Acta Histochemica 116 (2014) 1289–1300

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and peroxiredoxin 6 (Prdx6) proteins in healthy and pathologic placentas of human and rat

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ARTICLE INFO

Article history:
Received 12 June 2014
Received in revised form 22 July 2014
Accepted 23 July 2014

Keywords:
Human
IUGR
Nrf2
PE
Placenta
Prdx6
Rat

ABSTRACT

A relationship has been shown between preeclampsia (PE) and intrauterine growth restriction (IUGR) and oxidative stress (OS). Since such pregnancies experience OS, we aimed to detect the distribution pattern and expression levels of a transcription factor, Nuclear factor erythroid 2-related factor-2 (Nrf2) that has a role in the regulation of antioxidant enzymes, and peroxiredoxin 6 (Prdx6) an antioxidant enzyme, in human healthy, IUGR, PE and in groups of rat healthy and IUGR placentas using immunohistochemistry and Western blotting. Both Nrf2 and Prdx6 immunoreactivities were weaker in human and rat IUGR group placentas compared to human and rat control group placentas, respectively. Nrf2 and Prdx6 were immunostained in labyrinth trophoblasts, decidua, giant, glycogen and fetal vessel endothelial cells in rat control and IUGR group placentas. Nrf2 and Prdx6 immunoreactivities were seen in the decidua, syncytiotrophoblasts, villous stromal cells, and vascular endothelium in human control, IUGR and PE group placentas. Results of Nrf2 and Prdx6 Western blotting applied for rat and human placentas were compatible with the results of Nrf2 and Prdx6 immunohistochemical observations with regard to rat and human placentas. Down-regulation of Nrf2 and Prdx6 proteins in human and rat IUGR group placentas may have led to the formation of OS which may have impaired proliferation and invasion of cytotrophoblasts.

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Introduction

The placenta is a regulator organ for many metabolic activities between mother and fetus. It is a critical organ affecting the outcome of pregnancy. Fetal growth is directly related to the placental development. Abnormal placental development, which is among the most important causes of early embryonic death, lies at the core of many common complications of pregnancy, such as preeclampsia (PE) and intrauterine growth restriction (IUGR) (Hemberger and Cross, 2001).

PE is a complex multisystem disorder and complicates 7–10% of all gestations (Tug et al., 2003). It presents serious risk of both maternal and fetal morbidity and mortality (Sibai et al., 2005). PE is characterized by high blood pressure and proteinuria in the second half of pregnancy (Uzan et al., 2011). Pre-term delivery, low birth weight, fetal death and neonatal death due to complications of pre-term delivery are common perinatal outcomes associated with PE (Ware-Jauregui et al., 1999). Fetal delivery is the only definitive treatment for threatening manifestations of symptoms. Whereas the pathophysiology of PE in particular remains incompletely understood, it appears to result from poor development of placental blood vessels (Redman and Sargent, 2005), which may disrupt the normal pattern of blood flow into the intervillous space, potentially risking ischemia/reperfusion injury (Burton et al., 2009). Indeed, there is general agreement that the induction of

Abbreviations: ARE, antioxidant-responsive element; Cys, cystein; Dexamethasone, dexamethasone 21-acetate; GPx, glutathione peroxidase; HO-1, hemeoxygenase 1; IUGR, intrauterine growth restriction; Keap1, Kelch-like ECH-associated protein 1; LOX-1, lectin-like oxLDL receptor-1; NAD(P)H, nicotinamide adenine dinucleotide phosphate; NQO1, NAD(P)H quinone oxido-reductase-1; Nrf2, nuclear factor erythroid 2-related factor 2; OS, oxidative stress; oxLDL, oxidized low-density lipoprotein; PBS, phosphate buffered saline; Prdx, peroxiredoxin; PE, preeclampsia; ROS, reactive oxygen species; RSA, recurrent spontaneous abortion; SOD, superoxide dismutase; γ-GCS, γ-glutamyl cysteine synthetase; 4-HNE, 4-hydroxynonenal.

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http://dx.doi.org/10.1016/j.acthis.2014.07.012
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oxidative stress (OS) is a component of PE (Hung and Burton, 2006) supported by reports of increased pro-oxidant factors (Gandley et al., 2008; Many et al., 2000) and decreased anti-oxidants in the preeclamptic placenta (Many et al., 2000).

IUGR is defined as fetal weight below 10th percentile of a given population at the same gestational age (Roman et al., 2013; van Vliet et al., 2013). Vascular development of the placenta is inadequate in IUGR, resulting in impaired uteroplacental blood flow and poor nutrient and oxygen supply to the fetus (Aimot-Macron et al., 2013). Sustained hypoxemia and under-nutrition of the developing fetus can affect fetal programming of vital organs and an increased risk for disease later in life. IUGR due to placental insufficiency occurs in 5–10% of gestations and is a major determinant of perinatal morbidity and mortality (Neerhof et al., 2012; Aimot-Macron et al., 2013; Radulescu et al., 2013; van Vliet et al., 2013).

Although the architecture of the human and rodent placentas show minor differences, 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). 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 (Liggins and Howie, 1972; Trainer, 2002). 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).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a member of the CNC (cap' n' collar) family of regulatory proteins (Ikeda et al., 2004) and is a basic leucine zipper transcription factor that is essential for the regulation of antioxidant enzymes and the phase II detoxifying response (Perez-Leal et al., 2013). It plays an important role in inhibiting OS by up-regulating the Nrf2-regulated antioxidants hemeoxygenase 1 (HO-1), NAD(P)H: quinone oxidoreductase-1 (NQO1), γ-glutamylcysteine synthetase (γ-GCS) and Peroxiredoxin 6 (Prdx6) (Chowdhury et al., 2009; Wang et al., 2013). Under non-stress conditions, newly synthesized Nrf2 is captured by Kelch-like ECH-associated protein 1 (Keap1) and constitutively degraded through the cytoplasmic ubiquitin-proteasome pathway (Kobayashi et al., 2004). The presence of excess reactive oxygen species (ROS), nitric oxide, zinc and alkenals can modify cysteine residues on Keap1, thereby inactivating it (McMahon et al., 2010; Taguchi et al., 2012). Nrf2 is consequently stabilized and can translocate into the nucleus to promote the transcription of antioxidant genes via binding to the antioxidant response element (ARE) sequence (Kobayashi et al., 2004).

Prdx6 was initially discovered in yeast as a 25-kDa enzyme that protects against oxidative damage (Chae et al., 1994). Peroxiredoxins (Prdxs) are a widely distributed superfamily of peroxidases that use thiols to reduce hydrogen peroxide, a broad range of organic hydroperoxides, and peroxynitrite. There are six mammalian Prdx isoforms: 2-Cystein (Cys) Prdx group (Prdx1–4), atypical 2-Cys Prdx group (Prdx5), and 1-Cys Prdx group (Prdx6) (Kang et al., 1998; Rhee et al., 1999; Seo et al., 2000). Prdx1, 2 and 6 are localized particularly in the cytosol, Prdx 3 is expressed mainly in mitochondria, Prdx 4 is located in endoplasmic reticulum and also secreted extracellularly and Prdx5 is located in mitochondria and peroxisomes (Paula et al., 2013). Prdx6 is a sole 1-cys enzyme with both phospholipase A2 and peroxidase activities (Ahn et al., 2013) and is a unique antioxidant enzyme that can reduce phospholipid and other hydroperoxides (Chowdhury et al., 2009). Prdx6 is the only member of the 1-Cys Prdx group and catalyzes the reduction of phospholipid hydroperoxide (Manevich et al., 2002; Pak et al., 2002).

In this study we aimed to detect the immunolocalization and expression levels of Nrf2 and Prdx6 proteins in human healthy, PE and IUGR term placentas. We also intended to determine the immunolocalization and expression levels of Nrf2 and Prdx6 proteins in rat healthy and dexamethasone-induced IUGR group placentas to be able to conceive whether dexamethasone-induced intrauterine growth restricted rat placentas reflect similar results with human IUGR placentas and is a good model for studying intrauterine growth restriction mechanism.

Materials and methods

Animal tissues

Female Wistar rats 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. Dexamethasone injection was performed based on some studies in the literature (Levitt et al., 1996; Sugden and Langdown, 2001). On day 13 of pregnancy, rats were subcutaneously injected with a dose of 100 μg dexamethasone 21-acetate (dexamethasone) (D1881; 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 10% ethanol subcutaneously on corresponding days of pregnancy. Eight 10% ethanol injected (control group) and eight dexamethasone injected (IUGR group) female Wistar rats were sacrificed on day 20 of pregnancy and placentas were obtained. 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.

Human tissues

Term placentas from healthy women were obtained immediately after cesarean section and were used as control group ($n=6$). Control women had no history of pregnancies with PE, recurrent spontaneous abortion (RSA) or IUGR. Placentas from women with PE ($n=6$) and IUGR ($n=6$) were obtained immediately after cesarean deliveries. The gestational age of control group placentas ranged from 36 to 40 weeks. The gestational age of PE and IUGR specimens ranged from 35 to 40 weeks. The samples were supplied from the Department of Obstetrics and Gynecology at Akdeniz University, Medical Faculty after informed consent of patients. The Ethical Committees of Medical Faculty of Akdeniz University approved the consent forms and protocols. Pre-eclamptic cases were defined as persistent blood pressure above 140/90 mmHg and proteinuria of more than 0.3 g/24 h or 2+ or higher according to a dipstick test, developing after 20 weeks of pregnancy. IUGR was defined as birth weight below the 10th centile of customized birth weight for gestational age (van Vliet et al., 2013). Exclusion criteria of the study were pre-existing hypertension treated with antihypertensive drugs, diabetes mellitus, gestational diabetes, renal disease, heart

disease and fetal anomalies. Only singletons were included in the study.

Tissue preparation

Healthy and pathologic human and rat placentas were dissected and either fixed for immunohistochemistry or stored in liquid nitrogen for Western blotting. The placentas were fixed in 10% formalin (100 ml 37% formaldehyde, 900 ml distilled water, pH ~ 7) at room temperature for 12–18 h. Formalin was removed by several washings with tap water. This was followed by dehydration; immersion in 70%, 80%, 90% ethanol for 24 h each and 100% ethanol for 4 h. After dehydration, tissues were cleared in xylene and embedded in paraffin wax.

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 13 min (respectively 5, 4 and 4 min) for antigen retrieval and cooled for 20 min at room temperature. Then, sections were immersed in 3% hydrogen peroxide for 15 min to block endogenous peroxidase. Slides were then incubated in a humidified chamber with UltraV block (Lab-Vision, Fremont, CA, USA) for 7 min at room temperature. Excess serum was drained and sections were incubated with primary antibodies Nrf2 rabbit polyclonal antibody (sc-722; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:250 dilution; Prdx6 rabbit polyclonal antibody (LF-PA0011; Abfrontier, Republic of Korea) at 1:300 dilution for overnight at 4 °C in a humidified chamber. Isotype controls were performed by replacing the primary antibody with the non-immune IgG in the same dilutions as the specific antibodies. Next day the sections were washed three times for 5 min with phosphate buffered saline (PBS) and then incubated with biotinylated secondary antibody (K0609, HRPLSAB-2 System; DakoCytomation, Glostrup, Denmark) for 20 min then with peroxidase labeled streptavidin (K0609, HRP LSAB-2 System; DakoCytomation, Glostrup, Denmark) for 15 min. Then slides were washed three times for 5 min with PBS and the resulting signal was developed with diaminobenzidine (DAB) (K3466; DakoCytomation, Glostrup, Denmark). Sections were counterstained with hematoxylin, dehydrated, mounted in Kaiser's glycerin gelatin (OB514196; Merck, Whitehouse Station, NJ, USA) and examined by light microscopy (Zeiss, Oberkochen, Germany).

H-SCORE and semi-quantitative evaluations

The evaluations of the Nrf2 and Prdx6 immunohistochemical stainings of rat control and IUGR group placentas were done using H-SCORE (Acar et al., 2012). Briefly, stained sections were evaluated using an Axioplan microscope (Zeiss, Oberkochen, Germany) with a special ocular scale. Three randomly selected slides, each with five different fields using x20 objective were evaluated for the analysis of immunohistochemical staining. Staining was scored in a semi-quantitative fashion that included the intensity of specific staining in sections. The evaluations were recorded as percentages of positive stained cells of all types in each of four intensity categories which were denoted as 0 (no staining), 1+ (weak but detectable above control), 2+ (distinct), 3+ (intense). The evaluation was made by two blinded observers and for each tissue, an H-SCORE value was derived by summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining [H-SCORE = S·Pi(i + 1), where i is the intensity score and Pi is the corresponding percentage of the cells]. The H-SCORE values were graphed. The distributions of Nrf2 and Prdx6 positive immunoreactive cells in healthy and pathologic human and rat placentas were determined semi-quantitatively: 0 = negative; (+) = weak positive; + = positive; ++ = strong positive; +++ = very strong positive. After evaluations were done, photographs of sections were taken with a digital camera (Diagnostic Instruments Inc., Spot Insight Qe, Sterling Heights, MI, USA) using a Zeiss Axioplan 100 light microscope (Zeiss, Oberkochen, Germany).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting

Protein extraction and immunoblot analysis were performed as described previously (Acar et al., 2012). Healthy and pathologic human and rat placenta samples 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 −80 °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 (Amersham Pharmacia, Piscataway, NJ, USA) 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 (TA 125 TW; Lab-Vision, Fremont, CA, USA) in 0.14 mol/L Tris-buffered saline (TBS; pH 7.2–7.4) at +4 °C. Membranes were incubated with a 1:2000 dilution of rabbit polyclonal anti-Nrf2 antibody (sc-722; Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:1500 dilution of rabbit polyclonal anti-Prdx6 antibody (LF-PA0011; Abfrontier, Republic of Korea) overnight. After the washing steps, the membranes were further incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (172-1019; BioRad Laboratories, Hercules, CA, USA), diluted 1:2000 for both anti-Nrf2 and Prdx6 antibodies.

Immunolabeling was visualized by using the chemiluminescence-based Super Signal CL HRP Substrate System (34080; 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 an identical protocol for binding of a 1:5000 dilution of a mouse monoclonal anti-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, MD, USA).

Table 1
Semi-quantitative scoring of Nrf2 and Prdx6 immunolabeling intensities in human control, IUGR and PE group placentas.

	Decidua	Syncytiotrophoblast	Villous stromal cells	Vascular endothelium
Nrf2				
Control	++	–	+	++
IUGR	+	–	+	(+)
PE	++	++	+	+
Prdx6				
Control	++	+++	+++	++
IUGR	+	++	++	+
PE	+++	+++	++	++

0 = negative; (+) = weak positive; + = positive; ++ = strong positive; +++ = very strong positive.

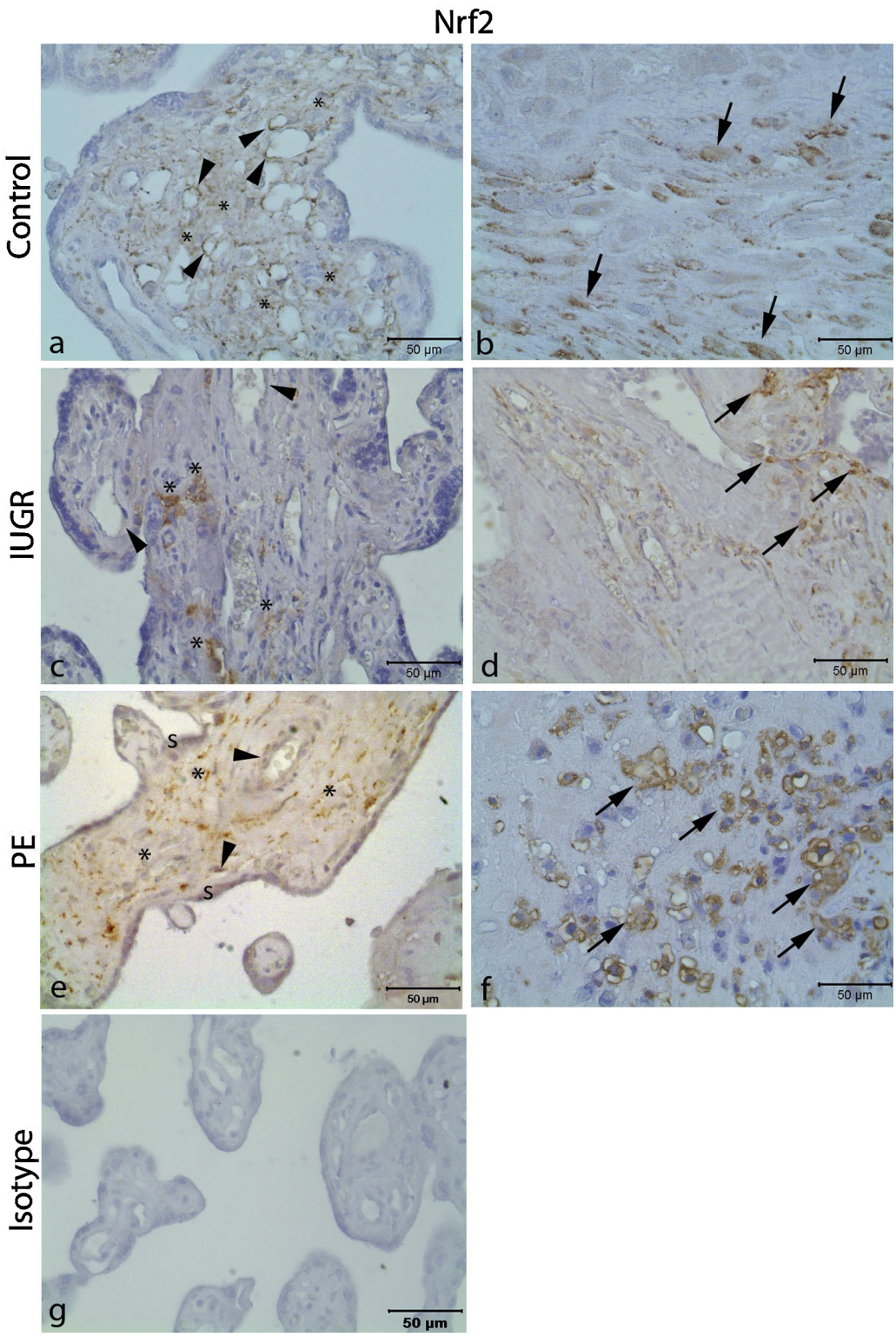


Fig. 1. Nrf2 immunostaining in human control (a, b), IUGR (c, d) and PE (e, f) group placentas. In control group placentas, Nrf2 immunoreaction was seen in vascular endothelium (arrowhead), villous stromal cells (asterisks) (a) and decidua (arrows) (b). Nrf2 immunoreaction was weaker in vascular endothelium (arrowhead), villous stromal cells (asterisks) (c) and decidua (arrow) (d) in IUGR group placentas compared to control group placentas. Unlike in control and IUGR group placentas there was Nrf2 immunoreactivity in syncytiotrophoblast of PE group placentas, generally expression intensity was slightly more intense compared to control group placentas: syncytiotrophoblast (s), vascular endothelium (arrowhead), villous stromal cells (asterisks) (e) and decidua (arrows) (f). Isotype control is seen in (g).

Statistical analysis

The following comparisons between rat control and IUGR groups were performed by Student's *t*-test: embryo and placenta weights and relative mathematical values of Prdx6 and Nrf2 H-SCORE. Nrf2 and Prdx6 Western blot band values of rat control and IUGR group placentas and human control, IUGR and PE group placentas were done by One-Way ANOVA tests followed by post

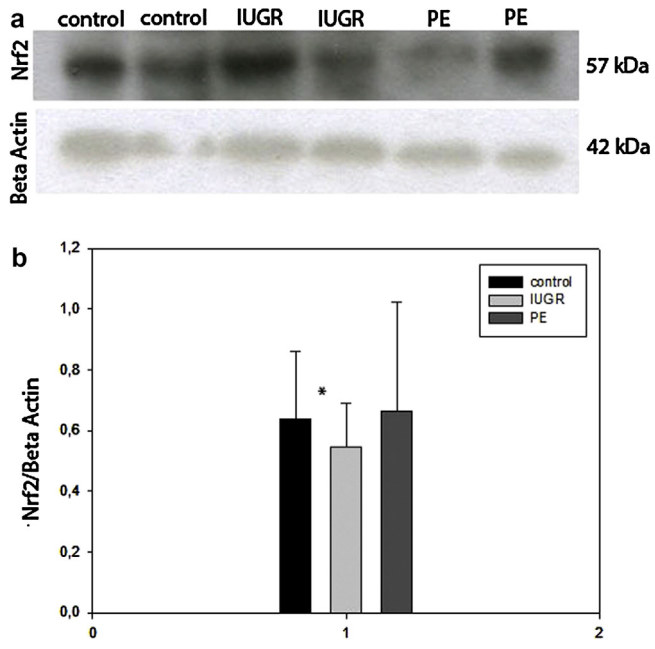


Fig. 2. (a) Western blotting results of Nrf2 in human control, IUGR and PE group placentas. Beta-actin was used as an internal control. (b) Quantitative analysis of Nrf2 Western blotting data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding beta-actin intensity value. The differences seen between control and IUGR group placentas were statistically significant (*). But the difference seen between control and PE; IUGR and PE group placentas were not statistically significant. The image is representative of three independent experiments.

hoc Tukey tests. Probability values of less than 0.05 were considered significant. All statistical analyses were performed using Sigma Stat 3.5 (Statcon, Witzenhausen, Germany).

Results

Immunohistochemical findings on human placentas

Semi-quantitative immunolabeling density distribution patterns of Nrf2 and Prdx6 on human control, IUGR and PE group placentas are shown in Table 1.

Nrf2 immunolocalization on human control, IUGR and PE group placentas

Nrf2 immunolocalization was both cytoplasmic and nuclear. In control group placentas there was no Nrf2 immunostaining on syncytiotrophoblasts, it was positive on villous stromal cells (Fig. 1a), and strongly positive on vascular endothelium (Fig. 1a), and decidua (Fig. 1b). There was a similar Nrf2 immunostaining pattern in IUGR group placentas but intensity was weaker. Nrf2 immunoreactivity was weak positive in vascular endothelium (Fig. 1c); positive in villous stromal cells (Fig. 1c) and decidua (Fig. 1d). In PE group placentas Nrf2 expression pattern was similar to control group placentas except in syncytiotrophoblasts. Although there was no Nrf2 immunoreaction in syncytiotrophoblasts of control and IUGR group placentas, there was strong positive Nrf2 immunoreactivity in syncytiotrophoblasts (Fig. 1e) of PE group placentas. Villous stromal cells and vascular endothelium (Fig. 1e) had positive and decidua (Fig. 1f) had strong positive Nrf2 immunoreactivity.

Nrf2 Western blotting on human control, IUGR and PE group placentas

Nrf2 Western blot results were compatible with Nrf2 immunohistochemical results. Nrf2 protein levels were lower in IUGR group placentas compared to control group placentas and it was statistically significant ($p = 0.036$). Nrf2 expression was slightly increased in PE group placentas compared to control and IUGR group placentas, but it was not statistically significant (Fig. 2).

Prdx6 immunolocalization on human control, IUGR and PE group placentas

Prdx6 immunolocalization was both cytoplasmic and nuclear. In the control group, Prdx6 expression was very strong positive in syncytiotrophoblasts, villous stromal cells and it was strong positive in vascular endothelium (Fig. 3a), and decidua cells showed strong Prdx6 immunoreactivity (Fig. 3b). In the IUGR group a similar Prdx6 immunostaining pattern was seen but intensity was weaker. Syncytiotrophoblasts and villous stromal cells had strong positive and vascular endothelium had positive Prdx6 immunoreactivity (Fig. 3c). Also decidua cells had positive Prdx6 immunoreactivity (Fig. 3d). PE group placentas had a similar, but a slightly more intense, immunostaining pattern than in control group placentas. It was very strong positive in syncytiotrophoblasts, and strong positive in villous stromal cells and vascular endothelium (Fig. 3e). Decidua cells had very strong positive Prdx6 immunostaining (Fig. 3f).

Prdx6 Western blotting on human control, IUGR and PE group placentas

Prdx6 Western blot results were compatible with Prdx6 immunohistochemical results. Prdx6 protein levels were lower in IUGR group placentas compared to control group placentas and it was statistically significant ($p = 0.018$). Prdx6 expression was slightly increased in PE group placentas compared to control and IUGR group placentas, but it was not statistically significant (Fig. 4).

Placenta and embryo weights of rat control and IUGR groups

When rats were sacrificed 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 ($p < 0.001$) (Fig. 5a). Mean embryo weights were lower in the IUGR group compared to the control group with statistically significant difference ($p < 0.005$) (Fig. 5b). Although embryo numbers were lower in the IUGR group compared to the control group it was not statistically significant (Fig. 5c). In conclusion, subcutaneously injected Dexamethasone caused reduction of embryo and placenta weights in the IUGR group, but did not affect embryo numbers.

Immunohistochemical findings on rat placentas

Semi-quantitative immunolabeling density distribution patterns of Nrf2 and Prdx6 on rat control and IUGR group placentas are shown in Table 2.

Nrf2 immunolocalization on rat control and IUGR group placentas

Nrf2 immunolocalization was both cytoplasmic and nuclear. In the control group Nrf2 was positive in decidua cells (Fig. 6a), weak positive in giant cells (Fig. 6b) and very strong positive in glycogen

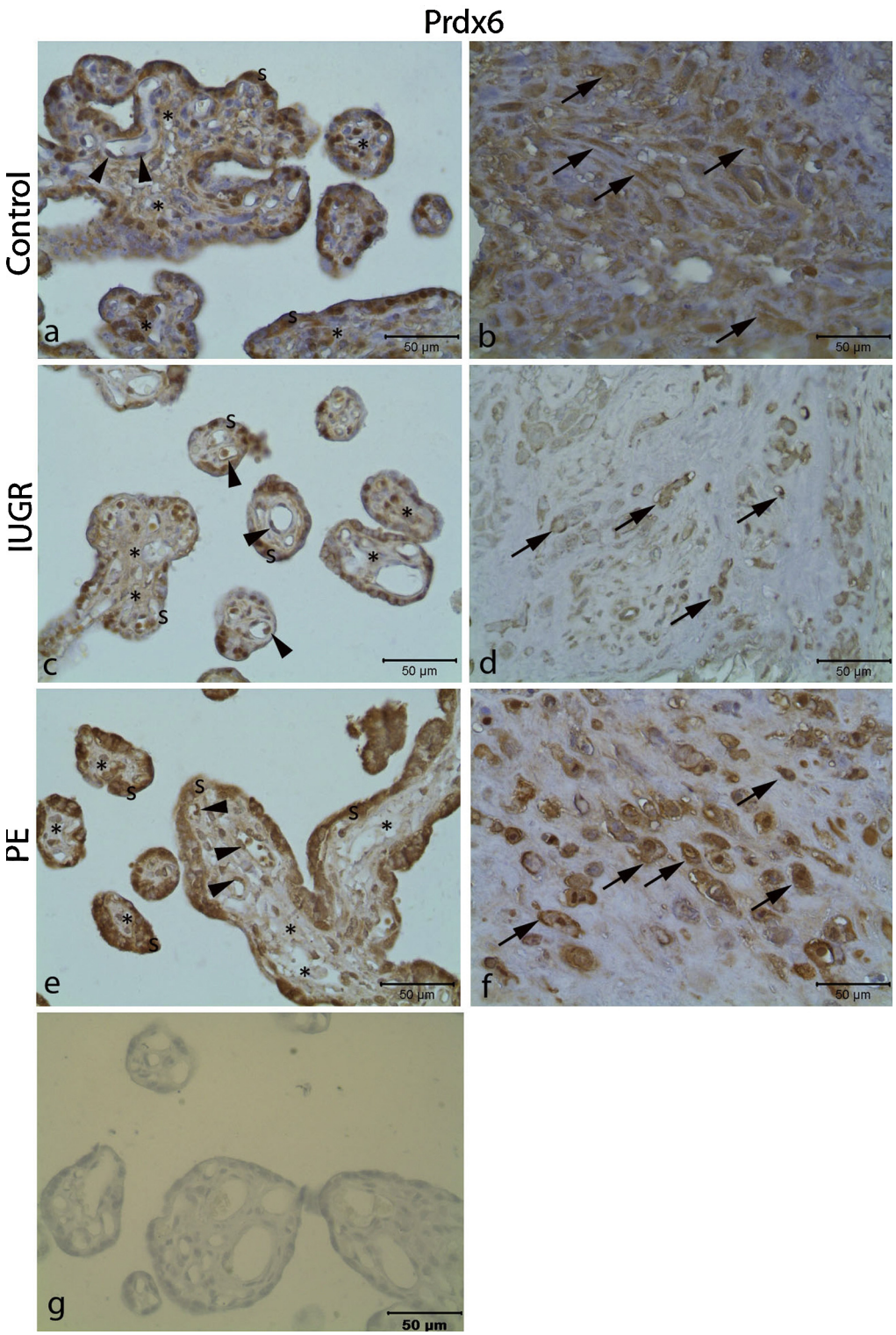


Fig. 3. Prdx6 immunostaining in human control (a, b), IUGR (c, d) and PE (e, f) group placentas. In control group placentas Prdx6 immunoreaction was seen in syncytiotrophoblast(s), vascular endothelium (arrowhead), villous stromal cells (asterisks) (a) and decidua (arrows) (b). Prdx6 immunoreaction was weaker in syncytiotrophoblast (s), vascular endothelium (arrowhead), villous stromal cells (astericks) (c) and decidua (arrow) (d) in IUGR group placentas compared to control group placentas. Prdx6 expression intensity was slightly more intense in PE group placentas compared to control group placentas, syncytiotrophoblast (s), vascular endothelium (arrowhead), villous stromal cells (asterisks) (e) and decidua (arrows) (f). Isotype control is seen in (g).

cells (Fig. 6c). Nrf2 expression was strong positive in labyrinth fetal vessel endothelial cells (Fig. 6d), positive in labyrinth trophoblasts (Fig. 6d) and spongiotrophoblasts (Fig. 6e).

Nrf2 immunolabeling intensity was lower in IUGR group placentas compared to control group placentas. Nrf2 expression was weak positive in decidua cells (Fig. 6f) and giant cells did not show any reactivity (Fig. 6g). There was a weak positive immunoreactivity in glycogen cells (Fig. 6h). Labeling intensity of Nrf2 was weak positive in labyrinth fetal vessel endothelial cells (Fig. 6i), labyrinth trophoblasts (Fig. 6i) and spongiotrophoblasts (Fig. 6j).

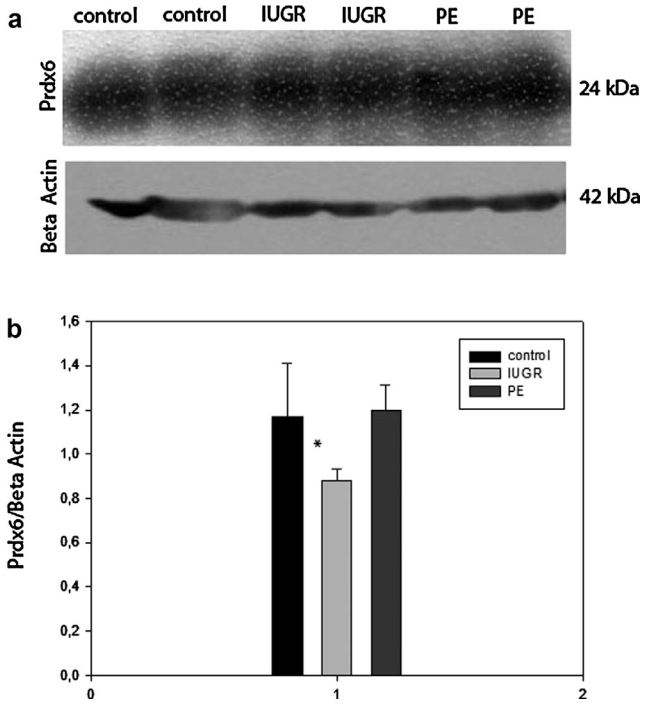


Fig. 4. (a) Western blotting results of Prdx6 in human control, IUGR and PE group placentas. Beta-actin was used as an internal control. (b) Quantitative analysis of Prdx6 Western blotting data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding beta-actin intensity value. The differences seen between control and IUGR group placentas were statistically significant (*). But the difference seen between control and PE; IUGR and PE group placentas were not statistically significant. The image is representative of three independent experiments.

When comparisons were made between control and IUGR group placentas via H-SCORE; Nrf2 expression was found to be lower in the IUGR group compared to the control group and this difference was statistically significant ($p < 0.001$) (Fig. 6k).

Nrf2 Western blotting on rat control and IUGR group placentas

Consistent with Nrf2 H-SCORE results, Nrf2 protein amount of IUGR group placentas were lower than control group placentas and this difference was statistically significant ($p = 0.029$) (Fig. 7).

Prdx6 immunolocalization on rat control and IUGR group placentas

Prdx6 immunolocalization was both cytoplasmic and nuclear. In the control group, Prdx6 reactivity was positive in decidua (Fig. 8a) and giant cells (Fig. 8b). Glycogen cells (Fig. 8c) and labyrinth fetal vessel endothelial cells (Fig. 8d) showed very strong positive reactivity. Labyrinth trophoblasts (Fig. 8d) and spongiotrophoblasts (Fig. 8e) showed strong positive immunoreactivity.

In IUGR group placentas, Prdx6 immunolabeling intensity was weaker compared to control group placentas. In the IUGR group, there was no Prdx6 reaction in decidua cells (Fig. 8f), it was weak positive in giant cells (Fig. 8g), glycogen cells (Fig. 8h), labyrinth trophoblasts (Fig. 8i), labyrinth fetal vessel endothelial cells (Fig. 8i), and spongiotrophoblasts (Fig. 8j) had positive Prdx6 immunoreactivity.

When comparisons were made between control and IUGR group placentas via H-SCORE; Prdx6 expression was found to be lower in the IUGR group and this difference was statistically significant ($p < 0.001$) (Fig. 8k).

Table 2
Semi-quantitative scoring of Nrf2 and Prdx6 immunolabeling intensities in rat control and IUGR group placentas.

	Labyrinth zone		Junctional zone			
	LFVEC	LT	ST	Gly.C	GC	Decidua
Nrf2						
Control	++	+	+	+++	(+)	+
IUGR	(+)	(+)	(+)	(+)	0	(+)
Prdx6						
Control	+++	++	++	+++	+	+
IUGR	+	+	+	+	(+)	0

LFVEC: labyrinth fetal vessel endothelial cells; LT: labyrinth trophoblasts; ST: spongiotrophoblasts; Gly.C: glycogen cells; GC: giant cells.
0 = negative; (+) = weak positive; + = positive; ++ = strong positive; +++ = very strong positive.

Prdx6 Western blotting on rat control and IUGR group placentas

Consistent with Prdx6 H-SCORE results, Prdx6 protein levels of IUGR group placentas were lower than control group placentas and this difference was statistically significant ($p = 0.01$) (Fig. 9).

Discussion

Adequate placental growth and function are fundamental to the well-being, growth and development of the fetus throughout gestation (Waddell et al., 2000). IUGR and PE share a common placental phenotype called "placental insufficiency" (Mando et al., 2014). Deficient remodeling of the uterine vessels in these placenta-mediated pregnancy complications leads to clinical maternal or fetal disorders in later pregnancy.

PE is an expression of the maternal manifestation of the disorder, while IUGR represents the fetal expression of this disease (Ness and Sibai, 2006). While the only known cure is delivery of the placenta for PE, the administration of synthetic glucocorticoids is one

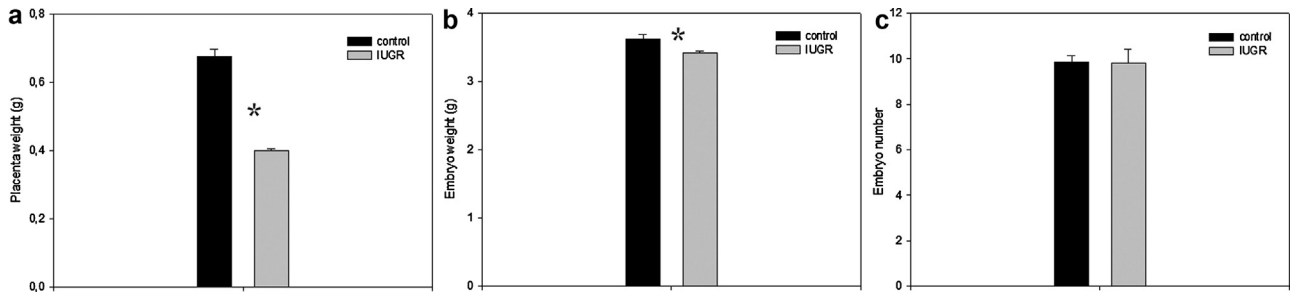


Fig. 5. Placenta and embryo weights, number of embryos for control and IUGR group rats. (a) Placenta and (b) embryo weights were lower in IUGR group and the difference was statistically significant ($p < 0.001$). (c) Embryo numbers were higher in control group compared to IUGR group but it was not statistically significant.

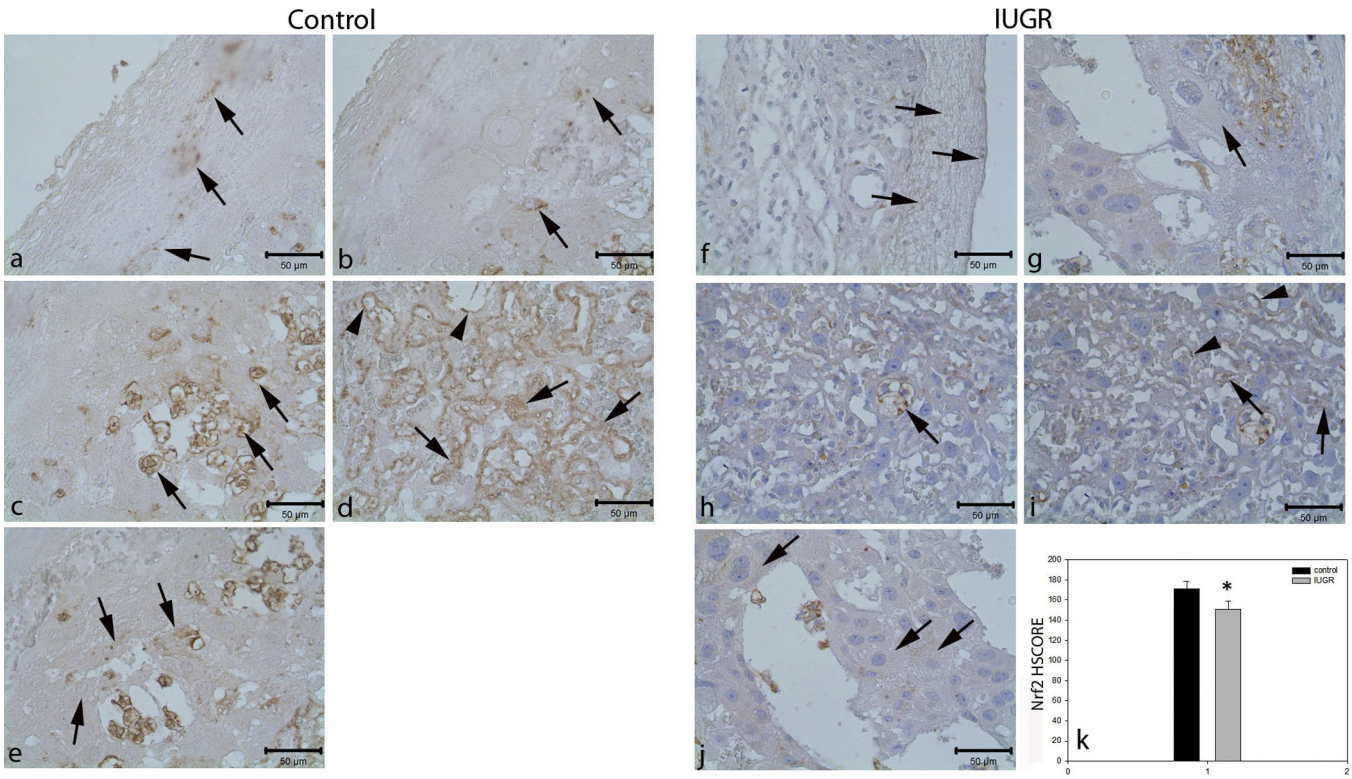


Fig. 6. Nrf2 immunostaining in rat control (a–e) and IUGR (f–j) group placentas. Nrf2 immunostaining in the IUGR group was weaker compared to the control group. In the control group decidua cells (arrows) were stained positive (a). Giant cells (arrows) were weak positive (b). Glycogen cells (arrows) were stained very strong positive (c). Labyrinth fetal vessel endothelial cells (arrowheads) and labyrinth trophoblasts (arrows) were stained strong positive and positive, respectively (d). Spongiotrophoblasts (arrows) revealed positive immunolabeling intensity (e). In the IUGR group, compared to the control group, reactivity was decreased in the decidua (f), giant (g) and glycogen cells (h), labyrinth trophoblasts and labyrinth fetal vessel endothelial cells (i), spongiotrophoblasts (j) (arrows and arrowheads). (k) H-SCORE analysis of Nrf2 in the control and IUGR group placentas.

of the most effective and important therapies in IUGR. 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 are the subject of ongoing controversy and uncertainty (Crowther and Harding, 2007; Newnham and Jobe, 2009). 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). 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).

OS is an alteration in the pro-oxidant–antioxidant balance in favor of the former that leads to potential damage. OS occurs when accumulation of ROS damages DNA, proteins and lipids, an outcome that is limited by antioxidant enzymes (Jones et al., 2010). OS is now recognized to play a central role in the pathophysiology of many different disorders, including complications of pregnancy such as PE and IUGR (Burton and Jauniaux, 2011). One source for OS is ischemia/reperfusion of the placenta by the inadequately developed spiral arterioles (Scifres and Nelson, 2009). Evidence of increased OS seen in pregnancies complicated by IUGR, and PE includes increased lipid peroxides and isoprostanes and decreased expression and activity of antioxidants (Myatt and Cui, 2004).

Nrf2 is a major OS activated transcription factor, upregulates antioxidative survival genes (Wruck et al., 2009; Kweider et al., 2012). Also, Nrf2 is a redox-sensitive transcription factor which upon activation by inflammatory stimuli, but also ROS, xenobiotics or electrophiles induces the transcription of a large set

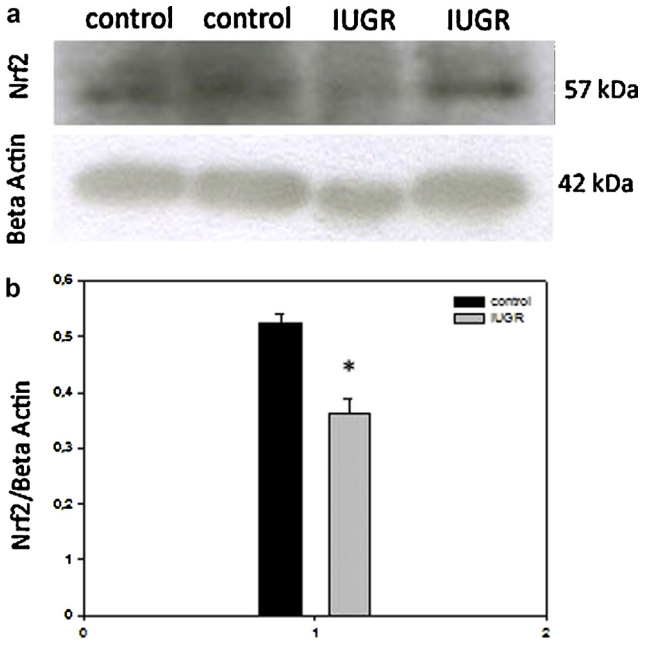


Fig. 7. (a) Western blotting results of Nrf2 in rat control and IUGR group placentas. Beta-actin was used as an internal control. (b) Quantitative analysis of Nrf2 Western blotting data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding beta-actin intensity value. The differences seen between control and IUGR group placentas were statistically significant (*). The image is representative of three independent experiments.

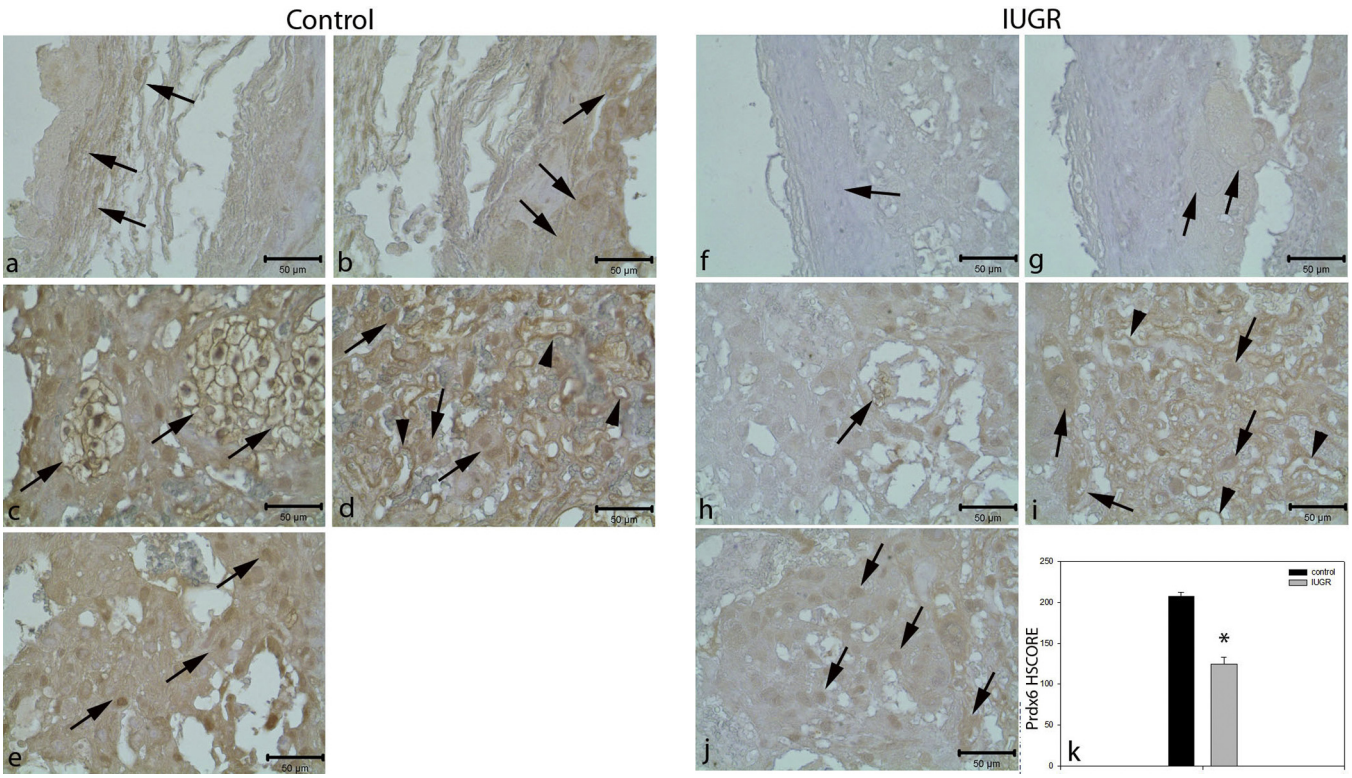


Fig. 8. Prdx6 immunostaining in rat control (a–e) and IUGR (f–j) group placentas. Prdx6 immunostaining in the IUGR group was weaker compared to the control group. In the control group, decidua cells (arrows) were stained positive (a) also giant cells (arrows) were weak positive (b). Glycogen (arrows) cells had very strong positive Prdx6 reactivity (c). Labyrinth fetal vessel endothelial cells (arrowheads) and labyrinth trophoblasts (arrows) showed very strong positive and strong positive Prdx6 immunoreactivity respectively (d). Spongiotrophoblasts (arrows) revealed strong positive immunolabeling intensity (e). In the IUGR group, compared to the control group, reactivity was decreased in decidua (f), giant (g) and glycogen cells (h), labyrinth trophoblasts and labyrinth fetal vessel endothelial cells (i), spongiotrophoblasts (j) (arrows and arrowheads). (k) H-SCORE analysis of Prdx6 in the control and IUGR group placentas.

of genes encoding various antioxidative proteins such as glutathione peroxidase 1 (encoded by GPX1), superoxide dismutase (encoded by SOD) and cytoprotective proteins [hemeoxygenase 1 (encoded by HO-1), NAD(P)H dehydrogenase, quinone 1 (encoded by NQO1), peroxiredoxin 6 (encoded by Prdx6), thioredoxin reductase 1 (encoded by TXNRD1), glucuronosyltransferase 1 family, polypeptide A1 (encoded by UGT1A1)] and thereby protects tissues from inflammatory damage and neutralizes ROS produced under pro-inflammatory conditions (Rosenbaum et al., 2012).

The results of studies identifying Nrf2 expression in normal and pre-eclamptic placentas are often contradictory. Wruck et al. (2009) found increased expression of Nrf2 in placentas of women with severe PE cases compared to control placentas (Wruck et al., 2009). Kweider et al. (2012) showed an increase of Nrf2 and 4-hydroxynonenal (4-HNE) which was produced by lipid peroxidation in placenta cells of women suffering from severe PE with IUGR compared to control ones (Kweider et al., 2012). In contrast, Chigusa et al. (2012) showed a decrease of Nrf2 expression in PE group placentas (Chigusa et al., 2012). They also showed the decreased expression of lectin-like oxLDL receptor-1 (LOX-1) that is one of the scavenger receptors for oxidized low-density lipoprotein (oxLDL) and antioxidant hemeoxygenase-1 (HO-1). Despite this, we found a slight increase in Nrf2 expression, but it was not statistically significant. It might have been caused by PE cases included in our study. The PE cases might have been mild and not as severe as the ones included in studies by Wruck et al. (2009) and Kweider et al. (2012). In our study, although there was not any Nrf2 expression in syncytiotrophoblast of control and IUGR group placentas, it might be important that there was strong positive Nrf2 expression in the PE group. The syncytiotrophoblasts in particular

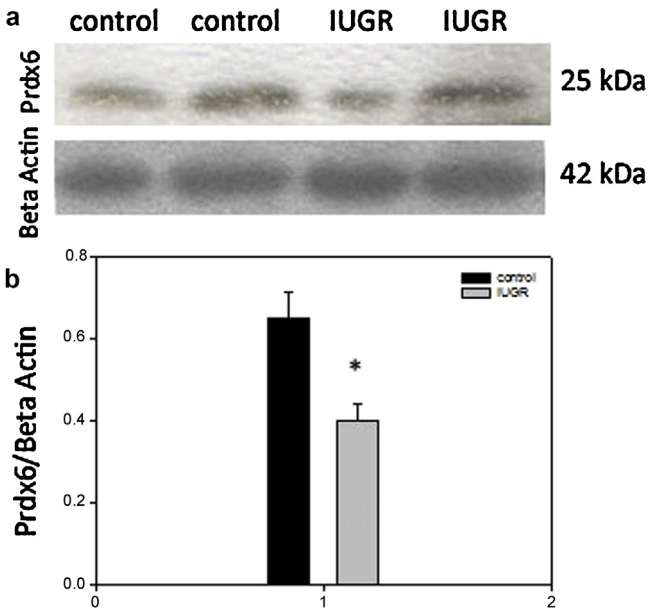


Fig. 9. (a) Western blotting results of Prdx6 in rat control and IUGR group placentas. Beta-actin was used as an internal control. (b) Quantitative analysis of Prdx6 Western blotting data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding beta-actin intensity value. The differences seen between control and IUGR group placentas were statistically significant (*). The image is representative of three independent experiments.

are sensitive to OS, partly because of their location in the outermost layer of placental villi, which are exposed to high oxygen concentrations (Perkins, 2006). Appearance of Nrf2 expression in syncytiotrophoblasts might be an adaptation to OS.

As far as we know, no study examined the expression of Nrf2 in human IUGR group placentas. We found that in IUGR group placentas Nrf2 expression was weaker compared to control and PE group placentas. Since IUGR is characterized by impaired invasion of the extravillous trophoblast, this limited invasion might have been caused by Nrf2 down-regulation.

Prdx6 is a unique non-redundant antioxidant that acts independently of other Prdxs and antioxidant enzymes. A study has shown that Prdx6-deficient (Prdx6−/−) mice have normal development, but paraquat-induced OS causes abnormal phenotypes such as lower survival rate, not resulting from changes in gene expression and/or activity of other antioxidant enzymes (Wang et al., 2003). Thus, Prdx6 is an important antioxidant against exogenous OS. Hirota et al. (2010) showed strong expression of Prdx6 in decidua and placenta in wild type mice and this suggests that decidual and placental Prdx6 has an antioxidant function and protects embryos against increased oxygen tension. To the best of our knowledge, this is the first study which shows Prdx6 expression in human and rat placentas. In parallel with Nrf2 expression, Prdx6 expression was weaker in IUGR group placentas compared to control and PE group placentas. Inadequate Nrf2 and Prdx6 expressions in human IUGR group placentas might be one of the possible mechanisms involved in IUGR pathogenesis. Insufficient production of Nrf2 and Prdx6 might have led to the formation of OS and OS might have prevented trophoblast invasion.

Our dexamethasone induced IUGR rat model enabled us to compare whether it is a good model to mimic intrauterine growth restriction in human. We had the possibility to compare the results we obtained from human IUGR group placentas and rat dexamethasone-induced IUGR group placentas. Our Nrf2 and Prdx6 results in the rat placenta were compatible with the human placenta. Maternal dexamethasone treatment was used as a model of glucocorticoid-induced IUGR. How dexamethasone inhibits placental growth is still unknown. The impact of glucocorticoid excess on placental oxidative stress is of particular interest since previous studies show that dexamethasone increases plasma F2-isoprostane concentrations in non pregnant rats (Zhang et al., 2004). This growth inhibition could be associated with imbalance between ROS and antioxidants. Balance between ROS and antioxidants is crucial for homeostasis of an organism. Since there are limited data about the effects of dexamethasone on OS, we investigated stress-activated transcription factor Nrf2 and antioxidant protein Prdx6 in control and dexamethasone induced growth retarded placentas in addition to human control, IUGR and PE group placentas. Both Nrf2 and Prdx6 expressions were weaker in rat IUGR group placentas compared to control group placentas according to our immunohistochemical and Western blotting results.

Dexamethasone is known to increase OS in various types of cells and tissues (Rajashree and Puvanakrishnan, 1998; Iuchi et al., 2003; Almeida et al., 2011). Lin et al. (2013) showed increase of Nrf2 expression via RT-PCR and Western blot in dexamethasone induced IUGR rat placentas (Lin et al., 2013). They found that superoxide dismutase (SOD) and glutathione peroxidase (GPx) gene expressions were increased while SOD and GPx activity were decreased after dexamethasone treatment. Although Lin et al. (2013) found increase of Nrf2 in rat IUGR group placentas we found a decrease. Since we used the same dexamethasone treatment protocol this inconsistency might have been caused by different rat strains. We used Wistar rats and Lin et al. (2013) used Sprague-Dawley strain rats. A failure of extravillous trophoblast invasion is correlated with the pathophysiology of IUGR (Cozzi et al., 2012). Insufficient Nrf2 and Prdx6 expressions in rat IUGR group placentas might have caused an increase in OS and this might have triggered inadequate trophoblast invasion. As a result a reduction in fetal and placental weights at term might have been triggered.

This study demonstrates the expressions of regulator of cellular resistance to oxidants – Nrf2 and antioxidant – Prdx6 proteins in healthy and pathologic rat and human term placentas. Both human and rat IUGR group placentas had lower expression levels of Nrf2 and Prdx6 compared to the control group placentas, this insufficiency might have led to impair proliferation and invasion of cytotrophoblasts. Our study may provide the basis for further studies to improve our understanding of factors leading to inadequate trophoblast invasion and increased OS, however, more detailed studies are required to explain the molecular mechanisms involved.

Acknowledgement

The authors would like to thank Histology and Embryology technician Sibel Ozden-Ozer for her technical assistance.

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