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Research Article

Oxidative Stress Elevates eNOS Expression and VSMCs Proliferation of the Umbilical Vein of GDM Mothers

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Abstract

Aim: Gestational diabetes mellitus (GDM) is associated with an increased risk of fetal and maternal complications, such astype 2 DM (diabetes mellitus) and cardiovascular disease (CVD). This study aimed to predict the potential for future vascular complications in mothers with GDM by evaluating oxidative stress, endothelial NO synthase (eNOS) expression, and vascular smooth muscle cell (VSMC) proliferation in the umbilical vessels of mothers with GDM.

Material and Methods: Subjects were divided into two groups: the normoglycemic control (NGC) group (n = 10) and the GDM group (n = 12). Expression of eNOS and production of reactive oxygen species (ROS) in human umbilical vein endothelial cells (HUVECs) were determined. The mitochondrial mass of HUVECs was evaluated by spectrofluorometry. VSMC proliferation was ascertained *in vitro* with an EdU cell proliferation assay. Advanced glycation end products (AGEs) accumulation was measured by ELISA and assessed by immunohistochemical staining

Results: VSMC proliferation, eNOS expression, and ROS production in HUVECs were significantly increased, and greater immunohistochemical staining to AGEs was observed in endothelium in GDM.

Conclusion: Increased oxidative stress, which elevates eNOS expression and VSMC proliferation in the umbilical vessels of mothers with GDM, may be a sign that mothers have a high potential for developing diabetes or cardiovascular disease in the future.

Keywords: GDM, oxidative stress, VSMCs, ROS, eNOS

INTRODUCTION

Gestational diabetes mellitus (GDM) is a condition of glucose intolerance with onset or first recognition during pregnancy (1). With the increase in obesity and diabetes mellitus (DM) cases, an elevation in the incidence of GDM is observed (2). Understanding the pathogenesis of GDM is essential for the precaution against the progression of type 2 DM and cardiovascular disease (CVD). In a long-standing hyperglycemic state in DM, protein glycation reactions lead to the formation of advanced glycation end-products (AGEs), which are thought to be the major causes of different vascular complications in DM (3). Oxidative stress generation in a variety of cells is induced by AGEs' interaction with a receptor for AGEs (RAGE) (4). The intermolecular collagen cross-linking caused by AGEs results in diminished arterial compliance and increased vascular stiffness (5). The deposition of AGEs has been known to progress at an accelerated rate under DM. AGEs are hardly broken down and remain in diabetic tissue for a long time, even with improved glycemic control (4). Cellular exposure to high glucose as seen in DM induces reactive oxygen species (ROS) production (6). The release of ROS and the generation of oxidative stress are considered critical factors for the

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pathogenesis of DM (7). ROS production in the vessel wall is considered a risk factor for atherosclerotic CVD as well (8). A marked increase in oxidative stress characterized by the overproduction of ROS has been observed in DM. A study has shown that oxidative stress brings about the downregulation of occludin (9). It has been reported that the downregulation of β-catenin in placental vessels is significantly associated with GDM (10).

Endothelial dysfunction is a common feature in GDM (11). Endothelial dysfunction determines future vascular disease complications (12) as well. Vascular smooth muscle cells (VSMCs) are crucial to maintaining the integrity of the arterial wall. VSMC proliferation contributes to vascular remodeling in CVD and diabetic vascular complications (13).

Studies have confirmed that endothelial nitric oxide synthase (eNOS) is an important factor in endothelial function. eNOS is involved in vascular development by promoting angiogenesis (14). A high level of glucose exposure increases eNOS expression (15). eNOS is the main weapon of endothelial cells to overcome vascular diseases. This study aimed to predict the potential for future vascular complications in mothers with GDM by evaluating oxidative stress, eNOS expression, and VSMC proliferation in the umbilical vessels of mothers with GDM.

MATERIAL AND METHOD

Subjects

The subjects were recruited from the Department of Gynecology and Obstetrics of Kocaeli University Hospital. The study protocol was approved by the Clinical Research Ethical Committee of Kocaeli University. An informative written consent was signed by all the subjects. The normoglycemic control women were selected as pregnancies without any history of illness, and no risk factor of GDM including a normal oral glucose tolerance test (OGTT) result. Women aged between 18-40 years, with a body mass index (BMI) of less than 30 kg/m^2 at the beginning of pregnancy were selected (Table 1). Diagnosis of GDM was made according to American Diabetes Association (ADA) criteria at the end of the OGTT after 75 g oral glucose load between 24 and 28 weeks of pregnancy (16).

Collection of Blood Plasma, Placenta, and Umbilical Cord Samples

Placentas and the umbilical cord samples were collected and transferred to the laboratory immediately. Fetal umbilical cord blood samples were collected from the umbilical veins and placed into heparinized tubes. An equal volume of Histopaq®-1077(SIGMA-ALDRICH®, USA) reagent was added and centrifuged at 20 ºC, 400 g for 30 min. The plasma samples were frozen at -80 ºC.

Isolation of Human Umbilical Vein Endothelial Cells

HUVECs were isolated through enzymatic digestion. Umbilical cords were stored at 4 ºC in Hank's Balanced Salt Solution (HBSS) with 1% Pen/Strep for a night. The umbilical cords were washed extensively with Dulbecco's Phosphate Buffered Saline (DPBS), one end of the vein was gripped with a clamp and the vein was filled with 0.1% collagenase (Gibco) prepared in HBSS. After incubating at 37 °C for 25 minutes, the endothelial cells in the enzyme solution were transferred into a falcon tube. After centrifugation (1500 rpm, 5 min), the cell pellet was washed with DPBS and finally resuspended in EGM-2 (Lonza) medium containing 5% fetal bovine serum (FBS) before being drawn into a gelatin-coated T25 culture flask. HUVECs were cultured until forming a 70% confluent monolayer and passaged afterward. HUVECs were immunostained for CD31 to prove the culture purity. HUVECs were seeded in 8-well slides (Ibidi, ibiTreat), and fixed with formaldehyde. For the primary antibody, anti-CD31 (Dako Denmark A/S, Glostrup, Denmark) antibody, and for the secondary antibody, Alexa Fluor 488 labeled secondary antibody (Molecular Probes, Life Technologies) were used. At last, for nuclear staining, DAPI was applied. The negative controls were cells that were treated with the same protocol but without the primary antibody.

Vascular Smooth Muscle Cell Isolation Through Explant Culture

The umbilical cord vein was opened with scissors, and the endothelial layer on its surface was removed. Then smooth muscle fibers are mechanically pulled out and immediately added to gelatin-coated culture wells. Vascular smooth muscle fibers are cultured in a minimal amount of culture medium (10% FBS, 1% Pen/Strep, and DMEM-F12 with 2 ng/ml bFGF) with keeping tissue attached to the surface. VSMCs were immunostained for alpha-smooth muscle actin (α-SMA) to prove purity. For the primary antibody, anti-α-SMA antibody (Abcam; ab5694), for the secondary antibody, Alexa Fluor 568 labeled secondary antibody (Molecular Probes, Life Technologies) were applied.

Collagen Isolation from Umbilical Cords

Umbilical cord tissues were cut into small pieces and treated with 0.2% sodium chloride for 3 days at +4°C with shaking at 90 rpm. After incubating, the liquid part was discarded by centrifugation at 10000 rpm for 10 minutes. Umbilical cord tissues were then treated with 0.1% pepsin prepared in 0.2 M acetic acid for 4 days at + 4°C. After centrifuging, the supernatant was drawn into the new tubes, and an equal volume of 1.8 M sodium chloride was added and left undisturbed overnight. The collagen fibers were retrieved by centrifugation at + 4ºC and 12000 g for 45 min. The collagen pellets were disintegrated in 0.5 M acetic acid and dialyzed at + 4 °C against 0.1 M acetic acid for 3 days. 0.1 M acetic acid was refreshed daily and then the collagen samples were dialyzed against distilled water at + 4 °C at 90 rpm for the whole day. Collagen solutions were collected and stored at + 4 °C in tubes for further analysis.

Detection of Reactive Oxygen Species

Intracellular production of ROS was measured using 2',7'-dichlorofluorescein diacetate (H2DCF-DA Sigma-

Aldrich). This non-fluorescent compound rapidly oxidizes to highly fluorescent DCF by interacting with cellular ROS. H2DCF-DA was added to the HUVECs seeded in a 96-well plate (10^4cells/well) at a final concentration of 5 µM and the cells were incubated at 37 ºC for 1h. Cells from all experimental groups were seeded on the same plate and treated simultaneously with the same H2DCF-DA stock solution. Cellular fluorescence was measured in a Flex Station3 spectrofluorometer with excitation and emission wavelengths set at 490 nm and 530 nm, respectively. The DNA content of each well was ascertained with Quant-iT PicoGreen dsDNA assay kit (Molecular Probes, Life Technologies, Eugene, Oregon, USA), and fluorescence emission/DNA content was calculated for normalization of the values.

Determination of Mitochondrial Mass

Mitochondrial mass was determined by incubating the HUVECs seeded in 96-well plate (10^4cells/well) with 100nM Mito Tracker Orange (MTO) (Molecular Probes, Life Technologies) prepared in EBM-2 medium for half an hour at 37 ºC. It was washed with warm DPBS and immediately fluorescence was read on a Flex Station3 spectrofluorometer with excitation and emission wavelengths set to 554 nm and 600 nm, respectively. The DNA content of each well was determined with the same dsDNA assay kit mentioned above.

Quantitative Real-time PCR for eNOS Expression in HUVECs

RNA was isolated from HUVECs by using the High Pure RNA Isolation Kit (ROCHE) and subsequently converted to cDNA with Transcriptor High Fidelity cDNA Synthesis Kit (ROCHE) according to the manufacturer's instructions. Quantitative real-time PCR analysis was performed on the Light Cycler 480-II (Roche Diagnostics, Rotkreuz, Switzerland) with appropriate cycle conditions using the Light Cycler 480 SYBR Green I Master kit (Roche Diagnostics) for amplification of eNOS and Real-Time Ready Single Assay (Roche) and Light Cycler 480 Probes Master kit for amplification of β-actin. Primer sequences used for the amplification of eNOS were as follows: forward 5'-AGGAACCTGTGTGACCCTCA-3', reverse 5'-CGAGGTGGTCCGGGTATCC-3'. Real-Time Ready probes (Roche Diagnostics, Assay ID: 143636, Config. No. 100069730) were used to amplify β-actin in each sample. Expression levels of eNOS were calculated using the 2^-ΔCt (ΔCt = Target Gene – Reference Gene) formula. β-actin was used as a reference gene.

Determination of Vascular Quality by Immunohistochemistry

Placentas and umbilical cords were fixed with formalin and embedded with paraffin, then sections were cut 4-5 µm thick. AGE, RAGE, occluding, and β-catenin in the placenta and umbilical cord tissues were determined by immunostaining. Placenta and umbilical cord sections were incubated with the primary antibodies against RAGE (Abcam; ab3611), AGE (Abcam; ab23722), Occludin (Abcam; ab31721), and β-catenin (Abcam; ab1605). After washing with DPBS, Biotinylated Goat Anti-Polyvalent, Streptavidin peroxidase, and AEC Chromogen were applied. The negative controls were sections that were treated with the same protocol but without the primary antibody. Immunohistochemical staining assessment was performed blindly. Immunohistochemically positive stained areas were scored by two pathologists by semi-quantitative method (0: no staining, 1: weak, 2: moderate, 3: high).

Vascular Smooth Muscle Cell Proliferation

EdU cell proliferation assay was performed to determine VSMC proliferation. VSMCs were seeded in 8-well slides (Ibidi, ibiTreat) at a density of 1.5 × 10^4cells/well and cultured in presence of EdU (Click-iT® EdU Alexa Fluor® 488 Imaging Kit, Invitrogen) with, a final concentration 1µl/mL for 5 days and fluorescently labeled for EdU after fixation in 4 % paraformaldehyde. Fluorescent images were obtained by fluorescence microscopy from 6 different areas of each sample and EdU positive cell number/total cell number was determined by counterstaining all cell nuclei with Hoechst. The proliferation index was analyzed according to the following formula:Proliferation Index=(EdU positive cell number/total cell number)x100

ELISA for Determination of Ages in the Umbilical Cord Blood and Umbilical Cord Collagen

Determination of the average level of AGEs in the umbilical cord blood and the accumulation number of AGEs in the umbilical cord collagen was performed by ELISA. The number of AGEs in the umbilical cord blood and the umbilical cord collagen were determined with OxiSelect™ Advanced Glycation End Product (AGE) Competitive ELISA Kit (Cell Biolabs, STA-317), respectively, according to manufacturers' instructions.

Statistical Analysis

The sample size of the present study was determined considering a power of 80% to detect the effect of a given test at the desired level of significance (based on a two-tailed alpha level of 0.05). Values for clinical parameters and *in vitro* assays were given as mean ± SD. Statistics were performed with the number of different biological samples and corresponding cell cultures with 2–4 replicates per experiment. Student's unpaired t-test and Fisher's Exact test were applied. JMP Start Statistics version 9 (Statistical Discovery Software SAS Institute, Cary, NC, USA) was used for analysis. $p<0.05$ was considered statistically significant.

RESULTS

Clinical characteristics for GDM and normoglycemic control groups are summarized in (Table 1). There were slight differences identified in maternal ages and gestational weeks at partum between GDM (n=12) and NGC group (n=10) ($p<0.05$). However, there were no significant differences in newborn weight and APGAR score between the two groups ($p>0.05$).

Effects of Maternal Diabetes on the Proliferation of VSMC

The proliferation of diabetic VSMC was higher than control VSMC cultured in DMEM-F12 with 10% FBS (p<0.001) (Figure 1). Both EdU positive and Hoechst-stained total nuclei numbers were counted and (EdU positive cell number/total cell number)x100 was calculated as the proliferation index.

Table 1. Comparison parameters between groups

Parameters	GDM (n=12) Mean±SD	NGC (n=10) Mean±SD	Statistical significance
Maternal age, years	35.83±4.43	29.80±4.48	p<0.05*
Gestational weeks at partum	37.30±0.35	38.70±0.39	p<0.05*
Newborn weight, g	3077±129	3166±135	p>0.05
APGAR score	7.90±0.25	8.20±0.27	p>0.05
AGEs in umbilical cord blood (µg/mL)	8.53±1.84	7.70±1.25	p>0.05
AGEs in umbilical cord collagen (µg/mg)	89.4±29.1	79.7±24.2	p>0.05

Abbreviations: APGAR, appearance, pulse, grimace, activity, and respiration; AGEs, advanced glycation end products; GDM, gestational diabetes mellitus; NGC, normoglycemic control; SD, standard deviation; $p<0.05$* was considered statistically significant

Maternal Diabetes Induces Oxidative Stress in HUVECs

The average ROS of HUVECs was significantly increased in the GDM (n=12) compared to the NGC (n=10) group ($p<0.05$) (Figure 2A).

Effects of Maternal Diabetes on the Mitochondrial Mass of HUVEC

The average mitochondrial mass of HUVECs was determined in the GDM (n=12) and NGC (n=10) group. There were no significant differences identified between groups ($p>0.05$) (Figure 2B).

Effects of Maternal Diabetes on the Expression of eNOS in HUVECs

Quantitative real-time PCR analysis revealed that eNOS mRNA levels were significantly increased in the GDM (n=12) compared to the NGC (n=10) group ($p<0.001$) (Figure 2C).

Effects of Maternal Diabetes on the Accumulation of AGEs in Umbilical Cord

Blood and Umbilical Cord Collagen

The result of ELISA showed that there were no differences in terms of the average amount of AGEs in umbilical cord blood and umbilical cord collagen of GDM (n=12) and NGC (n=10) ($p>0.05$) (Table 1).

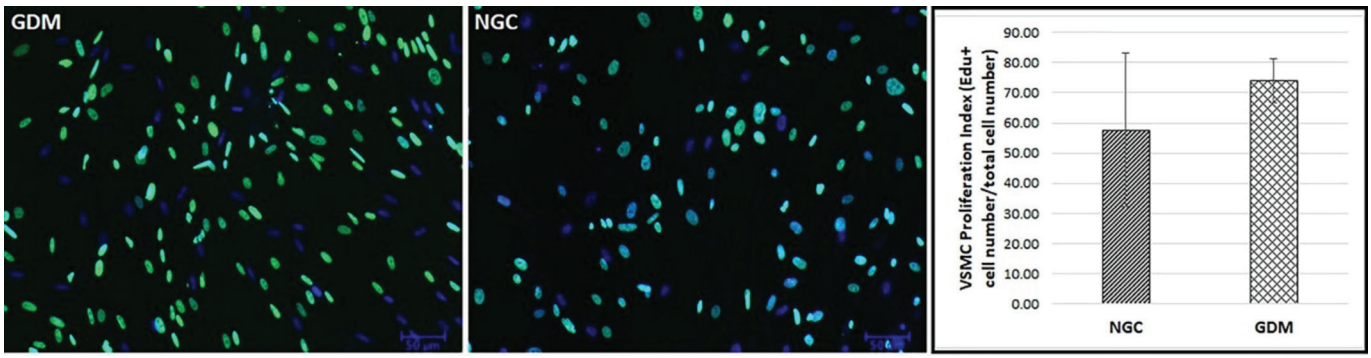


Figure 1. Proliferation index of VSMCs. Cell nuclei were stained for EdU and with Hoechst (Green: EdU positive proliferating cells, Blue: Hoechst stained nuclei). The proliferation index(EdU positive cell number/total cell number)x100 of VSMCs from the GDM group was determined to be significantly higher than the NGC group (p<0.05). Scale bars: 50 µm

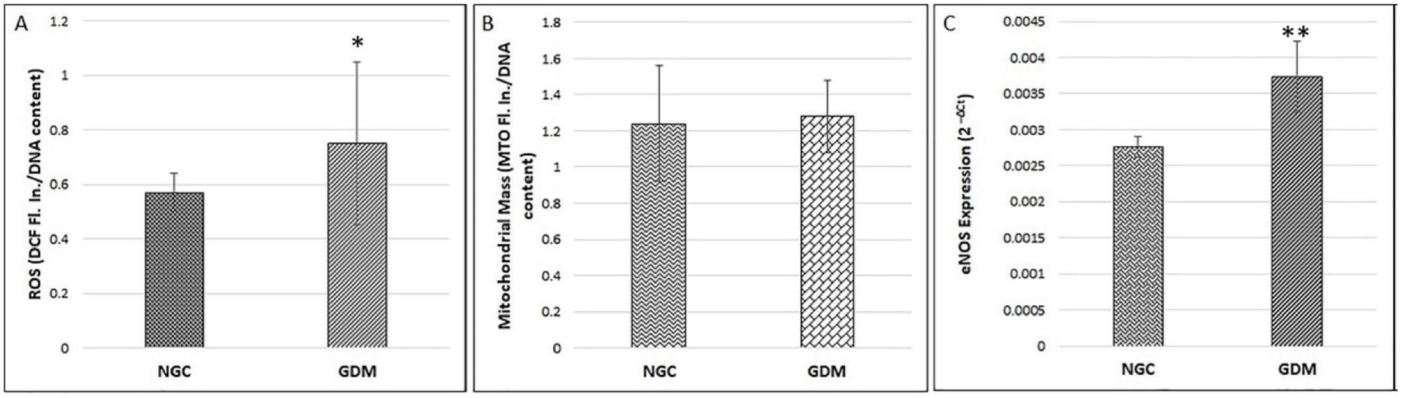


Figure 2. Comparison of ROS production, mitochondrial mass, and eNOS expression in HUVECs between groups. A. ROS production (DCF fluorescence intensity/DNA content), B. Mitochondrial mass (Mitotracker Orange fluorescence intensity/ DNA content), C. eNOS expression (2 −ΔCt). ROS production and eNOS expression were found to be significantly higher in HUVECs from the GDM group (*$p<0.05$ and **$p<0.001$, respectively)

Effects of Maternal Diabetes on the Expression of AGE, RAGE, and Vascular Junctional Proteins of Occludin and β-Catenin in the Placenta and Umbilical Cord

Immunohistochemical analysis showed that AGEs and RAGE (Figure 3) are localized in endothelial cells (shown by the arrows). The immunostaining positivity to AGEs and RAGE was present in endothelial cells of blood vessels in the chorionic villi of the placentas and umbilical cord. Higher immunohistochemical staining to AGEs was found in endothelial cells of umbilical veins in GDM ($p<0.05$). However, no significant differences were identified between the two groups concerning immunohistochemical positivity to RAGE (Table 2). Analysis of adherence and tight junctional proteins showed that β-catenin and occludin were immunolocalized to endothelial paracellular clefts of the placental and umbilical cord arteries and veins in both groups (shown by the arrows) (Figure 3). The result of the analysis showed a slight decrease in immunostaining for β-catenin and occludin in GDM. However, there were no significant differences identified in terms of immunohistochemical positivity to β-catenin and occludin between groups ($p>0.05$) (Table 2).

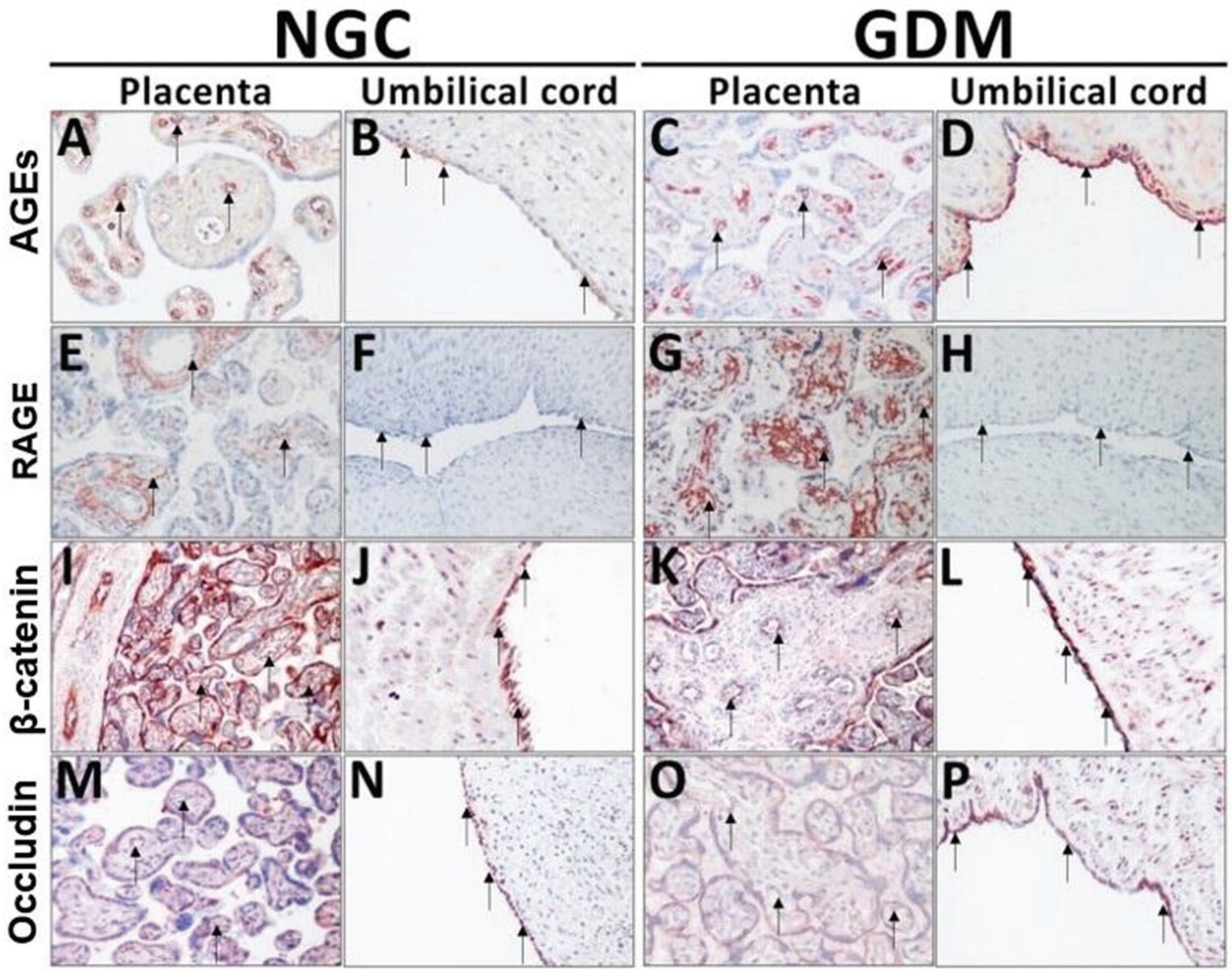
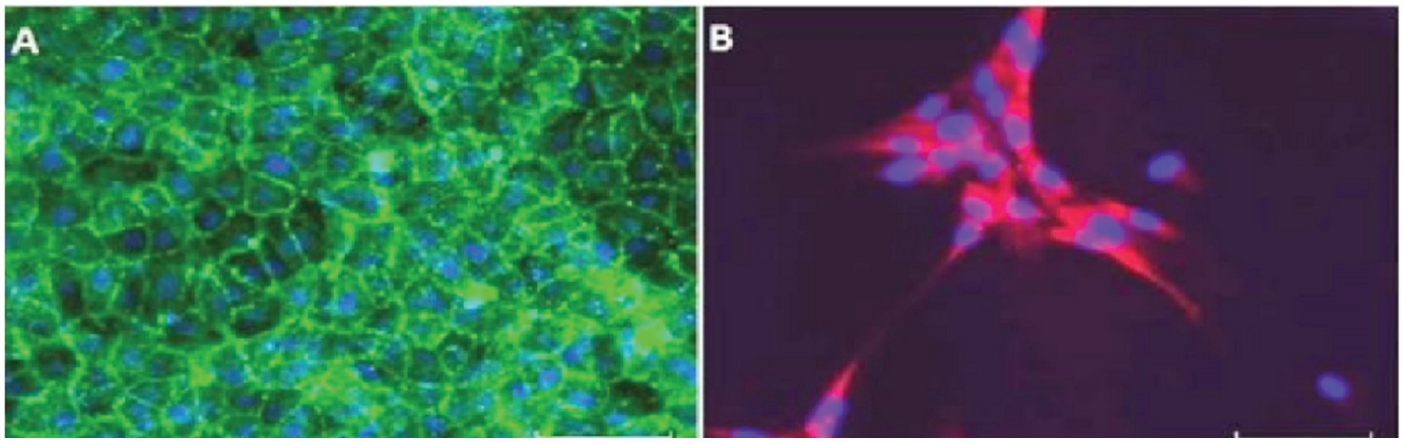


Figure 3. Representative immunohistochemical staining image of AGEs, RAGE, β-catenin, and Occludin in the placenta and umbilical cords. Anti-AGEs, RAGE, β-catenin, and Occludin immunohistochemical staining in endothelial cells of blood vessels in the chorionic villi of the placentas (A, E, I, M) and umbilical cord (B, F, J, N) in NGC (shown by arrow). Anti-AGEs, RAGE, β-catenin, and Occludin in endothelial cells of blood vessels in the chorionic villi of the placentas (C, G, K, O) and umbilical cord (D, H, L, P) in GDM (shown by arrow), respectively. Immunohistochemically positive stained areas were scored by the semi-quantitative method (0: no staining, 1: weak, 2: moderate, 3: high). All panels, 200× magnification

Table 2. Immunohistochemical staining data of the two groups

Variable		GDM group	NGC group	Statistical significance
Occludin staining (placenta)	≥ 1+ (positive)	8 (67%)	7 (78%)	p>0.05
	0 (negative)	4 (33%)	2 (22%)	
Occludin staining (artery)	≥ 1+ (positive)	9 (75%)	5 (63%)	p>0.05
	0 (negative)	3 (25%)	3 (37%)	
Occludin staining (vein)	≥ 1+ (positive)	9 (75%)	6 (67%)	p>0.05
	0 (negative)	3 (25%)	3 (33%)	
β-catenin staining (placenta)	≥ 1+ (positive)	9 (75%)	9 (100%)	p>0.05
	0 (negative)	3 (25%)	0 (0%)	
β-catenin staining (artery)	≥ 1+ (positive)	1 (75%)	6 (75%)	p>0.05
	0 (negative)	10 (25%)	2 (25%)	
β-catenin staining (vein)	≥ 1+ (positive)	3 (75%)	2 (67%)	p>0.05
	0 (negative)	8 (25%)	7 (33%)	
AGEs staining (placenta)	≥ 1+ (positive)	7 (70%)	5 (71%)	p>0.05
	0 (negative)	3 (30%)	2 (29%)	
AGEs staining (artery)	≥ 1+ (positive)	3 (25%)	0 (0%)	p>0.05
	0 (negative)	9 (75%)	8 (100%)	
AGEs staining (vein)	≥ 1+ (positive)	6 (55%)	0 (0%)	*p<0.05
	0 (negative)	5 (45%)	8 (100%)	
RAGE staining (placenta)	≥ 1+ (positive)	7 (70%)	5 (56%)	p>0.05
	0 (negative)	3 (30%)	4 (44%)	
RAGE staining (artery)	≥ 1+ (positive)	3 (33%)	4 (50%)	p>0.05
	0 (negative)	6 (67%)	4 (50%)	
RAGE staining (vein)	≥ 1+ (positive)	1 (11%)	1 (11%)	p>0.05
	0 (negative)	8 (89%)	8 (89%)	

Abbreviations: AGEs, advanced glycation end products; RAGE, the receptor for advanced glycation end products; GDM, gestational diabetes mellitus; NGC, normoglycemic control; *p<0.05 was considered statistically significant



Supplementary Figure. Fluorescence micrographs of HUVECs and VSMCs: A. HUVECs immunostained for CD31, B. VSMCs immunostained for α-SMA. Scale bar: 50 μm

HUVEC and VSMC morphology and culture purity

The morphology and purity of HUVECs and VSMCs were assessed through fluorescence microscopy. All cells in the HUVEC culture were positive for CD31 and all cells in the VSMC culture stained positive for α-SMA in both groups (Supplementary Figure).

DISCUSSION

We demonstrated in the present study that AGEs deposition was substantial within the umbilical cord vein of GDM women. It is supposed that maternal hyperglycemia-induced AGEs accumulation in the umbilical vein either takes place due to fluctuations in blood glucose level despite the insulin treatment or diet control applied afterward (17). In the present study, ROS production in HUVECs was identified as significantly higher in GDM. Our result of increased ROS production in response to hyperglycemia in GDM was consistent with previous reports (18). We also found that umbilical vein-derived VSMCs proliferation was considerably higher in GDM. Elevated ROS may be related to the proliferation and migration of VSMCs. This is parallel with recent studies that ROS promotes VSMCs growth by inducing autologous/paracrine growth mechanisms (19). In addition, ROS mediates the proliferation effect of hormones and growth factors on VSMCs (20). VSMC proliferation is associated with the pathogenesis of DM (21) and atherosclerotic CVD (22).

Moreover, in the current study, increased eNOS expression in HUVECs was identified as markedly different in GDM. ROS elevates the expression of eNOS through posttranscriptional and posttranslational modifications (23). Higher expression in eNOS in HUVECs associated with type 2 DM (24).

From another point of view, in the present study, there were indicators of oxidative stress such as elevated ROS generation and AGEs deposition in the tissue of the GDM mothers. Kostopoulou et al. showed that oxidative stress leads to the development of DM and its complications (25). Cristian E. indicated that GDM exposes the placenta to a hypoxic environment that would disturb vascular function on account of persistently increased oxidative stress. He suggested that hyperglycemia supports this pro-oxidant environment and leads to endothelial dysfunction. Regulation of vascular tone by endothelial cells could be distorted in favor of vasoconstriction and further tissue hypoxia. Continued hyperglycemia probably damages blood vessels and forces β-cells to secrete insulin intensively, causing metabolic and vascular disorders that predispose the mother to CVD in the long run (26).

Furthermore, we investigated whether GDM leads to alterations in the expression of tight and adherent junctional molecules involved in endothelial barrier function and angiogenesis (11). In this study, we found that there is a propensity for reduction of junctional protein expression. However, no significant decrease was observed. We suggest that three-dimensional visualization techniques may be required to confirm enhanced angiogenesis in the GDM placentae.

We characterized levels of RAGE protein expression in the umbilical vein during pregnancy. Interestingly, no difference was identified in the RAGE protein expression levels. Nevertheless, our results confirmed that there was a significant deposition of AGEs in an umbilical vein in GDM.

We could conclude that increased oxidative stress generated from ROS production and AGEs deposition probably elevates the predisposition to vascular endothelial dysfunction of the GDM mother in the long run.

Our study has been limited to a small number of participants. Thus, a high number of participants will result in a more sensitive analysis and more statistically significant conclusive results. Detailed evaluation of oxidative stress in GDM mothers would provide new targets for future research in the prevention of DM or CVD.

CONCLUSION

GDM mothers were under oxidative stress status when compared to the control group. In addition, high eNOS production by HUVECs and increase in proliferative capacity of VSMCs at time of delivery are indicative of a deepening of oxidative stress. Thus, there is a tendency to endothelial dysfunction and the probability of having CVD or type 2 DM increases in mothers with GDM in the long run.

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Conflict of Interest: *The authors declare that they have no competing interest.*

Ethical approval: *This study was conducted by the Declaration of Helsinki. Approval for the study was granted by the Local Ethics Committee of Kocaeli University.*

Authorship contributions: *WA: conceptualization, formal analysis, investigation, methodology, supervision, writing -original draft, writing - review & editing. CYO: data curation, formal analysis, investigation, methodology, validation, visualization, and writing. GKM, ED, VK, BM, and BO: data curation, formal analysis, investigation, methodology, validation, and visualization. ZC, BC, and IT: data curation, formal analysis, investigation, methodology, validation, visualization. HK: data curation, formal analysis, investigation, methodology, validation, visualization, writing-review & editing.*

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