European Review for Medical and Pharmacological Sciences 2019; 23: 2325-2331

Effect of miR-29b on rats with gestational diabetes mellitus by targeting PI3K/Akt signal

H.-Y. ZONG[1], E.-L. WANG[2], Y.-M. HAN[3], Q.-J. WANG[4], J.-L. WANG[5], Z. WANG[6]

[1]Department of Obstetrics and Gynecology, Juancheng People's Hospital, Heze, China.
[2]Department of Acupuncture, the People's Hospital of Zhangqiu Area, Ji'nan, China.
[3]Operating Room, Rizhao Hospital of TCM, Rizhao, China.
[4]Department of Nephrology, the People's Hospital of Zhangqiu Area, Ji'nan, China.
[5]Department of Radiology, the People's Hospital of Zhangqiu Area, Ji'nan, China.
[6]Department of Obstetrics and Gynecology, The 5th People's Hospital of Ji'nan, Ji'nan, China.

Abstract. – OBJECTIVE: To investigate the role of micro-ribonucleic acid-29b (miR-29b) in rats with gestational diabetes mellitus (GDM) through the phosphatidylinositol 3-kinase (PI3K)/serine/threonine kinase (Akt) signal and its mechanism by establishing rat models of GDM.

MATERIALS AND METHODS: Rat models of GDM were constructed, and then the expression levels of miR-29b, total PI3K, phosphorylated PI3K (p-PI3K), total Akt and phosphorylated Akt (p-Akt) in the model group and control group were measured via Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting assays, and the association between miR-29b expression and total PI3K expression was analyzed. In addition, miR-29b mimics and inhibitors were used to further explore the regulatory pathway, and the influences of miR-29b mimics and inhibitors on PI3K and Akt phosphorylation in GDM rats, characteristic indicators of oxidative stress such as superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) in liver tissues of GDM rats, and fasting blood glucose in GDM rats were studied.

RESULTS: Compared with those in the control group, miR-29b expression was lowered in rat models of GDM, while PI3K/Akt signal expression was increased. In rats with GDM, miR-29b expression was prominently negatively correlated with total PI3K expression (r=-0.777, p=0.007, p<0.01). MiR-29b mimics could reduce PI3K and Akt phosphorylation, increase SOD and CAT expression levels and decrease MDA content (p<0.05). Moreover, miR-29b mimics significantly lowered the blood glucose level in rats with GDM (p<0.05).

CONCLUSIONS: MiR-29b mimics can alleviate oxidative stress and reduce blood glucose by inhibiting the PI3K/Akt signal transduction.

Key Words:
MiR-29b, PI3K/Akt signaling pathway, Gestational diabetes mellitus, Influence.

Introduction

Gestational diabetes mellitus (GDM), a common pregnancy complication, refers to DM or glucose intolerance during the gestation period. GDM, of which the incidence rate is 1-14%, is an increasing problem[1]. As the burden of obesity on women of childbearing age increases, the incidence rate of GDM is elevating[2]. GDM is related to a large number of short-term and long-term poor health outcomes in mothers and offspring. Women with GDM have an increased risk of perinatal morbidity and elevated risks of impaired glucose tolerance and Type 2 DM in the years following pregnancy[3,4]. Female children with GDM are more likely to be obese and have impaired glucose tolerance and DM in childhood and early adulthood[5,6]. These data highlight the risk factors identifying this common pregnancy complication and preventing GDM of high-risk populations, especially the importance of modifiers[7].

Micro-ribonucleic acid-29b (miR-29b) is an important member of the miRNA family and has been proved to be disordered in many tumors[8,9]. In particular, it has been testified that miR-29b acts as a tumor suppressor inhibiting the proliferation of cancer cells[10]. MiR-29b shares the gene regulation function with other members of the miR-29 family. In AML cell lines, the overexpression of miR-29a, miR-29b or miR-2c overtly suppresses cell proliferation and promotes apoptosis by targeting the mRNAs of protein kinase B2 and cyclin D2 (two key signaling molecules). However, the molecular mechanism of miR-29b dysregulation in the diagnosis and prognosis of GDM remains to be further clarified.

This study aims to investigate whether miR-29b affects rats with GDM through the phospha-

Corresponding Author: Zhe Wang, MM; email: zjoo81@163.com

tidylinositol 3-kinase (PI3K)/serine/threonine kinase (Akt) signal and reveal the expression of miR-29b in rat models of GDM and its roles in the PI3K/Akt signal transduction and GDM.

Materials and Methods

Materials

Main Experimental Reagents

Streptozotocin (purchased from Sigma-Aldrich, St. Louis, MO, USA), miR-29b mimics and inhibitors (GenePharma, Shanghai, China), total PI3K, phosphorylated PI3K (p-PI3K), phosphorylated Akt (p-Akt) and total Akt antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), SYBR Green Polymerase Chain Reaction (PCR) Master Mix (Solarbio, Beijing, China), and kits for determining superoxide dismutase (SOD) and catalase (CAT) activity and malondialdehyde (MDA) content (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Experimental Animals

Animals used in the assays were female Wistar Albino rats (160-180 g, n=100) purchased from the Hubei Provincial Laboratory Animal Research Center-Hubei Provincial Center for Disease Control and Prevention. This study was approved by the Animal Ethics Committee of The 5th People's Hospital of Jinan Animal Center.

Methods

Establishment of Rat Models of GDM

Albino Wistar rats were raised in an IVC environment and given sufficient fodder and water. According to the references[12,13], rats in the model group were injected with streptozotocin diluted with citrate buffer before mating, to induce DM in pregnant rats, while those in the control group were only injected with citrate buffer. Next, fasting blood glucose test was employed to confirm the presence of GDM (fasting blood glucose level >130 mg/dL).

Test Protocol

To explore whether miR-29b influences GDM rats *via* the PI3K/Akt signal, established GDM rats were injected with different reagents: miR-29b mimic (100 nM), mimic control, miR-29b inhibitor (100 nM) and inhibitory control.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assay

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). After quantification, 10 ng total RNA was synthesized into complementary deoxyribose nucleic acid (cDNA) that was used for Real Time-Polymerase Chain Reaction (RT-PCR). Primers: miR-29b: forward: ACACTCCAGCTGGGGCTGGTTTCATATGGT, and reverse: TGGTGTCGTGGAGTCG, and PI3K: forward: ATTCCAGACGCATTTCCAC, and reverse: ATTCAGCCATTCATTCCACC. All assays were performed in triplicate. The relative difference between the control group and model group was calculated using the $\Delta\Delta$CT method.

Western Blotting Analysis

After tissues were lysed with radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China), total protein was extracted and centrifuged, and the supernatant was collected for Western blotting analysis. Briefly, the protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), blocked with Tris-Buffered Saline and Tween 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA) containing 5% skim milk powder, and incubated with diluted primary antibodies at 4°C overnight. Then, the membrane was washed and incubated with horseradish peroxidase-labeled mouse secondary antibody at room temperature for 1 h, followed by membrane washing. Next, chemiluminescence detection reagents were applied to observe immunoreactive proteins on an autoradiograph. In all cases, β-actin was used as a loading control.

Detection of SOD and CAT Activity and MDA Content Through Enzyme-Linked Immunosorbent Assay (ELISA)

After 72 h of drug treatment, liver tissues were collected, weighed and homogenized with tissue lysis buffer for 10 min, followed by centrifugation. Then, the supernatant was collected, and the SOD and CAT activity and MDA level were detected using a kit according to the instructions.

Determination of Blood Glucose Content

The glucose oxidase assay was performed to measure the blood glucose content using an automatic biochemical analyzer in strict accordance with the relevant standards for the biochemical analyzer.

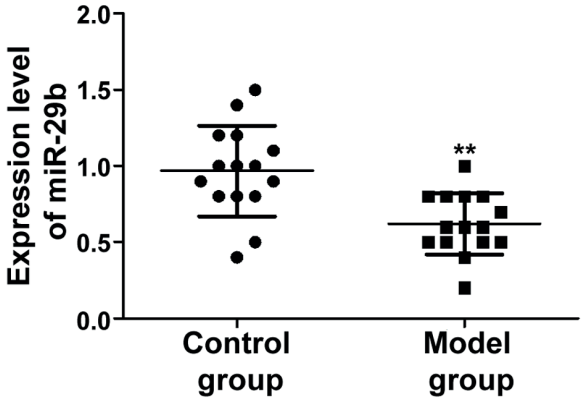

Figure 1. Expression level of miR-29b in rats detected through RT-PCR, **indicates that the difference is extremely evident (p<0.01).

Statistical Analysis

Student's t-test was used for statistical evaluation to compare the differences between groups. Data were expressed as mean ± SD (Standard Deviation). p<0.05 suggested that the difference was statistically significant.

Results

Expression of MiR-29b in GDM Rats

First, miR-29b in GDM rat model group and control group was determined, and the results revealed that the expression of miR-29b was down-regulated in the model group compared with that in the control group (p<0.01) (Figure 1). MiR-29b exhibited low expression in rat models of GDM.

Expression of PI3K/Akt Signal in GDM Rats and Its Correlation With MiR-29b Expression

In addition, the expression of the PI3K/Akt signal in rat models of GDM was analyzed. The results of Western blotting (Figure 2A) showed that the expressions of total PI3K and p-PI3K in the model group were significantly higher than those in the control group (p<0.01), the expression of total Akt showed no significant difference between the model group and control group, while the expression of p-Akt in the model group was markedly higher than that in the control group. Moreover, the relation between total PI3K expression and miR-29b expression in the model group was analyzed, and it was found that the miR-29b expression and total PI3K expression in GDM rats were negatively correlated (r=-0.777, p=0.007, p<0.01) (Figure 2C).

Influence of MiR-29b on PI3K/Akt Signaling Pathway in GDM Rats

MiR-29b mimics and miR-29b inhibitors were used to regulate the expression of miR-29b in GDM rats and validate the effects of miR-29b

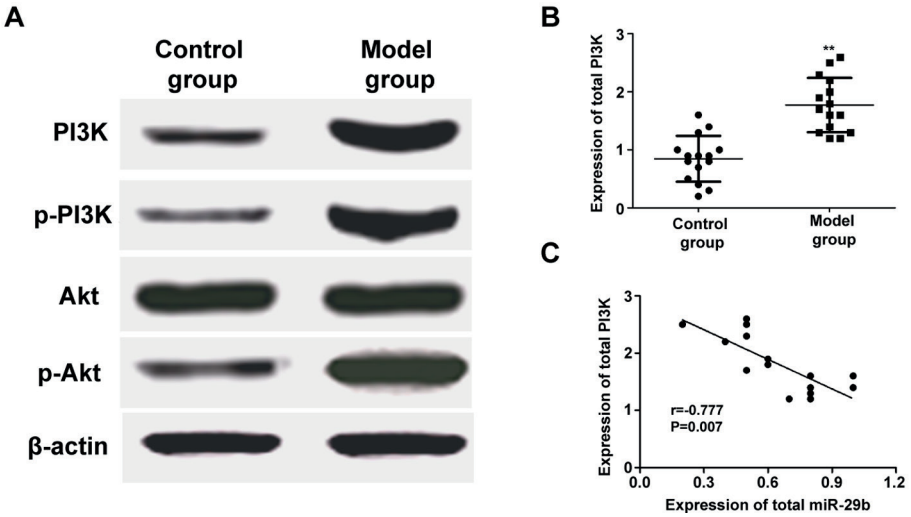

Figure 2. Expression of the PI3K/Akt signal in GDM rats. ***A,*** Expression levels of total PI3K, p-PI3K, total Akt and p-Akt in GDM rat model group and control group determined *via* Western blotting. ***B,*** Expression of total PI3K in 15 rats from model group and 15 rats from control group detected through RT-PCR. **Indicates that the difference is extremely significant (p<0.01). ***C,*** Correlation between total PI3K expression and miR-29b expression in 15 rats from GDM rat model group.

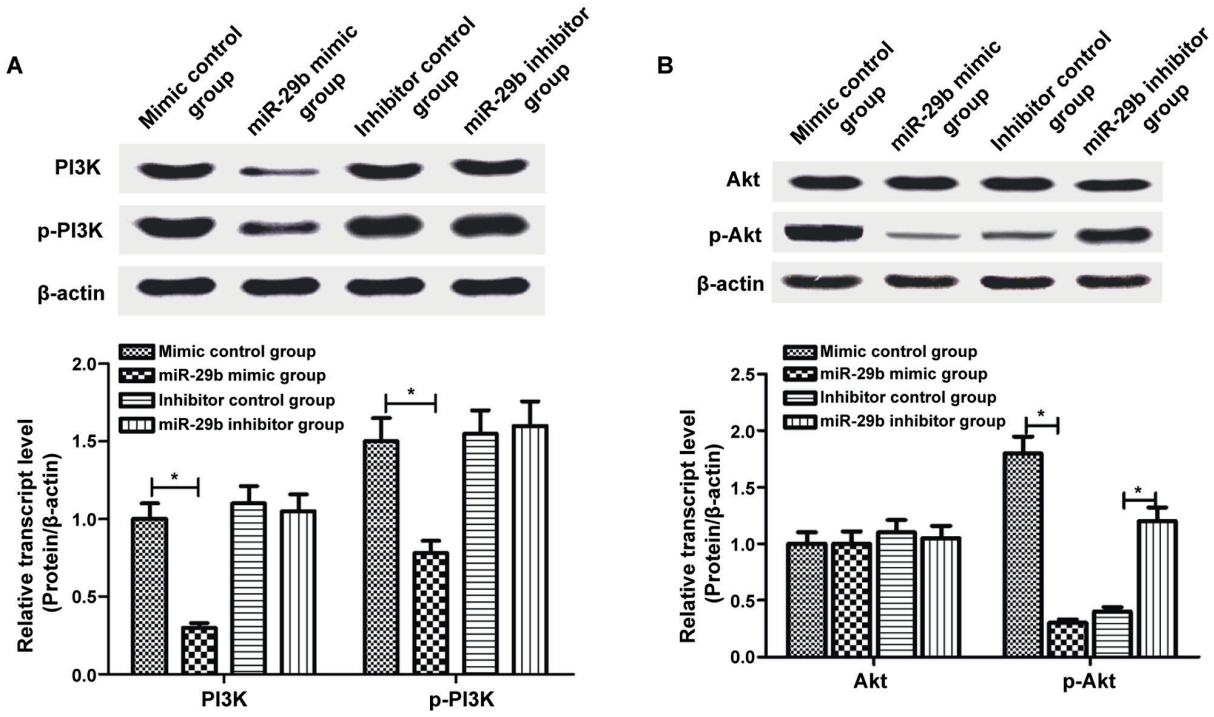

Figure 3. Influence of miR-29b on the PI3K/Akt signaling pathway in GDM rats. ***A,*** Expression levels of total PI3K and p-PI3K under different conditions detected by Western blotting. ***B,*** Expression levels of total Akt and p-Akt under different conditions detected *via* Western blotting. *Indicates that the difference is significant ($p<0.05$). Bands are subjected to densitometric analysis, expressed as the ratio of the density of target band to that of β-actin band.

expression changes on the PI3K/Akt pathway in GDM rats. The results (Figure 3) manifested that the expression levels of total PI3K and p-PI3K were markedly decreased in the miR-29b mimic group compared with those in the mimic control group, while they showed no significant difference between the miR-29b inhibitor group and inhibitor control group (Figure 3A). Besides, the miR-29b mimic group had a remarkably reduced p-Akt expression level in comparison with mimic control group ($p<0.05$), whereas the expression of p-Akt was markedly elevated in the miR-29b inhibitor group compared with that in the inhibitor control group ($p<0.05$). There was no significant difference in the expression of Atk among groups. The results suggest that miR-29b mimics have a specific effect of reducing Akt phosphorylation without any change in the total Akt level.

Effects of MiR-29b on SOD and CAT Activity and MDA Content in Liver Tissues of GDM Rats

Studies have revealed that MDA damage is correlated with lipid peroxidation damage in cell membranes and closely related to oxidative stress and liver function. Therefore, in this part, GDM rats were given different treatments, and the expression levels of SOD, CAT and MDA in their liver tissues were analyzed. The results displayed that, compared with the mimic control group, miR-29b mimic group had significantly elevated expression levels of SOD and CAT ($p<0.05$) and lowered expression level of MDA ($p<0.05$), while miR-29b inhibitors clearly decreased the expression levels of SOD and CAT ($p<0.05$) and increased the expression level of MDA ($p<0.05$) in comparison with the inhibitor control group (Figure 4).

Impact of MiR-29b on Fasting Blood Glucose of GDM Rats

Fasting blood glucose level is an important indicator for patients with GDM. Hence, in this part, different treatment methods were used for rat models of GDM, and the blood glucose level was measured using the biochemical analyzer. It was discovered that the miR-29b mimic group had an overtly reduced blood glucose level of GDM rats compared with that in the mimic control group, while the blood glucose level of GDM

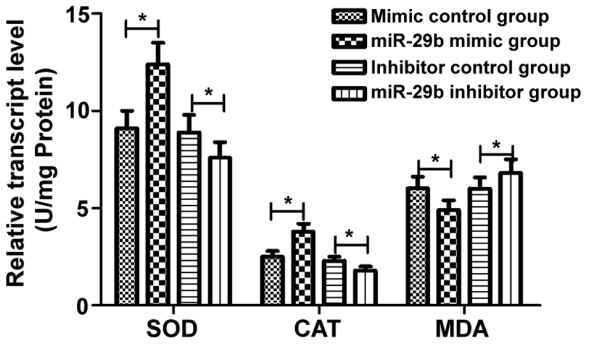

Figure 4. Influence of miR-29b on SOD/CAT activity and MDA content in liver tissues of GDM rats detected through ELISA. *Indicates that the difference is significant (p<0.05).

rats in the miR-29b inhibitor group was markedly higher than that in the inhibitor control group (Figure 5).

Discussion

GDM can lead to complications in mothers and children, probably resulting in fetal macrosomia and cesarean delivery[14]. In addition, obesity seems to be another long-term complication in offspring of mothers with GDM[15]. Capobianco et al[16] showed that the offspring of DM rats have abnormal changes in lipid characteristics and metabolic disorders that are able to affect the growth and metabolism of their offspring. Moreover, the offspring of women with GDM have increased risks of hypertension and other cardiovascular diseases[17]. Evidence suggests that the increased risk of Type 2 DM in the offspring is related to impaired glucose tolerance and prenatal exposure to the diabetic intrauterine environment[18]. Oxidative stress is one of the earliest abnormalities detected in DM patients[19]. Furthermore, the risk of oxidative stress is increased in fetuses of GDM mothers, subsequently triggering the production of highly reactive oxygen free radicals that are toxic to cells, especially to plasmalemmas[19]. In this work, the effect of miR-29b on rat models of GDM through the PI3K/Akt signal transduction was investigated, and it was observed in animal models that miR-29b was lowly expressed in rat models of GDM, while the PI3K/Akt signal transduction was highly expressed in rat models of GDM, and the miR-29b expression had a significantly negative relationship with the total PI3K expression in GDM rats (r=-0.777, p=0.007, p<0.01). Besides, miR-29b mimics markedly reduced the blood glucose level in GDM rats (p<0.05).

Song et al[20] showed that women with GDM had decreased adiponectin level and increased pro-inflammatory cytokine levels compared with those in women without DM. High levels of pro-inflammatory cytokines are also thought to be major mediators of demyelination of the central nervous system, which result in various inflammatory and neoplastic diseases and many other pathological complications. In addition, Lapolla et al[21] manifested that lymphocyte subset damage is detected in mothers with GDM and their newborn, which is more important for patients who are positive for autoantibodies or treated with insulin. Oxidative stress is one of the important indicators in GDM. The main roles of antioxidants are to scavenge free radicals, control the synthesis or release of nitric oxide, inhibit the production of reactive oxygen species and upregulate the activity of antioxidant enzymes in metabolism[22]. In this work, miR-29b mimics were able to reduce PI3K and Akt phosphorylation and MDA content, increase SOD and CAT expression levels and relieve oxidative stress. There are no studies on the roles of miR-29b and the PI3K/Akt pathway in GDM. In recent years, more research focuses on the role of the PI3K/Akt signaling pathway in human cancers. The regulatory effect of the miR-29 family on the proliferation and apoptosis of cells is dependent on the AKT pathway and p-Rb level[23].

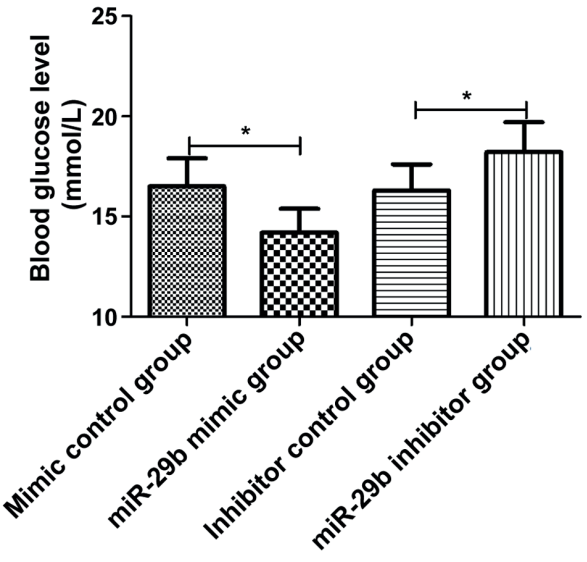

Figure 5. Impact of miR-29b on fasting blood glucose of GDM rats. *Indicates that the difference is significant (p<0.05).

In MM, the PI3K/AKT pathway is a negative regulator of miR-29b. In contrast, miR-29b acts as a negative regulator of the PI3K/AKT pathway by reducing AKT phosphorylation[24]. Chen et al[25] studied the effect of miR-29b on the angiogenesis in endometrial carcinoma by targeting VEGFA to regulate MAPK/ERK and PI3K/Akt signaling pathways and demonstrated that miR-29b negatively regulates MAPK/ERK and PI3K/Akt signaling pathways by targeting VEGFA to inhibit angiogenesis in endometrial carcinoma[25]. The negative regulation of miR-29b on the PI3K/Akt signaling pathway is consistent with that in this study.

Conclusions

We found that miR-29b exerts a regulatory effect in rat models of GDM and affects GDM *via* the PI3K/Akt signaling pathway. MiR-29b reduces blood glucose content and alleviates oxidative stress in rats with GDM by negatively regulating the PI3K/Akt signaling pathway.

Conflict of interest

The authors declare no conflicts of interest.

References

1) Sobande AA, Eskandar M, Archibong EI. Complications of pregnancy and foetal outcomes in pregnant diabetic patients managed in a tertiary hospital in Saudi Arabia. West Afr J Med 2005; 24: 13-17.
2) Fister K, Ivankovic D, Korsic M, Pavlekovic G, Milanovic SM, Vuletic S, Kern J. Prevalence and five-year cumulative incidence of abdominal obesity in Croatian women of childbearing age: the CroHort study. Coll Antropol 2012; 36: 77-81.
3) Jang HC, Cho NH, Min YK, Han IK, Jung KB, Metzger BE. Increased macrosomia and perinatal morbidity independent of maternal obesity and advanced age in Korean women with GDM. Diabetes Care 1997; 20: 1582-1588.
4) Shoham I, Wiznitzer A, Silberstein T, Fraser D, Holcberg G, Katz M, Mazor M. Gestational diabetes complicated by hydramnios was not associated with increased risk of perinatal morbidity and mortality. Eur J Obstet Gynecol Reprod Biol 2001; 100: 46-49.
5) Yang SJ, Kim TN, Baik SH, Kim TS, Lee KW, Nam M, Park YS, Woo JT, Kim YS, Kim SH. Insulin secretion and insulin resistance in Korean women with gestational diabetes mellitus and impaired glucose tolerance. Korean J Intern Med 2013; 28: 306-313.
6) Friedman JE, Ishizuka T, Shao J, Huston L, Highman T, Catalano P. Impaired glucose transport and insulin receptor tyrosine phosphorylation in skeletal muscle from obese women with gestational diabetes. Diabetes 1999; 48: 1807-1814.
7) Kale SD, Kulkarni SR, Lubree HG, Meenakumari K, Deshpande VU, Rege SS, Deshpande J, Coyaji KJ, Yajnik CS. Characteristics of gestational diabetic mothers and their babies in an Indian diabetes clinic. J Assoc Physicians India 2005; 53: 857-863.
8) Arner P, Kulyte A. MicroRNA regulatory networks in human adipose tissue and obesity. Nat Rev Endocrinol 2015; 11: 276-288.
9) Chen YL, Zhang ZX, Shou LH, Di JY. Regulation of DNA methylation and tumor suppression gene expression by miR-29b in leukemia patients and related mechanisms. Eur Rev Med Pharmacol Sci 2018; 22: 158-165.
10) Nijhuis A, Biancheri P, Lewis A, Bishop CL, Giuffrida P, Chan C, Feakins R, Poulsom R, Di Sabatino A, Corazza GR, MacDonald TT, Lindsay JO, Silver AR. In Crohn's disease fibrosis-reduced expression of the miR-29 family enhances collagen expression in intestinal fibroblasts. Clin Sci (Lond) 2014; 127: 341-350.
11) Mott JL, Kurita S, Cazanave SC, Bronk SF, Werneburg NW, Fernandez-Zapico ME. Transcriptional suppression of mir-29b-1/mir-29a promoter by c-Myc, hedgehog, and NF-kappaB. J Cell Biochem 2010; 110: 1155-1164.
12) Jawerbaum A, White V. Animal models in diabetes and pregnancy. Endocr Rev 2010; 31: 680-701.
13) Kurtz M, Capobianco E, Martinez N, Roberti SL, Arany E, Jawerbaum A. PPAR ligands improve impaired metabolic pathways in fetal hearts of diabetic rats. J Mol Endocrinol 2014; 53: 237-246.
14) Gao F, Wang G, Wang L, Guo N. Phytosterol nutritional supplement improves pregnancy and neonatal complications of gestational diabetes mellitus in a double-blind and placebo-controlled clinical study. Food Funct 2017; 8: 424-428.
15) Leng J, Li W, Zhang S, Liu H, Wang L, Liu G, Li N, Redman LM, Baccarelli AA, Hou L, Hu G. GDM Women's pre-pregnancy overweight/obesity and gestational weight gain on offspring overweight status. PLoS One 2015; 10: e129536.
16) Capobianco E, Pelesson M, Careaga V, Fornes D, Canosa I, Higa R, Maier M, Jawerbaum A. Intrauterine programming of lipid metabolic alterations in the heart of the offspring of diabetic rats is prevented by maternal diets enriched in olive oil. Mol Nutr Food Res 2015; 59: 1997-2007.
17) Kajantie E, Osmond C, Eriksson JG. Gestational hypertension is associated with increased risk of type 2 diabetes in adult offspring: the Helsinki Birth Cohort Study. Am J Obstet Gynecol 2017; 216: 281.
18) El HN, Schneider E, Lehnen H, Haaf T. Epigenetics and life-long consequences of an adverse nutritional and diabetic intrauterine environment. Reproduction 2014; 148: R111-R120.
19) Wei W, Liu Q, Tan Y, Liu L, Li X, Cai L. Oxidative stress, diabetes, and diabetic complications. Hemoglobin 2009; 33: 370-377.

20) Song X, Li Y, Liu J, Ji X, Zhao L, Wei Y. Changes in serum adiponectin in mice chronically exposed to inorganic arsenic in drinking water. Biol Trace Elem Res 2017; 179: 140-147.

21) Lapolla A, Dalfra MG, Sanzari M, Fedele D, Betterle C, Masin M, Zanchetta R, Faggian D, Masotti M, Nucera V, Plebani M. Lymphocyte subsets and cytokines in women with gestational diabetes mellitus and their newborn. Cytokine 2005; 31: 280-287.

22) Rakesh SU, Patil PR, Mane SR. Use of natural antioxidants to scavenge free radicals: a major cause of diseases. International J PharmTech Res 2010; 2: 1074-1081.

23) Trisciuoglio D, Iervolino A, Zupi G, Del Bufalo D. Involvement of PI3K and MAPK signaling in bcl-2-induced vascular endothelial growth factor expression in melanoma cells. Mol Biol Cell 2005; 16: 4153-4162.

24) Amodio N, Di Martino MT, Foresta U, Leone E, Lionetti M, Leotta M, Gulla AM, Pitari MR, Conforti F, Rossi M, Agosti V, Fulciniti M, Misso G, Morabito F, Ferrarini M, Neri A, Caraglia M, Munshi NC, Anderson KC, Tagliaferri P, Tassone P. miR-29b sensitizes multiple myeloma cells to bortezomib-induced apoptosis through the activation of a feedback loop with the transcription factor Sp1. Cell Death Dis 2012; 3: e436.

25) Chen HX, Xu XX, Tan BZ, Zhang Z, Zhou XD. MicroRNA-29b inhibits angiogenesis by targeting VEGFA through the MAPK/ERK and PI3K/Akt signaling pathways in endometrial carcinoma. Cell Physiol Biochem 2017; 41: 933-946.

.