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Evaluation of the Future Atherosclerotic Heart Disease with Oxidative Stress and Carotid Artery Intima Media Thickness in Gestational Diabetes Mellitus

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Background and objective. In this study our aim was to evaluate paraoxonase (PON1) activity and free sulfhydryl groups (–SH) as antioxidative parameters and lipid hydroperoxide (LOOH) as oxidative parameter in the serum of women with gestational diabetes mellitus (GDM) and determine their relation with the degree of subclinical atherosclerosis. *Material and methods*. Serum samples from 39 pregnant women complicated with GDM and 40 healthy pregnant women were collected for the analysis of oxidative markers. Common carotid artery intima media thickness (CIMT) was measured for both groups to assess future atherosclerotic heart disease risk. PON1 activity and –SH were measured spectrophotometrically. LOOH levels were measured by ferrous oxidation with a xylenol orange assay. *Results*. CIMT and LOOH levels were significantly higher ($p = 0.01$, $p < 0.001$, respectively) in GDM group compared to controls, whereas PON1 and –SH levels were significantly lower ($p < 0.001$ for both). CIMT values were significantly correlated with body mass index (BMI), 50 g oral glucose tolerance test (OGTT), and mean arterial blood pressure (MABP) ($p = 0.003$, $p = 0.02$, and $p = 0.03$, respectively). However, there was no correlation between CIMT and oxidative markers. *Conclusions*. Increased levels of LOOH and decreased levels of PON1 and –SH showed disturbance of antioxidative mechanisms in GDM. These changes were associated with increased BMI and MABP which may be relevant to GDM pathophysiology. Furthermore, increased CIMT values in GDM compared to healthy controls designate increased risk of future atherosclerotic heart disease.

Keywords Common carotid artery intima media thickness, Gestational diabetes mellitus, Oxidative stress, Paraoxonase, Free sulfhydryl groups, Lipid hydroperoxide, Subclinical atherosclerosis, Doppler ultrasonography, Glucose tolerance test

INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as carbohydrate intolerance of variable severity with the identification of onset during pregnancy (1). It is the most

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common metabolic disorder of pregnancy, occurring in 1–10% of all pregnancies (2). GDM has been considered as a "prediabetic" state, and clearly both diseases share common pathophysiology (3). Although most women with GDM regain normal glucose tolerance after delivery, it is documented that up to 70% of them possibly will develop type 2 diabetes mellitus (DM) later in their life (4). Also they are under risk of atherosclerosis and hypertension. Since DM plays a major role in the pathogenesis of atherosclerosis (5), it is an independent risk factor for the development of coronary artery disease and is also associated with the development of an atherogenic lipid profile and increased oxidative stress (6).

Common carotid artery intima media thickness (CIMT) is a noninvasive technique to evaluate subclinical atherosclerosis (7). It is a valid representative of morbidity and mortality risk of vascular diseases (8). Increased CIMT predicts the risk of cardiovascular disease in type 2 DM; furthermore, it is a marker of atherosclerosis in other vascular beds (7). On the other hand, reduction of postprandial hyperglycemia in type 2 DM patients had regression of CIMT (9). Therefore, hyperglycemia is generally accepted to be the major cause of microvascular atherogenesis (10).

Oxidative stress is an impaired balance between the production of reactive oxygen species and antioxidant defense mechanisms and related with tissue injury (11). Association of oxidative stress with DM is well described previously (12,13). However, limited data are available regarding the oxidative stress in GDM patients. CIMT in GDM patients is also investigated in a few studies (14,15).

The aim of this study was to evaluate paraoxonase (PON1) activity and free sulfhydryl groups (–SH) as antioxidative parameters and lipid hydroperoxide (LOOH) as oxidative parameter in the serum of women with GDM and determine their relation with the degree of subclinical atherosclerosis. The hypothesis to be tested here was that both oxidative stress and CIMT were significantly increased in GDM compared to nondiabetic controls.

METHODS

Study Population

This study was conducted in the Department of Obstetrics and Gynecology, School of Medicine, Harran University. Singleton pregnancies between 24 and 28 weeks of gestation were included into study between April 2009 and August 2010. Informed consent for study participation was obtained from all women. The study protocol conforms to the principles of the Helsinki Declaration and was approved by the Medical Ethics Committee of Harran University. The study group included 39 patients with GDM and the control group was composed of 40 healthy pregnant women who had a negative oral glucose tolerance test (OGTT). Exclusion criteria for all study participants included smoking, alcohol abuse, preeclampsia, multiple pregnancies, pregestational DM, and having family history of DM for control group. The study was performed in a cross-sectional design.

Gestational age determination was based on routine ultrasound examination performed before the end of the 19th completed gestational week. Body mass index (BMI) of patients was estimated during the 50 g OGTT.

OGTT Measurement

As a routine screening test, the 50 g OGTT was performed independent of the time of day or any previous meals (16). A 100 g, 3 h OGTT was recommended to all patients whose 1 h test result is over or equal to 140 mg/dL. OGTT was performed in the morning after at least 8 h fasting. Women who had two or more abnormal values according to the Carpenter–Coustan criteria were diagnosed to have GDM (17). Women whose

50 g OGTT results were greater than 190 mg/dL were also accepted to have GDM. OGTTs of 50 and 100 g were performed between 24th and 28th weeks of gestation, respectively. Since we performed 50 g OGTT for both groups, we used 1 h result of this test for the comparisons. After diagnosis of GDM, treatment was planned for each patient individually.

Serum Collections

All blood samples were obtained in the morning from the cubital vein into blood tubes after an overnight fast. Blood samples were immediately separated from the cells by centrifugation at 3000×*g* for 10 min and stored at −80°C until analyses.

CIMT Evaluation

All the sonographic examinations were performed by an examiner, who was unaware of the subject's clinical status (GDM vs. unaffected pregnancy) throughout the study. Each subject was studied in the morning hours (8:00–10:00). None of the participants was using vasoactive drugs. Studies were performed in a quiet, temperature-controlled room (20–25°C). Images were obtained with high-resolution Doppler ultrasonography (Logiq 7 Pro; General Electric, Milwaukee, WI, USA) with a 12 MHz linear-array transducer. Sonographic examinations were evaluated by the same investigator to avoid interobserver variations.

Bilateral assessment of wall thickness was made in the common carotid artery (CCA). CIMT was measured as the distance from the leading edge of the first echogenic line to that of the second echogenic line. The first line represents the lumen–intima interface, and the second line the collagen-containing upper layer of tunica adventitia. CIMT measurement of both the right and left CCAs was performed at three points on the far walls in each CCA from 2 cm proximal to the bifurcation of the CCA. Three locations were then averaged to produce the mean CIMT for each side. Average of two sides was evaluated as mean CIMT.

Serum PON1 Activity Analysis

Measurements of PON1 activities were performed in the absence of basal activity. The rate of paraoxon hydrolysis (diethyl-*p*-nitrophenylphosphate) was measured by monitoring the increase of absorbency at 412 nm at 37°C. The amount of generated *p*-nitrophenol was calculated from the molar absorptivity coefficient at pH 8, which was 17000/M/cm (18). PON1 activity was expressed as unit per liter serum. Phenylacetate was used as a substrate to measure the arylesterase activity. Enzymatic activity was calculated from the molar absorptivity coefficient of the produced phenol: 1310/M/cm. One unit of arylesterase activity was defined as 1μmol phenol generated per minute under the above conditions and expressed as unit per liter serum (19).

Serum –SH Level Analysis

Serum –SH levels were assayed according to the method of Elman (20) as modified by Hu et al. (21). Briefly, 1 mL of buffer containing 0.1 M Tris, 10 m M EDTA, pH 8.2, and 50 μL serum was added to cuvettes, followed by 50 μL of 10 mM 5,5-dithiobis 2-nitrobenzoic acid in methanol. Blanks were run for each sample as a test. After incubation for 15 min at room temperature, sample absorbance was interpreted at 412 nm on a Cecil 3000 spectrophotometer (Cecil Instruments, Cambridge, UK). Sample and reagent blanks were subtracted. The concentration of –SH groups was calculated using reduced glutathione as the free –SH group standard and the results were expressed as millimolars per liter. Coefficients of variation for measurement of serum –SH levels were 3.6%.

Serum LOOH Level Analysis

Serum LOOH levels were measured by the ferrous ion oxidation-xylenol orange (FOX-2) method as previously described (22).

Serum Lipid Profile and HbA_{1c}

The levels of triglycerides (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and fasting blood glucose (FBG) were determined using commercially available assay kits (Abbott®, Chicago, IL, USA) in an Aeroset autoanalyzer (Abbott). HbA_{1c} level was measured using commercially available kits (Roche®, Basel, Switzerland). The normal range was 4–6%.

Statistical Analysis

All analyses were conducted using SPSS 15.0 (SPSS for Windows 15.0, Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation (SD). Normality of distribution was evaluated with the Kolmogorov–Smirnov test. Homogeneity of the variances was evaluated with Levene test. Parameter comparisons were performed with Student's *t*-test and Mann–Whitney *U* test. The correlation between CIMT, BMI, 50 g OGTT, mean arterial blood pressure (MABP), and other serum parameters was assessed by Pearson's correlation test. Multiple linear regression analysis was performed to assess relations between measured parameters. A value of $p < 0.05$ was considered significant.

RESULTS

The demographic and clinical data of the study population are summarized in Tables 1 and 2. There was no statistical difference between the groups regarding maternal age, gestational age, parity, BMI, and lipid profile.

TABLE 1 Demographic and Clinical Characteristics of Patients with GDM and Controls

Parameters	GDM ($n = 39$)	Controls ($n = 40$)	p-Value
Age (years)	31.85 ± 7.59	29.55 ± 4.36	0.10
Gestational Age (weeks)	27.18 ± 2.68	27.00 ± 2.52	0.76
Parity	3.59 ± 2.54	2.70 ± 2.31	0.12*
BMI (kg/m^2)	31.83 ± 5.48	29.77 ± 5.67	0.10
MABP (mm/hg)	92.11 ± 12.69	85.06 ± 10.83	0.01
CIMT (mm)	0.54 ± 0.13	0.46 ± 0.05	< 0.001
Left CIMT (mm)	0.56 ± 0.15	0.47 ± 0.07	0.003
Right CIMT (mm)	0.54 ± 0.12	0.45 ± 0.05	< 0.001
FBG (mg/dL)	111.87 ± 51.31	83.67 ± 23.55	< 0.001*
50 g OGTT	181.49 ± 35.88	119.08 ± 24.08	< 0.001
Total cholesterol (mg/dL)	211.43 ± 49.92	203.55 ± 23.19	0.37
HDL-C (mg/dL)	49.95 ± 9.80	54.55 ± 14.50	0.10
LCL-C (mg/dL)	114.77 ± 41.15	107.96 ± 28.53	0.40
Triglyceride (mg/dL)	233.59 ± 91.78	205.20 ± 78.26	0.14
HbA_{1c} (%)	6.17 ± 1.27	5.09 ± 0.79	< 0.001*

Values are presented as mean ± SD. *Mann–Whitney *U* test.
Abbreviation: GDM, gestational diabetes mellitus; BMI, body mass index; MABP, mean arterial blood pressure; CIMT, carotid artery mean [(right side + left side)/2] intima media thickness; FBG, fasting blood glucose; OGTT, oral glucose tolerance test; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol.

TABLE 2 PON1, –SH, and LOOH Levels in Women with GDM and Controls

Parameters	GDM ($n = 39$)	Controls ($n = 40$)	p-Value
PON1 (U/L)	195.48 ± 84.68	262.10 ± 38.13	< 0.001
–SH (mmol/L)	0.19 ± 0.04	0.26 ± 0.02	< 0.001
LOOH (mmol H_2O_2 Equiv./L)	15.42 ± 4.06	11.36 ± 2.14	< 0.001

Abbreviation: PON1, paraoxonase; –SH, free sulfhydryl groups; LOOH, lipid hydroperoxide; GDM, gestational diabetes mellitus.

TABLE 3 Correlation Analysis between CIMT and Other Parameters in Patients with Gestational Diabetes

Parameters	r	p-Value
Age	0.13	0.42
BMI (kg/m^2)	0.46	0.003
Parity	0.06	0.69
Week of gestation	−0.02	0.89
FBG (mg/dL)	0.13	0.42
50 g OGTT	0.37	0.02
Total cholesterol (mg/dL)	−0.03	0.83
HDL-C (mg/dL)	−0.04	0.82
LCL-C (mg/dL)	−0.11	0.49
Triglyceride (mg/dL)	0.18	0.28
HbA_{1c} (%)	0.14	0.39
MABP (mm/hg)	0.34	0.03
PON1 (U/L)	−0.02	0.89
–SH (mmol/L)	−0.27	0.10
LOOH (mmol H_2O_2 Equiv./L)	−0.21	0.20

Values are presented with Pearson's coefficient and p-value.
Abbreviation: BMI, body mass index; MABP, mean arterial blood pressure; CIMT, carotid artery mean [(right side + left side)/2] intima media thickness; FBG, fasting blood glucose; OGTT, oral glucose tolerance test; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol.

MABP, CIMT, left CIMT, and right CIMT levels were significantly higher in women with GDM with regard to healthy pregnant controls ($p = 0.01$, $p < 0.001$, $p = 0.003$, $p < 0.001$, respectively; Table 1, Figure 1).

PON1 and –SH levels were significantly lower in the GDM group ($p < 0.001$ for both), while LOOH levels were significantly higher ($p < 0.001$; Table 2, Figure 2). Correlation analysis revealed that CIMT was significantly correlated with BMI, 50 g OGTT, and MABP ($p = 0.003$, $p = 0.02$, and $p = 0.03$ respectively; Table 3). However, there was no correlation between CIMT and oxidative markers (PON1,–SH, LOOH).

When we performed regression analysis using CIMT as a dependent variable and other demographic and clinical data (age, parity, MABP, gestational week, BMI, HDL-C, LDL-C, triglyceride, 50 g OGTT, HbA_{1c}, PON1, –SH, LOOH, FBG) as independent variable, we found only BMI ($\beta = 0.59$, $p = 0.005$) was related with CIMT in the GDM group.

DISCUSSION

In this study we aimed to find out whether there is an association between subclinical atherosclerosis and oxidative markers in the GDM patients. We determined increased

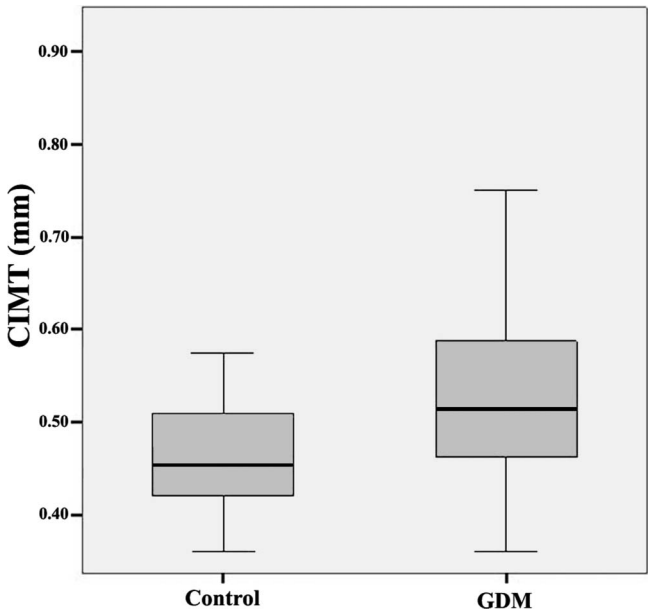

FIGURE 1 Mean value of common CIMT in women with gestational diabetes and unaffected controls. Boxes represent 1 SD and whiskers represent 2 SD.
Abbreviation: CIMT, carotid artery intima media thickness; SD, standard deviation.

risk of subclinical atherosclerosis and significant changes in the oxidative markers in the GDM group.

It has been shown that increased CIMT is related with subclinical atherosclerosis and elevated risk of stroke and coronary events (23). The increased CIMT values are also detected in type 1 and type 2 DM and GDM (9,14,24). These results were consistent with the data obtained from this study in which we found the increased levels of CIMT in the GDM. Several mechanisms are accused to cause the subclinical atherosclerosis process. Acute hyperglycemia impairs endothelial function even in healthy people (25) and evokes oxidative stress through protein kinase C, which eventually inhibits endothelium-derived nitric oxide delivery and interfering vasodilatation (26). Besides, hyperglycemia may directly lead to the formation of free radicals through glucose autoxidation (27). Thus, glucose level is strictly associated with increased CIMT, future risk of atherosclerosis, and coronary events.

Bo et al. (15) evaluated CIMT in nonpregnant women having previous GDM history and healthy controls and showed significantly higher measures in the former group. They also revealed that even not having current metabolic abnormalities, endothelial dysfunction, and higher CIMT values were consistent in the women having GDM history and commented as this finding to be related with future cardiovascular diseases. Our cross-sectional study also demonstrated significantly high CIMT values even in the second half of the pregnancy. This finding may arise several questions like: Whether this change has occurred in the pregnancy period or there is a previous change before pregnancy. As we excluded pregestational DM, we believe that this change has occurred during pregnancy. Another issue is whether increased CIMT in GDM patients is reversible or not in postpartum period. Bo et al. (15) have proved that this is a permanent pathophysiological process that remains even after 6.5 years after delivery.

Previously, it was documented that oxidant/antioxidant mechanisms are deteriorated in type 2 DM (13) and hyperglycemia may directly lead to the formation of free

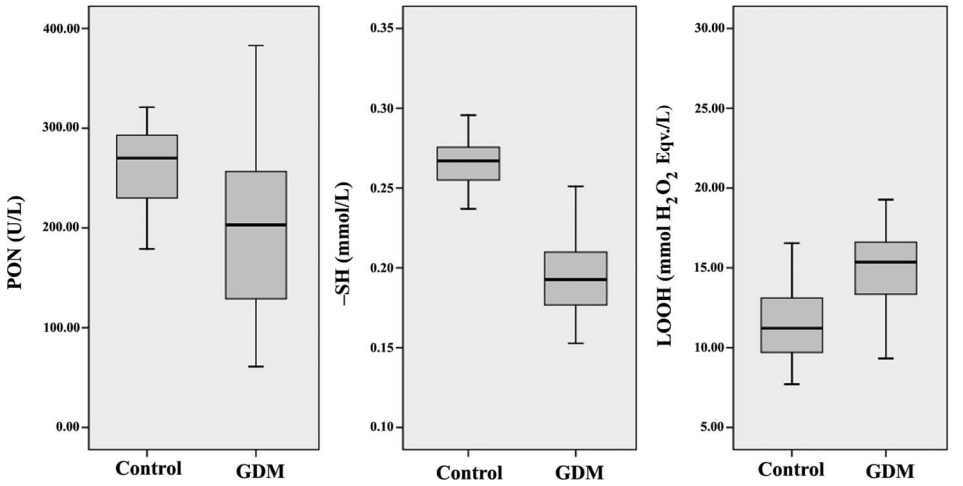

FIGURE 2 PON1,–SH, and LOOH levels in women with gestational diabetes and unaffected controls. Boxes represent 1 SD and whiskers represent 2 SD.
Abbreviation: PON1, paraoxonase; –SH, free sulfhydryl groups; LOOH, lipid hydroperoxide; SD, standard deviation.

radicals through glucose autoxidation (27). In the literature, few studies are present dealing with GDM and oxidative stress. Among them, Coughlan et al. (3) have demonstrated placental release of 8-isoprotostane as an oxidative marker is significantly increased in placenta obtained from women with GDM. Additionally, they found higher antioxidant levels; superoxide dismutase, protein carbonyl content, and glutathione peroxidase – except the last one, reaching significance level. They interpreted this state as a compensatory mechanism to the oxidative stress. Peuchant et al. (28) have also found disturbed oxidative/antioxidative balance with free malondialdehyde and glutathione peroxidase in women with GDM with respect to healthy controls. In this study, we found that serum levels of PON1 activity and –SH groups were significantly lower in patients with GDM than in healthy pregnant controls, while serum LOOH levels were higher. In other words, we found significantly decreased antioxidant levels and increased oxidative marker level in patients with GDM as similar to another study which was from our institute and have shown decreased PON1 activity and increased LOOH levels in women with GDM compared with healthy pregnant controls (6). Recent studies showed that PON1, a protective enzyme for LDL-C oxidation, is an important molecule in the pathogenesis of atherosclerosis (6,29,30). It has also been shown that PON1 activity is associated with coronary artery flow (31). Thus, in our opinion, the evaluation of the activity of this enzyme together with CIMT may be important in patients with GDM in consideration of the evaluation of coronary artery disease risks. This is the first study underlining this issue and reports decreased levels of PON1 activity and increased CIMT. Beyond these findings by the measurement of PON1, – SH, and LOOH, this study also supports that oxidative stress is increased with GDM on the base of increased CIMT. Free sulfhydryl groups of proteins are also an important antioxidant component of serum and both serum LOOH and –SH levels were shown to be associated with the presence and severity of coronary artery diseases (32). LOOH is a well-known marker of oxidative stress formed from unsaturated phospholipids, glycolipids, and cholesterol by peroxidative reactions under oxidative stress (33). Oxidized LDL, besides membrane-bound cholesterol-derived hydroperoxides, is the main form of LOOH to be responsible for the development of oxidative stress-related atherosclerosis (33).

Hyperglycemia has been involved in the formation of advanced glycation end-product and free radical formation (34). In our study, elevation of the HbA_{1c} in the GDM group also reflected poor glucose metabolism despite frequent controls and adequate treatments. This altered glucose metabolism may explain the enhancement of oxidant production.

GDM and control group exhibited elevated total cholesterol and triglyceride levels with no significant differences between the groups. This result supports the literature that has showed that TC and TG increased significantly throughout pregnancy and no significant difference existed between GDM and healthy pregnancy (5,35). On the contrary, Camuzcuoglu et al. (6) have shown that GDM group has significantly higher levels in our laboratory. Additionally, we have found significantly increased MABP in GDM group and significant correlation of CIMT with BMI, MABP, and 50 g OGTT. It is also well known that GDM and high blood pressure are closely related (36,37). Beyond this fact, we clearly identified significant correlation between MABP and CIMT by which we add another point of view to the future cardiovascular, hypertensive, and diabetic disease potential of pregnancy.

Based on the results of our current study, we can conclude that impairment of the antioxidative mechanisms in association with increased BMI and MABP are related to GDM pathophysiology. In this manner, we clearly demonstrated association of the PON1, –SH, and LOOH levels with GDM. As a cross-sectional study, our study may have a limitation that we could not evaluate before and after treatment values of both CIMT and serum parameters. However, along with the findings related with CIMT values, we can interpret increased atherosclerotic heart disease risk in the future of patients with GDM.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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