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Effect of miR-26b on gestational diabetes mellitus in rats *via* PI3K/Akt signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the influence of micro ribonucleic acid (miR)-26b on gestational diabetes mellitus in rats *via* the phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/Akt) signaling pathway.

MATERIALS AND METHODS: A total of 60 healthy pregnant female rats were randomly divided into three groups, including group A (normal group), group B (model group), and group C (model + miR-26b group). The differences in fasting blood glucose (FBG), C-reactive protein (CRP), and phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/AKT) among the three groups were analyzed *via* serum CRP test, morphological observation, quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR), and Western blotting, respectively.

RESULTS: The levels of FBG ad CRP were significantly up-regulated in group B when compared with group A ($p<0.01$). Meanwhile, they increased significantly in group C when compared with group B ($p<0.01$). Rats in group A exhibited smooth and flat thoracic aortic intimas, as well as neatly arranged smooth muscle cells at the media layer. However, rats in group B showed fractured intimas with enlarged junction gaps, as well as necrotic and detached endothelial cells. Compared with group B, group C exhibited extremely poorly arranged cells at all the layers, rough and rugged intimas, larger areas of necrotic and detached endothelial cells, and markedly worsened lesions. QRT-PCR results indicated that the expression of phosphorylated-PI3K (p-PI3K) was significantly lower in group B than that of group A ($p=0.04$). Meanwhile, it was markedly lower in group C than that in group B ($p=0.04$). The expression of p-Akt was remarkably lower in group B than group A ($p=0.04$), which was also significantly lower in group C than group B ($p=0.04$). Compared with group A, the expressions of p-PI3K and p-Akt in the thoracic aorta of group B were evidently down-regulated ($p<0.01$). Furthermore, they decreased markedly in group C when compared with group B ($p<0.01$).

CONCLUSIONS: MiR-26b accelerates the progression of gestational diabetes by inhibiting the PI3K/Akt signaling pathway.

Key Words:
Gestational diabetes mellitus, PI3K/Akt signaling pathway, MiR-26b.

Introduction

Gestational diabetes mellitus refers to the phenomena in which no dose of glucose fails to be completely absorbed by the body of pregnant women. Currently, gestational diabetes has become one of the most common complications during pregnancy worldwide[1,2]. It can increase the risk of macrosomia (20-30%) and fetal growth retardation (15-20%)[3], eventually affecting fetal development. If not corrected timely, gestational diabetes will cause stillbirth in severe cases. Meanwhile, it has been one of the major diseases threatening human health[4,5]. Gestational diabetes is characterized by polydipsia, polyphagia, and diuresis in pregnant women. It can further increase the risks of obesity, diabetes, and insulin resistance[6-9]. Therefore, the exploration of how to prevent and treat this disease has become the top priority in the medical community in recent years.

The regulatory subunit p85 and catalytic subunit p110 in the phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/Akt) signaling

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pathway, as well as PI3K/Akt signals, can function as regulators in cells[10]. Specifically, they are capable of participating in cell secretion and transport, thus playing important roles in the growth, development, and death of cells. The PI3K/Akt signaling pathway has been demonstrated involved in all cell activities in gestational diabetes. It not only features in glucose transport, synthesis, and decomposition, but also serves as a crucial regulator of blood glucose balance by insulin. Experiments have proved that up-regulating the activity of PI3K/Akt in diabetic patients can promote membrane translocation of glucose transporter 4 (GLUT4). This may help to reduce insulin resistance factors and alleviate gestational diabetes.

Currently, micro ribonucleic acid (miR)-26b has been found to participate in the growth and development of cell tissues. Meanwhile, it especially plays an important role in tumors, non-tumor diseases, and gestational diabetes[11,12]. The mechanism of inflammatory factors in gestational diabetes involves hypoxia-inducible factor (HIF)-1. As an extracellular glycoprotein, HIF-1 regulates several targeting factors. Furthermore, the inhibition of its expression can negatively regulate the PI3K/Akt signaling pathway. Thus, the inhibition of miR-26b can activate the expression HIF-1, thereby relieving the inflammation in gestational diabetes.

Materials and Methods

Basic Information of Rats

Healthy pregnant female rats aged about 6 weeks old and weighing 0.2-0.25 kg were purchased from the Hunan Provincial Center for Disease Control and Prevention. All rats were fed under the temperature of 20-25°C and relative humidity of 38-70% for 8 weeks as required. This investigation was approved by the Animal Ethics Committee of The Affiliated Hospital of Qingdao University Animal Center

Main Reagents and Instruments

SureStep Plus blood glucose meter (LifeScan, Seattle, WA, USA), vertical polyacrylamide gel electrophoresis and transfer systems, and E1×800 enzyme immunoassay instrument (Bio-Rad, Hercules, CA, USA), high-speed refrigerated centrifuge (Eppendorf, EP), ultra-low temperature refrigerator (SANYO, Osaka, Japan), ChemiDoc-It 610 chemiluminescence imaging system (UVP), C-reactive protein (CRP) enzyme-linked immunosorbent assay (ELISA) kit (Beijing China Ocean Technology Co., Ltd., Beijing, China), phosphorylated-PI3K (p-PI3K) primary antibody (Cell Signaling Technology Co., Ltd., Danvers, MA, USA), p-Akt (Ser473) primary antibody, LIJ goat anti-mouse secondary antibody and LIJ goat anti-rabbit secondary antibody (Beyotime, Shanghai, China), actin antibody and polyvinylidene difluoride (PVDF) membranes (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China), and bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA).

Animal Grouping and Modeling

A total of 60 healthy female rats were randomly divided into three groups, including group A (normal group), group B (model group), and group C (model + miR-26b group). Rats in group A were fed with a normal diet. However, rats in both group B and group C fasted for solids, with free access to water. Moreover, fasting rats in group B and C were injected with streptozotocin at a dose of 40 mg/kg *via* the left lower abdomen to establish the model of diabetes in rats. However, those in group A were intraperitoneally injected with the same dose of sodium citrate buffer. At 18 h after injection, blood samples were drawn from the veins of rats in the three groups, and blood glucose was measured once a day for 3 days. The model with blood glucose concentration over than 17.1 mmol/L was eligible. After successful modeling, rats in group C were intravitreally injected with the same volume of miR-26b, and they had no complications such as vitreous hemorrhage, cataract, and intraocular hypertension.

Collection of Specimens

After drug intervention, blood samples were collected from the tails to determine fasting blood glucose (FBG). The rats were first anesthetized *via* intraperitoneal injection of 2.5% pentobarbital sodium at a dose of 0.25 g/kg. Subsequently, 2-3 mL of blood was drawn from the abdominal aorta, followed by centrifugation at high speed and 10-20°C for 5-8 min. The serum was obtained and stored at –80°C for use. After blood sampling, the thoracic aorta was immediately separated and cut into two segments for hematoxylin-eosin staining (HE) staining and storage at –80°C, respectively.

Detection of FBG and Serum CRP

The level of FBG was determined by a blood glucose meter. Meanwhile, the level of serum CRP was measured according to the instructions of relevant kits.

Morphological Observation in Rats of the Three Groups

After removal, thoracic aortic tissues were first fixed with 0.8 mL of neutral formalin for 18 h. Subsequently, they were dehydrated using 5% ethanol, transparentized using xylene, soaked, and sealed in paraffin. Next, the tissues were sliced into 4 mm-thick serial sections and stained with HE. Finally, morphological changes were observed under a light microscope.

Expressions of PI3K/Akt Signals in Rats Via Quantitative Reverse Transcription-Polymerase Chain Reaction (QRT-PCR)

Cell membranes of rats were digested with trypsin, washed with phosphate-buffer saline (PBS) and added with 2 mL of miR-26b vector reagent. The total RNA was extracted as follows: 35 mg of cell tissues were placed in an Eppendorf (EP) tube and added with the miR-26b reagent. Extracted RNA was reverse transcribed into complementary deoxyribonucleic acids (cDNAs). QRT-PCR amplification was performed with miR-26b vectors as templates under the following condition: 98°C for 6 min, 98°C for 28 s, 75°C for 30 s, and 80°C for 4 min.

Detection of Phosphatidylinositol 3-Hydroxy Kinase/Protein Kinase B (PI3K/AKT) in Rats Via QRT-PCR

A total of 20 mg cell tissues were placed in an EP tube and added with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) reagent. Then, they were added dropwise with 0.9% sodium chloride solution and 2 mL of RNA extraction reagent to extract RNAs. Subsequently, extracted RNAs were reverse transcribed into cDNAs using the A3500 RT kit. The cDNAs were stained with nucleic acid gel dye to detect the expression of target genes using the CFX-96 qRT-PCR instrument. Primers were designed by NCBI Primer-BLAST. Specific reaction conditions were as follows: 98°C for 6 min, 98°C for 28 s, 75°C for 30 s, and 80°C for 4 min, for a total of 55 cycles. The primer sequences used in this study were shown in Table I.

Western Blotting

Cells in 6-well plates were digested with 100 μg of trypsin, followed by addition of 3 mL of medium to terminate the digestion. Subsequently, cell extract was placed in an EP tube, mixed with trypsin extract at 1:100, and frozen in a refrigerator for 10 min. After the cells were completely split into E solution, thoracic aorta tissue and 3mL trypsin extract were added into EP tubes at the ratio of 1:100 to fully split the cells into F solution. Next, E and F solution were mixed at the volume ratio of 80:1 and shaken evenly for preparation of the working solution. After the working solution was stored in an incubator at 37°C for 20 min and cooled, the protein concentrations of p-PI3K and p-Akt were calculated.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (SPSS, Chicago, IL, USA) software was used for all statistical analysis. The *t*-test was adopted for comparisons among the three groups. Measurement data were expressed as mean ± standard deviation. One-way analysis of variance was applied to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Table I. Primer sequences of transcriptional genes.

Gene		Primer sequences
P-PI3K	Forward	5'-GGCTGAGGGlTrAGTGAGCA-3'
	Reverse	5'-AGGGAGTTGGTGAAAGACATC-3'
P-Akt	Forward	5'-AGGGCAGAATCATGAGCAAGT-3'
	Reverse	5'-AGGGTCTGCATFGGATGGCA-3'
GAPDH	Forward	5'-CACCATIGGCAATGAGGGGTFC-3'
	Reverse	5'-AGGTCTTTGCGGATGTCCACGT-3'

Results

General Conditions of Rats

During the experiment, rats in group A grew well and had glossy hairs, with normal locomotor activity and no death. Rats in group B showed dull and yellow hairs, depression with less motion, increased water and food intake, as well as urine discharges. However, rats in group C exhibited significantly aggravated diabetes symptoms and extremely poor mental state without locomotor activity.

Levels of FBG and Serum CRP in Rats

The levels of FBG and CRP were markedly up-regulated in group B when compared with group A ($p<0.01$). Meanwhile, they were increased significantly in group C when compared with those in group B ($p<0.01$) (Figure 1).

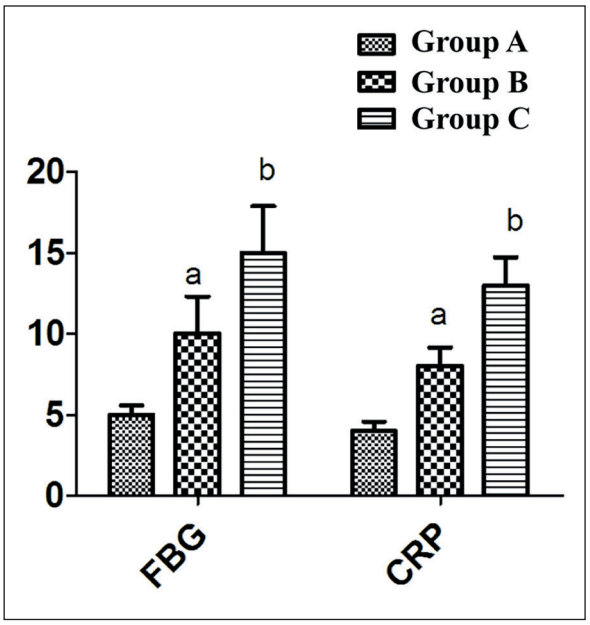

Figure 1. Levels of FBG and serum CRP in rats of the three groups. Note: a: $p<0.05$, *vs.* group A and b: $p<0.05$, *vs.* group B.

Endothelial Morphology of Rat Thoracic Aorta

Rats in group A showed smooth and flat thoracic aortic intimas, as well as neatly arranged smooth muscle cells at the media layer. However, rats in group B had fractured and rough intimas with widened junction gaps, as well as necrotic and detached endothelial cells. Compared with group B, rats in group C exhibited extremely poorly arranged cells at all the layers, rough and uneven intimas, larger areas of necrotic and detached cells, and evidently worsened lesions (Figure 2).

Expression of PI3K/Akt Signals in Rats Detected Via qRT-PCR

The expressions of p-PI3K and p-Akt were significantly lower in group B than that of group A. Meanwhile, they were also significantly lower in group C than that of group B ($p=0.04$) (Figures 3 and 4).

Protein Activity in Rats of the Three Groups of Detected Via Western Blotting

The active expressions of p-PI3K and p-Akt in blood cells in group B were significantly lower than those in group A ($p<0.01$). Moreover, they decreased remarkably in group C when compared with group B ($p<0.01$), with the highest level in group A (Figures 5 and 6).

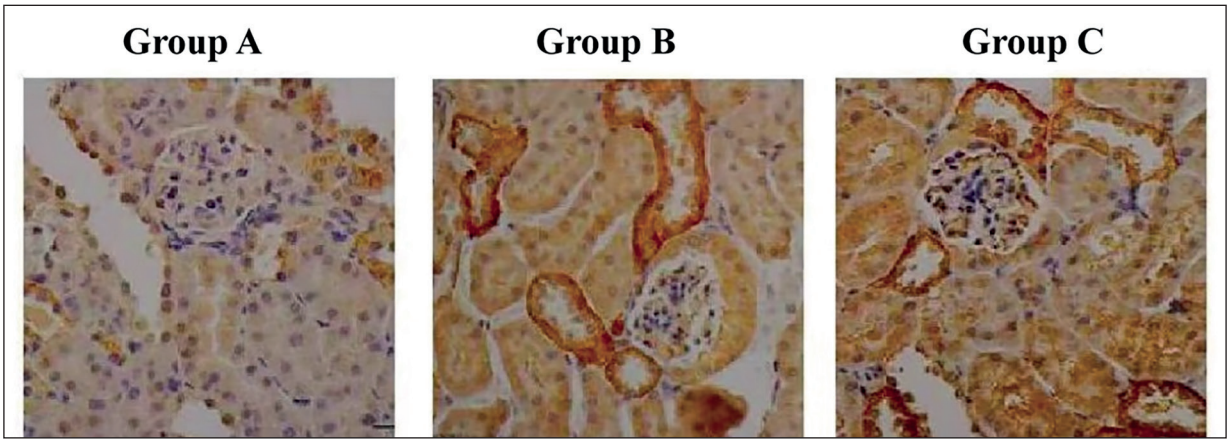

Figure 2. Endothelial morphology of rat thoracic aorta (magnification × 40).

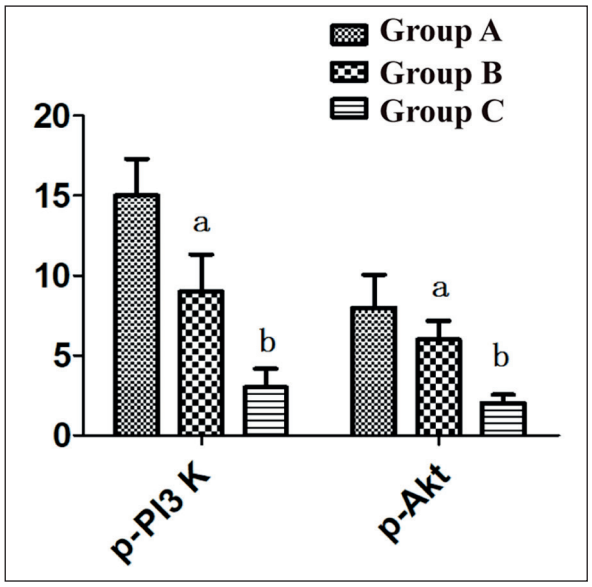

Figure 3. Active expressions of PI3K and Akt in rats of the three groups. Note: a: $p<0.05$, *vs.* group A and b: $p<0.05$, *vs.* group B.

Discussion

According to literature, China has become the country with the largest number of patients with diabetes worldwide. Gestational diabetes accounts for 30% of total patients. As a major chronic non-infectious disease, gestational diabetes can cause hyperglycemia and abnormal lipid metabolism, worsening injuries in the Akt insulin signaling pathway[13]. Gestational diabetes shows similar clinical manifestations to type 2 diabetes. It may ultimately develop into type 2 diabetes in about 1-5% cases[14]. Previous studies have suggested that the incidence rate of gestational diabetes is higher in some developed provinces in eastern China than that of central and western regions. This is largely related to the control of carbohydrates intake in pregnant women. Large fluctuation and increased blood glucose are supposed to be avoided. In particular, the intake of food that easily causes obesity, such as animal fat and carbohydrates, should be controlled[15]. This may help to enhance the ability of human bodies to control blood glucose, thereby reducing the risk of weight gain-induced gestational diabetes.

According to the results of the present study, the levels of FBG and serum CRP in rats were markedly up-regulated in group B when compared with those in group A ($p<0.01$). Meanwhile, they increased significantly in group C

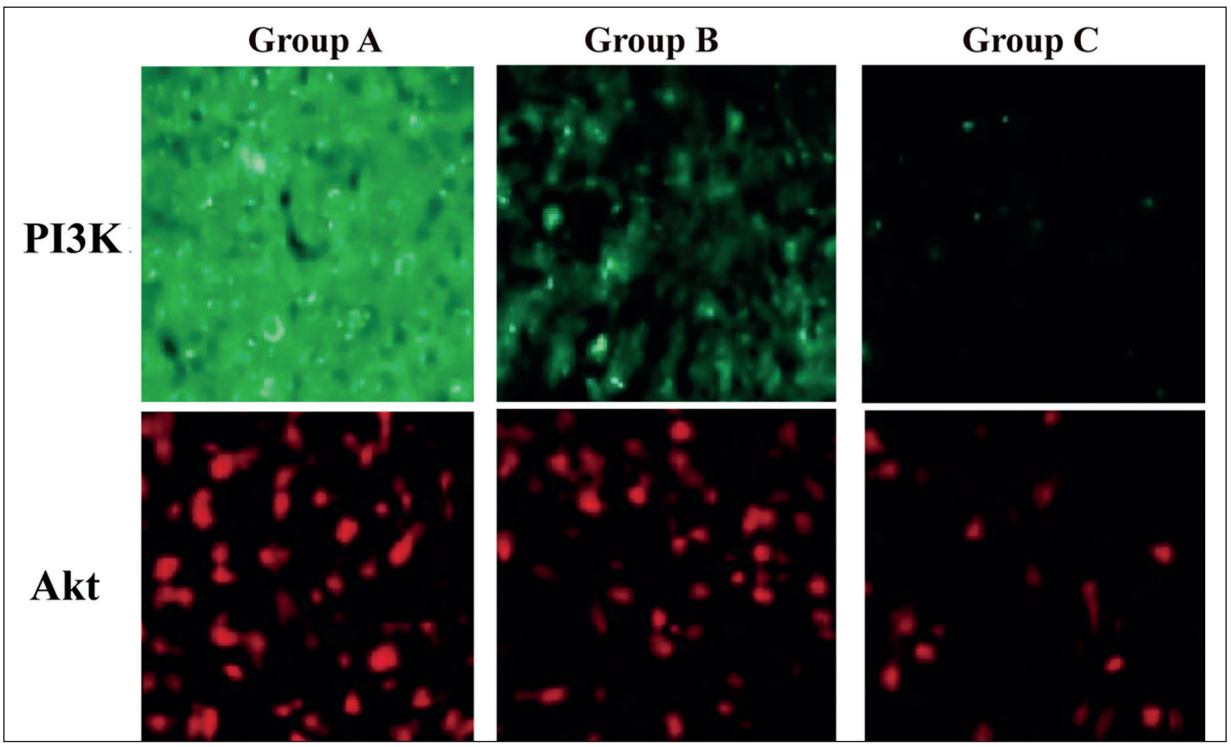

Figure 4. Active expressions of PI3K and Akt in cells of the three groups (magnification ×40).

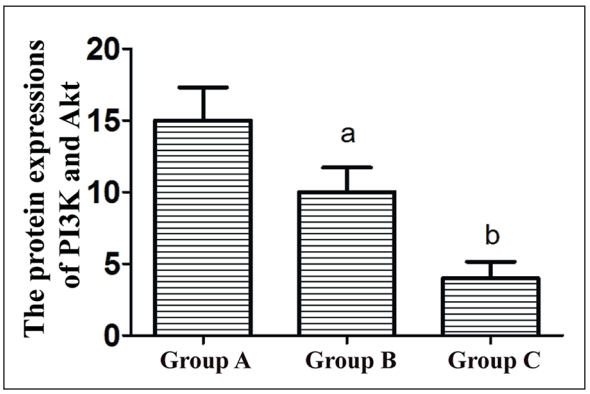

Figure 5. Protein expressions of PI3K and Akt in rats of the three groups. Note: a: $p<0.05$, *vs.* group A and b: $p<0.05$, *vs.* group B.

when compared with those in group B ($p<0.01$). Rats in group A showed smooth and flat thoracic aortic intimas, as well as neatly arranged smooth muscle cells at the media layer. However, rats in group B had fractured and rough intimas with widened cell junction gaps as well as necrotic and detached endothelial cells. Compared with group B, rats in group C exhibited extremely poorly arranged cells at all the layers, rough and uneven intimas, larger areas of necrotic and detached cells, and evidently worsened lesions. Hs-CRP and fibrinogen (Fbg) are acute reactive proteins, whose content can reflect the degree of inflammation in the body and cell tissue injuries. Meanwhile, they can also recognize some disease vectors and complements, such as active exogenous foreign matters and phagocytes. This is of great clinical significance for current research. Karaca et al[16] have suggested that inflammatory factors can cause cell function impairment, mainly in the form of disorder at the endothelial cell level. However, some scholars believe that CRP and Fbg can be used as non-specific inflammatory markers. Therefore, they are the most widely applied to detect inflammatory sensitivity in recent years. In gestational diabetes, hyperglycemia can up-regulate the levels of CRP and Fbg, disorder their activity, enhance the permeability of endothelial cells in the body, and impair body functions. Therefore, their levels in the serum are correlated with the occurrence of gestational diabetes. Literature reports[17] in China and beyond have corroborated each other, namely, the serum CRP and Fbg are higher in patients with gestational diabetes at acute exacerbation and stable stages than those in normal people. Moreover, they increase gradually with the aggravation of the disease. The above results are similar to those of the present work.

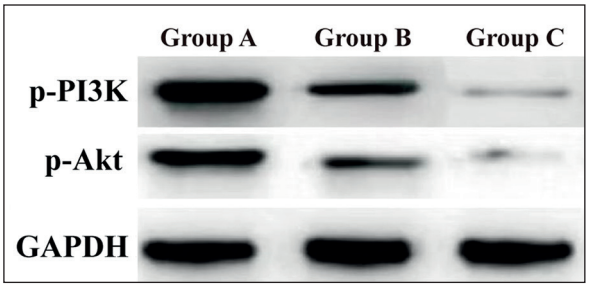

Figure 6. Protein expressions of PI3K and Akt in rats of the three groups.

QRT-PCR results indicated that the expressions of p-PI3K and p-Akt were significantly lower in group B than those in group A ($p=0.04$). Meanwhile, they were also lower in group C than those in group B ($p=0.04$). Western blotting results showed that the expressions of p-PI3K and p-Akt were significantly down-regulated in group B when compared with those in group A ($p<0.01$). Meanwhile, they were also evidently lower in group C than those in group B, with the highest level in group A ($p<0.01$). Akt is an important component in the PI3K/Akt signaling pathway. It can promote the expression of major downstream target proteins, while PI3K can bind to the IRS. Due to the stimulation by phosphatidylinositol, PI3K is activated to accelerate glucose ingestion in cells. During this process, glucose is transported into cells through mediating GLUT4. Fruman et al[18] have found that mice with the PI3K gene knocked out sill have fertility, with hypoglycemia and increased insulin sensitivity. Pederson et al[19] have demonstrated that down-regulating the expression of IRS-1 can suppress PI3K activity and glucose intake and weaken insulin sensitivity. Other studies[20-22] have revealed that the PI3K/Akt signaling pathway is involved in the transduction of insulin signals. Furthermore, high-concentration glucose can repress the expressions of Akt and PI3K in human umbilical vein endothelial cells, which are similar to the results of this research.

Conclusions

Summarily, miR-26 promoted gestational diabetes through inhibiting the PI3K/Akt signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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