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Ovarian stimulation perturbs methylation status of placental imprinting genes and reduces blood pressure in the second generation offspring

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ABSTRACT

Objective(s): Assisted reproductive technology (ART) is associated with DNA methylation dysfunction of offspring. However, it is unclear whether ovarian stimulation (OS) is responsible for DNA methylation dysfunction of offspring

Study design: We built the first-generation (F1) and second-generation (F2) offspring mice model of ovarian stimulation. Bodyweight of F1 and F2 were measured. Expression levels of several imprinted genes (*Impact*, *H19*, *Igf2*, *Plagl1*, *Mest*, and *Snrpn*) in F1 placenta were tested. Methylation status of Plagl1 and H19 promoters was examined with bisulfite sequencing. Glucose tolerance, blood pressure, and heart rate were evaluated in F2 mice.

Results: The OS F1 showed elevated bodyweights in the 2nd, 3rd and 4th weeks, but the difference disappeared in the 5th week. *Plagl1* was down-regulated in OS F1. Promoters of *Plagl1* and *H19* were also hypermethylated in OS F1. F2 of OS mice had the similar bodyweight and glucose tolerance compared with the control F2. However, F2 of OS ♂F1 + OS♀ F1 showed the decreased systolic pressure, diastolic pressure, and heart rate.

Conclusions: Ovarian stimulation perturbs expression levels and methylation status of imprinted genes in offspring. The effect of ovarian stimulation may be passed to F2.

Introduction

Assisted reproductive technology (ART) is an effective treatment for infertile couples. However, the consequences of manipulating germ cells and early embryos are not fully known. ART children are at the increased risks of intrauterine growth retardation, premature birth, low birth weight, and genomic imprinting disorders [1,2]. In the past decade, more evidences proved that children born via ART are susceptible to cardiometabolic disorders, specifically elevated systolic and diastolic blood pressures, higher fasting glucose, elevated triglycerides, increased body fat composition, and increased incidence of subclinical primary hypothyroidism [3–8].

Genomic imprinting is an epigenetic phenomenon of the non-equivalent expression of maternally and paternally derived alleles in an individual. Genomic imprinting affects several dozens of genes and one allele is usually suppressed during development. Aberrant imprinting disturbs development and causes various disease syndromes [9]. DNA methylation is the most important molecular mechanism in imprinting establishment and maintenance.

More evidences suggest that ART is associated with widespread epigenetic alterations [10–13] and even leads to transgenerational glucose intolerance [14]. It is unclear whether the altered methylation is directly associated with ovarian stimulation, in-vitro procedures with zygotes and embryos, or the characteristics of infertility. Ovarian stimulation (OS) is an ART procedure increasing oocyte production. It is well known that imprint marks are established before meiosis in sperm, but they are still establishing during meiosis in oocytes [10,15,16]. Therefore, ovarian stimulation which induces oocyte maturation and ovulation may perturb the proper acquisition of imprint marks during oogenesis. This study aims to explore the possible adverse influences of ovarian stimulation on offspring.

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Materials and methods

Ovarian stimulation, mice model establishment, and placenta collection

All animal protocols were reviewed and approved by the Animal Care and Use Committee of Zhejiang University. Placentas were obtained from the crosses of C57BL/6 females and C57BL/6 males. For ovarian stimulation, 10 IU of Pregnant Mare's Serum Gonadotropin (PMSG) were administered to female mice, followed by the 10IU of Human Serum Chorionic Gonadotropin in 48 h. Females were mated with males and pregnancy was determined according to the presence of a vaginal plug in the next morning (Day 0.5). In the control group, ovulation was determined daily by means of vaginal smears. Ten female mice and five male mice were used as F0 to produce F1 in each group.

Placentas were collected at 19 days post-coitus (dpc). Placentas were bisected transversely to allow DNA and RNA extraction from the same embryo.

The second generation of offspring (F2) was produced with the first generation of offspring (F1): OS F1♂-OS F1♀; OS F1♂-control F1♀; control F1♂-OS F1♀; control F1♂-control F1♀.

Both F1 and F2 experiments were performed according to the guidelines established by Women's Hospital Commission for Animal Care in School of Medicine, Zhejiang University.

All F1 and F2 mice were weighted in 8 weeks after birth, and male and female mice were distinguished and separated from each other after 5 weeks. Nineteen OS F1 and twenty-two Control F1 were measured for bodyweight. Eight F2 mice for each group were measured for bodyweight.

Measurements of blood pressure, heart rate and plasma glucose concentration

Eight F2 mice for each group were measured for blood pressure, heart rate and glucose concentration. Heart rate, systolic blood pressure, and diastolic blood pressure were measured with tail cuff in untrained conscious mice by using the BP-98A tail cuff system (Softron, China). Blood pressures were measured 5 times per day for 3 consecutive days, and a mean value was calculated for each individual mouse.

Introperitoneal glucose tolerance test (IPGTT) was performed in unrestrained conscious mice after a 12-h overnight fast. Plasma glucose concentration was taken at fasting and 30 min, 60 min and 120 min after intraperitoneal glucose injection (2 g/kg bodyweight). Blood samples were taken by cutting off tip of the tail by scissors. Whole-blood glucose levels were determined with a portable glucometer. Food was supplied immediately after the last time point.

RNA isolation, RT, and quantitative analysis of imprinting genes

Total RNA was extracted from placentas. The cDNA was reversely transcribed from total RNA with the PrimeScript RT Reagent Kit (TAKARA). Six biological replicates were used for mRNA expression analysis

ABI 7500 Real-Time PCR (Applied Biosystems) was used to analyze the mRNA levels of *Impact, H19, Igf2, Plagl1, Mest,* and *Snrpn* with the RT product of total RNA. These genes have well-defined Differentially Methylated Regions (DMRs), which showed gamete-specific differences in methylation [12,17–19]. Samples were analyzed in duplicate and the threshold cycle (CT) was normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The value of CT is set at 10 times the standard deviation of the mean baseline emission calculated for PCR cycles 3–10[20].The mRNA level in each sample relative to one control sample was calculated with the relative CT method.

Table 1
Primer sequences.

Genes	Primers (5′-3′)
Gapdh	F- CAGGGCTGCTTTTAACTCTGG
	R- TGGGTGGAATCATATTGGAACA
Impact	F- TTGCAGGACTGTGAAGATGATGG
	R- GTTGATGTGTTTGAAACGGTCAGG
H19	F-GCACTAAGTCGATTGCACTGG
	R-GCCTCAAGCACACGGCCACA
Igf2	F-TGTTGACACGCTTCAGTTTGTCTG
	R-GAAGCAGCACTCTTCCACGATG
Plagl1	F-CCACAGTTTCAGTTGCAGATCCA
	R-CTGCTGCTGAGGTTGCAGTTG
Mest	F-GGCCATTGGATCCTATAAATCCGTA
	R-GGTAGTGGCTAATGTGGTCATCCAG
Snrpn	F- GGCCATTGGATCCTATAAATCCGTA
	R-AGAACTTGGTTTCAATGACTGTGGA

Primer sequences are provided in Table 1.

DNA isolation and bisulfite sequencing

DNA was isolated from one half of bisected placentas and bisulfite treatment was carried out with EpiTect Bisulfite Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Six biological replicates were used for DNA methylation analysis. Previously reported gene-specific primers for nested PCR amplification of *H19* and *Plagl1* as well as PCR reaction conditions were adopted [18,21]. Firstly, 2 μL of the first round product of *H19* (diluted by 100 times) were seeded into the second round PCR reaction. Then 2 μL of the first round product of *Plagl1* were seeded into the second round PCR reaction. All the second round PCR products were subjected to electrophoresis in 1.5% agarose. Analysis of the methylation status of the Igf2 and *H19* DMR was determined by cloning and sequencing of bisulfite-treated DNA. The purified PCR products were cloned by using the pMD19-T vector system (TaKaRa, Dalian, China). The cloned sequence was analyzed with 3730 DNA Analyzer polymers (Applied Biosystems, Carlsbad, CA).

Ten clones of one placenta were sequenced for DNA methylation analysis. All successfully sequenced clones were included in the final analysis.

Statistical analysis

Bodyweight and mRNA expression levels on placenta of F1 mice between control and ovarian stimulation groups were analyzed using Student's *t*-test. Bodyweight, blood pressure, heart rate, glucose concentration of four F2 groups were firstly subjected to Levene's test in order to explore the homogeneity of variance. One-way ANOVA and L.S.D post hoc tests were performed for multiple comparisons between F2 groups. Bisulfite sequencing results were compared by Pearson Chi-Square test. All the statistical analyses were performed on SPSS 16.0.

Results

To evaluate F1 growth, bodyweight was measured for consecutive 8 weeks (Fig. 1A). Because of the similar male/female sex ratio between the control and ovarian stimulation group (11/11 for control group and 9/10 for ovarian stimulation group), we considered the two groups shared the same sex ratio and analyzed the bodyweight data in a genderless way. The ovarian stimulation offspring showed the increased bodyweight at the ages of 2 weeks,

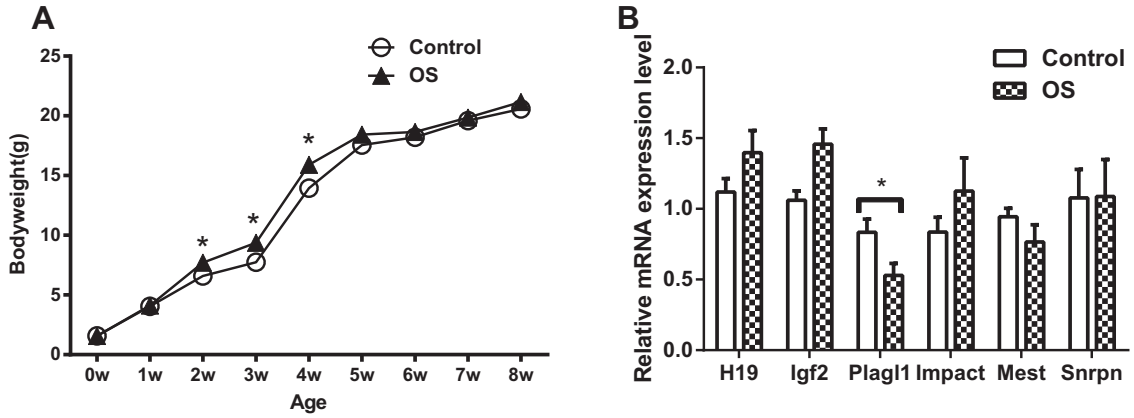

Fig. 1. Bodyweight of F1 and expression levels of imprinted genes in F1 placenta. (A) Bodyweight of Control and OS group.*, $P < 0.05$ (B) mRNA expression levels of imprinted genes in F1 placenta. *, $P < 0.05$.

3 weeks and 4 weeks, while the bodyweight was comparable among the groups at other ages. The mean litter size of OS group was 4.3. The mean litter size of OS group was 4.5. There were no significant difference between the two groups in litter size.

In order to examine the effects of ovarian stimulation on the expressions of imprinted genes, RNA was extracted from placenta tissue at 19 days postcoitus (dpc) and cDNA was generated, followed by relative RNA expression analysis. Six genes were assayed, including the paternally expressed genes (*Igf2, Plagl1, Impact, Mest,* and *Snrpn*) and the maternally expressed gene *H19* (Fig. 1B).

Plagl1 showed a half expression level after ovarian stimulation compared with the control group. The genes (*H19*, *Igf2*, and *Impact*) displayed up-regulation trends after OS without significant differences (P-value were 0.15, 0.07 and 0.36, respectively). The genes (*Mest* and *Snrpn*) did not alter their expression levels after OS.

The methylation status of *H19* and *Plagl1* by bisulfite sequencing is shown in Fig. 2A, B, and Table 2. *H19* DMR was highly methylated (554/700, 79%) after ovarian stimulation compared with the control group (628/860, 73%). The significance of difference was verified by Pearson's Chi-Square test ($P = 0.005$). *Plagl1* DMR was also highly methylated after ovarian stimulation (462/756, 61% for ovarian stimulation group; 275/613, 44% for control group, $P < 0.001$).

Table 2
Methylation status of *H19* and *Plagl1*.

	H19 control	*H19* OS	*Plagl1* control	*Plagl1* OS
Methylated CpG sites	628	554	275	462
Unmethylated CpG sites	232	146	337	294
Total sites	860	700	612	756

Trans-generational changes

We assessed the growth and long-term health profile of the second generation (F2) by measuring bodyweight, blood pressure, heart rate, and blood sugar. To determine the most powerful factor of F2 mice growth, we designed four F2 mice groups: Control♂ + Control♀, OS ♂ + OS♀, OS♂ +Control♀, and Control♂ + OS♀. The bodyweight data were taken according to the same protocol adopted in the measurements of F1 bodyweight. Four groups did

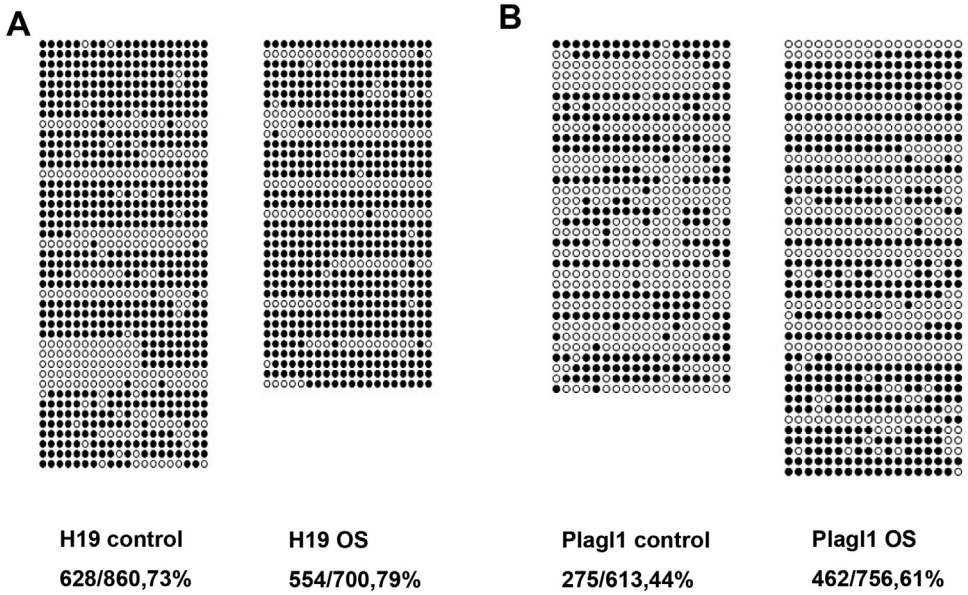

Fig. 2. Methylation status of *H19* and *Plagl1* DMRs in F1 placenta. Each line represents an individual clone; open circles denote unmethylated CpG sites; closed circles denote methylated CpG sites. Methylation rate was calculated below.

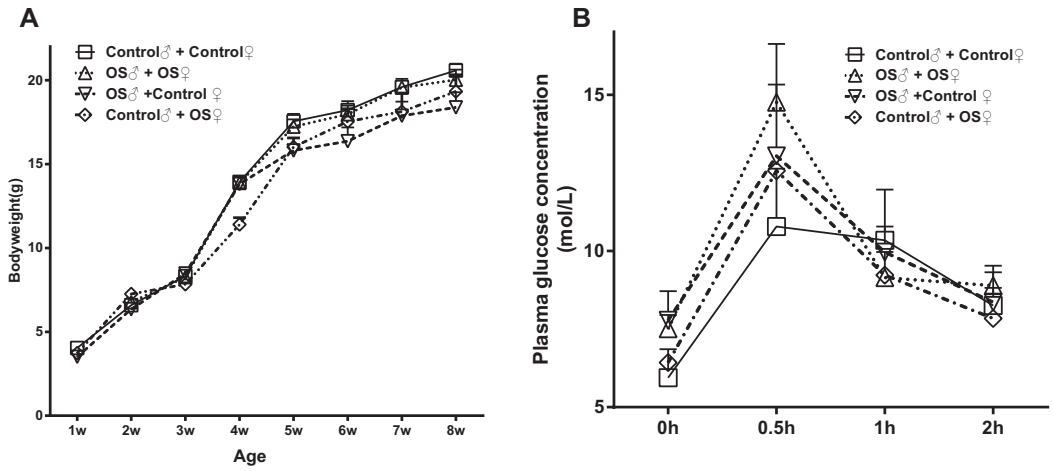

Fig. 3. Bodyweight and IPGTT of F2 mice. A) The bodyweight of F2 mice (OS F1♂-OS F1♀, OS F1♂-control F1♀, control F1♂-OS F1♀, control F1♂-control F1♀). B) Plasma glucose concentration after 0 h, 0.5 h, 1 h and 2 h during IPGTT. IPGTT, introperitoneal glucose tolerance test.

not show any statistically significant difference in bodyweight or IPGTT at any check time point (Figs. 3 and 4).

The F2 mice of group OS♂ + OS♀ displayed the significantly lower systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate compared with F2 of Control♂F1 + Control♀F1 ($P < 0.05$), suggesting that ovarian stimulation might also influence blood pressure regulation of F2 mice and that the influence was exaggerated by double ovarian stimulation parents origin. The OS♂ + Control♀ mice showed the similar lower heart rate. Ovarian stimulation has the skip-generation effect on the blood pressure and heart rate of middle-age F2 mice, but growth and glucose metabolism remain unchanged.

Discussion

Ovarian stimulation and other ART procedures have negative influences on offspring birth bodyweight [2,22]. However, the weight difference disappeared until the age of 12 as children grew up by "catch up" growth of the low birth weight infants [3]. Other studies on children born after IVF between birth and 18 months of age [23], at the ages of 12–45 months [24], at 5 years of age [25], between 6 and 13 years of age [26], and between 4 and 14 years of age [4] showed comparable bodyweight. A systemic review on post-natal health children born after ART also gave sufficient evidences supporting the idea that there was no significant difference between IVF and spontaneously conceived children [27]. The similar results of mice were previously reported [28] Up-lifted bodyweight in 2, 3 and 4 weeks of ovarian stimulation mice in our study indicated that influenced growth of offspring might come from ovarian stimulation, other than other ART procedures.

Plagl1 (Lost-on-transformation 1 gene (LOT1) and pleomorphic adenoma gene-like 1 gene) is a member of the novel subfamily of zinc-finger transcription factors, designated as PLAG family. *Plagl1* is maternally imprinted and linked to developmental disorders

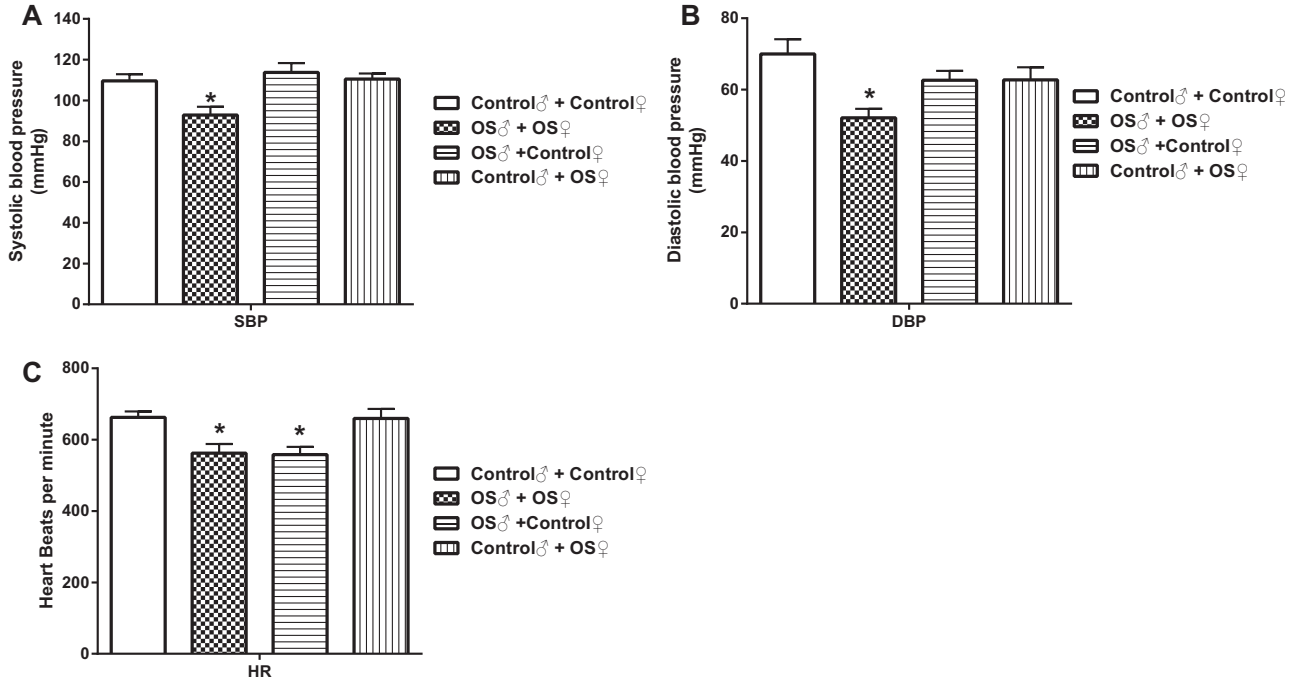

Fig. 4. Systolic pressure, diastolic pressure, and heart rate of F2 mice. *, $P < 0.05$, compared with control F1♂-control F1♀.

such as growth retardation and transient neonatal diabetes mellitus (TNDM). It encodes a growth suppressor protein and its expression loss is frequently observed in various neoplasms such as breast tumors [29]. It is reported that fetal growth can be influenced by altered expression of the *PLAGL1* gene network in human placenta[30]. In our study, *Plagl1* displayed nearly half loss of expression at 19-day post-coitus placenta of ovarian stimulation mouse compared with the control group. The loss of expression of *Plagl1*, a growth suppressor, promotes mouse growth. The loss of expression of *Plagl1* may produce a larger and nutrient-rich placenta and bigger baby mice as well. The increased bodyweight of ovarian stimulation mice also strengthened the hypothesis. Under the growth-inhibiting situation caused by ovarian stimulation, fetus and placenta start self-regulation via enhancing the functions of some genes related to growth like *Plagl1*.

The interference with oocyte development during ART may lead to imprinting abnormalities. In previous animal studies, the methylation status of *H19* was gained in developing oocytes after ovarian stimulation [31]. Another study demonstrated that ovarian stimulation perturbed genomic imprinting of both maternally and paternally expressed genes; loss of Snrpn, Peg3, and Kcnq1ot1 and gain of *H19* imprinted methylation were observed in a dose-dependent way [11]. Moreover, The ART placentae exhibited histomorphological alterations with defects in placental layer and the ART placentae were associated with increased methylation levels at imprinting control regions of H19[32]. However, Fortier AL et al. did not observe any significant DNA methylation perturbations in the differentially methylated regions of Snrpn or *H19* in mouse placenta after ovarian stimulation [12]. In human study, a study evaluating IGF2/*H19* DMR methylation in placenta following ovarian stimulation drew the same conclusion that gain of methylation was observed [33]. Other studies also gave a different conclusion that no significant difference in DNA methylation of *H19* was observed in mean methylation among all groups [34–36]. DNA methylation at the PLAGL1 differentially methylated region (DMR) was significantly higher in IVF cord blood[37]. A meta-analysis also showed that there was an increase in imprinting disorders in children conceived though IVF and ICSI[38]. Our bisulfite sequencing results indicated the gains in both *H19* and *Plagl1* methylation in late-gestation mouse placenta after ovarian stimulation. The hypermethylated *Plagl1* DMR well explained the loss of imprinted expression of gene *Plagl1*. In our study, the gain in *H19* DMR methylation might not be responsible for the insignificantly increased *H19* gene expression, indicating that another underlying epigenetic regulation other than methylation might be responsible for the *H19* expression up-regulation.

Emerging evidences indicated that IVF associated with offspring cardiovascular risks and glucose metabolism. In humans, Celeen et al. [3] reported that elevated systolic and diastolic blood pressure levels in IVF children were independent of early life factors and parental characteristics. Sakka et al. [4] also reported that IVF-born children had the higher systolic blood pressure and diastolic blood pressure than controls. Our study group has recently reported cardiovascular dysfunction in ART children[8]. Watkins et al. found the increased systolic blood pressure at 21 weeks in the first generation offspring mice whose embryos were cultured in vitro, compared with the mice whose embryos were developed in vivo[39]. However, our study further reported blood pressure alteration in F2, suggesting the trans-generational effect of ART.

Adult mice conceived in vitro underwent a hyperinsulinemic response after intraperitoneal glucose tolerance test (IPGTT) compared with mice conceived in-vivo [28]. The results indicated that adult mice born by ART which involved ovarian stimulation were resistant to insulin and independent of obesity [28]. In human study, Ceelen et al. reported the elevated fasting glucose levels other than the insulin levels, glucose to insulin ratio or the homeostasis assessment model (HOMA) in IVF adolescents compared with controls, independent of any early life factors or parental characteristics [3]. Sakka et al. did not detect any difference in weight, glucose, fasting insulin, fasting glucose-to-insulin ratio between IVF children and controls [4]. Another New Zealand cohort study revealed no difference in fasting glucose and insulin [40]. Oral glucose tolerance tests have not been performed in ART-conceived offspring and it is unknown whether IVF-born individuals are more resistant to insulin.

Surprisingly, systolic pressure and diastolic pressure as well as blood pressure measured from mouse tail in the OS♂ + OS♀ F2 group were lower than those in Control♂ + Control♀ F2 group. It is the first time to report trans-generational blood pressure regulation. The reduction of blood pressure may be caused by several factors, such as reproductive system disorder, genetic perturbation, and irregular uterine milieu of F1 ovarian stimulation mice, which deserve further well-designed research.

In conclusion, we reported the increased bodyweight in OS F1 mice and the decreased expression and elevated methylation of Plagl1 in OS placenta. Moreover, the trans-generational effect in blood pressure and heart rate was observed in OS F2 mice.

Conflict of interest

None.

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