Altered placental morphology and VEGF expression in diabetic rats

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Diabetes is a common problem in approximately 7.6% of pregnant women older than 20 years in Brazil. Results from human and rodent diabetic experimental models have suggested that the placenta is a compromised target, which largely suffers the impact of maternal diabetes. In view of the importance of diabetes for maternal and neonatal health, the objective of the present study was to assess maternal glycemia and body weight, placental morphology and VEGF distribution in the rat animal model for diabetes that shows a similar placental phenotype observed in human diabetic pregnancy. Diabetes was induced by a single injection of alloxan in saline solution on gestational day (gd) 8 in Wistar rats. On 17 gd, rats from control (n=5) and diabetic (n=5) groups were anesthetized, exsanguinated, and laparotomized to remove the uterine horns for weighing of fetuses and placenta collection. Placentas were sampled and processed for paraffin embedding, histological and immunohistochemical analysis. The female rats showed severe diabetes (329.3 ± 13.38 mg/dl), but the maternal weight was not different between the two groups (control: 209.4 ± 11.08 g, diabetic: 198.2 ± 8.76 g). Placentas from the diabetic group had an enlarged junctional zone (JZ) and a straight arrangement of fetal capillaries and maternal sinusoids in the labyrinth zone (LZ). VEGF expression was observed in different cell types in the JZ and LZ. A greater number of glycogen and trophoblast giant cells positive for VEGF were quantified in the diabetic group. In the LZ, more cells were positive for VEGF in the control group than in the diabetic group. Severe hyperglycemia increased the expression of VEGF in the JZ and decreased in the LZ. The morphological changes in the size and organization of the spongiotrophoblast and glycogen cells in rat models of diabetes suggests the JZ as the placental compartment most sensitive to diabetes condition. Moreover, the VEGF role in the JZ is not fully understood and may have another function other than capillary growth.

Keywords: diabetes; placenta; VEGF; rat

1. Introduction

Diabetes in pregnant women is associated with an increased risk of maternal and neonatal morbidity, and remains a significant medical challenge. Diabetes during pregnancy may be divided into clinical diabetes (in cases previously diagnosed with type 1 or type 2 diabetes) and gestational diabetes [1]. The prevalence of diabetes during pregnancy varies in a given population or ethnic group. In the Brazilian health public system, 7.6% of pregnant women older than 20 years are diabetic [2].

Despite treatments, diabetic pregnant women are susceptible to miscarriage, stillbirth, and congenital and placental malformations [3, 4, 5]. Frequent placental abnormalities associated with maternal diabetes indicate the occurrence of hypoxia [6, 7, 8]. Under hypoxic/ischemic conditions, hypoxia-inducible factor upregulates transcription of various cytokines, including erythropoietin and vascular endothelial growth factor (VEGF) [9] in many tissues, as well as the placenta.

The rat animal model for diabetes also shows a similar placental phenotype observed in human diabetic pregnancy. However, no immunohistochemical studies have been conducted to identify VEGF-producing cells in different placental regions of alloxan diabetic rats. In view of the importance of diabetes for maternal and neonatal health, the objective of the present study was to assess glycemia, body weight, placental morphology and VEGF expression in diabetic female rats.

2. Materials and Methods

2.1 Animals and diabetes induction

Twenty-five female and five male adult Wistar rats weighing 200-250 g were used for the mating. The morning when spermatozoa were found in the vaginal smear was designated gestational day 1 (1 gd) (Ethics committee - CEPA 86/2011). Diabetes was induced by a single injection of alloxan monohydrate (37mg/kg, i.v) in saline solution on 8 gd, after 12 hours of starvation. Animals in control group received identical volume of saline solution. Blood glucose concentration and body weight were verified at 8gd, 10gd and at 17gd. Animals exhibiting blood glucose level higher than 200mg/dl at 10 gd (Accu-Chek Performa test strips, Roche Diagnostic) were included in the diabetic group. On 17 gd, rats from control (n=7) and diabetic (n=7) groups were anesthetized, exsanguinated, and laparotomized to remove the uterine horns.
2.2 Tissue processing

Placental samples were fixed in 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4 for 24 hours. After dehydration in ethanol, tissues were cleared in xylene and embedded in paraffin wax. Subsequently, serial 5 µm sections were cut and mounted on gelatin coated slides. For histological analysis, sections were stained with haematoxylin and eosin (H&E).

2.3 Immunohistochemistry

Serial 5 µm sections of four placentas per animal per group (n=7) were cut and mounted on silane coated slides. Sections were deparaffinized, rehydrated and incubated with 3% H$_2$O$_2$ in 0.1M PBS pH 7.4 for 10 minutes to block endogenous peroxidases. The sections were then incubated with 1% BSA in 0.1M PBS pH 7.4 (30 minutes) and with primary polyclonal goat anti-mice VEGF antibody (diluted 1:30, Sc-1836, Santa Cruz Biotechnology) for 12 hours at 4°C. After primary antibody incubation, the sections were washed in PBS and incubated with secondary biotinylated donkey anti-goat IgG (diluted 1:300, Sc-2042, Santa Cruz Biotechnology) for 1 hour at room temperature. Subsequently, the material was washed in Tris-HCl buffer and treated with peroxidase streptavidin (diluted 1:300, Vector SA-5004). The peroxidase was revealed with 0.5mg diaminobenzidine tetrahydrochoride (DAB; Sigma) in 0.3% H$_2$O$_2$ in TBS. The slides were counterstained with Carrazzi’s Hematoxylin, dehydrated, and mounted with Entellan (Merck, Darmstadt). Negative controls were performed by exclusion of primary antibody incubation step.

2.4 Image capture and semi-quantitative analyze

Three sections of each placenta per animal (n=4) in each group were used for semi-quantitative analyze of VEGF positive cells. Sequential images of entire placental sections at 400x magnification (Leica DM1000 Microscope/Laz EZ 2.0 software) were captured. The selection of images was made per placenta area, discarding 1 image and analyzing the sequence one until totalize at least 50 images on junctional zone (JZ) and 64 images on labyrinth zone (LZ) of each section. In the LZ, VEGF positive cells were counted without the cell type specification because of the difficulty to identify different labyrinth trophoblast cells at light microscopic level. The cell counting was performed using the ImageJ Software (cell counter tool) and the data transferred for a spreadsheet Excel Software for posterior statistical analyze.

2.5 Statistical analysis

All data are expressed as means and standard errors of means (SEM). Comparisons between control and diabetic groups for glycemia, body weight and VEGF positive cells were performed by on way ANOVA followed by Tukey test. A value of p < 0.05 was considered significant.

3. Results

3.1 Maternal glycemia and body weight

In control rats, normoglycemia was confirmed at 17 gd with a mean glucose value of 86.29 ± 2.79 mg/dl. In the diabetic group, glucose levels were significantly higher (329.3 ± 13.38 mg/dl, p< 0.001) than those in the control group. There was no significant difference in body weight between the two groups (control: 209.4 ± 11.08 g, diabetic: 198.2 ± 8.76 g) at 17 gd.

3.2 Placental morphology

The rat placenta at 17 gd is composed of three compartments: the decidua (D), the junctional zone (JZ), and the labyrinth zone (LZ) (Fig. 1a, 1b). In the JZ, three differentiated cell types were observed, including trophoblast giant cells (TGC), spongiotrophoblast cells (STC), and glycogen cells (GC) (Fig. 1c, 1d). The histological analysis revealed that severe maternal diabetes results in enlarged JZ (Fig. 1b, 1d), with increased number of STC and GC. In the LZ, a straight arrangement of fetal capillaries and maternal sinusoids was observed in the diabetic group (Fig. 1f) instead of the sinuous pattern in the control group (Fig.1e).
3.3 VEGF expression

VEGF-positive cells were observed in different cell types of the JZ in both groups, but strong staining intensity was present in the diabetic group than in the control group. VEGF expression was detected in the cytoplasm of trophoblast giant cells (TGC), glycogen cells (GC), and in some spongiotrophoblast cells (STC). In the LZ, strong staining intensity was present in trophoblast cells in the control group compared with the diabetic group (Fig. 2).
Fig. 2  Placentas of control (a, c) and diabetic groups (b, d) at 17 gd, a and b) VEGF was detected in the cytoplasm of the trophoblast giant cells (arrowhead), glycogen cells (*) and spongiotrophoblast cells (arrow) in the junctional zone (JZ). c and d) In the labyrinth zone (LZ), trophoblast cells were positive for VEGF (arrow). VEGF immunoperoxidase, D (decidua), scale bars (a, b) 100 μm, (c,d, detail in b) 30μm.

Semi-quantitative analysis showed a greater number of GC (p < 0.05) and TGC (p < 0.05) expressing VEGF in the diabetic group compared with the control group. The number of STC positive for VEGF was not different between the two groups. In the LZ, more cells were positive for VEGF in the control group (p < 0.01) than in the diabetic group (Table 1).

Table 1  Semi-quantitative analyze of VEGF positive cells. Trophoblast giant cells (TGC), spongiotrophoblast cells (STC), glycogen cells (GC) and labyrinth cells (LC). Values are SEM, n=7/group, **p < 0.01, *p < 0.05.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Control group</th>
<th>Diabetic group</th>
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<tbody>
<tr>
<td>TGC</td>
<td>25.5 ± 4.5</td>
<td>53.67± 4.95*</td>
</tr>
<tr>
<td>STC</td>
<td>169.0 ± 21.5</td>
<td>195.3 ± 26.77</td>
</tr>
<tr>
<td>GC</td>
<td>483.3 ± 18.25</td>
<td>800.5 ± 70.16*</td>
</tr>
<tr>
<td>LC</td>
<td>556.3 ± 156.3**</td>
<td>159.0 ± 21.08</td>
</tr>
</tbody>
</table>

4. Discussion and conclusion

Animal models in diabetes and pregnancy are useful because of ethical concerns related to research on human pregnancy. The most often used experimental models are rodents (Wistar rats) because of their convenient maintenance, short length of pregnancy, and multiparity, thus enabling studies on multiple fetuses and generations [1]. Many studies have applied different doses of alloxan and streptozotocin in rats to induce diabetes during pregnancy, reaching glycemic levels between 275 to 353 mg/dl [10, 11, 12]. In our study, the mean blood glucose concentration in diabetic rats at 17 gd was 329.3±13.38 mg/dl, which is considered severe diabetes [13, 14].

Pregnancy is characterized by a progressive increase in maternal weight gain, derived from the fetus and placental annexes growth (approximately 40%) and by adaptations of the body (the remaining 60%), characterized by anabolism at the beginning and catabolism at the end of pregnancy [15]. In the present study, maternal weight was not reduced in female rats from the diabetic group compared with the control group. Similarly, Volpato and co-workers [16] studied diabetic pregnant rats and observed no difference in weight gain between sedentary and animals subjected to physical exercise. However, Sinzato [17] and Iessi and co-workers [14] observed a significant reduction in maternal weight gain.
in the diabetic group, induced during the neonatal period, compared with normoglycemic rats. These differences between studies may have occurred because of the interval between the day of induction and the day of sacrifice of animals (i.e., the time at which females remained hyperglycemic). In our model, we performed the induction of diabetes on 8 dg. Therefore, the rats remained hyperglycemic for 9 days until sacrifice, and this may not have been a sufficient time to cause a reduction in body weight.

Morphological analysis showed an enlarged JZ that is described as an increased amount of glycogen cells and giant trophoblast cells [18, 19]. Indeed, our previous stereological study confirmed a bigger spongiosotrophoblast/glycogen cells volume in diabetic animals compared with controls [20]. Also, some works show morphological changes in the size and organization of the spongiosotrophoblast and glycogen cells in rat models of diabetes [18, 21, 22], suggesting the JZ as the placental compartment most sensitive to diabetes condition [23, 20].

Placental glycogen accumulation in diabetes occurs in marked contrast to other tissues, such as maternal liver, from which glycogen disappears. Liver and muscle glycogenesis and glycogenolysis are under insulin control, by regulation of activity of glycogen synthase and phosphorylase. However, glycogen accumulation in the placenta of diabetic rats is related to the extent of maternal hyperglycemia and is independent of insulin [24]. The increased capacity of glucose uptake by placental cells could be related to the expression of glucose carrier transporter isoforms (GLUTs). GLUT1 is highly expressed in the junctional and labyrinth zones of the rat chorioallantoic placenta [25, 26]. GLUT1 expression in the JZ is highest at midgestation and actually decreases in concentration by the end of pregnancy [27, 28]. GLUT1 in the JZ is likely associated with metabolic requirements for rapid placental growth at midgestation rather than transplacental glucose transport [29]. Therefore, GLUT1 could be upregulated in the JZ of the diabetic placenta, enabling increased uptake of glucose by glycogen cells.

The straight arrangement of fetal capillaries and maternal sinusoids observed in the diabetic group was clearly different from the normal sinuous pattern in the control group. The significance of changes in the placental labyrinth should be investigated by further studies on capillary surface area, thickness, and capillary length/diameter, which are important factors for predicting interhemal membrane development and function [30]. Placental efficiency can be changed by alterations in the surface area for exchange, the thickness of the barrier between the maternal and fetal circulations, and/or in the density and architectural arrangements of the fetal and maternal vasculature within the placenta [31].

The maternal-placental oxygen supply is reduced in diabetes [32] and placental hypoxia is related to the thickness of trophoblast layers of the placental barrier, labyrinth disorganization, and reduced fetal capillarization in diabetic placentas [18, 19]. Moreover, under hypoxia, hypoxia-inducible factors (HIFs) bind to specific promoter elements that are present in the promoter region of VEGF regulating the production of this factor [33]. In our study, a greater number of TGC and CG positive for VEGF in the diabetic group compared with the control group indicates an increased production of this factor in the JZ under hyperglycemia. On the contrary, fewer labyrinth cells were positive for VEGF in the diabetic group. Hypoxia, which occurs in diabetes, appears to be harmful in the JZ compared to LZ. However, VEGF action in the JZ is not fully understood and may have another function other than capillary growth.

To better understand the potential function of VEGF in the placenta under hyperglycemic conditions is necessary to verify the target cells by VEGFR1 (Flt-1) and VEGFR2 (Flk-1) immunolocalization. In the human placenta, VEGF and VEGFR2 staining has been identified in trophoblast and vascular cells, and weak staining for VEGFR1 has been observed in trophoblast cells [34]. The staining pattern showed variations in the placenta at term (> 37 weeks of pregnancy) in patients with prior diabetes and gestational diabetes [34], supporting the involvement of VEGF in diabetic placental disorders.

VEGFR2 is the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF-A [35]. However, many conflicting reports about the function of VEGFR1 exist [34]. Migration of monocytes/macrophages and induction of growth factors from liver sinusoidal endothelial cells are some of VEGFR1 functional roles [36, 37, 38]. However, no studies have revealed the expression of VEGF receptors in the placenta of normal and diabetic rats. Further works are necessary to verify the distribution of VEGFR1 and VEGFR2 in different placental cell types to determine the role of VEGF in target cells, and their effects in the placenta of diabetic rats.

In this report, we investigated the negative effect of severe hyperglycemia on maternal weight, placental morphology and VEGF expression. More detailed studies are required to explain the cellular and molecular mechanisms underlying placentaion in diabetic pregnancy.

Acknowledgments This work was supported in part by CNPq and FAPITEC, Brazil.

References


