An *in vitro* Model to Study Placental Functions in Gestational Diabetes Mellitus

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Abstract

Background: Gestational diabetes mellitus (GDM) is a serious pregnancy complication that affects around 14 % of pregnancies worldwide. It not only causes short-term complications in the mother and child but also significantly increases the risks of several chronic diseases, including cardiovascular diseases and diabetes. An excess production of the soluble vascular endothelial growth factor (VEGF) receptor 1 in the placenta, which is a natural inhibitor of VEGF, has been linked to various pregnancy complications. However, placentas affected by GDM often exhibit increased vascularization, suggesting that the role of VEGF in GDM differs from its impact on typical pregnancy complications. Unfortunately, limited studies on placental functions in GDM have been conducted due to a lack of reliable culture models. Methods: In this study, we developed an *in vitro* model of GDM using the human choriocarcinoma trophoblast cell line BeWo and examined the effects of VEGF on glucose uptake. The BeWo cells were treated with a high glucose-containing (25 mM) syncytialization differentiation medium to produce insulin-resistant cells. Insulin resistance was assessed by a glucose uptake assay using the fluorescent glucose analog 2-NBDG and flow cytometry analysis. Results: The cells treated with high glucose exhibited a significant decrease in glucose uptake, suggesting the successful development of glucose resistance in these cells. However, VEGF treatment did not show a significant impact on glucose uptake in high glucose-treated cells. Conclusions: It is important to note that glucose homeostasis is a complex process involving multiple cell types, and further studies are necessary to fully understand the functions of VEGF on GDM placentas. Nevertheless, the *in vitro* model we have developed and validated will be a valuable tool for studying placental pathophysiology in GDM and evaluating the effectiveness of potential therapeutic agents.

Keywords: Gestational diabetes mellitus, Biomarker, Trophoblast, Insulin resistance, BeWo cells, VEGF, Flow cytometry

Introduction

Gestational diabetes mellitus (GDM) is a serious metabolic disorder occurring during pregnancy and affects around 14 % of human pregnancies worldwide. The rising prevalence of GDM has significant health implications for both the mother and the child. Maternal complications of GDM include pre-eclampsia, cesarean delivery, and postpartum hemorrhage, while fetal complications include macrosomia, birth trauma, and neonatal hypoglycemia. In addition, children of women with GDM are at increased risk of human diseases like obesity, cardiovascular diseases, and type 2 diabetes later in life [1]. Hyperglycemic condition during GDM, is shown to increase oxidative stress and inflammation in the placenta, impairs placental vascularization and nutrient transport, and alter the production of key placental hormones which adversely affect the fetal development [2-4]. The onset of GDM involves several metabolic changes, including insulin resistance and impaired insulin secretion. Insulin resistance is caused by the increased secretion of placental hormones such as human placental lactogen (hPL) and cortisol. These hormones induce insulin resistance by reducing insulin sensitivity in maternal tissues, such as adipose tissue, liver, and skeletal muscle. As a result, glucose uptake and utilization in these tissues are impaired, leading to hyperglycemia [5,6]. Furthermore, the progressive β-cell dysfunction exacerbates the GDM condition [7,8].

Vascular endothelial growth factor (VEGF) is found to have a potential role in the regulation of insulin sensitivity and glucose uptake. It seems to stimulate the glucose uptake in skeletal muscle and adipocytes, through the activation of the PI3K-Akt pathway [9,10]. It is a potent angiogenic factor involved in formation of new blood vessels, which improves tissue perfusion and nutrient delivery [11]. Study revealed that there is decreased expression of Flt-1 (VEGFR1) and increased KDR (VEGFR2) expression which represents a proangiogenic state during GDM with placental hyper-vascularization [12].

Trophoblasts are the primary cells in the placenta and are responsible for many of its functions, including nutrient and oxygen exchange between the mother and the fetus. Studies have shown that trophoblasts from women with GDM exhibit altered gene expression and impaired function compared to trophoblasts from healthy women [13,14]. *In vitro* study on the primary human trophoblasts, cultured under low glucose (5 mM) or high glucose (25 mM) conditions, showed perturbed biochemical pathways due to exposure of placenta to high glucose concentration [15,16]. Unfortunately, there are limited studies on the placental functions during GDM due to lack of suitable culture models. In our study, we have successfully developed an insulin-resistant BeWo cell line (choriocarcinoma cells), mimicking the placental features in GDM condition during early pregnancy in human. Besides, we observed the effect of VEGF on the insulin-resistant cells for any modulation in glucose uptake. We did not find significant change in glucose uptake upon addition of VEGF to the insulin-resistant cells. However, it is important to note that glucose homeostasis is a complex phenomenon and many cell types and regulatory molecules are involved to maintain the homeostasis, more studies are required to understand the role of VEGF in this context. Nevertheless, the insulin-resistant BeWo cell model we have developed, will be very useful in studying the pathophysiological changes occurring in placenta during GDM and evaluating potential therapeutic agents against the disease.

Methodology

Materials used

For culturing the BeWo cells, the following reagents were used: Ham's F12K cell culture medium (Gibco), fetal bovine serum (FBS) (MP Biomedicals, France), 1X Dulbecco's Phosphate Buffered Saline (DPBS), 0.25 % Trypsin-EDTA solution, and dimethyl sulfoxide (DMSO). T-25 flasks (Thermo Fisher Scientific Inc., USA); 10 mL serological pipettes and 96-well plates from Thermo Fisher Scientific Inc., USA (Nunc); XDFL series, SDPTOP, Sunny Instruments, China; Analysis Software ImageJ (Fiji) software V1.53c.

BeWo cell culture and differentiation

The BeWo cell line (procured from NCCS, Pune, India) was seeded and cultured using Ham's F12K culture media supplemented with 10 % FBS to get the adherent cells. The cells were plated in T-25 flasks (5×10^5 cells/mL) in growth medium and incubated overnight. The CO_2 incubator was maintained at 37 °C temperature, 5 % CO_2 level and 90 % relative humidity during the cell growth. After 3 passages, the cells were used to initiate the differentiation. For this purpose, fresh Ham's F12K growth medium, supplemented with 2 % FBS, was added with a combination of both 40 μM forskolin and 250 μM 8-Br-cAMP. The same treatment was continued on the 3rd day. The cells were observed to be effectively differentiated on day 5 with the development of syncytial cells [17].

Development of insulin-resistant cells and VEGF treatment

Once the differentiation is achieved, the normal growth medium was fluxed with 25 mM glucose and incubated for 72 h to induce insulin resistance. Once it was achieved (**Table 1**), 50 ng/mL VEGF was added to the Test + VEGF group only. All flasks were incubated for 18 h, and cells were observed under the microscope. At the end of the treatment, the medium from all the flasks were removed, and cells were washed with DPBS. The cells were trypsinized and harvested in 15 mL tubes. The tubes were centrifuged for 5 min. At 300 g at 25 °C, and the supernatant was discarded. Finally, the tubes containing the cells were resuspended and used for viability assay and glucose uptake assay.

Cell counting and viability

The BeWo cells were harvested through trypsinization, and 50 μL of cell suspension was taken, and an equal volume of 0.4 % Trypan blue was added to estimate the viable cells through dye exclusion method. The number of viable cells (seen as bright cells) and non-viable cells (stained blue) were counted, and ideally > 100 cells were counted to increase the accuracy of the cell count. The total number of cells under observation was 5.82×10^5 cells/mL. Out of which number of viable cell was around 5.8×10^5 and

2×10^4 cells/mL of dead cells were observed. The percentage of viability was estimated to be around 99.6 %.

Treatment groups

In this experiment, there were 3 groups (4 replicates of each group) of BeWo cell cultures such as control (5 mM glucose), Test (25 mM glucose) [35] and Test + VEGF group. After the cells became insulin resistant, VEGF was added at a concentration of 50 ng/mL for 18 h to the Test + VEGF group [16,17]. The results obtained from each group were then compared and analyzed to determine the effects of insulin resistance/glucose uptake and VEGF on BeWo cell cultures.

Glucose uptake assay using flow cytometry

After the incubation was completed, the spent medium was aspirated, and the cells were washed with DPBS. Low serum media without glucose, containing 0.5 % FBS, was added to all wells. After 2 h, the medium was aspirated, and the cells, excluding the negative control (without insulin treatment), were treated with 0.1 µg/mL insulin in 5 mL glucose-free culture medium, containing 100 µM 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose) (7 µL per mL used). The cells were then incubated for 2 h. At the end of the treatment, the medium was removed from all wells, and the cells were washed with DPBS. Trypsinization was done and the cells were harvested directly into 12×75 mm tubes. The tubes were then centrifuged for 5 min at 300× g at 25 °C, and the supernatant was carefully aspirated. The cells were resuspended in 0.5 - 1 mL of PBS, and the suspension was mixed well to ensure the separation of individual cells. Subsequently, the cells were used for flow cytometry analysis. The 2-NBDG is a fluorescent glucose analog that is commonly used as a probe to detect glucose uptake in cultured cells. It is taken up by cells in the same mechanism as glucose uptake. It is not metabolized, so it remains trapped within the cells and emits fluorescence upon excitation in a flow cytometer. The fluorescence of 2-NBDG is typically detected using optical filters designed for fluorescein, with excitation and emission maxima of 465/540 nm [18]. The cells were analyzed using flow cytometer to detect the fluorescence emitted by the 2-NBDG that has been taken up by the cells. The intensity of the fluorescence is proportional to the amount of glucose uptake by the cells.

Results

The findings of this study highlight the association between high glucose flux and the development of insulin resistance in BeWo trophoblast cells. We investigated the impact of insulin resistance and VEGF treatment on glucose uptake in BeWo cells which mimics the placental features in GDM during early pregnancy.

The BeWo cell culture (**Figure 1**) was established with suitable culture media and differentiated using 40 µM forskolin and 250 µM 8-Br-cAMP. **Figures 1A - 1E** shows the BeWo cells with increasing confluency at 10× magnification. The differentiation was initiated with monolayer cells with 70% confluency. The differentiation was marked by the fused syncytia (**Figure 1F**), and these cells were used for further development of insulin resistant model and VEGF treatment.

The glucose uptake assay revealed a 2.2-fold increase in 2-NBDG fluorescence in the control group (with insulin treatment) compared to the negative control cells. However, the test group and test + VEGF group exhibited resistance to insulin treatment, showing a decrease in glucose uptake with a 1.1-fold and 1.2-fold increase in 2-NBDG fluorescence, respectively, relative to the negative control cells. Additionally, the percentage of cells with high glucose uptake was lower in the test and test + VEGF groups compared to the control group (**Table 1**; **Figure 2**). Comparative analysis of the mean fluorescence intensity (MFI) for 2-NBDG uptake in BeWo cells showed the insulin-sensitized control group significantly higher MFI (9816 ± 126; fold change: 2.2) compared to the insulin-resistant test group (MFI: 4693 ± 74.2; fold change: 1.1). The inclusion of exogenous VEGF in the test group did not significantly impact glucose uptake (MFI: 5040 ± 76.7; fold change: 1.2).

Figures 3 - 6 show the flow cytometry analysis of different cell groups with 2-NBDG signal. The positive control group, representing normal cells with insulin treatment, showed a high percentage of cells (approximately 70 - 73 %) with high 2-NBDG signal (**Figure 3**). In contrast, the absence of insulin resulted in a significant decrease in glucose uptake, with only 38.2 % of cells displaying high 2-NBDG signal (**Figure 4**). Similarly, the test group and test + VEGF group showed reduced glucose uptake, with approximately 40.5 % (**Figure 5**) and 43.5 % (**Figure 6**) of cells exhibiting high 2-NBDG signal, respectively.

Overall, these results demonstrate that BeWo cells subjected to hyperglycemic condition exhibited insulin resistance resulting in impaired glucose uptake compared to the insulin-sensitized control group.
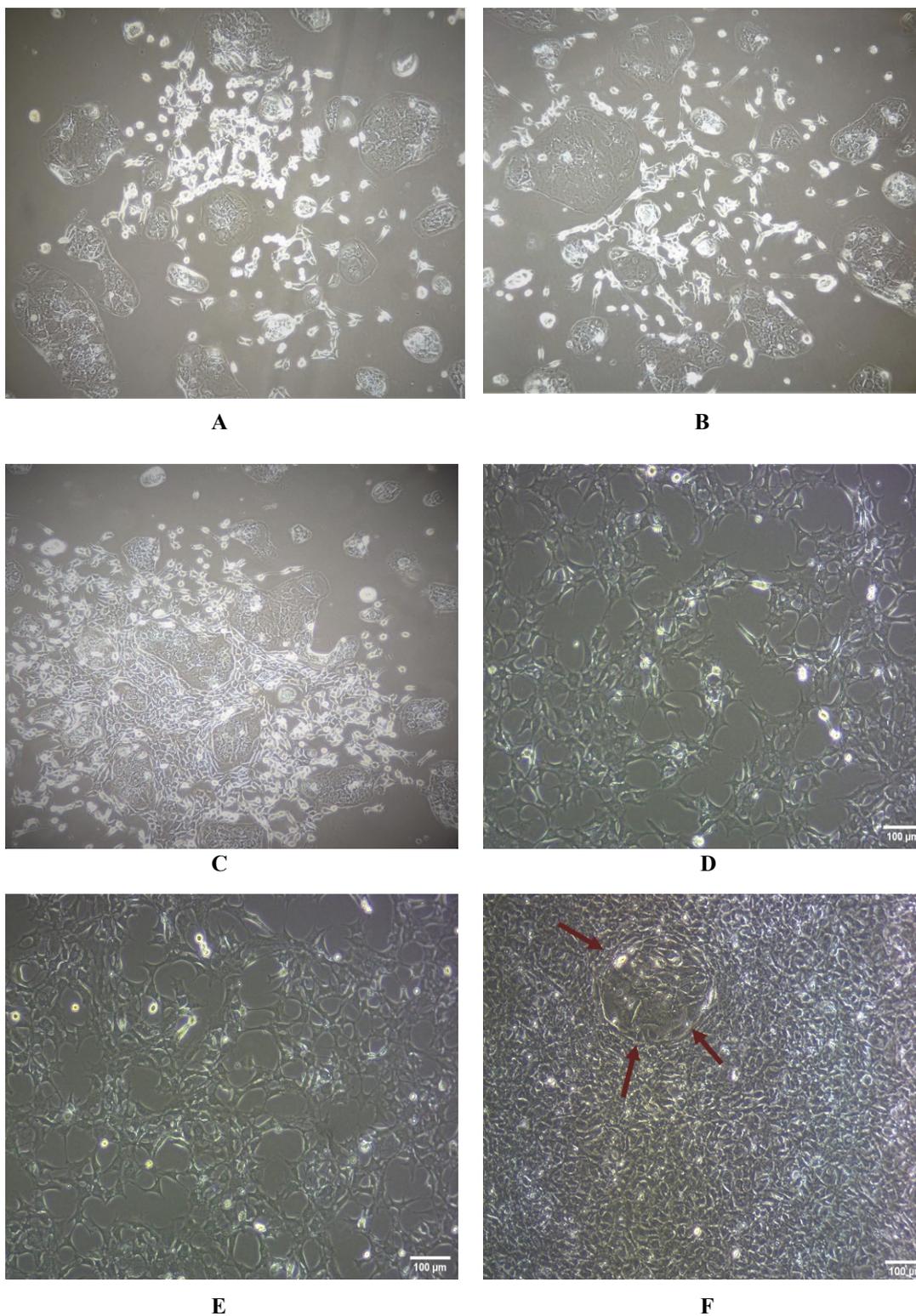

Figure 1 Images of BeWo cells at 10× magnification. A) and B) shows multiple small colonies at 40 % confluency. C), D) and E) shows monolayer culture at 70 % confluency. F) showed cellular differentiation marked with fused syncytia (red arrow), in the presence of 40 µM forskolin and 250 µM 8-Br-cAMP.

Table 1 Mean fluorescence intensity (MFI) and fold change of glucose uptake in various cell groups. The increase in Glucose uptake was detected by measuring the cells positive in 2-NBDG (green) fluorescence in the FL1 detector using a 525nm band pass filter.

|	Sample Name	Geometric mean fluorescence intensity (MFI) of NBDG (FL1-A parameter) ± CV	Fold change in glucose uptake	% of cells	
				NBDG low (Cells with lower conc. of 2-NBDG)	NBDG high (Cells with higher conc. of 2-NBDG)
	BeWo - assay controls for flow cytometry				
1	Negative control (without insulin)	4402 ± 75.9	1	61.8	38.2
2	Positive control (with 0.1 µg/mL insulin)	10431 ± 85.2	2.4	27.0	73.0
	BeWo - differentiated cells				
1	Control group (with 0.1 µg/mL insulin and 5 mM glucose)	9816 ± 126	2.2	29.2	70.8
2	Test group (with 0.1 µg/mL insulin and 25 mM glucose)	4693 ± 74.2	1.1	59.5	40.5
3	Test + VEGF group – (with 0.1 µg/mL insulin, 25 mM glucose, and 50ng/mL VEGF)	5040 ± 76.7	1.2	56.5	43.5
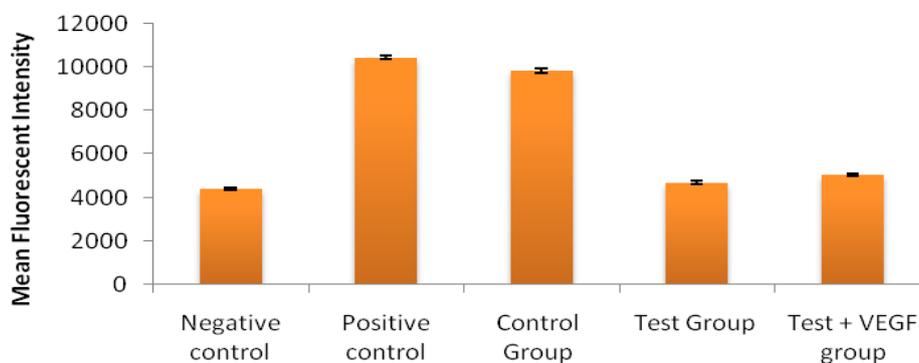
Figure 2 Comparative MFI for 2-NBDG uptake in BeWo cells for control and test groups. The above result shows the cells developing insulin resistance. Insulin resistant test group (MF1: 4693 ± 74.2; Fold change:1.1) was compared to insulin sensitized control group (MFI: 9816 ± 126; Fold change:2.2). VEGF with test group (MFI: 5040 ± 76.7; Fold change:1.2).

In **Figure 3**, Flow cytometry analysis of 2-NBDG uptake in BeWo cells. The plot on the left-hand side describes the forward scatter plot (corresponds to cell size) vs side scatter plot (corresponds to cellular complexity) for untreated positive control normal cells. Side scatter plot also defines the grouping of cells of similar size and granularity. The plot on the right side describes the count of cells which responds to 2-NBDG uptake (Geometric mean or Mean Fluorescence intensity).
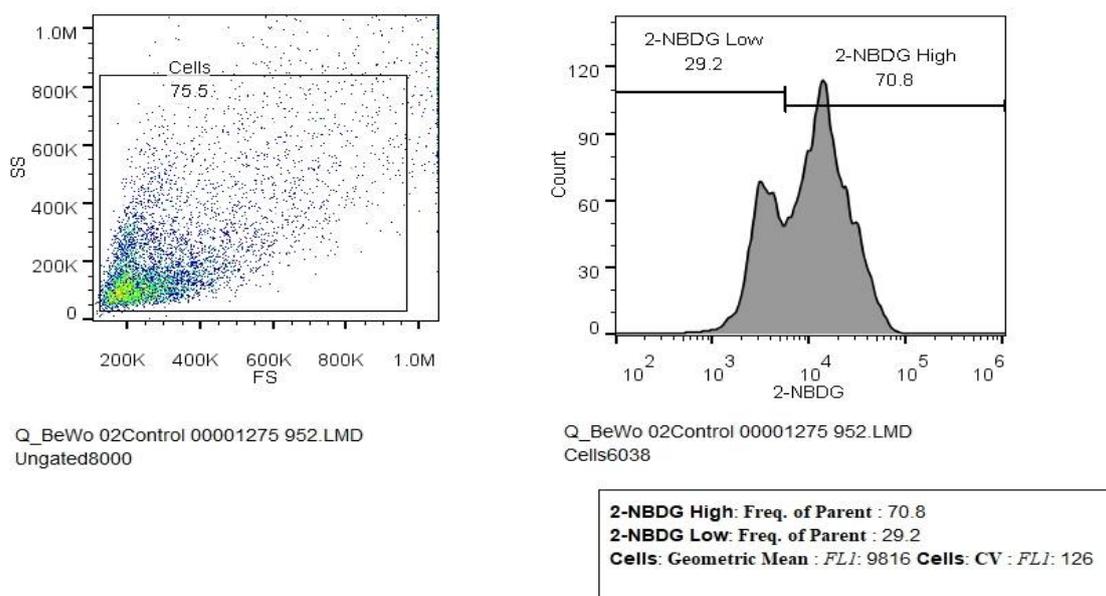
Figure 3 The following plot shows that 70.8 % of cells have 2-NBDG (Glucose analog) high with Geometric mean or Mean Fluorescence intensity: 9816 ±126 when insulin is induced to normal cells (Glucose conc: 5 mM), hence validates the result of positive control.
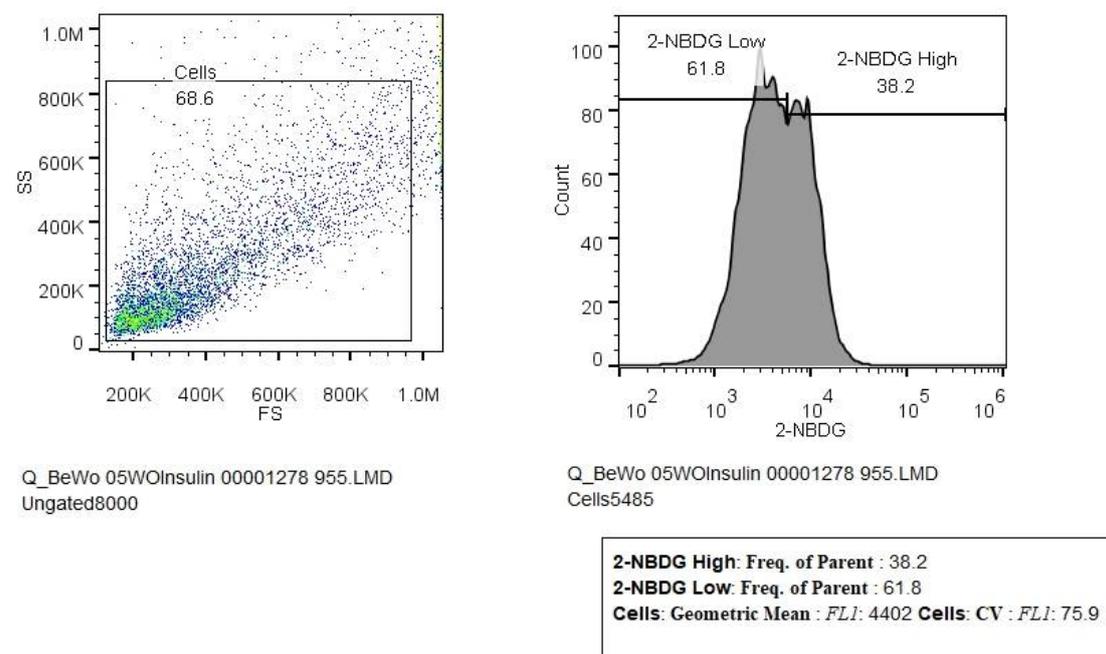

Figure 4 The following plot shows negative control where 38.2 % of cells have 2-NBDG (Glucose analog) high with Geometric mean or Mean Fluorescence intensity: 4402 ± 75.9 when no insulin is treated to normal cells (Glucose:6 mM). A 45 % drop in 2-NBDG uptake validates the role of insulin in glucose(2-NBDG) uptake in cellular system (BeWo cells).
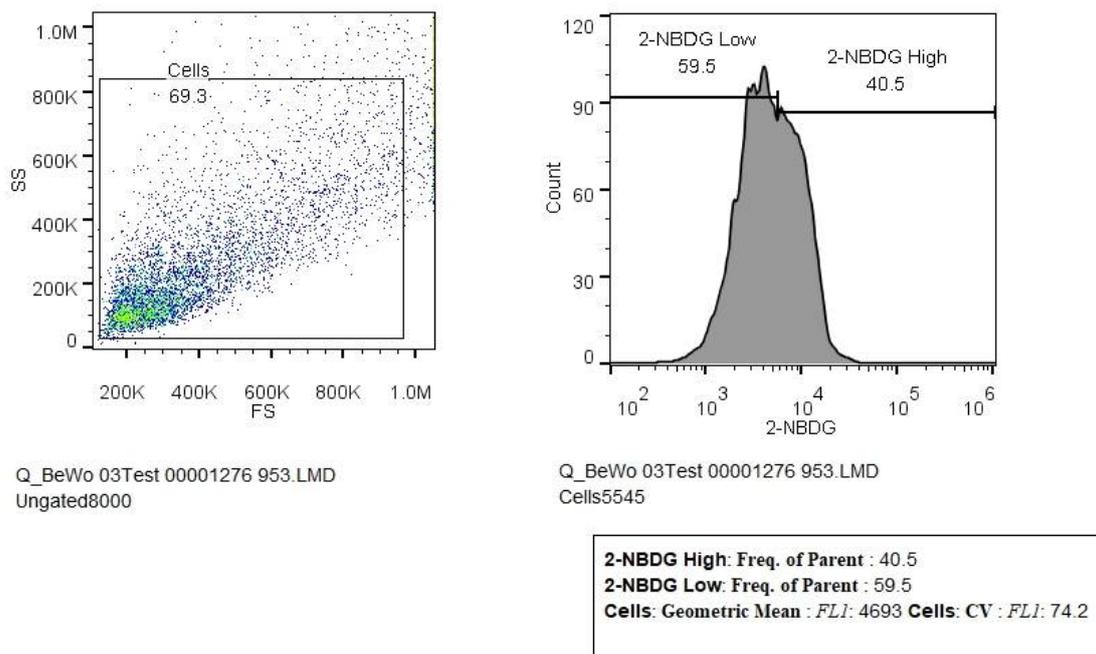
Figure 5 The following plot shows that 40.5 % of cells have 2-NBDG (Glucose analog) high with Geometric mean or Mean Fluorescence intensity: 4693 ± 74.2 under hyperglycemic condition (Glucose: 25 mM). A 47 % drop in 2-NBDG uptake validates the test condition in which cells becomes glucose intolerant or insulin resistant.
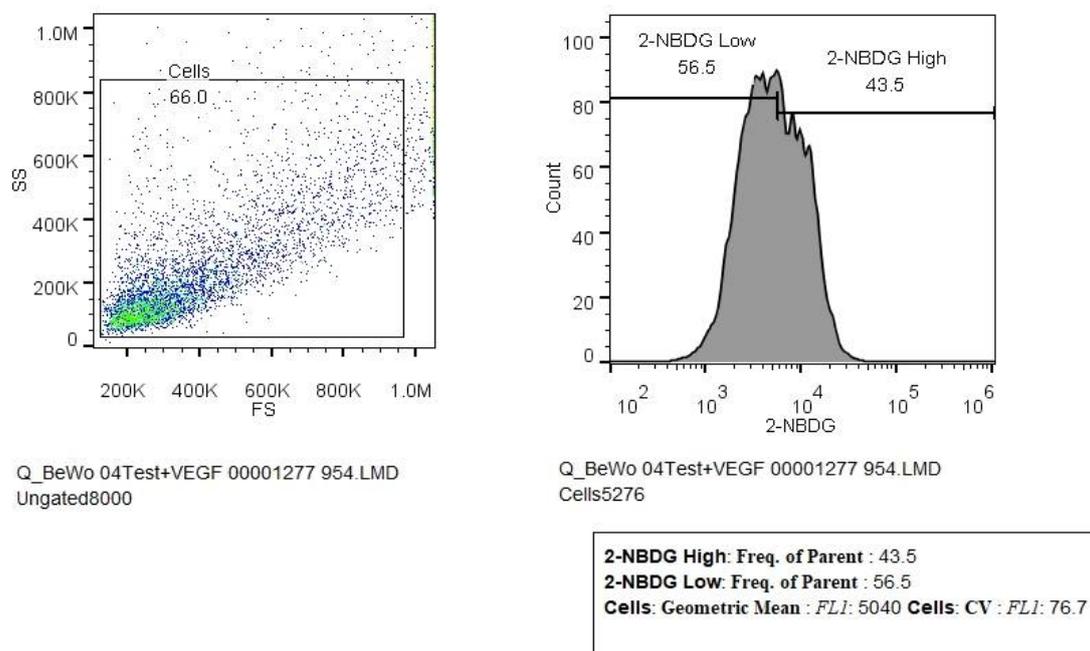

Figure 6 The following plot shows that 43.5 % of cells have 2-NBDG (Glucose analog) high with Geometric mean or Mean Fluorescence intensity: 5040 ± 76.7 under hyperglycemic test condition (Glucose: 25 mM) in the presence of exogenous VEGF. A 50 % drop in 2-NBDG uptake was observed, which is statistically equivalent to test cells.

Discussion

Hyperglycemia is a key factor in the development of GDM which develops during pregnancy and affects women worldwide. It can lead to complications such as pre-eclampsia, cesarean delivery, hypoglycemia, and an increased risk of developing type 2 diabetes. Babies born to women with GDM are at risk of macrosomia, respiratory distress syndrome, and jaundice. Cell culture technology can be used to study the effects of maternal diabetes on cells and tissues, which can help in the development of effective diagnostic methods and therapeutic compounds against GDM condition [19].

Pregnancies complicated by GDM exhibit maternal insulin resistance, low-grade inflammation, and endothelial cell dysfunction as central features. Research in recent decade, has unveiled candidate regulators of these phenomena within placental, maternal adipose tissue, and skeletal muscle. These regulators encompass nuclear factor κB (NF-κB), peroxisome proliferator-activated receptors (PPARs), sirtuins (SIRTs), 5′ AMP-activated protein kinase (AMPK), glycogen synthase kinase 3 (GSK3), PI3K/mTOR, inflammasome, and endoplasmic reticulum (ER) stress. They exert influence across these tissues, impacting insulin resistance, inflammation, and endothelial function [20]. Sterile inflammatory insults further exacerbate these complications by perturbing signaling pathways within placental cell system, adipose tissue, and muscle. This disruption leads to functional alterations within these cell systems, potentially contributing to adverse outcomes for offspring associated with GDM [21].

In *in-vitro* culture condition, the primary role of insulin is to facilitate the uptake of glucose into cells, where it is used for energy. In GDM condition, this process is hindered. Firstly, the initial step of insulin signaling is compromised [22]. The insulin receptor, which acts like a key, does not work properly, making it challenging for insulin to open the cellular door for glucose. This is due to reduced tyrosine phosphorylation, a crucial step in insulin signaling. Secondly, there is an excess of serine phosphorylation, a chemical change that acts like a road block, obstructing insulin action and preventing glucose uptake [23]. Inflammatory proteins, like TNF-α, increase serine phosphorylation, intensifying insulin resistance. Inside the cells, proteins such as mTOR and p70S6K1 contribute to excess serine phosphorylation and break down IRS-1, an essential protein in insulin signaling [24]. Additionally, an excess of a protein called p85-α disrupts insulin activation, which acts as a dominant roadblock. All these factors combined create a scenario where insulin struggles to facilitate glucose uptake into cells, resulting in hyperglycemic condition dominantly due to insulin resistance [25].

We investigated the robustness of the BeWo cells as an *in vitro* model for the evaluation of glucose uptake which mimics the placental environment during GDM in pregnant women. We developed the insulin-resistant cell model by exposing the cells to high glucose concentration. The findings were validated by flow cytometry analysis of various cell groups, which showed significant difference in glucose uptake by the resistant cells as compared to the control cells sensitized with insulin. The geometric mean fluorescence intensity (MFI) (**Table 1**) of NBDG (FL1-A parameter) and fold change (**Figure 2**) in glucose uptake for different experimental groups showed convincing results supporting the establishment of insulin-resistant model. Insulin is a hormone that plays a crucial role in glucose uptake and metabolism in the body. The negative control group without insulin showed the lowest glucose uptake with an MFI of 4402 ± 75.9. This group represents the baseline glucose uptake in the absence of insulin stimulation. The positive control group treated with 0.1 μg/mL insulin [26] showed highest glucose uptake with an MFI of 10431 ± 85.2, indicating a 2.4-fold increase in glucose uptake compared to the negative control group. The control group (5mM glucose) treated with 0.1 μg/mL insulin, showed an MFI of 9816 ± 126, indicating a 2.2-fold increase in glucose uptake as compared to the negative control group. These results suggest that the insulin treatment alone is sufficient to stimulate glucose uptake in trophoblastic placental cells. The test group which was exposed to 5 times the normal glucose concentration (hyperglycemic; 25 mM) in presence of 0.1 μg/mL insulin, showed an MFI of 4693 ± 74.2, indicating only a 1.1-fold increase in glucose uptake as compared to the negative control group.

Our findings showed that exposure to high glucose concentration (hyperglycemia) in the presence of insulin resulted in impaired glucose uptake in BeWo cells, resulting in the development of insulin resistance. This result is consistent with previous studies that have linked hyperglycemia and insulin resistance with adverse pregnancy outcomes such as GDM, preeclampsia, and fetal growth restriction [27,28].

Furthermore, as hypothesized, the same test group in presence of 0.1 μg/mL insulin and 50 ng/mL VEGF showed an MFI of 5040 ± 76.7, indicating a 1.2-fold increase in glucose uptake as compared to the negative control group. This result suggests that the VEGF has marginally increased the glucose uptake in the presence of insulin and improvised the sensitivity on glucose uptake. Although the presence of VEGF showed a 1.2-fold increase in glucose uptake compared to the negative control group, the difference was

not statistically significant. However, it is important to note that glucose homeostasis is regulated by multiple cell types and various regulatory molecules, hence further research is required to understand the role of VEGF in glucose uptake during GDM condition. Previous studies on the role of VEGF in glucose metabolism and insulin sensitivity, showed mixed findings. Study by Chen *et al.* [29] revealed that VEGF treatment improved glucose uptake and insulin sensitivity in diabetic mice, while a study by Luo *et al.* [30] reported that VEGF had no or insignificant effect on glucose uptake in skeletal muscle cells. In this context, further in-depth study is required to unravel the role of VEGF in glucose homeostasis during GDM.

The above experimental outcomes showed that the BeWo cells undergo insulin resistance under hyperglycemic condition and can serve an efficient cellular platform to investigate the gestational diabetic complications. Studying placental cells under hyperglycemic conditions will provide important insights into the mechanisms underlying gestational diabetic complications and further therapeutic approach.

Conclusions

BeWo cell line is a human placental cell line originated from choriocarcinoma cells and it is an ideal *in vitro* model to study the pathogenesis of GDM and explore the involvement of regulatory molecules in glucose homeostasis. We have developed the cellular model that mimic the placental pathophysiology in GDM condition in vivo and show insulin resistance. To attain the GDM model, cells were differentiated and further treated with high glucose concentration to develop insulin resistance. Flow cytometry was performed to assess the glucose uptake of insulin-resistant cells with convincing results. Besides, we observed the effect of VEGF on glucose uptake by insulin-resistant cells and found its role to be insignificant in glucose homeostasis. However, it needs further study to decipher the role of VEGF in glucose homeostasis during GDM. To conclude, we have successfully developed the *in vitro* GDM model which will further help the researchers to unravel the regulatory proteins in GDM pathogenesis and discover biomarkers for early diagnosis of GDM in pregnant mother and adopt therapeutic strategy to save from the ill effects of GDM on mother and the fetus.

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