Abstract. – OBJECTIVE: To compare the placental exosome levels of normal pregnant women and pregnant women with gestational diabetes mellitus (GDM) in different gestational stages, and further investigate the effects of exosomes on the release of cytokines from human umbilical vein endothelial cells.

PATIENTS AND METHODS: 20 pregnant women, including 13 normal pregnant women and seven pregnant women with GDM were selected. Blood samples were collected during the three gestational stages (from week 11 to week 14 in the first trimester, from week 22 to week 24 in the second trimester, and from week 32 to week 36 in the third trimester).

RESULTS: Our results showed that both gestational age and physical condition significantly affected the concentration of exosomes in plasma (p < 0.05). The concentration of exosomes in plasma increased with gestational age in both normal pregnant women and pregnant women with GDM, but were increased significantly in the plasma of pregnant women with GDM (2.2-fold, 1.5-fold, and 1.8-fold higher than in normal pregnant women in the first, second, and third trimester, respectively).

CONCLUSIONS: Exosomes extracted from the plasma of pregnant women with GDM significantly increased the release of inflammatory cytokines from endothelial cells. However, the function of exosomes in pregnant women with GDM needs to be fully elucidated. The detection of exosomes in plasma could serve as a diagnostic tool for asymptomatic GDM.

Keywords: Gestational diabetes mellitus, Exosomes, Gestational age, Cytokines.

Introduction

Gestational diabetes mellitus (GDM) is defined as diabetes onset or initial recognition of diabetes during pregnancy. GDM affects about 15% of pregnant women worldwide. The increase of morbidity is correlated with the increase of obesity and type 2 diabetes. The global morbidity rate of GDM is roughly 18% based on the new standards set by the International Association of Diabetes and Pregnancy. GDM is associated with increased acute complications of pregnancy, and is closely related to the increased risk of diseases in pregnant women and fetuses.

Over the course of pregnancy, the placenta plays a key role in the regulation of physiological changes in pregnant women and fetal development. Insulin enhances the release of placental hormones during pregnancy, although placental changes are not directly related to insulin resistance in pregnant women. Current studies emphasize the use of tissue-specific exosomes as markers for disease diagnosis and monitoring. Exosomes are small membrane-bound vesicles (about 40-120 nm in diameter) that are released from the cell membrane by multiple vesicles through exocytosis. Exosomes are enriched with specific intracellular membrane proteins including Tsg101, CD63, CD9, and CD81. It has been reported that the concentration of exosomes in the plasma of pregnant women is higher than in non-pregnant women. Exosomes are released by the placenta into the peripheral blood circulation of pregnant women during the first 6 weeks of gestation. However, to date, there is no report on the changes of placental exosome concentration in plasma of pregnant women with GDM. Therefore, in the present study, we compared the placental exosome concentration in plasma of pregnant women with GDM with that of normal pregnant women. In addition, the effects of exosomes extracted from the plasma of pregnant women with GDM on cytokines released from human umbilical vein endothelial cells were an-
analyzed to determine if changes in the concentration, composition, or biological activity of exosomes could serve as markers of early diagnosis of diabetes.

**Patients and Methods**

**Patients and Sample Collection**

Twenty pregnant women who met the experimental requirements from Xuzhou Maternity and Child Health Care Hospital in 2015 were included. We obtained the informed consent from subjects. The study was approved by the Xuzhou Maternity and Child Health Care Hospital Ethics Committee. Blood samples were collected from the pregnant women during the first trimester of pregnancy (from week 11 to week 14), second trimester of pregnancy (from week 22 to week 24), and third trimester of pregnancy (from week 32 to week 36). Blood samples were centrifuged, and plasma was stored at −80°C. The 20 subjects included 13 healthy pregnant women as controls, and seven pregnant women with GDM as the experimental group. According to the standards established by the World Health Organization, pregnant women with blood glucose level higher than 7.0 mmol/l (126 m/dl) or 140 mg/dl at 2 h after treatment with oral glucose (75 g) were classified as having GDM.

**Extraction of Exosomes**

Extraction of exosomes was performed as previously described. Briefly, plasma was diluted with an equal volume of phosphate-buffered saline (PBS, pH 7.4), and centrifuged at 2,000 × g for 30 min at 4°C. The supernatant was then centrifuged at 12,000 × g for 45 min. The supernatant (approximately 2 ml) was transferred to a centrifuge tube (Beckman Coulter, Brea, CA, USA, 10 ml), and centrifuged at 100,000 × g for 2 h at 4°C. After centrifugation, the pellet was suspended in PBS, filtered through a 0.22-μm filter, and centrifuged again at 100,000 × g for 2 h at 4°C. The pellet was then resuspended in 500 μl of PBS, resulting in a relatively pure exosome preparation, and stored at −80°C until use.

**Measurement of Total and Placental Exosomes**

Total and placental exosomes from the blood of pregnant women were quantified by CD63 and Placental Alkaline Phosphatase (PLAP) ELISA kits (Beyotime, Nanjing, China). PLAP is a syncytiotrophoblast-specific marker. Therefore, placental-derived exosomes can be quantified using PLAP.

**Isolation and Culture of Human Umbilical Vein Endothelial Cells**

Cultured human umbilical vein endothelial cells were used to assess the biological activity of exosomes extracted from the plasma of pregnant women. The tissue was enzymatically digested with type II collagenase, and primary human umbilical vein endothelial cells were isolated. The isolated cells were cultured in an incubator at 37°C with 5% CO₂ using a fetal bovine serum-containing basal medium with 2% exosomes.

**Cytokine Detection**

To evaluate the effects of extracted exosomes on human umbilical vein endothelial cells, endothelial cells were cultured in 96-well plates. The cells were visualized using a Real-time cell imaging system (IncuCyte™ live-cell ESSEN BioScience Inc, Ann Arbor, Michigan, USA) according to the manufacturer’s instructions (Corning Life Science, Tewksbury, MA, USA). Before experiments, human umbilical vein endothelial cells were seeded in 96-well plates (Corning Life Science, Tewksbury, MA, USA), and cultured with PBS basal medium containing 0.2% exosomes. Cell fusion and morphological changes were observed every hour. Exosomes (100 μg/ml) were co-cultured with human umbilical vein endothelial cells in medium containing 5 mM D-glucose in an environment with 8% O₂. The release of cytokines was quantified using a protein dissolution assay (BioPlex® 200, Bio-Rad, Hercules, CA, USA). Cytokine data are expressed as pg/10⁵ cells/24 h.

**Statistical Analysis**

Data are presented as mean ± standard error. Normal represents the control group, GDM represents the experimental group, early represents the first trimester of pregnancy, mid represents the second trimester of pregnancy, and late represents the third trimester of pregnancy. The effects of gestational age on the concentration of exosomes in plasma, exosome protein, and PLAP were analyzed by double factor variance analysis. Statistical differences between groups were analyzed using Turkey HSD method. Mann-Whitney U-test was used to analyze the distribution of independent data. Student’s t-tests were used to
compare the statistical differences between the two groups. \( p < 0.05 \) was considered statistically significant.

**Results**

**Clinical Characteristics of Patients**

A total of 100 women were enrolled in this study. None of the women were screened for either normal blood glucose or GDM. Finally, 20 women were selected for inclusion in the study. Among the 20 cases, 8 were from normal blood glucose healthy women, in which 39 blood samples were collected. In addition, there were seven cases with GDM, from which 21 blood samples were collected. Age, weight, body mass index (BMI), and gestational age of the 20 subjects are presented in Table I. The women who participated in the study did not smoke, did not drink alcohol, and did not use drug. In addition, all pregnant women selected for this study had no hypertension, no intrauterine infection, or medical or obstetric complications other than GDM. * represents significant difference compared with normal pregnant women, \( p < 0.05 \).

### Exosome Extraction and Characterization

Exosomes were extracted with gold standard and were purified by density gradient centrifugation. Western blot analysis showed positive expression of CD63 (Figure 1). There was no significant difference in vesicle size between the control group and GDM group (Table II).

### Table I. Clinical data of patients and neonates.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Normal (n=13)</th>
<th>GDM (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>24 ± 1.6 (18-36)</td>
<td>25.53 ± 7.1 (20-34)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>65 ± 4.3 (54-108)</td>
<td>60.44 ± 10.0 (50-86)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>158 ± 2.2 (149-173)</td>
<td>155.6 ± 5.1 (149-163)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.9 ± 1.2 (21-36)</td>
<td>26.86 ± 2.2 (22-30)</td>
</tr>
<tr>
<td>OGTT of first trimester of pregnancy (mg/dl)</td>
<td>73 ± 3.1 (50-90)</td>
<td>76 ± 5.0 (62-82)</td>
</tr>
<tr>
<td>OGTT of second trimester of pregnancy (mg/dl)</td>
<td>70 ± 2.6 (57-90)</td>
<td>70 ± 3.8 (58-70)</td>
</tr>
<tr>
<td>OGTT of third trimester of pregnancy (mg/dl)</td>
<td>95 ± 5.3 (61-124)</td>
<td>96.5 ± 10.5* (61-124)</td>
</tr>
<tr>
<td>First trimester of pregnancy (week)</td>
<td>12 ± 2.3 (11-14)</td>
<td>12.1 ± 2.3 (11-14)</td>
</tr>
<tr>
<td>Second trimester of pregnancy (week)</td>
<td>26 ± 3.0 (22-28)</td>
<td>27.2 ± 3.2 (22-28)</td>
</tr>
<tr>
<td>Third trimester of pregnancy (week)</td>
<td>35 ± 3.6 (30-38)</td>
<td>35 ± 3.6 (30-38)</td>
</tr>
<tr>
<td>Gestational age at delivery (week)</td>
<td>39 ± 1.9 (38-40)</td>
<td>39 ± 1.9 (38-40)</td>
</tr>
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</table>

**Systolic blood pressure (mm/Hg)**

- First trimester of pregnancy (week): 107 ± 3.0 (90-120) vs. 107 ± 3.0 (90-120)
- Second trimester of pregnancy (week): 106 ± 3.3 (90-120) vs. 110 ± 3.3 (100-120)
- Third trimester of pregnancy (week): 111 ± 2.2 (100-120) vs. 108 ± 2.2 (100-120)

**Diastolic blood pressure (mm/Hg)**

- First trimester of pregnancy (week): 65 ± 3.3 (50-80) vs. 65 ± 3.5 (60-80)
- Second trimester of pregnancy (week): 65 ± 3.0 (50-90) vs. 62 ± 2.6 (60-80)
- Third trimester of pregnancy (week): 65 ± 3.0 (50-70) vs. 65 ± 3.0 (50-70)

**Neonatal variables**

- Placental weight (g): 650 ± 30 (535-730) vs. 650 ± 30 (535-730)
- Fetal weight (g): 3369 ± 223 (2340-3800) vs. 3369 ± 223 (2340-3800)
- Fetal sex (male/female): 4/6 vs. 6/7

Note: Data are expressed as mean ± standard error. All pregnant women did not smoke, did not drink alcohol, and did not use drug. In addition, all pregnant women selected for this study had no hypertension, no intrauterine infection, or medical or obstetric complications other than GDM. * represents significant difference compared with normal pregnant women, \( p < 0.05 \).

**Oral Glucose Tolerance Test (OGTT)** measures the glucose content after 2 h of oral glucose treatment (75 g). Normal represents normal pregnant women, GDM represents pregnant woman with gestational diabetes mellitus.

**Figure 1.** The features of exosomes from pregnant women with GDM. Exosomes were extracted from the plasma of pregnant women with GDM and normal pregnant women during the first trimester of pregnancy (from week 11 to week 14), second trimester of pregnancy (from week 22 to week 28), and third trimester of pregnancy (from week 32 to week 38). Western blot was used to detect the expression of CD63, a marker of exosome enrichment.
Changes of Exosome Concentration in Pregnant Women at Different Gestational Stages

The average concentration of exosomes extracted from the plasma of normal pregnant women and pregnant women with GDM was $1.34 \times 10^{12} \pm 2.75 \times 10^{11}$ and $2.89 \times 10^{12} \pm 5.33 \times 10^{11}$/ml plasma, respectively (Figure 2A). Exosome concentration gradually increased with gestational age in both the control group and GDM group (Figure 2B). Gestational age and GDM had significant effects on the amount of exosomes ($p < 0.005$). The levels of exosomes were higher at each gestational stage (first, second, and third trimester of pregnancy) in pregnant women with GDM compared with the corresponding gestational stage in normal pregnant women. The concentration of total exosomes extracted from the normal and GDM groups at the first trimester of pregnancy was $1.27 \times 10^{12} \pm 4.97 \times 10^{11}$ and $2.77 \times 10^{11} \pm 6.20 \times 10^{10}$/ml plasma, respectively; the concentration at the second trimester of pregnancy was $2.85 \times 10^{12} \pm 7.78 \times 10^{11}$ and $1.37 \times 10^{12} \pm 4.14 \times 10^{11}$/ml plasma, respectively; and the concentration at the third trimester of pregnancy was $4.55 \times 10^{12} \pm 1.04 \times 10^{12}$ and $2.38 \times 10^{12} \pm 5.99 \times 10^{11}$/ml plasma, respectively. Fetal sex, maternal BMI, maternal age, and maternal weight and height had no significant effect on the concentration of exosomes.

The Changes of PLAP Concentration in Exosomes of Pregnant Women with GDM at Different Gestational Stages

There was a higher concentration of PLAP in the plasma of pregnant women with GDM compared with normal pregnant women. The concentration in the two groups was $276 \pm 33$ and $191 \pm 18$ pg/ml, respectively (Figure 3A). Placental exosome (PdE) concentrations of pregnant women

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Table II. The distribution of vesicle size during pregnancy.

<table>
<thead>
<tr>
<th>Gestational age</th>
<th>Normal</th>
<th>GDM</th>
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<tbody>
<tr>
<td>First trimester of pregnancy (from week 11 to week 14)</td>
<td>103 ± 41 nm</td>
<td>108 ± 47 nm</td>
</tr>
<tr>
<td>Second trimester of pregnancy (from week 22 to week 28)</td>
<td>107 ± 48 nm</td>
<td>109 ± 53 nm</td>
</tr>
<tr>
<td>Third trimester of pregnancy (from week 32 to week 36)</td>
<td>110 ± 51 nm</td>
<td>112 ± 53 nm</td>
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Notes: Vesicle size distribution was analyzed using a NanoSight NS500 instrument (NanoSight, Amesbury, UK) according to the manufacturer’s instructions. All data are expressed as mean ± standard deviation.

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**Figure 2.** Analysis of the concentration of exosomes in plasma of pregnant women with GDM. **(A)** The concentration of total exosomes in plasma of pregnant women with GDM. **(B)** The concentration of exosomes in plasma of pregnant women at different gestational stages. Data are expressed as mean ± standard deviation, white circles represent normal pregnant women, and black circles represent pregnant women with GDM. *$p < 0.05$.**
with GDM increased gradually with gestational age (Figure 3B). During the first trimester of pregnancy, PLAP levels in the GDM group were six times greater than those in the normal control group (128 ± 14 and 81 ± 7 pg/ml, respectively). During the second trimester of pregnancy, PLAP levels in the GDM group were 1.5 times higher than those in the normal control group (282 ± 24 and 188 ± 14 pg/ml, respectively). During the third trimester of pregnancy, PLAP levels in the GDM group were 1.3 times higher than those in the normal control group (418 ± 57 and 304 ± 29 pg/ml, respectively). Fetal sex, maternal BMI, maternal age, maternal weight, and height had no significant effect on PLAP concentration.

**The Effect of Exosomes on the Release of Cytokines from Endothelial Cells**

GDM is a syndrome closely related to the inflammatory response. Therefore, we analyzed the effects of exosomes extracted from the plasma of pregnant women on the release of inflammatory cytokines from endothelial cells. Compared with untreated cells, exosome treatment caused a significant increase in the release of cytokines from endothelial cells (p < 0.05) (Figure 4). GM-CSF, IL-6, IL-8, IFN-γ, and TNF-α levels were significantly increased in response to exosomes extracted from the plasma of normal pregnant women at the first, second, and third trimester of pregnancy (about 1.8-fold), while the exosomes extracted from the plasma of normal pregnant women had no significant effect on cytokine levels. Exosomes extracted from the plasma of pregnant women with GDM significantly increased the release of cytokines from endothelial cells (about 3.3-fold).

**Discussion**

Extracellular vesicles have long been recognized as important regulators of intercellular biological processes. According to their size and origin, extracellular vesicles are divided into microvesicles (50-1000 nm, produced by serosa by budding) and exosomes (40-130 nm, produced by endosomal exocytosis). Several scholars have demonstrated the presence of placental-derived extracellular vesicles in maternal blood circulation during pregnancy. The metabolic and immune status of the body may change the metabolism and function of the placenta during the first trimester of pregnancy. Inflammation in pregnant women is closely associated with GDM, indicating that an inflammatory environment can regulate maternal blood glucose. Moreover, this phenomenon may be closely related to the biological activity of placental and non-placental exosomes. Researches have shown that exosomes can increase the cell response to high glucose concentration during...
Placental exosomes in maternal circulation in gestational diabetes mellitus

Figure 4. Exosome-induced release of cytokines from endothelial cells. The effects of exosomes (100 μg/ml) extracted from plasma of normal pregnant women (EXO Normal) and pregnant women with GDM (EXO GDM) on the levels of (A) GM-CSF, (B) IL-4, (C) IL-6, (D) IL-8, (E) IFN-γ, and (F) TNF-α. Data were analyzed by double factor variance analysis. *p < 0.05 represents the comparison with all groups, and #p < 0.05 represents the comparison with the EXO normal group.
the first trimester of pregnancy, and the release of exosomes under this condition can increase the release of cytokines from endothelial cells. The effects of placental exosomes on cytokine release have not yet been reported, and further studies are still required.

Early diagnosis of GDM (i.e., during the first week of pregnancy) can reduce the long-term effects of GDM on pregnant women and the fetus29. However, if GDM is diagnosed at the second or third week of gestation, the reversion or restriction of the impact of the disease on the perinatal prognosis may be relatively difficult. Complications of pregnancy can adversely affect both pregnant women and fetuses, increase the risk of development of a metabolic syndrome (obesity and type 2 diabetes), and increase the risk of type II diabetes in pregnant women. The risk of females born from pregnant women with GDM acquiring GDM during their own future pregnancy will also be increased, thus forming a vicious circle. Diagnosing gestational diabetes within the first week of pregnancy allows for the opportunity to treat and improve the outcomes of pregnancy, and reduce the occurrence and severity of complications of pregnancy.

Therefore, the aim of this study was to assess the concentration and biological activity of exosomes in the plasma of pregnant women with GDM. Our results showed that compared with normal pregnant women, the concentration of exosomes in plasma of pregnant women with GDM increased by about 2-fold. Several studies showed that the concentration of both exosomes and placental exosomes in both normal pregnant women and pregnant women with GDM increased by about 2-fold. However, our study confirmed that the levels of placental exosomes in maternal plasma of the pregnant women at different gestational stages were higher than in normal pregnant women at the corresponding stage. In addition, our study confirmed that exosomes extracted from the plasma of pregnant women with GDM are biologically active and can regulate the release of proinflammatory cytokines from endothelial cells. These results suggest that it is feasible to diagnose asymptomatic GDM (from week 11 to week 14) and asymptomatic GDM (from week 24 to week 28) by measuring the concentration of exosomes in plasma. In addition, placental exosomes are associated with symptoms of maternal inflammation, which is a potential risk factor for the development of GDM in pregnant women.

Conclusions

We showed that there was a significant difference between the levels of placental exosomes in normal pregnant women and pregnant women with GDM. Exosomes can affect the function of endothelial cells, and participate in the development of the inflammatory state of GDM. The cytokines released by endothelial cells enhance the degree of the effects of exosomes on GDM. However, further studies are needed to elucidate the exact mechanism of effects of total exosomes and placental exosomes in normal pregnant women and pregnant women with GDM on the metabolism of pregnant women.

Conflict of Interest

The authors declare that they have no conflict of interests.

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