

RESEARCH ARTICLE



# Association of hyperglycaemia with the placenta of GDM-induced macrosomia with normal pre-pregnancy BMI and the proliferation of trophoblast cells

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## ABSTRACT

The aim of this study was to identify the effect of hyperglycaemia on placentas of gestational diabetes mellitus (GDM) women with macrosomia and normal pre-pregnancy body mass index (BMI), and uncover the molecular mechanism of hyperglycaemia on trophoblast cells *in vitro*. GDM women with normal pre-pregnancy BMI were divided into GM group (macrosomia,  $n = 30$ ) and GN group (normal birth weight,  $n = 35$ ). The study showed GM group had more adverse pregnancy outcomes and higher levels of gestational weight gain, blood glucose and triglyceride. After adjustment for confounding factors, just the fasting plasma glucose level and HbA1c percentage were related to the incidence of GDM-induced macrosomia with normal pre-pregnancy BMI. Meanwhile, the fasting blood glucose was closely related to the placental weight and placental PCNA expression. Furthermore, the *in vitro* model for placenta showed that hyperglycaemia significantly promoted trophoblast cell proliferation and activated ERK1/2 phosphorylation. ERK1/2 inhibitor markedly suppressed hyperglycaemia-induced trophoblastic proliferation. The fasting plasma glucose and placenta are closely related with the development of GDM-induced macrosomia with normal pre-pregnancy BMI. The mechanism may be hyperglycaemia promotes trophoblast cell proliferation via ERK1/2 signalling. It provides scientific evidence for optimising outcomes of GDM women with normal pre-pregnancy BMI.

## KEYWORDS

Hyperglycaemia; placenta; macrosomia; gestational diabetes mellitus; pre-pregnancy body mass index; ERK1/2 signal pathway



## IMPACT STATEMENT

- **What is already known on this subject?** Gestational diabetes mellitus (GDM) is one of the strongest risk factors correlated with macrosomia. The hyperglycaemic intrauterine environment affects not only the foetus but also the placental development and function in humans and experimental rodents. However, placental abnormalities associated with maternal diabetes have been inconsistently reported, possibly because of population differences in pre-pregnancy weight, diabetes types, glycemic control or pregnancy complication, and the molecular mechanism of hyperglycaemia on trophoblast cells *in vitro* was not clearly stated.
- **What do the results of this study add?** This is the first study to identify the effect of hyperglycaemia on placentas of gestational diabetes mellitus (GDM) women with macrosomia and normal pre-pregnancy body mass index (BMI), and uncover the molecular mechanism of hyperglycaemia on trophoblast cells *in vitro*.
- **What are the implications of these findings for clinical practice and/or further research?** Understanding placental changes in the environment of abnormal glucose metabolism which can establish the maternal-placental-foetal interface dysfunction as a potential source of adverse pregnancy outcomes is very necessary. Our study found the fasting plasma glucose and placenta are closely related with the development of GDM-induced macrosomia with normal pre-pregnancy BMI. The mechanism may be hyperglycaemia promotes trophoblast cell proliferation via ERK1/2 signalling. It provides scientific evidence for optimising outcomes of GDM women with normal pre-pregnancy BMI, and could be used for the following studies of relationship between placenta and childhood complications.

## 1. Introduction

Macrosomia, defined as a birth weight over 4,000 g or above the 90th percentile for gestational age, is a common pregnancy-associated disease (Walsh and McAuliffe 2012). It has been linked to an increased maternal and neonatal risk of

complications (Kc et al. 2015; Li et al. 2014; Beta et al. 2019). In addition, macrosomia is also at greater risk of childhood obesity, diabetes, cardiovascular diseases and some cancers later in life (Walsh and McAuliffe 2012; Mamun et al. 2009; Ghassibe-Sabbagh et al. 2019). Many studies have shown

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gestational diabetes mellitus (GDM) is one of the strongest risk factors correlated with macrosomia (Wang et al. 2017; Mohammadbeigi et al. 2013; He et al. 2015). Between 15 and 45% of newborns of mothers with GDM are macrosomic (Kc et al. 2015). GDM-induced macrosomia remains a major public health problem over the next few years.

The foetal growth is determined by numerous factors including the maternal uterine environment, the function of the placenta, and the availability of nutrients to mother and foetus. Glucose is the principal energy substrate for the placenta and the foetus (William and Hay 2006). Altered maternal glucose homeostasis is perhaps the most significant risk factor for foetal macrosomia. The hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study (Catalano et al. 2012), and a number that have subsequently followed, indicated a direct relationship between maternal glucose levels and infant birth weight (Walsh et al. 2011; Clapp 2002). The hyperglycaemic intrauterine environment affects not only the foetus but also the placental development and function in humans and experimental rodents (Aires and Dos Santos 2015). Understanding placental changes in the environment of abnormal glucose metabolism which may establish the maternal-placental-foetal interface dysfunction as a potential source of adverse pregnancy outcomes is very necessary.

Macroscopically, the diabetic placenta is characterised by increased size and weight leading to an increased placental-to-birth weight ratio (Ramos et al. 2016; Kucuk and Doymaz 2009). Further studies demonstrated that more active proliferation of trophoblast cells among diabetics may contribute to increased placental tissue (Chen et al. 2016; Unek et al. 2017). However, other authors found no differences of proliferative activity but increased apoptosis in trophoblast between normal and diabetic term placenta (Burleigh et al. 2004; Sgarbosa et al. 2006). Placental abnormalities associated with maternal diabetes have been inconsistently reported, possibly because of population differences in pre-pregnancy weight, diabetes types, glycemic control or pregnancy complication. Consequently, given pre-pregnancy obesity is an important risk factor for macrosomia (Dai et al. 2018), to examine the relationship between hyperglycaemia and placentas from GDM-induced macrosomia, our study focussed on GDM women with normal pre-pregnancy BMI. Moreover, the study also investigated the underlying mechanism of hyperglycaemia in trophoblast cells *in vitro*.

To our knowledge, there are several trophoblast pathways that have been already characterised, such as mitogen-activated protein kinase (MAPK) signalling (Knöfler et al. 2005), playing a crucial role in several key activities of the trophoblast. The extracellular-signal-regulated kinase 1/2 (ERK1/2) is a member of the MAPK family, which is activated by growth factors and have an important role in the regulation of cell proliferation and differentiation (Nishimoto and Nishida 2006). Uddin *et al.* reported ERK1/2 might play a role in human first trimester cytotrophoblast cell proliferation (Uddin *et al.* 2008). Nadeau *et al.* showed appropriate ERK/MAPK signalling in defined cell types is required for the proper growth, differentiation and morphogenesis of the placenta (Nadeau and Charron 2014). Additionally, many studies indicate ERK1/2 signal transduction pathway is involved in various diabetic

complications. High glucose activated the ERK1/2 pathway, which may contribute to the development of diabetic nephropathy (Pan et al. 2014), and in diabetes, ERK1/2 phosphorylation was reported to be increased in peripheral nerves (Stavniichuk et al. 2013). These researches suggest that ERK1/2 activation may be involved in the hyperglycaemia-induced trophoblast cell proliferation.

In the present study, we divided GDM women with normal pre-pregnancy BMI into GM group (GDM women with macrosomia) and GN group (GDM women with normal birth weight) according to the newborn's birth weight, to investigate the differences of maternal glucose level, serum lipid profile, gestational weight gain, umbilical cord diameter, pregnancy outcomes, total placental volume, placental weight and the expression level of placental cell proliferation between two groups, and to determine the correlation of maternal glucose with foetal birth weight, placental weight and placental cell proliferation in human placentas. Moreover, we explored the role of hyperglycaemia in cell proliferation and ERK1/2 pathway of BeWo cells *in vitro*, which is commonly used as model system for trophoblast cells (Chen et al. 2016), to uncover the molecular mechanism of hyperglycaemia involvement in the development of GDM-induced macrosomia.

## 2. Material and methods

### 2.1. Study subjects

This study was approved by the institutional review board of the Second Affiliated Hospital of Nantong University with written informed consent form being obtained from all participants. All participants did not have any acute or chronic complications before pregnancy, such as chronic hypertension, or other disorders affecting glucose metabolism, and without any dietary restrictions. Their pre-pregnancy body mass indexes (BMI) were all in 18.5–24.9 kg/m<sup>2</sup>. They all attended antenatal cares from January 2015 to December 2018 in the outpatient obstetrics of the Second Affiliated Hospital of Nantong University until delivery. During 24–28 gestational weeks, they underwent a 75-g oral glucose tolerance test (OGTT). The diagnosis of GDM was made when one of the following plasma glucose values in the OGTT was met or exceeded: Fasting plasma glucose 92 mg/dL (5.1 mmol/L); One-hour plasma glucose 180 mg/dL (10.0 mmol/L); Two-hour plasma glucose 153 mg/dL (8.5 mmol/L). Macrosomia is defined as a birth weight  $\geq 4000$  g. According to the OGTT results and newborn's birth weight, participants were stratified into two groups: GM group (GDM women with macrosomia) ( $n = 30$ ) and GN group (GDM women with normal birth weight) ( $n = 35$ ). Clinical data such as glucose profile and pregnancy outcomes are summarised in Table 1. According to the latest 2009 IOM recommendations (Yang and Yang 2012), the target weight gain of women with normal pre-pregnancy BMI during pregnancy was 11.5–16 kg. Maternal outcomes included caesarean delivery and postpartum haemorrhage. Neonatal outcomes were neonatal asphyxia (Apgar score  $\leq 7$  at one minute after delivery) and neonatal

**Table 1.** Maternal characteristics and pregnancy outcomes.

Variables	GN group	GM group	<i>p</i> Value
<i>N</i>	35	30	–
Age(years)	29.10 ± 2.28	30.20 ± 2.85	.354
Gravidity	1.75 ± 0.97	1.86 ± 1.09	.755
Parity	1.31 ± 0.49	1.42 ± 0.51	.586
Pre-pregnancy BMI(kg/m <sup>2</sup> )	21.91 ± 2.64	22.56 ± 3.01	.764
OGTT gestational week(weeks)	25.46 ± 0.81	25.27 ± 0.90	.485
Fasting-OGTT glucose (mmol/L)	4.64 ± 0.32	5.52 ± 0.58	.004
1h-OGTT glucose (mmol/L)	9.58 ± 1.16	11.12 ± 1.30	.011
2h-OGTT glucose (mmol/L)	8.04 ± 0.97	8.93 ± 1.16	.046
Fasting insulin (mU/L)	11.03 ± 3.35	14.16 ± 3.58	.009
Women with target gestational weight gain	18(71.43%)	13(43.33%)	.022
Gestational age at delivery (weeks)	39.45 ± 1.21	39.33 ± 1.10	.811
Fasting plasma glucose (mmol/L)	4.61 ± 0.31	5.76 ± 0.49	.009
2-h postprandial glucose (mmol/L)	6.68 ± 0.89	7.56 ± 1.48	.017
HbA1c percentage (%)	5.54 ± 0.33	5.94 ± 0.31	.010
Triglyceride (mmol/L)	2.94 ± 0.27	3.37 ± 0.43	.032
Total cholesterol (mmol/L)	6.33 ± 0.61	6.91 ± 0.74	.271
hPL(ug/ml)	6.33 ± 1.12	5.44 ± 1.18	.094
Birth weight (grams)	3392 ± 253	4244 ± 167	.000
Neonatal gender			.399
Male	15(42.86)	16(53.33)	
Female	20(57.14)	14(46.67)	
Neonatal asphyxia	1(2.9)	2(6.7)	.891
Neonatal hypoglycaemia	0(0.0)	6(20.0)	.019
Caesarean delivery	9(25.7)	16(53.3)	.023
Postpartum haemorrhage	0(0.0)	5(16.7)	.041

GN group: gestational diabetes mellitus women with normal birth weight and normal pre-pregnancy BMI; GM group: gestational diabetes mellitus women with macrosomia and normal pre-pregnancy BMI; BMI: body mass index; OGTT: oral glucose tolerance test; HbA1c: haemoglobin A1c; hPL: human placental lactogen.

All values in the table are given as the mean ± SD, except for women with target gestational weight gain, neonatal gender, neonatal asphyxia, neonatal hypoglycaemia, caesarean delivery and postpartum haemorrhage values given *n* (%).

hypoglycaemia (< 2.2 mmol/L). All pregnancy outcomes were recorded by specially-assigned persons.

## 2.2. Anthropometric indices and laboratory examination

Gestational age and pre-pregnancy BMI were calculated. The pre-pregnancy BMI was obtained by self-reported measurements and recorded when the participants received the first antenatal care before 12 gestational weeks. If the body weight before pregnancy was unknown, the weight before 12 gestational weeks was adopted. All plasma samples from 65 pregnant women were taken after >6 h fast before delivery. Plasma glucose levels were measured by the glucose oxidase method. Fasting plasma glucose, postprandial blood glucose, total cholesterol and triglyceride were measured using Hitachi Model 7600 Series Automatic Analyser. HbA1c was measured by column chromatography using Biorad Diastat kit. The serum hPL level was examined by the HPL-ELISA method. The umbilical cord diameter was measured to computer by Philips iU22 Colour Doppler Ultrasound before delivery.

## 2.3. Placental tissue collection

Human term placentas were collected and cleaned after deliveries immediately. First, we measured the placental weight and placental thickness (*t*). Then, the placental surface area was determined using Cavalier's point-counting method using this formula,  $A = \sum P \cdot A(p)$ , where *A* is estimation of the surface area,  $\sum P$  is the sum of the number of points landing on the surface of placenta, *A* (*p*) is the area

associated with each point in stereological grid and the total placental volume was estimated by this formula:  $V = A \cdot t$ , where *V* is estimation of the volume, *A* is surface of placenta and *t* is the mean thickness of placenta (Heidari et al. 2013; Heidari et al. 2015). Next, four pieces of 0.5 cm<sup>3</sup> fragments were sharply cut from placental central part on maternal side, immediately frozen at –80 °C and fixed with 4% paraformaldehyde at 4 °C separately.

## 2.4. Cell culture

Human trophoblast cell line BeWo was purchased from American Tissue Type Collection (Manassas, VA) and maintained in normal DMEM media (5.5 mM D-glucose) (Gibco, Life technologies, Shanghai, China) supplemented with 10% foetal bovine serum (Gibco, ThermoFisher, Waltham, MA, USA), streptomycin (0.1 mg/ml), penicillin (100 U/ml), and a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. For high glucose media, additional D-glucose was supplemented to the normal DMEM media with a final D-glucose concentration at 25 mM (Chen et al. 2014; Inadera et al. 2010). The osmotic control media was made by supplementing normal DMEM media with 19.5 mM mannitol. Cells were exposed to the following experimental conditions for 1–5 days: normal glucose medium, high glucose medium and high mannitol medium.

## 2.5. Rna extraction and quantitative real-time PCR (qRT-PCR)

The total RNA from BeWo cells and placental tissues was extracted using TRIzol reagent (Invitrogen) according to the

manufacturer's instructions. First-strand complementary DNA (cDNA) was reversed according to the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™, USA) with 1 ug of total RNA. Quantitative real-time PCR (qRT-PCR) was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, USA) with gene-specific primers. The primers were: PCNA, 5'-TGC CAT GGT TCT CAC TAA AAT G-3' (F), 5'-ATT TAG GCC AGC AAT AGT TCC A-3' (R), Cyclin D1, 5'-CTT CCT CTC CAA AAT GCC AG -3' (F), 5'-AGA GAT GGA AGG GGG AAA GA -3' (R) and GAPDH, 5'-AGG TGG TCT CCT CTG ACT TCA A-3' (F), 5'-TTC GTT GTC ATA CCA GGA AAT G-3' (R). All target gene transcripts were normalised to GAPDH, and the relative fold change in expression calculated using  $2^{-\Delta\Delta CT}$  formula. All the reactions were run in triplicate.

## 2.6. Western blotting

The placental tissues and cultured cells were homogenised with lysis buffer after being washed with cold PBS containing a protease inhibitor. Proteins were separated by SDS-PAGE on a 10% gel and were transferred to nitrocellulose (NC) membranes. After 2 h of blocking with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature, the membranes were incubated with primary antibody overnight at 4°C, followed by three 10 min washes in TBST and incubation with the corresponding secondary antibody for 2 h at room temperature. The protein bands were visualised in the membranes using an Enhanced Chemiluminescence Detection Kit (Pierce Biotechnology, USA), and Image J software (National Institutes of Health, USA) was used to measure the band intensities. The relative protein expression was normalised to GAPDH. Primary antibodies used and their dilutions are as follows: PCNA (1:2000), Cyclin D1 (1:1000) (Biogot technology, co, Ltd, Nanjing, China), p-ERK1/2(Thr202/204), ERK1/2 (Cell Signalling Technology, Inc., Danvers, MA, USA) and GAPDH (1:2000) (Bioss Biotechnology, Beijing, China). The secondary antibodies were goat anti-rabbit antibody or goat anti-mouse antibody (1:2000) (Biogot technology, co, Ltd, Nanjing, China).

## 2.7. Cell Counting kit-8 assay (CCK-8)

BeWo cells were plated in 96-well microtiter plates at a density of  $5 \times 10^3$  cells/well, and each plate was incubated for 12 h. Cells were then cultured under normal glucose, high glucose and high mannitol medium for 1–5 days, respectively. To find the effect of ERK pathway on the proliferation of BeWo under high glucose, cells were serum starved for 12 h and subsequently exposed to high glucose medium containing the selective ERK1 and ERK2 inhibitor U0126 (20uM, Sigma) for 1–3 days. Using Cell Counting Kit-8 (CCK-8) (Yeasen, Shanghai, China) according to the manufacturer's protocol, 10 ul of CCK8 reagent was added to each well, and the samples were incubated for 1.5 h before absorbance values were measured at 450 nm.

## 2.8. Flow cytometry analysis

For the cell cycle flow cytometry assay, cells were digested by trypsin-EDTA, harvested as many as  $1 \times 10^6$  cells, and fixed in 70% ethanol overnight at 4°C. Then cells were centrifuged (1,000 g, 5 min, 4°C) and resuspended in PBS. Thereafter, cells were incubated, stained with propidium(PI), RNase A and staining solution (Yeasen, Shanghai, China) at room temperature for 30 min in the dark, and analysed by flow cytometry (BD FACSVerser, BD Company) with ModFit software (Verity Software House, Inc.).

## 2.9. Statistical analysis

Data from at least three independent experiments were analysed using SPSS software version 17.0 (SPSS, Chicago). Continuous data, which were normal distribution data exhibited as mean  $\pm$  standard deviation (SD), were compared using the independent-samples T test between two groups. Categorical data were described as frequency and percentage, and the comparisons of them were done using Chi-square test or Continuity correction  $\chi^2$ . The logistic regression analysis was used to explore the influence of different clinical factors on the incidence of GDM-induced macrosomia with normal pre-pregnancy BMI. The Pearson Correlation analysis was to examine the correlation of placental weight and total placental volume and the association between maternal glucose with placental weight and placental cell proliferation in human placentas. A two-sided  $p$ -value  $< .05$  was considered to be statistically significant.

## 3. Results

### 3.1. Maternal glucose is the most significant risk factor for GDM women with normal pre-pregnancy BMI and macrosomia

Clinical characteristics of patients in GM group (GDM women with normal pre-pregnancy BMI and macrosomia) and GN group (GDM women with normal pre-pregnancy BMI and normal birth weight) are presented in Table 1. Mean age, gravidity parity, pre-pregnancy BMI, OGTT gestational week, serum hPL level, male/female foetal ratio and gestational age at delivery and neonatal asphyxia were similar in both groups ( $p > .05$ ). However, the GM group had significantly higher levels of fasting plasma glucose, 1- and 2-hour plasma glucose in OGTT and fasting insulin ( $p < .05$ ). The GM group had 13 women (43.33%) achieved target weight gain, while the GN group included 18 women (71.43%) with target weight gain. The gestational weight gain in GM group was controlled worse than that in GN group ( $p < .05$ ). The incidences of neonatal hypoglycaemia, caesarean delivery, and postpartum haemorrhage in GM group were 20.0% (6/30), 53.3% (16/30) and 16.7% (5/30), which were significantly higher than in GN group ( $p < .05$ ). Additionally, there was no difference in the serum total cholesterol level ( $p > .05$ ), but the fasting plasma glucose concentration, postprandial blood glucose, HbA1c percentage and triglyceride levels were obviously increased in GM group than in controls ( $p < .05$ ). However, after



adjustment for the degree of weight gain, triglyceride, HbA1c percentage, fasting and postprandial plasma glucose, just the fasting plasma glucose and HbA1c percentage were related to the incidence of GDM-induced macrosomia with normal pre-pregnancy BMI ( $p < .05$ ) (Table 2). Taken together, these data indicate that GDM women with macrosomia and normal pre-pregnancy BMI have obviously adverse pregnancy outcomes, which may be closely associated with hyperglycaemia.

**Table 2.** Logistic regression analysis of the impact of factors that affect GDM-induced macrosomia.

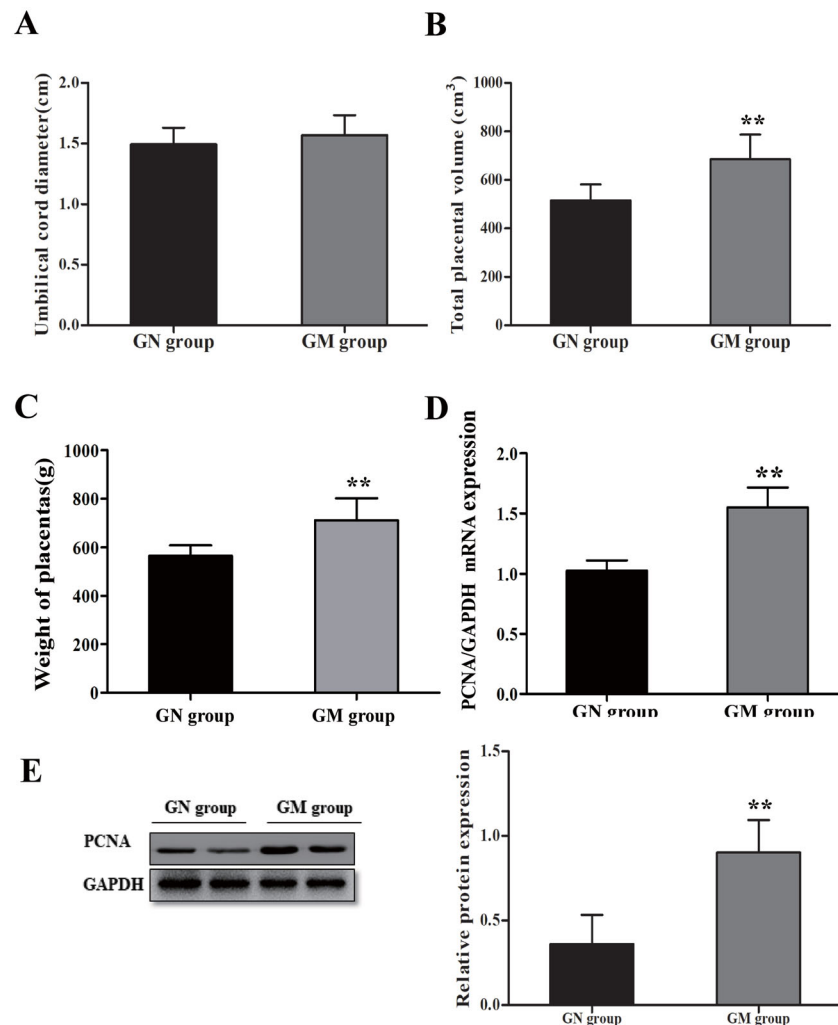
	B	S.E	Wald	Df	Sig.	Exp(B)
Degree of gestational weight gain	0.311	0.0.848	0.135	1	0.714	1.365
Triglyceride (mmol/L)	0.616	0.406	2.306	1	0.129	1.852
Fasting plasma glucose (mmol/L)	2.280	1.104	4.265	1	0.039	9.777
2-h postprandial glucose (mmol/L)	0.071	0.429	2.737	1	0.098	2.033
HbA1c percentage (%)	2.306	1.052	4.800	1	0.028	10.03

GDM: gestational diabetes mellitus; HbA1c: haemoglobin A1c.

Degree of gestational weight gain: undesirable gestational weight gain or target gestational weight gain.

### 3.2. Association between maternal glucose and placenta in GDM women with macrosomia and normal pre-pregnancy BMI

To evaluate the association between maternal glucose and placenta in GDM women with macrosomia and normal pre-pregnancy BMI, we firstly examined and analysed the diameter of the umbilical cord, the total placental volume and placental weight between two groups and found there was no significant difference in umbilical cord diameter between two groups ( $p > .05$ , Figure 1(A)), but a significant increase in placental volume and placental weight in GM group compared to the control group ( $p < .01$ , Figure 1(B,C)). Further analysis revealed that the placental weight was correlated to the placental volume ( $R = 0.511$ ,  $p = .011$ ). Then, we evaluated the proliferative activity of trophoblast cells in placenta of GDM-induced macrosomia with normal pre-pregnancy BMI. The results showed there was a significant increase in the placental PCNA mRNA expression in GM group than in controls ( $p < .01$ , Figure 1(D)). We also found GDM with macrosomia



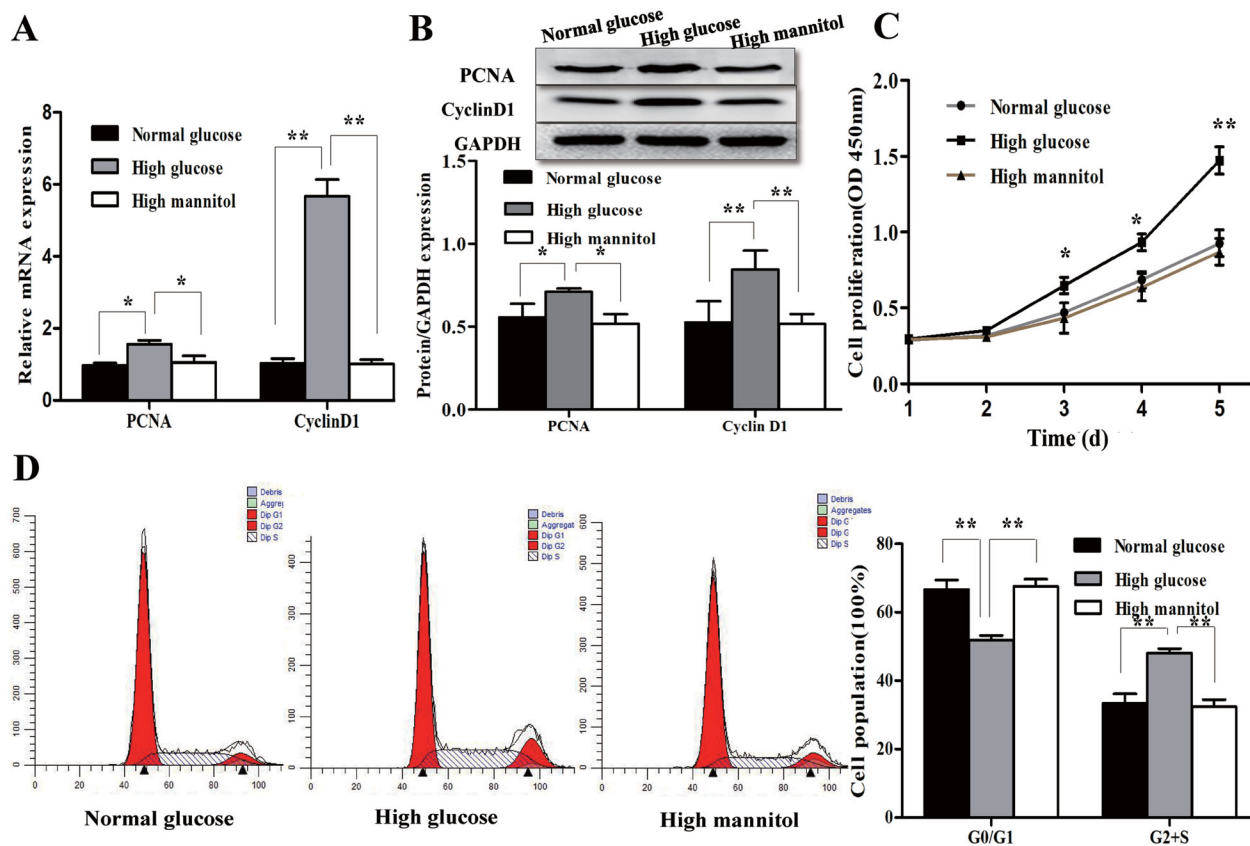
**Figure 1.** Heavier placental weight and more placental cell proliferation in GDM with normal pre-pregnancy BMI and macrosomia (A) The umbilical cord diameter of GN group was measured to computer by Philips iU22 Colour Doppler Ultrasound before delivery compared with GM group. (B) The total placental volume was estimated between two groups. (C) The placental weight of GN group compared with GM group. (D) Transcription of PCNA in placental tissues of GN group and GM group was determined by quantitative real-time PCR. (E) The protein level of PCNA in the placental tissues of two groups was detected by western blotting. GAPDH served as loading control. The graph on the right of figures E showed the quantification of the results. Qualified statistical data are the mean  $\pm$  SD of at least 3 separate experiments. \*\* $p < .01$ . GDM: gestational diabetes mellitus. BMI: body mass index. GN group: GDM women with normal pre-pregnancy BMI and normal birth weight. GM group: GDM women with normal pre-pregnancy BMI and macrosomia.

and normal pre-pregnancy BMI had significantly elevated PCNA expression in placenta in contrast with the controls at the protein level by Western blotting ( $p < .01$ , Figure 1(E)). Furthermore, a linear correlation test was performed using the fasting plasma glucose level and placental weight as well as the fasting blood glucose value and mRNA expression of PCNA in GDM women with normal pre-pregnancy BMI. The results suggested that the fasting plasma glucose was positively related to placental weight ( $R = 0.538$ ,  $p = .009$ ) and PCNA mRNA expression ( $R = 0.481$ ,  $p = .016$ ). Collectively, these data show that there is obviously higher cell proliferation in the placentas of GDM-induced macrosomia with normal pre-pregnancy BMI, which might be due to hyperglycaemia.

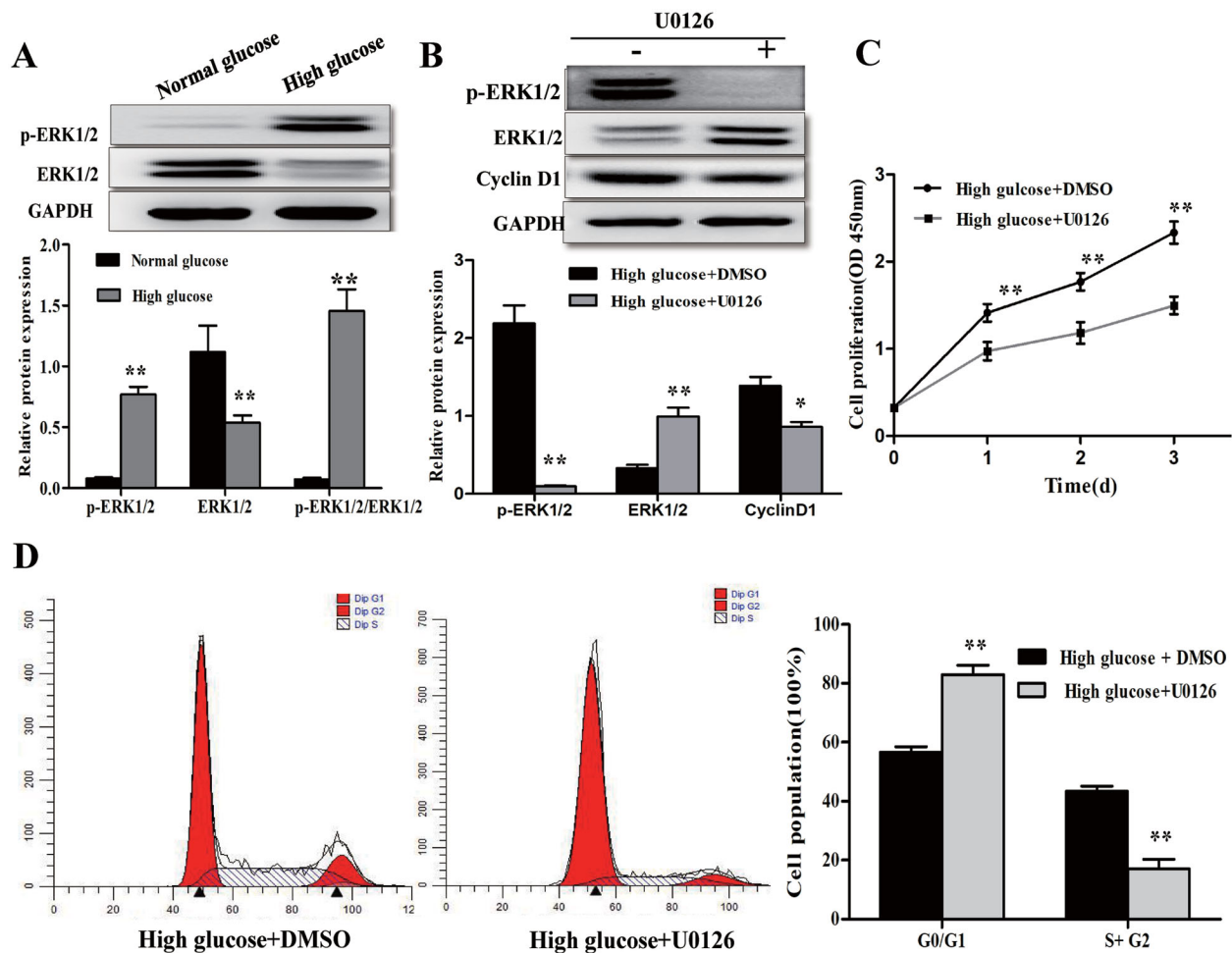
### 3.3. Hyperglycaemia promotes trophoblast cell proliferation *in vitro*

To investigate the effect of high glucose on the growth potential of human trophoblast cells, we chose a placental cell line - BeWo cell line which has been widely used as an *in vitro* model for the placenta. Considering high glucose-induced changes in the biological response can be caused by

osmotic changes, normal glucose and high mannitol were simultaneously set as the controls. We first measured the effect of normal glucose, high glucose and high mannitol on BeWo cell proliferation by measuring PCNA and Cyclin D1 expression via qRT-PCR and western blotting for 120 h. The mRNA and protein expressions of PCNA and Cyclin D1 were both obviously increased after exposure to high glucose in contrast with normal glucose and high mannitol (Figure 2(A,B)). Then, we tested CCK-8 assay to detect the effect of high glucose on BeWo cell growth for different culture time. Figure 2(C) shows high glucose markedly enhanced the proliferation potential of BeWo cells compared with normal glucose and high mannitol from 96 to 120 h. Moreover, flow cytometry was performed to analyse cell cycle distribution to further characterise the effect of hyperglycaemia on cell growth. As shown in Figure 2(D), there was a statistically significant decrease in the percentage of BeWo cells in the G0/G1 phase after exposure to high glucose for 120 h, as well as a remarkably increase in the percentage of BeWo cells in the S + G2 phase compared with normal glucose and high mannitol treated BeWo cells ( $p < .01$ ). Together, the results of the present study reveal that hyperglycaemia but not osmotic pressure is involved in the regulation of BeWo cell proliferation.



**Figure 2.** Hyperglycaemia promotes trophoblast cell proliferation (A) High glucose increased the expression of PCNA and Cyclin D1 as detected by quantitative real-time PCR in contrast with normal glucose and high mannitol, whereas there was no difference between normal glucose and high mannitol. (B) Western blot was performed to detect the protein expression of PCNA and Cyclin D1, which were significantly promoted by high glucose, rather than normal glucose and high mannitol. High mannitol was used as a hypertonic control. The lower panel showed the quantitative results. (C) High glucose promoted proliferation of BeWo cells as determined by the CCK-8 assay. Bar graph shows the extent of BeWo cell proliferation upon normal glucose, high glucose and high mannitol. No significant change was found under normal glucose and high mannitol condition. High glucose markedly promoted the proliferation of BeWo cells compared with normal glucose and high mannitol from 96 to 120 h. (D) The cell cycle was detected by flow cytometry with PI staining in BeWo cells after treated with normal glucose, high glucose and high mannitol. The right panel showed the quantitative results. GAPDH was used as an internal control. The data represent means  $\pm$  SD of three independent experiments each performed in triplicates. \* $p < .05$ . \*\* $p < .01$ .



**Figure 3.** Hyperglycaemia activates ERK1/2 signalling of trophoblast cells (a) Western blot analysis of ERK1/2 showed increased phosphorylation in high glucose-induced BeWo cell proliferation. (B) Western blot analysis of p-ERK1/2, ERK1/2 and CyclinD1 in BeWo cells treated with high glucose and 10  $\mu$ M U0126 (which specifically blocks ERK1/2 phosphorylation). U0126 inhibited high glucose-induced ERK1/2 phosphorylation and CyclinD1 expression. (C) U0126 inhibited proliferation of BeWo cells with high glucose treatment as determined by the CCK-8 assay. (D) The cell cycle was detected by flow cytometry with PI staining in BeWo cells with high glucose and 10  $\mu$ M U0126 treatments. The proportion of BeWo cells in the G2/M phase with U0126 and high glucose treatment was significantly lower than that only with high glucose treatment. GAPDH was used as an internal control. DMSO was used as a control of U0126. Mean  $\pm$  SD of 3 independent experiments done in triplicates is presented. \*  $p < .05$ ; \*\*  $p < .01$ .

### 3.4. Hyperglycaemia promotes trophoblast cell proliferation via activation of ERK signalling pathway

The ERK1/2 signal pathway regulates cell functions including cell proliferation, migration, invasion and apoptosis and is closely related to hyperglycaemia. Therefore, we tested whether high glucose stimulation of BeWo cell proliferation was mediated by ERK1/2 pathway. As shown in Figure 3(A), ERK1/2 phosphorylation was significantly up-regulated and ERK1/2 expression was obviously decreased in BeWo cells treated with high glucose compared with normal glucose ( $p < .01$ ). To confirm that the elevated p-ERK1/2 level contributed to the increased proliferation of BeWo cells, we blocked ERK1/2 activity using U0126, a selective inhibitor of ERK1 and ERK2. Figure 3(B) shows treatment with U0126 remarkably attenuated the glucose-induced upregulation of p-ERK1/2 ( $p < .01$ ) and CyclinD1 expression ( $p < .05$ ). In CCK-8 assay, treatment with U0126 also significantly alleviated the proliferation of BeWo cells ( $p < .01$ , Figure 3(C)). Furthermore, the percentage of BeWo cells in the G0/G1 phase with U0126 and high glucose treatment were significantly higher than

those with only high glucose treatment ( $p < .01$ , Figure 3(D)). Thus, these findings demonstrate that hyperglycaemia may have the ability to promote trophoblast cell proliferation via activation of ERK1/2 signalling.

## 4. Discussion

Normal development of the placenta is essential for foetal well-being, adequate foetal growth, and a good pregnancy outcome. Changes in the placenta itself may represent either beneficial adaptive responses or pathological changes in response to inadequate nutrient supply (James 2006). However, to our knowledge, the diabetic environment has a profound impact on placental and foetal development, and studies on relationship between hyperglycaemia and placentas of GDM women with pre-pregnancy BMI and macrosomia are limited. Moreover, the specific roles and underlying mechanisms of hyperglycaemia in trophoblast cell *in vitro* have not been clearly stated. In the present research, we found that GDM women with pre-pregnancy BMI and

macrosomia had more adverse pregnancy outcomes compared with GDM women with pre-pregnancy BMI and normal birth weight. After adjustment for confounding factors, we demonstrated the fasting plasma glucose level was associated with the incidence of GDM-induced macrosomia with normal pre-pregnancy BMI, and closely related to placental weight and the PCNA expression of placentas. Furthermore, we utilised human trophoblast cell line BeWo as an *in vitro* model for the placenta, and found that hyperglycaemia but not osmotic pressure significantly promoted human BeWo cell proliferation. In addition, we also showed that hyperglycaemia obviously activated ERK1/2 signalling pathway of BeWo cells, and ERK1/2 inhibitor U0126 significantly inhibited the hyperglycaemia-induced proliferation of trophoblast cells. These findings suggest that hyperglycaemia may contribute to the development of GDM-induced macrosomia through promoting trophoblast cell proliferation via ERK1/2 signalling pathway.

GDM is a relatively common gestational disease, which refers to any degree of glucose intolerance with onset or first recognition during pregnancy, with the prevalence of 17.5%~18.9% in China (Wei et al. 2014). It is a known clinical risk factor associated with foetal macrosomia and represents 90% of all types of diabetes occurring in pregnancy. In women diagnosed with GDM, the main complication is macrosomia (Kc et al. 2015; Yang et al. 2018). Several decades ago, Pedersen proposed maternal hyperglycaemia contributes to increased risk of macrosomia during pregnancy (Pedersen 1967). Many studies also have explored the impact of GDM on foetal macrosomia and shown a continuous relationship between maternal glucose and increasing birth weight (Walsh et al. 2011; Liu et al. 2014). Given the close relationship of maternal glucose with macrosomia, women with GDM represent an ideal population model to study these interrelationships *in vivo*. On the other hand, emerging evidence suggests maternal pre-pregnancy obesity is also an important determinant of macrosomia (Dai et al. 2018; Liu et al. 2014). Ehrenberg et al. demonstrated maternal obesity has a strong and independent effect on foetal macrosomia (Ehrenberg et al. 2004). Similar findings were found by Kc et al. (2015) and Langer (2000). Thus, we modified the study model of GDM women with normal pre-pregnancy BMI to exclude the impact of pre-pregnancy obesity on macrosomia. After adjustment of potential confounding factors, the study found the fasting plasma glucose and HbA1c percentage were closely related to the incidence of GDM-induced macrosomia with normal pre-pregnancy BMI, which was consistent with the previous reports that fasting hyperglycaemia (Ding et al. 2018; Araujo Júnior et al. 2017) and HbA1c (Delaney et al. 2015) had clear associations with macrosomia. However, other researches demonstrated no significant association between HbA1c and birthweight was observed (Weykamp et al. 2009) and foetal growth was mostly influenced by postload glucose levels, rather than the fasting plasma glucose (Yang et al. 2018). Contributing factors to these inconsistencies may include the sample size, diabetes status, variations in glucose testing protocols and treatment regimens. The study also demonstrated that macrosomia associated with hyperglycaemia significantly increased the

adverse pregnancy outcomes of GDM women with normal pre-pregnancy BMI, which needs us to explore the mechanism of hyperglycaemia involvement in the development of GDM-induced macrosomia.

The placenta is a complex organ that fulfils pleiotropic roles during foetal growth by mediating the efficient maternal-to-foetal transfer of gases, nutrients and waste products. Among placental cells, trophoblasts permit the embryo implantation and nutrition in the early pregnancy and thereafter they will contribute considerably to the development and function of the placenta. Exposure to a hyperglycaemic environment results in abnormalities of the placenta and trophoblast cells, which leads to maternal complications and poor foetal outcomes. Many studies have found placental weight in diabetic pregnancy was higher compared with normal pregnancy (Ramos et al. 2016; Kucuk and Doymaz. 2009). Dysregulation of trophoblast proliferation may contribute to abnormal growth of placenta. Fox found higher number of villous cytotrophoblast, endothelial cells and syncytiotrophoblast nuclei in diabete (Fox 1969). Unek et al. showed PCNA, Ki67 and cyclin D3 staining of villous cytotrophoblast, syncytiotrophoblast, villous stromal cells and foetal endothelial cells increased in diabetic placentas compared to controls (Unek et al. 2014). As in diabetic animals, it was also reported, increased proliferative activity in villous cytotrophoblasts compared to normal placentas (Zorn et al. 2011). Unlike previous studies on the placentas from patients with GDM and normal pregnancy, we specifically investigated the differences in placentas of GDM women with macrosomia and GDM women with normal birth weight, who all had normal pre-pregnancy BMI, and found placental abnormalities in GDM women with macrosomia and normal pre-pregnancy BMI were significantly increased placental weight and higher placental cell proliferation. The study also demonstrated fasting plasma glucose was significantly associated with placental weight and PCNA expression of trophoblast cells after adjustment for pre-pregnancy obesity and other diabetes types. Furthermore, we testified the biological role of hyperglycaemia on the proliferation of human trophoblast cell – BeWo cell line and confirmed that hyperglycaemia but not hyperosmolarity markedly enhanced the proliferation potential of trophoblast cells *in vitro*. These results indicated increased placental growth secondary to over cell proliferation by hyperglycaemia in GDM pregnancies. Meanwhile, it is also speculated that in the diabetic environment the placenta may grow first and then contribute to accelerated foetal growth by increased glucose as well as the transfer of other nutrients. As the pregnancy progresses, the increased placental weight and increased placental nutrient transport rate will contribute to the accumulation of foetal fat in GDM, eventually leading to the production of macrosomia.

The extracellular signal regulated kinase1/2 (ERK1/2) signalling pathway exists in various cell types and functions to convert extracellular stimuli into transcriptional programs. ERK1/2 as serine/threonine protein kinases, are critically involved in regulation of many biological processes, including cell differentiation, cell survival, migration and proliferation (Nishimoto and Nishida. 2006; McCubrey et al. 2007.). Previous studies showed that ERK1/2 pathway was activated



by high glucose and regulated the occurrence of diabetes and its complications (Pan et al. 2014; Stavniichuk et al. 2013). Additionally, ERK1/2 pathway played an important role in high concentration glucose-mediated proliferation of rat aortic vascular smooth muscle cells (Yang et al. 2017). Importantly, compelling evidence in the last years implicated ERK signalling pathway in placental development such as the growth, differentiation and morphogenesis of the placenta (Nadeau and Charron. 2014; Hatano et al. 2003). Therefore, to further delineate the mechanisms by which high glucose upregulate trophoblast cell proliferation, the ERK1/2 signalling pathway was next examined. We first examined the p-ERK of BeWo cells treated with high glucose and found ERK1/2 phosphorylation was significantly up-regulated than treated with normal glucose. Then, we blocked the ERK1/2 pathway by a specific inhibitor U0126, finding the stimulating effects of hyperglycaemia on ERK1/2 phosphorylation and the proliferation of BeWo cells were both markedly attenuated. Together, these results suggested hyperglycaemia may exert the promoting role in trophoblast cell proliferation through ERK1/2 signalling activation.

Still, there are several limitations in our study. Firstly, the sample size was relatively small. Secondly, the glucose fluctuation in addition to hyperglycaemia, is also important in influencing excessive foetal growth (Herranz et al. 2007), but we did not explore the effect of glycaemic variability on GDM women with pre-pregnancy BMI and macrosomia. Thirdly, in the present study, we also found GM group had significantly higher levels of fasting insulin, fasting plasma glucose, 1- and 2-hour plasma glucose in OGTT, which indicated insulin resistance and different time-point blood glucose levels may also have significantly joint effects on birth weight and risk of macrosomia in GDM women with normal pre-pregnancy BMI. It warrants further investigation. However, the present study focussed on GDM women with normal pre-pregnancy BMI and macrosomia, and showed the fasting blood glucose was significantly associated with the placental weight and placental PCNA expression, which was closely related to the incidence of GDM-induced macrosomia. Subsequently, the study demonstrates exposure to high glucose caused the increase of cell proliferation in cultured BeWo cells, thus forming a primary basis of an *in vitro* trophoblast model for GDM-induced macrosomia. Moreover, the study also provided evidence that aberrant ERK1/2 signalling activation may be involved in the hyperglycaemia induced trophoblast cell proliferation. Therefore, these findings may provide a novel understanding of GDM-induced macrosomia.

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## Ethical approval and consent to participate

All participants provided informed written consent, and the study protocol was by the Ethics Committee of the Second Affiliated Hospital of Nantong University.

## Disclosure statement

All authors have no conflicts of interest to declare.

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