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RESEARCH ARTICLE

Perinatal exposure to low doses of cypermethrin induce the puberty-related hormones and decrease the time to puberty in the female offspring

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Abstract

Pyrethroid insecticides are ubiquitously detected in environmental media, food, and urine samples. Our previous epidemiological study reported a correlation between increased pyrethroid exposure and delayed pubertal development in Chinese girls. In this study, we further investigated the effects of perinatal exposure to low doses of cypermethrin (CP) on pubertal onset and hypothalamic-pituitary-ovarian axis in the female mice offspring. The treatment of CP with 60 μg/kg/day from gestation day 6 (GD6) to postnatal day 21 (PND21) significantly decreased the time to puberty in the female offspring. Exposure of CP increased the serum levels of gonadotropin-releasing hormone (GnRH) and the expression of GnRH genes in a dose-dependent manner in the female offspring. CP also induced the serum levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), as well as the expression of gonadotropin subunit genes [LHβ, FSHβ, and chorionic gonadotropin α (Cgα)]. Furthermore, CP induced serum estradiol (E_2) levels and the expression of steroidogenesis-related genes [steroidogenic acute regulatory (StAR) and Cytochrome p 450, family 11, subfamily A, polypeptide 1 (CYP11A1)] in the ovary. In accordance with the in vivo tests, administration of CP (6.7, 20, and 60 μg/L) stimulated a dose-dependent increase in the synthesis and secretion of the puberty-related hormones in the explants of hypothalamus, pituitary, and ovary. The interference with calcium channels in the ovary may be responsible for CP-induced pubertal onset. Our study provided evidence that perinatal exposure to low doses of CP induced puberty-related hormones and decreased the time to puberty in the female offspring.

Keywords Pyrethroids · Cypermethrin · Pubertal onset · HPG axis · Female offspring

Introduction

Pesticides are widely used all over the world. With the phase-out of organochlorine pesticides and organophosphorus pesticides, pyrethroids are increasingly applied to residential, agricultural, horticultural, and public health sites. As one of the top ten most used pesticides, pyrethroids account for more than 30% of global insecticide usage (Fenner et al. 2013). The global sales of pyrethroids were up to 3.1 billion dollars in 2018. Such extensive use of pyrethroids might attribute to their environmental-friendly features such as the relatively low mammalian toxicity and good degradability (Zhu et al. 2020). However, human beings, especially the children and teenagers, are at the risk of being exposed to the ubiquitous pyrethroids (Ye and Liu 2019). For example, the detection rate of 3-PBA (a common metabolite of pyrethroids) in healthy children in Shanghai, China, was 82% and the geometric mean (GM) of urinary 3-PBA concentration was 0.39 μg/g creatinine (Ding et al. 2012). Urinary 3-PBA was detected in 98.8% of all the urine samples in pregnant women in Jiangsu, China, and the GM reached up to 1.53 μg/g creatinine (Qi et al. 2012). A recent study reported that a 100% detection rate of 3-PBA in Japanese children at 3 years old and the GM of urinary 3-PBA levels

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was 1.71 μg/g creatinine (Osaka et al. 2016). These studies showed that both children and pregnant women were exposed to the pyrethroid pesticides in daily life. Dietary ingestion and dermal absorption are two main exposure routes of pyrethroids (Pirard et al. 2020).

Although the acute toxicity was relatively low, as a class of endocrine-disrupting chemicals (EDCs), the endocrine-disrupting effects of pyrethroids cannot be neglected (Greenspan and Lee 2018). A number of studies have shown the endocrine-disrupting effects of pyrethroid insecticides (Ji et al. 2019; Liu et al. 2011; Pine et al. 2008; Ye and Liu 2019). It was reported that bifenthrin showed disrupting effects on ovulatory gene [cytochrome P450 side chain cleavage enzyme (P450scc), steroidogenic acute regulatory protein (StAR), and so on] expression patterns and prostaglandin synthesis in rat ovarian granulosa cells (Liu et al. 2011). Cypermethrin (CP) was found to be able to disturb the expression of steroid hormones–related genes and hormone secretion accordingly (Ji et al. 2019). In addition, perinatal exposure to fenvalerate affected the reproductive physiology and behavior in female rats (Moniz et al. 2005). Esfenvalerate was demonstrated to suppress the afternoon luteinizing hormone (LH) and delay puberty onset in female rats (Pine et al. 2008). Our previous study showed an association between urinary 3-PBA concentrations and gonadotropin levels in boys (Ye et al. 2017b). These studies suggested that pyrethroid exposure may affect the puberty time and secretion of puberty-related hormones.

Puberty is an important process for not only sexual maturation and reproductive health, but also social, cognitive, and behavioral development. Hypothalamic-pituitary-ovarian (HPO) axis plays a vital role in the growth, pubertal development, and reproduction in females. Recently, our epidemiological study indicated that pyrethroid exposure was negatively correlated with the risk of being advanced pubertal development in peripubertal girls (Ye et al. 2017a, b, c). However, how pyrethroids affect the pubertal timing, secretion of puberty-related hormones, and HPO axis in females are still poorly understood.

Cypermethrin, one of the top 3 pyrethroids in use, has become one of the ubiquitous insecticides all over the world. Although CP rapidly degrades in the environment, the detection frequency of CP on wipe samples collected from the vinyl floor and kitchen countertop in Boston was 24% with mean levels of 3.87 ± 10.4 μg/m^2 (Xia et al. 2013). It was reported that the highest concentration of CP in cabbage, green chili, and okra grown in the North and North–West region of India was 0.120, 1.310, and 0.30 mg/kg, respectively (Sharma et al. 2022). The certified values of apples collected from a field of Aomori Prefecture, Japan, were 1.55 ± 0.81 mg/kg for CP (Otake et al. 2013). These studies indicated that CP existed extensively in the environment and food. In this study, CP was used as a representative pyrethroid to investigate the effects and mechanisms of perinatal exposure to low doses of pyrethroids on the puberty-related hormones and the onset of puberty in the female offspring by in vivo and in vitro experimental rodent models.

Experimental methods

Chemicals

CP (purity ≥ 99%) and anhydrous magnesium chloride were obtained from Aladdin (Beijing, China). D-glucose and glycine were purchased from Sangon Biotech (Shanghai, China). L-Ascorbic acid was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Dulbecco's Modified Eagle Medium F-12 (DMEM/F12) and Medium 199 were purchased from Gibco (New York, NY, USA). Minimum Essential Media (MEM) was purchased from HyClone (Utah, UT, USA). Penicillin–streptomycin solution was purchased from Biosharp (Beijing, China). Dimethyl sulfoxide (DMSO) was purchased from Amresco (PA, USA).

Animals and treatment

ICR mice were purchased from Shanghai Laboratory Animal Research Center, Chinese Academy of Science (Shanghai, China). Mice were raised in climate-controlled rooms with the controlled photoperiod (light on at 0800 h and light off at 2000 h) and temperature (23 ± 1 ℃). All the animal procedures were approved by Zhejiang Chinese Medical University Animal Care and Use Committee. Pregnant mice were housed individually and assigned to different groups randomly and administered intragastrically every morning (0800 to 1100 h) from gestation day 6 (GD6) to postnatal day 21 (PND21). The exposure doses of CP were 0, 6.7, 20, and 60 μg/kg/day in corn oil [the maximum daily intake of CP through dietary exposure for adults in Zhejiang Province reported in reference was 6.7 μg/kg/day; the acceptable daily intake (ADI) of CP recommended by WHO was 20 μg/kg/day; the reference dose (RfD) of CP recommended by Environmental Protection Agency was 60 μg/kg/day]. The newborn females in every group were assigned to two groups on PND22 randomly: one group were sacrificed on PND22 to collect the trunk blood for the measurement of GnRH, LH, FSH, and E_2 as well as the hypothalamus, pituitary, and ovary for quantification of mRNA. The female mice in the other group were kept alive and observed every morning (0800 to 1100 h) from PND22 until the appearance of vaginal opening (VO), which indicated the puberty onset of the female mice.

Explants incubation of hypothalamus, pituitary, and ovary

PND21 mice were used for the in vitro experiments of hypothalamus, pituitary, and ovary explants. After decapitation, the hypothalamus, pituitary, and ovary were immediately dissected and incubated according to previous studies (Rasier et al. 2007; Ye et al. 2017a). In short, each well plate contained different 120 μL mixture for the culture of explants. For hypothalamus explants, the MEM was supplemented with 25 mM D-glucose, 1 mM $MgCl_2$, 10 mM glycine, and 1% penicillin–streptomycin solution (Ye et al. 2017a). For pituitary explants, each chamber contained Medium 199 with 1% penicillin–streptomycin solution (Baratta et al. 1994). For ovary explants, the DMEM/F-12 was supplemented with 284 mM L-Ascorbic acid and 1% penicillin–streptomycin solution (Zhao et al. 2014a, b). Explants were randomly divided into 0.1% DMSO and different concentrations of CP (6.7, 20, 60 μg/L) groups and incubated in a 5% CO_2 incubator for 24 h. The supernatant and explants were collected respectively and stored at − 80 ℃ for further analysis.

HPG axis–related hormone assays

After collection, the trunk blood was centrifuged at 4 ℃ and stored at − 80 ℃ until the measurement of GnRH, LH, FSH, and E_2. The levels of GnRH, LH, FSH, and E_2 were determined by the ELISA kits according to the protocol provided by the manufacturer (Jiangsu Meimian Industrial Co., Ltd. Jiangsu, China). The minimum detectable concentration of GnRH, LH, FSH, and E_2 were typically less than 0.1 mIU/L, 0.1 mIU/L, 0.1 mIU/L, and 0.1 pmol/L, respectively, with inter- and intra-assay coefficients of variation less than 10%.

Quantification of mRNA

Total RNA was isolated from the tissues by RNA-Quick Purification Kit according to the protocol provided in the Kits (ES Science, China). First-strand cDNA was synthesized by the reverse-transcription of 0.5–1.0 μg total RNA with BeyoRT™ II First-Strand cDNA Synthesis Kit with gDNA Eraser (Beyotime, China). Oligonucleotide primer sequences for mouse GnRH, KISS-1, LHβ, FSHβ, Cgα, StAR, CYP11A1, and GAPDH are listed in Table 1. Real-time polymerase chain reaction (RT-PCR) was used to determine the expression level of related genes. ABI7500 RT-PCR system (Thermo Fisher, Massachusetts, USA) and SYBR Green PCR master mix reagent (Thermo Fisher, Massachusetts, USA) were used for determination. The PCR system contained 10 μL SYBR Green master mix, 1 μL 10 μM Primer mix, 7 μL ddH_2O, and 2 μL cDNA. The relative amount of each gene transcript was calculated with the $2^{-\Delta\Delta CT}$ method and normalized with the reference gene GAPDH.

Table 1 Primer sequences used for real-time PCR assays

Gene	Primer sequences (5′-3′)
KISS-1	Forward: CTGGTGCAGCGGGAGAAG Reverse: GCGCAGGCCGAAGGA
GnRH	Forward: TGATCCTCAAACTGATGG Reverse: GTACATTCGAAGTGCTGG
LHβ	Forward: ATCACCTTCACCACCAGCAT Reverse: GACCCCCACAGTCAGAGCTA
FSHβ	Forward: GAAGAGTGCCGTTTCTGCAT Reverse: GTGCTGTCGCTGTCACACTT
Cgα	Forward: TCTGGTCATGCTGTCCATGT Reverse: GATATGCCCTGGAGAAGCAA
CYP11A1	Forward: AATGCTGTCTACCAGATGTTCC Reverse: TCGCTTCTGCCTTAAGTCC
StAR	Forward: AGAGGTGGCTATGCAGAAGG Reverse: TCTGCAGGACCTTGATCTCC
GAPDH	Forward: CATCACTGCCACCCAGAAGACT Reverse: GACACATTGGGGGTAGGAACAC

Determination of calcium concentration ($[Ca.^{2+}]_i$)

The concentration of $[Ca^{2+}]_i$ was determined according to previous studies (Usmani et al. 2010; Ye et al. 2017a). In short, ovary explants were incubated with HEPES-buffered saline containing 2 μM Fluo-4AM with or without 50 μM BAPTA-AM, 10 μM nimodipine or 3 mM EGTA. Ovary explants were placed into the Spark 10 M multimode microplate reader (Tecan Group, Switzerland) to start the kinetic cycle. The excitation and emission wavelengths were set at 490 nm and 525 nm, respectively. When the fluorescence reached a steady state, solutions containing CP with or without BAPTA-AM, nimodipine, or EGTA were immediately added to the ovary explants to measure the fluorescence for another 180 min. Relative fluorescence (ΔF/F) was calculated as fluorescence intensities divided by fluorescence intensity determined at the last time point before adding CP.

Statistical analysis

SPSS 22.0 was used for statistical analysis. All the data were presented as the mean ± standard error of the mean (SEM). The data in each group all followed the normal distribution by Kolmogorov–Smirnov and Shapiro–Wilk tests. One-way analysis of variance (ANOVA) was used to assess the differences among groups. If ANOVA revealed significant effects of treatments, the means were compared by Tukey's post hoc test, with $p < 0.05$ being considered statistically significant.

Results

Effects of CP exposure on pubertal onset in the female offspring

Pregnant mice were exposed to 6.7 to 60 μg/kg/day CP daily from GD6 to PND21. As shown in Fig. 1A, the time to puberty was decreased after CP exposure in a dose-dependent manner. The mean age of pubertal onset was decreased approximately 2 days in the female offspring administered with 60 μg/kg/day CP compared with control animals (Fig. 1A). The phenomenon was especially evident at PND 34, where all 60 μg/kg/day CP animals already displayed VO while only 50% VO displayed in the control animals (Fig. 1B). However, no significant decrease in the age of VO was observed in 6.7 and 20 μg/kg/day CP treatment animals compared with control animals, as illustrated by the fact that the mean age of pubertal onset in control group was similar with that in 6.7 μg/kg/day CP animals and about 0.5 day older with that in 20 μg/kg/day CP animals.

Effects of CP exposure on the hypothalamus

To determine the impacts of CP on the synthesis and secretion of GnRH in hypothalamus, both in vivo and in vitro experiments were assigned. Serum GnRH levels and the GnRH mRNA levels in hypothalamus were assessed in the PND22 female offspring whose mothers were exposed to 6.7 to 60 μg/kg/day CP from GD6 to PND21. As shown in Fig. 2A and B, both the serum GnRH levels and the GnRH mRNA levels in the hypothalamus of the PND22 female offspring were significantly increased in a dose-dependent manner.

The hypothalamus of PND21 female mice was dissected and exposed to 6.7–60 μg/L CP for 24 h to further explore the effects of CP on the hypothalamus. In accordance with the results in in vivo test, the GnRH concentration in the medium of hypothalamus was significantly higher in 60 μg/L group than that in the control group (Fig. 2D). As depicted in Fig. 2E, the relative levels of GnRH genes were significantly higher in 20 μg/L CP exposure group than that in control group.

The KISS-1 neuronal population is a key element to generate the GnRH pulse, which is the main driving force of HPO axis (Trevisan et al. 2018). Therefore, the relative levels of KISS-1 gene were quantified in both in vivo and in vitro test. As shown in Fig. 2C, the relative level of KISS-1 was higher in the 6.7 and 20 μg/kg/day groups than that in the control group. However, the relative level of KISS-1 gene in 60 μg/kg/day group was significantly lower than that in the control group, which might be attributed to the negative feedback of GnRH since the synthesis and secretion of GnRH were significantly higher compared with those in the control group. The relative levels of KISS-1 gene in CP treatment group were significantly higher than those in the control group (Fig. 2F). The relative levels of KISS-1 gene decreased with the increase of the exposure concentration of CP, which may be due to the negative feedback of GnRH.

Effects of CP exposure on the pituitary

In vivo exposure to 6.7 to 60 μg/kg/day CP from GD6 to PND21 induced the secretion of LH and FSH in the female offspring in a dose-dependent manner (Fig. 3A and B). Compared with the control group, 20 and 60 μg/kg/day CP significantly increased serum FSH levels while only 60 μg/kg/day CP significantly increased serum LH levels (Fig. 3A and B). Similarly, the expression of gonadotropin subunit genes (Cgα, LHβ, and FSHβ) in the pituitary of the female offspring were induced after in vivo exposure to 6.7 to 60 μg/kg/day CP from GD6 to PND21 (Fig. 3C).

The pituitary of PND21 female mice was dissected and exposed to 6.7–60 μg/L CP for 24 h to further explore the effects of CP on the pituitary in vitro. As shown in Fig. 4A and B, the secreting levels of LH and FSH in conditioned media of pituitary explants were both significantly higher in 60 μg/L group compared to the control group. In accordance with the secretion of gonadotropins, the

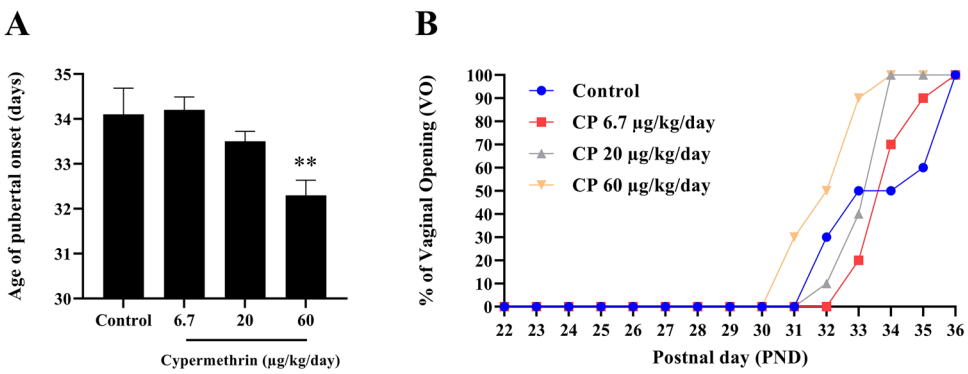

Fig. 1 Effects of perinatal cypermethrin exposure on age of pubertal onset in F1 female mice. **A** The average age of pubertal onset in F1 female mice exposed to 6.7–60 μg/kg/day CP from GD6 to PND21 (presented as mean ± SEM, $n = 10$ independent in vivo experiments). **B** Percentage curves of VO for each experimental group. ** $p < 0.01$ treatment vs. corresponding control

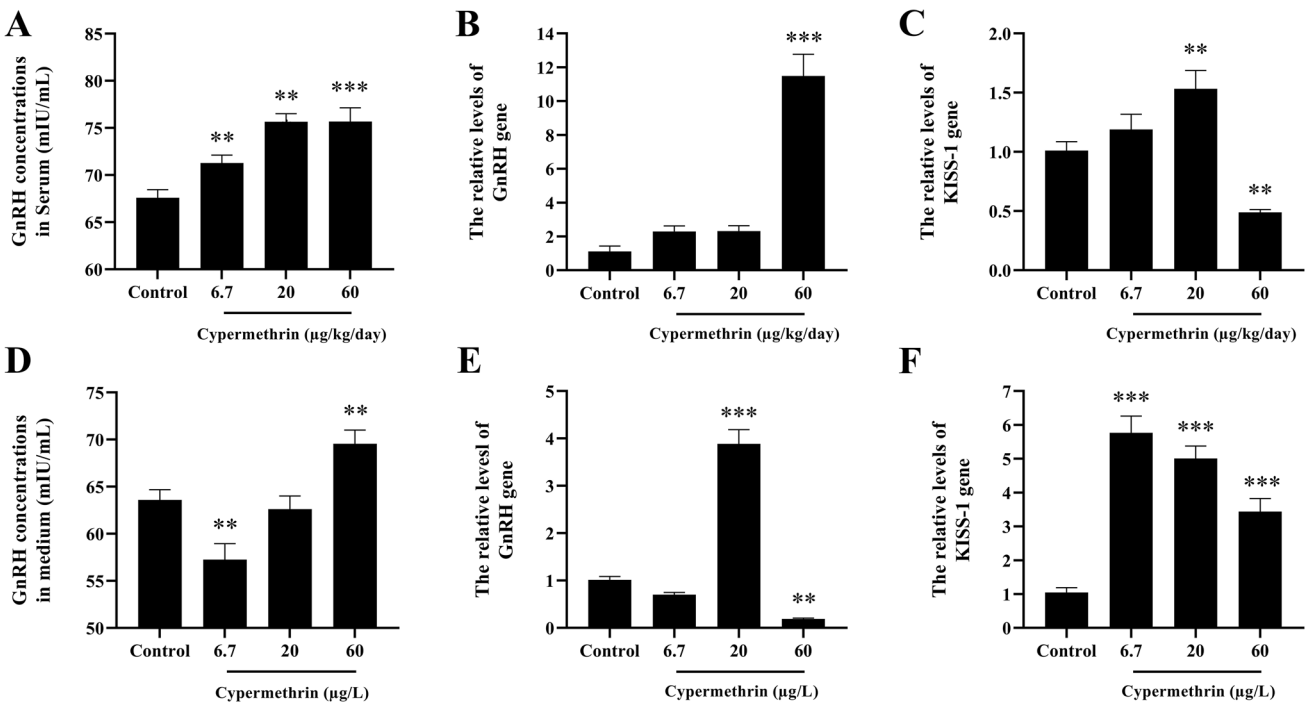

Fig. 2 Effects of perinatal cypermethrin exposure on the hypothalamus. **A** The serum levels of GnRH in F1 female mice exposed to 6.7–60 μg/kg/day CP from GD6 to PND21. **B** The relative expression level of GnRH mRNA in F1 female mice exposed to 6.7–60 μg/kg/day CP from GD6 to PND21. **C** The relative expression levels of KISS-1 mRNA in F1 female mice exposed to 6.7–60 μg/kg/day CP from GD6 to PND21. **D** The secreting levels of GnRH in conditioned media of hypothalamus explants exposed to 6.7–60 μg/L CP for 24 h. **E** The relative expression levels of GnRH mRNA of hypothalamus explant incubation with 6.7–60 μg/L CP for 24 h. **F** The relative expression levels of KISS-1 mRNA of hypothalamus explants incubation with 6.7–60 μg/L CP for 24 h. Values shown present as mean ± SEM ($n = 10$ independent in vivo experiments; $n = 4$–6 independent in vitro experiments). $^{**}p < 0.01$, $^{***}p < 0.001$ treatment vs. corresponding control

relative expression levels of LHβ, FSHβ and Cgα of pituitary explants incubating with 6.7–60 μg/L CP for 24 h were higher than those in the control group (Fig. 4C).

Effects of CP exposure on the ovary

In vivo exposure to CP from GD6 to PND21 induced the secretion of E_2 in the female offspring with a significant increase in 60 μg/kg/day CP group (Fig. 5A). An increase in StAR and CYP11A1 mRNA expression in the ovary was observed in all in vivo experiment groups (Fig. 5B and C). The ovary of PND21 female mice was dissected and exposed to 6.7–60 μg/L CP for 24 h to further explore the effects of CP on the ovary. In accordance with in vivo test, a dose-dependent increase was observed for 6.7–60 μg/L CP on E_2 secretion of the ovary in vitro test (Fig. 5D). Furthermore, in ovary explants, CP stimulated the expression of StAR and CYP11A1 mRNA genes in a dose-dependent manner too (Fig. 5E and F).

To further explore whether voltage-gated calcium channels (VGCCs) was modified by CP in the ovary, ovary explants dissected from PND21 female mice were pretreated with nimodipine, BAPTA-AM and EGTA to block L-VGCCs, chelate intracellular and extracellular Ca^{2+}, respectively. As shown in Fig. 6A, CP-stimulated secretion of E_2 was blocked by all these Ca^{2+} signaling inhibitors. CP-induced StAR and CYP11A1 gene expression was inhibited by pretreatment with nimodipine, BAPTA-AM and EGTA, which was in consistent with the results of E_2 secretion (Fig. 6B and C). CP exposure led to an increase in the concentration of Ca^{2+} in ovary explants, while pretreatment with VGCCs inhibition or calcium chelation attenuated the increase of $[Ca^{2+}]_i$ by CP (Fig. 6D).

Discussion

Pubertal onset is particularly sensitive to environmental factors, specifically endocrine-disrupting chemicals which mimic the secretion of hormones. Pyrethroids, as a typical kind of endocrine disrupting chemicals, could influence the time of pubertal onset in girls, as seen in our recent human study (Ye et al. 2017a, b, c; WHO 2020). CP, one of the most widely used pyrethroid, was applied as a typical pyrethroid insecticide in both in vivo and in vitro tests. The results of the present study showed that perinatal exposure to low

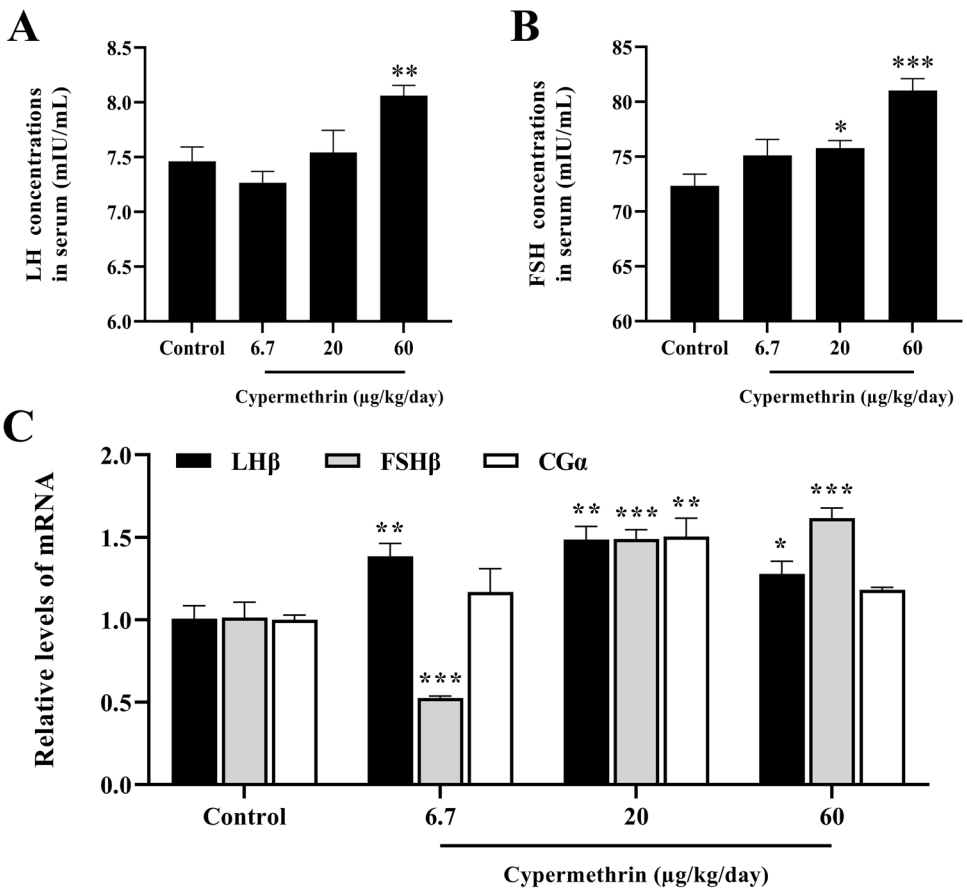

Fig. 3 Effects of perinatal cypermethrin exposure on the pituitary in F1 female mice. **A** The serum levels of LH in F1 female mice exposed to 6.7–60 μg/kg/day CP from GD6 to PND21. **B** The serum levels of FSH in F1 female mice exposed to 6.7–60 μg/kg/day CP from GD6 to PND21. **C** The relative mRNA expression levels of LHβ, FSHβ, and Cgα in F1 female mice exposed to 6.7–60 μg/kg/day CP from GD6 to PND21. Values shown present as mean ± SEM ($n = 10$ independent in vivo experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ treatment vs. corresponding control

doses of CP decreased the time to puberty as well as induced puberty-related hormones in the female offspring.

To the best of our knowledge, it is the first study to report that perinatal exposure to CP at environmental relative doses decreased the time to puberty in the female offspring. Although it was reported that 25 mg/kg CP treatment from GD6 to PND 21 could delay pubertal onset in female rats, the exposure doses were much higher than that in human beings (Singh et al. 2020). A number of biomonitoring studies have reported the urinary levels of 3-PBA in children at the range from < LOD to 141 μg/g creatinine with the median levels ranged from 0.16 to 2.5 μg/g creatinine, which reflected the internal exposure levels of pyrethroids in children (Babina et al. 2012; Barr et al. 2010; Ding et al. 2012; Naeher et al. 2010; Osaka et al. 2016). The exposure doses of CP (6.7, 20 and 60 μg/kg/day) which decreased the time to puberty in the female offspring in this study were similar with that of internal exposure levels in girls (Ye et al. 2017a, b, c). However, in the in vivo experiments, the intake of the female offspring after CP exposure to the female mice was uncertain and required more in-depth study to verify. Moreover, the results found in the present study were not consistent with that in our previous human study or in some other animal studies (Ye et al. 2017a, b, c). The divergence of the results may be caused by the differences in exposure, such as exposure doses, routes of administration, windows of exposure, and co-solvents. Studies on the effects of bisphenol A (BPA) or di-(2-ethylhexyl) phthalate (DEHP) on puberty reported opposite effects at different exposure doses. Franssen et al. suggested that postnatal exposure to very low doses (25 ng/kg BW/d) of BPA delayed puberty onset in female rats, whereas postnatal injections of very high doses (5 mg/kg BW/d) tended to advance the onset of puberty (Franssen et al. 2016). Another study showed that low dose (5 mg/kg) of DEHP decreased the time to puberty, while high dose (500 mg/kg) increased it (Yu et al. 2020). As reported by Fernandez, Losa-Ward, and Naule, even at the same exposure dose (50 μg/kg), differences in routes of administration and species can lead to opposite results (Fernandez et al. 2009; Losa-Ward et al. 2012; Naule et al. 2014). Furthermore, the combined effects of compounds, including additive, independent, synergistic, and antagonistic, may be responsible for the different results. Humans live in a complex natural environment and are subject to interference from different endocrine-disrupting chemical (EDCs), while animal studies are mostly limited to continuous exposure to one compound, which may lead to the divergence of results (Kahn et al. 2020; Kiess et al. 2021). Additionally, as mentioned in the limitations of our previous study, the epidemiological study was a cross-sectional study that may not

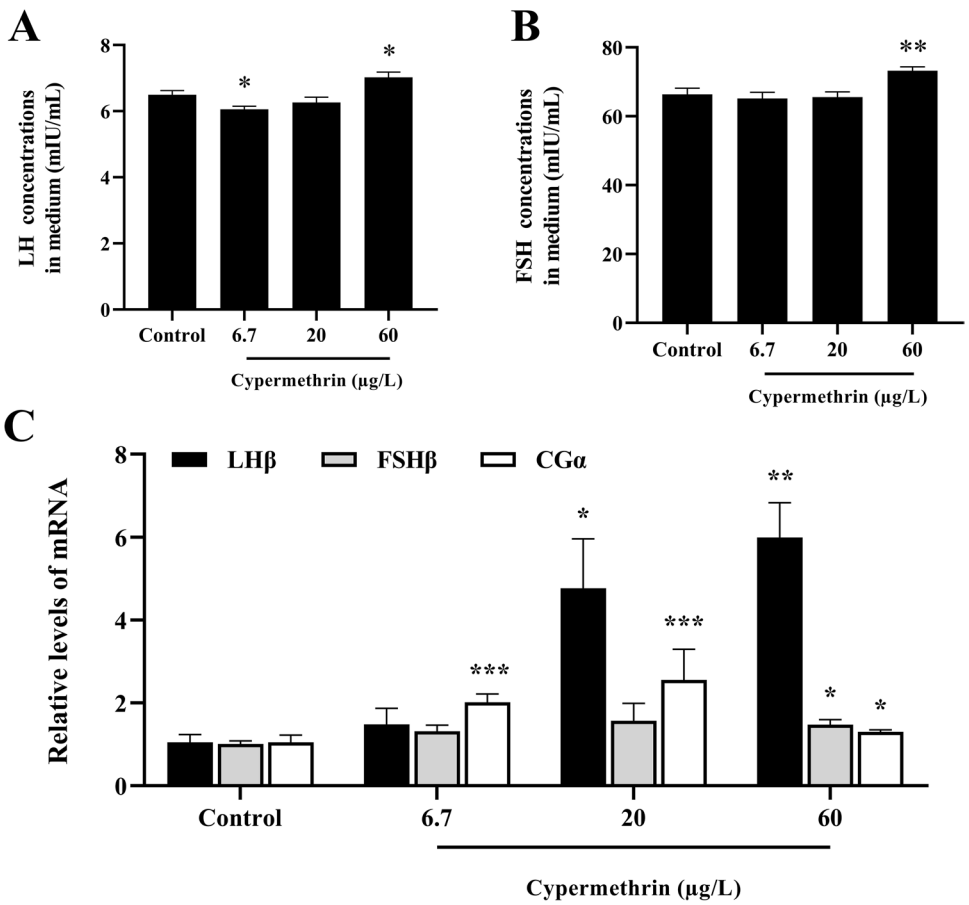

Fig. 4 Effects of cypermethrin exposure on the pituitary explants in PND21 female mice. **A** The secreting levels of LH in conditioned media of pituitary explants exposed to 6.7–60 μg/L CP for 24 h. **B** The secreting levels of FSH in conditioned media of pituitary explants exposed to 6.7–60 μg/L CP for 24 h. **C** The relative mRNA expression levels of LHβ, FSHβ, and Cgα of pituitary explant incubation with 6.7–60 μg/L CP for 24 h. Values shown present as mean ± SEM ($n = 4$–6 independent in vitro experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ treatment vs. corresponding control

adequately represent the long-term exposure levels, which may be one of the possible reasons for the discrepancy (Ye et al. 2017a, b, c). Apart from the above, distinct sensitivity, signal pathways, and individual genetic in different species may account for the multiple results. A population-based study suggested that BPA exposure may lead to delayed menarche in girls, while in some animal studies, BPA exposure resulted in earlier puberty in female mice or rats (Fernandez et al. 2009; Losa-Ward et al. 2012; Miao et al. 2017). As mentioned above, the differences between female rats and mice were also some of the reasons for the discrepancy (Fernandez et al. 2009; Losa-Ward et al. 2012; Naule et al. 2014). Last but not least, different environmental conditions, meteorological conditions, and animal cages will also affect the experimental results. Therefore, the effects of more compounds on animals of more species at real exposure conditions need investigation in the further study.

The axis of hypothalamus-pituitary–gonadal (HPG axis) plays an important role in the growth, development, and reproduction in mammalian. The onset of puberty is activated by gonadotropin-releasing hormone (GnRH) which was regulated by a complex neuronal and glial network in the hypothalamus (Parent et al. 2015). The release of GnRH activates the pituitary gonadotropes to secrete LH and FSH. The gonadotropins then drive the ovarian to produce the sex steroids which lead to the appearance of secondary sexual characteristics (Parent et al. 2015). The disrupted balance of pubertal onset treated with CP could be attributed to a direct effect on the ovary, or to an indirect disruption of the hypothalamus or pituitary. In this study, CP increased the secretion of GnRH and the expression of GnRH genes in the female offspring, which was consistent with the induction in the hypothalamus explants. In good agreement with the observed changes in GnRH levels, it was found that the synthesis of GnRH was induced by bifenthrin, another kind of widely used pyrethroid (Zhao et al. 2014a, b). Furthermore, CP induced the secretion of LH/FSH as well as the expression of gonadotropin subunit genes. LH and FSH are key hormones in regulating the pubertal development and reproductive function (Choi and Yoo 2013). LH and FSH are produced and excreted from the pituitary gonadotropin cells and act on the ovarian in females to regulate the development of ovarian and the excretion of E_2 (Farello et al. 2019). In accordance with this study, our previous case–control study also showed that pyrethroids exposure was positively associated with the secretion of LH and FSH (Li et al. 2018). The increased expression of gonadotropin subunit genes might stimulate the secretion of gonadotropin, finally leading to

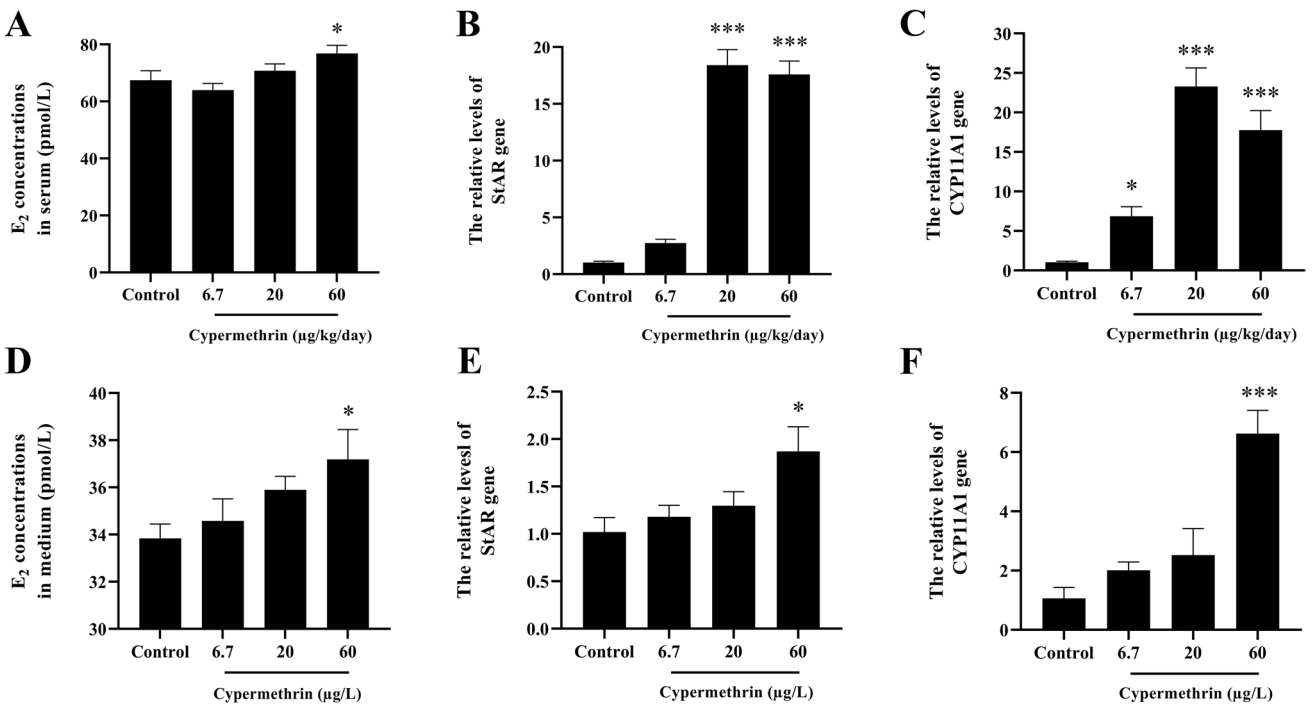

Fig. 5 Effects of perinatal cypermethrin exposure on the ovary in F1 female mice. **A** The serum levels of E_2 in F1 female mice exposed to 6.7–60 μg/kg/day CP from GD6 to PND21. **B** The relative expression levels of StAR mRNA in F1 female mice exposed to 6.7–60 μg/kg/day CP from GD6 to PND21. **C** The relative expression levels of CYP11A1 mRNA in F1 female mice exposed to 6.7–60 μg/kg/day CP from GD6 to PND21. **D** The secreting levels of E_2 in conditioned media of ovary explants exposed to 6.7–60 μg/L CP for 24 h. **E** The relative expression levels of StAR mRNA of ovary explant incubation with 6.7–60 μg/L CP for 24 h. **F** The relative expression levels of CYP11A1 mRNA of ovary explants incubation with 6.7–60 μg/L CP for 24 h. Values shown present as mean ± SEM ($n = 10$ independent in vivo experiments, $n = 4$–6 independent in vitro explants). *$p < 0.05$, ***$p < 0.001$ treatment vs. corresponding control

the increasing serum E_2 levels. In the present study, the increased levels of GnRH, LH, and FSH may induce the excretion of E_2. It was reported that early menarche (before 12 years) was correlated with significantly higher E_2 levels in the adolescent period (Vihko and Apter 1984). In this study, earlier age of VO in female mice may attribute to the increasing E_2 in the serum responding to the increasing synthesis and secretion of LH and FSH in the pituitary gonadotropes as well as GnRH in the hypothalamus. According to these data suggested in the present study, the advancement of puberty by CP may involve all three levels of the HPO axis, which was in line with the endocrine disruption by bifenthrin (Zhao et al. 2014a, b).

Our previous study in male mice showed that CP induced the synthesis and secretion of gonadotropin and testosterone by interference with L-type voltage-gated calcium channels (VGCCs) (Ye et al. 2017a). In addition, CP could cause an increase in $[Ca^{2+}]_i$ in pituitary cells and Leydig cells (Ye et al. 2017a). In accordance with that in male mice, CP also lead to an increase in $[Ca^{2+}]_i$ in the ovary. L-type VGCCs are required for steroidogenesis as well as the regulation of StAR and CYP11A1 genes in ovarian granular cells (Kunz et al. 2002). In the present study, we found that both chelation of Ca^{2+} and inhibition of VGCCs could attenuated CP-stimulated increase of E_2 as well as CP-induced the expression of StAR and CYP11A1 genes. Therefore, CP may increase E_2 secretion by interfering with VGCCs, so as to decrease the time to puberty.

The earlier onset of puberty may increase the risk of mental health problems in the adolescent period as well as diseases in adulthood. For instance, girls with precocious puberty are easier to be depressed because of the increasing possibility of peer rejection (Yu et al. 2019). It was reported that younger age at menarche was associated with increased risk of breast cancer in adulthood (Goldberg et al. 2020). Therefore, it is of crucial importance to assess the effects of pyrethroids exposure on the pubertal onset. The results in this study suggested that CP, a commonly used pyrethroid, was a potential risk factor of earlier pubertal onset in female mice. As the usage of pyrethroids is continuously growing, in further studies, the molecular mechanisms of the alteration of pubertal timing caused by CP exposure is needed to be identified imperatively. The effect of pyrethroids exposure on human beings is urgent to be studied by cohort study.

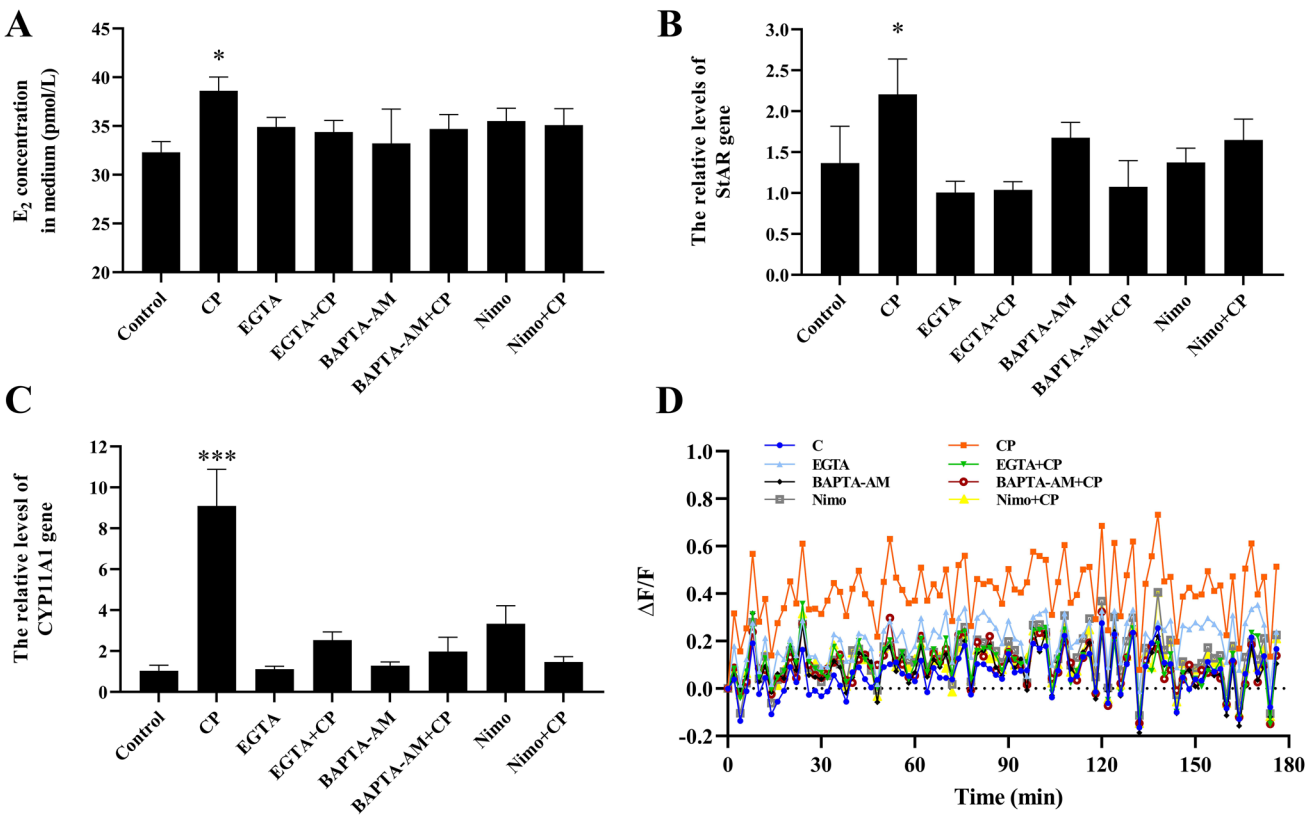

Fig. 6 Involvement of Ca^{2+} signaling in cypermethrin-stimulated E_2 secretion. **A** The secreting levels of E_2 in conditioned media of ovary explants exposed to 60 μg/L CP alone or in combination with 10 μM nimodipine (Nimo), 50 μM BAPTA-AM (BAPTA), or 3 mM EGTA for 24 h. **B** The StAR mRNA levels of ovary explants exposed to 60 μg/L CP alone or in combination with 10 μM Nimo, 50 μM BAPTA, or 3 mM EGTA for 24 h. **C** The CYP11A1 mRNA levels of ovary explants exposed to 60 μg/L CP alone or in combination with 10 μM Nimo, 50 μM BAPTA, or 3 mM EGTA for 24 h. **D** Time course of $[Ca^{2+}]_i$ changes induced in ovary explants exposed to CP alone or in combination with 10 μM Nimo, 50 μM BAPTA, or 3 mM EGTA. Values shown present as mean ± SEM ($n = 6$ independent in vitro experiments). $^*p < 0.05$, $^{***}p < 0.001$ treatment vs. corresponding control

Conclusion

In summary, the present study showed that perinatal exposure to environmentally relative CP may induce earlier pubertal onset and increase secretion of puberty-related hormones in the female offspring. In addition, CP could interfere with VGCCs to induce the synthesis and secretion of E_2 to decrease the time to puberty in the female mice. Taken together, these findings further highlight the endocrine-disrupting effects of CP on females as well as the need for more stringent management of pyrethroids. Moreover, co-exposure with other endocrine disruptors with more species of animals are needed in the further studies.

Author contribution Hongya Gan: Investigation, formal analysis, writing—original draft. Bingqi Zhu: Investigation, formal analysis, visualization. Fangmei Zhou: Visualization, formal analysis. Zhishan Ding: Resources, supervision. Jing Liu: Supervision. Xiaoqing Ye: Investigation, project administration, funding acquisition. Writing—review and editing.

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Data availability The data used to support the findings of this study are available from the corresponding author.

Declarations

Ethical approval We testify that this study is our original research with all data acquired by our examination and there is no plagiarism in it. We also affirm that this study has not been published elsewhere and is not under consideration by another journal.

For the animal study, the animals' care was in accordance with institutional guidelines [Approval number IACUC-20190812–02].

Consent to participate Not applicable.

Consent of publication Not applicable.

Conflict of interest The authors declare no competing interests.

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