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Prenatal androgen excess impairs beta-cell function by decreased sirtuin 3 expression

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Abstract

Prenatal androgen exposure induces metabolic disorders in female offspring. However, the long-term effect of maternal testosterone excess on glucose metabolism, especially on pancreatic beta-cell function, is rarely investigated. Our current study mainly focused on the effects of prenatal testosterone exposure on glucose metabolism and pancreatic beta-cell function in aged female offspring. By using maternal mice and their female offspring as animal models, we found that prenatal androgen treatment induced obesity and glucose intolerance in aged offspring. These influences were accompanied by decreased fasting serum insulin concentration, elevated serum triglyceride, and testosterone concentrations. Glucose stimulated insulin secretion in pancreatic beta cells of aged female offspring was also affected by prenatal testosterone exposure. We further confirmed that increased serum testosterone contributed to downregulation of sirtuin 3 expression, activated oxidative stress, and impaired pancreatic beta-cell function in aged female offspring. Moreover, over-expression of sirtuin 3 in islets isolated from female offspring treated with prenatal testosterone normalized the oxidative stress level, restored cyclic AMP, and ATP generation, which finally improved glucose-stimulated insulin secretion in beta cells. Taken together, these results demonstrated that prenatal testosterone exposure caused a metabolic disturbance in aged female offspring via suppression of sirtuin 3 expression and activation of oxidative stress in pancreatic beta cells.

Key Words
- prenatal androgen excess
- beta cell function
- aging
- Sirt3
- oxidative stress

Introduction

Polycystic ovary syndrome (PCOS), which affects 6–20% of reproductive-age women worldwide, is considered the most common cause of infertility (Dumesic et al. 2015). In addition to reproductive system disorders, it is also closely related to endocrine dysfunctions, such as type 2 diabetes (Wolf et al. 2018, Forslund et al. 2020). Hyperandrogenism, mainly caused by hypersecretion of androgen from an ovary, is an important feature in PCOS patients that can manifest in many aspects, including hirsutism and acne (Rosenfield & Ehrmann 2016). As a heritable disease, the transmission of PCOS-like features from mothers to their daughters was confirmed by many recent studies (Crisosto et al. 2019, Risal et al. 2019, Mimouni et al. 2021). Hyperandrogenism during the maternal stage was also reported to cause disorders in the endocrine system, reproductive system, and even nervous system of their offspring (Hu et al. 2015, Hakim et al. 2017, Huffman et al. 2017, Sun et al. 2020, Zhou et al. 2020). Androgen treatment during the late gestational stage produced female F1–F3 offspring with PCOS-like reproductive and
metabolic disorders, suggesting that androgen excess also has transgenerational effects (Risal et al. 2019). Our previous results confirmed that testosterone treatment during the late gestational stage induced disorders in sex hormone secretion of 3-month-old female offspring, including decreased estradiol and increased testosterone concentration, accompanied by reproductive system impairments (Zhou et al. 2020). However, the long-term effects of maternal androgen excess on the endocrine system of female offspring have been rarely investigated.

Pancreatic beta cells play critical roles during the maintenance of glucose homeostasis by synthesis and secretion of insulin. In response to glucose stimulation, ATP is produced inside the mitochondria through the tricarboxylic acid cycle and electron transport chain in pancreatic beta cells. Elevated ATP/ADP ratio causes the closure of the potassium channel and opening of the calcium channel which finally promotes the release of insulin. Pancreatic beta cells are extremely vulnerable to oxidative stress because of their intrinsically low antioxidant capacity (Lenzen 2008, Drews et al. 2010). As a mitochondrial deacetylase, the protective role of sirtuin 3 (Sirt3) against oxidative stress-induced apoptosis and dysfunction in pancreatic beta cells has attracted much attention recently (Caton et al. 2013, Zhou et al. 2017, Peterson et al. 2018). Although the phenotype remains normal in Sirt3 knock-out (KO) mice under basal condition (Lombard et al. 2007), the superoxide dismutase 2 (SOD2) acetylation level was elevated and glucose-stimulated insulin secretion (GSIS) was impaired in Sirt3 deficient islets. Moreover, the absence of Sirt3 aggravated lipotoxicity or hydrogen peroxide-induced pancreatic beta-cell dysfunction by over-activation of oxidative stress in pancreatic beta cells (Zhou et al. 2017, Peterson et al. 2018). On the other hand, over-expression of Sirt3 protected beta cells from high-fat diet (HFD)– or palmitate-induced dysfunction (Kim et al. 2015, Zhou et al. 2017). These results highlighted the pivotal role of Sirt3 during the protection of pancreatic beta-cell function against oxidative stress.

Maternal androgen treatment was reported to impair pancreatic beta-cell function and promote insulin resistance in both rats and sheep (More et al. 2016, Carrasco et al. 2020), but the underlying mechanism requires further elucidation. Based on these premises, our current study aimed to identify the long-term influences of late gestational androgen excess on the endocrine system in female offspring especially focused on glucose metabolism and pancreatic beta-cell function. We hypothesized that maternal androgen excess may induce disorders of glucose metabolism in aged female offspring due to impaired pancreatic beta-cell function, through decreased Sirt3 expression and increased oxidative stress. To test this hypothesis, we examined the long-term effects of maternal testosterone treatment on glucose metabolism and pancreatic beta-cell function of female offspring as well as the protective role of Sirt3 against these influences.

Materials and methods

Animal treatment

All animal protocols were approved by the Animal Care and Use Committee of the Model Animal Research Center of Nanjing Medical University. Female C57BL/6 mice were purchased from Nanjing Medical University and housed in temperature-controlled (21–22°C) pathogen-free conditions with 12 h light: 12 h darkness cycle and free access to water and food. All animals were acclimatized to the laboratory environment for at least 1 week before experiments.

Testosterone injection was performed as previously described (Zhou et al. 2020). The solvent was prepared by mixing sesame oil (S3547, Sigma) and benzyl benzoate (B6630, Sigma) in a ratio of 1:1. Testosterone (T1500, Sigma) was dissolved in a solvent to a final concentration of 1 mg/mL. A total of 16 pregnant C57BL/6 mice were divided into two groups (n=8 for each group), followed by daily subcutaneous injection with testosterone in a dose of 2.5 mg/kg body weight or equivalent solvent as control (e.g. 75 µL testosterone solution or solvent for a 30 g mouse) from days post coitum (dpc) 14 to 19. The body weight and fasting blood glucose concentration of female offspring were measured every 6 weeks starting from 6 to 48 weeks of age. In this study, 12-week-old mice were defined as the young group, and 48-week-old mice were defined as the aged group.

Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

OGTT and ITT were performed in both young and aged mice. For OGTT, all mice were fasted overnight before oral gavage of glucose in a dose of 2 g/kg body weight. Blood glucose concentrations were measured by a glucometer (Johnson & Johnson) at time points 0, 15, 30, 60, and 120 min after glucose administration. During OGTT, blood samples were collected at different time points for serum insulin concentration measurement. For blood sample collection, the tail tip (~1 mm) of the mouse was amputated.
by a scissor, then blood was moved to the tip of the tail by gently ‘milking’ the tail and collected in a capillary tube. Samples of different time points were drawn by removing the clot or re-cutting when necessary. About 30 µL of blood sample was collected each time.

For ITT, all mice were fasted 6 h before i.p. injection of recombinant human insulin (Novo Nordisk) at a dose of 0.5 IU/kg body weight. Blood glucose concentrations were measured by a glucometer (Johnson & Johnson) at time points 0, 15, 30, 60, and 120 min after insulin injection.

Insulin resistance was calculated using the following formula: homeostasis model assessment-estimated insulin resistance (HOMA-IR) index = fasting serum glucose concentration (mmol/L) × fasting serum insulin concentration (mU/L)/22.5 (Zaafar et al. 2014).

**Islet isolation and treatment**

Mouse pancreatic islets were isolated as previously described (Zhou et al. 2017). Briefly, islets were isolated from different mice by dissociation of the pancreas, followed by gradient centrifugation at 1360 g for 20 min. Islets were then selected under a stereomicroscope and cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and 100 IU/mL penicillin and 100 µg/mL streptomycin (Invitrogen), at 37°C in a humidified atmosphere with 5% CO2. All islets were cultured for 48 h before further treatment.

Testosterone was purchased from Sigma and dissolved in DMSO (D2650, Sigma). Islets were treated with testosterone in a final concentration of 10−6 mol/L for 48 h followed by Western-blot analysis or GSIS study.

Sirt3 over-expression adenovirus (AdSirt3) was purchased from GenePharma (Shanghai, China), Sirt3 DNA fragment (NM_022433.2) was synthesized and ligated to pADV-CMV-IRES-GFP vector before being transfected into 293A cells for virus production and amplification. For virus infection, mouse islets were isolated and infected with AdSirt3 or AdGFP as control (m.o.i. = 100) for 48 h before different experiments or analysis.

GSIS study was performed as previously described (Zhou et al. 2017). Briefly, mouse islets were incubated in KRKBH buffer containing 2.8 mM glucose for 1 h before stimulation with KRKBH buffer containing 16.7 mM glucose. The KRKBH buffer supernatant was collected after both basal (2.8 mM glucose) and stimulation (16.7 mM glucose) for insulin ELISA analysis and the islets were harvested for protein amount measurement.

Enzyme-linked immunosorbent assay (ELISA)

All ELISA kits used in our current research are commercially available and validated in published results by our group or other researchers. The mouse insulin ELISA kit was purchased from Mercodia (10-1247-01, Mercodia, Uppsala, Sweden. Sensitivity: 0.2 ng/mL) (Kleiner et al. 2018, Figueiredo et al. 2019, Zhou et al. 2020), mouse triglyceride ELISA kit was purchased from Abcam (ab65336, Abcam. Sensitivity: 2 nmol/mL) (Brekk et al. 2020, Drori et al. 2020, Zhou et al. 2020), mouse estradiol ELISA kit was purchased from BioVision (K3830, BioVision. Sensitivity: 2 ng/L) (Zhou et al. 2020, Mousavy Gharavy et al. 2021), mouse testosterone ELISA kit was purchased from Abcam (ab108666, Abcam. Sensitivity: 0.07 ng/mL) (Morgan et al. 2020, Zhou et al. 2020), The reactive oxygen species (ROS) level was measured by DCFDA Cellular Reactive Oxygen Species Detection Assay Kit (ab113851, Abcam) (Zhou et al. 2017, Lei et al. 2021). All experiments were performed according to the manufacturer’s instructions. ATP and cyclic AMP (cAMP) measurements were performed as previously described (Zhou et al. 2017). Briefly, islets were incubated in KRKBH buffer containing 2.8 mM glucose for 1 h, followed by stimulation with KRKBH buffer containing 16.7 mM glucose for 2 h. The supernatant was collected for ATP or cAMP measurement by the ATP Colorimetric/Fluorometric Assay Kit (K354, BioVision. Sensitivity: 1 nmol/mL) or Cyclic AMP ELISA Kit (581001, Cayman. Sensitivity: 0.1 pmol/mL) (Zhou et al. 2017). Intra-assay and inter-assay coefficient of variation (CV) of samples in different groups were shown in Table 1.

Quantitative RT-PCR and Western blotting analysis

Quantitative RT-PCR was performed as previously described (Zhou et al. 2017, 2020), details of primers are listed in Table 2.

Western blotting was performed as previously described (Zhou et al. 2017, 2020), and the following primary antibodies were used: Sirt3 (1:1000, 2627, Cell Signaling Technology), Ac-SOD2 (1:1000, Abcam), SOD2 (1:1000, Abcam), beta-actin (1:5000, Santa Cruz). Horseradish peroxidase (HRP)-linked anti-rabbit IgG (1:2000, Cell Signaling Technology) or anti-mouse IgG (1:5000, Cell Signaling Technology) was used as secondary antibodies. Sirt3 and Ac-SOD2 antibodies were validated by Western blotting in islets isolated from Sirt3 knock-out mice in our previous work (Zhou et al. 2017).
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Immunohistochemical (IHC) staining

IHC staining was performed as previously described (Zhou et al. 2017, 2020, Hua et al. 2020). Briefly, the mouse pancreas was fixed in 4% paraformaldehyde and embedded in paraffin after dehydration. Tissues were then cut into 4 μm sections, followed by rehydration and permeabilization. Insulin antibody (1:2000, Cell Signaling Technology), 4-hydroxynonenal (4-HNE) antibody (1:50, Abcam), and Sirt3 antibody (1:200, Cell Signaling Technology) were used as primary antibodies, and HRP-linked goat anti-rabbit antibody (1:400, Dako, Glostrup, Denmark) was used as the secondary antibody.

Statistical analysis

All data are presented as mean ± S.E.M. Differences between the groups were analyzed by a two-tailed unpaired Student’s t-test with Welch correction or one-way ANOVA, followed by the Tukey post hoc test where appropriate. Statistical comparisons were made using GraphPad Prism 6 (GraphPad Software). A P value < 0.05 was considered statistically significant.

Results

Prenatal testosterone exposure leads to glucose intolerance in aged female offspring

Testosterone treatment significantly increased the new-born body weight of these mice (Fig. 1A). In addition, prenatal testosterone treatment also induced obesity in adult female offspring (Fig. 1B and C). By week 24, the fasting blood glucose concentration was higher in the experimental group than in the control group. This influence continued and was exacerbated with increased age (Fig. 1D).

Both OGTT and ITT were performed to evaluate the glucose tolerance and insulin sensitivity of these female offspring mice at 12- and 48-weeks of age. Glucose tolerance and insulin sensitivity were disturbed in aged mice compared to the young groups (Fig. 2A, B, C and D). Insulin sensitivity was affected by testosterone treatment in the young group, but their glucose tolerance was not affected (Fig. 2A, B, C and D). Serum insulin concentrations at different time points in OGTT showed a marked reduction in glucose-induced insulin secretion in prenatal testosterone-treated aged mice, suggesting that pancreatic beta-cell function was impaired (Fig. 2E and F). In addition, the HOMA-IR index was also significantly increased in prenatal testosterone-treated aged mice compared to aged controls (Fig. 2G).
Prenatal testosterone exposure impairs hormone expression in aged female offspring

One week after OGTT, these mice were sacrificed, and blood samples were collected to measure the serum hormone concentration. Fasting serum insulin concentration was not affected in the young group but was significantly decreased in prenatal testosterone-treated aged mice compared to controls (Fig. 3A). Prenatal testosterone treatment induced obesity in female offspring, therefore, we analyzed serum triglyceride concentration in both young and aged mice. Serum triglyceride was slightly increased in young mice after prenatal testosterone treatment but dramatically elevated in aged mice compared to the control group (Fig. 3B). Consistent with our previous results (Zhou et al. 2020), prenatal testosterone treatment significantly inhibited serum estradiol (E2) concentration and increased serum testosterone in young offspring (Fig. 3C and D). However, serum E2 concentration was similar between the control group and the testosterone-treated group in aged mice (Fig. 3C). Although serum testosterone concentration decreased with age, it was still higher in prenatal testosterone-treated aged offspring compared to aged controls (Fig. 3D).

Pancreatic beta-cell function is affected after prenatal testosterone exposure

Because the glucose tolerance was disturbed and serum insulin concentration was decreased during OGTT in prenatal testosterone-treated aged offspring, we next evaluated pancreatic beta-cell area and function in these mice. Beta-cell area was not affected in young and aged mice regardless of treatments (Fig. 4A and B). However, the beta-cell function, which was reflected by GSIS in cultured islets, was significantly impaired in prenatal testosterone-treated aged mice compared to the aged control group (Fig. 4C).

Table 2 Details of primers for quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward (5′-3′) (bp)</th>
<th>Reverse (5′-3′) (bp)</th>
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<tbody>
<tr>
<td>Glut2</td>
<td>TCAGAAGACAAGATCACCGGA</td>
<td>GCTGGTGTGACTGTAAGTGGG</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>CCAAGACCAACGGCGTATCA</td>
<td>GTCAAGGGTCTGGACTTCTCT</td>
</tr>
<tr>
<td>Sirt3</td>
<td>ATCCCGGACTTCAAGATCCC</td>
<td>CAACATGAAAAAGGCTCTGGG</td>
</tr>
<tr>
<td>Insulin1</td>
<td>CCAGCTAAATACAGACCACTCAG</td>
<td>ACAAAAGCTGGTGGGTGT</td>
</tr>
<tr>
<td>Insulin2</td>
<td>GGAATCACTCGGCCTTAAA</td>
<td>CAAGTCTAAACTATGCGAGGA</td>
</tr>
<tr>
<td>Pdx-1</td>
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<td>CTCGGTTCCATCGGGAAAAGG</td>
</tr>
<tr>
<td>NF-κB</td>
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<td>TTGTTGACAGTTATTTCTGTGG</td>
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<tr>
<td>IL-1β</td>
<td>GCAACTTGGTCTTGAATCACA</td>
<td>ATCTTTGGGGTCCGTCACACT</td>
</tr>
<tr>
<td>Gapdh</td>
<td>TGGATTTGGACGCATGGTC (20)</td>
<td>TTTGCACCTGGTACGTGTGGAT (21)</td>
</tr>
</tbody>
</table>
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Figure 2
Prenatal testosterone (T) exposure leads to glucose intolerance in aged female offspring. (A and B) ITT and (C and D) OGTT were performed at 12-weeks (young) and 48-weeks (aged) of age (n = 6–16 for each group). (E and F) Serum insulin concentrations were measured at different time points during OGTT (n = 12–16 for each group). (G) Insulin resistance was estimated by HOMA-IR index (n = 12–16 for each group). Data are presented as mean ± s.e.m. AUC was calculated by GraphPad Prism 6, and the individual mouse data were integrated to Y = 0. Control: female offspring treated with sesame oil and benzyl benzoate mixture; treatment: testosterone-treated female offspring. *Treatment-young vs control-young, #Treatment-aged vs control-aged.

* or *P < 0.05, ** or **P < 0.01.

Figure 3
Prenatal testosterone (T) exposure impairs hormone expression in aged female offspring. Mice were sacrificed after OGTT, and blood samples were collected for (A) fasting serum insulin, (B) serum triglycerides, (C) serum E2, and (D) serum testosterone concentration measurement (n = 6–7 for each group). Control: female offspring treated with sesame oil and benzyl benzoate mixture; treatment: testosterone-treated female offspring. Data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01.
Sirt3 expression in pancreatic beta-cell is decreased after prenatal testosterone exposure

Elevated oxidative stress and disturbed mitochondrial function were reported to be involved in prenatal androgen treatment-induced pancreatic beta-cell dysfunction in adult offspring, thus we evaluated the oxidative stress level in pancreatic beta cells of these mice. The 4-HNE staining results suggested that oxidative stress in pancreatic beta-cell increased with age but was...
exacerbated in prenatal testosterone-treated aged mice (Fig. 5A and B).

Our previous data have already confirmed that mitochondria deacetylase Sirt3 protects the pancreatic beta-cell from oxidative stress-induced dysfunction (Zhou et al. 2017), we, therefore, analyzed the Sirt3 expression in islets of these mice. IHC staining showed that Sirt3 expression was decreased in prenatal testosterone-treated aged mice compared to other groups (Fig. 6A and B). Consistently, Western blot results also showed that Sirt3 expression was lower in islets of prenatal testosterone-treated aged mice (Fig. 6C and D). The acetylation level of superoxide dismutase 2 (SOD2), a well-known downstream substrate of Sirt3, was also detected. As shown in Fig. 6C and E, Ac-SOD2 was increased in islets of prenatal testosterone-treated aged mice compared to the control group.

In addition to Sirt3, prenatal testosterone treatment also affected mRNA expression of marker genes related to pancreatic beta-cell function (Fig. 6F). Although glucokinase and insulin mRNA expression were similar between the two groups, Glut2 and Pdx-1 expression were significantly decreased in islets of prenatal testosterone-treated aged mice. Furthermore, prenatal testosterone treatment also up-regulated inflammatory cytokines in islets of aged offspring, including NF-κB and IL-1β, which may also contribute to impaired beta-cell function (Fig. 6F).

**Elevated serum testosterone contributes to impaired Sirt3 expression and beta-cell function**

Increased testosterone was reported to induce pancreatic beta-cell dysfunction (Mishra et al. 2018, Navarro et al. 2018). In order to identify whether decreased Sirt3
expression and impaired GSIS was caused by up-regulated serum testosterone concentration in prenatal testosterone-treated aged offspring, we isolated islets from aged mice in the control group and treated these islets with testosterone. As shown in Fig. 7A, B and C, testosterone treatment significantly impaired Sirt3 expression and increased Ac-SOD2 level to a pattern similar to that in islets isolated from prenatal testosterone-treated aged offspring.

Consistently, the oxidative stress level was elevated after testosterone treatment in control islets, and their GSIS was decreased (Fig. 7D and E). Both oxidative stress and GSIS were not affected by DMSO treatment alone (Fig. 7F and G), which further confirmed that impaired cell function was caused by testosterone exposure. These results suggested that decreased Sirt3 expression and impaired pancreatic beta-cell function in prenatal testosterone-treated aged

**Figure 7**

*In vitro* testosterone (T) treatment down-regulates Sirt3 expression and impairs pancreatic beta-cell function in islets of normal aged mice. Islets were isolated from normal-aged mice and treated with 10^{-6} mol/L testosterone, DMSO, or empty control for 48 h. (A) Western-blot analysis of Sirt3 and Ac-SOD2 (K68) expression after *in vitro* testosterone treatment. (B) Quantification of Sirt3 protein expression level (n = 3). (C) Quantification of Ac-SOD2 protein expression level (n = 3). (D) ROS level measurement after *in vitro* testosterone treatment (n = 6). (E) GSIS study of islets after *in vitro* testosterone treatment (n = 6). (F) ROS level measurement after empty control or DMSO treatment (n = 6). (G) GSIS study of islets after empty control or DMSO treatment (n = 6). Data are presented as mean ± s.e.m. *P < 0.05.

**Figure 8**

Restoration of Sirt3 protects beta-cell from prenatal testosterone (T) treatment-induced dysfunction in aged offspring. Islets were isolated from prenatal testosterone-treated aged female offspring or control mice (offspring of sesame oil and benzyl benzoate treated aged female mice) followed by AdSirt3 or AdGFP infection for 48 h. (A) Western-blot analysis of Sirt3 and Ac-SOD2 (K68) expression after Sirt3 over-expression in islets isolated from prenatal testosterone-treated aged female offspring. (B) Quantification of Ac-SOD2 protein expression level (n = 3). (C) ROS level measurement after Sirt3 over-expression (n = 6). (D) ATP concentration measurement after Sirt3 over-expression (n = 6). (E) cAMP concentration measurement after Sirt3 over-expression (n = 6). (F) GSIS study in pancreatic islets after Sirt3 over-expression (n = 6). Data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001.
offspring was, at least partially, contributed by increased serum testosterone concentration.

**Restoration of Sirt3 protects beta-cell from prenatal testosterone treatment-induced dysfunction in aged offspring**

Since prenatal testosterone treatment impaired Sirt3 expression in pancreatic beta-cell of aged offspring, we sought to evaluate the therapeutic effect of Sirt3 in prenatal testosterone-treated aged mice. Islets were isolated from these mice and infected with Sirt3 over-expression adenovirus or control green fluorescent protein (GFP) virus. Over-expression of Sirt3 was confirmed by Western-blot (Fig. 8A). SOD2 acetylation was decreased after Sirt3 over-expression (Fig. 8A and B), which was accompanied by a downregulated ROS level (Fig. 8C). In the functional study of pancreatic islets, reduced ATP and cAMP produced by the islets of prenatal testosterone-treated aged mice were both reversed by Sirt3 over-expression (Fig. 8D and E). Insulin secretion was decreased in the prenatal testosterone-treated group, but Sirt3 over-expression partially restored this impairment (Fig. 8F). These results suggested that restoration of Sirt3 could protect pancreatic beta cells from prenatal testosterone treatment-induced dysfunction in aged female offspring through decreased oxidative stress and improved insulin secretion pathway.

**Discussion**

In our current study, we examined the long-term effects of prenatal androgen exposure on glucose metabolism of female offspring. Testosterone treatment during the late gestational stage induced obesity in aged female offspring, this was accompanied by impaired glucose tolerance and decreased GSIS, as well as disrupted serum hormone concentrations. Although the pancreatic beta-cell area was not affected, beta-cell function was significantly decreased. Using isolated islets as a cell model, we further confirmed that elevated serum testosterone concentration contributed to decreased Sirt3 expression, activated oxidative stress, and decreased insulin secretion in pancreatic beta cells. Finally, we demonstrated that negative effects caused by prenatal testosterone exposure could be partially restored by over-expression of Sirt3 in pancreatic islets isolated from aged female mice. Taken together, these results suggested that Sirt3 plays a key role in protecting pancreatic beta-cell function from maternal testosterone excess-induced impairments of glucose metabolism in aged female offspring.

Maternal androgen excess was reported to induce disorders in both endocrine and reproductive systems, two important features of PCOS-like phenotype (Ramezani Tehrani et al. 2014, Chappell et al. 2020). However, most studies have focused on defects in young offspring. Here, we demonstrated that negative effects of prenatal testosterone exposure on glucose metabolism were exacerbated with increased age, as impaired glucose tolerance and decreased GSIS could be detected only in elderly offspring. Although body weight was higher in prenatal testosterone-treated female offspring throughout the monitored period, increased serum triglycerides were observed only in aged mice, suggesting that fatty acid metabolism could be impaired in young litters but accumulated in aged offspring.

In our previous study (Zhou et al. 2020), we treated maternal mice with testosterone throughout pregnancy (dpc 1–19), but the parturition rate was dramatically decreased (25% vs 100%, treatment vs control). This result indicated that maintenance of normal testosterone concentration during the early gestational stage was critical for embryo development and elevated maternal testosterone may cause embryo loss. Based on these results and papers published by other groups (Ramezani Tehrani et al. 2013, 2014, Hu et al. 2015), we treated pregnant mice with testosterone during the late gestational stage and evaluated the long-term effects of maternal testosterone exposure on female offspring.

Impaired insulin sensitivity is one of the prominent features of both type 2 diabetes and PCOS (Pasquali 2018). Prenatal androgen treatment was reported to induce insulin resistance (Morisset et al. 2013, Puttabyatappa & Padmanabhan 2017, Ferreira et al. 2021). Different from the glucose tolerance test, the ITT results in our current study suggested that insulin sensitivity was decreased in both young and aged offspring, but exacerbated with increased age. Insulin resistance is closely related to obesity and is often accompanied by hyperinsulinemia in the early stage of type 2 diabetes (Shanik et al. 2008, Thomas et al. 2019). Although obesity was observed in those mice, fasting serum insulin and serum triglyceride were not affected in young offspring, suggesting that the influences of prenatal testosterone exposure were manifested in a mild way in young offspring but exacerbated with increased age, and finally predisposed these mice to type 2 diabetes.

It was well recognized that androgen has bidirectional regulatory effects on pancreatic beta-cell function between males and females. Testosterone deficiency impairs beta-
cell function which predisposes males to type 2 diabetes (Xu et al. 2019). In females, androgen exposure induces hyperinsulinemia and finally leads to beta-cell failure (Mishra et al. 2018, Navarro et al. 2018). Acute androgen treatment in pancreatic beta cells stimulates hypersecretion of insulin through cAMP- and mTOR-dependent pathways. Moreover, acute androgen excess also leads to elevated mitochondrial respiration and oxygen consumption in female islets. As a result, long-term androgen exposure in female mice leads to oxidative stress and mitochondrial dysfunction in pancreatic islets, which finally promotes beta-cell failure (Navarro et al. 2018). Because serum testosterone concentration was elevated in prenatal androgen treated female offspring, we hypothesized that activated oxidative stress and impaired pancreatic beta-cell function might be related to an aberrant serum testosterone concentration. We isolated islets from control aged mice and treated these islets with testosterone. As expected, in vitro testosterone treatment-induced oxidative stress and impaired GSIS of beta cells. In addition, Sirt3 expression in pancreatic islets was also decreased after testosterone treatment, which was accompanied by increased SOD2 acetylation level. These results were consistent with our in vivo data that prenatal testosterone-treatment impaired Sirt3 expression in pancreatic islets of 48-week-old female offspring. Interestingly, serum testosterone concentration was increased in both young and aged female offspring, but increased oxidative stress in pancreatic islets and glucose intolerance could be detected only in 48-week-old mice. These findings imply that young individuals may have a relatively higher antioxidant capacity than aged mice (Kregel & Zhang 2007, Liguori et al. 2018). This explanation was supported by the other finding in our current study that Sirt3 expression in islets of prenatal androgen treated aged mice was also decreased compared to young groups.

Sirt3 is a mitochondrial deacetylase that participates in a series of biological pathways, including fatty acid oxidation, glucose metabolism, and insulin secretion (Feldman et al. 2012, Singh et al. 2018). Through deacetylation of its downstream target SOD2, Sirt3 is supposed to ameliorate oxidative stress and further protect the pancreatic beta-cell from lipotoxicity-induced dysfunction (Zhou et al. 2017). Moreover, Sirt3 is also involved in the regulation of the aging process, as its expression was reported to be suppressed with aging (Brown et al. 2013), which is consistent with our current results. In addition, activation of Sirt3 was supposed to restore or prevent age-associated mitochondrial dysfunction (Kincaid & Bossy-Wetzel 2013). Our current data further demonstrated that over-expression of Sirt3 protected beta-cell function in prenatal testosterone-treated aged offspring through deacetylation of SOD2, downregulation of oxidative stress, and restoration of ATP generation.

Prenatal testosterone treatment induced pancreatic beta-cell dysfunction in aged female offspring which was accompanied by decreased Glut2, Pdx-1 mRNA expression, and activated inflammatory cytokines, such as NF-κB and IL-1β. Although GSIS was impaired, insulin mRNA expression was not affected, suggesting that the insulin secretion pathway might be impaired in these aged islets after maternal testosterone treatment. Furthermore, inhibition of glucose transportation and exacerbation of inflammatory responses may also be involved in this process (Ferreira et al. 2021). However, this hypothesis still needs further investigation.

In summary, our present study demonstrated that prenatal testosterone treatment induced glucose intolerance in aged female mice. This negative effect was contributed by elevated serum testosterone concentration, decreased Sirt3 expression, and activated oxidative stress in pancreatic islets. Over-expression of Sirt3 protected beta cell from prenatal testosterone treatment induced dysfunction, as evidenced by ameliorated oxidative stress and partially restored GSIS. These results suggested that Sirt3 may be a therapeutic target for maternal androgen excess-induced metabolic disorders. However, further clinical studies and investigations on human islets are necessary to confirm these findings.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Y Z, J C, and R J designed and performed the experiments, analyzed the data, and wrote the article. M G and Y L performed the experiments and analyzed the data. The manuscript was guaranteed by J C and R J.

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