High insulin impaired ovarian function in early pregnant mice and the role of autophagy in this process

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Abstract. Metabolic disorders, such as PCOS (polycystic ovarian syndrome) and T2DM (type 2 diabetes mellitus), are associated with menstrual dysfunction, anovulation, infertility, and early pregnancy loss. Ovarian dysfunction is not only related to low pregnancy rates but also to the increased risk of miscarriage. Women with PCOS or T2DM, characterized by hyperinsulinemia, commonly experience ovarian dysfunction. In this study, we first explored whether high insulin levels directly affected ovarian functioning during embryo implantation. Mice in the insulin-treated group were given a subcutaneous injection of recombinant human insulin. After insulin treatment, serum levels of E2 (estrogen), PROG (progesterone), LH (luteinizing hormone), and FSH (follicle-stimulating hormone) were obviously lower, and there was a significant decrement of ovarian GDF9 (growth differentiation factor 9) mRNA. H&E (hematoxylin and eosin) staining showed a greater number of immature follicles and less luteinization in the insulin group. Further autophagy was studied in this process. A significant increase of P62 (SQSTM1/Sequestosome1) and a decrease of Cathepsin B, BECN1 (Beclin 1), and ULK1 (Unc-51-like kinase 1) mRNA in ovary was found in the insulin group. Western blot analysis showed that the expressions of LC3 (microtubule-associated protein 1 light chain 3), BECN1, and Cathepsin B proteins in ovaries from insulin group were obviously reduced, while P62 proteins were significantly increased. All these results illustrated that insulin could directly impair ovarian function during embryo implantation and the imbalance of ovarian autophagy due to insulin. Autophagy could enhance the impaired ovarian function results from insulin.

Key words: Insulin, Autophagy, Ovary, Hyperinsulinemia, Embryo implantation

METABOLIC DISORDERS, such as PCOS (polycystic ovary syndrome) and T2DM (type 2 diabetes mellitus), are reported to be associated with high miscarriage rates, decreased pregnancy rates, and low live birth rates [1]. PCOS and T2DM are characterized by hyperinsulinemia [2]. PCOS is an endocrine and metabolic disorder with a prevalence ranging from 5 to 13% in women of reproductive age. Moreover, PCOS is often accompanied by menstrual dysfunction, anovulation, infertility, early pregnancy loss, and other complications of pregnancy [3]. In reproductive-age women, PCOS is the most common cause of anovulatory infertility, with a rate of 75% [4], and women with PCOS experience a 30 to 60% higher rate of early pregnancy loss than the general population [5]. Additionally, women with PCOS are thought to have a spontaneously reduced placental volume and weight, increased chorioamnionitis futilities, and villous immaturity [6].

Submitted Oct. 19, 2016; Accepted Feb. 23, 2017 as EJ16-0494
Released online in J-STAGE as advance publication Apr. 18, 2017
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A Japanese study reported that 67% of pregnant diabetic women had T2DM. Compared with the general pregnant population, women with T2DM tend to have worse pregnancy outcomes [7]. Women with T2DM deliver earlier and are at an increased risk of injuries from delivering a large infant [8]. Additionally, the live birth rates of women with T2DM were significantly lower than those of the general population [9]. However, the mechanism of this phenomenon is still unclear.

Embryo implantation is an important step for pregnancy. Failed implantation affects the development of the embryo and may lead to fetal anomaly and abortion. Perfect ovarian function is essential to the embryo implantation and development during early pregnancy. Ovarian dysfunction is not only related to low pregnancy rates but also to the increased risk of miscarriage. Some studies have reported that ovarianectomy at any time during pregnancy of mice will lead to abortion [10, 11]. Estrogen and progesterone, which are synthesized and secreted by the ovaries, are necessary to early pregnancy embryo implantation [12]. A successful embryo implantation requires both an implantation-competent blastocyst and a receptive uterus. These elements have been shown to be under the control of the steroid hormones estrogen and progesterone. Estrogens are associated with cell proliferation, and progesterone is associated with cellular differentiation [13]. Both of these hormones regulate uterine receptivity and implantation in early pregnancy [14]. Research has proven that PR knockout mice, lacking both PR-A and PR-B, displayed pleiotropic reproductive abnormalities and exhibited implantation failure [15]. Erα (estrogen receptor-α) knockout uteri are hypoplastic and unable to support implantation [16]. Pregnancy cannot occur without a successful implantation and the regulation of hormones.

Women with PCOS or T2DM are reported to have ovarian dysfunction, low implantation rates, slow development, and high miscarriage rates [1, 17-19]. However, the underlying mechanism of this phenomenon is still unknown. Women with PCOS and T2DM have some common characteristics, such as insulin resistance and high levels of insulin in serum [20-22]. However, there is little evidence showing that ovary function could be directly affected by high levels of insulin during embryo implantation. Autophagy is an intracellular degradation process, which is essential for cell growth, survival, differentiation, development, and protein homeostasis. Autophagy is reported to contribute to the maintenance of ovarian functioning in many ways [23-25]. Insulin is reported to be involved in the regulation of autophagy, and studies have shown that insulin can inhibit autophagy through activating mTORC1 (mechanistic target of rapamycin complex 1) and PKB (protein kinase B) [26]. Thus, in this study we first explored whether high levels of insulin would directly affect the functioning of ovaries during embryo implantation and further studied whether autophagy participated in this process.

**Materials and Methods**

**Animal**

Kunming female mice (6 weeks old) were purchased from the Animal Facility of Chongqing Medical University. All the mice were housed in plastic cages containing shavings as bedding material and were exposed to 12 h light/dark cycles at a constant temperature (22±2°C) and humidity (50%), with access to food and water ad libitum. All animal procedures were approved by the Ethics Committee of Chongqing Medical University in China.

**Animal model and tissue collection**

Female mice were randomly assigned to two groups: the insulin treated group and the control group. Human recombinant insulin (insulin glargine, SANOFI) was purchased from the hospital. The insulin intervention mouse model was established as described by Ou et al. [27]. Mice in the insulin-treated group were given a subcutaneous injection of insulin. Mice in the control group were injected with saline. For the insulin-treated group, 0.05 IU insulin was injected until day 16. Then, insulin was gradually increased until the daily dose reached 0.8 IU. After treatment with 0.8 IU of insulin daily for 3 days, the mice were given a maintenance dose of 0.2 IU as shown in Table 1. After that, all mice were mated with normal fertile males. The morning in which a vaginal plug was found after mating was recorded as the first day of pregnancy (D1). The ovary tissue on pregnant D4 was collected and stored at –80°C or fixed in 4% paraformaldehyde for further analyses. Blood samples were collected from the eyeball and centrifuged at 5,000 g for 5 min to obtain the serum. The serum was stored at –80°C for further analysis.
**Hematoxylin-eosin staining (H&E)**

The ovary was fixed in 4% paraformaldehyde for 4 h, then dehydrated with increasing concentrations of ethanol and embedded in paraffin. Serial cross-sections of 5 μm were prepared for future study. For H&E, the ovary tissue samples were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. The samples were stained with hematoxylin for 3 min and then with eosin for 30 s, followed by dehydration until they were transparent. Finally, the pieces were mounted with neutral gum.

**ELISA**

The serum for detection of INS (insulin), estrogen (E2), progesterone (PROG), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were collected from pregnant female mice on D4. The levels of INS, E2, PROG, FSH and LH were detected using an ELISA kit (Yan Hui Biological Technology Co. Ltd., Shanghai, China), according to the manufacturer’s recommended instructions.

**Real-time PCR (RT-PCR) analysis**

Total RNA was extracted from the ovary samples of mice from the insulin-treated and control group pregnant mice on D4 using RNAsio plus Reagent (Takara, Dalian, China) and reverse-transcribed into cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China). The primers used in this study are shown in Table 2. β-actin was used as an internal control for standardization. RT-PCR was performed with the SYBR Premix Ex Taq™ Kit (TaKaRa, Dalian, China). Experiments were performed in triplicate. Data obtained from RT-PCR were analyzed with the 2^\(-\Delta\Delta C_t\) method.

**Western blotting**

A tissue protein extraction kit (Beyotime, Shanghai, China) was used for protein preparation. Total proteins were extracted from the ovary samples of the insulin-treated and control group pregnant mice on D4. Protein samples were electrophoresed through 10% SDS (sodium dodecyl sulfate)–polyacrylamide gels and then transferred onto PVDF (polyvinylidene difluoride) membranes (Millipore, Billerica, Massachusetts). Membranes were blocked in 5% milk or 5% BSA for 1 h at room temperature, then appropriate primary antibodies were diluted in blocking buffer and antibody incubation (4°C, overnight). After several washes with PBST, the membranes were incubated with secondary antibodies for 1 h at room temperature. After several washes with PBST, the immunoreactive bands were visualized using ChemiDoc™ XRS+ (Bio-Rad) and chemiluminescence reagents (Millipore, WBKLS0500, Billerica, MA, USA). Protein expression levels were normalized against β-actin. The following primary antibodies were used: rabbit monoclonal anti-LC3 (1:1,000; cat. no. 3868; Cell Signaling Technology, CA, USA), rabbit polyclonal anti-P62 (1:500; cat. no. 5114; Cell Signaling Technology, CA, USA), rabbit monoclonal anti-ATG5 (1:500; cat. no. 12994; Cell Signaling Technology, CA, USA), rabbit Polyclonal anti-Cathepsin B (1:500; cat. no. WL01089; WanLei Biological Company, Shenyang, China), and mouse monoclonal anti-β-actin (1:1,000; cat. no. A5441; Sigma, USA).

### Table 1

<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>Treating dose</th>
</tr>
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<tbody>
<tr>
<td>1-16</td>
<td>0.05IU</td>
</tr>
<tr>
<td>17</td>
<td>0.2IU</td>
</tr>
<tr>
<td>18</td>
<td>0.4IU</td>
</tr>
<tr>
<td>19</td>
<td>0.6IU</td>
</tr>
<tr>
<td>20</td>
<td>0.8IU</td>
</tr>
<tr>
<td>21</td>
<td>0.8IU</td>
</tr>
<tr>
<td>22</td>
<td>0.8IU</td>
</tr>
<tr>
<td>23-</td>
<td>0.2IU</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5' → 3')</th>
</tr>
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<tbody>
<tr>
<td>ATG5</td>
<td>Forward: AGCCAGGTGATGATTCCAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGCTGGGGGACAATGCTAA</td>
</tr>
<tr>
<td>P62</td>
<td>Forward: CCTCTGAATCTCGGAATTTCA</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAATACTGACGTTGAGGC</td>
</tr>
<tr>
<td>CathepsinB</td>
<td>Forward: TCTTTGACCTCTTCTGTGC</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACAGTGCACACAGCTTCTC</td>
</tr>
<tr>
<td>BECN1</td>
<td>Forward: ATGGAGGGTGCTAAAGGGTCT</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCCCTCCTGAGTTAGCTCTC</td>
</tr>
<tr>
<td>ULK1</td>
<td>Forward: AAGTTCGGATCTCTGCAAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGATGTTTCTGTTGTTGTTTCC</td>
</tr>
<tr>
<td>GDF9</td>
<td>Forward: TCTTTAGATCTCTGCAGGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGTCAGTCCCATACAGCTGCA</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: TCGTGCGTGACATCAGAGCAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: CAAGAAGGAGGCTGGAAAA</td>
</tr>
</tbody>
</table>
Statistical analysis

Experiments involved in the study were replicated at least three times. The data were analyzed using SPSS statistical software. A t-test was used to analyze differences between groups. Differences were considered significant if \( p < 0.05 \). Data were expressed as the mean ± standard deviation.

Results

Construction of insulin mice model

In order to verify the validity of the mice model, the level of insulin in the serum of pregnant mice on D4 was detected using ELISA. As shown in Fig. 1, the level of insulin was significantly higher in the insulin group than in the control group \((** p<0.001)\).

Ovarian function was directly impaired on the effect of insulin

To investigate whether ovarian function was influenced by insulin treatment, we first tested the pregnant mice serum levels of steroid hormones, including E2, PROG, LH, and FSH using ELISA. Fig. 2A shows that the levels of E2, PROG, LH, and FSH were obviously lower in the insulin-treated group when compared with those of the control group \((p<0.05)\). As one of the oocyte secreted factors, GDF9 is crucial to follicular development. GDR9 plays an important role in follicular development, luteum formation, and embryo development [28, 29]. RT-PCR results showed a significant \((p<0.05)\) decrement of GDF9 mRNA on the effects of insulin (Fig. 2B). Morphological examination of ovaries on pregnant D4 was further examined by H&E staining. Changes in the histological structure of the ovary in the insulin-treated group were examined. More multiple immature follicles and less luteinization was observed in the insulin-treated group (Fig. 2C). We found that pregnancy rate was lower in the insulin-treated group (Table 3). These results showed that insulin could directly impair ovarian function during embryo implantation.

Following insulin treatment, the imbalance of autophagy was appeared in ovary on D4

The mRNA level of autophagy-related genes (LC3, BECN1) showed significant upregulation in ovarian endometriomas [30]. Loss of autophagy-related gene BECN1 or ATG7 within the perinatal ovary could result in the premature loss of female germ cells [31]. Therefore, we further investigated whether the autophagy balance was disrupted by insulin. The mRNA levels of autophagy-related genes, including ULK1, Cathepsin B, BECN1, P62, and ATG5 were first detected by RT-PCR. As shown in Fig. 3A, we found a significant increase of P62 \((p<0.01)\) and a decrease of Cathepsin B \((p<0.001)\), BECN1 \((p<0.01)\), and ULK1 \((p<0.001)\) mRNA in ovaries from insulin-treated mice on pregnancy D4. However, no significant difference was found in ATG5 mRNA \((p>0.05)\). In addition, the expressions of autophagy-related proteins LC3, P62, ATG5, BECN1, and Cathepsin B were detected by western blot. As shown in Fig. 3B and C, the expressions of LC3, BECN1, and Cathepsin B proteins in ovaries from insulin-treated mice on pregnancy D4 were significantly reduced compared to those from control mice \((p<0.05)\). However, the P62 protein level \((p<0.05)\) in ovaries was significantly increased in response to insulin, and no significant difference was found in ATG5 expression \((p>0.05)\) between these two groups. These data suggested that ovarian autophagy is imbalanced due to insulin on pregnancy D4.

Furthermore, FOXO3a gene silencing efficiently prevented autophagy-related gene expression and autophagosome formation [32]. We further detected protein FOXO3a. FOXO3a proteins in ovaries from insulin-treated mice on pregnancy D4 were significantly reduced \((p<0.01)\).
Fig. 2  Ovarian function was directly impaired due to the effects of insulin

(A) ELISA shows a decrease of E2 (** p<0.01), PROG (** p<0.01), LH (** p<0.001), FSH (*** p<0.001) (n=5).  (B) RT-PCR shows a decrease of GDF9 in insulin group (** p<0.01) (n=3).  (C) H&E shows a greater number of immature follicles and less luteinization in the insulin group.  Arrows indicate multiple immature follicles, and triangles indicate luteinization (n=3).

Table 3  Pregnancy rate of mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Pregnancy</th>
<th>Non-pregnancy</th>
<th>Pregnancy rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>17</td>
<td>3</td>
<td>85%</td>
</tr>
<tr>
<td>Insulin</td>
<td>20</td>
<td>14</td>
<td>6</td>
<td>70%</td>
</tr>
</tbody>
</table>
Fig. 3 Autophagy was affected by insulin
(A) Expressions of autophagy-related mRNA was affected (n=3). RT-PCR shows an increase of P62 mRNA (** p<0.01) and decreases in Cathepsin B mRNA (*** p<0.001), BECN1 mRNA (**) p<0.01), and ULK1 mRNA (*** p<0.001). No significant difference was found in ATG5 (p>0.05). (B) Western Blot showed an increase in P62 (* p<0.05) and decreases in LC3 (** p<0.01) Cathepsin B (* p<0.05), BECN1 (* p<0.05), and FOXO3A (**) p<0.01). No significant difference was found in ATG5 (p>0.05). (C) A quantitative representation of the results is shown in (B) (n=3).
Discussion

PCOS and T2DM were often accompanied by ovarian dysfunction and insulin resistance. These two metabolic disorders are also reportedly associated with high miscarriage rates, decreased pregnancy rates, and low live birth rates. Some research has proven that ovariectomy in mice could cause an increased level of insulin [33, 34]. The present study demonstrated that women with PCOS had lower serum FSH [18]. Embryos from PCOS women were found to have a slower development during the blastocyst stage [17]. In this study, we constructed the insulin intervention pregnant mouse model on D4. As expected, the level of insulin was significantly increased in the insulin-treated group compared with the control group. In order to confirm whether the ovarian function was influenced by insulin treatment, we further detected pregnant mice serum levels of steroid hormones (E2, PROG, LH, and FSH). As shown in results, we found that the serum level of E2, PROG, LH, and FSH were all significantly decreased in the insulin-treated group. We also detected GDF9 mRNA, an oocyte secreted factor that is crucial to follicular development. The mRNA level of GDF9 was significantly decreased in the insulin-treated group. Morphological examination of ovaries on pregnant D4 was also examined. An increased number of immature follicles and less luteinization were observed in the insulin-treated group. We also found a decreased pregnancy rate in the insulin-treated group, which may be caused by the dysfunction of ovary. Previous studies have focused on the impact of estrogen on insulin signals, while much less is known about the influence of insulin on estrogen [35]. Our study first clarified that the level of estrogen and related hormones decreased significantly under the treatment of insulin.

Autophagy is an intracellular degradation process, which is essential for cell growth, survival, differentiation, development, and protein homeostasis. A lack of the autophagy gene BECN1 in ovaries has been found to cause a reduction of circling progestogen and a preterm birth in pregnant mice [36]. LC3 expression is increased from the early to mid-luteal stages in mice [37]. High levels of BECN1 and LC3 in ovarian carcinomas have been found to indicate a better prognosis [38]. In our research, we found a significant increase of P62 and decreases of Cathepsin B, BECN1, and ULK1 mRNA in the ovaries of insulin-treated mice on pregnancy D4. The expressions of LC3, ATG5, BECN1, Cathepsin B, and FOXO3A proteins in ovaries from insulin-treated mice on pregnancy D4 were significantly reduced compared to those from control mice. However, the P62 protein level in the ovaries was significantly increased in response to insulin. These results indicate that insulin’s influence on ovarian functioning was autophagy-dependent. The balance of autophagy in ovaries on D4 was disturbed following insulin treatment.

Insulin administration to rats and to cultured cardiomyocytes in vitro following nutrient withdrawal, acutely suppressed autophagic signaling [39]. Study of heart tissue indicated that insulin treatment could restore the increase of autophagy caused by insulin deficiency, via regulating AKT and FOXO3a [40]. Another study of human mesenchymal stem cells indicated that, although diabetic sera induced autophagy at the early stage, it inhibited autophagy at a late stage. Inhibition of insulin on autophagy occurred through two mechanisms: by activating mTORC1 resulting in the inhibition ofULK1 and by activating protein kinase B, which inhibits FOXO3 [41, 42].

Pregnancy is a complex process, and a successful implantation is an essential factor. The embryo enters the uterus, which is prepared for implantation on pregnant D4. On pregnant D4, secretion of E2 showed a small peak, and PROG showed a sharp increase [12]. The imbalanced hormones on Day 4 result in abnormal embryo implantation later. Both PCOS and T2DM, which are characterized by hyperinsulinemia, are often accompanied by abnormal implantation. Our results showed that the balances of hormones were seriously disturbed after insulin treatment. However, we only collected data for mice on pregnant D4. A study carried by Julie S. Rhee reported that a decreased autophagy was observed in a high-fat/high-sugar diet-exposed mice deciduization [43]. More research on other pregnant days as well as the deciduization is needed.

In our study, we successfully constructed an insulin intervention mouse model, and we detected an imbalance of hormones in insulin-treated mice. We further detected the level of GDF9 mRNA and completed a morphological examination of ovaries on pregnant D4. We also detected the level of autophagy, and we found an imbalance of autophagy in insulin-treated mice. However, the molecular mechanism of these changes remains unclear. Further investigation is needed to reveal the mechanisms.
Conflict of Interest

The authors declared that they have no conflicts of interest to this work.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (No. 81300486), the Natural Science Foundation of Chongqing (No. cstc2015jcyjA10013), Scientific Research Program of Science and Technology Commission of Yuzhong District of Chongqing (No.20150104), and the Excellent Young Scholars of Chongqing Medical University (No.CYYQ201508).

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