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Gestational diabetes mellitus is associated with changes in the concentration and bioactivity of placental exosomes in the maternal circulation across gestation

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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 [illegible] 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 [illegible]ly 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 has not been fully elucidated. The detection of exosomes in plasma could serve as a diagnostic tool for asymptomatic GDM.

Key words:
Gestational diabetes mellitus, Exosomes, Gestational [illegible].

Introduction

Gestational diabetes mellitus (GDM) is defined as diabetes onset or initial recognition of diabetes during pregnancy[1]. GDM affects about 15% of pregnant women worldwide. The increase of morbidity is related with the increase of obesity and type 2 diabetes[2]. The global morbidity rate of GDM is roughly 18% based on the new standards set by the International Association of Diabetes and Pregnancy[3]. GDM is associated with increased acute complications of pregnancy, and is closely related to the increased risk of diseases in pregnant women and fetuses[4].

Over the course of pregnancy, the placenta plays a key role in the regulation of physiological processes 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[4]. Current studies[5,6] 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[6,7]. It has been reported that the concentration of exosomes in the plasma of pregnant women is higher than in non-pregnant women[5]. Exosomes are released by the placenta into the peripheral blood circulation of pregnant women during the first 6 weeks of gestation[8-10]. 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-

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alyzed 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 stan[illegible] established by the World Health Organiz[illegible] pregnant women with blood glucose level [illegible]er than 7.0 mmol/l (126 m/dl) or 140 mg/dl a[illegible] after treatment with oral glucose (75 g) were c[illegible]sified as having GDM.

Extraction of Exosome[illegible]

Extraction of exosom[illegible] plas[illegible] formed as previousl[illegible]. B[illegible]y, plasma was dilute[illegible]ith an [illegible] volume of phosphate-buffer[illegible]line (PBS, [illegible]7.4), and centrifuged at [illegible] for 30 min a[illegible]°C. The supernatant w[illegible] then [illegible]fuged at 12,000 × g and 4°C f[illegible]45 min. The[illegible]rnatant (approximately [illegible]) was transferred [illegible] centrifuge tube (Bec[illegible]n Coul[illegible] Brea, CA, USA, 10 ml), and ce[illegible]ed a[illegible]0,000 × g for 2 h at 4°C. After centri[illegible] the pe[illegible] was suspended in PBS [illegible]ml), [illegible]d thr[illegible] a 0.22-µm filter, and cen[illegible]d ag[illegible],000 × g for 2 h at 4°C. The pel[illegible] was the[illegible]spended in 500 µl of PBS, re[illegible] relatively pure exosome preparation, [illegible]sto[illegible]-80°C until use.

[illegible]asurement of Total and [illegible]ntal 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)[5,8]. PLAP is a syncytiotrophoblast-specific marker. Therefore, placental-derived exosomes can be quantified using PLAP.

Isolation and Culture of Hum[illegible] Umbilical Vein Endothelial [illegible]

Cultured human umbilical ve[illegible]othelial cells were used to assess[illegible] bio[illegible]y of exosomes extracted from[illegible] plasma [illegible]nant women. The ti[illegible] was [illegible]zymat[illegible] digested with type I[illegible]agen[illegible] and primary human umbilical [illegible]al ce[illegible] were isolated. The is[illegible]d ce[illegible] cult[illegible] in an incubator at [illegible] with 5% [illegible]g a fetal bovine ser[illegible]ing basal [illegible]m with 2% exosomes[illegible]

Cy[illegible] Detection

[illegible]evaluate the effects o[illegible]xtracted exosomes on h[illegible]an umbilical [illegible]n endothelial cells, endothelial [illegible] were cultu[illegible] in 96-well plates. The cells w[illegible]visualized [illegible]ng a Real-time cell imaging sys[illegible]M live-cell ESSEN BioScience Inc, A[illegible] Michigan, USA) according to the manufacturer's instructions (Corning Life Science, [illegible]bury, MA, USA). Before experiments, hu[illegible]an [illegible]bilical 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% O_2. The release of cytokines was quantified using a protein dissolution assay (BioPlex® 200, Bio-Rad, Hercules, CA, USA). Cytokine data are expressed as pg/10^5 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

Table I. Clinical data of patients and neonates.

Pregnant women		
Variables	Normal (n=13)	GDM (n=7)
Age (years)	24 ± 1.6 (18-36)	25.53 ± 7.1 (20-3[illegible])
Weight (kg)	65 ± 4.3 (54-108)	60.44 ± 10.9[illegible]
Height (cm)	158 ± 2.2 (149-173)	155.6 ± 5.[illegible]0-163)
BMI (kg/m^2)	25.9 ± 1.2 (21-36)	26.86 ± [illegible] (22-30)
OGTT of first trimester of pregnancy (mg/dl)	73 ± 3.1 (50-90)	76 ± 5.[illegible]4)
OGTT of second trimester of pregnancy (mg/dl)	70 ± 2.6 (57-90)	83 ± 3.8 ([illegible])
OGTT of third trimester of pregnancy (mg/dl)	95 ± 5.3 (61-124)	[illegible] 10.5* ([illegible])
First trimester of pregnancy (week)	12 ± 2.3 (11-14)	[illegible]1 ± 1.9 (11-14)
Second trimester of pregnancy (week)	26 ± 3.0 (22-28)	27.2 ± 2.[illegible] (22-28)
Third trimester of pregnancy (week)	35 ± 3.6 (30-38)	35 ± [illegible]0-38)
Gestational age at delivery (week)	39 ± 1.9 (38-40)	39 [illegible] (38-40)
Systolic blood pressure (mm/Hg)		
First trimester of pregnancy (week)	107 ± 3.0 (90-120)	[illegible]3.4 ([illegible]20)
Second trimester of pregnancy (week)	106 ± 3.3 (90-13[illegible]	11[illegible]-120)
Third trimester of pregnancy (week)	111 ± 2.2 (100[illegible])	108 ± [illegible]100-120)
Diastolic blood pressure (mm/Hg)		
First trimester of pregnancy (week)	65 ± 2.4 (50-80)	67 ± 3.5 (60-80)
Second trimester of pregnancy (week)	65 ± [illegible]0)	[illegible] ± 2.6 (60-80)
Third trimester of pregnancy (week)	68 [illegible] (50-70)	[illegible]0 ± 3.6 (50-70)
Neonatal variables		
Placental weight (g)	[illegible] 24 (501-731)	650 ± 30 (535-730)
Fetal weight (g)	3[illegible] 141 (2660-4[illegible]	3369 ± 223 (2340-3800)
Fetal sex (male/female)	4/4[illegible]	6/7

Note: Data are expressed as mean ± standard error[illegible]gnant women [illegible]smoke, did not drink alcohol, and did not use drug. In addition, all pregnant women selected [illegible]had no hypertension, no intrauterine infection, or medical or obstetric complications other than GDM. *represen[illegible]ifica[illegible]e compared with normal pregnant women, $p < 0.05$. Oral Glucose Tolerance Test (OGTT) measures the glu[illegible]conte[illegible] oral glucose treatment (75 g). Normal represents normal pregnant women, GDM represents pregnant wo[illegible]stational diabetes mellitus.

compare the statistical diffe[illegible]es [illegible]een the two groups. $p < 0.05$ was [illegible]dered [illegible]istically significant.

[illegible]esults

Clinical Cha[illegible]cteris[illegible]of Patients

A total [illegible]100 wome[illegible] enrolled in this study. T[illegible]e women were sc[illegible]d for either normal [illegible]d gluc[illegible]or GDM. Finally, 20 women we[illegible]cted [illegible]nclusion in the study. Among the 20 ca[illegible] were 1[illegible]n normal blood glucose [illegible]ealth[illegible]trols, [illegible] which 39 blood samples w[illegible]ollect[illegible]tion, there were seven cases wit[illegible]DM, fr[illegible] which 21 blood samples were co[illegible]. The age, weight, body mass index (BMI), [illegible]ges[illegible] age of the 20 subjects are presented [illegible] Table I. The women who participated in the study [illegible]ot smoke, and had no intrauterine infection, or an[illegible]her medical or obstetric complications other than GDM. All had normal blood pressure. There were no significant differences in neonatal weight or placental weight between the two groups ($p > 0.05$).

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).

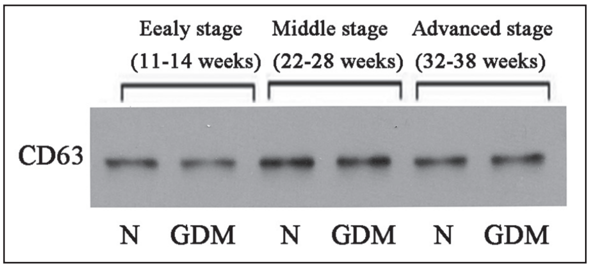

Figure 1. The features of exosomes from pregnant women with GDM. Exosomes were extracted from the plasma of pregnant women with GDM and normal pregnant women during the first trimester of pregnancy (from week 11 to week 14), second trimester of pregnancy (from week 22 to week 28), and third trimester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

Table II. The distribution of vesicle size during pregnancy.

Gestational age	Normal	GDM
First trimester of pregnancy (from week 11 to week 14)	103 ± 41 nm	108 ± [illegible]
Second trimester of pregnancy (from week 22 to week 28)	107 ± 48 nm	10[illegible]
Third trimester of pregnancy (from week 32 to week 36)	110 ± 51 nm	[illegible]53 nm

Notes: Vesicle size distribution was analyzed using a NanoSight NS500 instrument (NanoSight, Amesbury, U[illegible]ding to the manufacturer's instructions. All data are expressed as mean ± standard deviation.

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 [illegible]rd trimester of pregnancy) in pregnant wom[illegible] GDM compared with the corresponding [illegible]stational stage in normal pregnant women. [illegible] concentration of total exosomes extracted fr[illegible] the normal and GDM groups at [illegible] trimeste[illegible] of pregnancy was 1.27 × 10[illegible].9[illegible]10^{11} and $2.77 \times 10^{11} \pm 6.20$ × [illegible]ml pl[illegible], respectively; the concentrati[illegible] a[illegible]d trimester of pregnancy was [illegible] × 10[illegible]8 × 10[illegible]d $1.37 \times 10^{12} \pm 4.14$ [illegible]/ml plasm[illegible]ely; and the concent[illegible] the third t[illegible]er of pregnancy wa[illegible]55 × [illegible] ± 1.04×10^{12} and $2.38 \times 10^{12} \pm 5.99 \times 10^{11}$/m[illegible]ma, respectively. Fetal sex[illegible]al BMI, ma[illegible] age, and maternal w[illegible] and height had no significant effect on the [illegible]entration of [illegible]osomes.

T[illegible] Changes [illegible] PLAP Concentration in [illegible] Pregnant Women with GDM [illegible]rent Gestational Stages

There was a higher concentration of PLAP in [illegible]sma of pregnant women with GDM com[illegible]are[illegible]th normal pregnant women. The concentration in the two groups was 276 ± 33 and 191 ± 18 pg/ml, respectively (Figure 3A). Placental exosome (PdE) concentrations of pregnant women

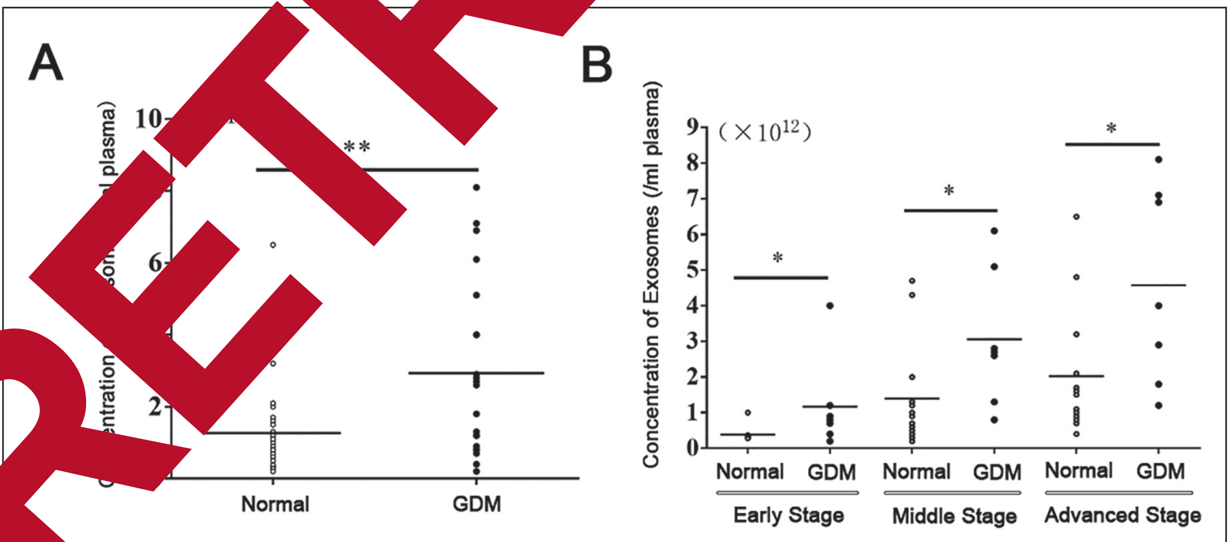

Figure 2. Analysis of the concentration of exosomes in plasma of pregnant women with GDM. ***(A)*** The concentration of total exosomes in plasma of pregnant women with GDM. ***(B)*** The concentration of exosomes in plasma of pregnant women at different gestational stages. Data are expressed as mean ± standard deviation, white circles represent normal pregnant women, and black circles represent pregnant women with GDM. *$p < 0.05$.

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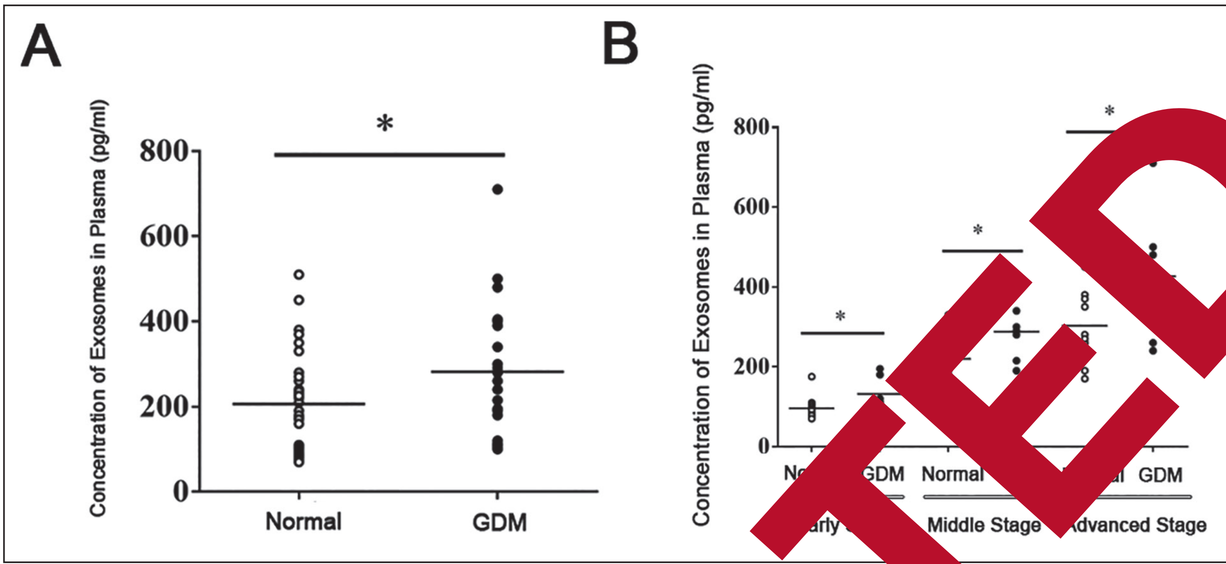

Figure 3. Analysis of the concentration of exosomes in plasma of preg[illegible]en with GDM. [illegible]e average concentration of placental exosomes in pregnant women with GDM. ***(B)*** The conc[illegible]ion of placental exoso[illegible] in pregnant women with GDM at different gestational stages. Data are expressed as mean ± [illegible]ard deviation, white circles represent normal pregnant women, and black circles represent pregnant women with GDM. *[illegible]0.05.

with GDM increased gradually with gestational age (Figure 3B). During the first trimest[illegible] of pregnancy, PLAP levels in the GDM gro[illegible] six times greater than those in the normal c[illegible]ol group (128 ± 14 and 81 ± 7 pg/ml, respectiv[illegible]. During the second trimester of pregnancy, PL[illegible] levels in the GDM group were [illegible]es high[illegible] than those in the normal c[illegible] g[illegible] (282 ± 24 and 188 ± 14 pg/ml, res[illegible]ively). [illegible]ring the third trimester of pregna[illegible] PLAP [illegible] the GDM group were 1.3 [illegible]es [illegible]an th[illegible] the normal control [illegible]p (418 [illegible]nd 304 ± 29 pg/ml, respectiv[illegible] Fetal sex, [illegible]nal BMI, maternal age, [illegible]eight, and [illegible]t had no significant ef[illegible] on PL[illegible]oncentration.

The E[illegible] of Exosomes [illegible]he Release of C[illegible]kines [illegible]m Endothelial Cells

[illegible] is a [illegible]rome closely related to the inflamm[illegible]ponse [illegible]refore, we analyzed the [illegible]ects o[illegible]omes [illegible]acted from the plasma of p[illegible]nt w[illegible]he release of inflammatory cyt[illegible]es fro[illegible]othelial cells. Compared with u[illegible] cells, exosome treatment caused a sig[illegible]an[illegible]se in the release of cytokines from [illegible]dothelial cells ($p < 0.05$) (Figure 4). GM-CSF, [illegible], IL-6, IL-8, IFN-γ, and TNF-α levels were sig[illegible]cantly 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 ext[illegible] fro[illegible]e plasma of normal pregnant women [illegible] significant effect on cytokine lev[illegible]ls. Exosomes extracted from the plasma of preg[illegible]men with GDM significantly increased [illegible]e [illegible]se 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[5,8,12-14] 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[15-17], 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[18-21] have shown that exosomes can increase the cell response to high glucose concentration during

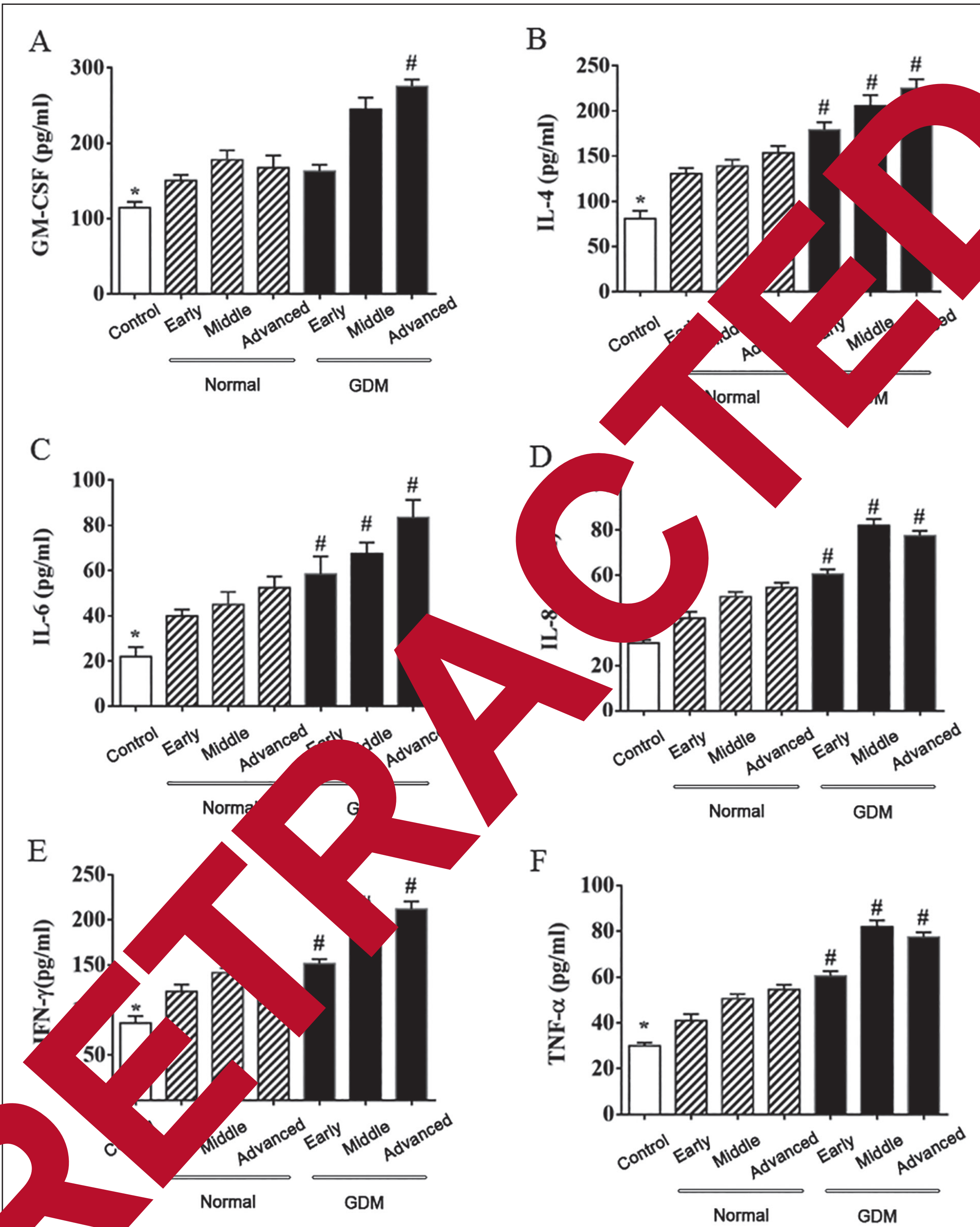

Figure 4. Exosome-induced release of cytokines from endothelial cells. The effects of exosomes (100 μg/ml) extracted from the plasma of normal pregnant women (EXO Normal) and pregnant women with GDM (EXO GDM) on the levels of *(A)* GM-CSF, *(B)* IL-4, *(C)* IL-6, *(D)* IL-8, *(E)* IFN-γ, and *(F)* TNF-α. 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 fetus[20]. 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 occurence and se[illegible] of complications of pregnancy.

Therefore, the aim of this study was [illegible]sess the concentration and biological act[illegible] of exosomes in the plasma of pregnant wom[illegible] with GDM. Our results showe[illegible] compare[illegible] with normal pregnant wome[illegible] c[illegible]ntration of exosomes in plasma of [illegible]gnant w[illegible]en with GDM increased by abo[illegible]fold. [illegible] studies showed that [illegible] c[illegible]on o[illegible] exosomes and pla[illegible] exos[illegible] of both normal pregnant wo[illegible] and pregna[illegible]men with GDM increase[illegible]t pregnanc[illegible]owever, the levels o[illegible]osome[illegible] plasma of the pregnant wom[illegible]t different g[illegible]ional stages were higher [illegible] in normal preg[illegible] women at the corr[illegible]nding [illegible]e. In addition, our study confir[illegible]hat e[illegible]mes extracted from the plasma of pre[illegible]omen w[illegible] GDM are biologically [illegible]ve a[illegible] regu[illegible] the release of proinflammatory cyt[illegible]m endothelial cells. These res[illegible] sugges[illegible]at it is feasible to diagnose ea[illegible]DM (from week 11 to week 14) and as[illegible]ptom[illegible]DM (GDM diagnosed from week [illegible] to week 28) by measuring the concentration [illegible]xosomes in plasma. In addition, placental ex[illegible]nes 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 exo[illegible] and exosomes derived from other tis[illegible] normal pregnant women and pre[illegible] women with GDM. Exosomes can affe[illegible]e function of endothelial cells, and participate [illegible]e development of the inflammatory [illegible]te of [illegible]. The cytokines released by end[illegible]ial cells ca[illegible] the degree of the effe[illegible] exoso[illegible]s on G[illegible]. However, further st[illegible] are r[illegible]red to elucidate the exact mec[illegible] [illegible] effects [illegible] total exosomes and p[illegible]ntal [illegible]es fr[illegible]ormal pregnant wom[illegible]nd pregna[illegible]ith GDM on the meta[illegible]f pregnant [illegible].

Co[illegible] Interest

Th[illegible]hors declare that they h[illegible]e no conflict of interests.

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