Effect of controlled ovarian hyperstimulation on puberty and estrus in mice offspring

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Abstract

Controlled ovarian hyperstimulation (COH) is widely used for the treatment of infertility, while the long-term effects of COH on the reproductive function in female offspring are currently unknown. Based on the fact that COH could cause high E2 levels in women throughout pregnancy and excess estrogenic exposure during fetal development is harmful to subsequent adult ovarian function, we assumed the hypothesis that COH disrupts reproductive function in female offspring. To test this hypothesis, COH was induced in mice to obtain female offspring by pregnant mare serum gonadotropin (PMSG) and HCG, and then we evaluated pubertal transition, serum levels of E2, anti-Müllerian hormone (AMH), FSH and LH, mRNA expressions of *Esr1*, *Amhr2*, *Fshr* and *Lhcgr* in ovaries, number of follicles and ovarian histology. We also investigated the apoptosis of follicles by TUNEL; the mRNA expressions of *Fas*, *FasL*, *Bax*, *Bcl2*, and caspase 3, 8 and 9 by quantitative real-time PCR; and the protein expressions of cleaved-caspase (CASP) 3, 8 and 9 by Western blot. Moreover, we further observed estrous cyclicity in young adult offspring, performed follicle counting and measured the level of AMH in both serum and ovary. COH could induce detrimental pregnancy outcomes, as well as delayed pubertal transition and irregular estrous cycle due to the aberrant growth and maturation of follicles in female offspring. Our novel findings add new evidence to better understand the potential risks of COH on the reproductive function in female offspring, raising the awareness that COH could exert adverse effects on female offspring, rather than just obtain more oocytes for fertilization.

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Introduction

Assisted reproductive technology (ART) is widely applied in infertility treatments. According to the European Society of Human Reproduction and Embryology (ESHRE), nearly 1269 cycles per million inhabitants were performed in 17 European countries and the number of treatment cycles is still growing in Europe, with more than 600,000 treatment cycles recorded in 2011 (European et al. 2016). Controlled ovarian hyperstimulation (COH), apart from being routinely applied to achieve more fully mature oocytes in ART procedures, is also used without oocytes fertilization and transference *in vitro*. Interestingly, the number of infants born in the United States with COH treatments in 2005 is 2–6 times higher than that conceived through ART ovulation treatments (Schieve et al. 2009). Consequently, in regard to the large number of patients undergoing COH treatment, the total impact of COH treatments should be given more attention.

Many studies have evoked concerns that COH might exert various detrimental effects on offspring. Clinical studies have found that the high maternal serum estradiol

environment during pregnancy as a result of COH can increase the risks of low birth weight and small-for-gestational-age birth, and induce elevated levels of total cholesterol and low-density lipoprotein cholesterol in newborns (Hu et al. 2014, Meng et al. 2015). A prospective, assessor-blinded follow-up research has revealed that higher systolic blood pressure (SBP) are presented in 4-year-old offspring born with COH (Seggers et al. 2014); subsequently, positive direct effects of COH on SBP percentiles and subscapular skinfold thickness have been found by the same research group (La et al. 2014). Moreover, animal experiments are also conducted with special emphasis on COH offspring. In mice, the methylation pattern of imprinted genes in the sperm of male offspring can be altered by COH in mother (Stouder et al. 2009), and the effect of COH on fatty acid composition in adipose tissue and liver of male offspring has been reported (Wang et al. 2013). However a series of studies have suggested no influence of COH on other different systems of offspring, including the risk of cancer (Klip et al. 2001, Brinton et al. 2004, Gao et al. 2014), dysmorphic features (Seggers et al. 2012), fertility and sexual behavior of male offspring (Wei et al. 2014),

and neurological outcome (Schendelaar *et al.* 2014). Since there is no consensus regarding the detrimental effects of COH, further investigation is needed for the improvement and understanding of the influences on offspring conceived through COH treatments for infertility.

Therefore, although a growing number of studies have attempted to explore the effects of COH on offspring and the underlying mechanisms, there are as yet no researches focused on the assessment of the long-term effect of COH on reproductive system in female offspring. However the research about the long-term influences of COH in human population is faced with many difficulties such as the follow-up of COH offspring and inadequate exploration of underlying mechanisms.

Thus, to test the hypothesis that COH might exert adverse effects on female offspring, we used mice as the study model to determine the effects of COH on pubertal transition and estrous cyclicity in female offspring from COH mothers, and explore the potential associated underlying mechanisms.

Materials and methods

Animals and treatments

Eight-week-old female mice and ten-week-old male mice of Kunming breed were obtained from the Laboratory Animal Center of Tongji Medical College, Huazhong University of Science and Technology. All experimental procedures were approved by the Ethics Committee, Tongji Medical College of Huazhong University of Science and Technology.

The overview of experimental design is shown (Fig. 1). The mice were housed under specific pathogen-free conditions with optimal temperature and humidity with 12 h light:12 h darkness rhythm and fed *ad libitum*. Upon arrival, female mice underwent one-week acclimation period without any experimental intervention. After the acclimation, the mice were observed daily for vaginal smears and only mice showing stable estrous cycles for two rounds were chosen to be superovulated with an intraperitoneal injection of 7.5 IU pregnant mare serum gonadotropin (PMSG; Hangzhou Animal Medicine Factory, China) in diestrus phase followed by 7.5 IU HCG (Lizhu Pharmaceutical Factory, China) 48 h later. Controls were injected with the normal saline at the appropriate times.

Figure 1 Overview of experimental design. Implantation sites were counted on D5 between control group and COH group. Offspring on PND1, PND21, PND28 and PND56 from the two groups were selected for detailed observation and analyses, and the numbers of animals for observations and analyses were presented.

After superovulation, the females were mated with fertile males. As for the control group, female mice were caged with fertile males in estrus phase. Mating was confirmed by the appearance of a vaginal plug the next morning and the day was designated day 1 of gestation.

The vaginal plug-positive females of the two groups were divided into two parts, one was for the analysis of the number of implantation sites on D5 ($n=8$), and the other was for the obtainment of offspring ($n=11$). As for the obtainment of offspring, the vaginal plug-positive females were kept individually in cages for gestation period. The spontaneous delivery day was determined through observation of pregnant mice twice daily. The day of delivery was considered as postnatal day 1 (PND1) of age of the neonates. The number of live/dead fetuses per pregnancy and the lengths of gestations were recorded, and the pregnancy loss rate was calculated.

All pups were weaned to an *ad libitum* diet until PND21, and female offspring were killed when pre-pubertal (PND28) or when young adult (PND56), blood samples were collected and centrifuged at 3000 g for 15 min at 4°C. After centrifugation, the serum was separated and stored at -80°C until assayed for hormones. Ovaries were removed for morphological analysis and snap frozen for RNA and protein extraction.

Evaluation of pubertal transition and estrous cyclicity

Since nutrition will affect the development of reproductive system (Chan et al. 2015), we measured BMI of female offspring on PND21 and PND28, and set the average of BMI (0.5 for PND21 and 0.6 for PND28) as the standard; average $\pm 10\%$ $\times$ average of BMI was considered to be the selection criteria for further comparison. Female offspring from the control group and COH group were selected based on similar BMI on PND21, and were observed every day for vaginal opening. After vaginal opening, BMIs of selected offspring were checked again according to the criteria on PND28, and were inspected daily for vaginal lavages in order to identify the age at first estrus. A true first estrus was considered to present the cornified cells in the vaginal lavages followed by at least 2 days of predominately leukocytes in lavages (Prevot et al. 2003). Furthermore, to assess the regularity of estrous cycle in young adult (PND56) female offspring, vaginal

smears of selected offspring were examined daily from PND56 for 4 weeks.

Quantitative analysis of ovarian follicles

For follicle counting, ovaries of pre-pubertal (PND28) female offspring were collected and fixed overnight in 4% paraformaldehyde (PFA), dehydrated, embedded in paraffin, and sectioned for hematoxylin and eosin staining. Five micrometer-thick sections were serially cut and every 10th section was observed for follicle counting in a blinded manner. Follicles with visible oocyte nuclei were counted. According to the previously described follicle classification (Durlinger et al. 1999, Detti et al. 2013), the number of follicles at different developmental stages was recorded; specifically, follicles were divided into 5 categories: primordial, primary, secondary, tertiary and atretic follicles. As the number of secondary and tertiary follicles ranges a lot with the follicle cycle selection, we only assess the number of primordial, primary and atretic follicles on PND56. Images were collected on an Olympus DP73 upright microscope (Olympus) with cellSens Standard acquisition software. Micrographs were analyzed by Adobe Photoshop CS6 software (Adobe Systems Inc., San Jose, CA, USA).

TUNEL assay

Apoptosis of follicle was evaluated in ovarian sections from PND28 female offspring, detected by TUNEL assay using In Situ Cell Death Detection Kit (Roche). The procedures followed the previous description (Grasa et al. 2015). Images were collected on an Olympus DP73 upright microscope (Olympus) with cellSens Standard acquisition software. Micrographs were analyzed by Adobe Photoshop CS6 software (Adobe Systems Inc.).

Analysis of serum hormone levels

Serum levels of anti-Mullerian hormone (AMH) in PND28 and PND56 female offspring, and FSH, LH and estradiol (E2) in PND28 female offspring were measured by commercially available ELISAs (Cloud-Clone Corp., Houston, USA) and radioimmunoassay (RIA) (Beijing North Institute of Biological

Table 1 Primer sequences.

Gene name	Sequence accession number	Forward (5'–3')	Reverse (5'–3')
<i>Amhr2</i>	XM_006520293	TGCGTTTCTCCAGGTAATCC	TACTGAGTCAAGTAGTGGCATAAGG
<i>Fshr</i>	NM_013523	CTCTGCCAAGATAGCAAGGTGAC	GGTCCCCAAATCCAGAAAATGA
<i>Lhcgr</i>	NM_013582	GGTGCTTTTACAAACCTCCCTC	CGAGATTAGCGTCGTCCTCA
<i>Esr1</i>	NM_007956	CAAGGTAATGTGTGGAAGGCA	ATGGGAAAGAATGAGAAGGAGC
<i>Fas</i>	NM_007987	TCCTGCCTCTGGTGCTTGCT	CAGTTTCACGAACCCGCCTC
<i>FasL</i>	NM_010177	GTGAGTTACCAACCAAAGCCT	GACCTTGTTGGTTTAGGGGCTG
<i>Casp8</i>	NM_001080126	CTTGAAGGAAGGGAAGAGTTGC	CAGTGTCTTGTCTCTTGGCGA
<i>Bax</i>	NM_007527	GCCTTTTGTCTACAGGGTTTCAT	TATTGCTGTCCAGTTCATCTCCA
<i>Bcl2</i>	NM_009741	TGACTTCTCTCGTCTACCGT	CCTGAAGAGTTCTCCACCACC
<i>Casp9</i>	NM_001277932	GAGGTGAAGAACGACCTGACTG	CTCAATGGACACGGAGCATC
<i>Casp3</i>	NM_009810	GTCTGACTGGAAGCCGAAAC	GACTGGATGAACCACGACCC
<i>Actb</i>	NM_007393	GTGACGTTGACATCCGTAAGA	GTAACAGTCCGCCTAGAAGCAC

Technology, Beijing, China) according to the manufacturer's recommended instructions.

Quantitative Real-Time PCR

Gene expressions of *Amhr2*, *Fshr*, *Lhcgr*, *Esr1*, *Fas*, *FasL*, caspase8, *Bax*, *Bcl2*, caspase9, caspase3 and β -actin as the reference had been detected in female offspring. Total RNA from ovaries were isolated by RNAiso Plus (TaKaRa) and their concentration and purity were measured by a Nucleic Acid/Protein Analyzer (Thermo Fisher Scientific). Then cDNA were synthesized with a PrimeScript RT reagent kit (TaKaRa) in a reaction volume of 20 μ L. Quantitative real-time PCR (qPCR) analysis was performed with SYBR Green qPCR kit (TaKaRa) on the Applied Biosystems StepOne Real-Time PCR System (Applied Biosystems) according to manufacturer's instructions. Forty cycles were set for reaction and each sample was run in triplicate ($n=6$). The amplification conditions were as follows: initial denaturation for 30s at 95°C, 40 cycles of 5s at 95°C, annealing and elongation for 30s at 60°C. β -Actin was used as the normalization control. The primer sequences were obtained from GenBank of National Center for Biotechnology Information (NCBI) database and verified, as well as provided in details (Table 1), and $2^{-\Delta\Delta CT}$ (Livak & Schmittgen 2001) method was used to calculate the fold change in gene expression.

Western blot analysis

We analyzed the protein expressions of cleaved-caspase3, cleaved-caspase8 and cleaved-caspase9 in PND28 female offspring, and AMH in PND56 female offspring. The ovarian tissues were lysed in RIPA lysis buffer (Goodbio Technology Co., Wuhan, China) supplemented with protease inhibitors (Goodbio Technology Co.), and the concentration of proteins were measured by bicinchoninic acid (BCA) assay kit (Goodbio Technology Co.). Then protein samples were loaded onto an SDS-PAGE and transferred onto PVDF membranes. 5% non-fat dry milk in 0.5% TBS-Tween was used to block

Table 2 The effects of COH on reproductive performance.

Groups	Control	COH
D5 implantation sites	13.75 $\pm$ 1.67	20.13 $\pm$ 2.03***
Gestation period (days)	20 (19, 20)	21 (20, 22)***
Total no. of live fetuses	140 ($n=11$)	99 ($n=11$)
No. of live fetuses	13 (10, 15)	12 (0, 16)
Total no. of dead fetuses	0 ($n=11$)	55 ($n=11$)
No. of dead fetuses	0	0 (0, 8)*
Pregnancy loss (%)	5.45 (13.75 – 13/13.75) $\times 100$	40.39 (20.13 – 12/20.13) $\times 100$

Data with a normal distribution represent mean $\pm$ S.D., and data with a skewed distribution represent medians (first quartile, third quartile). * $P<0.05$ and *** $P<0.001$ when compared to control group.

Figure 2 Female offspring of COH mice exhibited delayed first estrus. (A) Comparison of the age at vaginal opening between female offspring from control group and COH group. $n=18$. (B) Comparison of the age at first estrus between female offspring from two groups. $n=9-11$. (C) Comparison of the time between vaginal opening and first estrus between the two groups. $n=9-11$. * $P<0.05$ and ** $P<0.01$ when compared to the offspring from naturally conceived mice.

Figure 3 COH female offspring showed more atretic follicles on PND28. (A) Comparison of percentages of follicles at different developmental stages between control group and COH group. $n=6$, data represent mean $\pm$ s.d. * $P<0.05$ when compared with control group. PDF, primordial follicles; PMF, primary follicles; SEF, secondary follicles; TEF, tertiary follicles; ATF, atretic follicles; TF, total follicles. (B) Representative photographs of ovaries of offspring from control group, bar=200 μ m. (C) Representative photographs of ovaries of offspring from COH group, bar=200 μ m. Arrows indicate atretic follicles.

non-specific binding sites. cleaved-caspase3 (#9664; 1:1000 dilution; Cell Signaling Technology), cleaved-caspase8 (#8592; 1:1000 dilution; Cell Signaling Technology), cleaved-caspase9 (#9509; 1:1000 dilution; Cell Signaling Technology) and AMH (MAB1426; 1:500 dilution; R&D Systems) were detected by primary antibodies, which were followed by horseradish peroxidase (HRP)-conjugated anti-rat secondary antibody (GB23302; 1:3000 dilution; Goodbio Technology Co.). β -Actin was detected using primary antibody (GB13001-1; 1:1000 dilution; Goodbio Technology Co.) and HRP-conjugated anti-rabbit secondary antibody (GB23303; 1:3000 dilution; Goodbio Technology Co.). Bands were exposed by ECL kit (G2014; Goodbio Technology Co.). Finally, AlphaEaseFC software (Alpha Innotech Inc., California, USA) was used to analyze the relative protein expression.

Statistical analysis

All data were analyzed by SPSS 20.0 software package (SPSS). Continuous variables with a normal distribution were presented as means $\pm$ s.d. The unpaired Student's two-tailed t test was used for comparisons between two groups. The remaining continuous variables showing a skewed distribution were expressed as medians (first quartile, third quartile) and the intergroup differences were compared using Mann-Whitney U test. The statistical significance was considered as $P<0.05$.

Results

The effects of COH on reproductive performance

To determine the effects of COH on reproductive performance, we assessed the number of implantation sites on D5, the length of gestation period, the number of live and dead fetuses, and the percentage of pregnancy loss (Table 2). Compared to the control group, the number of implantation sites on D5 was increased in COH group (mean: 13.75 vs COH 20.13; $P<0.001$), but the number of live fetuses in COH group was no more

Table 3 CVs of qPCR.

Gene	CV (%)			
	D28 Control	D28 COH	D56 Control	D56 COH
<i>Amhr2</i>	27.27	29.55	21.62	35.74
<i>Fshr</i>	10.45	31.35	–	–
<i>Lhcgr</i>	37.21	30.43	–	–
<i>Esr1</i>	31.20	38.54	–	–
<i>Fas</i>	28.16	18.83	–	–
<i>FasL</i>	32.89	25.61	–	–
<i>Casp8</i>	25.86	9.91	–	–
<i>Bax</i>	34.31	19.99	–	–
<i>Bcl2</i>	35.84	24.91	–	–
<i>Casp9</i>	24.99	28.45	–	–
<i>Casp3</i>	23.96	28.64	–	–

– represents that genes were not evaluated in ovaries of D56 female offspring.

Figure 4 TUNEL analysis: genes and proteins detection in ovaries of PND 28 female offspring. (A) Representative images of ovary sections detected by TUNEL, bar=200 μ m. (B) Representative high-power photomicrographs of ovary sections detected by TUNEL, bar=50 μ m. White box refers to atretic follicles undergoing apoptosis of granulosa cells. (C) Ovarian expression levels of specific genes involved in apoptosis of granulosa cells (Fas, FasL, Caspase8, Bax, Bcl2, Caspase9 and Caspase3) in female offspring on PND28 from control group and COH group. Fold change value was normalized to β -actin. $n=6-8$, data represent mean $\pm$ s.d. * $P<0.05$ and *** $P<0.001$ vs the offspring from control group. (D) Protein expression levels of cleaved-caspase3, cleaved-caspase8 and cleaved-caspase9 in ovaries of PND28 female offspring ($n=5$). GAPDH was considered as the reference.

than that of the control group (median: 13 vs COH 12), and the number of live fetuses in COH group varied in a wide range. Apart from this, the large number of dead fetuses was discovered in COH mice, while no dead fetus was observed in naturally pregnant mice (total: 0 vs COH 55; $n=11$). Additionally, gestation period was significantly extended in COH group (median: 20 vs COH 21; $P<0.001$), and the comparison of pregnancy loss revealed that COH mice suffered a higher percentage of pregnancy loss (5.45% vs COH 40.39%).

The effects of COH on pubertal transition of female offspring

To investigate whether COH affected pubertal transition of female offspring, we assessed the age at vaginal opening and first estrus in female offspring. Between the two groups, no significant difference in the age at vaginal opening was found (Fig. 2A). However, compared to the control group, delayed first estrus was observed in the model group ($P<0.05$) (Fig. 2B). As a result, the time between vaginal opening and first estrus of the model group was significantly longer than that of the control group ($P<0.01$) (Fig. 2C).

To determine whether COH affected follicle development before pubertal transition, we performed follicle analysis in pre-pubertal female offspring on PND28 before the presentation of first estrus. Our results suggested that the number of atretic follicles was obviously increased in COH female offspring (Fig. 3A), and the quantitative evaluation was confirmed by the morphological analysis of ovaries of the two groups (Fig. 3B and C).

Apart from the fact that the number of atretic follicles was increased in COH female offspring, we explored whether the apoptosis of ovarian granulosa cells was

also enhanced in COH female offspring by TUNEL assay. As our data showed, there was an increase in TUNEL staining of atretic follicles of COH female offspring when compared to the control group (Fig. 4A and B). Furthermore, the expressions of relevant genes participating in apoptosis of granulosa cells were analyzed by qPCR; in contrast, the expressions of *FasL*, *caspase8*, *Bax*, *caspase9* and *caspase3* were significant higher in ovary of COH female offspring on PND28 (Fig. 4C). In our further study, we investigated the protein expression levels of cleaved-caspase3, cleaved-caspase8 and cleaved-caspase9 in ovaries of PND28 female offspring, and discovered that the expression of cleaved-caspase8 was increased, while no significant difference was found in the expressions of cleaved-caspase3 and cleaved-caspase9 (Fig. 4D).

In addition, to explore whether delayed first estrus in model group was associated with low hormone levels and reduced hormone receptor gene expressions, we measured serum AMH, FSH, LH and estradiol, and evaluated gene expressions of *Amhr2*, *Fshr*, *Lhcgr* and *Esr1* in ovaries of PND28 female offspring. The serum levels of LH and E2 were significantly reduced in model group, while FSH and AMH were not affected (Fig. 5A). For gene expressions, *Amhr2*, *Fshr*, *Lhcgr* and *Esr1* showed significant reduction in model group when compared with control group (Fig. 5B).

The effects of COH on estrus cyclicity in female offspring

We recorded the regularity of estrous cycle in young adult female offspring (PND56) to assess the long-term influence of COH. There was no statistical difference in average estrous cycle length (Fig. 6A). However,

Figure 5 Measurement of serum hormone levels and ovarian hormone receptor gene expressions of female offspring on PND28. (A) Circulating levels of AMH, FSH, LH and E2 in female offspring, $n=7-8$, * $P<0.05$ vs control group. (B) RT-PCR analysis of *Amhr2*, *Fshr*, *Lhcgr* and *Esr1* in ovaries of female offspring. Fold change value was normalized to β -actin. $n=6-8$, data represent mean $\pm$ s.d. * $P<0.05$ and *** $P<0.001$ vs the offspring of naturally conceived mice.

when compared with control group, the young adult female offspring in model group exhibited a growing percentage of days they spent in estrus and a decreased percentage of days in proestrus (Fig. 6B). Representative estrous cycles of the two groups are presented (Fig. 6C and D).

To explore follicle development of young adult female offspring, we performed follicle counting and morphological analysis of ovaries from PND56 female offspring of the two groups. Considering the obvious fluctuations in the number of secondary and tertiary follicles due to cycle selection, we only assess the number of primordial, primary and atretic follicles. Our data showed the ovaries of the offspring of naturally pregnant mice tended to have more primordial follicles ($P<0.05$) and primary follicles ($P<0.001$) than COH female offspring on PND56. However there was no significant difference in the number of atretic follicles (Fig. 7A). This quantitative evaluation was confirmed by

the morphological analysis of ovaries of the two groups (Fig. 7B and C).

What is more, overexpression of AMH in COH young adult (PND56) female offspring was found. We measured serum AMH, ovary AMH protein level, and *Amhr2* gene expression in offspring on PND56. In contrast, the serum AMH level (Fig. 8A, $P<0.001$) and the ovary AMH protein expression (Fig. 8B and C, $P<0.05$) were significantly elevated in model group, while the expressions of *Amhr2* gene were not statistical differences (Fig. 8D).

Discussion

Several studies have expressed concerns about the reproductive system being affected by COH in male offspring (Stouder *et al.* 2009, Wei *et al.* 2014). However, to our knowledge, there are few researches focusing on the potential detrimental influence of COH on the reproductive system of female offspring. The present study focuses on the long-term effects of COH on female offspring. We discovered, for the first time that COH in mother could adversely impact the pubertal transition and estrous cyclicity in female offspring.

In our study, we observed that COH mice exhibited poor pregnancy outcome, suggested by the evidence of higher percentage of pregnancy loss, increased gestational length and a large number of dead fetuses. These are consistent with earlier studies (Ertzeid *et al.* 1993, Ertzeid & Storeng 2001, Deng *et al.* 2013), which demonstrate the decreased implantation rate and the raised fetal mortality in COH mice. Also, prolonged gestation period is considered to be negative correlated with the number of live fetuses found on delivery day (Ertzeid *et al.* 1993), as we found a larger number of dead fetuses in COH group and the gestation period was significantly extended.

Based on the fact that COH could cause high E2 levels in women throughout pregnancy (Meng *et al.* 2015), and excess estrogenic exposure during fetal development is harmful to subsequent adult ovarian function (Abbott *et al.* 2006), we assumed that excessive E2 exposure to fetus caused by COH might impact on reproductive function of female offspring. Therefore, we first evaluated the pubertal transition and found that delayed puberty was clearly displayed in COH female offspring, manifesting in delayed first estrus, but not vaginal opening. Although still questionable, first estrus (but not vaginal opening) is considered to be a more reliable indicator of the age at first ovulation (vom Saal 1989), which signifies the transition of puberty and the ability to reproduce. The onset of puberty is driven by the activation of the gonadotropin-releasing hormone (GnRH) neurons from a state of quiescence, which is followed by an estrogen surge and vaginal opening; within a week of vaginal opening, a second peak in

Figure 6 Observation of estrus cyclicity of female offspring from PND56. (A) Comparison of average estrous cycle length of female offspring from control group and COH group. (B) Comparison of average percentage of time spent in proestrus, estrus and diestrus across 4 weeks. $n=8$, data represent mean $\pm$ s.d. * $P<0.05$ and *** $P<0.001$ vs the offspring of naturally conceived mice. (C) Representative estrous cycle of control group offspring. (D) Representative estrous cycle of COH group offspring. P, proestrus; E, estrus; D1, diestrus 1; D2, diestrus 2.

estrogen appears and subsequently the ovulation and first estrus occur, and the transitional period from juvenile to pubertal is completed. Hence, delayed first estrus in COH female offspring means that the completion of pubertal transition is affected.

At puberty onset, the pulse generator reawakens and induces endocrine changes, which stimulates pituitary gonadotropin secretion, as well as the secretion of gonadal steroid (Villanueva & Argente 2014). In concert with the modulations of E2 on the pituitary gland, the action of GnRH triggers the preovulatory surge of LH, which ultimately brings about the first ovulation (Herbison 2016). More specifically, FSH is modulated through pituitary, stimulated by GnRH, and then acts through *Fshr* to stimulate the proliferation and differentiation of granulosa cell. The responsive follicles produce E2 and express granulosa cell *Lhcgr*, paracrine signaling induced by FSH and LH maintain follicle growth and E2 secretion until an ovulation is triggered by the LH surge, LH then manages granulosa cell function, resulting in terminal rupture of the follicle wall and release of the oocyte (Hillier 2001). In this regard, we measured the hormone levels and gene expressions of ovarian receptors after the activation of the pulse generator (vaginal opening) but before the pubertal transition (first estrus), and found low levels of LH and E2, as well as the reduced mRNA expressions of *Fshr*, *Lhcgr* and *Esr1* in ovaries of COH female offspring. The insufficient levels of hormones and the expressions of their receptors indicate that the growth and maturation of the follicles are restricted, which prevents dominant follicle from ovulating, delaying the first ovulation defined as pubertal transition.

It is well known that the atresia of follicles is a hormonally controlled apoptotic process, and the

gonadotropins FSH and LH, the steroids progesterone and estrogen, and locally produced cytokines act as survival factors for follicle atresia via their receptors (Emilia *et al.* 2002). Accordingly, the reduced hormone levels and mRNA expression of receptors in ovaries of COH female offspring of PND28 might demonstrate that more follicle atresia might exist in COH group, which is coincident with our findings showing increased number of atretic follicles in COH female offspring. What is more, a recent novel study shows that AMH participates in the regulation of FSH secretion in immature females, which may be vital for pubertal timing and sexual differentiation (Garrel *et al.* 2016). No significant differences in FSH and AMH in COH female offspring may indicate that the regulation of FSH is not influenced. However we found that ovarian gene expression of *Amhr2* was reduced in COH female offspring; since AMH could reduce follicular atresia and *Amhr2* is necessary for AMH effect in ovary (Hayes *et al.* 2016), the decline in expression of *Amhr2* may attenuate the anti-atresia effect of AMH in COH female offspring, and lead to the increased percentage of atretic follicles.

The apoptosis of granulosa cells is necessary for the atresia of follicles, and one main death ligand-receptor system involved in the induction of apoptosis of granulosa cells is the FASL and FAS system. Female mice treated with FAS-activating antibody facilitated granulosa cell apoptosis and follicular atresia (Hakuno *et al.* 1996, Sakamaki *et al.* 1997). In human, the expression of FAS in the granulosa cells increases as atresia progresses (Kondo *et al.* 1996). Subsequent activation of intracellular signaling (Caspase8 and Caspase3) induces DNA fragmentation. Caspase8 could also activate the mitochondrial apoptotic pathway. BCL2 family members participate in the mitochondria-

Figure 7 COH female offspring presented less primordial follicles and primary follicles on PND56 than control group. (A) Comparison of percentages of follicles at different developmental stages between control group and COH group. $n=6$, data represent mean $\pm$ s.d. * $P<0.05$ and *** $P<0.001$ when compared with control group. PDF, primordial follicles; PMF, primary follicles; ATF, atretic follicles. (B) Representative photographs of ovaries of offspring from control group on PND56, bar=40 μ m. (C) Representative photographs of ovaries of offspring from COH group on PND56, bar=40 μ m. Arrows indicate primary follicles and arrowheads represent primordial follicles.

mediated apoptosis of granulosa cells, including both anti-apoptotic (BCL2, B cell lymphoma/leukemia X (BCLX), etc.) and proapoptotic proteins (BAX, BCL2L11, etc.). Overexpression of BCL2 protein decreases granulosa cells apoptosis and enhances folliculogenesis (Hsu *et al.* 1996). In human ovaries, the expression of BAX is increased in granulosa cells of atretic follicles than that of healthy follicles (Kugu *et al.* 1998). The same result was found in porcine follicles (Sai *et al.* 2011). The mitochondrial pathway of apoptosis involves the activation of caspase9, before the subsequent activation of caspase3. Caspase9 is expressed in granulosa cells and discovered to promote follicular atresia in mice and pig (Robles *et al.* 1999, Matsui *et al.* 2003). Interestingly, we found the significant increase in *FasL*, caspase8, *Bax*, caspase9 and caspase3 mRNA levels in female offspring of COH mice on PND28; however, the elevated protein expression was only detected in cleaved-caspase8, which is one of the main upstream initiators in apoptosis pathway. Apart from caspase3 and caspase9, other caspases such as caspase6 and caspase7 also act as executioner caspases (Stennicke *et al.* 1998); thus, it might be possible that other unevaluated caspases were activated by cleaved-caspase8, leading to the execution of apoptotic program. Furthermore, the functional activity of caspase should be considered regardless of the alteration of its quantity.

However, further investigation is needed to uncover the mystery behind the phenotype of delay pubertal transition. Since the hypothalamic-pituitary-gonad (HPG) axis develops during gestation (Herbison 2016), it is unclear whether the development of HPG axis is affected by the excessive exposure to E2 embryonically. Besides, thousands of genes are considered to be involved in the determination of pubertal timing (Lomniczi *et al.* 2013), and the disturbed patterns of DNA methylation could affect the gene expression coding for proteins vital for the epigenetic reprogramming, which subsequently leads to biological consequences (Stouder *et al.* 2009). Several studies have suggested that COH might induce abnormal methylation patterns in oocytes or embryos of mice (Shi & Haaf 2002, Fauque *et al.* 2007, Fortier *et al.* 2008). Thus, another possible reason for the delayed pubertal transition in female offspring might originate from DNA methylation errors in oocytes caused by COH in mother.

Beyond that, in our findings, disturbed estrous cycle in COH female young adult offspring reflects that COH exerts long-term effect on the reproductive system in female offspring. The estrus cycle in mice corresponds to the menstrual cycle in human, and the different stages of estrus cycle are indicative of underlying endocrine changes. During the proestrus phase, defined by a growing level of E2 before ovulation, follicle growth is stimulated by the secretion of FSH, until the peaks in FSH levels arrive it enters into estrus and signals ovulation. Following the estrus, E2 declines and prolactin level

Figure 8 COH female offspring overexpressed AMH. (A) Elevated serum level of AMH in COH group, $n=8$, *** $P<0.001$ vs control group. (B) Western blot showing increased expression of AMH in ovary of COH offspring. Three independent experiments were performed. (C) Relative quantity of AMH protein expression ($n=5$). (D) Gene expression of *Amhr2* in ovaries. Fold change value was normalized to β -actin. $n=8$, data represent mean $\pm$ S.D. ** $P<0.01$ and *** $P<0.001$ vs control group.

peaks; after the completion of ovulation, there is a rising trend in progesterone hormone level accompanied by a small surge in E2 level as the response to corpus luteum activation. Finally, the circulating progesterone level peaks with the entry into diestrus, and the regression of the corpus luteum results in a sharp decline in progesterone level (McLean *et al.* 2012). Thus, the disturbance of estrus cycle characterized by relatively shorter proestrus and prolonged estrus implicates the abnormal hormone changes in mice, which reflects the disturbed follicle growth in COH female offspring.

As FSH, LH, E2, prolactin and progesterone secretions fluctuate with the different stages of estrus cycle, we measured the circulating AMH level, which was strongly associated with follicle recruitment and relatively stable during the estrus cycle, and discovered elevated level of AMH in COH female offspring. As mentioned above, AMH could prevent follicles from atresia; therefore increased level of AMH reduces the atresia of follicles in PND56 female offspring of COH group, presenting no difference of atretic follicles between the two groups on PND56. In rodents, AMH expression is detected in pre-antral follicles, reaches its peak in antral follicles, and attenuates afterward till disappearing in preovulatory follicles (Durlinger *et al.* 2002). It is well known that AMH has an inhibitory effect on cycle recruitment of follicles, inhibiting the primordial follicles developing into primary follicles (Zehra *et al.* 2016); therefore the increased level of AMH in COH female offspring overdepresses the development of follicles. According to recent research, it is suggest that AMH promotes pre-antral follicle growth while restraining antral follicle maturation and dominant follicle selection in primates

(Xu *et al.* 2016). However, unlike in primates, AMH has inhibitory effects on both the pre-antral follicle growth and antral follicle maturation in mice (Durlinger *et al.* 2001). In light of this, we believe that the significant elevated level of AMH in COH female offspring implies the restriction of follicle growth and maturation, manifesting in disturbed estrus cycle pattern.

In conclusion, our present report indicates that COH could lead to poor pregnancy outcome and adversely affect follicle development in female offspring, resulting in delayed pubertal transition and subsequent irregular estrus cyclicity in adulthood. However, further investigations are necessary to explore the underlying mechanisms by which COH influences the reproduction system of female offspring, whether from the perspectives of imprinted genes expressions or excessive hormone exposure embryonically. And more generations could be involved in studies focused on the COH offspring. Despite the limitations of our study, our findings do raise the concern of better evaluation of the risks and effects of COH, especially for the reproductive well-being of COH female offspring.

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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