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Effects of gestational and lactational bisphenol AF exposure on female offspring reproductive function during two developmental stages

Yu-Jie Cao^{1,#}, Yu Ding^{2,#}, Min-Yan Chen³, Xi Liu², Yu-Li Sun², Yan Zhang¹, Rong Shi¹, Ming-Yue Ma⁴, Ping Xiao⁵, Ying Tian^{1,6,7}, Xin-Yu Hong², Yu Gao¹

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

²Institute 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.

³Yiwu Center for Disease Control and Prevention, Yiwu 322000, Zhejiang, China.

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

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

⁶State 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.

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

#Authors contributed equally.

Correspondence to: Dr. Xin-Yu 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, No. 1399, Shenhong Road, Shanghai 200336, China. E-mail: hongxinyu@scdc.sh.cn; Prof. Yu Gao, Department of Environmental Health, School of Public Health, Shanghai Jiao Tong University School of Medicine, No. 280, South Chongqing Road, Huangpu District, Shanghai 200025, China. E-mail: gaoyu_ciel@sjtu.edu.cn

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Abstract

Bisphenol AF (BPAF), a widely used substitute for bisphenol A (BPA), has been widely detected in pregnant women, raising concerns about its potential effects on reproductive health. However, studies on the reproductive toxicity of BPAF in mammals remain limited. This study aimed to investigate the reproductive toxicity and underlying mechanisms of BPAF exposure during gestation and lactation. Pregnant Sprague-Dawley rats were



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randomly assigned to four groups ($n = 10/\text{group}$) and administered BPAF by oral gavage (0, 2, 10, or 50 mg/kg/d in corn oil) from gestational day (GD) 0 to postnatal day (PND) 21. Female offspring [first filial generation (F1)] were examined at PND21 and PND70 for reproductive outcomes and steroidogenic alterations. Gestational and lactational BPAF exposure significantly reduced anogenital distance (AGD) at PND21, from 12.05 ± 0.59 mm in controls to 11.54 ± 0.50 mm in the 50 mg/kg group ($P < 0.001$). At PND21, serum estradiol (E2) levels were decreased in the 2 and 10 mg/kg BPAF groups compared with controls ($P < 0.05$). By PND70, all BPAF-exposed offspring showed reduced E2 and testosterone (T) levels ($P < 0.05$). In addition, BPAF exposure increased follicular atresia and downregulated the mRNA expression of key steroidogenic genes (StAR, CYP11A1, $17\beta\text{-HSD}$, and CYP19A1; $P < 0.05$) at PND70. Our findings indicate that BPAF exposure during gestation and lactation impairs reproductive function in F1 females and may exert long-term effects through disruption of ovarian steroidogenesis. Further research is warranted to confirm these outcomes.

Keywords: Bisphenol AF, reproductive development, female rat, estradiol, testosterone

INTRODUCTION

Bisphenol AF (BPAF), a structural analog and alternative to bisphenol A (BPA), has seen increasing application in the production of electronic materials, high-temperature composites, and other specialty polymers^[1,2]. Due to its widespread use, BPAF has been detected in various environmental media, including soil, surface water, sludge, and dust^[3-5]. It has also been found in human biological samples such as urine, serum, and breast milk^[6-8]. Importantly, BPAF can be transferred via cord blood and lactation^[9], posing a potential risk to fetuses and infants during the most sensitive early stages of development. The immature female reproductive system is particularly vulnerable to endocrine disruptors. Since BPAF shares a similar structure with BPA - already recognized as an endocrine disruptor - its potential reproductive toxicity in females, especially during pregnancy and lactation, as well as the underlying mechanisms, warrants closer investigation.

As an endocrine disruptor, BPAF may induce reproductive toxicity through several pathways, including oxidative stress, disruption of the hypothalamic-pituitary-gonadal (HPG) axis, and interference with steroidogenesis^[10]. The ovary has been identified as a primary target organ for bisphenol compounds^[11]. Given BPAF's potent estrogenic activity^[9,12-16] and the fact that ovarian granulosa cells are the primary site of estrogen receptor expression, it is plausible that BPAF contributes to reproductive dysfunction by altering granulosa cell-mediated steroidogenesis. This hypothesis provides the rationale for our current study. Disruption of steroid hormone production is a potential cause of abnormal reproductive development, infertility, and sterility^[17]. Previous *in vitro* and zebrafish studies have shown that BPAF can interfere with sex steroid hormone synthesis, thereby affecting ovarian follicle development^[18,19]. Cabaton *et al.*^[19] reported that female mice exposed to BPAF exhibited a decrease in the cumulative number of pups. Moreover, maternal BPAF exposure significantly impaired key reproductive outcomes, including embryo implantation, ovarian weight, and the expression of steroidogenic genes in the ovary^[20]. These findings suggest that disruption of steroid hormone synthesis may be a critical mechanism underlying the reproductive toxicity of BPAF. However, most existing studies have focused on maternal exposure or early postnatal effects in fish models, and very few have examined the long-term consequences of BPAF exposure in female mammals^[9,19].

Since BPAF can be transferred through cord blood and breast milk, it is essential to explore its impact on offspring during pregnancy and lactation. Furthermore, prepuberty and adulthood represent distinct developmental stages: the former precedes rapid reproductive system development, while the latter marks sexual maturity. To comprehensively evaluate female reproductive toxicity during prepuberty [postnatal day

(PND) 21] and adulthood (PND70), we exposed female rats to BPAF during gestation and lactation, followed their female offspring until PND70, and further explored the underlying mechanisms with a focus on steroid hormone synthesis.

EXPERIMENTAL

Chemicals

BPAF [257591, purity 97%, 2,2-bis(4-hydroxyphenyl) hexafluoropropane] was purchased from Sigma-Aldrich and dissolved in refined corn oil (Jinlongyu Pure Corn Oil, food-grade) by vortexing for 3 min, followed by sonication (40 kHz, 25 °C, 20 min). Fresh BPAF stock solutions (4 mg/mL in corn oil) were prepared weekly and stored at 4 °C.

Animal maintenance

A total of 140 pathogen-free Sprague Dawley SD rats (60 males and 80 females, 7-week-old) were obtained from Zhejiang Vital River Laboratory Animal Technology Co., Ltd (SCXK; Zhejiang, China, 2019-0001). Rats were housed in the animal facility of the Shanghai Municipal Center for Disease Control and Prevention (SYXK; Shanghai, China, 2018-0031) under controlled conditions (20-25 °C, 12:12-h light/dark cycle, relative humidity 40%-70%). Animals had free access to a commercial diet (Shanghai Zhouyu Biotechnology Co., Ltd., Shanghai, China) and water. All experimental procedures were approved by the Animal Ethics Review Committee of the Shanghai Municipal Center for Disease Control and Prevention (Protocol number: 20220008).

Experimental design

The experimental design is shown in [Figure 1](#). All mating procedures were conducted during the light-to-dark transition (16:00-18:00), which corresponds to peak rodent sexual activity. Individual nulliparous females were placed with sexually experienced males (1:1 ratio) and monitored for vaginal smears. The day sperm-positive smears were detected was designated gestational day (GD) 0. A randomized complete block design was used. Forty confirmed-pregnant females were stratified by body weight and randomly assigned to four exposure groups (10 dams/group). Pregnant rats received daily oral gavage of BPAF (Sigma-Aldrich; 0, 2, 10, or 50 mg/kg body weight dissolved in corn oil) from GD0 to PND20. Maternal body weight was recorded every three days to adjust the dose (4 mL/kg by gavage). The dosing regimen was based on the provisional Tolerable Daily Intake (TDI) of 4 µg/kg established by the European Food Safety Authority (EFSA) for BPA^[21]. Dose calculations followed the formula: Human Equivalent Dose (HED) × intraspecies uncertainty factor (UFH) × interspecies uncertainty factor (UFA) × toxicokinetic uncertainty factor (UFT)^[22,23]. Based on this calculation, 2 mg/kg was selected as the lowest dose, with 5-fold intervals defining the medium (10 mg/kg) and high (50 mg/kg) doses. According to the steady-state plasma concentration formula^[24] and parameters from a rat gavage model of BPAF^[25], the 2 mg/kg dose corresponded to a plasma concentration of 0.872 ng/mL, comparable to reported human plasma levels of BPAF (0.44 ng/mL)^[26].

At PND21 (prepuberty), parental generation (F0) females were euthanized, and one female pup from each litter was randomly selected for blood and organ collection ($n = 8-10/\text{group}$). The remaining first filial generation (F1) females received a standard diet without BPAF until PND70 (adulthood), when they were also euthanized for blood and organ sampling.

Pregnancy outcomes

At PND0, pregnancy outcomes including maternal weight gain, gestation length, litter size, sex ratio of pups, anogenital distance (AGD), and body weight of female pups were recorded. AGD was measured from the cranial edge of the anus to the caudal edge of the genital tubercle by an investigator blinded to group assignments.

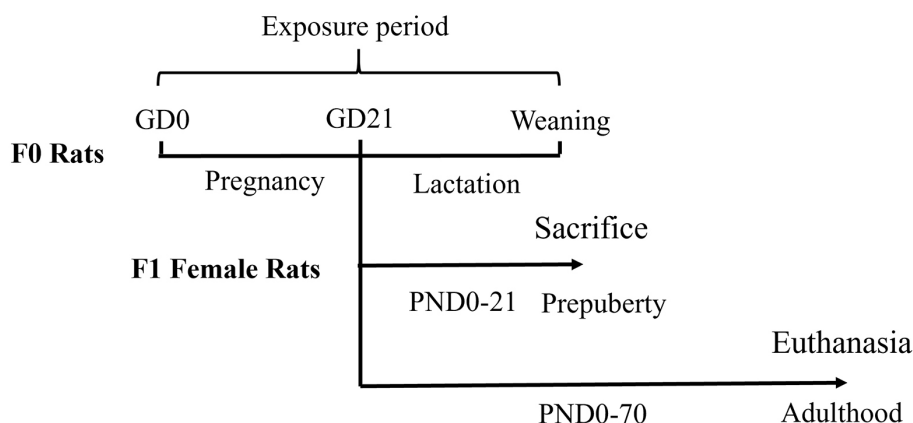


Figure 1. Schematic study design. GD: Gestation day; PND: postnatal day.

Physical development and reproductive function

From PND0 to PND21, when rapid physical development occurs in F1 females, the following developmental milestones were recorded daily: ear detachment, eruption of incisor teeth, and eye opening. At PND21, AGD was measured in F1 females. Vaginal opening was monitored by daily visual inspection from PND25. At PND45, when puberty has stabilized, estrous cyclicity was assessed by daily vaginal smears for two consecutive weeks to capture at least two full estrous cycles.

Organ weights and histological examination

The liver, spleen, uterus, and ovaries of F1 females were collected at PND21 and PND70. Each organ was weighed, and the organ coefficient (organ-to-body weight ratio $\times 100\%$) was calculated. Ovaries were fixed in 10% neutral buffered formalin for 3 days, embedded in paraffin along their longest axis, and sectioned at 4 μm thickness at 30- μm intervals. Sections were stained with hematoxylin and eosin (H&E), and histopathological evaluation was performed in a blinded manner using fluorescence microscopy. Analyses included follicular development and abnormal morphological changes. Follicular atresia was defined according to standardized criteria: presence of apoptotic bodies at the antral periphery, cell debris within the antrum, pyknotic granulosa and/or theca cell nuclei, and detachment of granulosa cells from the basement membrane.

Serum sample collection and hormone analysis

Blood samples from F1 females were collected at PND21 and PND70 and centrifuged at 2,000 rpm for 15 min to separate serum. The serum was used to measure estradiol (E2) and testosterone (T) levels using an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's protocol. Each well required 50 μL of serum, and each sample was assayed in duplicate. Absorbance was recorded at 450 nm using a microplate reader (Synergy H1M, BioTek, USA), and hormone concentrations were determined from the standard curve provided with the kit. The intra- and inter-assay coefficients of variation were both $< 10\%$. The minimum detectable concentrations for E2 and T were 1.56 pg/mL and 0.31 ng/mL, respectively. The ELISA kit has been validated in recent rodent studies^[27-29].

RNA extraction and ovarian qPCR

Total RNA was extracted from ovarian 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 columns, according to the manufacturer's instructions. RNA purity was assessed by spectrophotometry (Nanodrop 8000, Thermo, USA), with A260/A280 ratios close to 2.0, indicating high purity and minimal protein contamination. RNA

was reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio Inc., Otsu, Japan). PCR cycling conditions were 37 °C for 15 min and 85 °C for 5 s, repeated for 40 cycles. Real-time quantitative PCR (qPCR) was conducted to assess the expression of genes related to steroid hormone biosynthesis, including STAR, 3 β -HSD, CYP11A1, 17 β -HSD, CYP19A1, and CYP17A1. Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai), with sequences provided in [Supplementary Table 1](#). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the endogenous control. Gene expression was quantified using the comparative Ct method (2- $\Delta\Delta$ Ct) with efficiency correction, normalizing to GAPDH. Reference gene stability (CV < 5%) and amplification efficiency (90%-110%) were confirmed for all primer sets.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). Normality was tested using the Shapiro-Wilk test ($\alpha = 0.05$), and homoscedasticity was assessed with Levene's test. Log-transformation was applied when distributions were skewed. Outliers were identified using Grubbs' test ($\alpha = 0.05$) and excluded. To minimize litter effects, all specimens were derived from different litters, with litter considered the statistical unit. Intergroup differences were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Statistical significance was set at $P < 0.05$ and indicated by an asterisk in the figures. Analyses were performed using SPSS 26.0, and graphs were generated with GraphPad Prism 8 software.

RESULTS AND DISCUSSION

Pregnancy outcomes and physical development

Pregnancy outcomes for F0 females, including gestational length, gestational weight gain, litter size, pup sex ratio, and body weights of F1 females, are presented in [Table 1](#). Except for gestational length, which was significantly increased in the 50 mg/kg group compared with the control group, no other parameters differed significantly among treatment groups. Physiological development indicators, such as incisor eruption, ear detachment, and eye opening, are also shown in [Table 1](#). Compared with the control group, significant decreases were observed in weight gain in the 2 mg/kg BPAF group at PND21, in ovarian wet weight and ovarian coefficient in the 50 mg/kg BPAF group at PND21, and in spleen and uterine coefficients in the 2 and 10 mg/kg BPAF groups, respectively, at PND70. No significant differences were detected for other physiological development indicators [[Table 1](#), [Supplementary Table 2](#)].

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 PND0 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 [[Figures 2 and 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](#)].

Serum hormone levels

Serum hormone levels (E2 and T) of F1 females were measured at PND21 and PND70 [[Figure 4](#)]. At PND21, significantly lower serum E2 levels were observed in the 2 mg/kg (8.15 ± 2.24 pg/mL, $P < 0.05$) and 10 mg/kg (6.02 ± 2.07 pg/mL, $P < 0.01$) BPAF-exposed groups compared with the control group (10.90 ± 2.24 pg/mL). At PND70, serum E2 levels exhibited a dose-dependent decrease across treatment groups: 21.48 ± 3.45 pg/mL (control), 17.51 ± 1.85 pg/mL (2 mg/kg), 14.18 ± 2.30 pg/mL (10 mg/kg), and $13.76 \pm$

Table 1. Effects of BPAF exposure on pregnancy outcomes in F0 dams and physical development of F1 females

Dose (mg/kg/d)	0		2		10		50	
			<i>P</i> ^a		<i>P</i> ^b		<i>P</i> ^c	
Gestational weight gain (GD 1 to GD 20)		154.00 ± 31.56	165.50 ± 27.87	0.838	139.33 ± 62.62	0.871	134.88 ± 33.39	0.244
Gestational length		22.67 ± 0.50	22.88 ± 0.64	0.240	22.75 ± 0.46	0.183	23.63 ± 0.92 [*]	0.016
Pregnant dams (pup numbers)		9(111)	10(127)	0.861	8(108)	0.609	8(89)	0.581
Litter weight (g)		87.09 ± 28.79	89.88 ± 30.89	0.832	96.23 ± 28.82	0.518	79.98 ± 23.13	0.574
Litter size		12.33 ± 4.53	12.70 ± 4.42	0.861	13.50 ± 4.66	0.609	11.13 ± 4.26	0.581
Ratio of pup's sexes		0.96 ± 0.41	1.13 ± 0.60	0.475	1.31 ± 0.76	0.810	1.05 ± 0.50	0.926
Weights of female pups at PND1 (g)		7.23 ± 0.82	7.31 ± 0.48	0.519	7.19 ± 0.67	0.762	7.22 ± 1.12	0.945
The ages of the ears detachment (d)		2.11 ± 0.33	2.20 ± 0.35	0.620	2.50 ± 0.55	0.109	2.25 ± 0.46	0.485
Eruption of the incisor teeth (d)		8.22 ± 2.11	7.30 ± 0.46	0.195	7.38 ± 0.52	0.287	7.75 ± 0.89	0.566
Opening eyes (d)		13.33 ± 0.71	13.40 ± 0.76	0.839	13.25 ± 0.71	0.812	13.88 ± 0.64	0.120
Weight at PND21 (g)		68.92 ± 4.16	65.63 ± 5.15 ^{**}	0.009	70.51 ± 3.82	0.257	67.93 ± 5.46	0.578
Weight gain from PND1 to PND21 (g)		56.97 ± 3.56	54.24 ± 4.20 ^{**}	0.009	58.18 ± 3.18	0.304	55.77 ± 4.80	0.440
Organ coefficients at PND21 (g/100 g)	Liver	3.35 ± 0.27	3.24 ± 0.33	0.289	3.37 ± 0.17	0.752	3.23 ± 0.31	0.289
	Spleen	0.40 ± 0.09	0.36 ± 0.06	0.148	0.38 ± 0.08	0.642	0.36 ± 0.05	0.159
	Uterus	0.17 ± 0.11	0.16 ± 0.06	0.637	0.16 ± 0.06	0.701	0.14 ± 0.05	0.326
	Ovaries	0.09 ± 0.04	0.07 ± 0.03	0.177	0.07 ± 0.02	0.168	0.06 ± 0.02 [*]	0.040
Weight at PND70 (g)		292.22 ± 42.83	266.50 ± 37.50	0.173	293.25 ± 31.55	0.956	294.50 ± 32.15	0.904
Organ coefficients at PND70 (g/100 g)	Liver	3.02 ± 0.52	2.85 ± 0.17	0.354	2.95 ± 0.23	0.723	2.86 ± 0.19	0.424
	Spleen	0.19 ± 0.02	0.23 ± 0.03 ^{**}	0.003	0.23 ± 0.03 ^{**}	0.003	0.20 ± 0.03	0.451
	Uterus	0.18 ± 0.02	0.26 ± 0.06 ^{**}	0.003	0.27 ± 0.07 ^{**}	0.003	0.22 ± 0.08	0.219
	Ovaries	0.08 ± 0.02	0.08 ± 0.02	0.910	0.08 ± 0.02	0.506	0.07 ± 0.01	0.059

Weight gain of female pups (g) = weight of female pups at PND21 (g) - weight of female pups on PND4 (g). *P*^a: *P* values for 2 mg/kg vs. control; *P*^b: *P* values for 10 mg/kg vs. control; *P*^c: *P* values for 50 mg/kg vs. control. ^{*}*P* < 0.05, ^{**}*P* < 0.01. BPAF: Bisphenol AF; PND: postnatal day; GD: gestational day.

Table 2. Effects of gestational and lactational exposure to BPAF on anogenital distance, vaginal opening, and estrous cycle in female offspring

Dose (mg/kg/d)	<i>n</i>	AGD (mm)			Age of vaginal opening (d)	Estrous cycle length (d)
		PND0	PND21	PND70		
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

^{*}*P* < 0.05, ^{***}*P* < 0.001. BPAF: Bisphenol AF; AGD: anogenital distance; PND: postnatal day.

2.06 pg/mL (50 mg/kg). Similarly, serum T levels were reduced in the BPAF-exposed groups compared with the control group: 1.83 ± 0.10 ng/mL (control) vs. 1.54 ± 0.21 ng/mL (2 mg/kg), 1.48 ± 0.24 ng/mL (10 mg/kg), and 1.34 ± 0.35 ng/mL (50 mg/kg).

mRNA expression of steroidogenesis-related genes

The mRNA expression of steroidogenesis-related genes (STAR, 3β-HSD, CYP11A1, 17β3-HSD, CYP19A1, and CYP17A1) in the ovaries of F1 females was measured [Figure 5]. At PND21, no significant differences were observed for any of the genes in prepuberty F1 females. However, at PND70, significantly decreased mRNA expression of several steroidogenesis-related genes (StAR, CYP11A1, 17β3-HSD, and CYP19A1) was detected in BPAF-treated groups compared with the control group. Specifically, reduced mRNA levels were

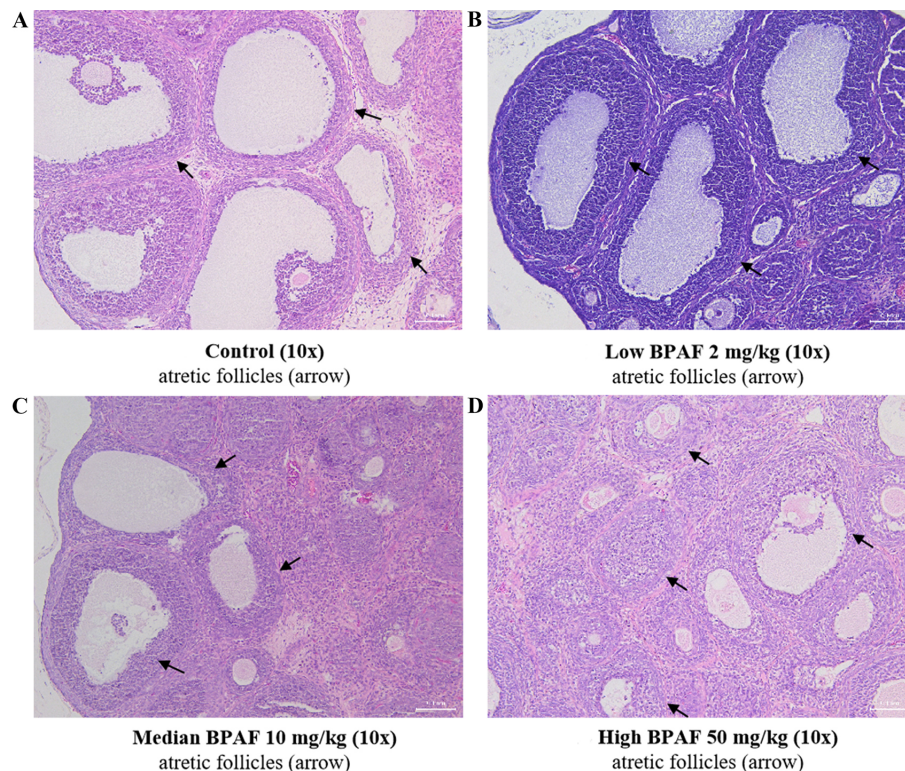


Figure 2. Effects of gestational and lactational BPAF exposure on ovarian histology of PND21 F1 females. (A) Control; (B) BPAF 2 mg/kg; (C) BPAF 10 mg/kg; (D) BPAF 50 mg/kg. Arrows indicate atretic follicles. BPAF: Bisphenol AF; PND: postnatal day.

observed for StAR in the 2 and 50 mg/kg BPAF groups, CYP11A1 in the 2 mg/kg BPAF group, 17 β -HSD in the 10 mg/kg/d BPAF group, and CYP19A1 in the 10 and 50 mg/kg/d BPAF groups.

Discussion

In this study, we found that exposure to BPAF during gestation and lactation may impair the reproductive system of F1 females, as evidenced by reduced AGD and serum sex hormone levels, increased follicular atresia, and decreased mRNA expression of genes involved in steroid hormone biosynthesis. Moreover, these changes varied across reproductive stages, with more pronounced effects observed at PND70, suggesting that early-life BPAF exposure may exert long-term effects on female reproduction.

With regard to ovarian steroid hormone synthesis, our results suggest several possible mechanistic pathways. BPAF exposure may downregulate mRNA expression of key genes involved in steroid hormone synthesis, including STAR, CYP11A1, 17 β -HSD, and CYP19A1, leading to decreased T and E2 levels. It is well exhibited that sex steroid hormone plays essential roles in follicle development and maintenance of regular estrous cycle^[30]. For example, reduced E2 levels may impair follicular development^[31], lower pregnancy and implantation rates^[32,33], while low serum T levels may be associated with reduced AGD^[34,35]. Similarly, previous research reported that BPA exposure decreases AGD in female rats^[36]. Epidemiological evidence also links shorter AGD with increased risks of endometriosis, polycystic ovary syndrome (PCOS), and hypospadias^[37-39]. In our study, we observed shortened AGD, lower serum E2 and T levels, and increased follicular atresia, supporting the hypothesis that disrupted steroid hormone synthesis is an important mechanism underlying BPAF-induced reproductive toxicity. Interestingly, the shortened AGD at PND21 that later recovered by PND70 might reflect the transient nature of this change following BPAF exposure ceased at weaning (PND21). Gradual recovery of AGD to normal levels by PND70 has also been

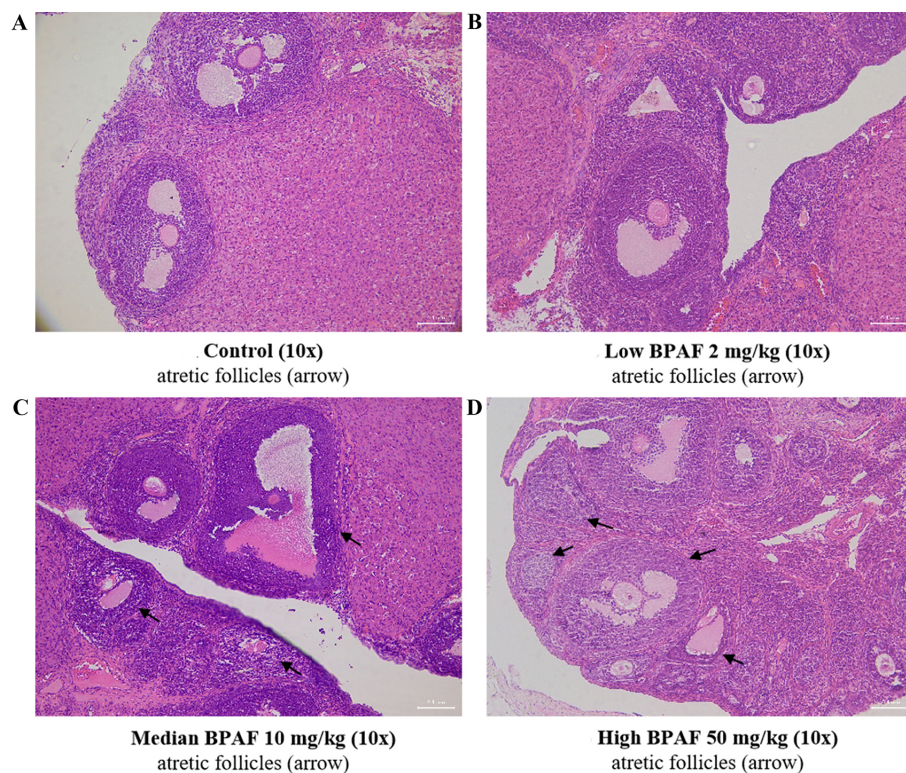


Figure 3. Effects of gestational and lactational exposure to BPAF on ovarian tissue in PND70 F1 females. (A) Control; (B) BPAF 2 mg/kg; (C) BPAF 10 mg/kg; (D) BPAF 50 mg/kg. Arrows indicate atretic follicles. BPAF: Bisphenol AF; PND: postnatal day.

reported in other studies^[40]. These findings suggest that the reproductive system possesses compensatory capacity against EED exposure, although long-term consequences may persist. In addition, the discrepancy between unchanged vaginal opening/estrous cycles and altered hormone/gene expression profiles may indicate differential sensitivity among biomarkers, as well as differences in sampling time (e.g., serum T and E2 were measured at PND21 and PND70, while AGD is primarily established during mid-to-late gestation in rats^[41]). Other unidentified factors may also contribute.

Relatively few studies have examined BPAF's effects on steroidogenesis *in vivo*. Consistent with our results, several studies have shown that BPAF alters steroid hormone levels by regulating expression of steroidogenic genes^[2,9,42–48]. For instance, LU S *et al.*^[20] reported that BPAF (3 and 30 mg/kg/d) during early gestation significantly downregulated ovarian StAR, CYP11A, 17 β -HSD, and CYP19A1 expression and decreased serum progesterone and E2 levels in mice. Another study showed that BPAF (100 mg/kg/d) exposure during gestation and lactation increased serum T levels in F1 male rats^[9]. Our study provides additional evidence that even relatively low-dose BPAF exposure (2 mg/kg/d) during gestation and lactation can disrupt steroid hormone synthesis and cause reproductive toxicity in female offspring. Collectively, our findings indicate that BPAF exerts reproductive toxicity similar to BPA, with both compounds disrupting steroid hormone synthesis. Previous studies have demonstrated that gestational and lactational exposure to BPA or BPAF reduces E2 and T levels and significantly suppresses mRNA expression of STAR, CYP11A1, and CYP19A1^[47,49,50]. Importantly, a comparative study demonstrated that BPAF produced stronger inhibitory effects on STAR, CYP11A1, and progesterone levels than BPA at equivalent doses (900 ppm)^[51]. *In vitro* studies further indicate that BPAF may have higher binding affinity for estrogen receptors (ERs) than BPA^[12,14]. Taken together, these findings suggest that although BPAF has been marketed as a substitute

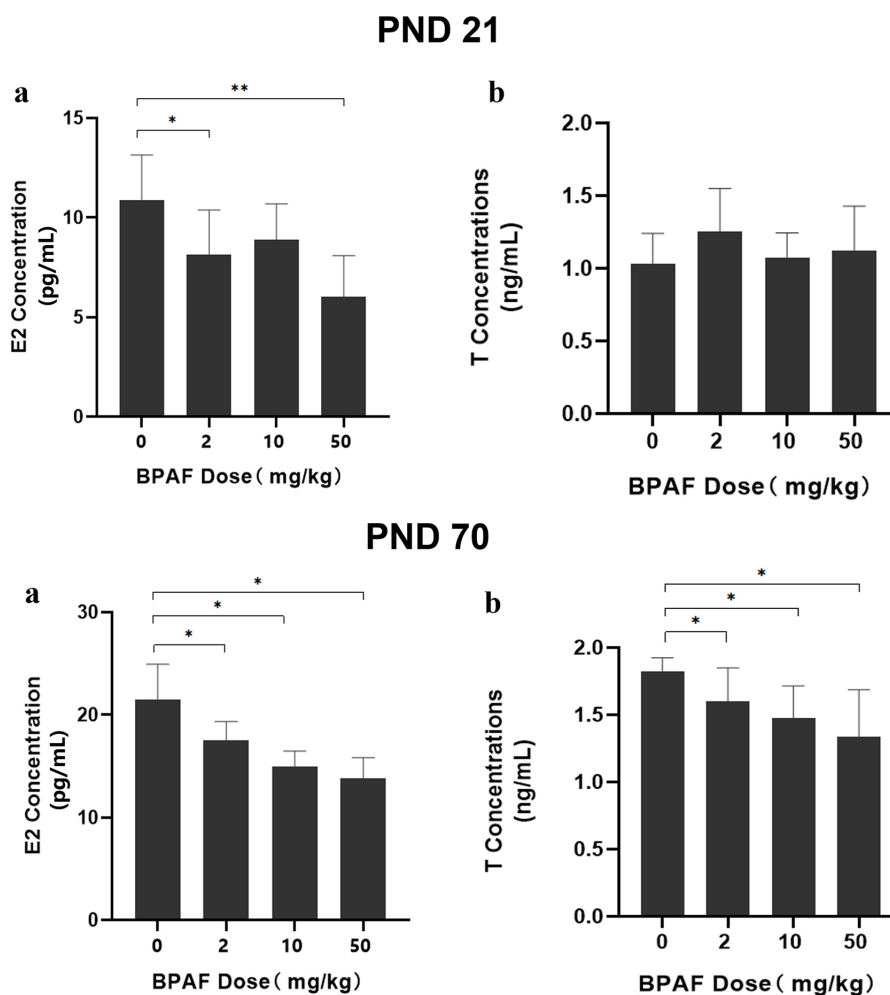


Figure 4. Effects of gestational and lactational exposure to BPAF on E2 and T levels in female rats at PND21 and PND70. * $P < 0.05$, ** $P < 0.01$. BPAF: Bisphenol AF; E2: estradiol; T: testosterone; PND: postnatal day.

for BPA, it may in fact exert stronger endocrine-disrupting effects and greater toxicity. Therefore, BPAF should not be considered a safe replacement for BPA, and its long-term reproductive effects warrant closer attention.

Figure 6 illustrates the possible pathways through which BPAF exposure may affect ovarian steroid hormone synthesis in adult female offspring. Our results suggested that gestational and lactational BPAF exposure reduced E2 and T levels and decreased ovarian mRNA expression of STAR, CYP11A1, 17 β -HSD, and CYP19A1 in F1 females (PND70). We therefore hypothesize that BPAF disrupts steroidogenesis by downregulating the expression of these key genes. In Figure 6, blue text indicates potential molecular targets of BPAF-induced reproductive toxicity identified in previous studies, while red boxes highlight E2 and T as key hormones affected. Based on the literature, we also included possible regulatory factors (gray boxes). For example, BPA can bind to ER α and upregulate ER α expression, thereby interfering with the steroidogenesis pathway and ultimately altering hormone levels. This imbalance may, in turn, modulate ER expression through negative feedback regulation^[28]. Additionally, recent evidence suggests that peroxisome proliferator-activated receptor γ (PPAR- γ) may participate in CYP19A1 transcriptional activation, thereby influencing steroid biosynthesis^[52].

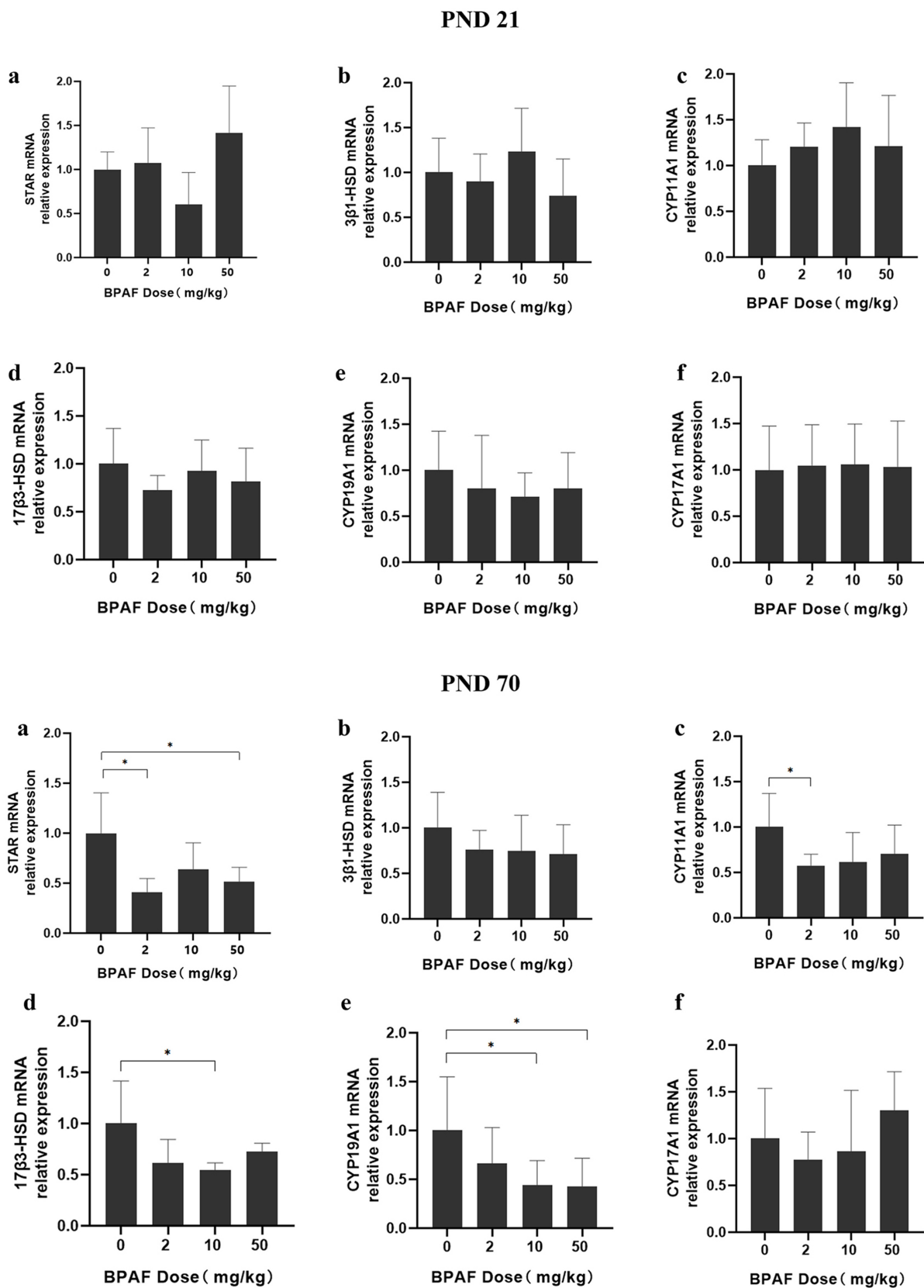


Figure 5. Effects of gestational and lactational exposure to BPAF on the mRNA expression of steroidogenic enzymes in F1 females at

PND21 and PND70. * $P < 0.05$. BPAF: Bisphenol AF; PND: postnatal day.

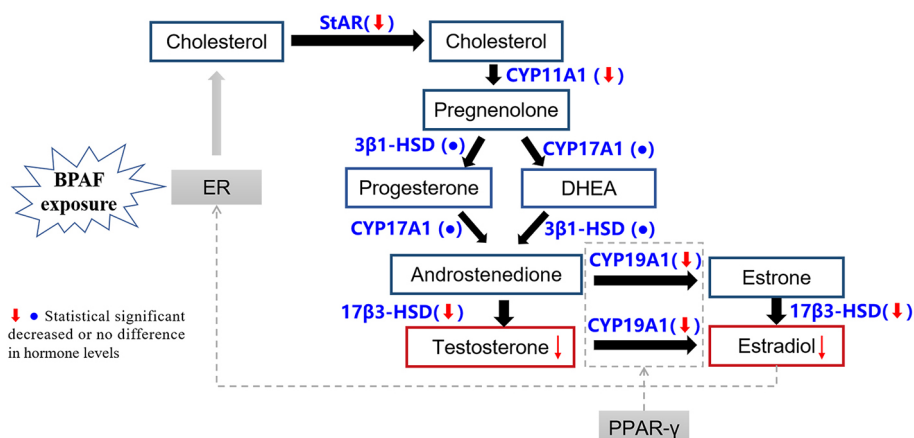


Figure 6. The key pathways of the impact of BPAF exposure on ovarian steroid hormone synthesis of adult female offspring based on the present study and literature. BPAF: Bisphenol AF; PND: postnatal day; PPAR- γ : peroxisome proliferator-activated receptor γ ; ER: estrogen receptor.

In this study, we compared outcomes at two reproductive stages and found that effects on reproductive function and steroidogenesis were more pronounced at PND70 than at PND21. For example, altered expression of STAR, CYP11A1, 17 β 3-HSD, and CYP19A1 was only observed at PND70, while sex hormone levels and ovarian histology also showed stronger changes at this stage. Similar findings have been reported in studies of BPA and BPAF exposure, with effects generally more evident after reproductive maturation than at puberty^[53,54]. For example, one study showed that oral BPA exposure (0.5, 20, and 50 μ g/kg) from GD11 until birth did not affect the timing of vaginal opening but increased the proportion of dead pups at three months in female offspring^[55]. Another study exposing F0 CD-1 mice to BPAF (5 mg/kg) between GD10 and GD17 found significant alterations in mammary gland morphology only in late adulthood (PND56)^[56]. A plausible explanation is that sexual organs remain immature during prepuberty^[9,56,57]. At this stage, hormone levels are relatively low and stable, steroid hormone synthesis is not yet fully established, and hormonal feedback mechanisms are relatively simple - factors that may mask immediate effects of environmental pollutants^[58]. Nonetheless, early subtle changes can lead to long-term reproductive toxicity, as demonstrated by alterations in morphology and hormone levels in adulthood^[9,56,57,59]. Although our study was limited to observations up to PND70 in the F1 generation, these results provide valuable insight into the potential reproductive and transgenerational consequences of BPAF exposure. Altered sex hormone levels may impair ovarian function and reduce fertility, consistent with previous studies showing that other endocrine-disrupting pollutants such as polystyrene nanoplastics^[60], cadmium^[61], and phthalate mixtures^[62] also impair F1 reproductive capacity following gestational exposure. Furthermore, steroidogenic disruption in F1 females may dysregulate gene expression in the second filial generation (F2)^[61], potentially leading to reduced primordial follicles in F2 females^[62]. These findings highlight the need for further research into the transgenerational effects of BPAF exposure.

A key strength of our study is the use of relatively low BPAF doses, while most previous studies^[9,42] examined much higher doses that may not accurately represent environmentally relevant human exposure. Another advantage is that we examined reproductive development at multiple stages in F1 females. Thus, compared with similar studies, our investigation provides a more continuous and comprehensive assessment of the reproductive toxicity of BPAF exposure.

Study limitations

Our study has several limitations. First, we primarily explored the mechanism of BPAF-induced reproductive and developmental toxicity from the perspective of hormone synthesis; however, other pathways may also contribute to its reproductive toxicity. Second, the study did not include validation of protein-level changes (e.g., by Western blot) to confirm transcriptional findings, which should be addressed in further research. Third, as BPAF concentrations in biological samples (e.g., serum or tissues) from experimental animals were not measured, internal exposure doses could not be determined. This limitation may affect the accuracy of exposure assessment. Future studies should quantify internal exposure levels to verify mechanistic pathways and improve the precision of risk assessment. Additionally, we did not quantify BPAF metabolites. Nevertheless, previous research has demonstrated that BPAF glucuronides (BPAF-G) - the primary metabolites in Sprague-Dawley (SD) rats after BPAF exposure^[63] - show negligible estrogenic activity^[64]. This evidence supports the validity of our findings despite the lack of metabolite measurements.

CONCLUSIONS

This study suggests that exposure to BPAF during gestation and lactation adversely affects reproductive function in female offspring, potentially through disruption of ovarian steroid hormone biosynthesis. The specific pathways and molecular targets underlying these toxic effects remain unclear, and further studies are needed to elucidate the mechanisms and assess long-term reproductive outcomes.

DECLARATIONS

Authors' contributions

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

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

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

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

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

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

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

Availability of data and materials

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

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Conflicts of interest

Gao, Y. is a Guest Editor of *Journal of Environmental Exposure Assessment*. Gao, Y. was not involved in any steps of editorial processing, notably including reviewer selection, manuscript handling, or decision making. The other 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 Control and Prevention (Protocol number: 20220008).

Consent for publication

Not applicable.

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