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Research Article

Bisphenol AF exposure during gestation and lactation and reproductive function of female offspring rats

Yujie Cao[1,#], Yu Ding[2,#], Minyan Chen[3], Xi Liu[2], Yuli Sun[2], Mingyue Ma[4], Ping Xiao[5],Ying Tian[1,6,7], Xinyu Hong[3,*], Yu Gao[1,*]

[1]Department of Environmental Health, School of Public Health, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China.

[2]Institute of Chemical Toxicity Testing/ State Environmental Protection Key Laboratory of Environmental Health Impact Assessment of Emerging Contaminants, Shanghai Municipal Center for Disease Control and Prevention, Shanghai 200336, China

[3]Yiwu Center for Disease Control and Prevention, Yiwu 322000, China

[4]Department of Toxicology, School of Public Health, Shenyang Medical College, Shenyang, Liaoning 110034, China.

[5]State Environmental Protection Key Laboratory of Environmental Health Impact Assessment of Emerging Contaminants, Shanghai Municipal Center for Disease Control and Prevention, Shanghai 200336, China

[6]State Environmental Protection Key Laboratory of Environmental Health Impact Assessment of Emerging Contaminants, School of Environmental Science and Engineering, Shanghai Jiao Tong University, Shanghai, 200240, China.

[7]Ministry of Education – Shanghai Key Laboratory of Children's Environmental Health, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

[#]These authors contributed equally to this work.

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***Correspondence to:** Xinyu Hong, Institute of Chemical Toxicity Testing/ State Environmental Protection Key Laboratory of Environmental Health Impact Assessment of Emerging Contaminants, Shanghai Municipal Center for Disease Control and Prevention, Shanghai 200336, China. Email: hongxinyu@scdc.sh.cn; Yu Gao, Department of Environmental Health, School of Public Health, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China. Email: gaoyu_ciel@sjtu.edu.cn

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Abstract

Bisphenol AF (BPAF), a substitute for bisphenol A (BPA), has been increasingly produced and used worldwide. It has been widely detected in pregnant women, and its potential effects on reproductive health warrant greater attention. However, still very few studies are available for female reproductive toxicity of BPAF in mammals. We aimed to examine the female reproductive toxicity and potential mechanism after exposure to BPAF during gestation and lactation. Pregnant SD rats were randomly divided into four groups (10 rats each group), and oral gavage daily from gestational day (GD) 0 until the weaning day (Postnatal Day 21, PND21). The control group was treated with corn oil, and the three selected doses of BPAF were 2,10 and 50 mg/kg/d. We followed up their F1 females at PND21 and PND70, and further explored the potential mechanism from the perspective of steroid hormone synthesis. The results showed that exposure to BPAF during gestation and lactation reduced anogenital distance (AGD) at PND21, reduced serum estradiol and Testosterone levels, increased follicular atresia, as well as decreased mRNA expression of genes related to steroid hormone biosynthesis (StAR, CYP11A1, 17β3-HSD and CYP19A1) at PND70. Our study suggested that exposure to BPAF during gestation and lactation can adversely affect reproductive function in F1 females and may have long-term effects, which may be related to interference with steroid hormone biosynthesis in ovaries. Further studies

are needed to verify our results and follow up the long-term reproductive outcomes.

Keywords: Bisphenol AF; Reproductive development; Female rat; Estradiol; Testosterone

INTRODUCTION

Bisphenol AF (BPAF), a substitute for bisphenol A (BPA), has been increasingly used in the production of electronic materials, high-temperature composites, and other specialty polymers[1,2]. Due to its widespread production and usage, BPAF has been detected in soil, surface water, sludge, dust and other environmental media[3-5]. BPAF has also been found in various human biological samples, such as urine, serum and breast milk[6-8]. Particularly, BPAF can transfer via cord blood and lactation[9], making it a potential threat to fetuses and infants during the most sensitive early stages of life to environmental stressors. It is well known that immature female reproductive system may be particularly vulnerable to endocrine disruptors. BPAF has the similar structure as BPA, which has already been recognized as an endocrine disruptor, therefore the potential female reproductive toxicity of BPAF, particularly exposure to BPAF during pregnancy and lactation and its underlying mechanism warrants more attention.

Disrupted steroid hormones are the primary causes of abnormal reproductive development, infertility, and sterility[10]. Although much less studied, several *in vitro* studies have shown that BPAF exhibit stronger binding activity for estrogen receptors (ERα and ERβ) than BPA, indicating that BPAF might have stronger endocrine-disrupting activity compared with BPA[9,11-15]. Previous in vitro and zebrafish studies suggested that BPAF can interfere with the synthesis of sex steroid hormones, influencing the follicle development in ovary[16,17]. Cabaton et al[17] reported that female mice exposure to BPAF revealed a decrease in the cumulative number of pups. In addition, maternal exposure to BPAF impaired embryo implantation, reduced ovarian weight and significantly down-regulated expression levels of ovarian steroidogenic

genes[18]. Therefore, disrupting steroid hormone synthesis may be the important mechanism which underlines the reproductive toxicity of BPAF. However, existing studies only focused on effects of exposure to BPAF on maternal or at early postnatal stage in model fish and very few studies are available for the long-term effects of exposure to BPAF in female mammals[9,17].

Given BPAF can transfer through cord blood and breast milk, it is crucial to explore its impact on offspring during pregnancy and lactation. Moreover, prepuberty and adulthood represent distinct developmental stages. The former is a period before rapid development of the reproductive system and the latter signifies the sexual maturity. In order to comprehensively evaluate the female reproductive toxicity during prepuberty (PND21) and adulthood (PND70), we exposed female rats to BPAF during gestation and lactation, followed up their female offspring until PND70, and further explored the potential mechanism from the perspective of steroid hormone synthesis.

MATERIALS AND METHODS

Chemicals

BPAF [257591, purity 97%, 2,2-bis(4-hydroxyphenyl) hexafluoropropane] was purchased from Sigma-Aldrich, and dissolved in corn oil to obtain 4 mL/mg stock solutions.

Rats maintenance

A total of 60 male and 80 female Sprague Dawley (SD) rats (7-week-old) were purchased from Zhejiang Vital River Laboratory Animal Technology Co., Ltd (SCXK; Zhejiang, China, 2019-0001). Rats were housed in the animal room of the Shanghai Municipal Center for Disease Control and Prevention (SYXK; Shanghai, China, 2018-0031) at 20-25 ℃ under a 12:12-h light-dark photoperiod and relative humidity of 40-70%. The rats were given a commercial diet (Shanghai Zhouyu Biotechnology Co., Ltd., Shanghai, China) and water ad libitum. All the procedures performed in this study

were approved by the Animal Ethics Review Committee of the Shanghai Municipal Center for Disease (Protocol number: 20220008) .

Experimental design

The experimental design is shown in Figure. 1. Natural mating was performed in the light-dark period of the cycle, putting one female in each male's box in the afternoon. On the next morning, the vaginal smear was collected, and the day when sperm-positive smears appeared was considered as the gestational day (GD) 0. The pregnant rats were randomly divided into four groups (10 rats in each group ultimately) following treatments with different doses (0, 2, 10 and 50 mg/kg body weight) of BPAF (Sigma-Aldrich) dissolved in corn oil by oral gavage from GD0 to the weaning day (Postnatal Day 20, PND20). The mother rats were weighed every three days for dose adjustment (Oral, 4 mL/kg body weight by gavage). Doses were selected based on our preliminary experiment as well as published BPAF toxicological studies[9,19]. We obtained the median lethal dose in female rats (Oral, LD_{50}) was 2535 mg/kg, and set the highest dose at 1/50 of LD50 (50 mg/kg) and retained the other doses as 2 and 10 mg/kg, which were set at a five-fold concentration gradient respectively. The daily intake of 50 mg/kg/day was equivalent to15 μg/mL in drinking water, which was comparable to the reported level of BPAF in water (15.3 μg/L)[20].

At PND21 (prepuberty), the F0 females were euthanized and one female pup from each litter was randomly selected and dissected (n = 8-10/group) to collect blood and internal organs for further analysis. From PND21 to PND70, the remaining F1 females continued to be given an ordinary diet (without BPAF exposure). At PND70 (adulthood), and they were also dissected to collect blood and organ samples.

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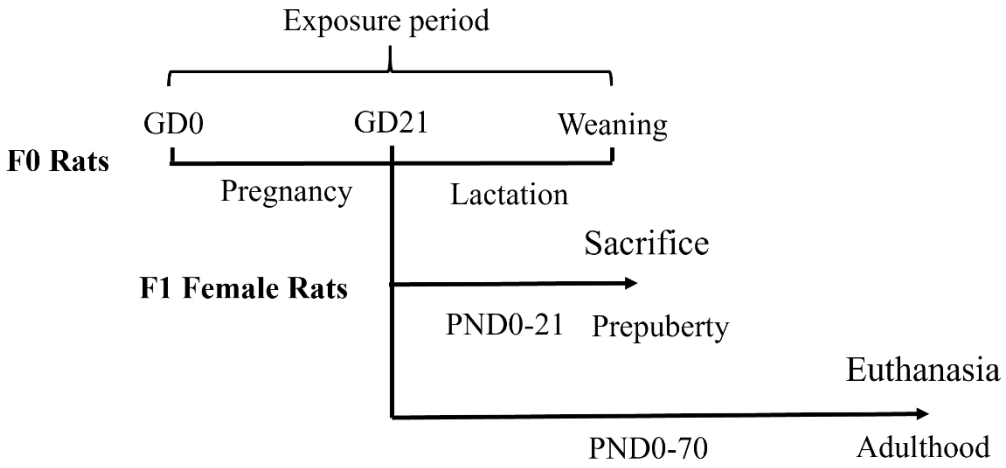

Figure 1. Schematic study design. GD: Gestation Day; PND: Postnatal Day.

Pregnancy outcomes

At PND0, pregnancy outcomes of rats such as gestational weight gain, gestational length, litter size, sex ratio of pups, anogenital distance (AGD) and weights of female pups were recorded. AGD measurements, which were taken from the cranial edge of the anal opening to the caudal edge of the genital tubercle, were conducted in a blinded manner.

Physical development and reproductive functions

From PND0 to PND21, when the physical development of F1 females was changing rapidly, the following parameters of F1 females were observed daily for the evaluation of their physical development: the ages of the ears detachment, eruption of the incisor teeth, and opening eyes. At PND21, the sexual organs of F1 females began to develop rapidly and the AGD of F1 females was measured. Vaginal opening of female pups was observed daily by visual examination of the vulva from PND25. At PND45, when puberty development stabilized, estrous cyclicity was monitored by daily vaginal smears for 2 consecutive weeks to cover at least two complete estrous cycles.

Organ weights and histological examination

The liver, spleen, uterus, and ovaries of F1 females were removed at PND21 and

PND70, respectively, and the wet weight of each organ was weighted. The organ coefficient was then calculated for each organ (organ weight/body weight*100%). Then, the ovaries of female offspring rats in each group were fixed and stored in 10% neutral formalin for 3 days, and were embedded along their longest axis in paraffin and sliced into 4 μm thick sections every 30-micrometer interval. Finally, these sections were stained with HE and a detailed histopathological examination was conducted under a fluorescence microscope in a blind method, including the assessment of follicular development as well as any abnormal changes. Any or all of the following features may be present and indicate follicular atresia: pyknotic granulosa and/or theca cell nuclei, apoptotic bodies at the periphery of the antrum, cell debris in the antrum and detachment of granulosa cells from follicular basement membrane.

Serum sample collection and serum hormone analysis

The blood samples of F1 females were collected at PND21 and PND70 and centrifuged at 2000 rpm for 15 min to separate serum, which were used for detection of estradiol (E2) and testosterone (T) levels by the enzyme linked immunosorbent assay (ELISA) kit, following the manufacturer's protocol (Elabscience Biotechnology Co., Ltd., Wuhan, China). The procedure calls for 50 μL samples per well and the measurement was repeated twice for each serum sample. The absorbance was recorded at 450 nm using a micro-plate reader (Synergy H1M, BioTek, USA) and the hormone levels were calculated by established standard curve from the ELISA kit. The intra- and inter-assay coefficients of variation were less than 10%. The lowest detectable levels for E2 and T were 1.56 pg/mL and 0.31 ng/mL.

RNA extraction and qPCR of ovaries

The total RNA was extracted from ovaries samples of F1 females at PND21 and PND70 (n = 6 per group) using the RNAeasy™ Animal RNA Isolation Kit (Beyotime Biotech. Inc, China) with Spin Column according to the manufacturer's instructions. The purity of RNA was analyzed by spectrophotometry (Nanodrop 8000, Thermo, USA). The

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A260/A280 ratio was about 2, indicating RNA samples were pure and protein-free. The RNA was reverse-transcribed into cDNAs using a Prime Script RT reagent kit (Takara Bio Inc., Otsu, Japan). The PCR cycling parameters were 37 ℃ for 15 min and 85 ℃ for 5 s for 40 cycles. Real Time quantitative PCR (qPCR) was conducted to analyze the mRNA expressions of genes related to steroid hormone biosynthesis, including STAR、3β1-HSD、CYP11A1、17β3-HSD、CYP19A1 and CYP17A1. All primers were synthetized and ordered from Sangon Biotech Co., Ltd. (Shanghai), and the sequences of the primers are shown in Supplementary Table 1. The gene encoding ribosomal protein GAPDH was used as the internal reference gene for each tested sample. The Ct-based relative quantitative expression value of each gene with an efficiency correction normalized to GAPDH was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were shown as mean ± standard deviation (SD). In all experiments, intergroup differences were assessed using one-way analysis of variance (ANOVA) with Dunnett t-Test. The level of statistical significance was set at $p < 0.05$, which was marked with an asterisk in the figures. All statistical analyses were performed using SPSS 26.0 software and plotted by GraphPad Prism 8 software.

RESULTS

Pregnancy outcomes and physical development

Pregnancy outcomes for F0 females, including gestational length, gestational weight gain, litter size, sex ratio of pups, and body weights of F1 females, are presented in Table 1. Except gestational length, which was significantly increased in 50 mg/kg group compared with the control group, no significant difference was found between the control and treatment groups. Physiological development indicators such as the ages of the ears detachment, eruption of the incisor teeth and opening eyes are also shown in Table 1. Compared with the control group, significant decreases were found for weight gain in the 2 mg/kg BPAF group at PND21, for ovaries wet weight and coefficient in the

50 mg/kg BPAF group at PND21, and for spleen and uterus coefficients in the 2 and 10 mg/kg BPAF groups respectively at PND70. No difference was found for other physiological development indicators (Table 1, Supplementary Table 2).

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Table 1. The impact exposure to BPAF on pregnancy outcome of F0 dams and physical development of F1 females.

Dose(mg/kg/d)		**0**	**2**	**10**	**50**
Gestational weight gain （GD 1 to GD 20）		154.00 ± 31.56	165.50 ± 27.87	139.33 ± 62.62	134.88 ± 33.39
Gestational length		22.67 ± 0.50	22.88 ± 0.64	22.75 ± 0.46	23.63 ± 0.92*
Pregnant dams (pup numbers)		9(111)	10(127)	8(108)	8(89)
Litter weight (g)		87.09±28.79	89.88±30.89	96.23±28.82	79.98±23.13
Litter size		12.33±4.53	12.70±4.42	13.50±4.66	11.13±4.26
Ratio of pup's sexes		0.96±0.41	1.13±0.60	1.31±0.76	1.05±0.50
Weights of female pups at PND 1 (g)		7.23±0.82	7.31±0.48	7.19±0.67	7.22±1.12
The ages of the ears detachment (d)		2.11 ± 0.33	2.20 ± 0.35	2.50 ± 0.55	2.25 ± 0.46
Eruption of the incisor teeth (d)		8.22 ± 2.11	7.30 ± 0.46	7.38 ± 0.52	7.75 ± 0.89
Opening eyes (d)		13.33 ± 0.71	13.40 ± 0.76	13.25 ± 0.71	13.88 ± 0.64
Weight at PND21(g)		68.92±4.16	65.63±5.15**	70.51±3.82	67.93±5.46
Weight gain from PND 1 to PND21 (g)		56.97 ± 3.56	54.24 ± 4.20**	58.18 ± 3.18	55.77 ± 4.80
Organ coefficients at PND21 (g/100g)	Liver	3.35±0.27	3.24±0.33	3.37±0.17	3.23±0.31
	Spleen	0.40±0.09	0.36±0.06	0.38±0.08	0.36±0.05

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	Uterus	0.17±0.11	0.16±0.06	0.16±0.06	0.14±0.05
	Ovaries	0.09±0.04	0.07±0.03	0.07±0.02	0.06±0.02*
Weight at PND70(g)		292.22±42.83	266.50±37.50	293.25±31.55	294.50±32.15
	Liver	3.02±0.52	2.85±0.17	2.95±0.23	2.86±0.19
	Spleen	0.19±0.02	0.23±0.03**	0.23±0.03**	0.20±0.03
Organ coefficients at PND70 (g/100g)	Uterus	0.18±0.02	0.26±0.06**	0.27±0.07**	0.22±0.08
	Ovaries	0.08±0.02	0.08±0.02	0.08±0.02	0.07±0.01

Weight gain of female pups(g) = weight of female pups at PND21(g)- weight of female pups on PND 4(g); $^{*}p<0.05$; $^{**}p<0.01$.

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Reproductive function and morphology

AGD of F1 females were measured at PND0, PND21 and PND70 repeatedly. Compared to the control group, significantly reduction of AGD was found in all treatment groups (2, 10 and 50 mg/kg) at PND21, but not at PND 0 and PND70. Additionally, no significant difference was found for vaginal opening and estrous cycle length among all the treated groups, although an increasing trend was observed (Table 2).

In addition, ovarian morphology was also examined (Figure 2 and Figure 3). At PND21, no difference in ovarian morphology was found significantly among all the BPAF treated groups when compared with the control group (Figure 2). At PND70, a noticeable trend towards an increased incidence of follicular atresia was found only in the 50 mg/kg BPAF(Figure 3).

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Table 2. The impact of gestational and lactational exposure to BPAF on vaginal opening time, estrous cycle and AGD of female offspring.

Dose	n	AGD (mm)			Age of vaginal opening (d)	Estrous cycle length
(mg/kg/d)		PND 0	PND21	PND70		(d)
0	9	5.64±0.46	12.05 ± 0.59	16.84 ± 0.68	31.33 ± 1.73	4.44 ± 0.73
2	10	5.73±0.38	11.51 ± 0.67***	16.20 ± 1.88	31.70 ± 2.47	4.90 ± 1.20
10	8	5.67±0.40	11.75 ± 0.54*	17.17 ± 1.31	30.63 ± 4.24	4.88 ± 1.06
50	8	5.58±0.45	11.54 ± 0.50*	17.33 ± 1.20	32.38 ± 1.41	5.11 ± 0.74

AGD, anogenital distance; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

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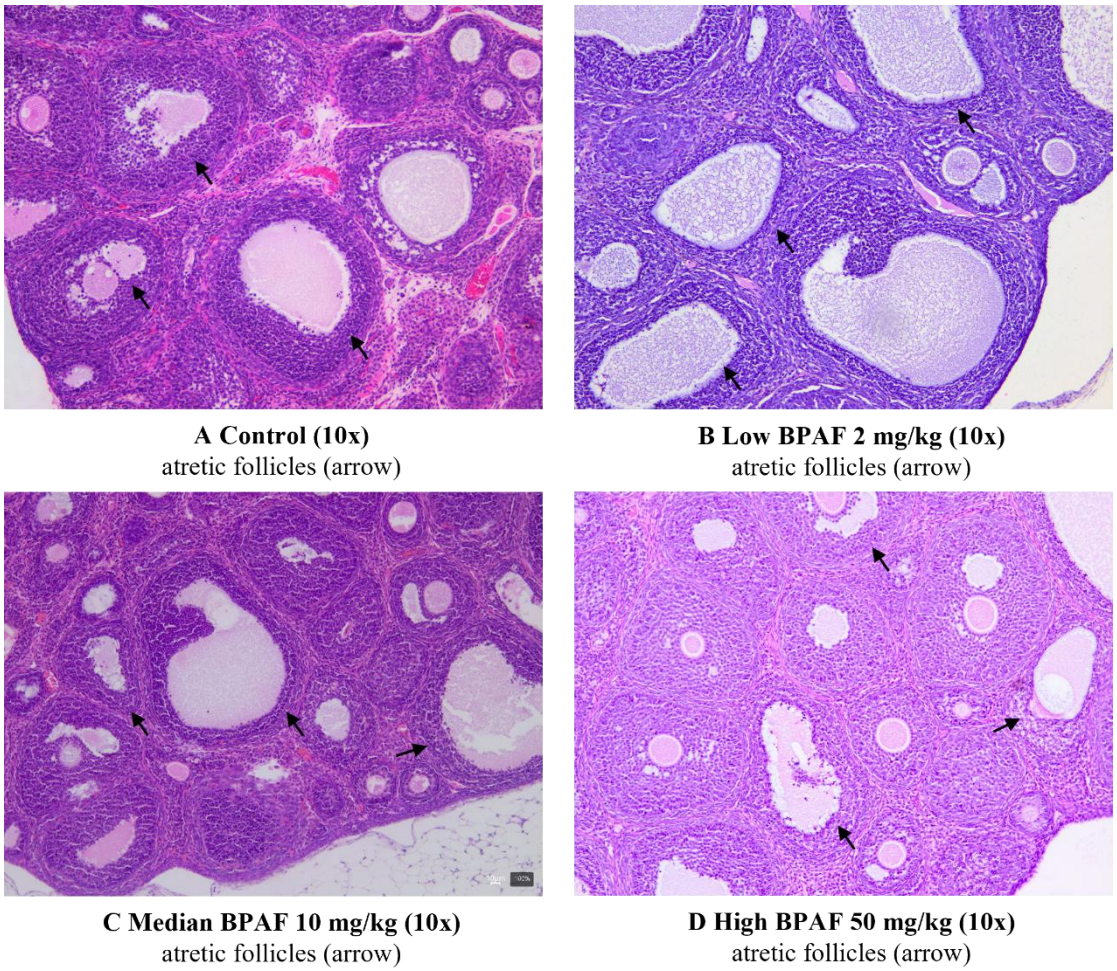

Figure 2. The impact of gestational and lactational exposure to BPAF on ovary histology of PND21 F1 females. (A) Control; (B) BPAF 2 mg/kg; (C) BPAF 10 mg/kg;(D) BPAF 50 mg/kg. Arrows indicate atretic follicles.

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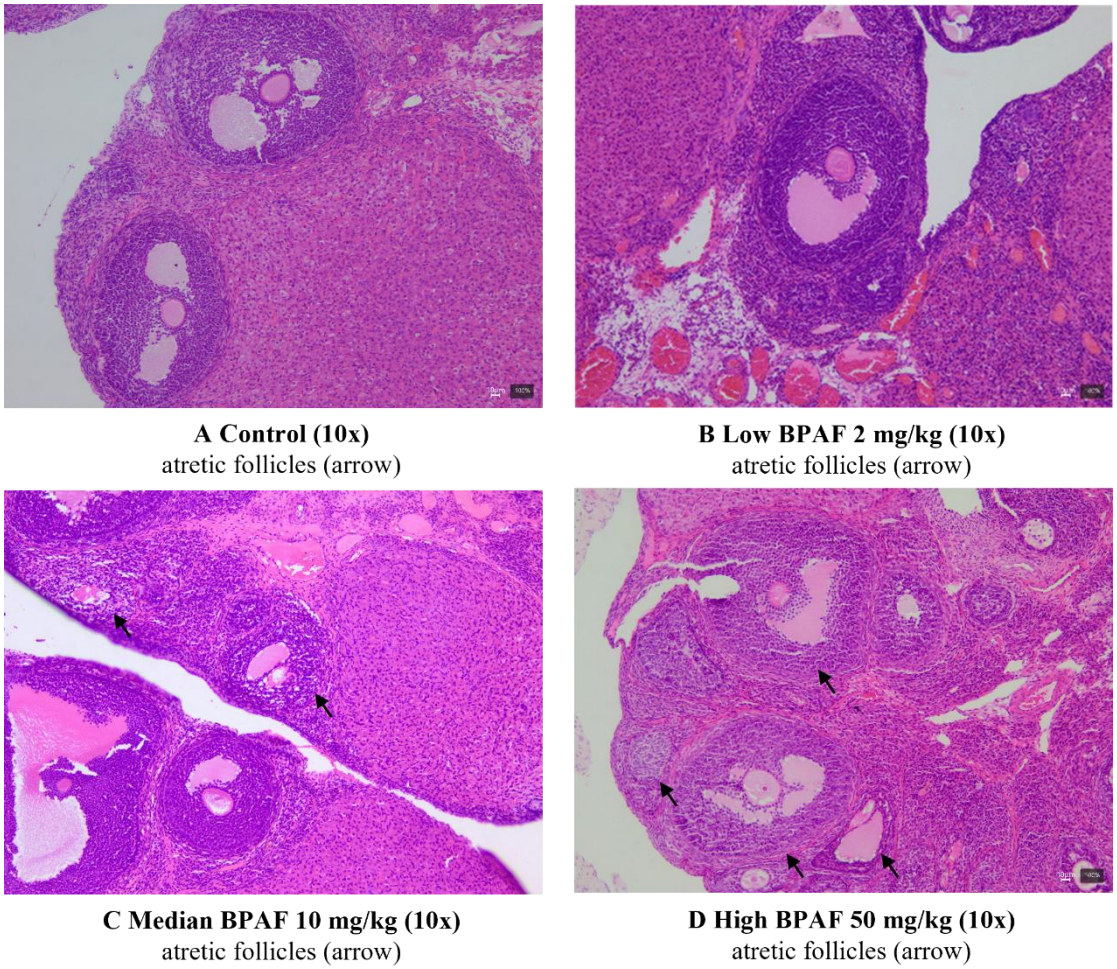

Figure 3. The impact of gestational and lactational exposure to BPAF on ovarian tissue of PND70 F1 females. (A) Control; (B) BPAF 2 mg/kg; (C) BPAF 10 mg/kg; (D) BPAF 50mg/kg. Arrows indicate atretic follicles.

Serum hormone levels

Serum hormone levels (E2 and T) of F1 females were measured at PND21 and 70 (Figure 4). At PND21, only lower serum E2 levels were found in the 2 and 10 mg/kg BPAF groups compared to the control group. At PND70, both serum E2 and T levels significantly decreased in all BPAF treated groups and showed in a dose-dependent relationship.

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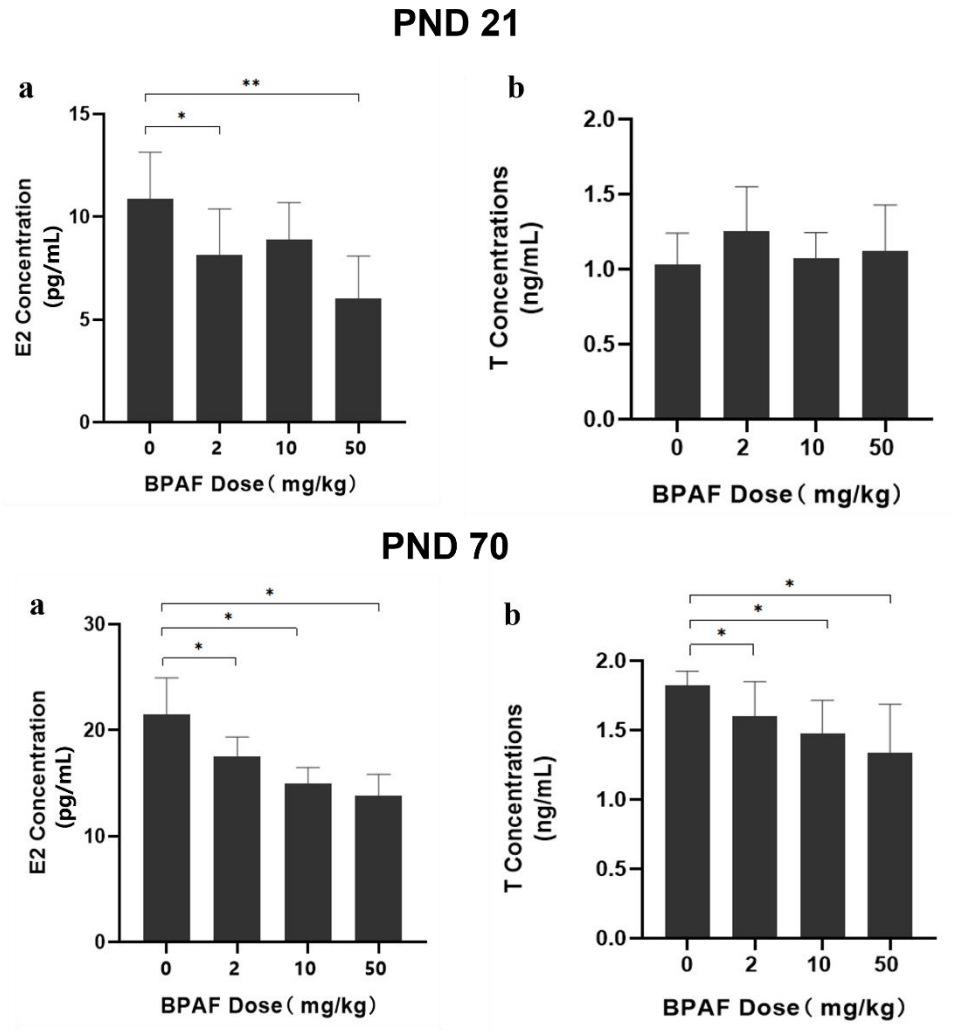

Figure 4. The impact of gestational and lactational exposure to BPAF on E2 and T hormones levels at PND21 and PND70 female rats. * $p<0.05$; ** $p<0.01$

mRNA expression in steroidogenesis genes

Steroidogenesis related genes (STAR, 3β1-HSD, CYP11A1, 17β3-HSD, CYP19A1 and CYP17A1) of F1 females' ovaries were measured for their mRNA expression, as shown in Figure 5. At PND21, no significant difference was observed for all genes in prepuberty F1 females, however, at PND70, significantly decreased levels of mRNA of several steroidogenesis related genes (StAR, CYP11A1, 17β3-HSD, and CYP19A1) were found among different treated BPAF groups compared to the control group, as shown decreased mRNA levels of StAR in 2 and 50 mg/kg BPAF groups, CYP11A1 in

2 mg/kg BPAF group, 17β3-HSD in 10 mg/kg/d BPAF group and CYP19A1 in 10 and 50 mg/kg/d BPAF groups.

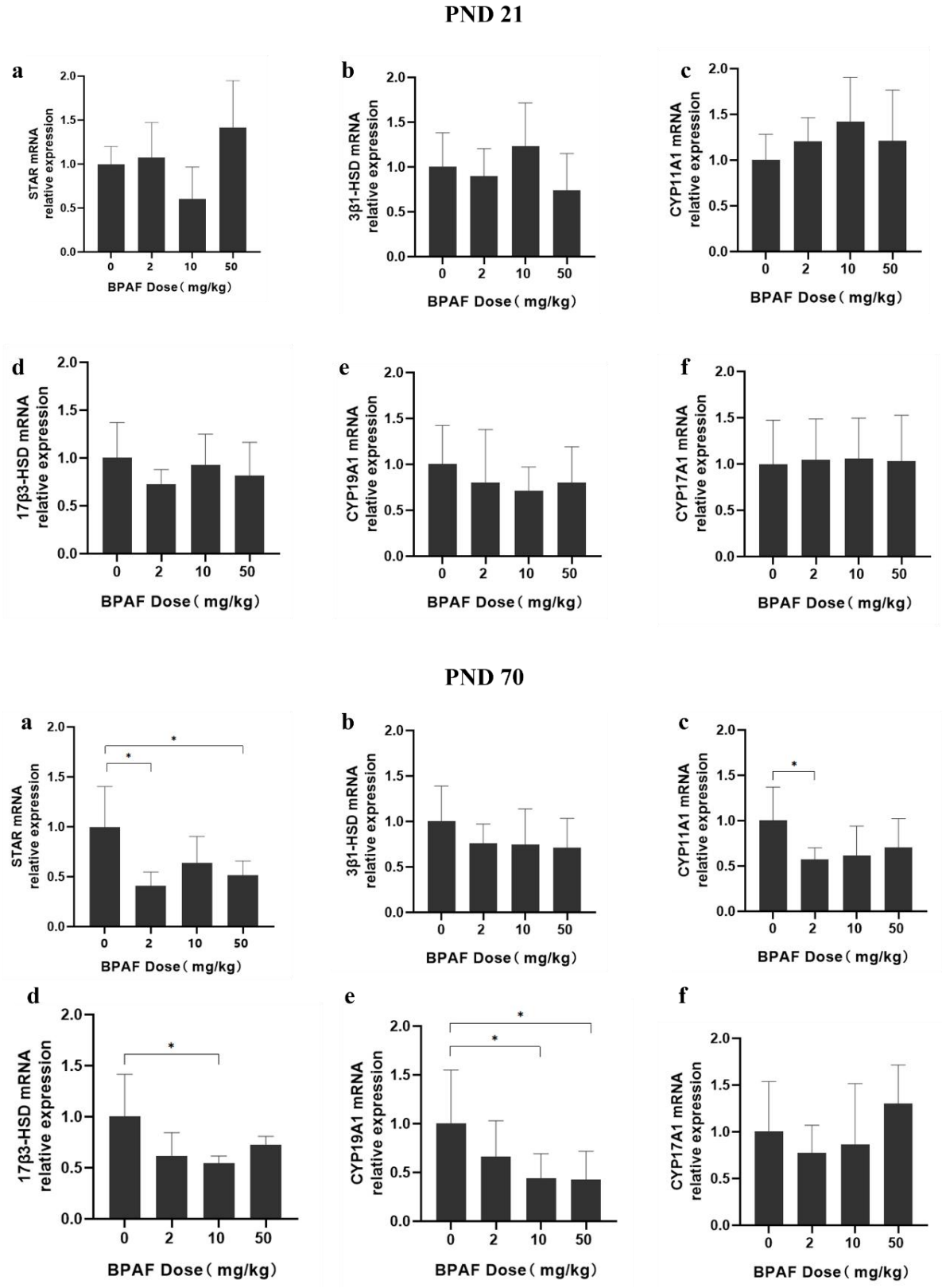

Figure 5. The impact of gestational and lactational exposure to BPAF on the mRNA

expression of steroidogenic enzymes of PND21 and PND70 F1 females. * $p<0.05$

DISCUSSION

In this study, we found that exposure to BPAF during gestation and lactation may affect reproductive system of F1 females, as evidenced by reduced AGD and serum sex hormone levels, increased follicular atresia, as well as decreased mRNA expression of genes related to steroid hormone biosynthesis. Moreover, above changes varied at different reproductive stages, and more prominent effects were observed at PND70, suggesting that BPAF exposure during the early life may have long-term effects on female reproduction.

Regarding to the effects of exposure to BPAF on ovarian steroid hormone synthesis, we speculate the following possible mechanistic pathways based on our results. Exposure to BPAF may down-regulate mRNA expression of several critical genes related to steroid hormone synthesis, including STAR, CYP11A1, 17β3-HSD, and CYP19A1, and lead to decreased T and E2 (Figure 6). It is well known that sex steroid hormone plays an important role in the development of follicles and maintaining regular estrous cycle[21], such as low E2 level may result in abnormal follicular development[22], decrease pregnancy rate and embryo implantation rate[23,24], and low serum T level may be related with reduced AGD[25,26]. One research also found that BPA exposure could reduce AGD of female rats[27]. In our study, we observed reduced AGD and serum E2 and T levels, as well as increased follicular atresia, which supported our hypothesis that disruption of steroid hormone synthesis may be the underlying important mechanism contributing to the reproductive toxicity of BPAF. However, serum T and E2 levels were measured at PND21 and PND70, while the main critical window for establishing AGD is in the mid-to-late gestation period in rats[28]. There may also be other explanations for the changes in this indicator. Still relatively few studies have examined the effects of exposure to BPAF on steroidogenesis *in vivo*. Consistent with our study, some studies

suggested that BPAF can affect steroid hormone levels by regulating mRNA expression levels related to steroid hormone synthesis[2,9,19,29-34]. For example, LU S et al[18] reported that BPAF(3 and 30 mg/kg/day) treatment during early gestation significantly down-regulated expression levels of ovarian StAR, CYP11A, 17β3-HSD and CYP19A1 mRNA, and decreased serum progesterone and E2 levels in mice. Another study showed exposure to BPAF(100 mg/kg/day) during gestation and lactation increased serum T levels in F1 male rats[9]. Our study provided additional evidence that exposure to BPAF even at relatively low dose (2 mg/kg/day) during gestation and lactation may disrupt steroid hormone synthesis and lead to reproductive toxicity in female offspring. Previous *in vitro* studies have showed that BPAF even has stronger binding activity for estrogen receptors compared to BPA[35], which has already been recognized as an endocrine disruptor, therefore BPAF may not be a safety substitute of BPA, and the long-term effects of its reproductive toxicity merits more attention.

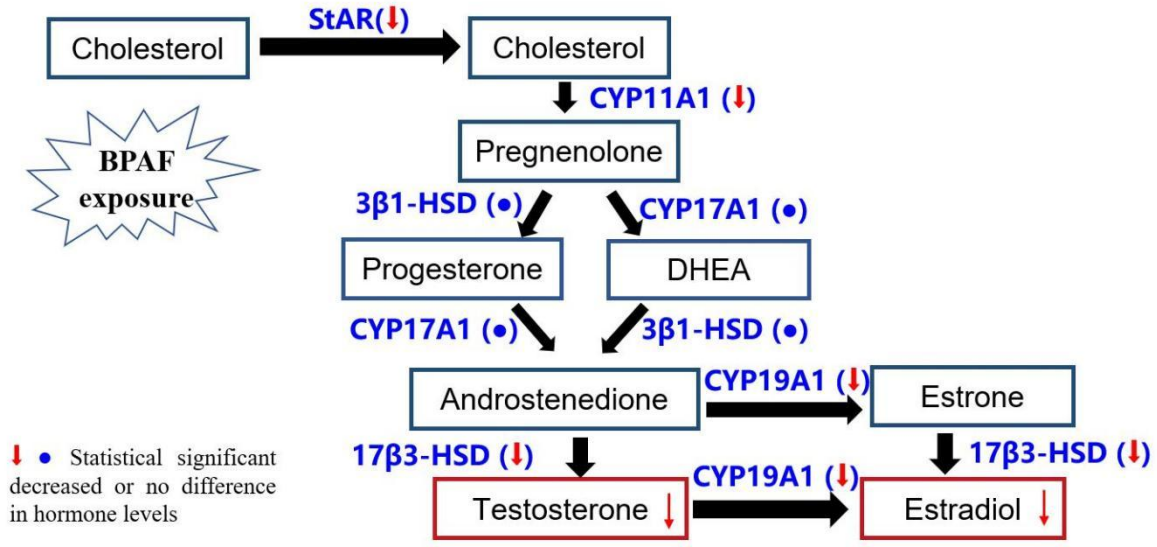

Figure 6. The pathways of the impact of BPAF exposure on ovarian steroid hormone synthesis of adult female offspring from the present study.

This figure described the possible pathways of the effects of BPAF exposure on ovarian steroid hormone synthesis in adult female offspring. The results suggested that BPAF

exposure during gestation and lactation reduced estradiol and testosterone levels and decreased the ovarian mRNA expression of STAR, CYP11A1, 17β3-HSD, and CYP19A1 in adult female offspring (PND70). Thus, we hypothesized that BPAF may disrupt steroid hormone (E2 and T) by down-regulating the mRNA expression of steroid hormone synthesis (STAR, CYP11A1, 17β3-HSD, and CYP19A1). The blue text in the figure indicated the possible targets of the reproductive developmental toxicity of BPAF from the experimental literature. The red text boxes indicated the two steroid hormones that BPAF may interfere with, including estradiol (E2) and testosterone (T).

In this study, we compared the changes at two different reproductive stages, and more prominent effects on reproductive function as well as steroidogenesis were observed at PND70 compared to PND21. For example, changes in mRNA expression of genes of steroid hormone synthesis (STAR、CYP11A1、17β3-HSD and CYP19A1) were only observed at PND70, and sex hormones and ovarian histology showed more significant changes also at PND70 compared to PND21. Some studies also explored the effects of BPA and BPAF exposure on F1 offspring at different stages and changes were more pronounced after reproductive maturation than puberty, which were similar to ours[20,36] For example, one study found that pregnant mice exposure to BPA via oral gavage (0.5, 20 and 50μg/kg) from GD11 until birth did not result in significant effects like the changes of the age of vaginal opening, but increased the percentage of dead pups at three months of age in female offspring[37]. Another study exposed F0 CD-1 mice to BPAF (5 mg/kg) via oral gavage between GD10 to GD17, and mammary glands exhibited significantly changed morphology in comparison with controls only by late adulthood (PND56)[36]. A plausible explanation for this phenomenon might due to the sexual organs are still immature during prepuberty[9,38,39]. Consequently, hormone levels are relatively low and stable, the steroid hormone synthesis is unstable at this stage and the hormonal feedback mechanism is relatively simple, on which effects of environmental pollutants may not immediately manifest[40]. Although the early changes

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may not be obvious, they can lead to long-term reproductive toxicity, as demonstrated by morphological and sex hormone changes during adulthood[9,38,39,41]. It is worth noting that the absence of notable changes in reproductive hormones or functions during puberty may be easily overlooked without long-term followed up. Therefore, it is necessary to pay more attention to the long-term effects of BPAF on offspring reproduction.

The advantages of this study are that we explored effects of low dose of BPAF exposure, while previous studies[9,19]on exposure to BPAF were conducted at relatively high doses, which may not accurately reflect the impact of human exposure to its environmental concentration. And we also observed the reproductive development of F1 females at different stages. Thus, compared with similar studies, the reproductive toxicity of exposure to BPAF on F1 females may be examined more continuous and comprehensive. Moreover, we only primarily explored the mechanism of reproductive and developmental toxicity of BPAF from the perspective of hormone synthesis, there may also be other pathways by which BPAF contributes to reproductive toxicity.

This study suggested that exposure to BPAF during gestation and lactation can adversely affect reproductive function in female offspring, which may be related to interference with steroid hormone biosynthesis in ovaries. The pathway and specific target of BPAF causing these toxic effects are not clear, and further studies are needed to underline the potential mechanism and follow up the long-term reproductive outcomes.

DECLARATIONS

Authors' contributions

Writing – original draft: Y.J. Cao, M.Y. Chen, X.Y. Hong, Y. Gao.

Writing – review & editing: Y.J. Cao, Y. Zhang, R. Shi, M.Y. Ma, P. Xiao, Y. Tian, X.Y. Hong, Y. Gao.

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Methodology, Resources : Y. Ding, X. Liu, Y.L. Sun.

Visualization, Formal analysis: Y.J. Cao, M.Y. Chen.

Conceptualization: Y.J. Cao, Y. Ding.

Data curation: Y.J. Cao, Y. Ding, M.Y. Chen

Supervision: P. Xiao, Y. Tian, X.Y. Hong, Y. Gao.

Availability of data and materials

Information related to this article can be found in Supplementary Materials. Further data are available from the corresponding authors upon reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All the procedures performed in this study were approved by the Animal Ethics Review Committee of the Shanghai Municipal Center for Disease (Protocol number: 20220008) .

Consent for publication

Not applicable.

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