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Is there a relationship between PCNA expression and diabetic placental development during pregnancy?

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Western blot

Summary

We aimed to investigate the distribution pattern of proliferating cell nuclear antigen (PCNA) by immunohistochemistry and Western blot in placentas of control and diabetic rats at different stages of pregnancy. It is still not clear how proliferation is coordinated and how this coordination is affected by diabetes in the placenta. Diabetes was induced by streptozocin on the first day of pregnancy. Animals were sacrificed on days 11, 13, 17 and 21 of pregnancy. In control placentas immunolabeling intensity of PCNA was the highest on days 11 and 13 of pregnancy and decreased with progression of pregnancy. In the diabetic groups immunolabeling was less intense on days 11 and 13 of pregnancy compared to controls. However, in parallel with placental weights, PCNA immunopositivity was more intense in diabetic groups than control groups on days 17 and 21 of pregnancy, and the difference was statistically significant on day 17. According to Western blot data, on days 11 and 13 of pregnancy the amount of PCNA was greater in control groups than in the diabetics, whereas it was greater in diabetic groups than the controls on days 17 and 21 of pregnancy. We conclude that PCNA may play a role in abnormal placenta formation resulting from diabetes.
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Introduction

The placenta promotes the delivery of nutrients and oxygen from the maternal circulation to the fetus and is essential for fetal growth. It forms the surface for transport of nutrients and gas exchange

Abbreviations: AMT, allantoic mesenchymal tissue; AVEC, allantoic vessel endothelial cell; GC, giant cell; Gly C, glycogen cell; LFVEC, labyrinth fetal vessel endothelial cell; LT, labyrinth trophoblast; PEC, parietal endoderm cell; ST, spongiotrophoblast; VEC, visceral endoderm cell.

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and directs maternal metabolism, ovarian function and growth and blood supply to the uterus (Hemberger and Cross, 2001). The rat placenta is of the hemochorial type (Yan et al., 2005), as is the human placenta, and is therefore regularly used as an animal model to study pregnancy-related problems in humans, such as those associated with diabetes and hypertension (Vercruysse et al., 2006).

Maternal diabetes increases the risk of placental dysfunction and diseases in both the neonate and in the later life of the offspring. *Diabetes mellitus* is a disorder that affects the normal development of pregnancy and may result in increased spontaneous abortion, congenital abnormalities and increased neonatal morbidity and mortality rates. In addition, diabetic pathology leads to structural and functional disturbances in the placenta that can result in deleterious consequences to the developing embryo. Maternal diabetes increases the risk of neonatal respiratory distress and the development of cardiovascular anomalies and glucose intolerance during the later life of the offspring (Pustovrh et al., 2005). Placental enlargement is a common feature of human diabetic pregnancies.

Although placental development depends on careful coordination of trophoblast proliferation and differentiation, little is known about the mitotic regulators that play key roles in synchronizing these events (Genbacev et al., 2000).

Pregnancy in the streptozocin (STZ)-induced diabetic rat is also characterized by placentomegaly and varying degrees of fetal growth retardation (Robinson et al., 1988). The laboratory rat, rendered diabetic by STZ, has been widely used as a model for studying the pathophysiological effects of maternal diabetes. Fetal growth retardation is a consistent feature of these diabetic pregnancies. As such, this rat model closely reflects the early events in human diabetic pregnancies and may be useful for studying the etiology of delayed somatic growth (Canavan and Goldspink, 1988).

Proliferating cell nuclear antigen (PCNA) is a marker for the cell cycle. It is an accessory protein that is necessary for deoxyribonucleic acid (DNA) synthesis in mammalian cells (Jonsson and Hubscher, 1997), whereas lack of this protein prevents cell division (Bravo and Macdonald-Bravo, 1987). PCNA loads onto DNA through the action of replication factor C complex and provides a scaffold for consecutive attachment of various DNA nucleases, DNA polymerases, DNA ligases and others, and is involved in DNA replication, recombination and repair (Paunesko et al., 2001). PCNA also plays a role in DNA repair and control of the cell cycle (Cayli et al., 2002).

On the basis of the literature, we hypothesized that the mitotic activity of the rat placenta under diabetic conditions might show alterations as pregnancy progresses. In the present study, we aimed to examine the distribution patterns of PCNA and to determine the relationship between placental development and PCNA in normal and diabetic rat placentas.

Materials and methods

Animals

Sixty-four female and 32 male adult Wistar rats (*Rattus norvegicus*) weighing 200–250 g were used in the study and none of them had mated previously. All rats were allowed free access to standard rat laboratory diet and tapwater and were maintained on a 12:12 h light/dark cycle. Two females and one male were kept overnight in one cage. A sperm-positive vaginal smear observed on the following morning was considered to indicate successful copulation. The sperm-positive day was designated as day 0 of pregnancy. Pregnant animals were caged separately and were randomly assigned to either the control or diabetic groups. Diabetes was induced in the appropriate group of rats by a single injection of STZ on the first day of pregnancy. Rats in the diabetic-induced group were injected intraperitoneally with a single dose of 50 mg/kg of STZ (Sigma-Aldrich Co., St. Louis, MO, USA; 63178) freshly prepared in 0.1 M citrate buffer (pH 4.5). Animals in the control group received an identical volume of citrate buffer.

Blood glucose concentrations were measured 48 h after STZ injection to confirm that diabetes had been induced. Blood samples were obtained from the tail vein. Animals exhibiting blood glucose level higher than 200 mg/dl (Accu-Chek Active test strips, Roche Diagnostic GmbH Mannheim, Germany, 3146332) were considered to be diabetic. Animals that did not develop diabetes were excluded from the experiments.

Control and STZ diabetic mothers were anesthetized with ether, sacrificed by cervical dislocation, and embryos, fetuses and placentas were taken on days 11, 13, 17 and 21 of gestation. Fetal and placental weights were recorded. Results are presented as mean $\pm$ SEM (standard error of the mean). The experimental protocols were approved by the Animal Care and Usage Committee of Akdeniz University and were in accordance with the guidelines of the International Association for the Study of Pain.

Tissue processing

Placental samples were fixed in Holland's fixative consisting of 4% formaldehyde, 5 ml glacial acetic acid, 4 g picric acid and 2.5 g cupric acetate in 100 ml distilled water. After dehydration in ethanol, tissues were cleared in xylene and embedded in paraffin wax. Subsequently, serial 5-μm-thick sections were cut and mounted on poly-L-lysine-coated slides (Sigma-Aldrich, St. Louis MO, USA, P8920).

PCNA immunohistochemistry

The immunohistochemical procedure has been described elsewhere (Ustunel et al., 2003). Briefly, sections were deparaffinized in xylene and rehydrated through a graded series of ethanol. To unmask antigens, an antigen retrieval procedure was performed by treating the samples in 10 mM citrate buffer, pH 6.0, in a microwave oven at 750 W for 5 min, twice. After cooling for 20 min at room temperature, the sections were washed in phosphate buffered saline (PBS; pH 7.4). Endogenous peroxidase activity was blocked by incubation in methanol containing 3% H_2O_2 for 30 min and sections were then washed with PBS three times. Afterwards, sections were incubated in a blocking serum (Ultra UV Block, LabVision Corporation, Fremont, CA, USA; TA-125UB) for 7 min at room temperature to block non-specific binding. Mouse monoclonal anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-56) was applied at a 1:200 dilution for 1 h at room temperature. Negative controls were performed by replacing the primary antibody with the appropriate non-immune IgG. Sections were rinsed three times in PBS and incubated with biotinylated secondary antibody (DAKO, Glostrup, Denmark; LSAB-2 system HRP K0609-ready to use) and a peroxidase labeled streptavidin (DAKO, Glostrup, Denmark; LSAB-2 system HRP K0609-ready to use), respectively, for 30 min, with each of the steps being followed by three rinses in PBS. Peroxidase activity was visualized with 3-amino-9-ethylcarbazol (AEC) (Lab Vision Corporation, Fremont, CA, USA; TA-125-HAC) chromogen in large-volume AEC substrate (Lab Vision Corporation, Fremont, CA, USA; TA-125 HAS) as one drop chromogen in 1 ml substrate for 4 min. All dilutions were performed using PBS unless otherwise stated. Sections were counterstained with hematoxylin, dehydrated, mounted in Kaisers glycerin gelatin (Merck, NJ, USA; OB514196) and examined by light microscopy. Tissue sections from different pregnancy days were evaluated for protein localization and immunolabeling intensity. All samples were treated with exactly the same protocol.

H-SCORE and semi-quantitative evaluations

The evaluation of the immunohistochemical labeling of PCNA in samples from both diabetic and control groups was performed using H-SCORE (Celik-Ozenci et al., 2004). Briefly, sections were evaluated using an Axioplan microscope (Zeiss, Oberkochen, Germany) with a special ocular scale. Three randomly selected slides, each of five different fields (two maternal and three fetal zones) at 200 × magnification, were evaluated for immunohistochemical labeling of PCNA. The labeling was scored in a semi-quantitative fashion that included the intensity of specific labeling in sections. The evaluations were recorded as percentages of labeled cells of all types in each of four intensity categories, denoted as 0 (no labeling), 1+ (weak labeling but detectable above control), 2+ (distinct labeling) and 3+ (intense labeling). For each tissue, an H-SCORE value was derived by summing the percentages of cells that were labeled at each intensity multiplied by the weighted intensity of the labeling: $\text{H-SCORE} = \sum P_i\ (i{+}1)$, where i is the intensity score and P_i is the corresponding percentage of the cells. Two observers blinded to the experimental groups performed the H-SCORE evaluations, and the average score was used. The distribution of immunoreactive cells in all experimental groups was also determined semi-quantitatively: 0 = negative; (+) = weak positive; + = positive; ++ = strong positive; +++ = very strong positive.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and Western blotting

Protein extraction and immunoblot analysis were performed as described previously (Hahn et al., 2001). Four placenta samples for each group were weighed and put into a homogenization buffer supplemented with CompleteR protease inhibitor cocktail (Boehringer, Mannheim, Germany). After homogenization, samples were centrifuged at 10,000*g* for 10 min. Supernatants were collected and stored at −70 °C.

The protein concentration was determined by Lowry assay (Lowry et al., 1951), and 50 μg of protein was applied per lane. Samples were subjected to SDS polyacrylamide gel electrophoresis (30% acrylamide in 7.5% gel) at 100 V for approximately 1.5 h and were then transferred onto

nitrocellulose membranes (Pharmacia, Kirkland, Quebec, Canada) in a buffer containing 0.2 mol/L glycine, 25 mmol/L Tris and 20% methanol overnight, at +4 °C, under 32 V and 65 A. The membranes were blocked for 1 h with 5% non-fat dry milk (BioRad Laboratories, Hercules, CA, USA) and 0.1% Tween 20 (Sigma-Aldrich, St Louis, MO, USA) in 0.14 mol/L Tris-buffered saline (TBS; pH 7.2–7.4) at 4 °C. Membranes were incubated with a 1:750 dilution of mouse monoclonal anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-56), overnight. After the washing steps, the membranes were further incubated with goat anti-mouse IgG horseradish peroxidase conjugate (BioRad Laboratories, Hercules, CA, USA; 170-6516), diluted 1:4000, for 1 h at room temperature.

Immunolabeling was visualized by using the chemiluminescence-based SuperSignal CL HRP Substrate System (Pierce, Rockford, IL, USA) prepared according to the manufacturer's instructions, and the membranes were exposed to Hyperfilm (Amersham, Bucks, UK; 28906837). Membranes were also labeled by identical protocol for binding of a 1:6000 dilution of an anti-mouse β-actin antibody (Abcam, Cambridge, UK; ab 6276-100) as an internal control to confirm the equal loading of the samples. The bands were quantified using NIH image analysis software (Image J Version 1.36b, National Institutes of Health, USA).

Statistical analysis

The following comparisons between control and diabetic groups were performed by Mann–Whitney rank sum test and Wilcoxon signed-rank test: embryo and placenta weights, H-SCORE values for PCNA immunohistochemistry, PCNA Western blot band values. Mann–Whitney rank sum test comparisons were made between control and diabetic groups samples taken from the same day. Wilcoxon signed-rank test comparisons were made between days for the same group, i.e. between control group day 11 and control group day 13. Values are presented as mean $\pm$ SEM. All statistical analyses were performed using SPSS (statistical package for the social sciences 10.0 for Windows, Chicago, IL, USA).

Results

Embryo weights

Embryo weights increased both in control and diabetic groups with the progression of pregnancy. Embryo weights of the diabetic group were found to be lower than those of the control group. Embryo weights, for control and diabetic groups, respectively (in g), were 0.174 ± 0.01 and 0.152 ± 0.01 for day 11 of pregnancy; 0.384 ± 0.02 and 0.249 ± 0.02 for day 13 of pregnancy; 0.913 ± 0.02 and 0.679 ± 0.02 for day 17 of pregnancy; 5.138 ± 0.12 and 4.859 ± 0.10 for day 21 of pregnancy. These decreases were statistically significant on days 13 and 17 of pregnancy ($p < 0.001$) but not on day 11 and 21 of pregnancy (Mann–Whitney rank sum test) (Figure 1a).

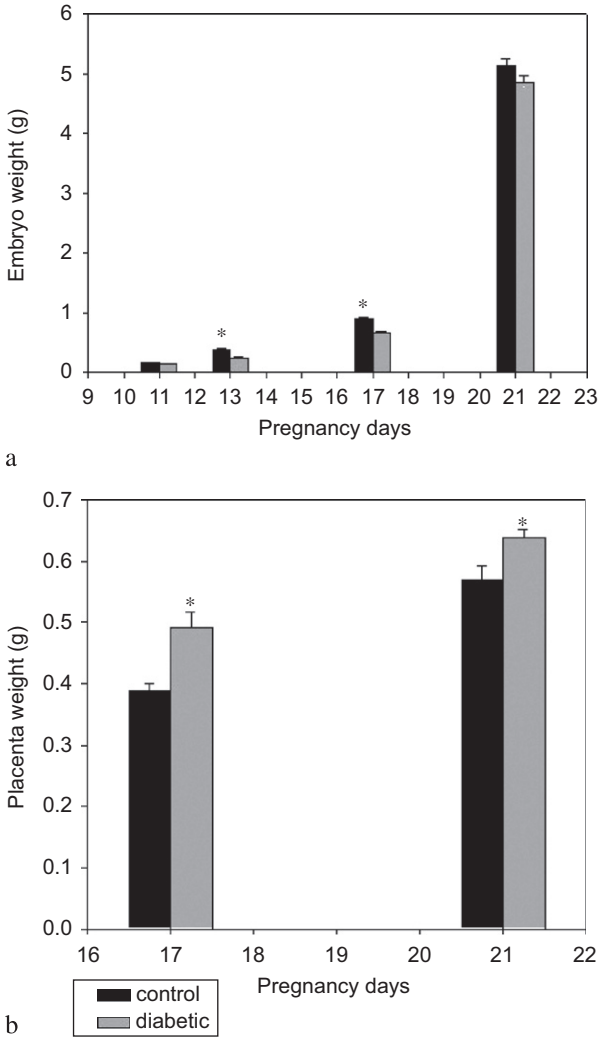


Figure 1. Embryo and placenta weights. (a) Embryo weights in control and diabetic groups. Control group embryos were heavier than the diabetic group embryos. The difference was statistically significant on days 13 and 17 ($p < 0.001$). (b) Placental weights in control and diabetic groups. Diabetic group placentas were heavier than the control placentas. The differences were statistically significant. *$p < 0.001$.

Table 1. Semi-quantitative scoring of PCNA immunolabeling intensities in control and diabetic group rat placenta zones.

Gestation days	Allantois				Labyrinth		Junctional zone		
	VEC	PEC	AVEC	AMT	LFVEC	LT	ST	Gly.C	GC
Control									
11	+++	+++	+++	+++	+++	+++	+++	x	+++
13	+++	++	+++	+++	+++	+++	+++	x	+++
17	+++	+	++	+	+	+	++	(+)	++
21	+	(+)	(+)	(+)	(+)	(+)	++	0	+
Diabetic									
11	+++	+++	+++	+++	++	++	++	x	+++
13	++	+	++	++	++	++	++	x	+++
17	+++	+	++	++	++	++	+++	(+)	++
21	++	(+)	+	+	+	(+)	+++	0	+++

0 = negative; (+) = weak positive; + = positive; ++ = strong positive; +++ = very strong positive; X = this zone does not exist.

Placenta weights

On days 17 and 21 of pregnancy, the placenta weights in the diabetic group were greater than the controls. Placental weights (in g) for control and diabetic groups, respectively, were 0.389 ± 0.01 and 0.491 ± 0.03 for day 17 of pregnancy; and 0.569 ± 0.02 and 0.638 ± 0.01 for day 21 of pregnancy. The difference was 26.2% and 12.1%, respectively, for day 17 and day 21 of pregnancy and the differences were statistically significant (Mann–Whitney rank sum test) (Figure 1b).

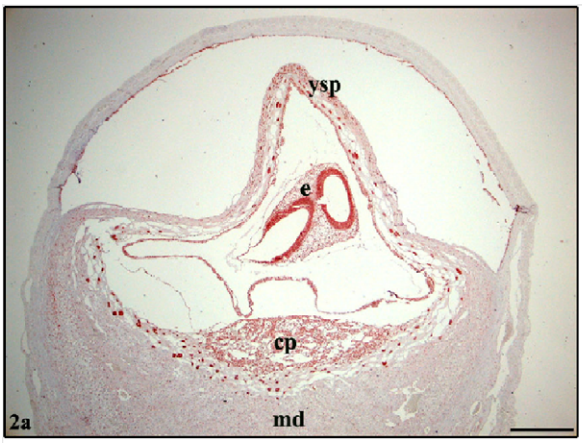
Immunohistochemical labeling of PCNA

The distribution pattern of PCNA immunolabeling intensity in control and diabetic group placentas is summarized in Table 1.

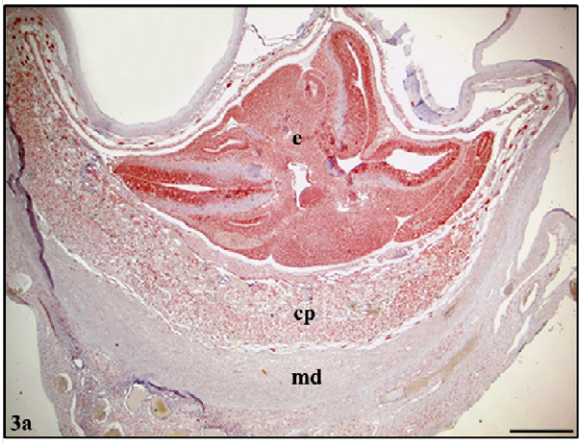
On day 11 of pregnancy, PCNA immunolabeling was intense in all the placenta zones (allantois, labyrinth and junctional zone) of the control group (Figure 2a). PCNA localization was nuclear. On the same day in the diabetic group, PCNA immunolabeling intensities decreased compared to the control group (Figure 2b).

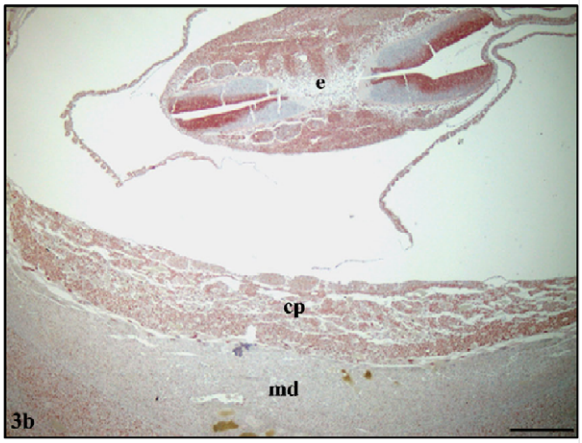
On day 13 of pregnancy, in the control group, visceral endodermal cells, allantoic vessel endothelial cells, allantoic mesenchymal tissue, labyrinth trophoblasts, labyrinth fetal vessel endothelial cells, spongiotrophoblasts and giant cells revealed very strong PCNA immunolabeling intensity; parietal endodermal cells were labeled strongly positive. In the diabetic group, PCNA immunolabeling intensities decreased compared to the control group, except for the giant cells in the junctional zone. Giant cells in the junctional zone were labeled very strong positive, similar to the giant cells in the junctional zone of the control group. The overall general appearance of immunolabeling in these groups is indicated in Figure 3a, b.

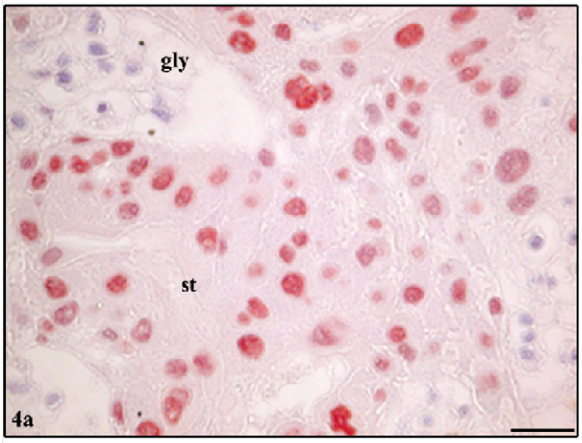
Figures 2–5. PCNA immunolabeling on day 11 of pregnancy. (a) Very strong positive PCNA immunolabeling intensity was seen in all zones of day 11 control group rat placenta. Scale bar = 500 μm. (b) PCNA immunolabeling intensity was decreased in all zones in the diabetic group compared to the control group on the same day. ysp: yolk sac placenta, e: embryo, cp: chorioallantoic placenta, md: mesometrial decidua. Scale bar = 500 μm. **3**. PCNA immunolabeling on day 13 of pregnancy. (a**)** PCNA immunolabeling intensity in control group on day 13 of pregnancy. Overall appearance is seen. Scale bar = 1.5 mm. (b) PCNA immunolabeling intensity was decreased in diabetic group compared to control group on the same day. Overall appearance is seen e: embryo, cp: chorioallantoic placenta, md: mesometrial decidua. Scale bar = 350 μm. **4**. PCNA immunolabeling on day 17 of pregnancy. (a) Strong positive PCNA immunolabeling intensity in spongiotrophoblast of control group placenta on day 17 of pregnancy. st: spongiotrophoblast, gly: glycogen cells. Scale bar = 25 μm. (b) PCNA immunolabeling intensity was increased in spongiotrophoblasts (arrows) compared to the same day control group–very strong positive. Scale bar = 25 μm. **5**. PCNA immunolabeling on day 21 of pregnancy. (a) PCNA immunolabeling intensity in control group on day 21 of pregnancy. While strong positive PCNA immunolabeling intensity continued in spongiotrophoblasts (st), it was positive in giant cells (arrow) and there was no reaction in glycogen cells (gly). Scale bar = 25 μm. (b**)** Stronger PCNA immunolabeling intensities both in spongiotrophoblasts (st) and giant cells (arrow) were observed in the diabetic group on day 21 of pregnancy compared to the same day control group. Scale bar = 25 μm.

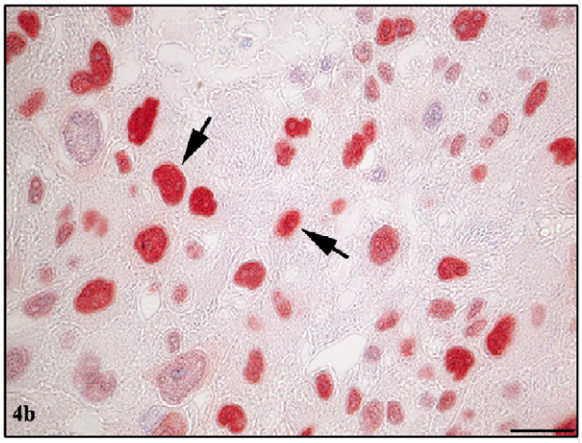

ysp
e
cp
md
2a

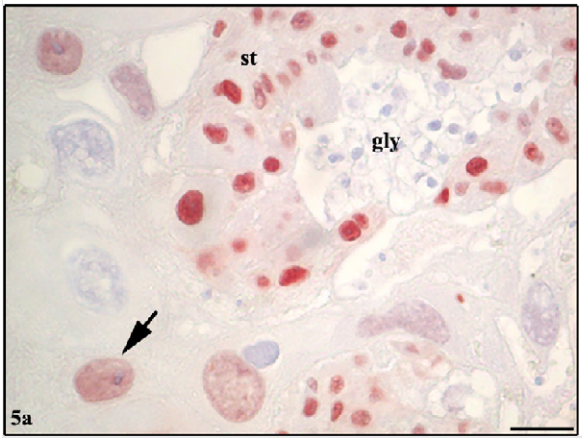

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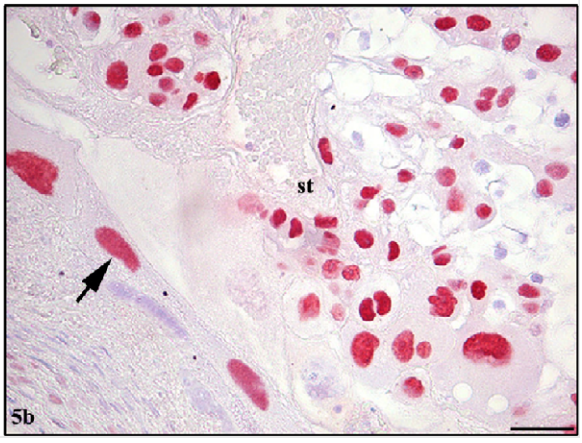

e
cp
md
3a


e
cp
md
3b


gly
st
4a


4b


st
gly
5a


st
5b

On day 17 of pregnancy in the control group, PCNA immunolabeling intensity decreased in the allantoic mesenchymal tissue and in giant cells compared to control groups of days 11 and 13 of pregnancy. Labeling intensity in the visceral endodermal cells was the same as that of the day 13 control group. Labeling intensity in spongiotrophoblasts decreased and there was a weak positive immunoreactivity in glycogen cells (Figure 4a). When the same day diabetic group was compared to the control group it was seen that PCNA immunolabeling was similar in the visceral endodermal cells and giant cells and increased in the labyrinth trophoblasts and spongiotrophoblasts (Figure 4b).

On day 21 of pregnancy in the control group, PCNA immunolabeling intensity was observed as strong positive in spongiotrophoblasts, positive in visceral endodermal cells and giant cells. There was no PCNA immunoreactivity in glycogen cells, and all other cell types were labeled weakly positive (Figure 5a). On the same day, the diabetic group PCNA immunolabeling intensity increased in the visceral endodermal cells, allantoic vessel endothelial cells, allantoic mesenchymal tissue, spongiotrophoblasts and giant cells (Figure 5b).

The H-SCORE analysis revealed that PCNA immunolabeling intensity in the control group placentas was higher than that in the diabetic group placentas on days 11 and 13 of pregnancy. On day 17 and day 21 of pregnancy, PCNA immunolabeling intensity was higher in the diabetic group compared to the control group placentas. H-SCORE values, for control and diabetic groups, respectively, were 240 ± 8 and 225 ± 9 for day 11 of pregnancy; 254 ± 4 and 236 ± 1 for day 13 of pregnancy; 137 ± 1 and 191 ± 8 for day 17 of pregnancy; 125 ± 2 and 135 ± 2 for day 21 of pregnancy. These differences were statistically significant on day 17 ($p < 0.05$).

Wilcoxon signed rank test showed that in the control group, the decrease in PCNA immunolabeling intensity between day 11 and days 17 and 21; and between day 13 and days 17 and 21 was statistically significant ($p < 0.05$). In the diabetic group, the decrease in PCNA immunolabeling intensity between day 11 and days 17 and 21, between day 13 and days 17 and 21, and between day 17 and day 21 was statistically significant ($p < 0.05$) (not shown).

Western blot results

On day 11 and 13 of pregnancy, PCNA protein amounts appeared to be higher in control group placentas; however, these differences were not statistically significant ($p = 0.60$ and $p = 0.42$, respectively). On day 17 and 21 of pregnancy, PCNA protein amounts appeared to be higher in the diabetic group placentas compared to control group placentas; however, these differences were not statistically significant ($p = 0.31$ and $p = 0.69$, respectively) (Figure. 6a, b).

Discussion

The placenta, which forms the interface between the embryo/fetus and the mother, is a critical determinant of the outcome of pregnancy. The placenta is the first organ to function during development (Genbacev et al., 2000). Adequate placental growth and function are fundamental to the well-being, growth and development of the fetus throughout gestation (Waddell et al., 2000).

The altered metabolic environment in maternal diabetes during pregnancy interferes with normal fetal development (Padmanabhan and al-Zuhair, 1987). Abnormally elevated glucose levels in maternal blood, which lead to increased glucose transport to the embryo, are responsible for the teratogenic effects of maternal diabetes. Consequently, expression of genes that control essential developmental processes are disturbed (Loeken, 2005), although the precise molecular mechanisms underlying malformations still remain unknown (Reece et al., 2005).

Placental enlargement is a feature of human diabetic pregnancies. Pregnancy in the STZ-induced diabetic rat is also characterized by placentomegaly and varying degrees of fetal growth retardation (Robinson et al., 1988). Whereas macrosomia often occurs in infants of diabetic women, growth retardation is almost a rule in spontaneous and experimental diabetes in animals. The functional significance of placentomegaly in the presence of maternal diabetes in rats and other species is unclear (Husain et al., 2001), and it is not clear when the growth inhibition starts or how placental pathology might affect fetal growth in maternal diabetes (Padmanabhan and Shafiullah, 2001). Some researchers hypothesized that hyperglycemia leads to a relative immaturity of rat placentas by providing a stimulus for continuous growth and cell division-delayed maturation (Gewolb et al., 1986).

Caluwaerts et al. (2000) showed that on day 9 of pregnancy the weight of the decidual swellings in diabetic rats and the mitotic index in the ectoplacental cone were lower compared to controls ($p < 0.0001$ and $p < 0.05$, respectively). In the study reported here, we observed that placental weights

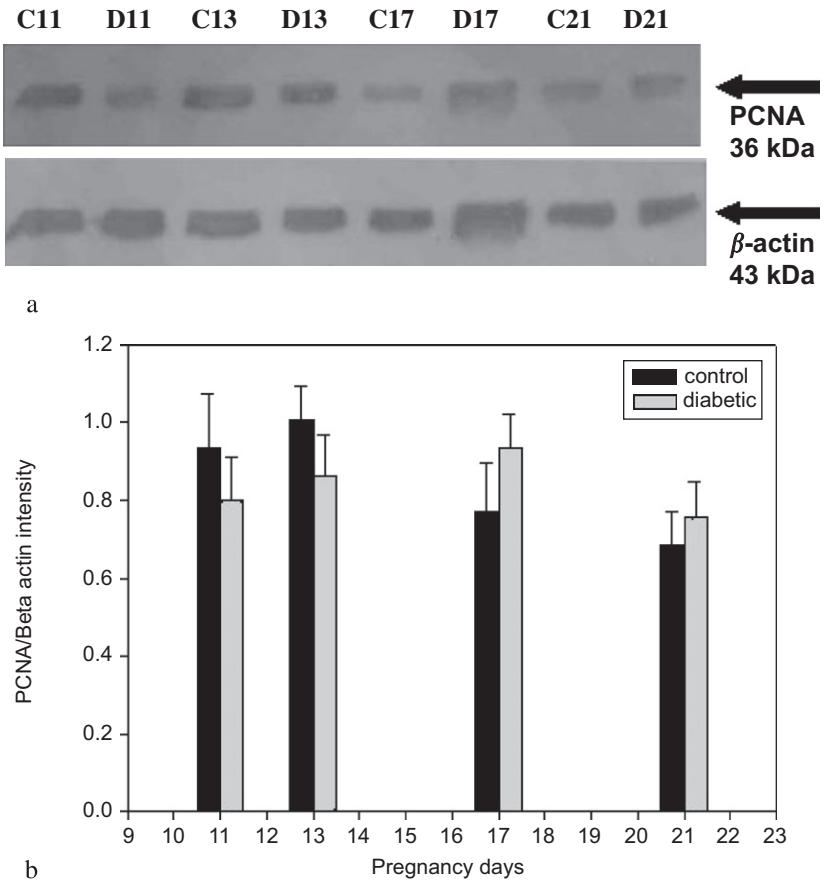


Figure 6. (a) Western blot results of PCNA in control and diabetes-induced group. β-Actin was used as an internal standard. (b) Quantitative analysis of PCNA Western blot data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding β-actin intensity value. The differences seen in (a) and (b) were not statistically significant. Abbreviations: C11, C13, C17 and C21 for control group on days 11, 13, 17 and 21 of pregnancy, respectively; D11, D13, D17 and D21 for diabetic group on parallel days of pregnancy, respectively.

in the diabetic groups were less than in control groups on days 11 and 13 of pregnancy, while higher placental weights were recorded on days 17 and 21 of pregnancy, in accordance with Caluwaerts et al. (2000) as well as some other studies (Padmanabhan and Shafiullah, 2001; Robinson et al., 1988; Padmanabhan and al-Zuhair, 1987). Moreover, although either pregnancy-induced or pre-existing macrosomia is seen in diabetic pregnancies, Pedersen and Molsted-Pedersen (1979) reported that there appears to be a subgroup of cases in which fetal growth is retarded early in pregnancy, leading to low birth weight and possibly a higher incidence of congenital malformations. We have also observed low embryo weights in our study as early as day 11 of rat pregnancy. Furthermore, the embryo weights in the diabetic groups were lower than that of the controls on all days examined.

In a study performed by Robinson et al. (1988), it was demonstrated that on days 14 and 16 of pregnancy placental weights of animals in the diabetic group were less than those in the control group, but the weight of placentas increased after day 18 of pregnancy. In this study, they also showed that although in the early stages of pregnancy placentas of animals in the diabetic group had lower amounts of protein, RNA (ribonucleic acid), DNA and cell number were similar to levels seen in the control group. After day 18 of pregnancy, placentas had more protein, RNA, DNA and cells compared to control group placentas. Glycogen storage increased in the diabetic group placentas starting from day 16 of pregnancy, but it decreased slowly with advancing pregnancy in control group placentas (Robinson et al., 1988). Consistent with this study, we also observed an increase in placental weights after day 17 of rat pregnancy, suggesting that this increase might be due to increased protein synthesis and cell proliferation since we have shown a significant increase in PCNA on this day. Our findings regarding placental weight and diameter (data not shown) are consistent with

previous studies. These data indicate that diabetes may contribute to fetal growth retardation and placentomegaly starting in the third trimester in rats.

PCNA, which is a ring-like protein involved in the major DNA replication and repair machinery of the cell, is expressed during the last 5% of G1 phase and the first 35% of S phase of the cell cycle (Takahashi and Caviness, 1993). Maruo et al. (2001) reported that PCNA is most abundantly expressed in very early placenta, and is less abundant in mid-term placenta and the least abundant in term placenta in humans. In our study we found that PCNA immunolabeling intensity decreased after days 13 and 17 in normal and diabetic placentas, respectively. On day 17 of pregnancy, PCNA protein intensity/amount appeared to be higher in the diabetic group than in the control group as shown by both immunohistochemistry and Western blot analysis. According to our semi-quantitative evaluations of immunolabeling, this difference may arise from spongiotrophoblasts. Indeed this cell type was observed to maintain its very strong immunolabeling intensity until day 21 of pregnancy. On the contrary, in a study by Mark et al. (2006) it was emphasized that during the final third of rat pregnancy the basal zone exhibits apoptosis and shows little, if any, growth, whereas the labyrinth zone grows substantially and apoptosis is very low over this period. This difference might be due to spongiotrophoblasts being affected much more than the labyrinth trophoblasts under diabetic conditions.

We observed greater PCNA immunolabeling intensity in diabetic group placentas on days 17 and 21 of pregnancy when compared to the controls. Likewise, Hinck et al. (2003) reported that when they cultured Rcho-1 cell line in high glucose concentration the number of cells were higher. This suggests that high glucose disrupts the progression through the endocycle, perhaps by impairing the cyclin/cyclin-dependent kinase complexes controlling each step of the endocycle. It was also reported in an ultrastructural study that giant cells in diabetic rat placenta proliferated to a greater degree compared to those of control (Padmanabhan and Shafiullah, 2001).

There are relatively few studies on the role of mitotic regulators involved in rat placental development. In conclusion, small placenta in the early stages of pregnancy and placentomegaly in later stages of pregnancy in diabetic rats may occur as a result of disturbed cell cycle pathways with the participation of PCNA and other mitotic regulators. Our study may provide the basis for further studies to improve our understanding of factors leading to abnormal placentation; however, more detailed studies are required to explain the molecular mechanisms of diabetic placentation.

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