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Maternal Diabetes Causes Alterations of DNA Methylation Statuses of Some Imprinted Genes in Murine Oocytes

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ABSTRACT

Maternal diabetes has adverse effects not only on oocyte quality but also on embryo development. By using streptozotocin (STZ)-induced and nonobese diabetic (NOD) mouse models we investigated the effect of maternal diabetes on DNA methylation of imprinted genes in oocytes. Mice which were judged as being diabetic 4 days after STZ injection were used for experiments. In superovulated oocytes of diabetic mice, the methylation pattern of Peg3 differential methylation regions (DMR) was affected in a time-dependent manner, and evident demethylation was observed on Day 35 after STZ injection. The expression level of DNA methyltransferases (DNMTs) was also decreased in a time-dependent manner in diabetic oocytes. However, the methylation patterns of H19 and Snrpn DMRs were not significantly altered by maternal diabetes, although there were some changes in Snrpn. In NOD mice, the methylation pattern of Peg3 was similar to that of STZ-induced mice. Embryo development was adversely affected by maternal diabetes; however, no evident imprinting abnormality was observed in oocytes from female offspring derived from a diabetic mother. These results indicate that maternal diabetes has adverse effects on DNA methylation of maternally imprinted gene Peg3 in oocytes of a diabetic female in a time-dependent manner, but methylation in offspring’s oocytes is normal.

INTRODUCTION

Previous studies suggest that fetuses of poorly controlled diabetic mothers display a higher incidence of malformations, primarily neural tube defects and skeletal/cardiovascular abnormalities and fetal death than those of nondiabetic pregnant mothers [1–5]. Furthermore, their offspring are susceptible to obesity, glucose intolerance, and type 2 diabetes [6, 7]. These adverse effects may be the result of lower oocyte quality and a disturbed uterine environment. Diabetic mice have been shown to exhibit uterine atrophy [8, 9], reduced mating ability [10], and alterations of the hypothalamic-pituitary-ovarian axis [11]. The ovarian function is impaired, and also the ovulation rate is lower in type 1 diabetic mice than in nondiabetic mice [9, 12].

Numerous studies have shown that if oocytes are exposed to diabetic conditions during folliculogenesis and meiotic maturation, meiotic maturation and subsequent developmental potential are negatively affected [12, 13]. Evidence suggests that the mitochondrial function, glucose metabolism pathways, and communications between cumulus cells and the oocyte are all changed in follicles of maternal diabetic mice [14–17].

The maternal diabetic state is also detrimental to pre- and postimplantation embryo development in rodents and in humans. Preimplantation embryo development is significantly delayed in both chemically induced and spontaneous diabetic models, and the embryo has a high incidence of degeneration and fragmentation [18–20]. Even one-cell embryos isolated from diabetic mice and transferred to nondiabetic mice still experience malformations and growth retardation in the process of development [21].

In order to know the mechanisms underlying these abnormal phenomena, genetic factors are of interest. Several studies have shown that gene expression in embryos is affected by maternal diabetes. [22–26]. However, we do not know whether the methylation status of imprinted genes in oocytes is influenced by maternal diabetes.

Epigenetics presents a link between gene and environment, and it has an important role in embryo development and diseases [27]. A series of experiments showed that diploidy alone is not sufficient for normal embryo development, but proper regulation of both parental genomes is required as well [28, 29]. Maternal or paternal expression is regulated by genomic imprinting being dependent on whether a parental allele is inherited from the spermatozoon or oocyte [30, 31]. Monoallelic expression is the result of DNA methylation at the cytosine residue of the CpG site in the regulatory domain of the imprinted gene [30, 32]. Modification of DNA methylation in imprinted genes in the oocyte is established during embryo development and oocyte growth [33]. Many studies show that establishing improper DNA methylation in imprinted gene is important for embryo development and disease [34–39]. Moreover, the DNA methylation re-establishment process of imprinted gene is susceptible to being affected by environment. Thus, we hypothesized that maternal diabetes might disturb the DNA remethylation process during oogenesis and oocyte maturation in diabetic mice. In the present study, streptozotocin (STZ)-induced and nonobese diabetic (NOD) diabetic models were selected to analyze methylation patterns of differential methylation regions (DMR) of the maternally methylated genes.
**Peg3 and Sirp**n and paternally the methylated gene H19 in oocytes.

**MATERIALS AND METHODS**

All CD-1 mice were provided by the Beijing Vital River Experimental Animals Centre and maintained at 12D:12L in a temperature-controlled room. NOD mice (NOD/ShiLtJ) were supplied by Model Animal Research Centre of Nanjing University. All procedures described were reviewed and approved by the ethical committee of the Institute of Zoology, Chinese Academy of Sciences.

**Production of Diabetic Mice**

To generate a diabetic model, female CD-1 mice (age 6–7 weeks) received a single intraperitoneal injection of STZ at a dose of 230 mg/kg [40, 41] (Fig. 1A). Four days after injection, the glucose concentration was checked after cutting the tip of the tail, using a glucometer (Accu-Chek active; Roche Diagnostic). If glucose level was higher than 17.0 mmol/L, the mouse was used for further study. Mice of similar age injected with buffer were selected as control animals.

**NOD Mice**

NOD mice were nonobese and spontaneous diabetic individuals and exhibited polyuria and glucosuria accompanied by rapid weight loss [42]. All NOD mice (NOD/ShiLtJ, Nanjing, China) had glucose checked at approximately 13–14 weeks by using a tail blood sample. If glucose level was more than 14.0 mmol/L, the mouse was considered to have the mutation. Age-matched controls were NOD females with glucose levels ~9.0 mmol/L.

**Oocyte Collection**

Oocytes were collected on Days 15, 25, and 35 after injection of STZ or buffer. Female mice were superovulated (Fig. 1A) by intraperitoneal injection of 8 IU of equine chorionic gonadotropin, followed by injection with 8 IU of human chorionic gonadotropin (hCG; Tianjin Animal Hormone Factory) 46–48 h later. At 13–14 h after injection of hCG, female mice were killed, and oocytes were recovered from oviductal ampullae; cumulus cells were removed by brief incubation in 1 mg/ml hyaluronidase. The oocytes were then washed with M2 medium (Sigma) until no cumulus cells were observed. Oocytes from offspring of diabetic and nondiabetic mice were also collected for analysis as described above.

**Bisulfite Treatment and PCR Amplification**

Bisulfite treatment of oocyte DNA was conducted according to previous procedures described by our laboratory [23]. Briefly, oocytes collected from diabetic and nondiabetic mice and offspring from diabetic and nondiabetic mice were divided into PCR tubes, each containing 5 oocytes. Samples were then digested in lysis solution (0.5 M EDTA, pH 8.0, 2 mg/ml proteinase K; Amresco) at 37°C for 37 min and denatured with 0.3 M NaOH at 37°C for 15 min. A 15-μl portion of melted 2% low-melting point agarose (Sigma) was added into the tube and mixed (total volume, <25 μl). The mixtures were placed in chilled mineral oil and incubated for 15 min on ice allowing formation of beads. The beads were carefully removed to another new 2-ml Eppendorf (EP) tube and then 500 μl of fresh bisulfite solution (2.5 M sodium metabisulfite, Merck; 125 mM hydroquinone, Sigma; pH 5) was added. The tube was covered with 200 μl of mineral oil followed by incubation at 50°C for 4 h in the dark. The reaction was stopped by transferring the beads to a new 2-ml tube, adding 1 ml of TE (Tris-HCl and EDTA) and incubation at room temperature for 15 min, and this treatment was repeated three times. Following desulfonation twice with 0.5 ml 0.3M NaOH for 15 min each, washing twice for 15 min each with TE and water, respectively, the beads were used for amplification by PCR.

Single oocytes isolated from diabetic and nondiabetic mice were placed into individual PCR tubes with a small amount of M2 medium, and bisulfite treatment was performed as described above with only slight modifications. The time of digestion with lysis solution was 30 min, and the final concentration of NaOH during the denaturation stage did not exceed 0.3 M. All manipulations were carried out with the necessary care.

The bisulfite-modified DNA was used as template for PCR according to previous reports [23]. Briefly, nested PCR was carried out using one bead or 0.5-μl solution with modified DNA in the first-round PCR and 2 μl of product of first-round PCR was added into the second reaction mixture system as template. All reaction mixtures contained 0.4 mM primers, 0.2 mM of all dNTPs, 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2, and 1.25 units of Taq Hotstart polymerase (TaKaRa). The reaction conditions were: the first-round PCR were 6 min at 94°C, followed by 35 cycles of PCR for 1 min at 94°C, 2 min at annealing temperature, and 2 min at 72°C and then extended 7 min at 72°C. The conditions for the second-round PCR were 4 min at 94°C, followed by 35 cycles of PCR for 1 min at 94°C, 1 min at annealing temperature, and 1 min at 72°C and then extended 7 min at 72°C. The annealing and primers [33] are shown in Table 1. To verify the specification of PCR amplification, products of each second-round PCR were checked by 1.5% agarose gel electrophoresis.

**Determination of Methylation Status by Combined Bisulfite Restriction Analysis (COBRA) and Sequencing**

To analyze the methylation patterns of DMR, the products of second-round PCR were digested by one or two restriction endogenous enzymes. These enzymes had different recognition sites at CpG loci located in DMRs of imprinting genes, e.g., *Tay1* (T/Sca), *Rsul* (GTAC), and *BstUI* (CGCG). The digested products were analyzed by 2.5% agarose gel electrophoresis. According to COBRA analysis, we cloned the second round PCR products to T vector and sequenced (Invitrogen, Beijing, China) to further investigate the methylation status.

**Generation of Embryos and Offspring**

Four days after injection with STZ or buffer (Fig. 1B), the estrous diabetic/nondiabetic mice were mated with normal male mice within 15 days. On the next morning, if a vaginal plug was observed after mating, it was recognized as gestational 0.5 day. A portion of the pregnant mice was killed by cervical dislocation at 10.5 days postcoitum (dpc), and the embryos were collected. Others were fed until the offspring were born.

**RNA Purification and Quantitative Real-Time PCR**

RNA was extracted from oocytes by using RNeasy micro kit (Qiagen) according to the manufacturer’s instructions. The first cDNA strand was synthesized using Superscript II (Invitrogen). Quantitative real-time PCR (qRT-PCR) was carried out using fast real-time PCR systems (ABI). Triple samples were analyzed for each gene, and the housekeeping gene of peptidylprolyl isomerase A (*Ppia*) was used as control gene. The expression level was evaluated by 2^-ΔΔCt_. Primers are shown in Table 1.

**Statistical Analysis**

Data are presented as means ± SD, and the significance between groups was compared by one-way ANOVA. Pregnancy rate was analyzed by chi-square test. A probability level of <0.05 was considered significant.
RESULTS

Ovulation Rate Is Lower in Diabetic Mice

The average number of MII oocytes was similar at different time points in the diabetic or nondiabetic group, but the ovulation rate was significantly lower in the diabetic group than in nondiabetic group (diabetes vs. nondiabetes, 9.2 vs. 23.5, 15 days; 12.6 vs. 27.2, 25 days; 10.1 vs. 28.0, 35 days; \(P < 0.01\)) (Fig. 2A).

Maternal Diabetes Affects DNA Methylation Re-establishment in DMRs of Maternally Imprinted Genes in Oocytes in a Time-Dependent Manner

To investigate whether the methylation re-establishment process was affected, we observed the methylation patterns of DMRs of the maternally imprinted genes Peg3 and Snrpn and the paternally imprinted gene H19 in oocytes by using COBRA and sequencing. A total of 90–120 MII oocytes per imprinted gene were analyzed in each group. The products of H19, amplified by nested PCR, were digested using nucleic acid restriction endonuclease TagI and RsaI (Fig. 3A). Data showed that all of the products were undigested by both enzymes in all groups. This indicates that the methylation pattern of DMR of the paternally imprinted gene H19 is not affected by maternal diabetes.

Products of the maternally imprinted genes Peg3 and Snrpn were digested by TagI, Bstul, or Bstul, respectively. For Peg3 (Fig. 3C), the COBRA results showed that most samples were completely digested by both enzymes except for a few samples (Fig. 3C, red arrow) for 15-day and 25-day groups. However, some samples were partially digested by both enzymes (Fig. 3C) for 35 days, and these undigested bands (Fig. 3C, red arrows) indicated that some CpG loci of Peg3 DMR were unmethylated for Snrpn, some samples were also partially digested by Bstul (Fig. 3C, red arrows) for 25 days and 35 days, but similar results were not observed for 15 days (Fig. 3B). Because there was only one enzyme which could be used to digest the products of Snrpn, the unmethylated CpG loci may only locate at the recognition sites of the enzyme.

To further reveal the detailed methylation of Peg3 and Snrpn at 15 days, 25 days, and 35 days after STZ injection, we pooled the uncut and/or cut samples together according to the COBRA results and sequenced the samples (Fig. 3, D and E).

Proportion of Oocytes in Which the Methylation Status of Maternal Imprinting Genes Is Changed as Evaluated by COBRA

To further determine the rate of oocytes in which the DMRs’ methylation status was changed on Day 35 after STZ injection, the sample was analyzed by COBRA. Part of the COBRA results are shown in Figure 2C. For the maternally methylated gene Peg3, the rate (total undigested samples/total samples) of unmethylated oocytes was significantly higher (\(P = 0.001 < 0.01; 21.98\%\), \(n = 91\)) in the diabetic group than in the nondiabetic group (6.2\%, \(n = 113\)). For Snrpn, the rate of unmethylated oocytes was slightly higher (\(P = 0.352 > 0.05; 7.5\%\), \(n = 80\)) in the diabetic group than in the nondiabetic group (4.0\%, \(n = 75\)) at 35 days after STZ injection (Fig. 2B).

Expression Levels of DNMTs Are Decreased in a Time-Dependent Manner in Oocytes from Diabetic Mice

To investigate how maternal diabetes affects the methylation patterns of some imprinted genes in a time-dependent manner, we analyzed the expression levels of DNMTs in oocytes by using qRT-PCR. As shown in Figure 4, their expression levels were significantly lower in diabetic group than in the control (\(P < 0.05\)). On Days 15 and 25, the expression levels of DNMTs in oocytes were similar in diabetic mice but were significantly decreased on Day 35 compared to Days 15 and 25 (\(P < 0.05\)).

TABLE 1. Oligonucleotides and annealing temperatures used for nested PCR.

<table>
<thead>
<tr>
<th>Primer name and/or amplification</th>
<th>Forward</th>
<th>Reverse</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H19</td>
<td>5'-GAGTAATTAGAGGTATATAGATT-3'</td>
<td>5'-ATCAAAATCTACAATAACCCCT-3'</td>
<td>55</td>
</tr>
<tr>
<td>Snrpn</td>
<td>5'-TATGTAATATAGATAGTTAGAAATAG-3'</td>
<td>5'-AATAAACCCAAATCTAAATTGTATCAATTC-3'</td>
<td>55</td>
</tr>
<tr>
<td>Peg3</td>
<td>5'-ATATTATATATGTTAGGT-ACTGAGGAGG-3'</td>
<td>5'-AAATCCTAAAACTTTATCTTACC-3'</td>
<td>45</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>5'-GCCGTCACTCTGCAAGCCACTCA-3'</td>
<td>5'-AATAGTGATGCTTTCCTCCTC-3'</td>
<td>45</td>
</tr>
</tbody>
</table>

Because there were no samples of Peg3 being completely undigested by both enzymes for 25 days and 15 days, we pooled all of the samples together at each time point and sequenced them, respectively. For the samples of Peg3 on Day 35, the uncut and cut samples were separately pooled and sequenced. As to Snrpn, because only one enzyme was used to digest products of PCR, all the samples of 15 days, 25 days, and 35 days were separately pooled and sequenced, respectively. Sequencing results showed that the methylation patterns were properly acquired on Days 15 and 25 for Peg3 in oocytes from diabetic mice, but on Day 35, the methylation status in DMR of Peg3 was significantly affected by maternal diabetes (Fig. 3D). For Snrpn, the methylated status was similar between the two groups (Fig. 3E).
To determine whether maternal diabetes disturbs the DNA methylation reprogramming process in oocytes of offspring of diabetic mice (OD), DNA methylation patterns of DMRs in MII oocytes were assessed by COBRA and sequencing. A total of 90–120 MII oocytes per imprinted gene were analyzed in each group. We observed that some samples were not completely cut by \textit{BstU} for \textit{Snrpn} (Fig. 5B, red arrow). This indicates that some CpG sites were not remethylated in oocytes of OD. Because only one enzyme was used, the unmethylated CpG locus may be located at the recognition sites of the enzyme. To investigate in more detail, we pooled all the samples together and sequenced them. The sequencing results showed that only a few CpG sites were unmethylated (Fig. 5E), and they were located at the enzyme recognition loci, and the total methylation rate was similar between the two groups. However, at the loci (Fig. 5E, red arrows), the methylation rate was significantly lower than that of ON (offspring from nondiabetic mice). For the paternally imprinted gene \textit{H19}, the COBRA result showed that several samples were partially digested by \textit{TaqI} (Fig. 5A), but the sequencing results showed that there was only a slight ($P > 0.05$) increase in the total methylation rate of \textit{H19} (Fig. 5D).

Pregnancy Rate and Embryo Development Are Affected by Maternal Diabetes

As shown in Figure 6, we observed that the pregnancy rate was only 73.68% (14 of 19) in diabetic mice in which vaginal plugs were observed, and the death rate of embryos at 10.5 dpc was 16.56% (26 of 157) in diabetic females, while in nondiabetic females, the pregnancy rate and embryo death rate were 100% and 0, respectively. The average number of live embryos per litter in diabetic group was significantly ($P < 0.05$) lower (9.36, 131 of 14) than that of the nondiabetic mouse (16.0, 192 of 12) at 10.5 dpc. We generated 86 pups that were born by 11 diabetic females, and the birth rate per mouse...
was 7.82 (86 of 11), which was significantly ($P < 0.001$) lower than that in nondiabetic groups (13.7, 164 of 12). A total of 43.02% (37 of 86) of pups died in 10 days of birth in diabetic groups, significantly ($P < 0.01$) higher than that of non-diabetic groups (13.41%, 22 of 164). These results suggest that embryo development was adversely affected by maternal diabetes and that spontaneous abortion occurred at an earlier than the mid-gestation period.

**NOD Genetic Diabetic Mice Show Altered Methylation Pattern of Peg3 DMR in a Time-Dependent Manner**

To test whether methylation alteration in Peg3 DMR in oocytes from STZ-induced diabetic mice was caused by STZ itself rather than maternal diabetes, we examined the methylation pattern of Peg3 DMR in NOD mouse model. Approximately 80–110 ovulated MII oocytes (not including failed samples) were analyzed by COBRA at each time point and similar results were obtained. On Days 15 and 25, only a few samples were undigested by one or two enzymes (Fig. 7, B and C) like in controls (Fig. 7A). On Day 35, the undigested
FIG. 5. Methylation patterns in DMRs of imprinted genes in oocytes from offspring of nondiabetic (ON) and diabetic (OD) females. ON and OD mice (7–8 weeks old) were superovulated, and oocytes were collected and treated with bisulfite and then amplified by nested PCR. Each sample included 5 oocytes and the number column on the left of the pictures showed the bands’ size of the marker. A–C The products of PCR were digested by enzymes and analyzed with 2.5% agarose gel electrophoresis. Red arrowheads indicate samples that were not completely digested by enzymes. D and E Methylation rates in DMRs of Peg3, Snrpn, and H19 were evaluated by sequencing. E) Red arrows point to the CpG loci methylation rates in Snrpn. Black cycle, methylated; white cycle, unmethylated; blank loci, CpG lost.

FIG. 6. Effects of maternal diabetes on embryonic development and pregnancy parameters. A) Diabetic and nondiabetic mice were mated with normal males within 15 days of STZ injection and the embryos were collected at 10.5 dpc of gestation. Data are presented as means ± SD (n = 14 diabetic, 6 nondiabetic). B) Diabetic and nondiabetic females were mated with normal males and pups were born. Data are means ± SD (n = 11 diabetic, 6 nondiabetic). C) Pregnancy rate was evaluated as the percentage of pregnant animals within the total number of animals with vaginal plugs. Death rate after birth was evaluated as percentage of dead pups of the total number of pups within 10 days. **P < 0.01.
samples (Fig. 7D) were apparently more than the other time points and controls.

**DISCUSSION**

The genomic DNA methylation is subjected to two demethylation and remethylation processes during gametogenesis and embryo development [43]. If imprinting is not properly acquired in oocytes and/or not maintained during preimplantation development, fetal development may be abnormal [44, 45]. It is well known that germ cell quality and fertility are adversely affected by diabetes mellitus, however, whether imprinting methylation is affected in germ cells is not known. In this study, by using a STZ-induced mouse model, which was used by many reports where the authors obtained similar results compared to those of a mutation mouse model [14, 46], we for the first time investigated whether DNA methylation of imprinted loci are disturbed by maternal diabetes in oocytes of diabetic females and their offspring. We showed that maternal diabetes mellitus caused adverse effects on DNA methylation imprinting in oocytes of diabetic females (Fig. 3) but did not evidently affect imprinting pattern in oocytes of their offspring (Fig. 5).

Previous studies have demonstrated that oocyte quality is adversely affected by maternal diabetes [14–17]. During oogenesis, a crucial event is to establish proper genome-wide DNA methylation for later embryo development and survival. In mice, parthenogenetic and androgenetic embryos die before Day 10 of gestation and have distinctive phenotypes: 25-somite embryos with poor extraembryonic tissue and retarded embryos with proliferated trophoblasts, respectively [29]. This may be related to improper genomic imprinting in oocytes. In diabetic females, the oocyte quality is compromised, indicated by reduced glucose metabolism, compromised communication between cumulus cells and oocytes, mitochondrial malfunc-

![FIG. 7. DMR methylation status of Peg3 analyzed by COBRA in NOD mouse model. DNA from ovulated MII oocytes from NOD mice was modified by bisulfite and amplified using nested PCR. The products were digested by enzymes. Control (A), Day 15 (B), Day 25 (C), Day 35 (D). Red arrows show undigested samples. Blank column, sample lost.](https://bioone.org/journals/Biology-of-Reproduction)
normal male mice at 15 days after injection of STZ, and pups were produced. Our data showed that the methylation patterns of DMRs of imprinted genes were not significantly altered in offspring oocytes. Thus, diabetic intrauterine environment may not affect DNA imprinting re-establishment during oogenesis in offspring of diabetic mice.

In summary, maternal diabetes causes adverse effects on imprinting patterns in oocytes, but no evident imprinting abnormality is observed in oocytes from female offspring derived from a diabetic mother.

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