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Increased placental expressions of nuclear factor erythroid 2–related factor 2 and antioxidant enzymes in gestational diabetes: Protective mechanisms against the placental oxidative stress?

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ABSTRACT

Objective: Gestational diabetes mellitus is associated with increased oxidative stress. Oxidative stress may contribute to the risk for pregnancy pathologies associated with gestational diabetes mellitus. In this study we investigated the expression of placental nuclear factor erythroid 2–related factor 2 (Nrf2) and antioxidant enzymes of gestational diabetes mellitus and healthy pregnant women and correlated them with the maternal and cord plasma as well as placental tissue oxidative stress parameters.

Study design: A cross sectional study was carried out in a South Indian Tamil population. Forty healthy pregnant women and forty gestational diabetes mellitus patients in the gestational age of 32 ± 4weeks were recruited. Maternal plasma, cord plasma and placental oxidative stress parameters were measured. Placental expression of Nrf2, phospho Nrf2, catalase and superoxide dismutase 1(SOD1) were analyzed by western blotting and immunohistochemistry.

Results: Placental expression of Nrf2, catalase and SOD1 were found to be significantly higher in gestational diabetes mellitus. The maternal plasma, cord plasma and placental tissue oxidative stress parameters, total antioxidant status (TAS) levels were significantly lower; whereas MDA (malondialdehyde) and MDA/TAS levels were significantly higher in gestational diabetes mellitus. Placental Nrf2 expression correlated positively with the placental catalase expression and negatively with placental TAS levels in both groups.

Conclusion: Maternal, fetal and placental oxidative stress was observed in gestational diabetes mellitus. The gestational diabetic placenta had an increased Nrf2 protein expression. The activated placental Nrf2/ antioxidant response element (ARE) pathway might have led to an increased expression of antioxidant enzymes SOD1 and catalase. This may be viewed as a protective mechanism in placenta from the further onslaught of oxidative stress.

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Introduction

Gestational diabetes mellitus is defined as a condition where glucose intolerance is first recognized during pregnancy. It complicates about 2–4% of pregnancies. Prevalence of gestational diabetes mellitus in India is about 16% [1]. It increases the risk of developing type 2 diabetes mellitus and cardiovascular complications in both mothers and their offspring later in life. Fetuses of pregnant women with maternal diabetes are at an increased risk for developing congenital anomalies including phocomelia, cardiac malformations, macrosomia and central nervous system malformations [2,3].

Reactive oxygen species (ROS) in pregnancy is required for normal embryonic and fetal development. Increased and sustained ROS production affects placental function and fetal growth, resulting in priming of future diseases in the offspring [4,5]. The increase in ROS, together with the impaired antioxidant activity are related to the induction of congenital malformations in

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pre-gestational diabetic pregnancies [6]. Increased oxidative stress and an impairment of antioxidant defense have been reported in maternal and cord plasma of women with gestational diabetes mellitus [7–9]. In gestational diabetic placenta, there is an increased oxidative stress compared with healthy pregnant women [10]. However, the simultaneous increase in antioxidant enzyme activities compensates for the increased placental oxidative stress [11–13].

Nuclear factor erythroid 2–related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) pathway plays a crucial role in transcriptional activation of antioxidant defence genes and restoration of redox homeostasis. Nrf2 is kept as an inactive complex in the cytoplasm by a repressor molecule, Keap1. However in response to oxidative stress, Nrf2 separates from Keap1 and is translocated into the nucleus for binding to the antioxidant response element (ARE) [14]. The activated Nrf2/ARE pathway leads to induction of numerous genes encoding antioxidant and phase-2 detoxifying enzymes and related proteins, such as superoxide dismutases, catalase, UDP-glucuronosyltransferase, heme oxygenase- 1, glutamate cysteine ligase, glutathione S-transferase, and thioredoxin [15]. In this role, Nrf2/ARE activation is one of the main defense mechanisms against oxidative stress in cells and tissue.

Dysregulation of Nrf2 has been shown to be involved in the etiology of diabetes and its complications [16–18]. Placenta of women with pre-existing type 2 diabetes showed a decreased expression of Nrf2 and thereby decreasing the expression of hemoxygnase-1 [19]. The placenta of gestational diabetic rats and pre gestational diabetic mouse showed an increased Nrf2 expression [20,21]. Increased Nrf2 expression has protective role in diabetic cardiomyopathy [22] and diabetic nephropathy [23]. Increased catalase expression has shown protective effects against the maternal diabetes induced perinatal programming of renal disease and hypertension [24]. Overexpression of Cu-Zn SOD1 in mice reduces the occurrence of fetal abnormalities and protects against diabetes-associated embryopathy [25].

We hypothesised that increased Nrf2 expression in placenta of gestational diabetic women has protective effect against the oxidative damage caused by the maternal hyperglycaemia. The present study was designed to investigate the expression of placental Nrf2 and antioxidant enzymes in gestational diabetes mellitus and healthy pregnant women and to correlate them with the maternal and cord plasma as well as placental tissue oxidative stress parameters.

Materials and methods

Subjects

The study was approved (JIP/IEC/2016/25/825) by the Institute ethics committee (Human), JIPMER hospital, Puducherry, one of the premier tertiary care centres in South India. The study subjects were recruited from the Department of Obstetrics and Gynaecology, Women's and Child Health block of the institute. A total of 80 primi pregnant women were recruited for this cross-sectional study. Women with gestational diabetes mellituswere diagnosed based on the International Association of Diabetes and Pregnancy Study Groups criteria [26]. Primi gravida pregnant women with gestational diabetes mellitus on insulin therapy (n = 40) and healthy pregnant women without any complications (n = 40) of age group 18–30 were included in the present study. Women with Gestational diabetes mellitus who were only on medical nutrition therapy, hypothyroidism, pre-existing glucose intolerance, pregnancy-induced hypertension, polycystic ovarian syndrome, major vascular complications, known infections in current pregnancy and autoimmune disorders were excluded from the study. Maternal age at delivery, pre-pregnancy body mass index (BMI), maternal weight gain, gestational age at delivery, birth weight and length, Apgar scores were noted. Ponderal Index is calculated by the formula: Ponderal Index = Birth weight (g)/Birth length3 (cm3) x 100.

Collection of blood samples

After obtaining an informed consent from all the study subjects, 5 ml of fasting maternal venous blood was collected in the third trimester at 32 ± 4 weeks of pregnancy. Umbilical cord blood was collected at delivery. Plasma was separated by centrifugation at 3500 rpm for 10 min and stored immediately at $-40\,^{\circ}C$ until analysis.

Collection of placental tissues

Human placentae were obtained from a total of 24 pregnant women (11 placentas from healthy pregnant women and 13 placentas from gestational diabetic women) who delivered healthy, singleton infants at term undergoing elective Caesarean section. Placentas were obtained within 10 min of delivery. A placental lobule (cotyledon) was removed from the central region of the placenta. The basal plate and chorionic surface were removed from the cotyledon. Visible connective tissue and calcium deposits were removed, blotted dry on filter paper, snap frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until further analysis.

Measurement of maternal and cord blood oxidative stress parameters

Estimation of malondialdehyde

Plasma MDA was measured by the method of Yagi, et al [27]. The assay was based on the reaction of TBARS mainly MDA with thiobarbituric acid (TABA) to form a pink colored product. The intensity of the color was measured at 535 nm which is directly proportional to the MDA concentration in the sample. The results are expressed in terms of μM/L.

Estimation of Total antioxidant status

Total antioxidant status in plasmawas measured by commercially available kits (Bioassay systems, Hayward, CA) in which the CU^{++} is reduced by antioxidant to CU^{+}. The resulting Cu^{+} specifically forms a colored complex with a dye reagent. The color intensity is measured at 570 nm by microplate reader (SpectraMaxPlus 384, Molecular devices, San Jose, CA), which is direcly proportional to TAS in the sample. The results were expressed in terms of μM/L. The index of oxidative stress was calculated by using the formula MDA/ TAS [28,29].

Measurement of placental tissue oxidative stress parameters

For the estimation of placental oxidative stress parameters such as MDA and TAS, the tissues were homogenized in phosphate buffered saline (PBS, pH 7.6). The homogenate was then centrifuged at 4 °C for 20 min at 11,000 rpm. From the supernatant, MDA and TAS were measured and the values were expressed as μmol/mg of protein.

Western blot analysis

The snap-frozen placental tissue was homogenized with a radio immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) containing the protease inhibitor cocktail (Sigma-Aldrich). Samples containing equal amounts of protein were loaded and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE) (Mini-Protein II System, Bio-Rad, Hercules, CA, USA). The separated proteins were then transferred onto the nitrocellulose membrane/ PVDF membrane (Sigma-Aldrich, St. Louis, MO, USA) using Trans SD semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% bovine serum albumin (BSA) or 5% non-fat dry milk. Then, it was incubated with the primary antibodies of rabbit anti-Nrf2 antibody (1:1000), rabbit anti-phospho Nrf2 antibody (1:5000), rabbit anti-Superoxide dismutase -1 antibody (1:2000) (Abcam, Cambridge, MA, USA) and rabbit anti-catalase antibody (0.5 μg/ml) (Novus Biologicals USA) at 4 °C overnight. Subsequently, the membranes were treated with goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The enhanced chemiluminescence reagent mixture was used to detect the signal (Thermo Fischer Scientific, Waltham, MA, USA). The expressed bands were imaged using the ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA), and the signals were quantified using Image Lab software version 5.1 (Bio-Rad, Hercules, CA, USA).

Assessment of syncytial knots

Placental tissues were fixed in neutral phosphate-buffered 4% formaldehyde solution and then embedded in paraffin. Sections (5 mm) were prepared from wax-embedded tissue. Following deparaffinisation, the sections were counterstained with haematoxylin and eosin. Using a 20 X objective, the number of syncytial knots was counted manually in 10 fields per placental tissue [30].

Immunohistochemistry

For immunohistochemical analyses, serial sections (4 μm) were cut, mounted on silane coated glass slides and incubated at 65 °C for 30 min. Sections were deparaffinised with xylene and rehydrated with a graded series of ethanol. Then slides were immersed in citrate buffer (pH 6.0, 10 mM) and heated in a microwave oven for antigen retrieval. Endogenous peroxidases were inactivated by incubation in H_2O_2. Non-specific binding sites were blocked (2.5% normal horse serum) for 20 min. The sections were incubated for 1 h in primary antibody (Nrf2 at 1:100 dilution, SOD1 at 2 μg / ml dilution and catalase at 5 μg / ml dilution) diluted in 2.5% normal horse serum. Secondary staining at a dilution of 1:2500 was then added for a period of 30 min at room temperature. DAB chromogen (Vector Laboratories, Burlingame, CA) was added for 30 s at room temperature. Sections were finally stained with hematoxylin, mounted and dried ready for viewing. Rabbit IgG were used as negative controls.

Statistical analysis

All statistical analyses were done using Statistical Package of Social Service (SPSS) version 19.0 software for Windows. Results are shown in mean ± S.D. The results were analyzed by unpaired t-test. Pearson's correlation was employed to assess the correlation between the parameters. A p-value less than 0.05 was considered as statistically significant.

Results

Maternal and neonatal characteristics

Tables 1 and 2 shows maternal and neonatal characteristics of all participants involved in this study. All the 80 women enrolled were primi gravida.There was no significant difference in the mean age, pre-pregnancy body mass index (BMI), maternal weight gain, Apgar Scores between the groups. However, birth weight, birth length and ponderal index of the baby was significantly higher ($P < 0.05$) among gestational diabetic women in comparison with healthy pregnant women.The mean gestational age at delivery for the two groups was 39.36 ± 1.05 and 38.52 ± 1.06 respectively which was not significantly different. Insulin therapy was started if sugars were not controlled with medical nutrition therapy. The mean insulin requirement at term was 20.45 ± 7.53. Most of these women were induced at 38 week of pregnancy if they did not have spontaneous labor.

Table 1
Maternal characteristics of the study groups.

Characteristics	Controls (n = 40)	Gestational diabetes mellitus (n = 40)
Maternal age (years)	24.26 ± 2.17	24.14 ± 2.46
Prepregnancy BMI (kg/m2)	22.19 ± 1.00	22.14 ± 2.58
Maternal weight gain (kg)	9.95 ± 0.97	10.38 ± 0.86
Gestational age at diagnosis	NA	24.82 ± 3.60
Gestational age at delivery (weeks)	39.36 ± 1.05	38.52 ± 1.06
Type of labor(n (%)		
Spontaneous	25 (62.5%)	09 (22.5%)*
Induced	04 (10%)	18 (45%)*
Mode of delivery(n (%)		
Spontaneous vaginal delivery	27 (67.5.5%)	23 (57.5%)
Instrumental delivery	02 (5%)	04 (10%)
Caesarean section	11 (27.5%)	13(32.5%)
Fasting blood glucose (mM)	3.82 ± 0.31	5.46 ± 0.28*
Mean insulin requirement at term	NA	20.45 ± 7.53

* $p < 0.05$, considered as statistically significant. Data are expressed as mean ± S. D. BMI - body mass index.

Table 2
Neonatal characteristics of the study groups.

Characteristics	Controls (n = 40)	Gestational diabetes mellitus (n = 40)
Birth weight (g)	2888 ± 449	3158 ± 465*
Birth length (cm)	46.2 ± 0.5	48.6 ± 0.4*
Ponderal index (g/cm3)	2.71 ± 0.37	3.04 ± 0.47*
Apgar 5' score	8.11 ± 0.32	7.90 ± 0.54
Apgar 10' score	9.11 ± 0.32	8.95 ± 0.38
AGA newborn (n (%))	38 (95%)	33 (82.5%)
LGA newborn (n (%))	02(5%)	07(17.5%)
Admission to NICU	00	02 (5%)
Birth asphyxia	00	00
Respiratory distress syndrome	00	00
Neonatal hypoglycaemia (n (%))	01(2.5%)	05(12.5%)
Fetal mortality	00	00

* $p < 0.05$, considered as statistically significant. Data are expressed as mean ± S. D. AGA – adequate for gestational age; LGA- large for gestational age; NICU – neonatal intensive care unit.

Maternal, cord plasma and placental oxidative stress parameters

Total antioxidant status in maternal plasma, cord plasma and placental tissues showed a significant decrease in patients with gestational diabetic women. When compared with healthy pregnant women, maternal plasma, cord plasma and placental tissue MDA levels of gestational diabetic women were significantly increased. The MDA/TAS ratio indicates the oxidant status, which was found to be higher in gestational diabetic women compared with the healthy pregnant women in maternal plasma, cord plasma and in placental tissues. The results are shown in Table 3.

Table 3
Maternal, cord blood and placental tissue oxidative stress parameters of the study groups.

Parameters	Controls (n = 40)	Gestational diabetes mellitus (n = 40)
Maternal blood		
MDA (μMol/L)	113.30 ± 37.33	148.30 ± 24.94*
TAS (μMol/L)	256.91 ± 38.91	174.83 ± 34.23*
MDA/TAS ratio	0.63 ± 0.27	0.86 ± 0.22*
Cord blood		
MDA (μMol/L)	30.72 ± 14.62	48.50 ± 11.53*
TAS (μMol/L)	209.46 ± 51.36	174.28 ± 52.30*
MDA/TAS ratio	0.16 ± 0.03	0.27 ± 0.17*
Placental tissue	**Controls (n = 11)**	**Gestational diabetes mellitus (n = 13)**
MDA (μMol/mg of protein)	0.63 ± 0.11	1.07 ± 0.27*
TAS (μMol/mg of protein)	10.62 ± 3.19	5.52 ± 1.86*
MDA/TAS ratio	0.06 ± 0.02	0.21 ± 0.07*

* $p < 0.05$, considered as statistically significant. Data are expressed as mean ± S. D. MDA – Malondialdehyde; TAS – Total anti-oxidant status.

Assessment of syncytial knots

Hematoxylin and eosin staining of gestational diabetic placenta (Fig. 2) showed a markedly increased number of syncytial clumps or knot on the villous surface. A syncytial knot was defined as a multi-layered aggregation of at least 10 syncytiotrophoblast nuclei protruding from the villous surface that was not in direct contact with adjacent villi [33].

Protein expression of Nrf2 and p-Nrf2 in human full-term placentas

The placental expression of Nrf2 was significantly higher in gestational diabetes mellitus compared to healthy pregnant women (Figs. 1). However the p-Nrf2 expression did not differ between the groups (Fig. 1B). Immunohistochemical staining of Nrf2 displayed intense staining in the syncytiotrophoblast layer of the gestational diabetic placenta. No staining was observed in the stromal villi and fetal blood vessels (Fig. 3B).

Protein expression of catalase in human full-term placentas

The placental expression of catalase (Fig. 1C) was significantly higher among patients with gestational diabetes mellitus when compared with healthy pregnant women. Immunohistochemical staining of catalase displayed intense staining in the syncytiotrophoblast layer of the gestational diabetic placenta. (Fig. 3D).

Protein expression of SOD1 in human full-term placentas

The placental expression of SOD1 (Fig. 1D) was significantly higher in gestational diabetes mellitus compared to healthy pregnant women (Fig. 1D). Immunohistochemical staining of SOD1 displayed intense staining in the syncytiotrophoblast layer of the gestational diabetic placenta. Minimal staining was observed in the stromal villi. (Fig. 3F)

Correlation of placental Nrf2 protein expression with oxidative stress parameters and antioxidant enzymes

The correlation analysis was carried out with the both groups. The placental TAS levels negatively correlated with Nrf2 expression ($r = -0.524$, $p < 0.037$) and catalase expression ($r = -0.788$, $p < 0.020$). Placental Nrf2 expression positively correlated with expression of catalase and birth weight of baby (Table 4). Placental catalase expression is positively correlated with maternal TAS levels ($r = 0.714$, $p < 0.042$) whereas negatively correlated with cord TAS levels ($r = -0.786$, $p < 0.036$).

Comment

Normal human pregnancy is associated with a state of enhanced oxidative stress; it plays central roles in embryo development, implantation, placental development and function, fetal development [4,31]. However, pathologic pregnancies, including gestational diabetes mellitus are associated with an

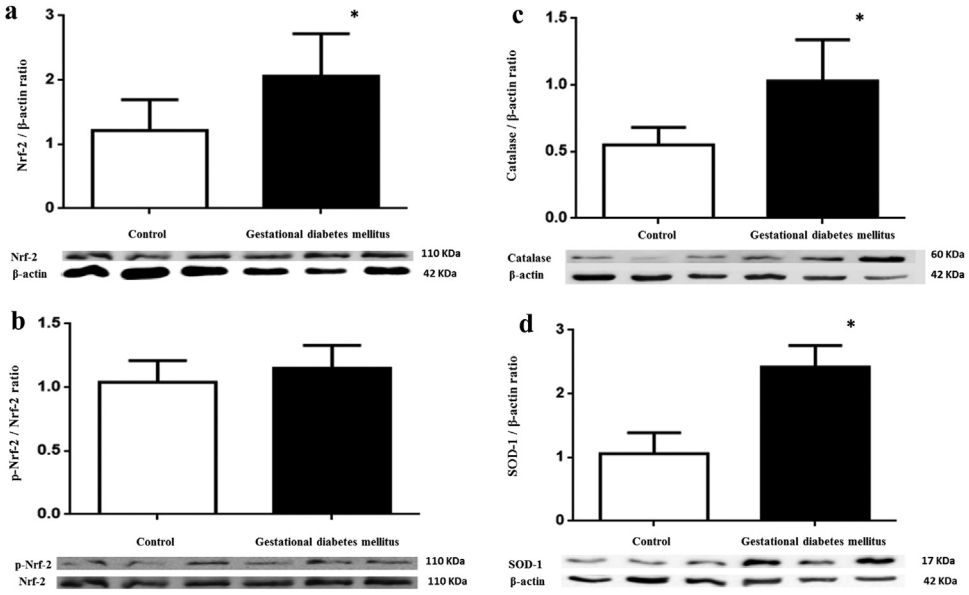

Fig. 1. Western blot analysis of A) Nrf-2 B) p-Nrf-2 C) catalase D) SOD1 in placental tissues collected from healthy control (n = 11) and gestational diabetic pregnant women (n = 13). Data were expressed as mean ± S.D. *$p < 0.05$ considered as statistically significant. A representative of n = 3 from healthy pregnant women and gestational diabetic pregnant women are represented in the figure.\

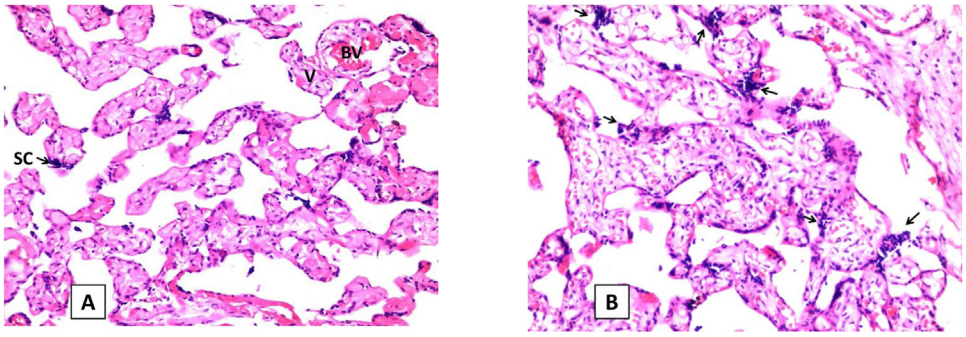

Fig. 2. A photomicrograph of H &E stained section of full-term placenta. A) Control placenta B) Gestational diabetic placenta showing: V – villous; BV – blood vessel; SC – syncytial clumps or knots. Gestational diabetic placenta shows plenty of syncytial knots (arrowhead) on the surfaces of villi, Images were captured using Olympus BX 43 bright-field microscope with 20 × magnification.

increased level of oxidative stress [32]. This increased oxidative stress is related to the pathological complications of the mother and new born; in addition to affecting placental function. Increased ROS is suggested to induce congenital anomalies including phocomelia, cardiac malformations, and central nervous system malformations [2,3]. Further, it involved in the fetal programming for future cardiovascular complications and diabetes in the offspring of diabetic mothers.

In the present study, we evaluated the oxidative stress parameters in maternal, fetal and placental compartments of gestational diabetes mellitus and healthy pregnant women. Recent studies have shown that there was an increased maternal plasma, cord plasma, and placental MDA, whereas decreased maternal plasma, cord plasma, and placental antioxidant potential in gestational diabetes mellitus [9]. In our study, we found an increased maternal plasma, cord plasma and placental tissue oxidative stress in gestational diabetic patients. This is reflected by elevated levels of MDA and MDA/TAS ratio, and a decreased TAS levels in maternal plasma, cord plasma and placental tissue of gestational diabetic women.

In gestational diabetes mellitus, altered glucose tolerance and insulin resistance results in hyperglycemia. Hyperglycaemia is directly implicated in the formation of free radicals by various pathways, which leads to oxidative stress in gestational diabetes mellitus [32]. MDA is a marker of lipid peroxidation and its level has been found to positively correlate with blood glucose concentration [33]. Lappas et al, found that gestational diabetic placenta is less responsive to exogenous oxidative stress than tissues obtained from normal pregnant women. They also found that gestational diabetic placenta is characterised by increased antioxidant gene expression [10]. Thus, gestational diabetic placenta is better adapted to the maternal ROS and its oxidative challenge. Recent studies have shown that oxidative stress could cause damage to developing fetus. It induces congenital anomalies in diabetic pregnancy. Cord blood of gestational diabetic mother shows increased oxidative stress. In utero developmental priming under oxidative stress increases the risk of disease in offspring. In utero exposure to first onset of gestational diabetes mellitus during pregnancy associated with an increased risk of vascular disease and/or insulin resistance in mother and child in later life. In particular, studies report that obesity in offspringgestational diabetic women has a strong association with development of insulin resistance [34,35].

In gestational diabetes mellitus, excessive ROS production could cause changes at the micro-anatomical and molecular level in placental tissues. Syncytial knots are aggregations of syncytiotrophoblast nuclei protruded to surface of villi. Nuclei contained within syncytial knots show features of advanced degeneration, with pyknosis, peripheral chromatin condensation and fused nuclear membranes [36]. These features are similar to those described in apoptosis and these nuclei are transcriptionally inactive and unable to replicate [37]. Our histological results have shown that syncytial knots were markedly increased in gestational diabetic placenta. Similarly, an in-vitro study shown that increased number of syncytial knots may be induced by exposure of the placenta to hypoxia, hyperoxia or oxidative stress [30]. Upregulation of Nrf2-dependent antioxidant defences in cytotrophoblasts and syncytiotrophoblasts have contributed to the increased shedding of syncytial knots in pre-eclampsia and it is implicated in maternal hypertension [38]. Further, studies are needed to explain the role of these syncytial knots in placental dysfunction in maternal diabetes.

Nrf2 together with its negative regulator Keap1 are considered as key proteins of cellular protective mechanisms to overcome cellular oxidative stress. They also have an important role in the transcriptional regulation of phase II detoxifying enzymes and antioxidant status [14,39]. Several endogenous enzymes have antioxidant functions, which include SOD, catalase, heme oxygenase-1, NADPH-Quinone Oxidoreductase-1, and glutamate cysteine ligase. They have been reported to be the downstream targets of Nrf2 [40,41]. These target genes are up-regulated when there is binding of Nrf2 to the antioxidant response element found in the promoters of these genes [42] as a result of increased cellular oxidative stress.

Dysregulation of Nrf2 has been shown to be involved in the etiology of diabetes, such as pancreatic islet beta cell dysfunction and insulin resistance and its complications [16–18]. Recent studies suggest that the dysregulation of Nrf2 caused by maternal diabetes impairs embryogenesis and placenta development. Nrf2 signalling is involved in maternal diabetes-induced defects in the development of the mouse placenta [20]. Placenta of women with pre-existing diabetes showed a heightened oxidative stress and decreased expression of the Nrf2/ARE pathway. This results in decreasing the expression of downstream antioxidant enzymes [19]. The placenta of gestational diabetic rats and pre gestational diabetic mouse showed an increased Nrf2 expression [20,21]. It appears that the early stages of pre gestational diabetes there may be an initial elevation in Nrf2 levels to counteract oxidative stress, whereas chronic diabetes results in decreased expression.

Several studies investigated the protective role of Nrf2 in diabetic cardiomyopathy and in diabetic nephropathy. Increased Nrf2 expression has a role in protecting against the severity of the disease. Human renal mesangial cells when exposed to high glucose resulted in the induction of ROS production which enhanced the expression of Nrf2 and its downstream genes [23]. In their study they found that activation of Nrf2 resulted in

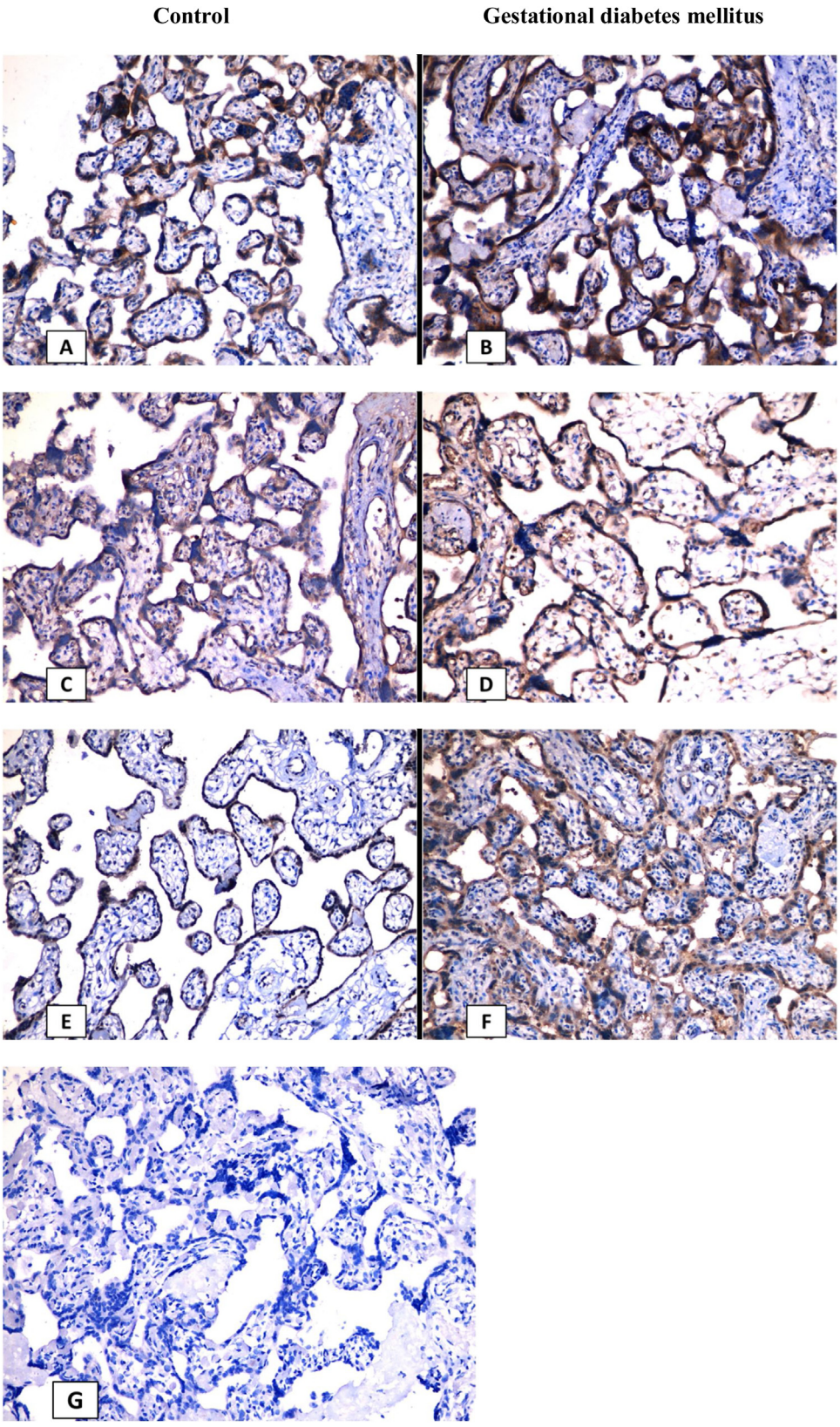

Fig. 3. Representative images of immunohistochemical staining of Nrf-2, catalase and SOD1 in control (n = 11) and gestational diabetic placenta (n = 13). (A & B: Nrf-2 protein expression in control and gestational diabetic placenta; C & D: Catalase protein expression in control and gestational diabetic placenta; E & F: SOD1 protein expression in control and gestational diabetic placenta; F: G: Rabbit IgG negative control). Images were captured using Olympus BX 43 bright-field microscope with 20 × magnification.

Table 4
Correlation of placental Nrf2 protein expression with oxidative stress parameters and antioxidant enzymes.

Nrf2 expression		
	Pearson's coefficient (r)	'p' value
Catalase	0.595	0.01
Placental TAS	−0.524	0.03
Birth weight of baby	0.761	0.02

the inhibition of the promoter activity of TGF-beta1, whereas knockdown of Nrf2 by siRNA increased TGF-beta1 transcription. The same investigators also found oxidative stress and elevated Nrf2 levels in the glomeruli of human diabetic nephropathy patients. Studies have reported that upstream kinases such as phosphatidylinositol-3-kinase (PI3K), MAPKs (p38, ERK, JNK), protein kinase C (PKC) and glycogen synthase kinase favor the release of Nrf2 from its inhibitory protein, Keap1 by directly or indirectly phosphorylating it. Most of these observations are based on in vitro studies. We did not find increased phosphorylation of Nrf2 in the placenta of gestational diabetic women. This could be explained by the fact the Nrf2 once subjected to phosphorylation is marked for degradation [43]. Therefore the stage of the disease is an important factor which dictates the levels of phosphorylated Nrf2 in the whole tissue like placenta.

This is first study to examine the nexus between placental Nrf2 expression and placental oxidative stress and anti-oxidant enzymes expression in gestational diabetic placenta. From the above we hypothesized that Nrf2 has a protective role in gestational diabetic placenta by increasing the downstream antioxidant enzymes and prevents the placenta from the heightened oxidative stress. The present study investigated the levels of expression of Nrf-2, p- Nrf-2 and anti-oxidant enzymes such as SOD1 and catalase in the placenta of gestational diabetic women and healthy pregnant women. Discrepancies in the levels of SOD in gestational diabetic placenta have also been reported. Studies reported that SOD levels are decreased [12] or increased [10]. This may be related the severity of the disease and variation in the population of the study. In our study, we found an increased SOD1 protein expression in placental tissues of gestational diabetic patients compared to controls. It has been reported that catalase activity is decreased [9] with an increased mRNA expression in placenta from women with gestational diabetes mellitus [10]. We have found an increase in catalase protein expression in gestational diabetic placentas when compared to control placentas.

The present study showed an increased total Nrf2 protein expression in gestational diabetic placentas when compared to control placentas. We found no differences in p-Nrf2 protein expression between the gestational diabetes mellitus and healthy pregnant women. Placental Nrf2 expression is positively correlated to catalase expression and negatively correlated to placental TAS levels. It appears that gestational diabetic placenta responds to the oxidative stress by promoting a concomitant increase in the expression of antioxidant enzymes via Nrf2 protein expression. Several Nrf2 activators have been reported which activate Nrf2 signalling through different mechanisms. These have been verified in both in vitro and in vivo models of diabetes. These activators prevented the development and progression of diabetic complications [44]. Chang et al. [24] have shown that triggering of Nrf2–HO-1 defense system causes over expression of catalase. It ameliorates the maternal diabetes–induced perinatal programming of renal disease and hypertension. Over expression of SOD1 in mice reduces the occurrence of fetal abnormalities and protects against diabetes-associated embryopathy [25].From the above it is clear that activated Nrf2 pathway increases the levels of anti-oxidants enzyme such as catalase and SOD. These increased enzymes decrease the fetal abnormalities associated with the maternal diabetes. The present study suggests that dietary or therapeutic activation of Nrf2 could be beneficial for the improvement of maternal and fetal outcomes in maternal diabetes.

Conclusion

In summary, in gestational diabetes mellitus, there is a maternal and placental oxidative stress as a result of maternal hyperglycaemia. The gestational diabetic placenta has a higher level of Nrf2 protein expression. The increased ROS levels in the placenta of gestational diabetic women may activate Nrf2/ARE pathway which results in increased expression of antioxidant enzymes SOD1 and catalase. This might have protected the placenta from the onslaught of oxidative stress.

Conflict of interest

The authors declare that they have no conflict of interest related to the publication of this article

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