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Maternal Type 1 diabetes activates stress response in early placenta

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ABSTRACT

Introduction: Human pregnancy and in particular the first trimester, is a period highly susceptible towards adverse insults such as oxidative stress, which may lead to inadequate embryonic and fetoplacental development. Diabetes mellitus is associated with increased oxidative stress caused by hyperglycemia, reactive oxygen species (ROS) production and inflammatory signals. In pregnancy, diabetes elevates the risk for early pregnancy loss, preeclampsia and fetal growth restriction, pathologies that origin from early placental maldevelopment. We hypothesized that maternal Type 1 diabetes mellitus (T1DM) induces oxidative stress in the first trimester human placenta.

Methods: We quantified stress induced, cytoprotective proteins, i.e. heat shock protein (HSP)70 and heme oxygenase (HO)-1 and determined protein modifications as markers for oxidation and glycation, i.e. levels of 4-hydroxynonenal (HNE) or Nε-(carboxymethyl)lysine (CML) modified proteins. Moreover, we measured expression levels of enzymes involved in antioxidant defense in the first trimester (week 7–9) placenta of normal and T1DM women by immunoblot and real-time qPCR. Primary human trophoblasts were isolated from first trimester placenta and the effects of oxygen, hyperglycemia and the pro-inflammatory cytokine tumor necrosis factor (TNF)-α on levels of HSP70 and HO-1 were analyzed.

Results: HSP70 (+19.9± 10.1%) and HO-1 (+63.5± 14.5%) were elevated ($p < 0.05$) in first trimester placenta of T1DM women when compared to normal women. However, levels of HNE or CML modified proteins were unchanged. Also, expression of most antioxidant enzymes was unchanged, with only superoxide dismutase 3 (*SOD3*) being upregulated by 3.0-fold ($p < 0.05$). In isolated primary trophoblasts, HSP70 and HO-1 were upregulated by increasing oxygen tension, but not by hyperglycemia or TNF-α.

Conclusion: Although protein oxidation and glycation was not elevated, we infer that T1DM increases placental cellular stress in the first trimester. Elevated stress in early placenta of T1DM women may contribute to disturbances in placental development.

Oxidative stress occurs when there is an imbalance between formation of reactive oxygen species (ROS) and antioxidant defense [1]. ROS are formed through a variety of extracellular and intracellular actions. ROS are generated as byproduct of mitochondrial oxidative metabolism, formed as part of signal transduction pathways induced by pro-inflammatory cytokines, or as cell defense mechanism (reviewed by Zhang et al. [2]). In order to resist excessive formation of ROS, antioxidant proteins are produced which gradually neutralize oxygen radicals [3]. An increase in ROS levels or a decrease in antioxidant system may lead to oxidative stress. Oxidative stress provokes modification of cellular proteins, lipids and DNA, and thereby affects not only cell behavior and cell differentiation, but can also induce apoptosis and cell damage [1,3].

Antioxidant defense strategy is of particular importance for embryonic and fetal tissue development, which is characterized by high activity of proliferation and differentiation processes. Thus, the first trimester of human pregnancy is a period highly sensitive and susceptible towards oxidative stress. Therefore, during early pregnancy the placenta and the embryo develop in a low oxygen environment which is thought to protect the embryo from oxidative damage. At the end of the first trimester, utero-placental blood

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flow is established and placental oxygen concentration raises rapidly [4,5]. This oxidative burst is an important signal for certain placental and fetal developmental processes. Hence, both, hypoxia at the beginning and the subsequent oxidative burst at the end of the first trimester are implicated as regulators that drive normal placental morphogenesis and function [5].

Diabetes mellitus is a condition associated with increased oxidative stress due to increased formation of ROS [6], with hyperglycemia as the major underlying cause. On one hand, hyperglycemia enhances activity of the respiratory chain and stimulates ROS production [7]. On the other hand, hyperglycemia leads to protein glycation, forming so-called advanced glycation end products (AGE) which further elevate cellular ROS production [7]. Also a pro-inflammatory environment stimulates the formation of ROS due to cytokine signalling [8].

The presence of increased oxidative stress in the human placenta at the end of diabetic pregnancies is well established [7,9–12]. Placental tissue from pregnancy terminations in diabetic pregnancies is enormously difficult to avail. Therefore, studies investigating the effect of maternal diabetes on placental development in the first trimester of pregnancy are almost entirely missing, with few exceptions [9,10]. One found ultrastructural alterations in mitochondria in the syncytio- and cytotrophoblast in T1DM. These may reflect changes in mitochondrial activity, which in turn could entail changes in ROS generation in this condition. Pregnancies complicated by T1DM have an increased risk for placenta-associated pregnancy pathologies, such as preeclampsia, fetal growth restriction and early pregnancy loss [11–13], suggesting a detrimental effect of T1DM on early placental development, to which oxidative stress as adverse consequence of T1DM may contribute. Thus, we hypothesized that maternal T1DM is associated with an oxidative stress response in the early placenta.

We have established a small placenta tissue bank from first trimester T1DM pregnancies. This enabled us to measure the formation of 4-hydroxynonenal (HNE) modified proteins as an indicator for lipid peroxidation [14], levels of Nε-(carboxymethyl)lysine (CML) modified proteins as marker for AGE formation [15] and protein levels of heat shock protein 70 (HSP70) and heme oxygenase-1 (HO-1) as markers for intracellular stress [16,17], in age matched first trimester placental tissue from healthy and T1DM mothers. In addition, we compared placental expression of key antioxidant enzymes between T1DM cases and controls.

1. Methods

1.1. First trimester placental samples

The study was approved by the institutional review board and ethical committee of the Medical University of Graz (23–435 ex 10/11). The characteristics of the Caucasian subjects are shown in Table 1. Patients were examined using endovaginal sonography with a high-resolution digital scanner GE Logic 400 with a 5- to 7.5-MHz vaginal probe. Embryonic heart movements were registered and the crown rump length (CRL) was measured in the plane of maximal resolution. The gestational age was calculated based on the patient's last menstrual period. Patients were only included if the sonographic gestational age as determined from CRL was equal to ± 1 days to the menstrual gestational age. After legal elective pregnancy termination for psycho-social reasons placenta samples were obtained with written informed consent, washed immediately in PBS, snap-frozen and stored for further analyses.

Table 1
Subject characteristics.

	Control	T1DM
Number of subjects	9	8
Maternal age [range]	32.8 ± 7.3 [21–40]	30.4 ± 5.8 [22–38]
BMI (kg/m^2)	24.4 ± 2.5	23.1 ± 1.4
HbA_{1C} (%)	≤6*	7.8 ± 1.7
Gestational week [range]	7.9 ± 0.8 [7–9]	8.1 ± 0.8 [7–9]

Data are presented as mean ± SD unless stated otherwise; * cut-off value in routine laboratory.
BMI: body mass index, HbA_{1C}: glycosylated hemoglobin A.

1.2. Isolation and culture of primary first trimester trophoblasts

Primary trophoblasts were isolated from first trimester villous placental tissues between gestational weeks 7–9 from 11 different healthy women by enzymatic digestion with trypsin/dispase. Percoll gradient centrifugation and negative magnetic bead immunopurification with the anti-leukocyte marker CD45 (Thermo Fisher Scientific, Invitrogen, Rockford, IL) and anti-fibroblast marker CD90 (Dianova, Hamburg, Germany) were performed as described earlier [18]. Purity was determined by immunocytochemical staining for cytokeratin 7 (CK7, Dako, Glostrup, Denmark). After isolation the first trimester trophoblasts were resuspended in keratinocyte medium (Thermo Fisher Scientific, Gibco, Carlsbad, CA) supplemented with the keratinocyte SFM kit (Thermo Fisher Scientific, Gibco) containing epidermal growth factor (EGF1-35), bovine pituitary extract (BPE) and FBS. Cells were seeded on plastic dishes and cultured at 37 °C. In experiments testing the effect of oxygen, the cells were exposed to different oxygen concentrations (5, 12 and 21%) for 48 h (n = 7). For hyperglycemic treatments, cells were grown in normoglycemic (5.5 mM D-glucose) or hyperglycemic (12 and 20 mM D-glucose) concentrations for 48 h (n = 4). The presence of hyperglycemia throughout the treatment was determined by glucose measurements of the culture medium (Glucose 201, HemoCue, Ängelholm, Sweden). To investigate the effect of pro-inflammatory environment, TNF-α (Reliatech, Wolfenbüttel, Germany) was added to a final concentration of 25 ng/ml and cells were cultured for 24 h.

1.3. RNA isolation and quantitative real-time PCR

Total RNA from T1DM and control placentas was isolated by using a homogenizer (Ultra-Turrax, IKA T10) and 1 ml TriReagent (MRC, Cincinnati, OH) according to the manufacturer's instructions. One μg isolated total RNA was reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, Invitrogen) according to the manufacturer's instructions. cDNA was subjected to quantitative real-time PCR (qPCR) using carboxy-fluorescein-dye (FAM) labeled TaqMan Gene Expression Assays (Applied Biosystems, Branchburg, NJ) for catalase (*CAT*, Hs00156308_m1), glutathione reductase (*GSR*, Hs00167317_m1) glutathione peroxidase 1 (*GPX1*, Hs00829989_gH), 3 (*GPX3*, Hs00173566_m1), 4 (*GPX4*, Hs00989766_g1), glutathione superoxide dismutase 1 (*SOD1*, Hs00533490_m1), 2 (*SOD2*, Hs00167309_m1), and 3 (*SOD3*, Hs00162090_m1) using the TaqMan Universal PCR Mastermix (Applied Biosystems). The expression of the ribosomal protein L30 (*RPL30*, Hs00265497_m1) and *HPTR1* (Hs01003267_m1) was shown to be stable a reference genes in the placenta [19] and was used as house-keeping genes by calculating the mean of their Ct values for analysis. The components were mixed according to the manufacturer's instructions and amplified in 20 μl total volume/well (96 well plates, Roche, Germany) using an ABI7900 (Applied Biosystems) real-time cycler. Ct values were automatically generated by the software (SDS2.2, Applied Biosystems) and relative gene expression was calculated by the standard $2^{-\Delta\Delta Ct}$ method. Statistical analyses used the ΔCt values.