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Mitochondrial DNA Copy Number and Oxidative DNA Damage in Placental Tissues from Gestational Diabetes and Control Pregnancies: A Pilot Study

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SUMMARY

Background—Available evidence supports the role of reactive oxygen species in the pathogenesis of placental insufficiency, gestational diabetes mellitus (GDM), and other pregnancy complications. Abnormal placental mitochondrial function resulting from reactive oxygen species may also be an important precedent of adverse perinatal outcomes.

Methods—We investigated the association of placental oxidative stress with placental mitochondrial DNA (mtDNA) copy number, an indicator of placental mitochondrial density and possible mitochondrial dysfunction, using samples collected from GDM cases and controls. 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, was measured in placentas of 19 GDM cases and 21 controls using a competitive immunoassay. Placental mtDNA copy number was determined using real-time quantitative PCR. Bivariate and multivariable linear regression procedures were used to evaluate associations of these two biomarkers.

Results—Placental DNA oxidation was positively associated with mtDNA copy number in both GDM and control placentas. After adjusting for maternal age, pre-pregnancy body mass index and gestational age at delivery, mtDNA copy number increased ($\beta=67.0$; 95% CI 27.8–106.2, $p=0.001$) for every 0.1 ng/ μ g increase of placental 8-OHdG among GDM cases and controls.

Conclusions—These cross sectional data suggest an association of placental mtDNA copy number with oxidative stress. The consequences of placental oxidative stress and mitochondrial dysfunction on the course and outcomes of pregnancy remain to be elucidated in larger prospective studies.

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Declaration of Interests

The authors report no competing financial interests.

Keywords

Mitochondrial DNA (mtDNA); placenta; gestational diabetes mellitus; pregnancy; oxidative DNA damage; oxidative stress; 8-hydroxy-2'-deoxyguanosine (8-OHdG)

INTRODUCTION

The placenta, a regulator of nutrient composition and supply from mother to fetus, is the source of hormonal signals that affect maternal and fetal metabolism. The appropriate development and functioning of the placenta is crucial to normal fetal programming, growth, and development [1]. Consequently, placental development and function requires increasing mitochondrial capacity to meet the increasing needs of the embryo. Placental mitochondria might play an important role in the maintenance of pregnancy and neonatal development through regulation of metabolic activity and adenosine triphosphate (ATP) production [2], hormone synthesis [3], and trophoblast oxygen sensing [4]. Mitochondrial dysfunction, induced by environmental toxins such as tobacco smoke [5] or maternal medical conditions such as preeclampsia [6], may cause cell damage and death by compromising ATP production, calcium homeostasis and increased oxidative stress.

Mitochondria, ubiquitous membrane-enclosed organelles, are recognized as the site of aerobic oxidation of metabolic fuels [7]. Mitochondria are at the crossroads of several crucial activities including ATP generation via oxidative phosphorylation; the biosynthesis of heme, pyrimidines and steroids; calcium and iron homeostasis; and programmed cell death [7–8]. By releasing several proteins that incite programmed cell death, mitochondria are thought to act as “executioners” in apoptosis [9]. Results from studies that have focused on understanding mitochondrial biology (primarily studying mitochondria isolated from muscle) have shown that smoking, hypercholesterolemia, and obesity can induce mitochondrial DNA damage and dysfunction [10]. Recently investigators have shown that hyperglycemia and diabetes may also contribute to mitochondrial dysfunction and oxidative stress [11]. This observation, coupled with findings from both experimental and clinical studies, suggest that there may be a close link between oxidative stress, impaired mitochondrial function and the pathogenesis of type 2 diabetes [12–13]. Hyperglycemia, for instance, is known to induce an overproduction of reactive oxygen species (ROS) and also attenuate antioxidant defense mechanisms thus contributing to oxidative stress.

Limited attention has been given to the role of mitochondrial dysfunction and oxidative stress in the etiology of gestational diabetes mellitus (GDM), a condition that is biochemically and epidemiologically similar to type 2 diabetes [14–15]. The limited available data suggest that oxidative DNA damage may be predictive of GDM [16]. For instance, in an earlier study of 55 GDM cases and 43 controls, Qiu and colleagues reported that maternal early pregnancy urine 8-OHdG concentrations were 26% higher in women who went on to develop GDM as compared to women who did not develop the disorder (mean $\pm$ standard deviation: 7.98 ± 4.12 vs. 6.31 ± 2.49 ng/mg creatinine; p -value=0.02). In view of the importance of mitochondria in oxidative glucose metabolism, and increased oxidative stress in the pathogenesis of type 2 diabetes and GDM [12–13, 16], we postulated

an association between mtDNA density (copy number) and oxidative stress in the placenta. We used data and DNA samples remaining from an earlier study [17] to preliminarily test our hypothesis.

MATERIALS AND METHODS

Study Population and Data Collection

Study methods, described earlier [17–19] were briefly as follows. Participants were enrolled in a study designed to examine differential placental gene expression associated with pregnancy complications [17, 19]. Participants were recruited among women who delivered at Swedish Medical Center, Seattle, WA. GDM was defined by the presence of 2 of the following four oral glucose tolerance test results based on the American Diabetes Association (ADA) criteria [20]: fasting 5.3 mmol/L (95 mg/dL); 1-hour 10.0 mmol/L (180 mg/dL); 2-hour 8.6 mmol/L (155 mg/dL); 3-hour 7.8 mmol/L (140 mg/dL) [20]. Controls, frequency matched to cases for gestational age and mode of delivery, were selected among participants who had a pregnancy uncomplicated by GDM. Women with a history of pre-gestational diabetes were excluded from this study. Among eligible women, 19 GDM cases and 21 controls consented, and provided placental samples at delivery. Information on risk factors, pregnancy history and outcome was obtained from medical records. All study procedures were approved by the Institutional Review Board of Swedish Medical Center. All participants provided written informed consent.

Placental Sample Collection

Placentas were weighed, double bagged and transported in coolers. The chorionic plate and overlying membranes were removed and tissue biopsies (approximately 0.5 cm³ each) were obtained from 16 sites (8 maternal and 8 fetal) using a grid system [17, 19]. For this analysis, biopsy samples taken from the fetal side, which consisted of the intervillous tissues and chorionic villi, were evaluated. Biopsies were placed in cryotubes containing RNAlater (Qiagen Inc, Valencia, CA), at 10µL per 1 mg of tissue and stored at –80°C.

Placental DNA Extraction

Approximately 25 mg of placental tissue (in aggregate from the 4 biopsy sites) was homogenized using a Tissue Tearor (Biospec Products Inc., Bartlesville, OK) in a lysis buffer from the Qiamp DNA Mini Kit (Qiagen Inc, Valencia, CA) with added Proteinase Kt. DNA was extracted using a standardized protocol adapted from Qiamp DNA Mini Kit (Qiagen Inc, Valencia, CA).

Placental Mitochondrial DNA (mtDNA) Determination

Placental DNA was used as a template in real-time quantitative polymerase chain reaction (PCR) analysis using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). The RNase P gene was used as an endogenous control (catalog # 4316844; Applied Biosystems), and Applied Biosystems MT-7S (catalog # Hs02596861_s1) encoding the D-loop of the mitochondrial DNA as the target gene. RNase P is a single-copy nuclear gene, MT-7S is the replication start site for mtDNA. Experiments were performed using 10 ng placental DNA in a 20µL reaction made up of 10µL 2X TaqMan universal PCR master

mix, 1μL primer, and nuclease-free water in a 96-well reaction plate. MT-7S and RNase P reactions were run in duplicate in separate wells. Cycling conditions were: 50° C, 2 minutes; 95° C, 10 minutes; followed by 40 cycles of 95° C, 15 seconds and 60° C, 1 minutes. Data were analyzed by using the comparative Ct method, where Ct is defined as the cycle number in which fluorescence first crosses the threshold. Ct was determined by subtracting the RNaseP Ct values from the MT-7S Ct values. The result was applied to the term $2^{(-Ct)}$.

Placental 8-OHdG Determination

Levels of 8-OHdG in placental DNA were measured using the New 8-OHdG Check Enzyme-Linked Immunosorbent Assay Kit (Catalog #KOG-200SE Genox Corp., Baltimore, MD). This ELISA method offers a valid and comparatively simple alternative to more technically demanding HPLC-EC or GC-MS techniques for the quantitative assessment of oxidative DNA damage [21]. Moreover, investigators have reported excellent correlation between HPLC and ELISA methods ($r = 0.96$) [22]. DNA samples were pretreated in a digest with Nuclease P1 using the Wako 8-OHdG Assay Preparation Reagent Set (Catalog # 292-67801 Wako Chemicals, Richmond, VA) and then applied in duplicate to the New 8-OHdG Check ELISA. The intra-assay coefficient of variation (CV) was 6.4%. All assays were performed without knowledge of pregnancy outcome.

Statistical Analysis

We examined the frequency distributions of maternal socio-demographic characteristics and medical and reproductive histories according to GDM case-control status. We also examined the distribution and differences of placental mtDNA copy number or 8-OHdG levels according to case and control status. Associations of maternal placental mtDNA copy number and 8-OHdG levels were estimated, for GDM cases and controls subjects separately, using Pearson's correlation coefficients (CC). The two biomarkers were approximately normally distributed, therefore parametric statistics were used. Multivariate linear regression model was used to examine the relationship between placental mtDNA copy number (outcome variable) and placental 8-OHdG concentrations (exposure variable). We evaluated all the covariates in Table 1 as potential confounders. To assess confounding, we entered variables into a linear regression model one at a time and then compared coefficients. Final linear regression models included covariates that altered unadjusted coefficients by at least 10%, as well as those covariates of *a priori* interest (e.g., maternal age and race/ethnicity). The final model included maternal age, pre-pregnancy body mass index and gestational age at delivery. The unadjusted and adjusted R^2 values are reported for each model and represent the total variation of placental mtDNA copy number explained by covariates in bivariate and multivariable models, respectively. All analyses were performed using Stata 9.0 (Stata, College Station, TX). All reported confidence intervals were calculated at the 95% level and all reported p-values are two-tailed.

RESULTS and DISCUSSION

Characteristics of GDM cases and controls are summarized in Table 1. GDM cases and controls were similar with regards to maternal age, parity, pre-pregnancy adiposity,

gestational age and mode of delivery. Cases were more likely than controls to be in the race/ethnicity group other than non-Hispanic White.

Because the distribution of placental mtDNA copy number and 8-OHdG concentrations were approximately normally distributed, we examined differences in mean concentrations between cases and controls using the Student's t test. Mean placental mtDNA copy number was similar for GDM cases and controls (mean $\pm$ standard deviation: 397 ± 121 vs. 412 ± 117 ; p-value=0.69). Mean placental 8-OHdG concentrations were also similar for GDM cases and controls (mean $\pm$ standard deviation: 0.30 ± 0.11 vs. 0.31 ± 0.07 ng/ μ g DNA; p-value=0.71). In bivariate analyses, placental mtDNA copy number was statistically significantly and positively correlated with placental 8-OHdG concentrations among GDM cases (Pearson CC: $\rho=0.56$; p-value=0.01). Similar positive correlations of placental mtDNA with placental 8-OHdG were noted in controls, but these values did not reach statistical significance (Pearson CC: $\rho=0.39$; p-value=0.08) (Figure 1A). Given that correlation coefficients were of similar directions and magnitudes, we combined cases and controls and repeated analyses. From this combined analysis (Figure 1B) we found that placental mtDNA copy number was statistically significantly and positively correlated with placental 8-OHdG concentrations (Pearson CC: $\rho=0.48$; p-value=0.002).

We next sought to further explore the association between placental mtDNA copy number and placental 8-OHdG concentrations in unadjusted and adjusted linear regression models after controlling for potential confounding factors. On the basis of our bivariate analyses documenting similar magnitudes of associations for placental mtDNA copy number and 8-OHdG concentrations in GDM or controls, we completed all multivariable analyses of GDM cases and controls combined. This strategy allowed for greater statistical power without concern about heterogeneity. In an initial simple unadjusted linear regression model, we noted that mtDNA copy number increased ($\beta=61.4$, 95% CI 26.3–96.5, p-value=0.001) for every 0.1 ng/ μ g increase in placental 8-OHdG concentration. We also noted that 23% of the variation in placental mtDNA copy number was accounted for by placental 8-OHdG concentrations in this simple model. The positive association of placental mtDNA copy number with placental 8-OHdG remained after we adjusted for potential confounders. After adjusting for maternal age, pre-pregnancy BMI and gestational age at delivery, mtDNA copy number increased ($\beta=67.0$; 95% CI 27.8–106.2, p=0.001) for every 0.1 ng/ μ g increase of placental 8-OHdG. The adjusted $R^2=38\%$.

This pilot study showed that placental mtDNA copy number was positively associated with a biomarker of oxidative DNA damage in placentas delivered from women with pregnancies complicated by GDM and those without GDM. The magnitude and direction of associations were similar for the two groups. Though we are unaware of any published studies of this topic in pregnancy, our finding is largely consistent with the report by other research team using different sample or tissue [23, 24]. For example, Liu et al first reported that 8-OHdG content in human leukocytes were positively correlated with the copy number of mtDNA isolated from leukocytes (p-value = 0.003) [23]. Their results also indicated that the copy number of mtDNA in leukocytes changed with age in a biphasic manner that fits in a positively quadratic regression model ($P = 0.001$). In another study of lung cancer tissue biopsies, Lin et al reported that relative mtDNA copy numbers were significantly correlated

to 8-OHdG formation in mtDNA (Pearson correlation coefficient=0.40, p-value=0.03) [24]. The finding from our pilot study extends the literature to include assessment of mitochondrial dysfunction and oxidative DNA damage to another tissue, the placenta. If confirmed in an independent sample, our findings suggest that alternations in placental mtDNA copy number may reflect placental DNA oxidative stress in both GDM and uncomplicated pregnancies. Our small sample size, however, did not allow for adequate statistical power to evaluate the functional and clinical consequences of placental oxidative DNA damage and mtDNA copy number alterations in GDM versus control pregnancies. Future larger, prospective cohort studies with more detailed information elicited and comprehensively measures of other markers of mitochondrial function and electron transport chain activity will help to clarify the possible causal relationship between mitochondrial dysfunction, reactive oxygen species production, mtDNA modifications, placental insufficiency and adverse pregnancy outcomes.

Increased oxidative stress in pregnancy has also been implicated in the pathogenesis of a number of pregnancy complications and adverse outcomes including gestational diabetes mellitus [16], preeclampsia [25–26], preterm birth [27], intrauterine growth retardation [25] and low birth weight deliveries [28]. Stein et al [27] reported that elevated urine 8-OHdG concentrations measured in early pregnancy were associated with reduced infant birth weight and with shortened length of gestation. Using information and urine specimens gathered as part of a prospective cohort study of women receiving prenatal care before 20 weeks gestation, Qiu and colleagues examined the association between early-pregnancy urine 8-OHdG concentrations, normalized using creatinine concentrations, with the subsequent risk of GDM. The authors found that the relative risk of developing GDM was 3.8-fold higher for mothers with high urine 8-OHdG concentrations (8.01 ng/mg creatinine) as compared with mothers who had lower concentrations (<4.23 ng/mg creatinine) (OR=3.79; 95%CI 1.03–14.00) [16].

Mitochondria are both the major intracellular source and primary target of oxidative stress. Investigators have previously shown that cells challenged by oxidative stress synthesize more copies of their mtDNA and increase their mitochondrial density or abundance to compensate for damage, and to meet increased respiratory demand required for clearance of reactive oxidative species [29]. Conversely, oxidative stress resulting from increased mitochondrial abundance may contribute to oxidative damage to mitochondria and other intracellular constituents including nuclear DNA, RNA proteins and lipids. In summary, our preliminary investigation showed that placental mtDNA copy number is statistically significantly and positively associated with increased oxidative stress. Alterations in mtDNA copy number in placental tissues may be an important biomarker of placental oxidative stress and bioenergetic changes in the mitochondria.

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Figure 1.

Relationship between placental mitochondrial DNA (mtDNA) copy number and placental oxidative DNA damage in GDM (**RED**), control (**BLUE**) and the groups combined (**GREEN**). Total DNA isolated from placental tissues were quantified by real-time quantitative PCR analyses using the RNase P as an endogenous control. Data were analyzed using comparative Ct method and are expressed as 2^{-Ct} . Placental 8-OHdG is expressed as ng/ μ g DNA.

Table 1

Socio-demographic and other characteristics of the study subjects, Seattle, Washington, USA

Characteristics	GDM Cases (N=19)	Controls (N=21)	p-value
	n (%)	n (%)	
Maternal Age, years *	32.8 ± 4.3	34.2 ± 4.8	0.32
25–34	12 (63.2)	11 (52.4)	0.49
35	7 (36.8)	10 (47.6)	
Maternal Race/Ethnicity			
White	9 (47.4)	17 (81.0)	0.13
African American	1 (5.3)	0 (0.0)	
Asian	5 (26.3)	3 (14.3)	
Other	2 (15.8)	1 (4.7)	
Missing	2 (10.5)	0 (0.0)	
Nulliparous	8 (42.1)	5 (23.8)	0.22
Twin Pregnancies	2 (10.5)	2 (9.5)	1.00
Delivery Mode			
Vaginal	7 (36.8)	7 (33.3)	0.82
C-Section	12 (63.2)	14 (66.7)	
Labored Before Delivery	13 (68.4)	8 (38.1)	0.06
Gestational Age at Delivery (weeks) *	37.7 ± 2.9	38.4 ± 1.7	0.36
Preterm Delivery (<37 weeks)	4 (21.1)	2 (9.5)	0.40
Pre-gestational Body Mass Index (kg/m ²) *	28.7 ± 6.8	25.9 ± 7.2	0.23
Infant Birth Weight (g) *	3484 ± 1049	3546 ± 513	0.81
Placental Mitochondrial DNA (2 ^{-ct}) *	397 (121)	412 (117)	0.69
Placental 8-OHdG Concentration (ng/μg DNA) *	0.30 (0.11)	0.31 (0.07)	0.71

* Mean ± standard deviation (SD); Abbreviations: BMI: body mass index; kg/m²: kilogram/meter²