Original Article

Gestational Diabetes Alters the Expression of Genes Involved in Sertoli Cells Maturation in Testis Tissue from Adult Rat Offspring

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Abstract

Introduction: Previous Studies indicate that both type 1 and 2 diabetes mellitus have adverse effects on the male reproductive system. Furthermore, it has recently shown that induced gestational diabetes significantly reduces the Sertoli cells number in the rat offspring. This study was done to evaluate the effect of gestational diabetes mellitus (GDM) on the expression of genes involved in Sertoli cell proliferation and maturation in the adult rat offspring. **Material and Methods:** To test this hypothesis, 12 Wistar rat dams were randomly allocated to control and diabetic groups. The diabetic group received 40 mg/kg/body weight of streptozotocin on day 0 of gestation. After delivery, six offspring of each group at the age of 12 weeks scarified and testis tissue harvested. After RNA extraction, the expression of p27kip1, A-kinase anchoring protein 9, CX43, and aromatase genes in both groups was evaluated by quantitative real-time polymerase chain reaction. **Results:** Our data showed that the expression of all examined genes which are important in Sertoli cells maturity and function were lower in GDM offspring. p27kip1 and aromatase were significantly downregulated in GDM offspring by 57% and 41%, respectively ($P < 0.05$*). **Discussion and Conclusion:** In summary, we provide evidence that GDM may adverse effects on the male reproductive system in the offspring by alterations in the expression of genes responsible for Sertoli cell proliferation and differentiation.

Keywords: *Gene expression, gestational diabetes, offspring, Sertoli cells*

Introduction

Gestational diabetes mellitus (GDM) is a major public health problem that affects up to one in seven pregnancies worldwide.[1,2] GDM is associated with an increased risk of developing long-term complications, such as obesity and type 2 diabetes in the offspring throughout life.[3-6] Our previous studies indicate that GDM causes structural alteration in some tissues such as pancreas, brain cortex, hippocampus, dentate gyrus, cerebellum, and retina in the offspring.[7-14] Several studies reported that induced diabetes in animals causes an adverse effect on the reproductive system including structural alterations of seminiferous tubules and disruption of spermatogenesis.[15-19] Sertoli cell proliferation and establishment of the adult Sertoli cell population occur in postnatal development. Recently, our group reported that GDM causes a significant reduction in total Sertoli cell numbers and nonsignificant reduction in the Leydig and spermatogonial cells in the adult rat offspring.[17] Currently, little is known about how GDM exposure contributes to the testis dysfunction in the offspring.

p27kip1 (also named Cdkn1b), a known marker of Sertoli cell maturation, is a cell cycle inhibitor that has typically low expression in neonatal testis and high expression in adult testis.[20] A-kinase anchoring protein 9 (Akap9) is a protein-coding gene that its mutations inhibit maturation of Sertoli cells, resulting in disruption of male fertility.[21]

Gap junction protein Connexin43 (Cx43) is associated with blood-testis barrier that located between Sertoli cells and also between Sertoli and germ cells.[22] Cx43 participates in Sertoli and germ cell differentiation, migration, and survival.[23] The cytochrome P450 aromatase (P450arom) is a major component of a microsomal enzymatic complex named aromatase, the essential multienzyme complex for converting androgens into estrogens in the testis.[24]

If either gene mentioned above are important regulators of Sertoli cell

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proliferation and function, then changes in expression of either or all of these should result in changes in Sertoli cell differentiation and sperm maturation. Hence, the aim of this study was to examine the effects of induced GDM on p27kip1, Akap9, Cx43, and aromatase genes expression in the testis tissue from 12-week-old rat offspring.

Material and Methods

Subjects

For this study, a total of 45 female and 15 male Wistar rats (aged 12 weeks) were used. The rats were maintained under controlled conditions of temperature 21°C and a 12 h alternating light/dark cycle. Animal procedures followed the guidelines and standards set by the Institutional Animal Care and Use Committee at the Golestan University of Medical Sciences.

Generation of the diabetic rat model

Female Wistar rats were separately placed with a proven breeder male for mating. Observation of vaginal plaques was assigned as gestational day 0. Pregnant rats were equally randomized into control and diabetic groups. On day 0 of gestation, the diabetic group received a single intraperitoneal injection of streptozotocin (STZ) solution (40 mg/kg body weight prepared with normal saline 0.85%) while control group received an equal volume of normal saline. Three days after STZ administration, tail incision method was used to measure fasting blood glucose level using a glucometer (ACCU-CHEK, Roche Diagnostics, Germany). Rats with blood glucose level between 120 and 250 mg/dl were labeled as GDM model. After spontaneous delivery, pups were allowed to mature for 12 weeks. Male offspring of GDM and normal groups were sacrificed, testis tissues harvested and stored at −80°C until further mRNA extraction.

Blood glucose level measurements

Blood glucose level of GDM and control group mothers (on day 0 and 3 of gestation) and also adult offspring of both groups was obtained through tail vein and was measured with a glucometer.

RNA extraction and quantitative polymerase chain reaction

Total RNA from testis tissue of each group was extracted using the total RNA purification kit (Jena Bioscience, Germany) according to the manufacturer's instructions. RNA quantity and purity were determined using a NanoDrop. For cDNA synthesis, RNA samples were amplified using prime script RT kit (Takara). Primers for respective genes (S18, p27kip1, Akap9, Cx43, and aromatase) were designed using OLIGO software and synthesized by Metabion company. The sequences of primers used for real-time polymerase chain reaction (PCR) are listed in Table 1. q-PCR was performed using the SYBR-Green kit (Bioneer) on an ABI-7300 real-time PCR system (Applied Biosystems, UK). In this experiment, S18 used as housekeeping gene and cDNA from control group offspring used as calibrator. The relative mRNA levels were calculated using the comparative CT method ($2^{-\Delta\Delta Ct}$). Every q-PCR experiment was conducted in duplicate.

Statistical analysis

Data from mRNA expression and also blood glucose level are expressed as the mean ± standard error of the mean (SEM). All data were analyzed with one-way ANOVA using SPSS 16.5 statistical analysis software (SPSS Inc., Chicago, IL, USA). Student's "*t*-test" was used to compare two independent groups and $P < 0.05$ was considered statistically significant.

Results

Serum glucose levels

Fasting blood glucose concentration in diabetic pregnant rats compared to normal rats is depicted in Figure 1. Student's *t*-test indicated a significant difference between two groups at 72 h after gestation ($P < 0.001$). In STZ-induced diabetic group, the mean ± SEM of blood glucose levels on day 0 and 3 of gestation were 94.20 and 208.23 mg/dl, respectively. While in control dams, the mean ± SEM of blood glucose level at day 0 and 3 of gestation were 96.33 and 93.42 mg/dl, respectively.

Results of real-time reverse transcription-polymerase chain reaction

After puberty of the offspring, we assessed whether the expression of Sertoli cell proliferation and maturation markers were affected by GDM. Expression of all examined genes (p27kip1, Akap9, Cx43, and aromatase) was detected in both diabetic and control groups. Figure 2a and b shows the q-PCR results and gel electrophoresis of amplified products, respectively. Our result showed that the mRNA expression levels of all studied genes which are important in Sertoli cells maturity and function were lower in the

Table 1: Real-time polymerase chain reaction primer name, sequences, size, and GenBank accession number

Genes	Forward primer	Reverse primer	Product size	GenBank accession number
p27kip1	AAATGTTTCAGACGGTTCCC	TGCTTTCTCAGTGCTTATACAG	218	NM_031762.3
Akap9	AAACACAGGATGGAAATGAC	CAACATCTAACAGGATCTTCAG	165	XM_006236023.3
Cx43	TTTCCTTTGACTTCAGCCTC	TATGAAGAGCACTGACAGCC	179	NM_012567.2
Aromatase	CCTGGACGAAAGTTCTATTGTG	TCTCATACTTTCTGTAGAGCCA	116	NM_017085.2
S18	CTTCCGCAGGTTCACCTAC	TGCCCTTTGTACACACCG	267	XM_021174474.1

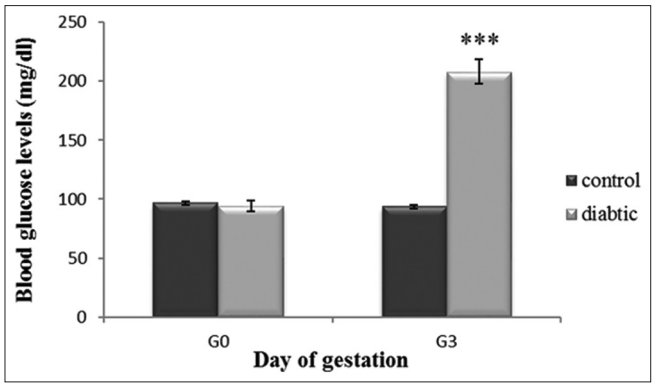

Figure 1: Serum glucose levels in gestational diabetes mellitus and control dams before and 72 h after streptozotocin administration. Values are means ± standard error of the mean. *(*P* < 0.001)**

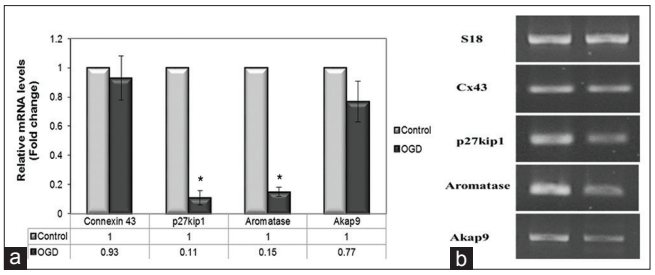

Figure 2: (a) Real-time polymerase chain reaction analysis of p27kip1, A-kinase anchoring protein 9, Connexin43, and aromatase mRNA expression in the testis tissue of control and diabetic rat offspring. (b) Agarose gel electrophoresis of polymerase chain reaction products following real-time polymerase chain reaction amplification. S18 gene used as internal control. Mean ± standard deviation, *P* < 0.05*. OGD: Offspring of gestational diabetes

offspring of diabetic mothers compared to controls. The analysis of the mRNA levels of p27kip1 and aromatase showed significant alterations in the diabetic group compared to controls ($P < 0.05$*). Furthermore, the expression of Cx43 and Acap9 was downregulated in testis tissue of diabetic group compared to control group, but the difference was not statistically significant.

Discussion

In this study, we present evidence that GDM may disrupt the Sertoli cell terminal differentiation program, resulting in decreased maturation of Sertoli cells and increased Sertoli cells numbers in adult offspring. It is well-documented that diabetes mellitus results in deleterious consequences to the reproductive system. Studies have demonstrated that testis, seminal vesicle, epididymis, Sertoli/blood-testis barrier function is disrupted in diabetic animal models. Several animal model studies have shown that GDM causes reduction of Leydig cells in male adult rats.[17,20,25] Türk *et al*., showed that GDM has negative effects on reproductive efficiency in Wistar Albino male offspring.[26] Based on our recently published data, GDM causes a significant reduction in the density of the Sertoli cells.[17] In continuation of our previous studies, we studied the effect of GDM on the expression of Sertoli cell maturation markers included p27kip1, Akap9, Cx43, and P450arom.

p27kip1 as a marker of Sertoli cell maturation has high expression in adult testis. A study of Schimenti *et al*., showed that mice lacking p27Kip1 exhibits increased Sertoli cell proliferation, indicating that this factor participates in the halt of mitogenesis and starting maturation of Sertoli cells in adult mice.[21] Our result showed that GDM significantly downregulate p27kip1 expression levels in Sertoli cells, suggesting that decreases in this cell cycle inhibitor in response to GDM may cause Sertoli cells to continue the proliferative phase and do not differentiate. This result is in agreement with our resent result that showed a significant increase in total number of Sertoli cells in offspring of GDM rats.[17] A-kinase anchor proteins (AKAPs) play critical roles in varying stages of spermatogenic development. One major AKAP family member, Akap9, has an important role in Sertoli cells maturation. Mutation of Akap9 leads to a high number of immature Sertoli cells and irregular gap and tight junctional protein localization in blood–testis barrier. There is evidence that Akap9 disruption causes failure of p27Kip1 expression.[21] Hence, in this study, downregulation of p27kip1 in offspring of GDM group may be a response to Akap9 inhibition. In addition, our other data suggest that GDM may disrupt sperm maturation. It has shown that in adult rats, the aromatase gene has been localized in Sertoli cells, Leydig cells, germ cells, and spermatids. Recent studies highlight the involvement of aromatase not only during the development but also in the acquisition of motility and final maturation of spermatozoa.[24] Results showed a decreased aromatase expression in offspring of GDM rats compares to controls. Furthermore, it has shown that in Akap9 mutant, Cx43 improperly localized in testis. In this study, we observed nonsignificant reduction of Cx43 mRNA in testis tissue from offspring of GDM rats.

Conclusion

Using quantitative RT-PCR method, we have evidenced that intrauterine exposure to hyperglycemia extends Sertoli cell proliferation and may disrupt the maturation of these cells in the offspring. Differential gene expression was most notable for p27kip1 and P450arom. Downregulation of these genes possibly increases the number of immature Sertoli cells and subsequently decreases differentiation potential of Sertoli cells, germ cells, and spermatids in the testis of GDM-exposed offspring. Male reproductive system complications caused by diabetes mellitus could be mediated through insufficient insulin hormone production, insulin resistance, oxidative stress, and excessive-free radical generation. Further studies are necessary to determine the exact molecular mechanism for inducing male reproductive system complications in the offspring by GDM.

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Conflicts of interest

There are no conflicts of interest.

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