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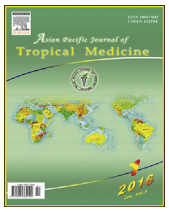

Original research http://dx.doi.org/10.1016/j.apjtm.2015.12.018

Effect of insulin and metformin on methylation and glycolipid metabolism of peroxisome proliferator-activated receptor γ coactivator-1A of rat offspring with gestational diabetes mellitus

Ai-Qin Song*, Li-Rong Sun, Yan-Xia Zhao, Yan-Hua Gao, Lei Chen

Department of Pediatric Hematology, Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong, China

ARTICLE INFO

Article history:
Received 15 Oct 2015
Received in revised form 20 Nov 2015
Accepted 3 Dec 2015
Available online 18 Dec 2015

Keywords:
Insulin
Metformin
Gestational diabetes mellitus
Peroxisome proliferator-activated receptor γ coactivator-1A
Methylation
Glycolipid metabolism

ABSTRACT

Objective: To discuss the effect of insulin and metformin on a methylation and glycolipid metabolism of peroxisome proliferator-activated receptor γ coactivator-1A (PPARGC1A) of rat offspring with gestational diabetes mellitus (GDM).

Methods: A total of 45 pregnant rats received the intraperitoneal injection of streptozotocin to establish the pregnant rat model of GDM. A total of 21 pregnant rats with GDM were randomly divided into three groups, with 7 rats in each group, namely the insulin group, metformin group and control group. Rats in the insulin group received the abdominal subcutaneous injection of 1 mL/kg recombinant insulin glargine at 18:00 every day. Rats in the metformin group received the intragastric infusion of metformin hydrochloride at 18:00 every day, with the first dose of 300 mg/kg. The doses of two groups were adjusted every 3 d to maintain the blood glucose level at 2.65–7.62 mmol/L. Rats in the control group received the intragastric infusion of 1 mL normal saline at 18:00 every day. After the natural delivery of pregnant rats, 10 offspring rats were randomly selected from each group. At birth, 4 wk and 8 wk after the birth of offspring rats, the weight of offspring rats was measured. The blood glucose level of offspring rats was measured at 4 wk and 8 wk, while the level of serum insulin, triglyceride and leptin was measured at 8 wk.

Results: The weight of offspring rats at birth in the insulin group and metformin group was significantly lower than the one in the control group ($P < 0.05$), and there was no significant difference at 4 wk and 8 wk among three groups ($P > 0.05$). The fasting blood glucose and random blood glucose in the insulin group and metformin group at 4 wk and 8 wk were all significantly lower than ones in the control group ($P < 0.05$); there was no significant difference between the insulin group and metformin group ($P > 0.05$). The expression of *PPARGC1A* mRNA in the insulin group and metformin group was significantly higher and the methylation level of PPARGC1A was significantly lower than the one in the control group ($P < 0.05$); but there was no significant difference between the insulin group and metformin group ($P > 0.05$). Insulin and leptin at 8 wk in the insulin group and metformin group were significantly higher, while triglyceride was significantly lower than the one in the control group ($P < 0.05$); triglyceride level in the insulin group was significantly higher than the one in the metformin group ($P < 0.05$). There was no significant difference in insulin and leptin level between the insulin group and metformin group ($P > 0.05$).

Conclusions: GDM can induce the methylation of PPARGC1A of offspring rats to reduce the expression of *PPARGC1A* mRNA and then cause the disorder of glycolipid metabolism when the offspring rats grow up; the insulin or metformin in the treatment of pregnant rats with GDM can reduce the methylation level of PPARGC1A and thus improve the abnormal glycolipid metabolism of offspring rats.

*Corresponding author: Ai-Qin Song, M.M., Deputy Attending Physician, Department of Pediatric Hematology, Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong, China.
Tel: +86 18661807987
E-mail: 18661807987@163.com

Peer review under responsibility of Hainan Medical College.
Foundation project: It was supported by Shandong Natural Science Fund (Y2008c170).

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1. Introduction

The gestational diabetes mellitus (GDM) refers to the hyperglycemia of pregnant woman who has no diabetes mellitus before the pregnancy. A survey indicated that [1] over 80% of pregnant women with diabetes mellitus was GDM, while the diabetes mellitus combined with pregnancy occupied less than 20%. As GDM usually occurs in the third trimester, the long-term hyperglycemia could increase the risk of metabolic diseases for the fetus [2,3]. A foreign scholar [4] reported that the peroxisome proliferator-activated receptor γ coactivator-1A (PPARGC1A) was the main transcription factor involved in the regulation of mitochondria metabolism, while the methylation degree of PPARGC1A was related to the occurrence and development of metabolic diseases of the offspring. According to a previous research [5], the effect of PPARGC1A on the secretion of human insulin was controlled by the genetics and external environment. Thus the external stimulation to change the methylation of PPARGC1A is of critical importance in controlling the occurrence of metabolic diseases. In this study, GDM rats received the interventional treatment of insulin and metformin respectively to discuss the methylation of PPARGC1A of the offspring rats and the effect of earlier intervention of hypoglycemic drugs on the metabolic diseases of offspring rats from the aspect of action mechanism. The findings are shown as follows.

2. Materials and methods

2.1. Laboratory animals

A total of 45 Sprague–Dawley rats were purchased from the laboratory animal of hospital, including 30 females and 15 males. All of them were unmated rats aged 8 wk. The weight of male rats was 280–310 g, with the average of (303.5 ± 10.4) g; while the weight of female rats was 200–240 g, with the average of (228.7 ± 9.7) g. All rats were fed with diet and water freely. The room temperature was maintained at 20–22 °C; the feeding was taken under 12 h of lighting and 12 h of darkness. The experiment was performed 1 wk later.

2.2. Methods

2.2.1. Rat model of GDM

The female and male rats were mated in the cage by the ratio of 2: 1. They were observed in the next morning. The observed vaginal suppository of female rats indicated Day 1 of pregnancy. A total of 27 rats in this group had successful pregnancy. Female rats with the success of pregnancy began the fasting for 12 h at Day 7 of pregnancy. The pregnant rats were given the intraperitoneal injection of streptozotocin (which was purchased from Shanghai Shifeng Biological Technology Co., Ltd., batch No. 130921), with the dose of 30 mg/kg. The blood was collected from the tails 48 h later using Accu-Chek Blood Glucose Monitoring Meter (Roche Diagnostics Shanghai Ltd.) to detect the blood glucose level of rats. The blood glucose ≥14 mmol/L was regarded as the successful rat modeling of GDM. A total of 21 rats in this group had successful modeling. Pregnant rats with GDM were randomly divided into three groups, with 7 rats in each group, namely the insulin group, metformin group and control group.

2.2.2. Drug administration

Insulin group: rats were given the abdominal subcutaneous injection of 0.1 mL/kg recombinant insulin glargine (which was purchased from Gan & Lee Pharmaceuticals, batch No. 121103, 3 mL: 300 units/piece) at 18:00 every day. The fasting blood glucose was detected in tails at 8:00 the next day. Referring to the related researches [6], the insulin dose was adjusted according to the blood glucose level every 3 d and the blood glucose level was maintained at 2.65–7.62 mmol/L. Metformin group: rats were given the intragastric infusion of metformin hydrochloride (which was purchased from Shanghai Sine Tianping Pharmaceutical, batch No. 130125, 0.25 g/tablet) at 18:00 every day, with the first dose of 300 mg/kg. The fasting blood glucose was detected in tails at 8:00 the next day. The metformin dose was adjusted according to the blood glucose level every 3 d and the blood glucose level was maintained at 2.65–7.62 mmol/L. Control group: rats were given the intragastric infusion of 1 mL normal saline at 18:00 every day and the blood glucose level was detected. In this study, only 1 rat in the insulin group and 2 rats in the metformin group had the blood glucose level before the delivery that beyond the standard range; while 2 rats in the control group died because of the high blood glucose. Pregnant rats with unqualified blood glucose level or died ones were excluded from this study. The remained 16 rats had the normal delivery. The offspring rats were fed by female rats regularly. The normal breeding began 3 wk later, while the experiment was taken 8 wk later. 1 offspring rat (insulin group) with the high birth weight (>10 g) and 2 offspring rats (1 in the metformin group and 1 in the control group) with the low birth weight (<5 g) were excluded from this study. Ten offspring rats were randomly chosen from each group for the experiment.

2.2.3. Offspring rat

The blood glucose and weight of offspring rats were measured at birth, 4 wk and 8 wk after the birth. The electronic balance (model: FA2004N, Nanjing Kehang Experimental Instruments Co., Ltd.) was employed to detect the body mass of offspring rats. The tail-docking blood sampling method was employed to detect the blood glucose at the 3rd week, while the heart puncture was adopted to detect the blood glucose at the 8th week.

2.2.4. Detection of serum biochemical indicator

At the 8th week, the offspring rats were anesthetized with the chloral hydrate. With the conventional thoracotomy, the venous blood was sampled by 2 mL and then centrifuged at 12 000 r/min for 10 min. The collected serum was stored in the refrigerator at −80 °C for the detection. Meanwhile, the pancreatic tissues were collected from offspring rats and then stored in the refrigerator at −80 °C for the detection. After sampling the serum from offspring rats, the enzyme-linked immunosorbent assay was employed to detect the level of serum insulin (INS), triglyceride (TG) and leptin (LP) in offspring rats. The related testing kit was provided by Shanghai Pumai Biotechnology Co., Ltd. The detection should be performed in accordance with the instruction manual of kit.

2.2.5. Detection of PPARGC1A mRNA

Trizol (Nanjing SenBeiJia Biological Technology Co., Ltd.) was employed to extract the total RNA from the pancreatic tissues and the reverse transcription kit was used to transcript it

Table 1
Primer sequence and product size.

Gene	Primer sequence (5′–3′)	Product size (bp)
PPARGC1A	Upstream: CTCGTCACATCGAGGCGAATTC Downstream: GTGCGCAAATGATTATGAGTTATGATCTG	185
Methylation of *PPARGC1A*	Upstream: GTCGTCACTAGCAGGCGATTAG Downstream: GCCGCAGGCCAATTCATTTAG	189
GAPDH	Upstream: GAGTCTAGCCCGCTGCTGAATC Downstream: GCAGGATGAATGCTGCATGATA	160

into cDNA. The real-time fluorescent quantitative polymerase chain reaction (PCR) was adopted to detect the *PPARGC1A* mRNA. The primers were synthesized by Shanghai Generay Biotech Co., Ltd. The PCR reaction system was: 10 μL Power SYBR Green PCR Master Mix, 1 μL primers, 3 μL cDNA and 15 μL ddH_2O; taking *GAPDH* as the reference, the primer sequence was shown in Table 1. PCR reaction conditions included: predegeneration at 95 °C for 5 min, degeneration at 97 °C for 30 s and annealing at 60 °C for 30 s, with 35 cycles in total. After PCR reaction, the melting curve was adopted for the analysis. Each sample had 3 repeated holes to calculate the average. The relative expression of target gene = $-2^{-\triangle Ct}$.

2.2.6. Methylation of PPARGC1A DNA

DNA extraction kit (Nanjing SenBeiJia Biological Technology Co., Ltd.) was used to extract the total DNA from pancreatic tissues of pregnant rats. The restriction enzyme *Hin*II (Thermo) that was sensitive to the methylation was employed to digest the sample DNA. DNA templates before and after the restriction endonuclease digestion were amplified with *PPARGC1A* and *GAPDH* respectively. The PCR reaction system was: 10 μL Power SYBR Green PCR Master Mix, 1 μL primers, 2.5 μL DNA templates and 15 μL ddH_2O. PCR reaction conditions included: predegeneration at 95 °C for 5 min, degeneration at 97 °C for 30 s and annealing at 60 °C for 30 s, with 35 cycles in total. The relative expression of target gene was calculated using $\triangle$Ct method, where $\triangle Ct = \triangle Ct_{PPARGC1A} - \triangle Ct_{GAPDH}$ and methylation index = $-2^{-\triangle\triangle Ct}$.

2.3. Statistical analysis

SPSS 19.0 was employed for the statistical analysis. The measurement data was expressed by mean ± standard deviation. The analysis of variance was chosen for the multiple-group comparison, while *LSD* test was used for the comparison between means. $P < 0.05$ indicated the significant difference.

3. Results

3.1. General information of offspring rats

There were 31 offspring rats in the insulin group, 29 in the metformin group and 37 in the control group. Except 7 offspring rats died in the control group, all other offspring rats survived normally. The offspring rats in each group had no obvious deformity in the appearance, with the good situation of growth and development.

3.2. Comparison of body mass and blood glucose level

The body mass for offspring rats at birth in the insulin group and metformin group was significantly lower than the one in the control group ($P < 0.05$). There was no significant difference in the body mass 4 wk and 8 wk after the birth of offspring rats between three groups ($P > 0.05$). The fasting blood glucose and random blood glucose for offspring rats in the insulin group and metformin group at 4th week and 8th week after the birth were significantly lower than ones in the control group ($P < 0.05$). There was no significant difference in the blood glucose level between the insulin group and metformin group ($P > 0.05$), as shown in Table 2.

3.3. Comparison of expression of PPARGC1A mRNA and methylation level

The expression of *PPARGC1A* mRNA for offspring rats in the insulin group and metformin group was significantly higher than the one in the control group, while the methylation level was significantly lower than the one in the control group ($P < 0.05$). There was no significant difference in the expression of *PPARGC1A* mRNA and methylation level between the insulin group and metformin group ($P > 0.05$), as shown in Table 3.

Table 2
Comparison of body mass and blood glucose level between three groups.

Group	Cases	Body mass (g)			Fasting blood glucose (mmol/L)		Random blood glucose (mmol/L)	
		At birth	4th week	8th week	4th week	8th week	4th week	8th week
Insulin group	10	5.5 ± 0.5	49.7 ± 7.6	257.4 ± 21.3	5.37 ± 1.03	6.01 ± 1.32	7.09 ± 0.85	7.58 ± 0.59
Metformin group	10	5.6 ± 0.7	48.9 ± 5.7	248.5 ± 17.4	5.24 ± 0.79	5.98 ± 1.17	6.94 ± 0.78	7.39 ± 0.43
Control group	10	7.5 ± 0.3	51.2 ± 9.3	245.8 ± 14.6	8.18 ± 0.85	10.65 ± 1.85	14.02 ± 3.49	15.43 ± 4.42
F value		18.362	0.231	1.143	13.753	13.274	14.531	12.593
P value		0.001	0.795	0.335	0.002	0.002	0.001	0.003

Table 3
Comparison of expression of *PPARGC1A* mRNA and methylation level.

Group	Cases	Expression of *PPARGC1A* mRNA	Methylation level of *PPARGC1A*
Insulin group	10	1.218 ± 0.219	0.027 ± 0.010
Metformin group	10	1.197 ± 0.176	0.026 ± 0.008
Control group	10	0.487 ± 0.038	0.739 ± 0.174
F value		64.620	66.713
P value		0.000	0.000

Table 4
Comparison of serum biochemical indicator.

Group	Cases	INS (mU/L)	TG (mmol/L)	LP (μg/L)
Insulin group	10	8.21 ± 1.37	1.96 ± 0.37	7.89 ± 0.79
Metformin group	10	8.27 ± 1.25	1.59 ± 0.41	8.07 ± 0.81
Control group	10	6.03 ± 0.69	2.31 ± 0.76	5.43 ± 0.28
F value		12.482	4.406	48.043
P value		0.001	0.022	0.000

3.4. Comparison of serum biochemical indicator

INS and LP for offspring rats at 8 wk in the insulin group and metformin group were significantly higher than ones in the control group, while TG was significantly lower than the one in the control group ($P < 0.05$). TG level in the insulin group was significantly higher than the one in the metformin group ($P < 0.05$). There was no significant difference in INS and LP level between the insulin group and metformin group ($P > 0.05$), as shown in Table 4.

4. Discussion

According to a previous research [7], the abnormal intrauterine environment would induce the fetus to form the certain genetic memory and then cause the significantly higher incidence of some metabolic diseases in adulthood. The gestational diabetes mellitus is the common complication for pregnant women. The epidemiological survey [8] indicated that the blood glucose level for the newborns delivered by GDM pregnant women would be higher than the normal infants, with the increased risk of diseases such as the diabetes mellitus in adulthood. Luo *et al*. [9] adopted the streptozotocin to prepare the rat model of GDM. The results showed that the birth weight of offspring rats of GDM was about 2.1 times heavier than the one of common offspring rats. Besides, the birth weight of offspring rats of GDM would be significantly increased during the observation. In consequence, the decrease in the blood glucose level of GDM pregnant women would be of critical importance for the prognosis of the newborns. The effect mechanism of GDM on the fetus has not been fully understood for the moment. Though some domestic research [10] reported that the metabolic disorder of offspring might be related to the malnutrition or hypernutrition of fetus, some evidence [11] indicated that the mechanism of epigenetic regulation played a key role in the metabolic diseases of fetus caused by GDM. The methylation of DNA is the main way to regulate the epigenetics, which is mainly involved in regulating the integrity of DNA, genetic mutation and chromatin modification. However the methylation of DNA was extremely sensitive to the external environmental factors [12], as the proper external stimulation could regulate the methylation of DNA. *PPARGC1A* is the main transcription factor to regulate the mitochondria metabolism. Gillberg [13] found that in the human pancreatic tissues, the methylation level of CpG island in the promoter of *PPARGC1A* could reflect the metabolic regulation of body. Therefore, this study proposed the following hypothesis in the beginning of design: the intervention of hypoglycemic drugs in the gestation of GDM pregnant women could reduce the methylation level of *PPARGC1A* in GMD patients to regulate the hyperglycemia environment in vivo and then improve the prognosis of offspring.

To prove the above hypothesis, GDM rats were given the subcutaneous injection of insulin and intragastric infusion of metformin respectively. Compared with the control group, the results showed that the body mass of offspring rats at birth in the insulin group and metformin group was significantly lower than the one in the control group, but there was no significant difference in the weight of offspring rats at the 4th and 8th week after the birth between three groups. During the gestation of GDM pregnant rats, the embryo was in the condition of hyperglycemia to cause the accumulation of subcutaneous fat and increase of weight. The drug intervention could reduce the condition of hyperglycemia of GDM pregnant rats, while the birth weight of embryo in the relatively normal environment would not be increased significantly. After the delivery, the offspring rats left the environment of hyperglycemia and then the increase in the weight was slowed, which indicated that the weight of offspring might be decided by the external factors and intrauterine environment. The further study on the blood glucose level of offspring rats showed that the fasting blood glucose and random blood glucose for offspring rats at the 4th and 8th week after the birth in the insulin group and metformin group were significantly lower than ones in the control group. Zeng *et al*. [14] reported that the β cell damage during the gestation of pregnant rats would cause the β cell damage of offspring rats and such damage might be continued to the adulthood and form the permanent insulin resistance of offspring rats. Lei *et al*. [15] also found that even the offspring rats left the environment of hyperglycemia, its β cells could not be recovered to the normal level. Referring to the findings of this study, the drug intervention could reduce the degree of β cell damage to improve the intrauterine hyperglycemia level, then the β cell function of offspring and finally the insulin resistance of offspring.

The long-term intrauterine hyperglycemia level of GDM would change the epigenetics through the methylation of *PPARGC1A* DNA [16,17], which would thus increase the risk of diabetes mellitus for offspring rats. In this study, the results showed that the expression of *PPARGC1A* mRNA for offspring rats in the insulin group and metformin group was significantly higher than the one in the control group, while the methylation level of *PPARGC1A* was significantly lower than the one in the control group, which indicated that the drug intervention could reduce the methylation of *PPARGC1A*, increase the expression of *PPARGC1A* mRNA, and thus reduce the intrauterine hyperglycemia environment of GDM pregnant rats, reverse the epigenetics and finally improve the metabolic level of offspring rats. Franks [18] also

reported that the expression of *PPARGC1A* mRNA in the pancreatic tissues and skeletal muscle of patients with diabetes mellitus was significantly lower than the one in the normal group, while the expression of *PPARGC1A* mRNA was closely related to the methylation level of such gene. It indicated that the decrease in the methylation of *PPARGC1A* and improvement on the intrauterine blood glucose were of significance to reduce the risk of metabolic diseases of offspring such as the diabetes mellitus. LP is the protein product of obese gene released by the adipose tissue, while LP level is negatively correlated to the fat storage. Morteza [19] reported that the absence of LP and receptor could increase the incidence of early obesity. In this study, LP level in the insulin group and metformin group was significantly higher than the one in the control group, which indicated that the drug intervention could increase the secretion of LP to reduce the birth weight, which was in consistent with the finding that the birth body weight for offspring rats in the insulin group and metformin group was significantly lower than the one in the control group. Su [20] found that GDM pregnant women would have the higher incidence of high TG of offspring. It was because there was the steep concentration gradient of TG in the blood circulation of GDM pregnant women and TG was accelerated to be transferred to the fetal tissue through the placenta. In this study, TG level in the insulin group and metformin group was significantly lower than the one in the control group, which proved that the drug intervention could reduce the transfer of TG from the pregnant rats to the offspring. Besides, it also found that the TG level in the insulin group was significantly higher than the one in the metformin group, which might be related to the different mechanism of insulin and metformin. The metformin can reduce the plasma TG and cholesterol level and can also act on the offspring rat through the placental barrier after the intragastric infusion to obviously reduce the TG level in offspring rats [21].

In conclusion, GDM can induce the methylation of *PPARGC1A* in offspring rats to reduce the expression of *PPARGC1A* mRNA; it will then cause the disorder of glycolipid metabolism of offspring in the adulthood. The treatment of insulin or metformin in GDM pregnant rats can reduce the methylation level of *PPARGC1A* and thus improve the abnormal glycolipid metabolism of offspring rats. At present, there are limited researches on such drug intervention in the human, because the drug intervention during the pregnancy may have the certain risk. The effect of drug intervention on the safety of offspring will be deeply analyzed in the further studies.

Conflict of interest statement

We declare that we have no conflict of interest.

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