Original Article
Aberrant expression of oxidative stress related proteins affects the pregnancy outcome of gestational diabetes mellitus patients

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Abstract: Gestational diabetemellitus (GDM) is a condition whereby a mother's glucose tolerance is impaired with onset or first recognition during pregnancy which is not either type 1 or type 2 diabetes mellitus. Oxidative stress plays an essential role in diabetes, however, whether it also includes in GDM has not been fully clarified. Therefore, we investigated the changes of oxidative stress biomarkers and their relationship with pregnancy outcomes in patients with GDM. The serum and placenta were collected for absorbance-based assay and immunohistochemistry assay (IHC). The patients' clinical information was collected and the pregnancy outcome was tracked. It was found that elder age is a risk factor to result in GDM. Moreover, GDM patients showed poor clinical factors or outcomes including higher prepregnancy weight and BMI value, premature delivery, higher rates of cesarean delivery, macrosomia, premature rupture of fetal membranes (PROM). Increasing serum MDA level and decline GSH and SOD levels were observed in GDM patients. Meanwhile, HO-1, Nrf2 and NQO1 overexpressed in GDM placental tissues. In the GDM group, MDA level was negatively associated with prepregnancy weight, while, SOD level was positively correlated with neonatal birth weight. We found an intensive relationship between SOD content and preterm birth in the GDM group. There is no significant difference between the level of MDA/GSH and neonatal birth weight as well as preterm birth. MDA, GSH and SOD levels were not associated with an increased risk of cesarean delivery or PROM. This study indicates aberrant expression of oxidative stress related proteins affects the pregnancy outcome of GDM patients.

Keywords: GDM, oxidative stress, MDA, SOD, GSH

Introduction

Gestational diabetes mellitus (GDM) is defined as diabetes diagnosed after the first trimester of pregnancy that is not either type 1 or type 2 diabetes mellitus [1]. The global prevalence of GDM ranges from 2% to 14% [2, 3], with that of the Chinese population being 2.4% to 6.8% [4], and this rate continues to rise each year. Pregnancy and delivery complications, including preeclampsia, cesarean delivery, preterm birth and infant macrosomia, are commonly happen in women with GDM [5]. Pregnancies

with GDM are also associated with miscarriage, premature rupture of fetal membranes (PROM), and other anomaly rates that are higher than in a nondiabetic gestation. Furthermore, children delivered by GDM mothers are at high risk for developing obesity and type 2 diabetes when they grow up [6]. For instance, it was reported that women with previous GDM were older and reported higher body weight 2 years postdelivery [7]. At 2-year follow-up, 14.1% of participants with GDM had developed diabetes after delivery. Compared with the normal babies, the body weight of the babies labored by the GDM

mother is potently higher not only on the birth but also two years old [7]. However, the detailed mechanism how GDM occurs remains unclarified.

Previous study addressed that oxidative stress plays an essential role in GDM occurrence [49]. Increasing evidence supported that the imbalance between the oxidation and antioxidant system contributes to the development of GDM and its complications. It was reported that pregnant women with GDM exhibit a hyperglycemia-induced increase in circulating oxidative stress and a reduction in the antioxidative enzymes [8, 9]. Increased oxidative stress may result in adverse effects to both the mother and fetus [10]. Malondialdehyde (MDA), the product of lipid peroxidation, is generated by the reactive oxygen species (ROS)-induced degradation of phospholipids under pathological conditions, such as diabetes mellitus [11, 12]. It is easily measured in plasma and commonly used to indicate lipid peroxidation and oxidative stress [13]. Glutathione (GSH) has 2 forms of reducible and oxidized forms: the reduced GSH is the predominant form and is one of the most prevalent antioxidants. It has been demonstrated that women with diabetic preeclampsia were found to have significantly lower levels of reduced GSH in blood compared with healthy pregnant controls, indicating increased oxidative stress [14]. Superoxide dismutase (SOD) is an enzymatic antioxidant that can detoxify superoxide and can be considered a measure of the oxygen radical absorbance capacity or the capacity of the sample to inhibit an oxidant reaction [15]. It was observed that SOD activities remarkablely decreased in GDM patients' liver tissues when compared with the normal-pregnancy control group [16]. While, Heme oxygenase-1 (HO-1) is one of the most easily induced protein, and various factors such as cytokines, oxidative stress and inflammatory factors can induce the expression of HO-1. The up regulation of HO-1 gene expression and increased HO-1 enzyme activity are thought to play protective roles against the development of diabetic complications [17]. Nuclear factor erythroid 2-related factor-2 (Nrf2) is activated following the induction of oxidative stress. This protein has been demonstrated to be involved in the regulation of cytoprotective genes that are mediated by oxidative stress [18]. Nrf2 is recognized as an essential upstream transcription factor that regulated HO-1 [19]. NAD (P) H: quinone oxidoreductase (NQO1) is a down-

stream regulator of Nrf2-ARE pathways, its expression level is related to the developing of a variety of cancers such as cervical cancer, lung cancer, ovarian cancer, pancreatic cancer, et al. [20, 21]. Aldo-keto reductase family 1 member c1 (AKR1C1) is a member of the aldo-keto reductase superfamily, which expressed up-regulated in cervical cancer [22, 23], but down-regulated in gastric cancer [24]. However, rarely study examined the association between AKR1C1 and GDM. Therefore, it is of great significance to evaluate the level of oxidative stress in GDM patients. Therefore, the aim of this study is to investigate the changes in markers of oxidative stress and evaluate the association between these markers and pregnancy outcomes in GDM patients.

Materials and methods

Patients and sample collection

175 pregnant women between 21 to 41 years old who came to the obstetric clinic at the Songjiang Maternal and Child Health Care Hospital from march to october of 2017 for antenatal care were enrolled in this study. These patients were devided into 2 groups according to their plasma glucose level: (1) 93 patients who developed gestational diabetes (GDM group) and (2) 82 healthy pregnant women (control group). These women suffered with hypertension, preeclampsia, hypothyroidism and placenta previa were excluded. The GDM patients with insulin therapy history were also excluded. In addition, GDM was defined according to the result of oral glucose tolerance test (OGTT) between 24 and 28 weeks of gestation Women whose plasma glucose met one of the following criteria [25] were considered to have GDM: fasting, $\geq$ 5.1 mmol/L; 1 h, $\geq$ 10 mmol/L; 2 h, $\geq$ 8.5 mmol/L. Blood samples were collected from these 175 pregnant women when they did the OGTT test. Serum was removed, placed into a centrifuge tube, and then stored at -80°C in a refrigerator in the laboratory of Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, until further processing. We then tracked all patients' pregnancy outcomes. Of the total sample, 16 individuals in the GDM group were excluded owing to intrauterine fetal death (n = 1), abortion (n = 1), and being lost to follow-up (n = 14). In the control group, 17 women were lost to follow-up, so they too were excluded. After exclusions, 142 blood samples (77 sam-

Table 1. Clinical characteristics of pregnant women from the GDM (*n* = 77) and control (*n* = 65) groups

Group	Age (year)	Prepregnancy weight (kg)	BMI (kg/m^2)	Gestational weeks at delivery (week)
GDM	31.32 ± 0.53	56.49 ± 0.95	22.15 ± 0.36	38.43 ± 0.25
Control	28.62 ± 0.50	52.44 ± 0.80	20.45 ± 0.30	39.35 ± 0.12
t	3.658	3.170	3.541	3.099
P	0.0004*	0.0019*	0.0005*	0.0024*

*, P<0.05.

Table 2. Comparison of pregnancy outcomes between the GDM and control groups, *n* (%)

Group	Cesarean delivery	Macrosomia	PROM	Preterm
GDM	40 (51.95%)	8 (10.39%)	14 (18.18%)	6 (7.79%)
Control	24 (36.92%)	2 (3.08%)	8 (12.31%)	1 (1.54%)
x^2	3.214	2.879	0.929	2.941
P	0.073	0.090	0.335	0.086

ples from the GDM group and 65 from the control group) were tested. We then randomly selected 10 placental tissues (5 samples from the GDM group and 5 from the control group) for observing markers of oxidative stress expression in placenta by immunohistochemical assay.

Determination of reduced GSH

GSH was assessed with an absorbance-based assay. Assay kits were purchased from Shanghai Cablebridge Biotechnology Co. The microplate reader (Thermo Scientific Varioskan Flash) was preheated for 30 min, and the wavelength was adjusted to 412 nm. Reagent 2 was heated in water at 37°C for 30 min, and then 20 µL of distilled water, 140 µL of reagent 2, and 40 µL of reagent 3 were placed into 96-well plates. After mixing for 2 min, the absorbance A1 at 412-nm wavelength was measured by Varioskan Flash as a blank tube. The steps above were repeated by replacing distilled water with serum in the test tube. We then read absorbance A2 at 412-nm wavelength. Finally, the density of GSH (µmol/mL) was calculated using the formula 6.67 × (A2-A1).

Assessment of SOD

Plasma SOD was determined by the microdilution method (assay kits by Shanghai Cablebridge Biotechnology Co.). Reagents 1, 2, and 4 were kept at room temperature for 30 min. Then, 45 µL of reagent 1, 100 µL of reagent 2,

2 µL of reagent 3 (enzyme, on ice when used), 18 µL of blood sample, and 35 µL of reagent 4 were inserted into 96-well plates serving as the test tube. In the control tube, distilled water was substituted for the blood sample. All mixtures were kept at room temperature for 30 min. The absorbance of the mixture was then measured spectrophotometrically (Thermo Scientific Varioskan Flash), at 560-nm wavelength. Levels of SOD were calculated according to the instructions. The values are expressed as U/mL of SOD.

Determination of MDA

To determine oxidative stress, the amount of MDA was assessed by the spectrophotometric method (assay kits by Shanghai Cablebridge Biotechnology Co.). We transferred 0.3 mL of reagent 1 to 1.5-mL centrifuge tubes and added 0.1 mL of serum, mixing well. We then heated the mixture in water at 95°C for 30 min and placed the tubes in an ice bath. After cooling, components were separated by centrifuge at 10,000 *g* and 25°C for 10 min, and we placed 200 µL of the supernatant into 96-well plates. Absorbance at 532 nm (= A532) and 600 nm (= A600) was determined by the microplate reader (Thermo Scientific Varioskan Flash), with ΔA = A532-A600. The MDA unit used in our lab was nmol/mL. Accordingly, the formula in our MDA model was MDA = 51.6 × ΔA.

Detection of the expression of SOD, HO-1, Nrf2, NQO1 and AKR1C1 in placenta by imunohistochemistry

Within 15 min after delivery, 1 piece of full-thickness placenta, 1 × 1 × 1 cm in size, were taken. Areas with the edge, calcification, hemorrhage and necrosis were avoided when collecting the samples. Fix the placental tissues immediately in 10% formalin, embedded in paraffin, making pathological section for imunohistochemistry (IHC). IHC staining was applied to evaluated the localization and expression of SOD, HO-1, Nrf2, NQO1 and AKR1C1 protein in two groups of placenta tissue. Staining intensity was graded on 0 to 3 scale, 0 for unstained,
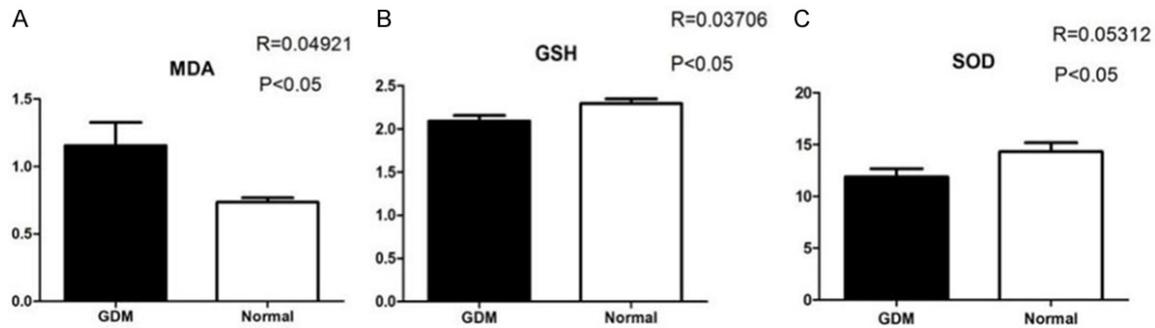
Figure 1. ROS related biomarkers' serum level in GDM and healthy pregnant women. MDA, GSH and SOD expression patterns in serum level between GDM and normal group. Data are shown as mean ± SEM. Statistical analysis was performed using an independent *t* test. *, P < 0.05 compared with the normal group. GDM, gestational diabetes mellitus. MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase.

1 for buffy, 2 for yellow, 3 for brown. The extent of the staining scored as follows: < 25% of trophoblast cells stained (0); 25-50% of the trophoblast cells stained positive (1); 50-75% of the trophoblast cells stained positive (2); and > 75% of the trophoblast cells stained positive (3). The stained cell can not be observed at high power (0); The stained cells can be observed at high power (1); The stained cells can be observed at medium power (2); The stained cells can be observed at low power (3). Total all the scores to determine the expression intensity: 0 for no staining (-), 1-3 for week immunoreactivity (1+); 4-6 for intermediate immunoreactivity (2+); and 7-9 for strong immunoreactivity (3+).

Statistical analysis

The results were analyzed with SAS 9.1 and GraphPad Prism5 software. Chi-square test, *t* test, and Pearson correlation coefficient analysis were used. Results are presented as the arithmetical mean with standard error of mean (mean ± SEM) and mean with standard deviation (mean ± SD). Values were considered statistically significant if *P* < 0.05. The detailed statistical analysis method for each experiment was described in the corresponding figure legend.

Results

Association between GDM and clinical factors

As shown in **Table 1**, GDM was more commonly happened in the elder pregnant women. The

GDM group shows increasing prepregnancy weight and BMI value compared with the control group. On the contrary, GDM resulted in shortening gestational weeks before delivery, even premature labor (*P* = 0.0024).

The effect of GDM on pregnant outcome

As shown in **Table 2**, there is a slightly increase rates of cesarean delivery, macrosomia, PROM and preterm birth in GDM group compared with the control group. However, no significant difference was observed.

The serum level of oxidative stress markers in normal and GDM patients

Oxidative stress molecules play an important role in GDM occurrence. It could be observed that the serum level of MDA in GDM group is higher than that of normal group (**Figure 1A**). Reversely, decrease pattern of GSH and SOD serum level was presented in GDM patients when compared with control group (**Figure 1B** and **1C**). The GDM group showed a lower servum GSH level (2.09 ± 0.06 vs. 2.3 ± 0.06 µmol/mL, *P* < 0.05) and SOD level (11.87 ± 0.78 vs. 14.31 ± 0.86 U/mL, *P* < 0.05).

The expression profiles of oxidative stress markers in placentas tissue

Since oxidative stress proteins shows potently changes in healthy pregnant women and GDM patients, we further want to know whether there is any variety in placentas tissue. The IHC
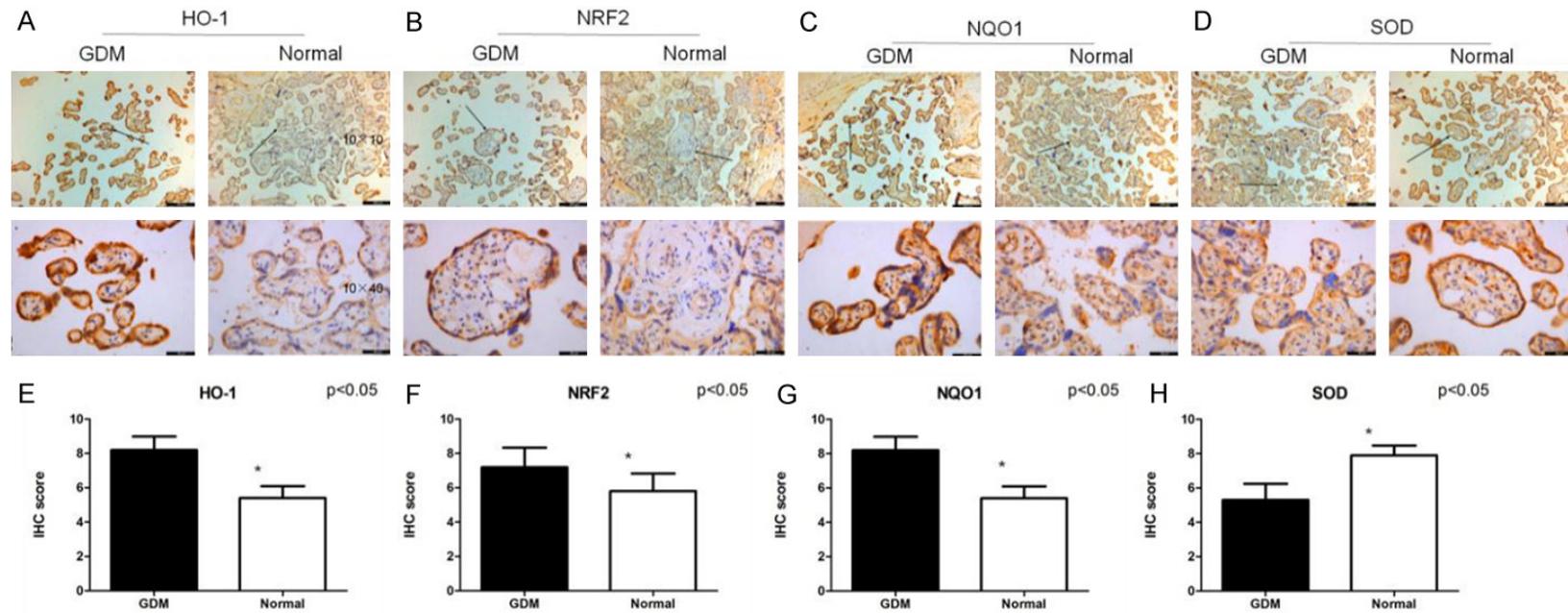
Figure 2. ROS related molecules' expression profiles in placenta tissue of GDM and normal pregnant women. The IHC was performed to detect HO-1 (A), Nrf2 (B), NQO1 (C) and SOD (D) staining in placenta. Representative images shown were captured at 100 × (upper panel) and 400 × (down panel) magnification. The corresponding statistical analysis of changes in HO-1 (E), Nrf2 (F), NQO1 (G) and SOD (H) protein by IHC were using an independent t test. *P < 0.05, compared with normal pregnant women.
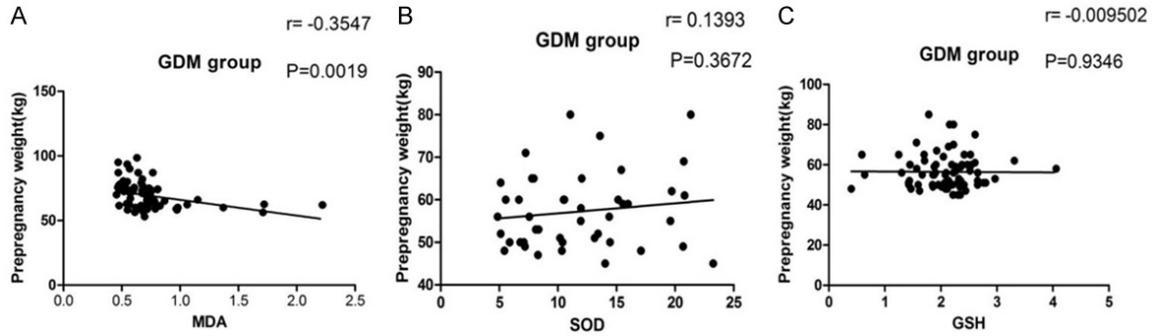
Figure 3. The association between pregnant body weight of GDM group and the levels of MDA, SOD and GSH. Statistical significance was determined using Pearson correlation coefficient analysis. A. Relationship between MDA and prepregnancy weight in the GDM group. r = -0.3547, P = 0.0019. B. Relationship between SOD and prepregnancy weight in the GDM group. r = 0.1393, P = 0.3672. C. Relationship between GSH and prepregnancy weight in the GDM group. r = -0.009502, P = 0.9346.
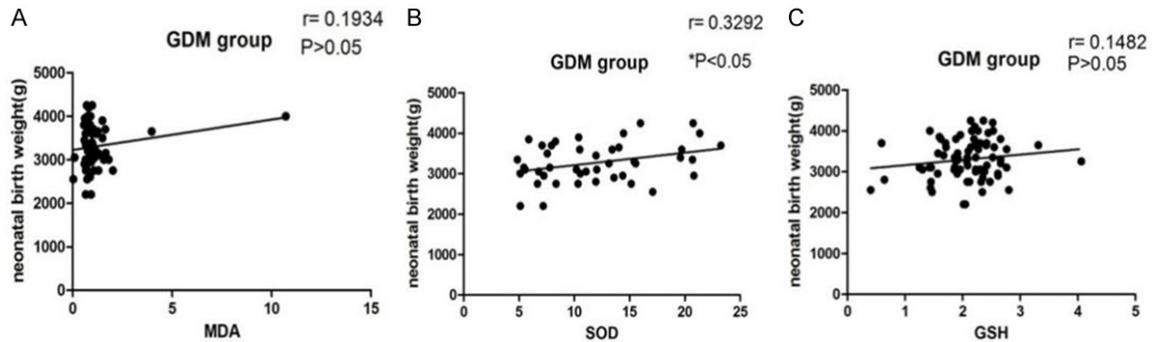

Figure 4. Correlation between MDA, SOD, and GSH serum levels and neonatal birth weight in the GDM group. Statistical significance was determined using Pearson correlation coefficient analysis. A. The correlation between MDA and neonatal birth weight in the GDM group. r = 0.1934, P = 0.1354. B. The correlation between SOD and neonatal birth weight in the GDM group. r = 0.3292, P = 0.0311. C. The correlation between GSH and neonatal birth weight in the GDM group. r = 0.1482, P = 0.2045. *, P < 0.05 indicated significant.
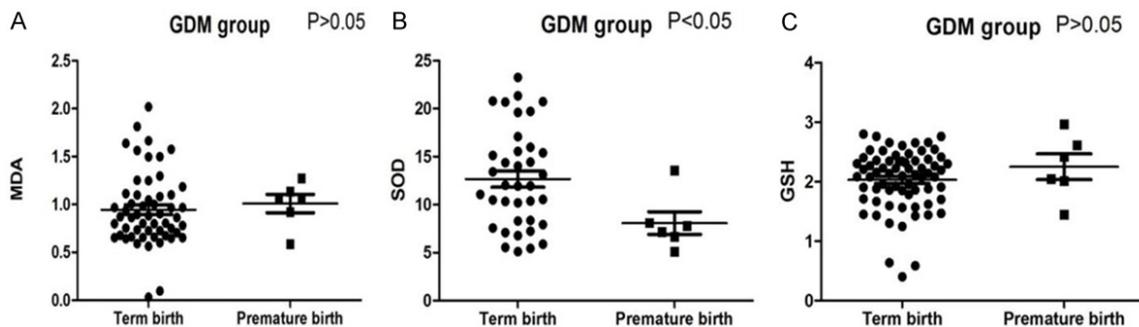

Figure 5. The effect of ROS related biomarkers' serum level on term or premature birth in GDM group. Data are given as the mean ± SEM. Statistical significance was determined using an unpaired t test. The detected biomarkers as indicated. *, P < 0.05 indicated significant.

assay was performed, the results showed that oxidative stress molecules HO-1, Nrf2 and NQO1 overexpressed in GDM placentas when compared with that of healthy pregnant women (**Figure 2A-C**), the reverse expression pattern was observed in SOD protein (**Figure 2D**). The staining mainly located in the cytoplasm of the trophoblast cells. All the statistical analysis was summarized in **Figure 2E-H**.
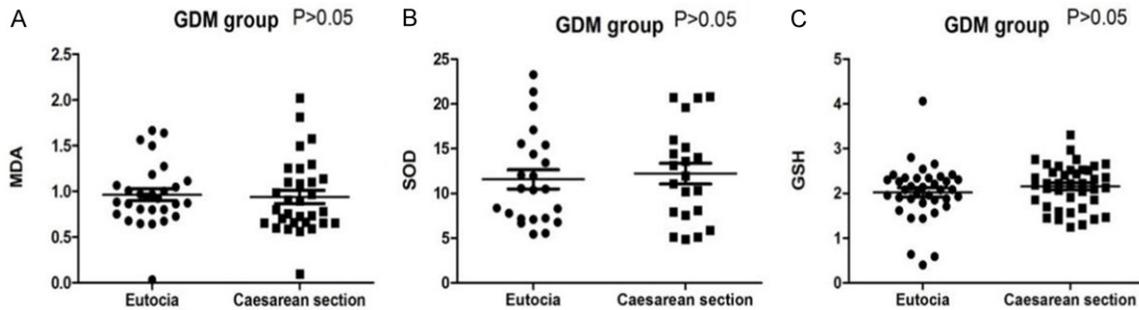
Figure 6. The association between the levels of MDA, SOD, GSH and delivery way in the GDM group, including eutocia and cesarean delivery. Data are given as the mean ± SEM. Statistical significance was determined using an unpaired *t* test. A. Comparison of MDA value between eutocia and cesarean delivery in the GDM group. *t* = 0.2430, *P* = 0.8088. B. Comparison of SOD value between eutocia and cesarean delivery in the GDM group. *t* = 0.4065, *P* = 0.6865. C. Comparison of GSH value between eutocia and cesarean delivery in the GDM group. *t* = 1.066, *P* = 0.2898.
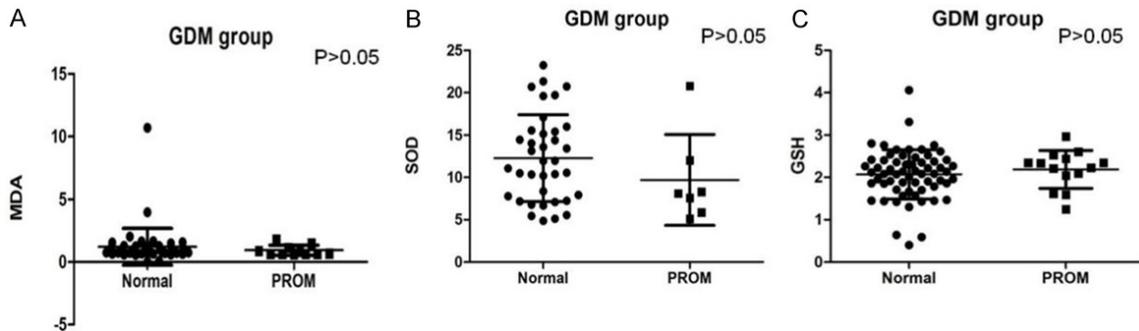

Figure 7. The effect of ROS related biomarkers' serum level on PROM in GDM group. Data are given as the mean ± SEM. Statistical significance was determined using an unpaired *t* test. A. Comparison of MDA value between healthy and PROM in the GDM group. *t* = 0.5893, *P* = 0.5579. B. Comparison of SOD value between healthy and PROM in the GDM group. *t* = 1.217, *P* = 0.2304. C. Comparison of GSH value between healthy and PROM in the GDM group. *t* = 0.6967, *P* = 0.4882. PROM, premature rupture of membranes.

Association between oxidative stress marker serum level and clinical pregnant outcome in GDM patients

There was an significant changes of oxidative stress markers in GDM patients, therefore, the effect of the variety on clinical pregnant outcome was further evaluated. The inverse association between the prepregnancy weight and MDA serum level was observed (r = -0.3547, P < 0.05, **Figure 3A**), indicating high level of MDA may be benefit to control body weight during the pregnancy. No significant correlation was found between the serum levels of GSH (r = -0.009502, P > 0.05) or SOD (r = 0.1393, P > 0.05) and prepregnancy weight (**Figure 3B** and **3C**). However, SOD serum level was positively correlated with neonatal birth weight (r = 0.3292, P < 0.05). In contrast, the MDA and GSH serum level has no any association with the neonatal birth weight (r = 0.1934 and r =

0.1482, P > 0.05, respectively; **Figure 4**). Interesting, it was found that lower level of serum SOD level has a risk of premature birth (12.68 ± 0.85 vs. 8.08 ± 1.18, P < 0.05). While, similar association has not been observed in MDA and GSH molecules (0.95 ± 0.05 vs. 1.01 ± 0.1, P > 0.05, and 2.03 ± 0.06 vs. 2.25 ± 0.22, P > 0.05; **Figure 5**). Further evaluation has not found any association between MDA, GSH, and SOD levels and cesarean delivery or PROM (P > 0.05, **Figures 6, 7**).

Discussion

Oxidative stress is an imbalance between ROS and antioxidant defense, which can lead to tissue damage. Although the exact mechanism of GDM is unknown, previous study addressed that oxidative stress involves in in GDM [26]. In current study, we found that some of the oxidative stress proteins overexpressed in GDM

patients, moreover, the aberrant levels of these molecules in serum associated with poor clinical outcome.

MDA is an end-product of lipid oxidation by peroxidation mediated by ROS. In the present study, it was observed that MDA serum level is higher in patients suffered with GDM than that in healthy pregnant women. Our result consistent with previous studies [27-29] SOD and GSH are antioxidants ubiquitously existing in human body and keeping the balance of redox reaction. The disfunction of these molecules will result various disease. Whether they also contribute to GDM development is not clear. Our result indicated that SOD and GSH levels are significantly decreased in patients with GDM. Similar result has been observed by López-Tinoco et al. [10, 30]. The expression pattern of SOD and GSH in GDM patients' placental issue is similar with the trend of both molecules' serum levels. As an inducible stress protein, HO-1 is widely accepted to be a highly sensitive and reliable marker of oxidative stress [31]. HO-1 was observed in villous trophoblastic cells of placenta [32]. As expect, HO-1 overexpressed in GDM patients' placental issues. The elevated HO-1 expression may be antagonize oxidative stress to ensure enough blood flowing to the placenta for the protection of the body and the fetus. Nrf2 is known as the upstream regulator of HO-1, which is an emerging regulator of cellular resistance to oxidants [33]. Martin et al. found that activation of HO-1 expression paralleled with increasing Nrf2 protein levels [34]. NQO1 is a downstream regulator of Nrf2-ARE pathways, Xinghua et al. reported that overexpression of Nrf2 significantly increased basal Nrf2 and NQO1 expression in GDM cells [35]. The Nrf2 antioxidant defense pathway may provide a therapeutic target for ameliorating oxidative stress associated with diabetes [36]. Our study also showed that Nrf2 and NQO1 proteins in GDM group were higher than that in control group, which is consistent with HO-1 expression pattern. AKR1C1 is a member of the aldo-keto reductase superfamily, which expressed up-regulated in cervical cancer but down-regulated in gastric cancer [22-24], There's rarely study examined the association between AKR1C1 and GDM, In our study, There was no significant difference in AKR1C1 expression between the GDM group

and control group. These data suggest that oxidative stress and its related molecules play an essential role in GDM.

Whether the aberrant changes of these molecules affect the clinical outcome remains unclarified. In the present study, we found that prepregnancy weight was significantly greater than that in the control group, moreover, MDA serum level was positively correlated with prepregnancy weight. More important, we observed that the rate of infants born with macrosomia was higher with 10.39% in the GDM group than that in the control group with 3.08%. Multiple studies have shown that patients with GDM are at increased risk of adverse perinatal outcomes, whether their infants with or without macrosomia, including intrauterine fetal demise, neonatal death, shoulder dystocia, and preeclampsia [37-39]. SOD levels were associated with neonatal birth weight in our study. Oussama et al. also found that GDM and macrosomia were associated with impaired SOD activities [40]. Globally, total serum antioxidant defense status in diabetic mothers and their macrosomic babies was diminished as compared with control subjects [40]. In favor of proving the previous point, Meriem et al. found that erythrocyte SOD activity was significantly increased in large-for-gestational-age newborns. These data confirm the existence of oxidative stress in fetal macrosomia [41]. Our study showed that there is no obvious correlation between MDA and neonatal birth weight. This result may be unexpected, because a previous study found that during pregnancy complicated by intrauterine growth restriction (IUGR), the concentration of MDA in amniotic fluid was significantly higher than in healthy pregnancy, and determination of MDA can be used as a biochemical test in parental diagnosis of IUGR [42]. The difference between those results and ours may be that we measured MDA in plasma, but the other authors measured MDA in amniotic fluid. We found an intensive relationship between SOD contents and preterm birth in the GDM group. In this context, it is notable that Maqusood et al. found SOD to be significantly higher in the placenta of women with preterm delivery compared with those delivering at full term [43], which was consistent with our findings. However, Pathak et al. demonstrated that maternal blood MDA levels were increased at birth in women with preterm

deliveries as compared with women delivering at full term [44]. On the contrary, our study suggested that MDA has nothing to do with preterm birth, which may be a result of the small number of premature births in our sample. As such, further studies need to be performed.

Our study showed that in the GDM group, the rates of cesarean delivery, macrosomia, preterm birth, and PROM were higher than that of the control group. Several other studies confirm our findings [45-47]. GDM increases the odds of adverse outcomes associated with a birth weight of 4000 g or greater, particularly shoulder dystocia [48]. This also indirectly increases the cesarean delivery rate. These biomarkers of oxidative stress we measured were not associated with cesarean delivery. This may be related to social factors and the fact that the number of women with a history of cesarean delivery is increasing.

In conclusion, it is obvious that there is oxidative stress in GDM. GSH, MDA, SOD, HO-1, Nrf2 and NQO1 could be regarded as useful markers for assessing oxidative stress in GDM. Some markers are closely associated with maternal weight and pregnancy outcomes in GDM, which tell us we should not only monitor blood glucose and control weight but also detect changes in oxidative stress levels with GDM. More experiments are needed to verify whether oxidative stress markers during pregnancy can be used as predictors of the occurrence of GDM. It is possible to establish targeted treatment for GDM through oxidative stress markers in the future. We therefore suggest follow-up studies on the oxidant and antioxidant status of GDM to improve the prognosis of pregnant women and fetuses.

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Disclosure of conflict of interest

None.

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