Alterations of transcriptome expression, cell cycle, and mitochondrial superoxide reveal foetal endothelial dysfunction in Saudi women with gestational diabetes mellitus

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Abstract. Gestational diabetes mellitus (GDM) affects one in four Saudi women and is associated with high risks of cardiovascular diseases in both the mother and foetus. It is believed that endothelial cells (ECs) dysfunction initiates these diabetic complications. In this study, differences in the transcriptome profiles, cell cycle distribution, and mitochondrial superoxide (MTS) between human umbilical vein endothelial cells (HUVECs) from GDM patients and those from healthy (control) subjects were analysed. Transcriptome profiles were generated using high-density expression microarray. The selected four altered genes were validated using qRT-PCR. MTS and cell cycle were analysed by flow cytometry. A total of 84 altered genes were identified, comprising 52 upregulated and 32 downregulated genes in GDM.HUVECs. Our selection of the four interested altered genes (TGFB2, KITLG, NEK7, and IGFBP5) was based on the functional network analysis, which revealed that these altered genes are belonging to the highest enrichment score associated with cellular function and proliferation; all of which may contribute to ECs dysfunction. The cell cycle revealed an increased percentage of cells in the G2/M phase in GDM.HUVECs, indicating cell cycle arrest. In addition, we found that GDM.HUVECs had increased MTS generation. In conclusion, GDM induces persistent impairment of the biological functions of foetal ECs, as evidenced by analyses of transcriptome profiles, cell cycle, and MTS even after ECs culture in vitro for several passages under normal glucose conditions.

Key words: Endothelial dysfunction, Mitochondrial superoxide, Cell cycle, Gestational diabetes, Transcriptome profile

GESTATIONAL DIABETES MELLITUS (GDM) is a metabolic disorder that occurs during pregnancy and is characterized by hyperglycaemia and insulin resistance [1], both of which are common features in type 2 diabetes (T2D). Saudi Arabia has the highest prevalence (18.5%) of diabetes among Arab world as of 2017, as reported by the International Diabetes Federation [2]. One in four Saudi women develops GDM during pregnancy and is subsequently at higher risk of developing T2D [3]. The majority of women with GDM revert to normal glycaemic status after delivery; however, GDM enhances the risk of Cardiovascular Diseases (CVD) for both mother and offspring [4]. Whether GDM progresses to T2D depends on many factors, including family...
history, obesity, and need for insulin treatment [5]. Epidemiological studies suggest a link between foetal exposure to maternal diabetes in utero and the propensity to develop a variety of diseases, including diabetes and atherosclerosis, later in life [6, 7].

Early in the pathogenesis of these vascular diseases, damage occurs to the endothelium, a single layer of Endothelial Cells (ECs) lining the blood vessels, causing endothelial cell dysfunction [8]. ECs is the main regulator of vascular homeostasis through performing several important physiological functions include vasodilation, angiogenesis, and the inflammatory response [9-12]. Several growth factors secreted by endothelium contribute to its vasodilatory, growth, and angiogenesis functions, including Transforming Growth Factor (TGF), Insulin Growth Factor-1 (IGF-1), Insulin Growth Factor Binding Protein (IGFBP), Epidermal Growth Factor (EGF), and Vascular Endothelial Growth Factor (VEGF) [13]. Endothelial dysfunction is defined as alterations in physiological ECs functions that lead to reduced vasodilation and increased cellular adhesion. Dysfunctional endothelium has been reported previously in patients with GDM [14-16]; however, the mechanisms are not fully understood.

Four hypotheses have been proposed to explain how hyperglycaemia causes endothelial dysfunction, which involve the polyol pathway (or sorbitol pathway), formation of Advanced Glycation End products (AGEs), activation of Protein Kinase C (PKC) and increased hexosamine pathway flux [17]. Hyperglycaemia is thought to stimulate these pathways to produce excess Reactive Oxygen Species (ROS), leading to oxidative stress and potential impairment of antioxidant defence [18, 19]. ECs are an important target of hyperglycaemia-induced superoxide production. Hyperglycaemia increases endothelial cell apoptosis, reduces ECs proliferation and disturbs the cell cycle, leading to endothelial dysfunction [20].

Mitochondria are considered the primary source of energy for metabolism and respiration, and their dysfunction severely affects tissue homeostasis. ROS, hydroxyl radicals, and superoxide dismutase are mainly produced by mitochondria. Oxidative stress generated under hyperglycaemic conditions can damage the structure and function of mitochondria [21]. Alteration of mitochondrial content and function has been evidenced in patients with GDM [22, 23] and T2D [24]. Mitochondrial dysfunction promotes the accumulation of ROS, leading to endothelial dysfunction.

Oxidative stress is the key initiator of glycaemic memory or epigenetic change [25]. An increasing number of studies have reported that the damaging effects of hyperglycaemia in ECs are maintained even after several generations in culture with normal glucose concentrations [26-28]. In this study, we analysed transcriptome profiles using microarray and investigated mitochondrial superoxide (MTS) production and cell cycle distribution to examine differences between HUVECs derived from GDM mothers (GDM.HUVECs) and healthy HUVECs as control cells (C.HUVECs) under normal glucose conditions.

Materials and Methods

All materials were purchased from UFC biotechnology (Riyadh, KSA) unless otherwise stated.

Sample collection

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the King Abdulaziz University Hospital, Jeddah, Saudi Arabia (approval number: HA-02-J-008). Umbilical cords were collected from a total of 11 GDM women (experimental group, GDM.HUVECs) and 6 healthy women (control group, C.HUVECs) immediately after full-term delivery. The women in the C.HUVECs group were free from medical disorders such as hypertension and were non-smokers. The women in the GDM.HUVECs group had a positive OGTT according to WHO criteria [29]. Venous blood samples for biochemical analyses of HbA1c and random plasma glucose were collected on the day of delivery. All women with GDM had no other obstetric complications (ex. pre-eclampsia) and were on a recommended diet. At approximately 12 weeks of pregnancy, basic anthropometric parameters, including weight, height, and body mass index (BMI), were recorded. For microarray analysis and qRT-PCR, samples were obtained from a minimum of three to four participants from each study group.

Isolation and culture of HUVECs

Primary HUVECs were isolated from the control group and experimental group by using collagenase enzyme treatment as described in Eccles [30] and stored at 4°C in Phosphate in phosphate buffered saline (PBS) containing heparin (10 U/mL), fungizone (2.5 μg/mL), penicillin, and streptomycin (100 U/100 mg/mL) until subsequent processing. The isolated HUVECs were then cultured in complete culture medium M199 containing 5 mM glucose, 20% foetal bovine serum, fungizone (2.5 μg/mL), L-glutamine (2 mM), and penicillin/streptomycin (100 U/100 mg/mL) at 37°C under 95% air and 5% CO2 humidified conditions. The cells were used up to the fourth passage.
Transcriptome profile analysis using microarray

Total RNA (250 ng/sample) was labelled and amplified using the GeneChip™ Whole Transcript PLUS kit (Affymetrix, Santa Clara, CA, USA). The biotinylated cRNA was then hybridized to Human Gene 2.0 ST arrays (Affymetrix, CA, USA). Washing and staining was performed with the Affymetrix GeneChip Hybridization, Wash and Stain Kit. Next, the arrays were scanned on an Affymetrix GeneChip Scanner 3000 7G according to the manufacturers’ instructions. Finally, probe cell intensity data (CEL files) were generated using GeneChip Command Console Software (AGCC).

Microarray data analysis

Data were analysed with Partek Genomics Suite version 6.6 (Partek Inc., MO, USA) following background correction and normalization. Differentially expressed genes (DEGs) were identified based on a cut-off fold change ≥1.5 and p-value <0.05. For functional analysis, the expression data of DEGs were uploaded into Ingenuity Pathways Analysis (IPA) programming (Ingenuity Systems Inc., Redwood City, CA, USA) software to identify the most significant altered biological networks based on statistical scores. Canonical pathway and Gene Ontology (GO) analyses were also performed using IPA software. The GO enrichment was utilized to group DEGs with significant p-value into functional categories. The Fisher’s Exact Test p-value was used to determine the level of DEGs in a functional category.

Extraction of RNA

The Qiagen RNeasy Mini Kit (Qiagen, Crawley, UK) was used to isolate total RNA from the C.HUVECs and GDM.HUVECs samples. RNA quantity was confirmed by spectrophotometric analysis (OD260/280) using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent, Edinburgh, UK).

qRT-PCR validation

Total RNA (1 μg) was reverse-transcribed into cDNA by ImProm-II Reverse Transcription System Kit (Promega, Southampton, UK) according to the manufacturer’s instructions. Real time qRT-PCR experiments were performed using the QuantiTect SYBR Green PCR kit (Qiagen, Manchester, UK) on an iCycler iQ Real-time PCR Detection System (Applied Biosystems, Cheshire, UK), following the manufacturer’s instructions. The expression of four selected genes based on functional assay of the microarray data was validated by qRT-PCR using β-actin as a reference housekeeping gene. The primer sequences used for qRT-PCR are shown in Supplementary Table S1. Rest 2009 software version 2.0.13 was used for calculating gene expression [31].

Cell cycle

ECs were washed with PBS, harvested by trypsin treatment and incubated with the staining solution FxCycle™ PI/RNase (Molecular Probes, Life Technologies) as per the manufacturer’s instructions. Flow cytometry analysis was performed on a FACS Aria III flow cytometer (BD Biosciences, San Jose, USA); at least 35,000 events were captured per analysis. Cell cycle analysis was performed using Flowlogic™ software (version 7.2).

Measurement of MTS level

MitoSOX™ Red dye (Invitrogen, Carlsbad, CA) was used to detect the MTS levels in cells from the GDM.HUVECs and C.HUVECs groups. Briefly, ECs were incubated with 2 μM MitoSOX Red in Hank’s buffer at 37°C for 30 min according to a previously described protocol [32]. Non-stained cells were used as negative controls. After the cells were washed three times with PBS, the MitoSOX™ Red fluorescence was detected using a FACS Aria III flow cytometer (BD Biosciences, San Jose, USA).

Statistical analysis

Unpaired data were analysed using Student’s t-test. The data are presented as the mean ± SEM. A p-value less than 0.05 was considered statistically significant.

Results

Subject characteristics

As depicted in Table 1, random plasma glucose and HbA1c were significantly higher in the patients with GDM (p < 0.05). However, there were no significant differences in age and BMI between the subjects with and without GDM.

DEGs between the GDM.HUVECs and C.HUVECs groups

A total of 84 DEGs were identified, comprising 52 upregulated and 32 downregulated genes in GDM.HUVECs samples compared with C.HUVECs samples (Table 2). The most significantly upregulated genes in the GDM.HUVECs group were listed in Table 2. Hierarchical cluster analysis of the 84 DEGs between the GDM.HUVECs and C.HUVECs groups was performed (Fig. 1A).
Functional analysis of DEGs

The top four merged networks in GDM.HUVECs involved the functional categories: cellular function and proliferation, lipid metabolism, embryonic development, cellular assembly and organization (Supplementary Table S2).

GO enrichment analysis of the DEGs identified by microarray revealed enrichment in the following categories: biological phase, locomotion, biological adhesion, immune response, cell aggregation, and growth (Fig. 2). Pathway analysis using IPA revealed that VDR/RXR activation was the most significant pathway associated with the DEGs between the GDM.HUVECs and C.HUVECs groups ($p = 1.91 \times 10^{-2}$; data not shown).

mRNA expression levels measured by qRT-PCR

Functional network analysis using IPA software revealed that these DEGs belonging to network involved in cellular function and proliferation and may play roles in the pathogenesis of endothelial dysfunction associated with GDM. Based on that, four interested altered genes ($KITLG$, $TGFB2$, $NEK7$, and $IGFBP5$) overexpressed in GDM in this highest score network were selected for validation by qRT-PCR. The results of qRT-PCR were consistent with the microarray results.

Cell cycle distribution

We next examined the effects of GDM on cell cycle phases in HUVECs. As shown in Fig. 3, the GDM.HUVECs group exhibited a greater proportion of cells in cell cycle arrest in the G2/M phase than did the C.HUVECs group ($p < 0.05$).

MTS production

Mitochondrial ROS (mtROS) is a sensitive dye that can be used to evaluate ROS generation by mitochondria. Comparison between the GDM.HUVECs and C.HUVECs groups revealed that GDM significantly ($p < 0.01$) enhanced MTS production in HUVECs (Fig. 4).

Discussion

GDM is associated with future offspring risk of T2D and obesity [33-35]. One mechanism underlying these associations has been hypothesised to be mitochondrial dysfunction associated with oxidative stress and subsequent endothelial dysfunction [24, 36, 37]. The production of excess ROS from dysfunctional mitochondria under hyperglycaemic conditions is believed to be the predominant cause of glycaemic memory or epigenetic changes [38-40]. The phenomenon of glycaemic memory is conceptualised as the persistence of damaging effects of hyperglycaemia in ECs, even after glucose normalisation [41]. In the present study, GDM affected the global transcriptome profile, induced cell cycle arrest, and increased MTS production of foetal ECs derived from GDM patients with Saudi ethnicity. These findings are in line with previous studies reporting deleterious effects of maternal gestational diabetes on foetal cells [14-16].

Transcriptome analysis performed using high density microarray technology showed that 84 genes were differentially expressed between the GDM.HUVECs and C.HUVECs groups, with 52 upregulated and 32 downregulated genes in GDM (Table 2). Four interested upregulated genes ($KITLG$, $TGFB2$, $NEK7$, and $IGFBP5$) were selected based on their involvement in the highest enrichment score associated with cellular function and proliferation (Supplementary Table S2), which may play roles in the pathogenesis of GDM associated with endothelial dysfunction. The qRT-PCR validation of four selected genes showed similar trends as the microarray results.

The transforming growth factor-beta (TGFB) signalling in ECs is important for the preservation of vascular
Table 2  List of selected differentially upregulated genes in GDM.HUVEC versus C.HUVEC

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold-Change</th>
<th>p-value</th>
<th>Gene name</th>
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<td>L1 cell adhesion molecule</td>
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<td>cytokine like 1</td>
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<td>NRK</td>
<td>3.13</td>
<td>0.046</td>
<td>Nik related kinase</td>
</tr>
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<td>IGFBP5</td>
<td>3.02</td>
<td>0.040</td>
<td>insulin like growth factor binding protein 5</td>
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<td>SNAPC1</td>
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<td>small nuclear RNA activating complex polypeptide 1</td>
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<td>2.27</td>
<td>0.045</td>
<td>reelin</td>
</tr>
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<td>0.044</td>
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<td>0.042</td>
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<td>0.038</td>
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<td>microRNA 4725</td>
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<td>0.036</td>
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<td>ubiquitin conjugating enzyme E2Q family member 2-like</td>
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<td>NEK7</td>
<td>1.57</td>
<td>0.008</td>
<td>NIMA-related kinase 7</td>
</tr>
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integrity [42]. TGFB family consists of three isoforms, namely, TGFB1, TGFB2, and TGFB3. These proteins exert diverse roles, participating in the regulation of ECs proliferation, migration, apoptosis, ROS production and cell cycle [43-45]. They are also involved in the pathogenesis of CVD such as diabetes and atherosclerosis [46, 47]. Increased expression of TGFB2 has been shown to enhance fibrosis in the kidney of streptozotocin (STZ)-induced diabetic rats [48] and inhibits ECs proliferation [49], suggesting its participation in the pathogenesis of diabetic complications. Moreover, in line with our findings, it has been shown that TGFB2 expression was increased in HUVECs from GDM subjects via a glucose-dependent epigenetic mechanism involving the differential methylation of its promoter [13].

NEK7 is one of 11 members of the NEK (NIMA-related kinase) family. NEK7 is required for cell cycle regulation [50], especially spindle formation during mitosis [51], and binds to NLRP3 to promote an NLRP3-dependent inflammatory response [52]. It is interesting that excessive ROS production by dysfunctional mitochondria, as occurs in diabetes, has been shown to be necessary for the binding of NEK7 to NLRP3 and subsequent inflammasome activation [53, 54]. Moreover, a low expression of NEK7 has been shown to inhibit the proliferation of retinoblastoma cells through induction of cell cycle arrest [55], whereas its overexpression is associated with increased proliferation of hepatocellular carcinoma [56]. Further, overexpression of NEK6 protein, which shares structural and functional similarities with NEK7, suppresses the cell growth arrest induced by TGFB in tumour cells [51]. Therefore, it can be
Argo enrichment analysis of the DEGs between the GDM.HUVECs and C.HUVECs groups identified by microarray analysis and ranked according to their Fisher’s Exact test $p$-value.

**Fig. 3** Cell cycle analysis of HUVECs. (A). Representative image of the quantification of cell cycle phase using flow cytometry. ECs were fixed overnight with ethanol and labelled with FxCycle™ PI/RNase stain, and the cell cycle distribution was determined by flow cytometry. (B). The percentage of HUVECs in each phase was quantified for the C.HUVECs ($n = 6$) and GDM.HUVECs groups ($n = 11$). Data are presented as the means ± SEMs. * $p < 0.05$ versus controls.
speculated that a similar scenario occurs in our model, although further investigation is crucial for substantiating this.

IGF stimulates cell growth and differentiation by binding to IGFBPs [57]. However, IGFBP5 could lead to dissociation of IGF from IGF receptors with subsequent suppression of mitogenesis, differentiation, and other IGF-related processes [58]. This is supported by a report that IGFBP5 stimulates the proliferation suppressing activity of TGFβ in porcine embryonic myogenic cell cultures [59]. Additional in vivo studies using diabetic animal models have shown that diabetes enhances the expression of IGFBP5 [60, 61]. The increase in IGFBP5 expression in ECs observed in the GDM.HUVEC group in the present study is consistent with the overexpression of IGFBP5 reported earlier in HUVECs [16] and serum [62] derived from GDM patients. Therefore, the aberrant expression of IGFBP5 could be proposed to repress cell proliferation in response to elevated glucose levels.

Stem cell factor or c-Kit binds to KITLG (c-Kit ligand) and promotes ECs survival and migration through the Akt signalling pathway [63]. On the other hand, high levels of KITLG has been shown to promote vascular ECs apoptosis through integrin β4 phosphorylation [64]. Interestingly, under pathogenic conditions, such as inflammation, c-Kit stimulates the recruitment of endothelial progenitor cells to the site of inflamed endothelium [65]. To the best of our knowledge, the present study is the first to demonstrate that GDM can alter the expression of KITLG in HUVECs. Interestingly, increased expression of KITLG has been reported in islets of T2D model db/db mice based on RNA-sequencing analysis [66] and in muscle tissue and activated T-lymphocytes of T2D patients based on microarray experiments [67]. These observations suggest that alteration of KITLG expression may be involved in the development of diabetic complications.

Mitochondria of ECs play an important role in controlling the intracellular levels of ROS, calcium, and ATP, all of which are important for normal ECs function, including vascular tone [68]. The exposure of ECs in womb to GDM, characterised by hyperglycaemia, has been shown to induce mitochondrial dysfunction associated with increased ROS levels [24]. This might be an irreversible effect and could explain our finding that the GDM.HUVEC group had greater mitochondrial ROS generation compared to the C.HUVEC group under normal glucose conditions (5 mM), supporting the notion that oxidative stress induces glycaemic memory or epigenetic changes. In addition, there are a number

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**Fig. 4** MTS generation from HUVECs. (A). A representative image of the MTS quantification by flow cytometry. Cells were labelled with MitoSOX Red stain, and the cell superoxide level was measured by flow cytometry. (B). Percentage of superoxide generation in cells of both the C.HUVEC ($n = 6$) and GDM.HUVEC ($n = 11$) groups was quantified. The results are expressed as the means ± SE. **$p < 0.01$ versus controls.**
Fig. 5  Schematic illustration showing the induction of persistent mitochondrial dysfunction, characterised by increased reactive oxygen species (ROS) levels, on exposure of endothelial cells (EC) to gestational diabetes mellitus (GDM) associated with hyperglycaemia in womb. This could lead to aberrant global gene expression, mitochondrial oxidative stress, and cell cycle arrest in EC derived from GDM patients (GDM.EC) even after glucose normalisation in vitro for several passages. All these alterations in GDM.EC collectively impaired endothelial functions. Question mark (?) represents a hypothetical pathway. (−) denotes inhibition and (+) denotes stimulation. (Arrow up) indicates increase in values. (---) dashed line denotes the possible effect of transcripts on cell cycle and proliferation, as reported earlier.
of studies investigated the effect of high glucose on ECs function to mimic diabetes environment and they found similar results [69, 70].

Further, the deleterious effects of endothelial mitochondrial oxidative stress cause delays in ECs cycle phases [71]. Interestingly, when we compared GDM.HUVECs samples with C.HUVECs samples, we found that GDM significantly increased the proportion of HUVECs in the G2/M phase, indicating that elevated glucose induced oxidative stress arrests the shift from the G2 phase to the M phase, thereby affecting the proliferation of ECs. It is possible that arrest in G2/M phase allows DNA repair before mitosis in HUVECs of GDM subjects, as the high glucose associated with oxidative stress induces DNA damage [72]. As mentioned above, we also suggest that in GDM.HUVECs, the alteration of gene expression involved in cell cycle regulation, such as NEK7 and TGFB2 is induced by oxidative stress, which in turn may affect the cell cycle distribution.

Altogether, as depicted in Fig. 5, under diabetic conditions, the exposure to high glucose in womb induces persistent damage effects on ECs function. In the present study, there were no significant differences in clinical characteristics between the donor groups except for significantly increased glucose and HbA1c levels in patients with GDM, suggesting alterations induced by hyperglycaemia. There are currently no treatments and seems that diet control is not enough to reverse the damaging effects of hyperglycaemia on the endothelium of mothers and offspring at an earlier stage. In conclusion, this study found alterations in global gene transcripts, cell cycle distribution, and MTS generation in foetal ECs derived from Saudi GDM patients under normal glucose conditions which may involve in the pathogenesis of GDM-induced endothelial dysfunction. The present findings enhance current understanding of this complex metabolic disease and may aid the development of new pharmacological therapies to reverse dysfunctional endothelium at an earlier stage.

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Disclosure of Interests

The authors declare that they have no competing interests.

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