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Determination of PCNA, cyclin D3, p27, p57 and apoptosis rate in normal and dexamethasone-induced intrauterine growth restricted rat placentas

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ABSTRACT

Intrauterine growth restriction (IUGR) is a major clinical problem, which causes perinatal morbidity and mortality. One of the reasons for IUGR is abnormal placentation. In rats, fetal–placental exposure to maternally administered glucocorticoids decreases birth weight and placental weight. Proper placental development depends on the proliferation and differentiation of trophoblasts. Our knowledge about the mitotic regulators that play key roles in synchronizing these events is limited. Also the mechanisms underlying the placental growth inhibitory effects of glucocorticoids have not been elucidated. The aim of this study was to investigate the immunolocalization, mRNA and protein levels of proliferating cell nuclear antigen (PCNA), cyclin D3, p27 and p57 in normal and dexamethasone-induced IUGR Wistar rat placentas by reverse transcriptase polymerase chain reaction (RT-PCR), immunohistochemistry and Western blot. We also compared apoptotic cell numbers at the light microscopic level via terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) and transmission electron microscopy. Glucocorticoid levels were higher in IUGR rats than in control rats after 60 and 120 min of injection. We showed reduced gene and protein expressions of PCNA and cyclin D3 and increased expressions of p27 and p57 in IUGR placentas compared to control placentas. Apoptotic cell number was higher in the placentas of the IUGR group. In brief we found that maternal dexamethasone treatment led to a shift from cell proliferation to apoptosis in IUGR placentas. Dexamethasone induced placental and embryonal abnormalities which could be associated with reduced expressions of PCNA and cyclin D3, increased expressions of p27 and p57 and increased rate of apoptosis in IUGR placentas.
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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 and directs blood supply to the uterus (Hemberger and Cross, 2001). Although the architecture of the human and rodent placentas show minor differences, however, their overall structure and the molecular mechanisms of placental development are thought to be very similar. As a result, the rat placenta is increasingly used as a model to investigate the mechanisms of placental development (Rossant and Cross, 2001) and pregnancy-related problems in humans, such as those associated with diabetes, hypertension and IUGR (Vercruysse et al., 2006).

The eukaryotic cell cycle is regulated by the coordinated activity of a family of cyclin-dependent kinases (CDKs). These are positively and negatively regulated by the cyclin and CDK inhibitor (CKI) families, respectively (Sherr and Roberts, 1995; Xiong, 1996).

Based on the timing of their appearance in the cell cycle, cyclins can be divided into two groups, the mitotic cyclins A and B, and the

Abbreviations: AEC, 3-amino-9-ethylcarbazole; AI, apoptotic index; CDK, cyclin dependent kinases; CKI, CDK inhibitor; Dex, dexamethasone; ELISA, enzyme-linked immunosorbent assay; IUGR, intrauterine growth restriction; JZ, junctional zone; LZ, labyrinth zone; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcriptase polymerase chain reaction; TEM, transmission electron microscopy; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling.

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G1 cyclins of the D and E family (Lew et al., 1991). D-type cyclins (cyclins D1, D2, or D3) promote progression from mid to late G1 via CDK4 and CDK6 activation (Sherr and Roberts, 1995) and are required for S-phase entry, providing a link between the cell cycle, signal transduction and proliferation. Cyclin E-CDK2 complexes are also active during late G1 phase and are implicated in G1/S progression (Koff et al., 1992). Cyclin E, one of the G1 cyclins, is expressed during mid- to late-G1 phase. Kinase activities of cyclin E/CDK2 complexes are at maximum levels before S-phase entry.

Proliferating cell nuclear antigen (PCNA) is a marker for the cell cycle, its expression peaks in late G1 and S phases of the cell cycle (Takahashi and Caviness, 1993). It is an accessory protein that is necessary for deoxyribonucleic acid (DNA) synthesis in mammalian cells (Murphy et al., 1974), whereas lack of this protein prevents cell division (Braun et al., 2007). It is an essential regulator of p21 activity by binding to the carboxy terminus of p21, which then results in the inhibition of PCNA-dependent DNA replication. Proliferating cell nuclear antigen 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 (Goland et al., 1995).

Cell cycle arrest and activation of differentiation processes require inhibition of CDKs by CKIs (Rao et al., 1994; Skapek et al., 1995, 1996). In mammalian cells, there are at least two distinct families of CKIs: the INK4 and the Cip/Kip inhibitors. INK4 family members include p15, p16, p18, and p19. Cip/Kip members include p21, p27, and p57 (el-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Polyak et al., 1994; Toyoshima and Hunter, 1994; Lee et al., 1995). Both families play regulatory roles during the G1/S cell cycle checkpoint (Hunter and Pines, 1994). Because of their broader panel of CDKs with which they interact (Harper and Elledge, 1996), the inhibitors of the Cip/Kip family control other checkpoints as well. Also, p27 has the capacity to arrest cells in G2 (Niculescu et al., 1998). p57 (Kip2) inhibits cyclin A- and E-associated CDKs and therefore regulates G1/S transition and completion of S phase (Lee et al., 1995) and is primarily expressed in terminally differentiated cells (Yan et al., 1997).

IUGR is a major cause of perinatal death and neonatal morbidity and mortality. There are numerous causes of IUGR. Glucocorticoid induced IUGR is highly relevant because administration of synthetic glucocorticoids, principally dexamethasone, to women threatened by premature labor is widely used in clinical practice. Fetal growth is directly related to placental growth and development.

Synthetic glucocorticoids, including dexamethasone, have been used for over three decades to mature fetal lungs and prevent respiratory distress syndrome in pregnancies where delivery is likely before 34 weeks (Liggins and Howie, 1972; Trainer, 2002). Glucocorticoids play an essential role in normal fetal development and are important for the maturation of fetal tissues in preparation for extra-uterine life (Fowden et al., 1998). Although glucocorticoids promote lung maturation, these actions are not without negative side effects. Exposure to glucocorticoids retards fetal growth in animal models and in humans (Benediktsson et al., 1993; Seckl, 1994; Gluckman, 2001; Sugden et al., 2001; McDonald et al., 2003) together with an increased risk of subsequent hypertension, cardiovascular disease and glucose intolerance in the adult offspring (Barker, 1997). The mode of action of dexamethasone in placental growth inhibition has still not been determined (Babat et al., 2005).

The placenta plays a pivotal role in fetal growth. Failure of placental growth and development during early and mid-pregnancy is directly associated with decreased fetal growth during late pregnancy (Hay et al., 1997). Despite the fact that placental development requires the coordinated action of trophoblast proliferation and differentiation, little is known about the mitotic regulators that play key roles in synchronizing these events (Genbacev et al., 2000). In this study, we aimed to shed light on the possible roles of cell cycle related proteins and cell cycle inhibitors in normal and growth retarded rat placentas. Moreover it is still not known how coordination mechanisms of proliferation and differentiation are influenced by dexamethasone-induced IUGR in the placenta. Therefore we aimed to examine the spatial and temporal immunolocalization, mRNA and protein levels of proliferating cell nuclear antigen (PCNA), cyclin D3, p27 and p57 in normal and dexamethasone-induced IUGR rat placentas. In addition we compared apoptotic cell numbers at light and electron microscopic levels to investigate the effects of maternally administered dexamethasone.

Materials and methods

Animals

Female Wistar rats (*Rattus norvegicus*) weighing 250–300 g used for all experiments were maintained under standard conditions and exposed to 12/12 h light–dark cycles. After mating, the presence of sperm in the vaginal smear the following morning was designated as day 0 of pregnancy. On day 13 of pregnancy, rats were subcutaneously injected with a dose of 100 μg dexamethasone 21-acetate (Dexamethasone) (Sigma–Aldrich, St. Louis, MO, USA) in 0.1 ml 10% ethanol. The animals subsequently received daily injections of 200 μg/kg dexamethasone on days 14–19 of pregnancy. Control animals were injected with 0.9% saline solution subcutaneously on corresponding days of pregnancy. Eight 0.9% saline injected (control group) and eight dexamethasone injected (IUGR group) female rats were sacrified on day 20 of pregnancy. 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 preparation

The placentas were dissected and fixed in 4% formaldehyde (100 ml 37% formaldehyde, 900 ml distilled water, pH 7) at room temperature approximately for 12 h for immunohistochemistry and TUNEL staining.

Immunohistochemistry

Formalin-fixed paraffin-embedded samples were cut into 5 μm sections and placed on slides coated with poly-L-lysine. After deparaffinization, slides were boiled in citrate buffer (pH 6.0) for 10 min for antigen retrieval and cooled for 20 min at room temperature. Then, sections were immersed in 3% hydrogen peroxide for 20 min to block endogenous peroxidase. Slides were then incubated in a humidified chamber with UltraV block (Labvision, Fremont, CA, USA) for 15 min at room temperature. Excess serum was drained and sections were incubated with primary antibodies PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-56) mouse monoclonal antibody at 1:1000 dilution; cyclin D3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-6283) mouse monoclonal antibody at 1:400 dilution; p27 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-1641) mouse monoclonal antibody at 1:200 dilution; p57 (sc-1040; Santa Cruz Biotechnology, Santa Cruz, CA, USA) rabbit polyclonal antibody at 1:200 dilution for 1 hour at room temperature in a humidified chamber. Isotype controls were performed by replacing the primary antibody with the appropriate serum or non-immune IgG in the same dilutions as the specific antibodies. The sections were washed three times for 5 min with phosphate buffered saline (PBS) and then incubated with biotinylated secondary antibody (K0609; HRP LSAB-2 system, DakoCytomation, Glostrup, Denmark) for 30 min then

with peroxidase labeled streptavidin (K0609; HRP LSAB-2 system, DakoCytomation, Denmark) for 30 min. Then slides were washed with PBS and peroxidase activity was visualized with 3-amino-9-ethylcarbazole (AEC) (K3461; DakoCytomation, Denmark) for 3–5 min. Sections were counterstained with hematoxylin, dehydrated, mounted in Kaiser's glycerin gelatin (OB514196; Merck, Rahway, NJ, USA) and examined by light microscopy. The distribution of immunoreactive cells in all experimental groups was determined semi-quantitatively: − = negative; + = weak positive; ++ = positive; +++ = strong positive; ++++ = very strong positive.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling staining (TUNEL)

Apoptosis in placenta tissue was detected by enzymatic labeling of DNA strand breaks by using TUNEL. 5-μm thick paraffin wax sections from placenta tissues were cut and placed on slides coated with poly-l-lysine, and after drying, the slides were left in the incubator at 45 °C overnight and at 60 °C for 1 h. After deparaffinization and rehydration, slides were washed twice in phosphate-buffered saline for 5 min.

After incubation of slides with the permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 8 min at 4 °C and washing twice with phosphate-buffered saline for 5 minutes, the labeling reaction was performed by using 50 μL of TUNEL reagent for each sample, except negative control, in which reagent without enzyme was added and incubated for 1 h at 37 °C. After phosphate-buffered saline washings, slides were incubated with converter reagent for 30 min at 37 °C. After washing, color development for localization of cells containing labeled DNA strand breaks was performed by incubating the slides with Fast Red substrate solution for 10 min. Labeling with TUNEL was conducted with using an In Situ Cell Death Detection kit (1684809; Roche, Mannheim, Germany) and was performed according to the manufacturer's instructions.

TUNEL stained sections were evaluated using an Axioplan microscope (Zeiss, Oberkochen, Germany) with a special ocular scale. Five randomly selected slides, each with four different fields at ×200 magnification were evaluated for the analysis of TUNEL. Positively stained cell numbers in the fields were counted in a blind study by two observers. Mean positive cell numbers were calculated and then were rated to 100. Positively stained apoptotic cell numbers (apoptotic index) in the tissue were indicated as percent (%).

Western blotting

Protein extraction and immunoblot analysis were performed as described previously (Smink et al., 2003). Four placenta samples for each group were weighed and put into a homogenization buffer supplemented with Complete R 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 (Lab Vision, TA 125 TW, UK) in 0.14 mol/L Tris-buffered saline (TBS; pH 7.2–7.4) at +4 °C. Membranes were incubated with a 1:500 dilution of mouse monoclonal anti-PCNA antibody (sc-56; Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:400 dilution of mouse monoclonal anti-cycD3 antibody (sc-6283; Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1: 100 dilution of mouse monoclonal anti-p27 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-1641), 1:100 dilution of rabbit polyclonal anti-p57 antibody (sc-1040; Santa Cruz Biotechnology, Santa Cruz, CA, USA), overnight. After the washing steps, the membranes were further incubated with either goat anti-mouse IgG horseradish peroxidase conjugate (170-6516; BioRad Laboratories, Hercules, CA, USA), diluted 1:2000 for PCNA, cycD3 and p27 antibodies or goat anti-rabbit IgG horseradish peroxidase conjugate (172-1019; BioRad Laboratories, Hercules, CA, USA), diluted 1:2000 for p57 for 1 h at room temperature.

Immunolabeling was visualized by using the chemiluminescence-based SuperSignal CL HRP Substrate System (Thermo Scientific, Waltham, MA, USA) prepared according to the manufacturer's instructions, and the membranes were exposed to Hyperfilm (28906837; Amersham, Bucks, UK). Membranes were also labeled by identical protocol for binding of a 1:5000 dilution of an anti-mouse beta actin antibody (ab 6276; Abcam, Cambridge, UK) 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, Bethesda, MD, USA).

Gene expression analysis of cell cycle proteins by reverse transcriptase polymerase chain reaction (RT-PCR)

RNA isolation and cDNA synthesis

Total RNA was isolated from placentas using Trizol Reagent (15596-018; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA pellets were eluated in RNase-free water and stored at −80 °C until the time of the experiment. Each RNA sample was quantified using UV spectrophotometer. Preparation of cDNA was carried out from 2 μg of total RNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

Semi-quantitative RT-PCR

cDNA was amplified in a 50 μl reaction volume containing 0.2 mM dNTPs, 10 mM specific primers, 1× PCR buffer and 1 U of Platinum® Taq DNA Polymerase (201203; Qiagen, Valencia, CA, USA). After initial denaturation at 94 °C for 10 min, PCR was carried out for 35 cycles with denaturation for 30 s at 94 °C, annealing for 30 s at 53 °C for PCNA, 55 °C for cyclin D3, p27, and beta-actin, 58 °C for p57 and extension for 1 min at 72 °C followed by a final extension of 10 min at 72 °C. The sequences of primers are shown in Table 1. The PCR products were visualized by electrophoresis with an ethidium bromide-stained 1.5% agarose gel. All results were normalized to beta-actin (*Actb*). And the intensity of bands were quantified using NIH image analysis software (Image J Version 1.36b, NIH, Bethesda, MD, USA).

Primer sequences

	Forward primer	Reverse primer	Product (bp)
PCNA	5′-GGCGTGAACCTACAGAGCAT-3′	5′-CACAGGAGATCACCACAGCA-3′	388
cyc D3	5′-TGCATCTATACGGACCAAGC-3′	5′-TCCACTTCAGTGCCTGTGAT-3′	341
p27	5′-GCACTGCCGAGATATGGAAG-3′	5′-AGAATCTTCTGCCGCAGGTC-3′	340
p57	5′-TCTGAGCAGGTCTCTGAGCA-3′	5′-CTGGTCCTTCAGCTCCTGAT-3′	240
Beta-actin	5′-CATGAAGATCCTGACCGAGC-3′	5′-CAGACAGCACTGTGTTGGCA-3′	335

Glucocorticoid assay

To verify whether dexamethasone injection was done correctly, glucocorticoid levels were measured in plasma. For this, blood samples were collected from tail veins before and 60, 120 and 180 min

Table 1
Semi-quantitative scoring of PCNA, cyclin D3, p27 and p57 immunolabeling intensities in control and IUGR group rat placenta zones. – = negative; + = weak positive; ++ = positive; +++ = strong positive; ++++ = very strong positive.

		Junctional zone			Labyrinth zone	
		Spongiotrophoblast cells	Glycogenic cells	Trophoblast giant cell	Trophoblast cells	Fetal endothelial cells
PCNA	Control	++++	–	++++	++++	++
	IUGR	++	–	++	++	++
Cyclin D3	Control	++	–	++++	+++	++
	IUGR	+	–	+++	++	+
p27	Control	++	–	++	++	++
	IUGR	+++	–	+++	+++	+++
p57	Control	++	–	+	+	++
	IUGR	+++	–	++	++	+++

–, negative; +, weak positive; ++, positive; +++, strong positive; ++++, very strong positive.

after physiological saline and dexamethasone injection for control and dexamethasone injected groups, respectively. For this assay blood samples were collected in cooled tubes at 4 °C, after which they were centrifuged to remove blood cells and obtain serum. Following centrifugation for 10 min at 4 °C and 1200 × *g*, blood plasma was collected and stored at −80 °C until assay. The plasma levels of corticosterone were determined with an enzyme-linked immunosorbent assay (ELISA) (900097; Assay Designs, Ann Arbor, MI, USA) according to the manufacturer's specifications. The samples were thawed in room temperature and 10 μl of samples were pipeted to new tube and were diluted 100 times with assay buffer. 100 μl of samples and standard were pipeted to an ELISA plate. 50 μl of conjugate and 50 μl of antibody were added to each well and the plate was incubated for 2 hour in room temperature, then the plate was washed 3 times by washing buffer and evaporated in room temperature. Each well was added with 200 μl of pNPP substrate and incubated for 1 hour. Finally, 50 μl of stop solution was added to each well and the absorbance of each well was read in an ELISA reader at 405 nm. The sensitivity of the assay is 27 pg/ml, and the intra- and inter-assay coefficient of variation amounted to 7.7% and 9.7%, respectively.

Transmission electron microscopy (TEM)

Rat placenta tissues were fixed by immersion in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 4 h and were postfixed in 1% phosphate-buffered osmium tetroxide for 2 h. The specimens were dehydrated in a graded series of ethanol and embedded in Araldite epoxy resin (E006; TAAB, Reading, UK). Semi-thin (1 mm) and ultrathin sections were cut using a Nova ultramicrotome (LKB, Bromma, Sweden). Ultrathin sections were collected on coated grids and stained with uranyl acetate (1 g uranyl acetate in 100 ml absolute ethanol) and lead citrate (sodium citrate 2.137 g and lead nitrate 1.33 g dissolved in 30 ml water and then added 8 ml 1 N sodium hydroxide and made to 50 ml with distilled water) prior to examination using a transmission electron microscope (LEO 906E TEM, Zeiss, Oberkochen, Germany).

Statistical analysis

ImageJ software from NIH Image was used to compare the density of bands on agarose and polyacrylamide gels. Comparisons between relative mathematical values of PCNA, cyclin D3, p27 and p57 RT-PCR and Western blotting, glucocorticoid assay results and apoptotic cell numbers of control and IUGR groups were compared by Mann–Whitney rank sum test. All statistical analyses were performed using Sigma Stat 3.5 (Statcon, Witzenhausen, Germany).

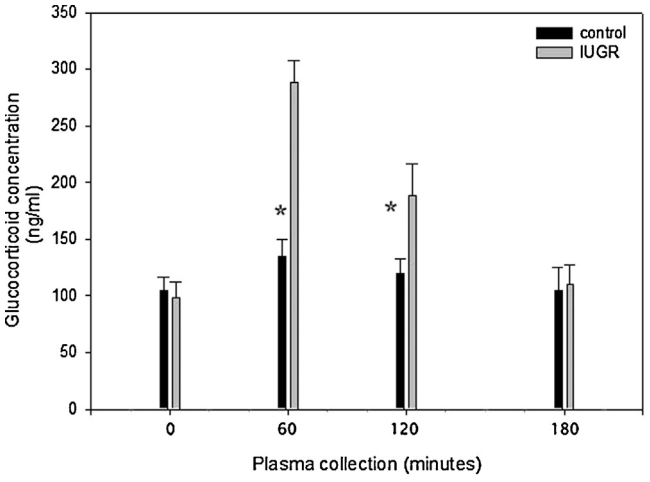


Fig. 1. Changes in plasma glucocorticoid concentrations of control and IUGR group rats (mean ± SE) following injection of dexamethasone after 0, 60, 120 and 180 min. Maternal dexamethasone treatment triggers the increase of glucocorticoid concentration in the IUGR group after 60 and 120 min with a statistically significant difference compared to the control group ($p < 0.001$). Glucocorticoid levels returned to normal in the IUGR group 180 min after injection.

Results

Glucocorticoid assay results

According to the glucocorticoid assay there was no change of glucocorticoid concentration in control rats during 180 min; however in Dexamethasone injected rats after a statistically significant increase after 60 and 120 min ($p < 0.001$), glucocorticoid levels returned to normal in 180 min (Fig. 1). According to these results injected dexamethasone was metabolized approximately in 3 h and showed its effects during this time.

Placenta and embryo weights

When rats were sacrified on day 20 of pregnancy, the numbers and weights of embryos and placentas were recorded. Mean placenta weights were lower in the IUGR group compared to the control group with statistically significant difference ($p < 0.001$). Also mean embryo weights were lower in the IUGR group compared to the control group with statistically significant difference ($p < 0.005$) (Fig. 2). Although embryo numbers were lower in the IUGR group compared to the control group it was not statistically significant (Fig. 2). In conclusion, subcutaneously injected dexamethasone caused weight loss of embryos and placentas but did not affect the number of embryos (Fig. 2).

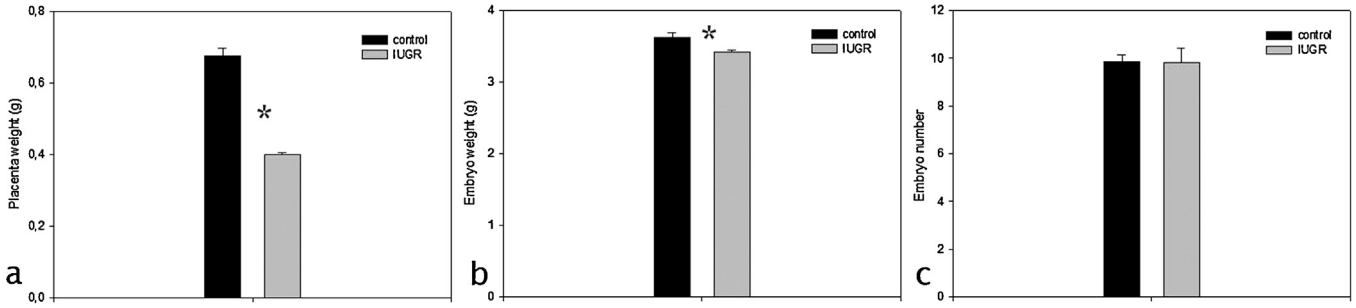


Fig. 2. Placenta and embryo weights, number of embryos for control and IUGR group rats. (a) Placenta (b) Embryo weights were lower in IUGR group and the difference was statistically significant ($p < 0.001$). (c) Embryo numbers were higher in the control group compared to IUGR group but it was not statistically significant.

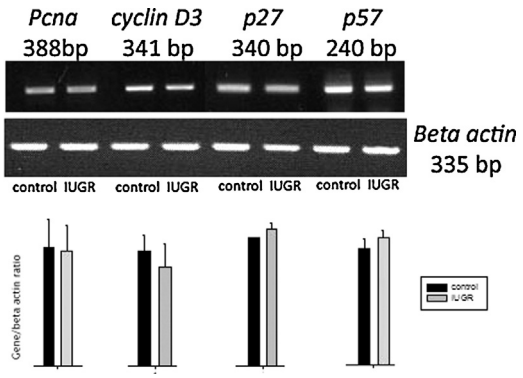


Fig. 3. RT-PCR results of *PCNA*, *cyclin D3*, *p27* and *p57* mRNAs. Expressions of *PCNA* and *cyclin D3* were higher in the control group than in the IUGR group, expressions of *p27* and *p57* were higher in the IUGR group. Actin was used as an internal control. The values in the bar graphs were obtained by dividing the mRNA intensity value by its corresponding b-actin intensity value Agarose gel picture (upper) and graphic of the intensity of bands (below) are seen.

Light microscopic evaluation of placentas

Two zones were recognized on placenta sections of control and IUGR groups after hematoxylin-eosin staining: the labyrinth and junctional zones. There were fetal and maternal blood cisternae in the labyrinth zone. Spongiotrophoblast zone and trophoblast giant cells were located next to the labyrinth zone towards the maternal decidua. There were only maternal blood islands in the spongiotrophoblast zone. Dexamethasone injected rat placentas were significantly smaller than control group rat placentas. The general appearance of the labyrinth zone was normal. Cell number in the labyrinth zone seemed less compared to the control group. The dominant cell type was the trophoblast. In addition to spongiotrophoblast and trophoblast giant cells, there were also some glycogenic cells in the junctional zone (not illustrated).

RT-PCR results

The semi-quantitative measurement of mRNA levels by RT-PCR showed that there was no significant difference between the control and the IUGR groups regarding the expressions of *Pcna*, *cycD3*, *p27* and *p57*. *PCNA* and *cycD3* mRNA expression levels were higher in the control group than in the IUGR group. *p27* and *p57* mRNA expression levels were higher in the IUGR group than in the control group, but the differences were not statistically significant (Fig. 3).

Immunohistochemical results

PCNA

Proliferating cell nuclear antigen immunolocalization was nuclear. While PCNA immunoreaction of labyrinth trophoblasts was very strongly positive in the control group, it was positive in the IUGR group. Proliferating cell nuclear antigen immunoreaction was positive in fetal endothelial cells of labyrinth trophoblasts, in both control and IUGR groups. Proliferating cell nuclear antigen immunoreaction in spongiotrophoblast and trophoblast giant cells of the junctional zone was very strong positive and positive in control and IUGR groups, respectively (Table 1 and Fig. 4).

Cyclin D3

Cyclin D3 immunolocalization was nuclear. Cyclin D3 was positive in spongiotrophoblasts and fetal endothelial cells of the control group placentas and it was weak positive in the same cell groups in the IUGR group. Immunoreactivity of cycD3 in trophoblast giant cells was very strongly positive in the control group and strongly positive in the IUGR group. Trophoblast cells showed a strong positive and positive immunoreaction in control and IUGR groups, respectively (Table 1 and Fig. 4).

p27

p27 immunolocalization was nuclear. Labyrinth trophoblasts of placentas of control and IUGR groups were stained positive and strong positive, respectively for p27 antibody. Fetal endothelial cells of control and IUGR groups showed strong positive and positive p27 immunoreactivity, respectively. Spongiotrophoblast and trophoblast giant cells of the junctional zone showed positive p27 immunoreaction in control and strong positive p27 immunoreaction in the IUGR group (Table 1 and Fig. 4).

p57

p57 immunolocalization was nuclear. p57 immunoreaction was positive in spongiotrophoblasts and fetal endothelial cells of the control group and these cell types showed strong positivity in the IUGR group. Trophoblast and trophoblast giant cells were weak positive in control and positive in the IUGR group (Table 1 and Fig. 4).

Western blot results

According to Western blot results, PCNA and cyclin D3 expressions in the control group were higher than in the IUGR groups and these differences were statistically significant. Expressions of p27 and p57 were higher in the IUGR group than in the control group. The difference between control and IUGR groups for p27 expression was statistically significant, but not for p57 expression (Fig. 5).

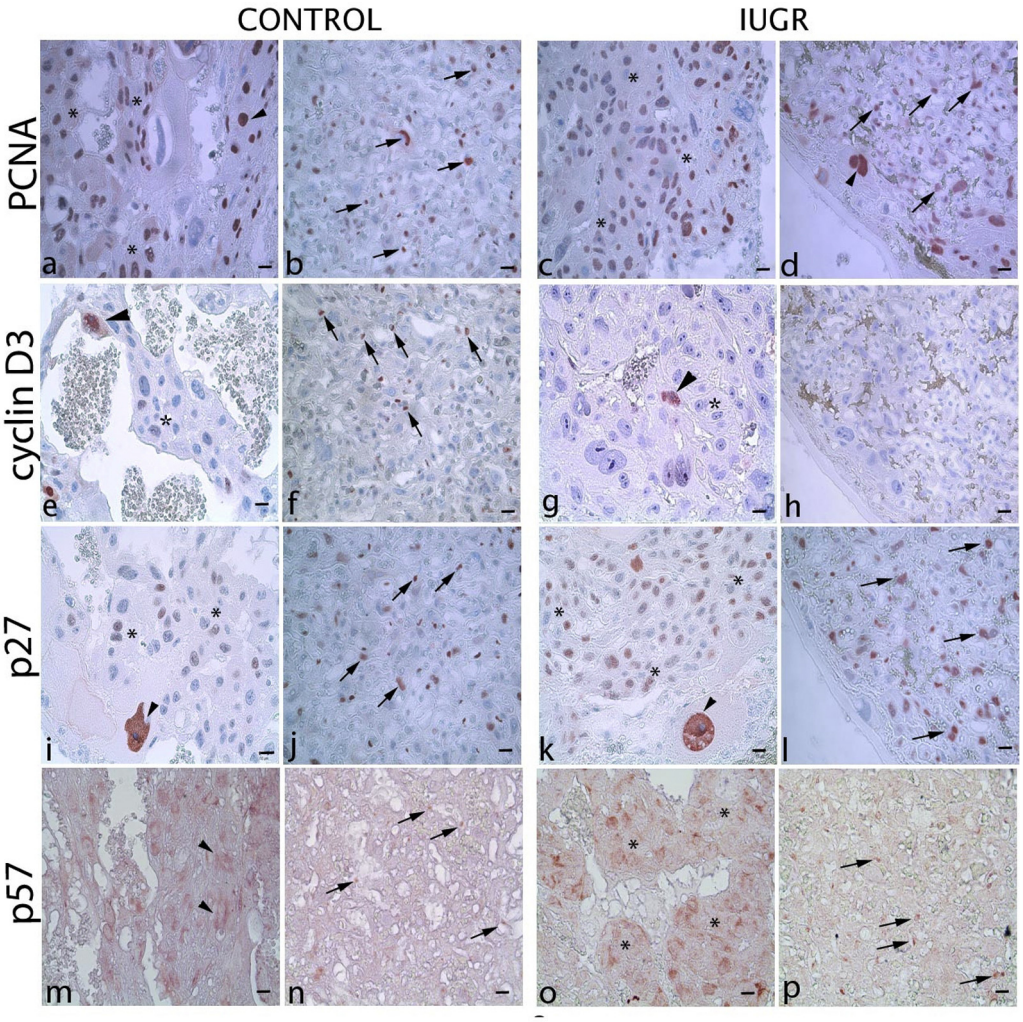


Fig. 4. PCNA immunolabeling in (a) junctional and (b) labyrinth zones of control group; (c) junctional and (d) labyrinth zones of the IUGR group. PCNA immunoreactivity was weaker in IUGR placentas. Scale bar = 10 μm. Cyclin D3 immunolabeling (e) junctional and (f) labyrinth zones of control group; (g) junctional and (h) labyrinth zones of IUGR group. Cyclin D3 immunoreactivity was weaker in IUGR placentas. Scale bar = 10 μm. p27 immunolabeling (i) junctional and (j) labyrinth zones of control group; (k) junctional and (l) labyrinth zones of the IUGR group. p27 immunoreactivity was stronger in IUGR group placentas. Scale bar = 10 μm. p27 immunoreactivity was stronger in IUGR placentas. p57 immunolabeling (m) junctional and (n) labyrinth zones of control group; (o) junctional and (p) labyrinth zones of IUGR group. p57 immunoreactivity was stronger in the IUGR placentas. Scale bar = 10 μm. Asterisks show spongiotrophoblasts; arrow heads show giant cells; arrows show labyrinth trophoblasts.

TUNEL

Apoptotic cell percent was calculated at 200× magnification by two observers counting the apoptotic cell numbers of maternal and fetal compartments of control and IUGR group placentas. Both control and IUGR group placentas had higher TUNEL positive cell numbers (apoptotic index (AI)) in the maternal compartments than in the fetal compartments and the difference was statistically significant ($p < 0.001$).

All values of the maternal and fetal compartments of the IUGR group were higher than maternal and fetal compartments of the control group and the difference was statistically significant ($p < 0.001$) (Fig. 6).

Transmission electron microscopy

Thin sections of placentas of the control and IUGR groups were examined in a transmission electron microscope (LEO 906E TEM, Zeiss, Oberkochen, Germany). The morphology of spongiotrophoblasts in the junctional zone; trophoblasts and fetal endothelial cells in the labyrinth zone was normal in the control group (Fig. 7a and b). Unlike the control group, some spongiotrophoblasts and trophoblasts showed nuclear changes characteristic of apoptosis in the IUGR group. These cells which were shrunken and dark stained had intense chromatin structure at the periphery of nucleus and had cytoplasm rich in vacuoles. Apoptotic bodies having fragments of nucleus chromatin was striking (Fig. 7c and d). Results showing apoptotic cell morphology at the electron microscope level were compatible with TUNEL results at the light microscope level.

Discussion

In this report of a rat placenta model, we have shown that use of prenatal dexamethasone leads to changed expressions of PCNA, cyclin D3, p27, p57 on mRNA and protein levels and also a change in apoptosis rate. Additionally, prenatal dexamethasone use was associated with reduction in fetal and placental weights at term and morphological changes in the placenta.

Adequate placental growth and function are fundamental to the well-being, growth and development of the fetus throughout gestation (Waddell et al., 2000). Glucocorticoids play a fundamental role in pregnancy with effects on decidualization, implantation, placental development, fetal brain development, lung maturation and parturition, but fetal-placental exposure to maternally

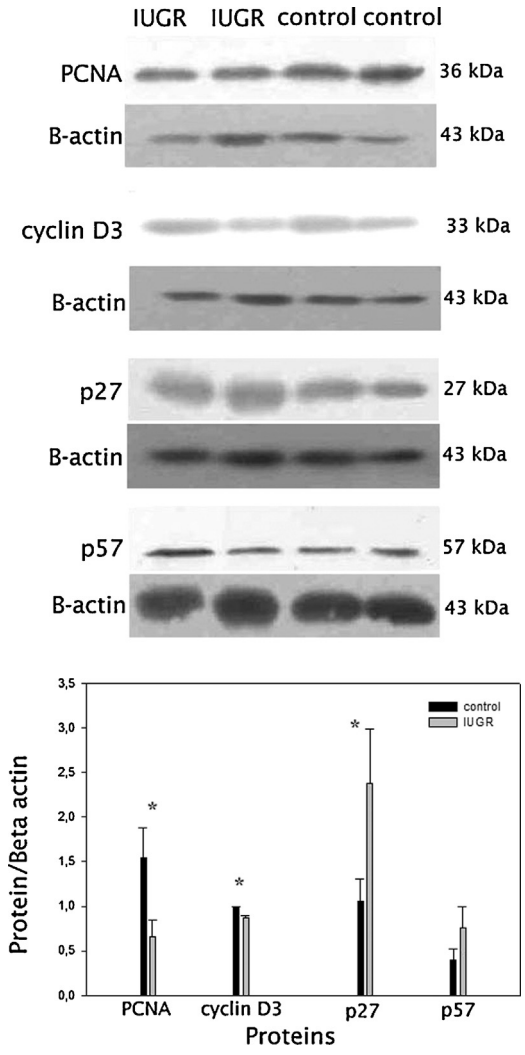


Fig. 5. Upper: Western blot results of PCNA, cyclin D3, p27 and p57 in control and IUGR group placentas. Actin was used as an internal control. Below: Quantitative analysis of Western blot data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding b-actin intensity value. PCNA and cyclin D3 were found to be lower in IUGR group with a statistically significant difference; p27 and p57 were found to be higher in IUGR group.

administered glucocorticoids may lead to abnormalities of fetal and placental growth (Benediktsson et al., 1993; Sugden et al., 2001; McDonald et al., 2003; Unek et al., 2012). The morphology and function of the placenta can be altered by maternal glucocorticoid administration (Murphy et al., 1974; Johnson et al., 1979; Sugden et al., 2001; Kerzner et al., 2002; Babat et al., 2005; Baisden et al., 2007; Braun et al., 2007; Gatford et al., 2008; Audette et al., 2011) and as the mediator between the mother and the fetus; it is likely that the placenta may play a role for respective developmental alterations (Jansson and Powell, 2007).

Exposure to glucocorticoids retards fetal growth in animal models and in humans (Benediktsson et al., 1993; Seckl, 1994; Gluckman, 2001; Sugden et al., 2001; McDonald et al., 2003; Unek et al., 2012) together with an increased risk of subsequent hypertension, cardiovascular disease and glucose intolerance in the adult offspring (Barker, 1997). How dexamethasone causes placental growth inhibition is still not known. This could be associated with proliferation and apoptosis mechanisms, which are crucial for development of both fetus and placenta. Since there are only scarce data about the effects of dexamethasone on proliferation and apoptosis mechanisms, we decided to investigate cell cycle and apoptosis mechanisms in control and dexamethasone induced growth retardated placentas. The hypothesis that the coordinated expression of cell cycle regulators and inhibitors determine whether cytotrophoblasts proliferate or undergo cell cycle arrest or cell cycle exit allowing subsequent apoptosis was tested by our team with glucocorticoid administration in rat pregnancy.

In our previous study we have shown that the cell populations in the placenta stain for a number of cell cycle related proteins and that these staining patterns change as a function of both gestational age and abnormal placentation. We found reduced expressions of PCNA and Ki67 and increased expressions of p27 and p57 in the dexamethasone-induced IUGR placentas compared to control placentas during gestational days from 13 to 21 (Unek et al., 2012).

Dexamethasone treatment led to a decrease in PCNA, cyclin D3 and an increase in p27, p57 expressions. And it also triggered an increase on apoptosis rates. Besides structural abnormalities were observed by electron microscopy. So we can comment that maternally administered glucocorticoids cause placental cell proliferation and apoptosis to be affected unfavorably and it results in reduced placental and fetal weight.

Cortisol, the predominant form of active glucocorticoid interacts with the glucocorticoid receptor (GR) in target cells to ensure functional maturation of fetal organs such as lung, liver, gut, and kidney which are necessary for survival of the newborn (Morrison et al., 2012). Compatible with our results Goland et al. (1995) showed that the mean fetal cortisol concentration was significantly higher in pregnancies complicated by uteroplacental insufficiency, than in the normotensive pregnancies (Goland et al., 1995).

The eukaryotic cell cycle is regulated by the successive activation and deactivation of a family of serine/threonine kinases composed of a positive regulatory subunit, termed cyclin, and a catalytic subunit, known as a CDK. Baisden et al. (2007) showed that dexamethasone caused a decrease in the expression of genes involved in cell division such as cyclins A2, B1, D2, CDK 2, CDK 4 and M-phase protein kinase in mouse placenta (Baisden et al., 2007). In another study done PCNA was shown to be severely reduced in the tibial growth plate of dexamethasone-induced IUGR mice (Smink et al., 2003).

According to our RT-PCR and Western blot results, cyclin D3 mRNA and protein levels were decreased in theIUGR gro p compared to control group. Since cyclin D3 takes role in transition from G1 to S phase of the cell cycle, this shows that cells of IUGR group placentas entered the cell cycle less. We also found down-regulated expression of PCNA mRNA and protein in IUGR rat placentas compared to control rat placentas. Decreased cyclin D3 and PCNA expressions may be one of the possible reasons of the lower placenta and embryo weights of IUGR rats. Several researchers reported multiple organ hyperplasia, abnormal placental development in mice when p27 and/or p57 were knocked-out (Nakayama et al., 1996; Zhang et al., 1998; Takahashi et al., 2000). p27 mRNA, but not p57 mRNA, was shown to be upregulated in the dexamethasone-induced JEG cells (Kim et al., 2005).

The expressions of p27 and p57 proteins in a cell cause cell cycle arrest leading to either differentiation or apoptosis (Zhang et al., 1997). We found up-regulated expressions of both p27 and p57 mRNA and proteins in IUGR rat placentas compared to control rat placentas. Additionally our TUNEL results showed that apoptotic cell number was higher both in junctional and labyrinth zones of IUGR rat placentas compared to the same zones of control rat placentas. Both in control and IUGR group apoptotic cell number was higher in junctional zones with respect to labyrinth zones. It seems that spongiotrophoblasts are affected much more than the labyrinth trophoblasts when Dexamethasone is injected. Dexamethasone-induction might have resulted in p27 and p57 upregulation and increased p27 and p57 expressions might have triggered the increase of apoptosis in the IUGR group. This may

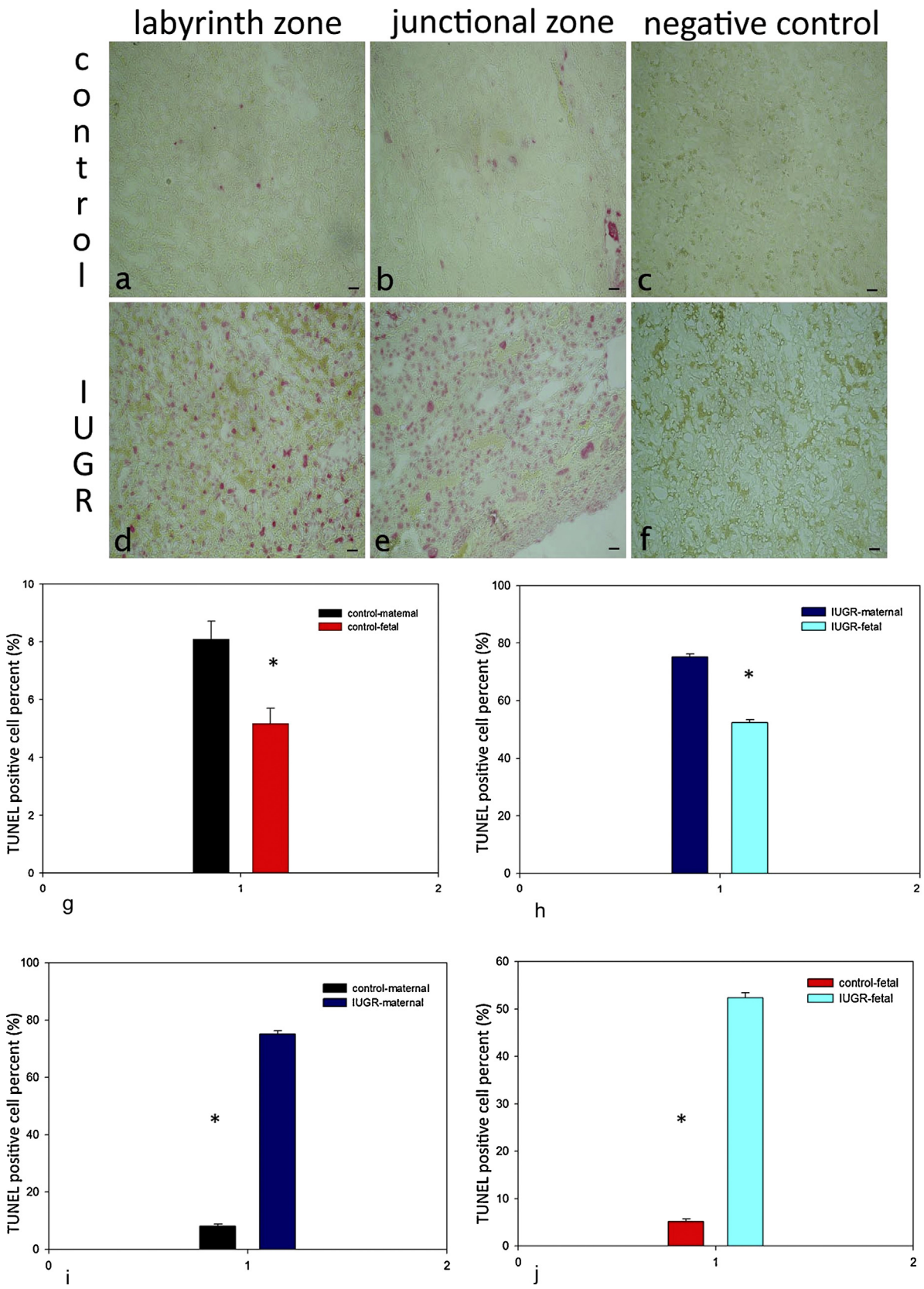


Fig. 6. TUNEL staining in control (a, b, c) and IUGR (d, e, f) group placentas. (g, h) Both control and IUGR group placentas had higher TUNEL positive cell percent in maternal compartments than in the fetal compartments with a statistically significant difference ($p < 0.001$). (i, j) TUNEL positive cell percents of maternal and fetal compartments of IUGR group were higher than maternal and fetal compartments of control group and the difference was statistically significant ($p < 0.001$). Scale bar: 10 μm.

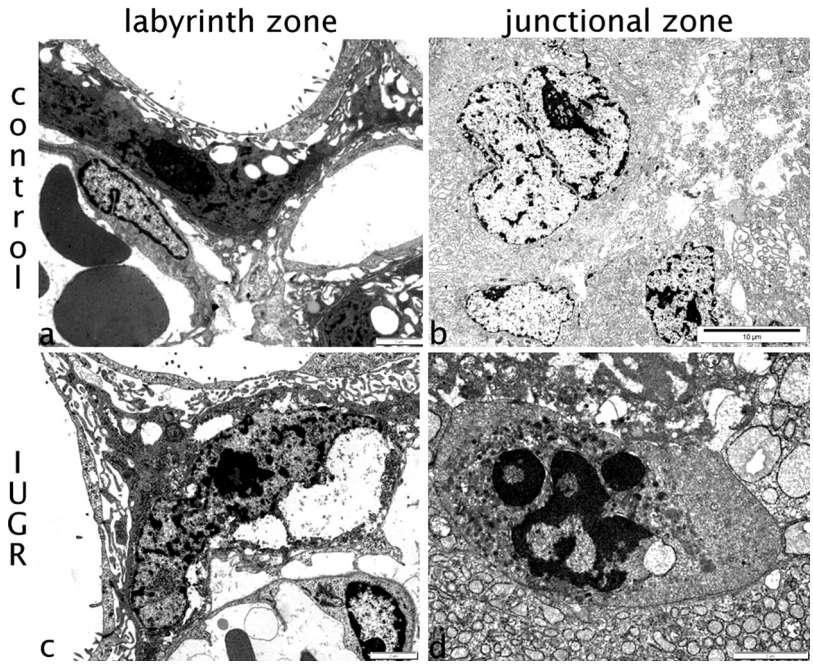


Fig. 7. Transmission electron microscope (TEM) images of control and IUGR group placentas. Compared to labyrinth (a) and junctional (b) zones of control group, IUGR labyrinth (c) and junctional (d) zones showed apoptotic cell characteristics. Scale bars: (a) 10 μm; (b–d) 2 μm.

be one of the possible reasons of the lower placenta and embryo weights of IUGR rats.

Smith et al. (1997) and Axt et al. (1999) also showed the increase of apoptosis in the placental tissue from IUGR gestation (Axt et al., 1999; Smith et al., 1997). Baisden et al. (2007) reported that antenatal dexamethasone use was associated with morphological changes in the placenta. Dexamethasone-treated placentas showed swollen trophoblast cells in both the junctional and labyrinth zones (Baisden et al., 2007), compatible with our TUNEL and TEM results.

Proper development of the placenta is dependent on the formation of trophoblasts. Trophoblast proliferation is tightly controlled, because it is important for normal proliferation, differentiation, and function of the decidual trophoblastic unit. Therefore, there must be a balance between the positive cell cycle factors and the negative cell cycle inhibitors (Zhang et al., 1997). Adequate placental growth and function are fundamental to the well-being, growth and development of the fetus throughout gestation (Waddell et al., 2000). Epidemiological data show that diabetes, hyperlipidemia, hypertensive disease and coronary vascular disease are more common among adults who were smaller than normal at birth and experienced IUGR (Hay et al., 1997). The administration of synthetic glucocorticoids is one of the most effective and important therapies in perinatal medicine. Treatment results in improved neonatal respiratory function and a decrease in morbidity and mortality (Roberts and Dalziel, 2006). However, repeated administration of glucocorticoids and its effects on fetal development and long term health remain the subject of ongoing controversy and uncertainty (Crowther and Harding, 2007; Newnham and Jobe, 2009). Dexamethasone may alter placental gene expression that controls essential developmental processes although the precise molecular mechanisms underlying malformations still remain unknown. Inappropriate exposure of the fetus to glucocorticoids has been proposed as a mechanism for 'fetal programming' with increased disease risk in later life (Seckl, 1997). Previous studies in humans have reported varying effects of antenatal glucocorticoids on birth weight and head circumference (French et al., 1999; Bloom et al., 2001; Thorp et al., 2002).

Our results might explain the relationship between the metabolism of glucocorticoids and proliferation, cell arrest or apoptosis in the placenta. Our study may provide the basis for further studies to improve our understanding of factors leading to reduced placental and fetal weight; however, more detailed studies are required to explain the molecular mechanisms of dexamethasone induced placenta and fetus weight reduction.

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