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The PI3K/Akt and MAPK-ERK1/2 pathways are altered in STZ induced diabetic rat placentas

Aslı Ozmen[1], Gozde Unek[1], Dijle Kipmen-Korgun[2] and Emin Turkay Korgun[1]
[1]Department of Histology and Embryology and [2]Department of Biochemistry, Medical Faculty, Akdeniz University, Antalya, Turkey

Summary. Diabetic pregnancy is associated with complications such as early and late embryonic death, fetal growth disorders, placental abnormalities, and embryonal-placental metabolic disorders. Excessive apoptosis and/or changes of proliferation mechanisms are seen as a major event in the pathogenesis of diabetes-induced embryonic death, placental weight and structural anomalies.

Akt and ERK1/2 proteins are important for placental and fetal development associated with cellular proliferation and differentiation mechanisms. The mechanism underlying the placental growth regulatory effects of hyperglycemia have not been elucidated. Moreover, it is still not determined how Akt and ERK1/2 proteins related proliferation and apoptosis mechanisms are influenced by Streptozotocin (STZ) induced diabetic rat placental development.

The aim of this study was to investigate the expression levels and spatio-temporal immuno-localizations of Akt, p-Akt, ERK1/2 and p-ERK1/2 proteins in normal and STZ-treated diabetic rat placental development. In order to compose the diabetic group, pregnant females were injected with a single dose of 40mg/kg STZ intraperitonally seven days before their sacrifice at 12th, 14th, 16th, 18th and 20th day of their gestation.

We found that maternal diabetic environment led to a decrease in ERK1/2 and Akt phosphorylation during rat placental development. It could be said that MAPK-ERK1/2 and PI3K/Akt cell signaling pathways are affected from hyperglycemic conditions in rat placentas.

In conclusion, hyperglycemia-induced placental and embryonal developmental abnormalities could be associated with reduction of Akt and ERK1/2 phosphorylation.

Key words: Placental development, Akt, ERK1/2, Diabetes, Rat

Offprint requests to: Prof. Dr. Emin Turkay Korgun, Department of Histology and Embryology, Medical Faculty, Akdeniz University, 07070 Antalya, Turkey. e-mail: korgun@akdeniz.edu.tr

Introduction

The placenta transports nutrients and oxygen between the maternal and fetal circulation and is essential for fetal growth (Hemberger and Cross, 2001). Human and rodent placentas differ a little in their details but their overall structures and the molecular mechanisms of placental development are thought to be very similar (Rossant and Cross, 2001). The rat placenta is of the hemochorial type (Yan et al., 2005) as is the human placenta, and therefore is 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).

Gestational diabetes is a syndrome characterized by glucose intolerance with onset or first recognition during pregnancy (Sobrevia et al., 2011). Diabetic pregnancy is associated with complications such as early and late embryonic death, fetal growth disorders (Mammon et al., 2005) placental abnormalities, and embryonal-placental metabolic disorders (Pustovrh et al., 2009).

The importance of Akt and Extracellular signal-

Regulated Kinase 1/2 (ERK1/2) proteins in placental and fetal development associated with cellular proliferation and differentiation has been shown in several studies (Meloche et al., 2004; Navarrete Santos et al., 2004; Daoud et al., 2005; Laviola et al., 2005; Riley et al., 2005; Forbes and Westwood, 2008; Prast et al., 2008).

ERK1/2 is the most activated Mitogen-Activated Protein Kinase (MAPK) in mammals and activation of ERK1/2 predominantly occurs through mitogenic stimuli such as growth factors and hormones (Pawson, 2002). The MAPKs control a wide range of biological processes including cell growth, development, differentiation, inflammation, and apoptosis (Kolch, 2000; Luttrell, 2003; Pollheimer and Knofler, 2005; Dickinson and Keyse, 2006; Binetruy et al., 2007).

On the other hand, the Ser/Thr protein kinase Akt (Protein kinase B) is a downstream target of Phosphoinositide 3-Kinase (PI3K) pathway (Liang and Slingerland, 2003; Mitsiades et al., 2004; Garcia et al., 2006; Jiang and Liu, 2008) and phosphorylates a wide range of other target proteins that control proliferation, survival, cell size, cell migration, differentiation and apoptosis (Franke et al., 1997; Hajduch et al., 2001; Kim and Chung, 2002).

Excessive apoptosis is seen as a major event in the pathogenesis of diabetes-induced embryonic death and structural anomalies (Moley, 2001). On the other hand, the inhibition of cell proliferation in embryos during organogenesis may also be another factor (Mammon et al., 2005). Besides, altered placental weight has been reported for diabetic rats (Padmanabhan and Shafiullah, 2001; Salbaum et al., 2011). Padmanabhan et al. (1988) reported an increase in giant cell number in Junctional Zone (JZ), and in Labyrinth Zone (LZ) cystic structures with excess leucocyte, perivascular fibrosis, vacuole and edema. Changes in the placental weights could be a result of altered apoptosis and/or proliferation mechanisms. Therefore Akt and ERK1/2 proteins are good candidates for studying these processes.

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. Our goal was to investigate how Akt, phospho-Akt (p-Akt), ERK1/2 and phospho-ERK1/2 (p-ERK1/2) proteins are affected in a diabetic environment during rat placental development. Therefore we aimed to examine the spatio-temporal immunolocalizations and expression levels of these proteins during rat placental development by immunohistochemistry and Western blot techniques in normal and diabetic rat placentas.

Materials and methods

Animals and tissue preparation

Female Wistar rats were used for all experiments. The animals were kept under standard conditions. Light/dark cycle was 12/12h. After mating, the presence of the sperm in the vaginal smear the following morning was designated as day 1 of pregnancy. The pregnant rats were sacrificed on 12th, 14th, 16th, 18th and 20th day of their gestation. In order to compose the diabetic group, pregnant females were injected with a single dose of 40mg/kg Streptozotocin (STZ, N-Methyl Nitroso-carbamoyl-D-glucosaminel, Sigma, S-0130) intraperitonally seven days before their sacrification. After 48 hours of STZ injection, blood glucose levels were measured from tail vein. Blood glucose levels of 200 mg/dl or more indicated diabetes. The control groups were injected the same dose of isotonic salt solution. All procedures in this study were approved by the Akdeniz University Animal Experiments Local Ethical Committee.

One part of the samples was frozen in liquid nitrogen on collection and stored at minus 196°C until further assessments by Western blot analysis. For immunohistochemical studies, one part of the dissected placentas was fixed in Holland 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) (Romeis, 1989; Korgun et al., 2003) at room temperature for 4 hours.

Immunohistochemistry

Holland-fixed paraffin-embedded samples were cut into 5-μm sections. After deparaffinization, slides were boiled in sodium-citrate buffer (pH 6.0) in a microwave oven for 21 minutes (3x7 minutes) for antigen retrieval and cooled for 30 minutes at room temperature. Then, sections were incubated with Levamisol for 30 minutes to block endogenous alkaline phosphatase in a humidified chamber. Slides were then incubated with Large Volume Ultra V block (Labvision, TA 125-UB) for 10 minutes at room temperature. Afterwards, excess serum was drained and sections were incubated with primary antibodies with the concentrations of 0,153 μg/ml Akt (Cell Signaling Technology,4685-11E7), 0,5 μg/ml p-Akt Ser473 (Cell Signaling Technology, 4051-587F11), 0,16 μg/ml p44/42 MAP Kinase (ERK1/2) (Cell Signaling Technology, 4695-137F5), 2 μg/ml p-ERK1/2 (Santa Cruz Biotechnology, E-4, sc7383) overnight at 4°C temperature in a humidified chamber.

The sections were washed three times for 5 minutes with Tris Buffer Solution, 0.1% Tween 20 (TBS-T) and then the antigen-antibody complex was detected by using an avidine-biotin-alkalen phosphatase kit (K0610, Dako). Fast Red (K0699, Dako) was used as the chromogen. Sections were counterstained with Mayer's hematoxylin (S3309, Dako) and mounted with Kaisers Glycerin Gelatin (MERCK, OB 514196). In order to perform control staining, for p-ERK1/2, mouse IgG2a (X0943, Dako), for p-Akt, mouse IgG2b (X0944, Dako) and for ERK1/2 and Akt, rabbit Ig fraction (X0903, Dako) were used as isotype controls with the same concentrations as the specific antibodies.

SDS PAGE and Western Blotting

Protein extraction and immunoblot analysis were performed as described previously by (Ozmen et al., 2011). Briefly, placental tissues were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 100 μM EDTA, 100 μM EGTA, 1% NP-40, 0.1% SDS, 0.1% desoxycholic acid) supplemented with protease inhibitor cocktail tablets (Complete; Roche, 11697498001). After homogenization, samples were centrifuged at 10.000g for 10 minutes. Supernatants were collected and stored at minus 80°C. Protein concentrations were determined for each sample using the Lowry assay (Lowry et al., 1951). Samples were boiled for 5 minutes at 95°C. 30 μg/ml of protein for each sample were applied per lane and were separated by SDS polyacrylamide gel electrophoresis at 100V 30mA for approximately 1.5 hours and then transferred onto nitrocellulose membrane (RPN 203D, Amersham) +4°C overnight under 30V 90 mA. The membranes were blocked for 1 hour with 5% w/v nonfat dry milk (BioRad Laboratories, 170-6404) 1X TBS-T, 0.1% Tween 20 at room temperature. Membranes were incubated with a concentration of 0.0115 μg/ml Akt, 0.05 μg/ml p-Akt, 0.16 μg/ml ERK1/2 and 0.8 μg/ml p-ERK1/2 antibodies +4°C overnight. After washing in TBS-T membranes were incubated with Horseradish Peroxidase conjugated goat anti-rabbit IgG and goat anti-mouse IgG (BioRad 170-6515, 170-6516 respectively) diluted 1:6000 in blocking solution for two hours at room temperature. After washing with TBS-T, bound antibodies were detected by chemiluminescence-based SuperSignal CL HRP Substrate System (Pierce, 34080T). The membranes were exposed to Hyperfilm (Amersham, 28906837) which was subsequently analyzed using an Alpha DigiDoc 1000 gel documentation unit (Alpha Innotech Corporation, CA, USA).

Statistical analysis

The following comparisons between control and diabetic groups were performed by Student's t- test: embryonal and placental weights and Western blot band densities for Akt, p-Akt, ERK1/2 and p-ERK1/2 proteins. Values are presented as mean ± SEM. All statistical analyses were performed using SigmaStat 3.5.

Immunohistochemical stainings were evaluated by two investigators in blind studies. The labelings were scored in a semi-quantitative fashion that included the intensity of labeling in sections. Evaluations were scored as (-) negative; (+/-) weak positive; (+) positive; (++) strong positive; (+++) very strong positive.

Results

Placenta and Embryo weights

Placental and embryonal weights of each group were measured (Tables 1-3). On day 12 of gestation placenta, embryo and uterus was evaluated together as it was difficult to separate them operationally. On the other days of gestation (Days 14, 16, 18, and 20) placental and embryonal weights were measured separately. Placenta, embryo and uterus weight was significantly (71.2%, p≤0.001) decreased in the experimental group on day 12 (Fig. 1a). The STZ treated group had significantly smaller embryos (Fig. 1b) than control on day 14 (26.3%, p≤0.001) and day 20 (8.2%, p≤0.001) of gestation and had smaller placentas (Fig. 1c) on experimental day 14 (45.5%, p≤0.001) and day 16 (7.5%, p≤0.05) of gestation. Besides, placental weights of diabetic group increased after day 16. At day 18 (18.8%, p≤0.05) and day 20 (12.3%, p≤0.05) of gestation diabetic group placentas were bigger than control group.

It was observed that glycogenic cell numbers altered in diabetic placentas compared to control. At day 16 of gestation, glycogenic cells in JZ and maternal decidua were fewer than control group placentas. On the other hand, at the bigger placentas at days 18 and 20, it was seen that glycogenic cell number increased in diabetic placentas at JZ and maternal decidua.

Table 1. Mean and SEM (Standard Errors of Mean) values belonging to embryo + placenta + uterus weights of control and diabetic groups.

Embryo + placenta + uterus weights (g)	Mean ± SEM
Control Group	0.448±0.09 (n=32)
Diabetic Group	0.129±0.09 (p≤0.001)* (n=31)

Table 2. Mean and SEM (Standard Errors of Mean) values belonging to embryo weights of control and diabetic groups.

	Embryo weights (g) , Mean ± SEM	
Gestational Days	Control Group	Diabetic Group
14	0.289±0.08 (n=32)	0.213±0.01 (p≤0.001)* (n=36)
16	0.787±0.02 (n=30)	0.811±0.03 (p=0.691) (n=31)
18	2.020±0.08 (n=31)	1.879±0.04 (p=0.158) (n=30)
20	5.200±0.06 (n=40)	4.773±0.06 (p≤0.001)* (n=34)

Table 3. Mean and SEM (Standard Errors of Mean) values belonging to placenta weights of control and diabetic groups

	Placenta weights (g), Mean ± SEM	
Gestational Days	Control Group	Diabetic Group
14	0.343±0.02 (n= 32)	0.187±0.08(p≤0.001)** (n=36)
16	0.400±0.02 (n=30)	0.370±0.01 (p=0.04) * (n=31)
18	0.450±0.02 (n=31)	0.535±0.02 (p=0.006)* (n=30)
20	0.561±0.02 (n=40)	0.630±0.01(p=0.04) * (n=34)

Immunolabelings of Akt, p-Akt, ERK1/2 and p-ERK1/2

The distributions of Akt, p-Akt, ERK1/2 and p-ERK1/2 proteins in the control and diabetic rat placenta during gestational days 12, 14, 16, 18 and 20 are detailed in Table 4 and 5. Major findings are presented below.

Gestational Day 12

Akt immune reaction was positive at decidual cells, giant cells, spongiotrophoblast cells, maternal endothelial cells, labyrinth giant cells, labyrinth trophoblasts, visceral endodermal cells, chorionic mesenchymal cells (CMCs), fetal endothelial cells and negative for parietal endodermal cells in the control and diabetic group placentas. For immune reaction densities see Table 4. In control group, p-Akt immunolabelings were strong at decidual and giant cells and negative for all other cell types. In diabetic group placentas, different from control group, p-Akt immune reaction was strong positive for fetal endothelial cells and negative for giant cells, and the rest of the cells were negative for p-Akt immune staining (Table 4).

ERK1/2 immune reactions were positive for all cell types in both groups. For immune reaction densities see Table 5. p-ERK1/2 immunolabelings were positive for decidual cells, giant cells, maternal endothelial cells, spongiotrophoblast cells, CMCs and fetal endothelial cells and negative for labyrinth giant cells, labyrinth trophoblasts, parietal and visceral endodermal cells in the control group. In the diabetic group placenta immune reactions were similar to control group placentas (Table 5).

Gestational Day 14

In the control and diabetic group placenta, except parietal endodermal cells in diabetic group, Akt immune reactivity was positive for all cell types. For immune reaction densities, see Table 4 and Fig. 2a-d. p-Akt immunolabelings were strong positive at giant cells, spongiotrophoblast cells (Fig. 3a), and positive at decidual cells, weak positive at parietal endodermal and fetal endothelial cells and negative at glycogenic cells, maternal endothelial cells, labyrinth trophoblasts, labyrinth giant cells, chorionic mesenchymal cells and

Table 4. Intensity of Akt and p-Akt labeling in the control and diabetic rat placentas.

Gestational Days	Decidual cells	Giant Cells	Spongiotrophoblast Cells	Glycogenic Cells	Maternal Endothelial Cells	Labyrinth Giant Cells	Labyrinth Trophoblasts	Parietal Endoderm	Visceral Endoderm	Chorionic Mesenchymal Cells	Fetal Endothelial Cells	Experimental Groups	Signal Transduction Proteins
12	++	+++	++	0	++	+	++	-	+/-	+	+++	Diabetes	Akt
	++	+++	++	0	++	+	++	-	+	+	+++	Control	
14	+	+++	+++	+	+	+++	+++	-	++	++	+++	Diabetes	
	++	+++	+++	+	++	++	+++	+/-	++	++	+++	Control	
16	+++	+++	+++	+	+++	++	+++	+/-	++	+++	+++	Diabetes	
	+++	+++	+++	-	+++	++	+++	+	+++	+++	++	Control	
18	++	+++	+++	+	++	+++	+++	+	++	+++	+++	Diabetes	
	+++	+++	++	+	+++	++	+++	+	++	+++	+++	Control	
20	+++	++	++	+	+	++	++	+	++	++	++	Diabetes	
	+++	+++	+++	+	++	+++	+++	+	+++	+++	+++	Control	
12	++	++	-	0	-	-	-	-	-	-	-	Diabetes	p-Akt
	++	-	-	0	-	-	-	-	-	-	++	Control	
14	+	+	+/-	-	-	-	-	-	-	+	+	Diabetes	
	+	++	++	-	-	-	-	+/-	-	-	+/-	Control	
16	+	+	+	-	-	-	-	-	-	-	-	Diabetes	
	+	++	++	-	-	-	++	+	+/-	+	++	Control	
18	+	+++	++	-	-	-	+/-	-	-	+/-	+/-	Diabetes	
	+	+++	+++	-	-	+/-	+/-	+/-	+	+	+/-	Control	
20	+	++	+	-	-	-	+	-	-	+	+	Diabetes	
	+	++	++	-	-	-	-	-	-	-	+++	Control	

(0), not determined; (-), negative; (+/-), weak positive; (+), positive; (++), strong positive; (+++), very strong positive

visceral endodermal cells in the control group (Table 4). Immune positivity was weaker at giant cells (Fig. 3e) and spongiotrophoblast cells in the diabetic group placentas. Besides, there was a positive p-Akt staining at CMCs in diabetic group as in a different manner in contrast to control groups (Table 4).

It was seen that ERK1/2 staining was similar between diabetic and control group placentas and all cell types were stained positively with ERK1/2 antibody. For immune reaction densities, see Table 5 and Fig. 4a-d. Except for glycogenic cells and labyrinth giant cell; p-ERK1/2 staining was positive for all cell types and immune reactivity was similar between groups (Fig. 5a,e). For p-ERK1/2 immune reaction densities, see Table 5.

Gestational Day 16

Akt immune positivity was similar between groups and except for glycogenic cells in control group, all cell types were positive for Akt immune reaction. For the Akt staining densities see Fig. 2e-h and Table 4. p-Akt labelings were strong positive at giant cells, spongiotrophoblast cells (Fig. 3b), fetal endothelial cells; positive at decidual cells, parietal endodermal cells and CMCs; weak positive at visceral endodermal cells and negative at glycogenic cells, maternal endothelial cells and labyrinth giant cells in the control group placentas (Table 4). p-Akt immune reactivity was decreased in the diabetic group placentas. p-Akt immune reactivity was positive for decidual cells, giant cells and spongiotrophoblast cells (Fig. 3f), and negative for the other cell types (Table 4).

ERK1/2 immunolabelings were similar between the STZ treated and control group placentas (Fig. 4e-h, Table 5). It was detected that p-ERK1/2 reactivity was only negative at glycogenic cells and labyrinth giant cells in both groups. The other cell types were positively stained for p-ERK1/2 and the immune reaction densities were similar between groups (Table 5, Fig. 5b,f).

Gestational Day 18

In the control and diabetic group placentas Akt immune reaction was positive for all cell types, and similar between groups (Fig. 2i-l and Table 4). p-Akt

Table 5. Intensity of ERK1/2 and p-ERK1/2 labeling in the control and diabetic rat placentas.

Gestational Days	Decidual cells	Giant Cells	Spongiotrophoblast Cells	Glycogenic Cells	Maternal Endothelial Cells	Labyrinth Giant Cells	Labyrinth Trophoblasts	Parietal Endoderm	Visceral Endoderm	Chorionic Mesenchymal Cells	Fetal Endothelial Cells	Experimental Groups	Signal Transduction Proteins
12	++	++	++	0	+	+	+++	++	++	+	++	Diabetes	ERK1/2
	+++	+++	++	0	+	+	+++	++	++	+	+++	Control	
14	+++	+++	+++	+/-	++	++	+++	++	+++	++	+++	Diabetes	
	+++	+++	+++	+/-	+	++	+++	+++	+++	++	+++	Control	
16	+++	+++	+++	+/-	++	++	+++	++	+++	+	+++	Diabetes	
	+++	+++	+++	+/-	++	++	+++	++	+++	+	+++	Control	
18	+++	+++	+++	+/-	++	++	+++	++	+++	++	+++	Diabetes	
	+++	+++	+++	+/-	++	++	+++	++	+++	++	+++	Control	
20	+++	+++	+++	+	++	++	+++	+	+++	++	++	Diabetes	
	+++	+++	+++	+/-	++	++	+++	+	++	++	+++	Control	
12	++	++	+/-	0	+/-	-	-	-	-	-	++	Diabetes	pERK1/2
	++	++	+/-	0	+/-	-	-	-	-	+/-	++	Control	
14	+++	+++	++	-	+/-	-	+	+/-	+	+	+++	Diabetes	
	+++	+++	++	-	+/-	-	+/-	+	++	++	+++	Control	
16	+++	+++	+++	-	+	-	+	+	++	++	+++	Diabetes	
	+++	+++	+++	-	+/-	-	+	+/-	++	++	+++	Control	
18	+++	+++	+++	-	+/-	+	+	+	++	+	++	Diabetes	
	+++	++	++	-	+/-	-	+/-	+/-	+/-	++	+++	Control	
20	+++	+++	+++	-	+	-	+	++	++	++	+++	Diabetes	
	+++	+++	+++	-	+/-	-	+/-	+/-	+/-	+++	+++	Control	

(0), not determined; (-), negative; (+/-), weak positive; (+), positive; (++), strong positive; (+++), very strong positive

immunolabelings were weaker in the diabetic group. Labelings were negative only for glycogenic cells and maternal endothelial cells in control group and in STZ treated diabetic group also negative for labyrinth giant cells, parietal and visceral endodermal cells. See Table 4 and Fig. 3c,g for immune reaction densities.

At the STZ treated placentas, ERK1/2 immune positivity was similar to control group placentas (Fig. 4i-l, Table 5). p-ERK1/2 labelings were positive for all cell types except glycogenic and labyrinth giant cells in the control group placentas (Fig. 5c). And for the diabetic group, p-ERK1/2 immune reaction was only negative for glycogenic cells. Immune labeling densities are mentioned in Table 5 and Fig. 5g.

Gestational Day 20

There was not any difference for Akt immunolabelings between control and diabetic group placentas. All cell types were positive for Akt immune reaction (Table 4, Fig. 2m-p). In the control group placentas, p-Akt immunolabelings were detectable in JZ but only for fetal endothelial cells in LZ. Labelings were positive for spongiotrophoblast cells (Fig. 3d), giant cells and decidual cells but negative for other cell types except fetal endothelial cells (Table 4). In the diabetic group placentas, p-Akt immune reactivity was similar in JZ (Fig. 3h). But in LZ, it was seen that labyrinth trophoblasts and CMCs were also positive for p-Akt labeling (Table 4).

ERK1/2 immune reactivity in the diabetic placentas was similar to control group placentas (Fig. 4m-p, Table 5). p-ERK1/2 immune staining was negative for glycogenic cells and labyrinth giant cells in both groups and positive for all the other cell types. For immune reaction densities see Table 5 and Fig. 5d. There was an increase in p-ERK1/2 labeling at the STZ treated diabetic group at parietal and visceral endodermal cells (Table 5), but for the other cell types, p-ERK1/2 immune reactivity was similar (Fig. 5h).

Western blot analyses

Akt activation was decreased in STZ treated diabetic group placentas and results were statistically significant on day 14 ($p \leq 0.05$), day 16 ($p \leq 0.05$), day 18 ($p \leq 0.001$) and day 20 ($p \leq 0.05$) of gestation (Fig. 6a). Total Akt protein expression was not significantly affected by the treatment (Fig. 6b). Similarly, there was a significant decrease in ERK1/2 activation on day 14 ($p \leq 0.05$) and

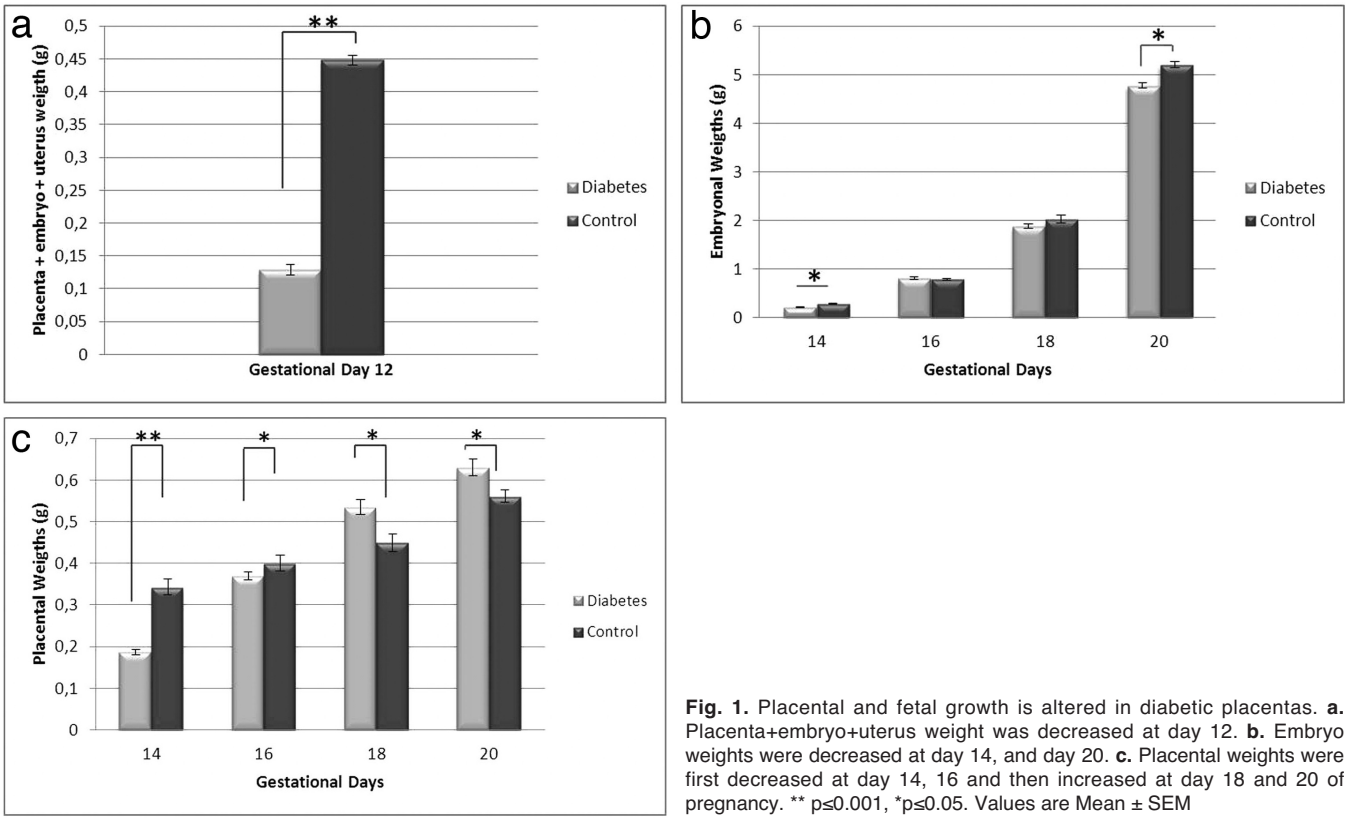

Fig. 1. Placental and fetal growth is altered in diabetic placentas. **a.** Placenta+embryo+uterus weight was decreased at day 12. **b.** Embryo weights were decreased at day 14, and day 20. **c.** Placental weights were first decreased at day 14, 16 and then increased at day 18 and 20 of pregnancy. ** $p \leq 0.001$, * $p \leq 0.05$. Values are Mean ± SEM

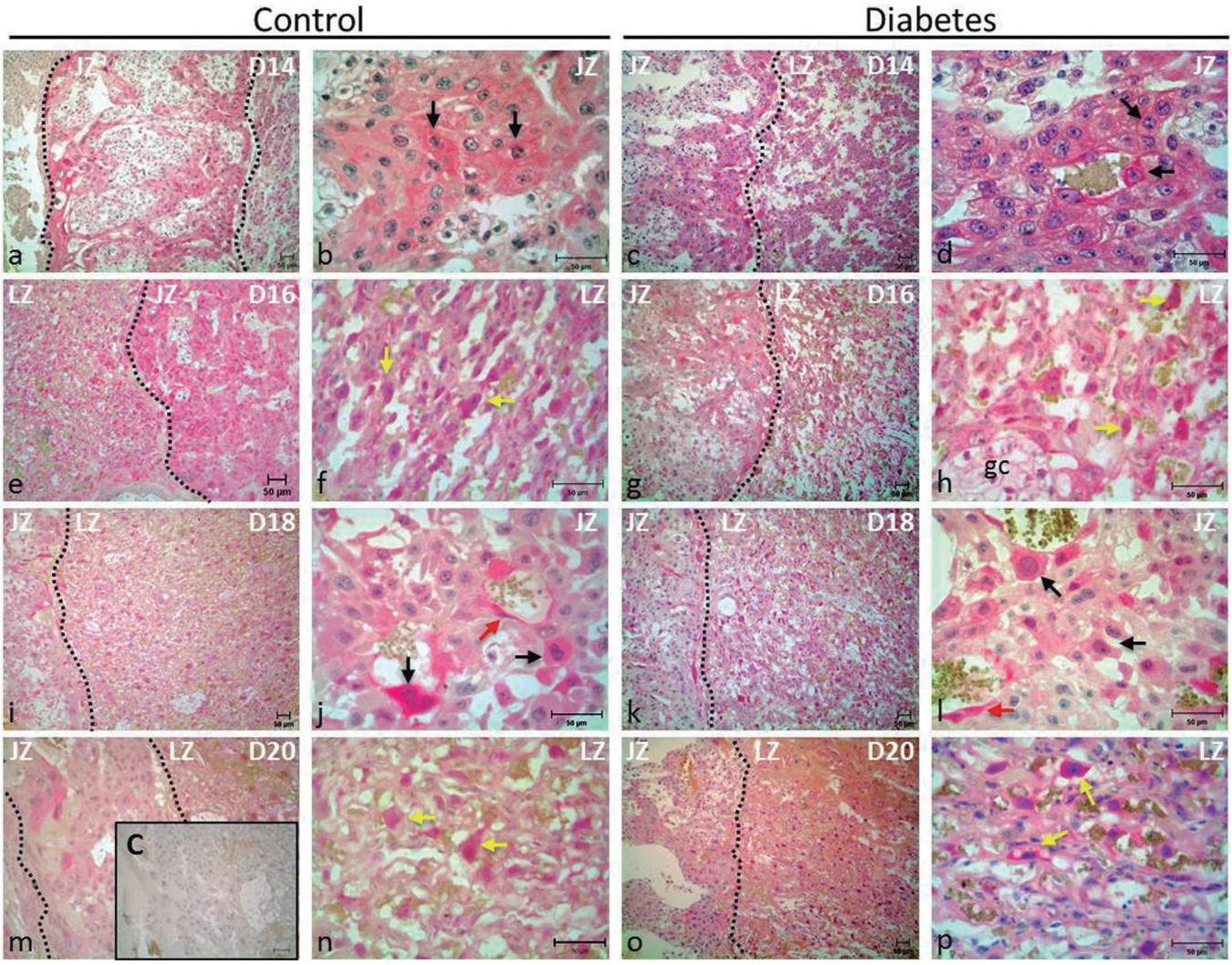

Fig. 2. Akt immunolabelings of the control and diabetic group placentas at gestational day 14, 16, 18 and 20. Control group Akt staining is represented in the left panels, and diabetic group Akt staining is represented in the right panels. Akt staining was similar between the groups for all days studied. Insert in m: IgG isotype control; control staining shows no immunoreactivity. Black arrows: spongiotrophoblast cells. Red arrows: maternal endothelial cells. Yellow arrows: labyrinth trophoblasts. LZ: Labyrinth Zone. JZ: Junctional Zone. C: Control. D: Day. gc: glycogenic cells. Scale bar: 50 μm

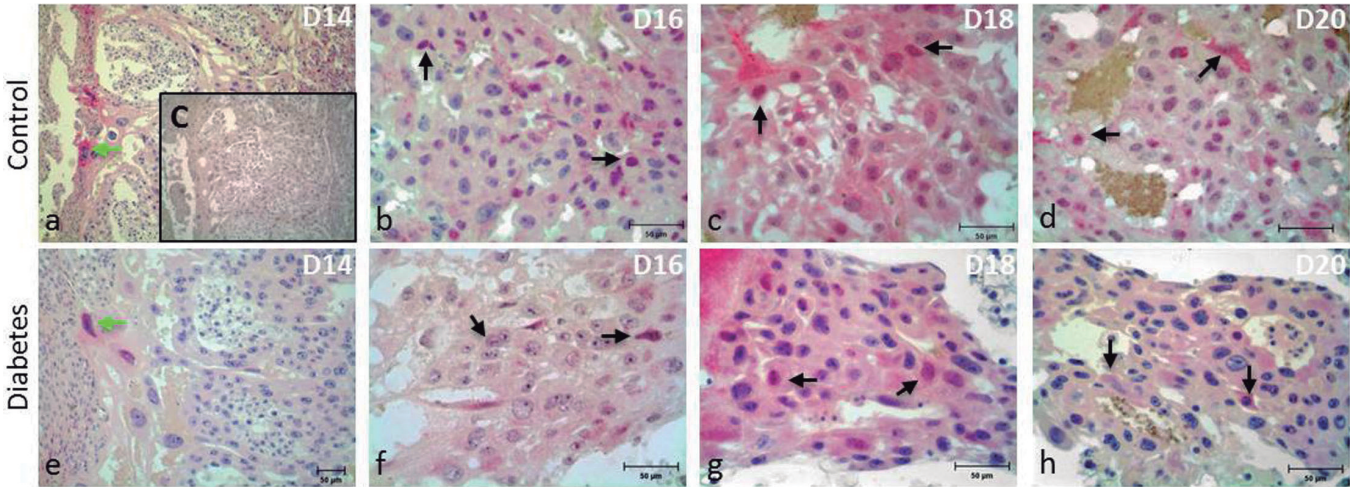

Fig. 3 p-Akt immunolabelings of the control and diabetic group placentas at gestational days 14, 16, 18 and 20. Control group placentas **(a-d)** and diabetic group placentas **(e-h)** are seen respectively. p-Akt immune reaction is weaker at giant cells **(e)** and spongiotrophoblast cells **(f)** in diabetic group placentas at day 14 and day 16 respectively. Insert in a: IgG2b isotype control; control staining shows no immunoreactivity. Black arrows: spongiotrophoblast cells. Green arrows: giant cells. C: Control. D: Day. Scale bar: 50 μm.

day 18 (p≤0.05) of gestation in diabetic group (Fig. 6c). On the other hand total ERK1/2 protein expression was significantly increased in STZ treated group on day 14 (p≤0.05), day 16 (p≤0.05) and day 18 (p≤0.05) (Fig. 6d).

Discussion

In this study, we have shown that hyperglycemia leads to a change in Akt and ERK1/2 activation during pregnancy. Besides, overexposure to glucose was associated with a reduction in fetal weight and changes in placental weights. Furthermore, we addressed the differences of spatial and temporal immunolocalizations of Akt, p-Akt, ERK1/2, p-ERK1/2 proteins in the normal and diabetic placentas.

Pregnancy in the STZ-induced diabetic rat is characterized by placentomegaly and varying degrees of fetal growth retardation (Robinson et al., 1988). In this study, we observed that placenta+embryo+uterus weights at day 12 and placental weights at day 14 and day 16 of gestation in the diabetic groups were decreased according to the control groups, while higher placental weights were recorded at day 18 and day 20, in accordance with (Caluwaerts et al., 2000; Acar et al., 2008; Korgun et al., 2011; Zorn et al., 2011) as well as some other studies (Padmanabhan and Al-Zuhair, 1987; Robinson et al., 1988; Padmanabhan and Shafiullah, 2001)

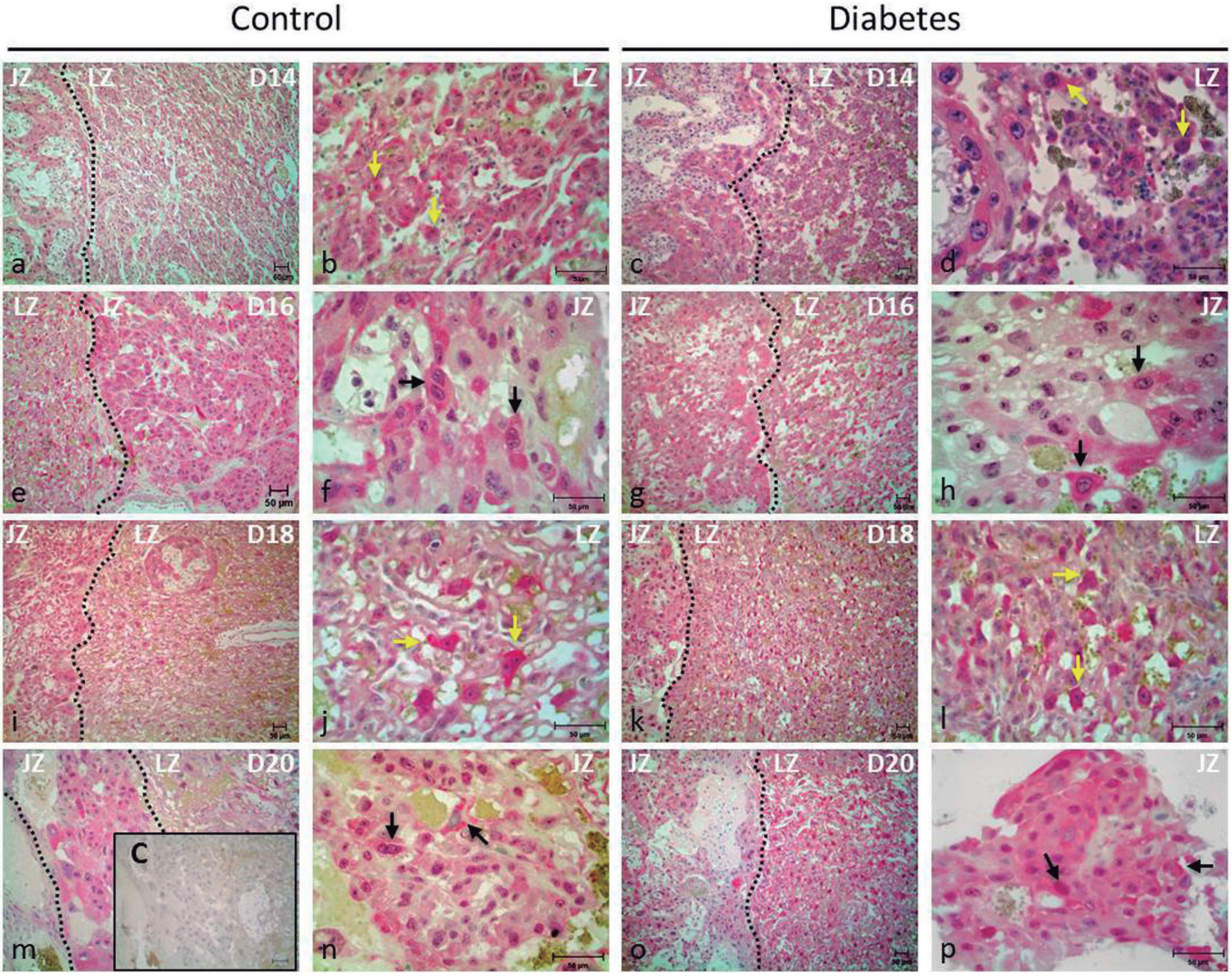

Fig. 4. ERK1/2 immunolabelings of the control and diabetic group placentas at gestational day 14, 16, 18 and 20. Control group ERK1/2 stainings are represented in the left panels, and diabetic group ERK1/2 stainings are represented in the right panels. Immunoreactivity was similar for all days studied. Insert in m: IgG isotype control; control staining shows no immunoreactivity. Black arrows: spongiotrophoblast cells. Yellow arrows: labyrinth trophoblasts. LZ: Labyrinth Zone. JZ: Junctional Zone. C: Control. D: Day. Scale bar: 50 μm.

Whereas macrosomia often occurs in infants of diabetic women, growth retardation is almost a rule in spontaneous and experimental diabetes in animals. 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. Here in our study, we have also observed low embryo weights at day 14 and day 20 of pregnancy in STZ-induced diabetic rats in accordance with previous studies (Padmanabhan and Shafiullah, 2001; Mammon et al., 2005; Koh et al., 2007; Acar et al., 2008; Korgun et al., 2011).

Akt and ERK1/2 are important proteins which have indispensible roles in placental and fetal development related with cellular proliferation and differentiation mechanisms during gestation (Meloche et al., 2004; Navarrete Santos et al., 2004; Daoud et al., 2005; Riley et al., 2005; Forbes and Westwood, 2008; Prast et al., 2008). The majority of the studies (Giroux et al., 1999; Rossant and Cross, 2001; Yang et al., 2003; Watson and Cross, 2005; Baisden et al., 2007) depend on knock out models and are related with gene expression. So, there have been limited numbers of studies relevant to Akt and ERK1/2 expression, during rat placental development at protein level. Furthermore, there is scarce data about alterations of these proteins in a diabetic environment. Therefore, we decided to study Akt and ERK1/2 protein expression levels during rat placental development and aimed to observe whether there is a different or not in diabetic groups.

In our previous study (Ozmen et al., 2011), we reported that in dexamethasone (a synthetic glucocorticoid) induced Intrauterine Growth Restricted (IUGR) placentas p-Akt and p-ERK1/2 protein expression levels were decreased. So, we mentioned that smaller placentas in IUGR groups would be a consequence of decreased Akt and ERK1/2 activation. Therefore, it was concluded that these proteins were not only important at normal placental development but also altered the placental growth in pathological conditions.

It was previously reported that Akt mutant mouse placentas have structural abnormalities. In the Akt-/- placentas there was a significant hypotrophy, with reduction of the decidua basalis and nearly complete loss of glycogen containing cells in the spongiotrophoblast, and the vascularization was decreased. Akt-/- placentas also showed significant reduction of phosphorylation of Akt (Yang et al., 2003). Our results show that p-Akt immune positivity was strong at giant cells and spongiotrophoblast cells in the control groups and p-Akt immune positivity was decreased in the STZ treated placentas at days 14 and 16.

The relationship between the PI3K/Akt pathway and diabetes/hyperglycemia in many different tissues is mentioned in several studies (Farese et al., 2005; Kobayashi et al., 2005; Zdychova and Komers, 2005; Riley and Moley, 2006). Also, there is a growing body of data revealing the importance of Akt protein for placental development (Kamei et al., 2002; Riley et al., 2006; Somanath et al., 2006; Straszewski-Chavez et al., 2010). But, to the best of our knowledge there is not any study determining the p-Akt levels in diabetic placentas during rat placental development. In this study, for the first time we showed that Akt phosphorylation was decreased in STZ treated diabetic rat placentas during development. Concerning the placental growth changes in diabetic pregnancies during gestation, it could be expected to see a positively correlated difference in Akt

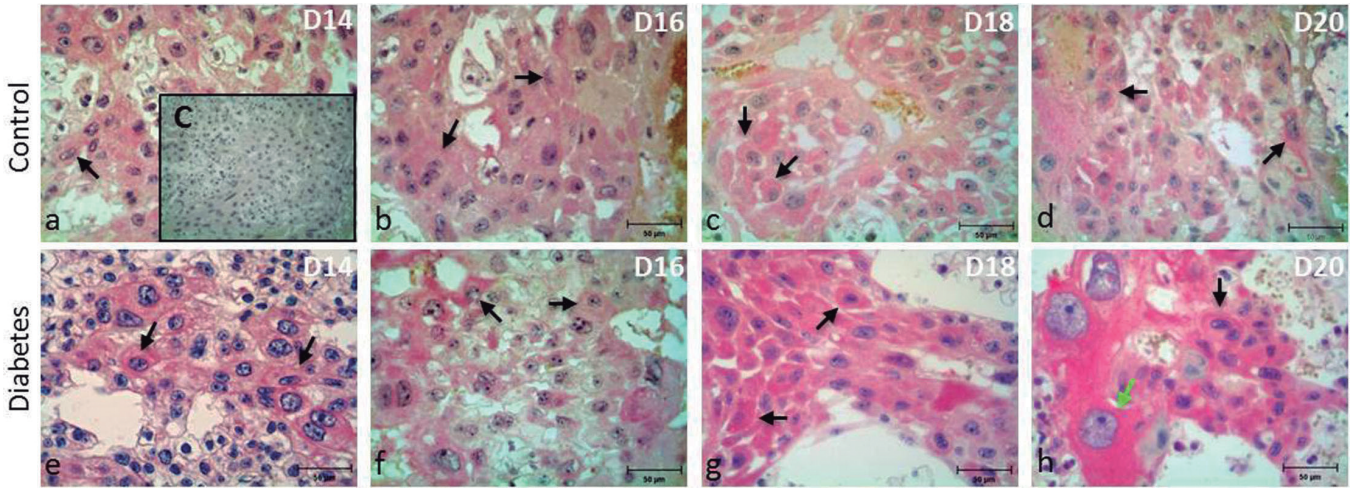

Fig. 5. p-ERK1/2 immunolabelings of the control and diabetic group placentas at gestational days 14, 16, 18 and 20. Control group placentas **(a-d)** and diabetic group placentas **(e-h)** are seen respectively. p-ERK1/2 immune reaction is similar at spongiotrophoblast cells for all days studied. Insert in a: IgG2a isotype control; control staining shows no immunoreactivity. Black arrows: spongiotrophoblast cells. Green arrow: giant cell. C: Control. D: Day. Scale bar: 50 μm.

activation; first a decrease from GD12-16, afterwards an increase from GD18 to term. In a recent study, (Zorn et al., 2011) mentioned that during the course of pregnancy, the number of proliferating cells decreased in both control and diabetic rat placentas. However, starting from day 17 of pregnancy, the number of proliferating cells in the labyrinth and spongiotrophoblast regions was higher in diabetic rat placentas as compared to control. In our study, however, there was a decrease in Akt phosphorylation at all days studied in diabetic group. This decrease would be a compensatory mechanism of the placenta against the hyperglycemic conditions to keep the placental and fetal development at normal sizes.

Besides proliferation, differentiation and apoptosis mechanisms, PI3K/Akt pathway also has important roles in angiogenesis mechanisms (Okumura et al., 2012;

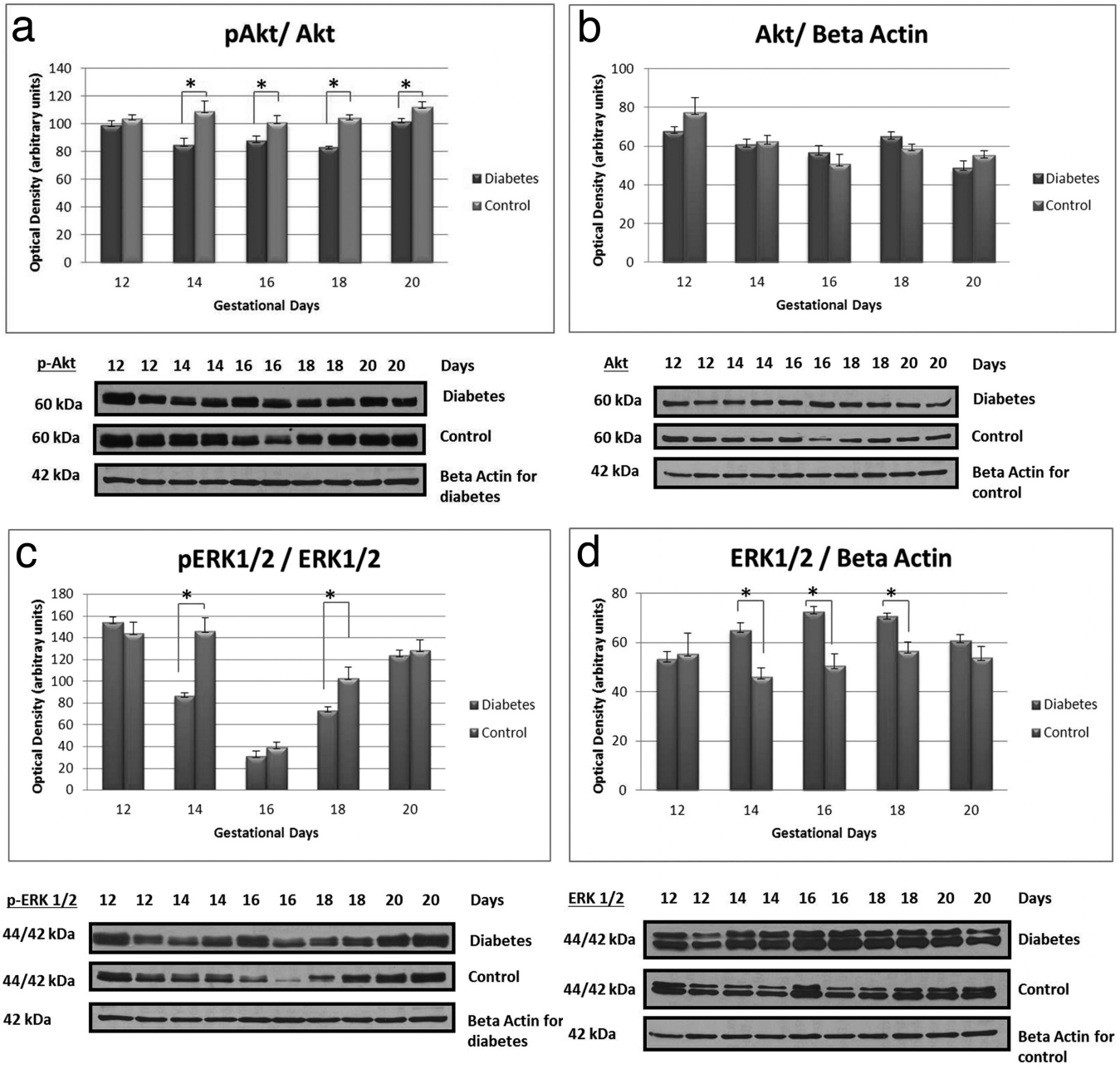

Fig. 6. Western Blot analyses of p-Akt, Akt, p-ERK1/2 and ERK1/2 proteins in diabetic and control groups. Densitometric measurements for p-Akt/ Akt **(a)** Akt/ Beta Actin **(b)** p-ERK1/2/ ERK1/2 **(c)** ERK1/2 / Beta Actin **(d)** are presented for control and diabetic groups. Beta actin bands represent diabetic groups **(a, c)** or control groups **(b, d)**. *p≤0.05.

Wang et al., 2012; Lee et al., 2013). The altered placental development in STZ- treated rat placenta could be a result of altered angiogenesis mechanisms. Varma et al. (2005) and Yu et al. (2006) reported in their study that high glucose decreased both Human Umbilical Vein Endothelial Cell (HUVEC) proliferation and Akt phosphorylation in a dose-dependent manner. Besides, Yu et al., also mentioned that high glucose both decreased the migration rate of endothelial cells and the numbers of total area of vascular bed, average tubule length, number of capillaries, and number of vessel branch point formed on the Matrigel dose-dependently (Yu et al., 2006). Therefore, smaller placentas seen in diabetic group could be a result of insufficient nutrient transport and afterwards insufficient proliferation/ survival will occur.

In contrast, there are some contradictory studies reporting that high glucose induces some angiogenesis related proteins. In the study of (Zhao et al., 2004) retinal microvascular endothelial cells were exposed to varying concentrations of glucose and Placental Growth Factor (PlGF) expression measured by RT-PCR and Western blotting. In endothelial cells, glucose (15 mM) induced an increase in expression of PlGF at both the mRNA and protein levels. It was also mentioned that MAPK pathway is one of the other signal transduction pathways which have roles in angiogenesis mechanisms. VEGF-stimulated PlGF expression was largely inhibited by PD 98059, an inhibitor of MAPK, indicating that VEGF up-regulates PlGF expression via the MAPK signaling pathway (Zhao et al., 2004).

It is clearly shown that MAPK-ERK1/2 signal transduction pathway is important for murine placental development (Giroux et al., 1999; Qian et al., 2000; Mikula et al., 2001). It was reported by (Hatano et al., 2003) that Erk1 mutant mice survived and fertile besides, while Erk2 mutant mice showed weaker vascularization and had smaller LZ. According to our findings, there is a strong p-ERK1/2 immune reaction in the fetal endothelial cells and chorionic mesenchymal cells in the LZ and in spongiotrophoblast cells and giant cells in the JZ of the control placentas. It was indicated by (Saba-El-Leil et al., 2003), that Erk2 mutant embryos were not able to form the ectoplacental cone and extra-embryonic ectoderm and (Bissonauth et al., 2006) reported that MEK1 (MAP kinase/ERK kinase 1) knockout mice resulted in fetal mortality and labyrinth defects. In this study, we determined changes at ERK1/2 phosphorylation in diabetic groups especially in LZ according to our immunohistochemistry results. These alterations in ERK1/2 activity would be a cause or consequence of altered placental vasculature development. And this could be one of the reasons for abnormal placental and fetal growth.

In many studies (Lappas et al., 2007; Tang et al., 2007; Takata et al., 2009; Li et al., 2010; Wang et al., 2010) it is clearly shown that MAPK is one of the signaling pathways affected by hyperglycemia. But there are no studies reporting p-ERK1/2 levels in diabetic placentas during rat placental development. With this report, for the first time we showed that ERK1/2 phosphorylation was decreased in diabetic group placentas during gestation. Likewise, decreased Akt activity and decreased ERK1/2 activity would also be a defense mechanism of the placenta against the hyperglycemic conditions to create the optimal conditions for normal placental and fetal growth. Unlikely, these alteration in p-Akt and p-ERK1/2 proteins would result with decreased fetal growth. The differences in the placental growth, first a decrease from GD12-16 and then an increase from GD18 to term need to be studied in more detail to explain these changes according to the cell signaling mechanisms' alterations.

In diabetic pregnancies, maternal Insulin and Insulin like Growth Factor (IGF) levels are elevated and Insulin/IGFs show their effects on several intracellular signal transduction pathways (Hiden et al., 2009). In vitro, IGFs regulate proliferation and survival of cytotrophoblasts in a human first trimester explant model. Both effects are mediated via two different signaling pathways, i.e. proliferation is stimulated via the MAPK pathway whereas rescue from apoptosis is regulated by the PI3K pathway (Forbes and Westwood, 2008). There is also a growing body of evidence from various studies to suggest either a direct or an indirect effect of these growth factors on placental growth. Based on histochemical and morphometric evidence, villous growth in the third trimester, when placental growth is predominantly characterized by mass expansion, is driven by capillary growth and proliferation (Mayhew, 2002). Therefore, it can be suggested that the placental growth differences in diabetic rats would be related to different effects of diabetic microenvironment both in a cell type dependent and time dependent manner. Also, it should be considered that hyperglycemia will have different effects even in the same type of cell in different niches. In their study Duffy et al., reported that hyperglycemia differently affected the macrovascular and microvascular endothelial cells. It was determined that high glucose levels reduced cell viability and induced apoptosis in human aortic endothelial cells, which may contribute to macrovascular complications associated with diabetes. In contrast, high-glucose increased viability in human retinal endothelial cells and inhibited apoptosis, which may contribute to the development of diabetic retinopathy (Duffy et al., 2006).

(Hiden et al., 2009) mentioned that in the first trimester pregnancies IRs are predominantly expressed on the microvillus membrane of the syncytiotrophoblast, directed to the maternal circulation and, hence, maternal insulin. But at term, in contrast, IRs are mainly expressed on the placental endothelium directed to the fetal blood. Therefore, IR expression shifts throughout pregnancy from the surface facing the maternal circulation to facing the fetal circulation (Desoye et al., 1994, 1997; Hiden et al., 2006). According to our results, both Akt and ERK1/2 phosphorylation were decreased during all gestational days of rat pregnancy, in

a placental weight independent manner. These results would be a consequence of both maternal and fetal metabolism reactions for hyperglycemia/ hyperinsulinemia and the cellular effects will be different depending on the gestational age.

In summary, it could be mentioned that MAPK-ERK1/2 and PI3K/Akt cell signaling pathways are affected by hyperglycemic conditions in rat placentas. Therefore, hyperglycemia-induced placental and embryonal developmental abnormalities could be associated with reduction of Akt and ERK1/2 phosphorylation. The results warrant further detailed studies into the complex mechanisms regulating Akt and ERK1/2 proteins during normal and diabetic placenta development.

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