Comparisons of the oxidative stress biomarkers levels in gestational diabetes mellitus (GDM) and non-GDM among Thai population: cohort study

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Abstract

Objective: The primary objective of this study was to compare the levels of oxidative stress biomarkers between pregnancies with gestational diabetes mellitus (GDM) and normoglycemic pregnancies.

Materials and methods: A prospective study was conducted on pregnant women at average risk for GDM. The participants were screened for GDM with glucose challenge test and confirmed by 100 g, 3-h oral glucose tolerance test and categorized into the control (non-GDM) and GDM groups. Maternal blood was collected from all participants at gestational age (GA) 24–28 weeks and early labor and fetal cord blood was collected for measurements of 8 Isoprostane (8Isop) (oxidative stress marker), TNF-α (inflammatory marker) and IL-10 (anti-inflammatory marker) and were followed up for maternal and neonatal outcomes.

Result: A total of 62 women, 30 in GDM and 32 in control group, met the inclusion criteria. At 24–28 weeks of gestation, maternal serum 8Isop and TNF-α levels were significantly higher in GDM group ($P=0.032$ and $P=0.047$), in spite of good glycemic control. At early labor, maternal 8Isop levels were significantly higher in GDM ($P=0.001$). The biomarkers in the cord blood as well as maternal and neonatal outcomes in both groups were not significantly different.

Conclusion: GDM is significantly associated with inflammatory process when compared to normal pregnancy, as indicated by higher oxidative stress and apoptosis markers. However, such levels were not correlated with the pregnancy outcomes. An increase in oxidative stress could not be prevented by good glycemic control. Cord blood biomarker levels in pregnancy with GDM were not changed, suggesting that the placenta could be the barrier for the oxidative stress and cytokines.

Introduction

Gestational diabetes mellitus (GDM) is the most common metabolic disease in pregnancy, characterized by abnormal blood sugar levels, leading to several maternal and neonatal adverse outcomes (1). Currently, the prevalence of GDM is continuously increased, especially in Asia. This is associated with more advanced maternal...
age in the recent years and higher risk of metabolic disorders (2). In 2015, the International Diabetes Federation estimated that the prevalence of pregnancy with hyperglycemia was about 16.2%, probably as high as 24.5% in South-East Asia, including GDM in 85.1% of cases (3). GDM is clinically important because of its association with increased adverse pregnancy outcomes, which is correlated with blood glucose levels (3, 4, 5, 6), including gestational hypertension and preeclampsia, fetal macrosomia, shoulder dystocia and cesarean section rate (7, 8, 9). Additionally, GDM carries a 50% risk of diabetes mellitus type 2 (DM type 2) in 22–28 years after pregnancy (4).

GDM is a metabolic disorder involving insulin resistance, like DM type 2, due to hormonal change during pregnancy (3). The pathophysiology of GDM is not clearly understood, but chronic subclinical inflammation induced by hormonal change during pregnancy probably plays an important role of clinical manifestation. GDM may probably be related to oxidative stress in insulin resistance pathway, as documented in DM type 2 (10). Nevertheless, the relationship between GDM and oxidative stress is unclear. The levels of oxidative stress in GDM such as xanthine oxidase, lipid peroxides, malondialdehyde, 8-isoprostane (8Isop), TNF-α and IL-10 have been studied in a very limited number of reports, in which the results are contradictory, though some showed higher levels of oxidative stress markers in pregnancies with GDM (11, 12). Moreover, most previous studies compared the oxidative stress markers only once in pregnancy, mostly at the time of diagnosis. We hypothesized that pregnancies with GDM had higher levels of oxidative stress markers. Therefore, we conducted this study to compare the levels of maternal oxidative stress markers between normal pregnant women and women with GDM at 24–28 weeks of gestation, before delivery and in the cord blood just after birth, as the primary objective and to determine the correlation between such markers and maternal and neonatal outcomes as the secondary objective.

Materials and methods

A prospective cohort study was conducted on pregnant women, attending the antenatal care clinic, Department of Obstetrics and Gynecology, Chiang Mai University, between July 2016 and August 2017. The study was ethically approved by Chiang Mai University Review Board. All participants were enrolled with informed written consent. Inclusion criteria were (1) singleton pregnancies, (2) maternal age of 20–50 years, (3) average risk of GDM, (4) gestational age of 24–28 weeks and (5) no other underlying disease. Exclusion criteria were preexisting diabetes (overt DM), other underlying disease such as chronic hypertension, chronic renal disease, autoimmune disease, heart disease and smoking. Diagnosis of GDM was based on the two-step approach as follows. (1) The women were screened with 50g glucose challenge test (GCT), using cut off at 140mg/dL. (2) The women with positive GCT underwent the standard 100g oral glucose tolerance test (OGTT) for diagnosis of GDM, using the criteria recommended by National Diabetes Data Group Conversion, diagnosed if two or more of the following thresholds were met (fasting 105mg/dL, 1 h 190mg/dL, 2 h 165mg/dL, 3 h 145mg/dL). Accordingly, the women were categorized into two groups, the control group (negative GCT or OGTT) and the GDM group. Both groups received standard antenatal care and were followed up until delivery for maternal and neonatal outcomes. The women with GDM were controlled for blood glucose either by diabetic diet or insulin.

Blood samples

Maternal blood samples for oxidative stress markers were taken at 24–28 weeks of gestation and at the time of admission for delivery. The umbilical cord blood was also collected before placental delivery after birth. All of the 3 mL blood samples were drawn in heparin tube then plasma was separated by centrifugation at 1036 g for 10min. The separated plasma samples for measurement of 8Isop, TNFα and IL-10 levels were stored at −80°C for subsequent testing in batches.

Measurement of anti-inflammatory markers IL-10

The Hu IL-10 standard is prepared according to the protocol and then 10μL of this 100x concentrated solution is diluted with 1 mL streptavidin-HRP diluent for each 8-well strip used in the assay. This product is labeled as streptavidin-HRP working solution. Mix thoroughly between steps. Add 100μL standard diluted buffer to zero wells and then add 100μL of standards, samples or controls to the appropriate microtiter wells. The plates are covered with the plate cover and incubated for 2h at room temperature. Thoroughly aspirate or decant solution from wells and discard the liquid and then wash wells four times. The biotinylated anti-IL-10 (biotin conjugate) solution is pipetted 100μL into each well except the chromogen blank(s) and tapped gently on the side of the plate to mix.
The plates are covered with the plate cover and incubated for 2 h at room temperature. Thoroughly aspirate or decant solution from wells and discard the liquid and then wash wells four times. Add 100 μL streptavidin-HRP in working solution to each well except the chromogen blank(s). Plates will be covered with the plate cover and incubated for 30 min at room temperature. Thoroughly aspirate or decant solution from wells and discard the liquid and then wash wells four times. 100 μL stabilized chromogen has been added to each well.

The liquid in the wells started to turn blue. The liquid was incubated for 30 min at room temperature and in the dark. The incubation time for chromogen substrate was often determined by the microtiter plate reader. One hundred microliters of stop solution were added to each well. Side of plate was tapped gently to mix and then the absorbance of each well was read at 450 nm of absorbance reader.

**Measurement of inflammatory markers TNF-alpha**

The TNF-α ELISA (Invitrogen) is used for the quantitative determination of Hu TNF-α in human. The standard Hu TNF-α is prepared by following the protocol of ELISA kit. Determine the number of eight well strips needed for the assay. Insert these strips in the frame(s) for current use. Add 50 μL incubation buffer to the wells containing standards and serum/plasma samples, or 50 μL standard diluent buffer to the wells containing cell culture samples, then add 100 μL standard diluent buffer to zero wells. The standard solutions of 100 μL are added to sample or control and then cover plated and incubated for 2 h at room temperature. Discard the solution liquid and wash well four times. Biotinylated anti-TNF-α (biotin conjugate) solution of 100 μL are added in the well, then incubated in room temperature for 1 h and then solution is removed and washed well 4 times again. Stabilized chromogens of 100 μL are added to each well and then incubated for 30 min at room temperature and in the darkness. The stop solution of 100 μL is added to each well. Tap side of plate gently to mix.

The solution in the wells starts to change from blue to yellow. The absorbance of each well was read at 450 nm of absorbance reader. Software for curve fitting was used to generate the standard curve.

**Measurement of 8-iso-Prostaglandin**

The OxiSelect 8-iso-Prostaglandin F2α ELISA Kit (STA-337), created by Cell Biolabs, Inc (San Diego, CA, USA) was used. The plasma/serum was prepared by the following protocol, 1 part of 10 M NaOH was used for every 4 parts of liquid sample. After incubation at 45°C for 2 h, 100 μL concentrated (10 M) HCl per 500 μL hydrolyzed sample was added. The sample turned milky after this addition. The samples were centrifuged for 5 min at 13,201 g in a microcentrifuge. The clear supernatant could be used immediately in the assay or stored at −20°C or below for future use. Before assaying, check to be sure that each neutralized sample was in the pH range of 6–8. The tests were started by adding 100 μL diluted Anti-8-iso-PGF2α antibody to the Goat Anti-Rabbit antibody-coated plate. The sample was incubated for 1 h at 25°C on an orbital shaker and then the antibody solution was removed from the wells. The samples were washed 5 times with 300 μL 1× wash buffer per well. After the last wash, emptied the wells and tapped microwell plate on absorbent pad or paper towel to remove the excess wash solution. The 55 μL 8-iso-PGF2α standard or sample was combined with 55 μL 8-iso-PGF2α-HRP conjugate in a microtube and mixed thoroughly. And then 100 μL combined solution were transferred per well. A well containing sample diluent could be used as a control. After incubation for 1 h at 25°C on an orbital shaker, the combined solutions were removed from the wells and washed 5 times with 300 μL of 1× wash buffer per well.

After the last wash, emptied wells and tapped microwell plate on absorbent pad or paper towel to remove excess wash solution and 100 μL substrate solution were added to each well. The samples were incubated at room temperature for 10–30 min on an orbital shaker. The enzyme reaction was stopped by adding 100 μL stop solution to each well. Results should be read immediately (color were fade over time). The absorbance of each well was read on a microplate reader using 450 nm as the primary wave length.

Based on the mean (±S.D.) concentrations of 8isoP in maternal plasma and cord blood shown in the previous study reported by (12) to test whether the means of the two groups are equivalent, this study needed a sample size at least 19 cases in each group to gain power of 90% at 95% confidence interval and testing margin of 1%.

**Statistical analysis**

Statistical analyses were performed by SPSS, version 21. Quantitative analyses were expressed as mean± standard error of mean (S.E.M.). Student's t test, Mann–Whitney test and one-way ANOVA analyses were performed as appropriate. The statistic significant value was accepted with P<0.05.
Results

During the study period, a total of 224 pregnant women at average risk of GDM were recruited to the study. Of them, 62 women, 30 in the GDM group and 32 in the control group, were completely followed up, and final outcomes were available.

All of the baseline characteristics of the two groups were not significantly different (P value >0.05), as shown in Table 1. Note that nearly all women in this study had Thai ethnicity.

At 24–28 weeks of gestation, the levels of 8Isop and TNFα among women with GDM were significantly higher than those in the controls (P value 0.032 and 0.047, respectively), as presented in Table 2. During early labor, the levels of 8Isop were still significantly higher in the GDM group, whereas the levels of TNFα tended to be increased but did not reach the significant level (Table 2). All the three biomarkers in cord blood were not significantly different between the two groups (P value >0.05), though the levels in the GDM group had a trend to be increased (Table 2). In both groups, the levels of 8Isop and TNFα were significantly increased with advancing gestational age, when compared the levels at 24–24 weeks and those at early labor (Wilcoxon signed-rank test; P value <0.05). However, the levels of IL-10 did not significantly change (P value >0.05), though they also tended to increase.

Regression analysis showed no significant correlation between the levels of the three biomarkers and neonatal birth weight (Pearson’s correlation; P value >0.05).

Of the women with GDM, at 24–28 weeks of gestation, HbA1c levels in the GDM group were 5.02±0.45 mg/dL. Mean ± s.d. fasting blood glucose and 2-hour postprandial glucose in the GDM group in late pregnancy were 87±7 mg/dL and 119±38 mg/dL, respectively. The levels of 8Isop, TNFα and IL-10 were not significantly correlated with the levels of HbA1c (Pearson’s correlation; P value >0.05).

Nearly all of the women with GDM (27 cases; 90%) had satisfactory glucose control with diabetic diet. Only four cases needed insulin administration. Note that the pregnancy outcomes (Table 3) and neonatal outcomes (Table 4) between the two groups were significantly different (P value >0.05). The prevalence of preeclampsia and fetal macrosomia were not significantly higher in the GDM group. Additionally, the levels of 8Isop, TNFα and IL-10 were also not significantly associated with birth weight (P value >0.05) and gestational age of delivery (P value >0.05).

| Table 1 | Baseline characteristics. |
|---------|--------------------------|
|         | Control (N=32) mean±s.d. | GDM (N=30) mean±s.d. | P value* |
| GA at first collection | 25.9±1.3 | 26.2±1.2 | 0.267 |
| Maternal age | 31.2±5.8 | 31.3±4.7 | 0.914 |
| BMI | 22.7±3.1 | 21.5±3.9 | 0.172 |
| Height | 158.6±4.7 | 157.6±6.4 | 0.499 |
| Weight | 56.9±9.6 | 53.7±10.7 | 0.202 |
| Hemoglobin | 12.3±1.0 | 11.9±1.0 | 0.143 |
| Hematocrit | 37.2±2.5 | 36.0±2.6 | 0.098 |
| Hemoglobin A1C | 4.9±0.3 | 5.0±0.5 | 0.728 |
| Parity | | | 0.477 |
| Nulliparous | 19 | 19 | |
| Parous | 13 | 11 | |
| Ethnic | | | 0.738 |
| Thai | 31 | 29 | |
| Other | 1 | 1 | |
| Education | | | 0.748 |
| No | 1 | 1 | |
| Primary school | 0 | 1 | |
| High school | 12 | 8 | |
| High vocational certificate | 4 | 3 | |
| Bachelor of arts | 13 | 15 | |
| Master of arts | 3 | 1 | |
| Income (Thai Baht) | | | 0.107 |
| ≤10,000 | 12 | 20 | |
| 10,001–15,000 | 14 | 5 | |
| 15,001–20,000 | 3 | 2 | |
| ≥20,000 | 3 | 2 | |

*Student’s T test or Chi-square as appropriate.
Table 2 Oxidative stress and biomarkers.

|                           | Control median (IQR) | GDM median (IQR) | P value* |
|---------------------------|----------------------|------------------|----------|
| Maternal blood at GA 24–28 weeks |                      |                  |          |
| 8Isop                    | 249.1 (47.7–997.2)   | 737.5 (584.9–1811.5) | 0.032    |
| TNF-α                    | 1.75 (0.10–1.65)     | 4.70 (2.42–6.91)  | 0.047    |
| IL-10                    | 0.73 (0.44–1.08)     | 0.88 (0.41–1.22)  | 0.773    |
| Maternal blood at early labor |                      |                  |          |
| 8Isop                    | 104.8 (39.2–373.4)   | 666.4 (454.5–1528.8) | 0.001    |
| TNF-α                    | 3.47 (1.21–6.59)     | 5.80 (3.83–7.45)  | 0.093    |
| IL-10                    | 1.08 (0.63–2.12)     | 1.13 (0.55–2.58)  | 0.640    |
| Cord blood               |                      |                  |          |
| 8Isop                    | 74.3 (44.1–109.2)    | 82.1 (30.9–233.8) | 0.842    |
| TNF-α                    | 12.16 (8.31–15.19)   | 14.06 (10.49–16.52) | 0.261    |
| IL-10                    | 0.79 (0.59–1.76)     | 0.825 (0.37–1.40) | 0.954    |

*Mann–Whitney test.

Discussion

Insight gained from this study is that oxidative stress markers like 8Isop were significant higher in pregnant women with GDM. Similarly, TNFα levels, representing inflammatory marker, are also increased both at 24–28 weeks of gestation and early labor, when compared to the normal control. However, cord blood levels of those biomarkers were comparable between both groups, suggesting that GDM with good control be unlikely to affect fetal or neonatal oxidative status. Notably, the higher levels of oxidative stress markers at early labor might possibly be caused by labor effect. Nevertheless, those levels in the GDM group were still significantly higher than those in the control group. Surprisingly, our study did not show a significant increase in IL-10 levels when compared to the control. The finding suggests that the intense of inflammatory process in GDM might not as much as seen in DM type II. An increase in inflammation related to GDM, as indicated by increased TNFα levels, was not severe enough to provoke intense response by anti-inflammatory process.

We found no significant correlation between HbA1c levels and oxidative markers, unlike the finding found in some previous studies (13). The contradictory results need to be elucidated by further study. HbA1c levels in the women with GDM in this study were in normal limits, while the oxidative stress markers were significantly increased, indicating that the oxidative stress markers may be more sensitive than HbA1c in confirmation of GDM. It is possible that HbA1c is reflexive of glucose control status rather than the indicator of oxidative stress.

Additionally, the levels of the biomarkers of the GDM group in late pregnancy or early labor were still significantly higher than those of the control group in spite of the fact that the women with GDM were well controlled for blood glucose levels. The finding strongly suggests that diabetogenic diet or insulin can effectively control the blood glucose levels but it cannot much affect oxidative stress marker levels. The findings signified that adverse pregnancy outcomes caused by GDM could be avoided by glucose control but cardiovascular risk associated with unhealthy vessels secondary to oxidative stress might have not been simply prevented by glucose control.

The pregnancy outcomes and neonatal outcomes, especially fetal macrosomia, of both groups were comparable, probably associated with good control of GDM. Nevertheless, though clinical course of GDM was under good control as indicated by the comparable rates of adverse outcomes in both groups and glucose levels within normal limit, oxidative stress biomarkers were higher than normal control in spite of good glucose control or subtle clinical course. Accordingly, GDM, even under good control, the subclinical risk related to oxidative stress might have existed. Based on our findings, GDM may be classified into two subgroups; GDM with and without increased oxidative stress markers. As previously mentioned, half of the women with GDM, though diabetogenic state disappears after birth, will develop overt diabetes mellitus later in life or 20 years later (4). Therefore, we hypothesize that women with GDM with high oxidative stress markers, even in the case of good glycemic control, may have a higher chance of developing DM type 2 later in life than GDM with normal levels of oxidative stress markers. Our findings pave the way for further studies to determine whether GDM with higher oxidative stress markers is predictive of future DM type 2 or not. It has been already known that oxidative stress markers like 8Isop and TNFα and...
anti-inflammatory cytokine (IL-10) are increased in patients with DM type 2 (4, 10).

Interestingly, though the oxidative stress markers were higher in the women with GDM, such biomarkers in the neonates were similar to those in the controls. The findings suggest that the placenta may have defensive mechanism to prevent oxidative stress in the fetus or neonate. Accordingly, maternal GDM is unlikely to place the neonate at a higher risk caused by maternal oxidative stress. However, whether the oxidative stress levels in poorly controlled GDM mothers are increased or not is yet to be elucidated.

The strengths of this study included (1) the levels of biomarkers were measured both in the second and in the third trimester, as well as in the umbilical cord blood; (2) Prospective nature of the study on the highly homogeneous population (Thai ethnicity). The weakness of this study included (1) Too small sample size for some secondary outcomes especially that only four cases of the women with GDM required insulin treatment. Thus, the correlation between oxidative stress markers and insulin requirement could not be reliably analyzed. Likewise, the correlation between oxidative stress markers and birth weight could not be demonstrated, due to under power because of too small sample size. (2) Levels of those biomarkers during postpartum period were not measured. Thus, we did not know whether the higher levels of such markers persisted or not. (3) Because of intervention or diabetic control in the GDM group leading to good pregnancy outcomes, the effect of oxidative biomarkers on such outcomes could not be interpreted.

In conclusion, women with GDM had significantly higher levels of oxidative stress markers and inflammatory markers than normal pregnancies. Nevertheless, the inflammatory process may not be as serious as seen in DM type II. Additionally, in cases of good diabetic control, they could not predict adverse pregnancy outcomes. The levels of biomarkers in the cord blood were comparable between the women with GDM and normal ones. Importantly, women with GDM had higher oxidative stress levels despite good control of glycemia, implying that the women with GDM may possibly take subtle risk associated with oxidative stress such as cardiovascular disease or DM type II in the future. Further studies should be conducted to determine whether high oxidative stress markers among women with GDM can be predictive of the development of overt DM later in life or not.

Declaration of interest
The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
H R, P J and T T conceived and designed the study; H R and P J performed the clinical experiment; S K, R T J and N C performed laboratory experiment; T T analyzed the data; H R and P J wrote the manuscript and T T and N C edited the manuscript.

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