demonstrated that maternal diabetes increases oocytes apoptosis (LIN et al., 2010) and follicular atrophy (GARRIS et al., 1985) in mice. CHANG et al. (2005) demonstrated that both models of maternal hyperglycaemia and hypoinsulinemia may have a detrimental effect on oocyte maturation and development, as detailed by the smaller sizes of oocytes and developing ovarian follicles, the lowered percentage reaching germinal vesicle breakdown, and the greater amount of apoptosis. Stillbirths are a phenomenon found in pregnancies complicated by pregestational diabetes (CUNINGHAM et al., 2005).

The transverse diameter of the spinal cord was lower in FDMs compared to the control group at days 17 to 20 of pregnancy, whereas the vertical diameter of the spinal cord was higher in FDMs compared to the control. Neuropathy of several nerves, such as the sciatic nerve, has been reported in diabetics (ARTICO et al., 2002). Maternal diabetes leads to white matter hyper intensities and gray matter density changes in the foetus (MUSEN et al., 2006). In addition, white matter microstructure deficits were seen in type 1 diabetic subjects that correlate with impaired performance in neurocognitive tests, which are thought to be associated with white matter function (KODL et al., 2008). Northam and coworkers have shown type 1 diabetic subjects, relative to control subjects, had decreased amounts of gray matter in some parts of the CNS (NORTHAM et al., 2009). Statistical analysis showed a significant decrease in neuronal density in neonates from diabetic mothers compared to neonates in the control group (TEHRANIPOUR and KHAKZAD, 2008); those changes can be due to cell apoptosis as Li and coworkers have shown (LI et al., 2002). MARTINEZ-TELLEZ et al. (2005) explained that diabetes mellitus may in part affect the dendritic morphology in some areas of the CNS. Our previous study demonstrated that maternal diabetes has significant deleterious effects on brachial enlargement of neonates' spinal cord, and changes the shape of the spinal cord (KHAKSAR et al., 2010).

The number of cells in the gray matter and white matter in FDMs demonstrated a decrease compared to that of the control group on all days. One study has shown that hyperglycaemia leads to disturbance in the proliferation and an increase in the cell death of neural progenitors in mouse embryonic spinal cords (GAO and GAO, 2007). Chappell et al. demonstrated that insufficient expression of the genes that regulate the viability of the

progenitor cells is responsible for the apoptosis (CHAPPELL et al., 2009). We have shown that diabetes leads to a decrease in the number of neurons and glial cells of the brachial enlargement of offsprings' spinal cord (KHAKSAR et al., 2010). Hyperglycaemia effectively makes more substrate available for aerobic glycolysis, leading to acidosis (BIESSELS et al., 1994) and enhanced oxygen free radical formation, by a reduction in levels of protective endogenous antioxidants (BAYDAS et al., 2002). These radicals contribute to increased neuronal death by oxidizing proteins, damaging DNA, and inducing the lipoperoxidation of cellular membranes (HAWKINS and DAVIES, 2001). MOORADIAN et al. (1997) explained that diabetes is associated with changes in both the blood brain barrier and the transport functions of the microvessels of the CNS. Structural changes in the microvessels may account for some of the observed changes. Also, the reduction in Na+,K(+)-ATPase activity in diabetic nerves may be an important factor in the pathogenesis of diabetic neuropathy (SCARPINI et al., 1993). Studies have shown that obstruction of the feeding vessels of nerves due to diabetes causes the death of nerve bundles and myelin destruction (HARRISON et al., 2000). GULERIA et al. (2006) have shown that hyperglycaemia inhibits retinoic acid, which prevents differentiation of neurons, and causes Oxidative Stress in a rat model of diabetic pregnancy. These reasons could be considered for the malformation of this region, that is due to maternal diabetes.

Conclusions

Maternal diabetes has significant deleterious effects on the foetal spinal cord and leads to a decrease in the number of cells and ratio of gray matter to white matter, as well as altering the diameters of white matter and gray matter.

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SAŽETAK

Cilj istraživanja bio je procijeniti učinke dijabetesa majke na kralježničnu moždinu ploda. Šesnaest odraslih štakorica bilo je podijeljeno u dvije jednake skupine. U jednoj skupini dijabetes je bio izazvan aloksanom. Obje skupine štakorica ostale su gravidne nakon parenja. Tijekom 17., 18., 19. i 20. dana gravidnosti prikupljene su kralježnične moždine plodova od svih štakorica te su tom prilikom utvrđeni podatci za masu tijela i broj plodova. Uporabom rutinskih histoloških tehnika utvrđeni su različiti histološki pokazatelji. Rezultati su pokazali da je kod plodova od majki s dijabetesom u usporedbi s kontrolnom skupinom došlo do smanjivanja transverzalnog dijametra kralježnične moždine, transverzalnog dijametra središnjeg kanala, broja stanica u bijeloj i sivoj supstanciji te povećanje vertikalnog dijametra kralježnične moždine i vertikalnog dijametra središnjeg kanala. Tjelesna masa fetusa od majki s dijabetesom bila je statistički značajno veća ($P<0,05$) u odnosu na kontrolnu skupinu, a broj plodova kod istih majki bio je statistički značajno manji ($P<0,05$) u odnosu na kontrolnu skupinu. Hiperglikemija majki imala je štetan učinak na kralježničnu moždinu tijekom fetalnog života, što je imalo utjecaj na njezin oblik, strukturu i broj stanica.

Ključne riječi: dijabetes majke, štakor, plod, aloksan, kralježnična moždina