

Effects of Prenatal Exposure to Hexafluoropropylene Oxide Dimer Acid on Rat and Offspring Mammary Gland Development and Associated Hormone Levels

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1 **ABSTRACT**

2 **Background:** This study investigated the effects of prenatal exposure to
3 hexafluoropropylene oxide dimer acid (HFPO-DA), a replacement for
4 perfluorooctanoic acid (PFOA), on mammary gland development of both
5 pregnant rats and their offspring, as well as its influence on related
6 hormone levels.

7 **Methods:** Pregnant Sprague-Dawley (SD) rats were orally exposed to
8 HFPO-DA at doses of 0, 1, 10, and 100 mg/kg/day from gestation day (GD)
9 0.5 to GD 19.5. On GD 19.5, half of the pregnant rats from each dose group
10 underwent caesarean section, while the other half gave birth naturally. The
11 offspring from the rats that gave birth naturally were raised until they
12 reached postnatal day (PND) 21. The serum levels of progesterone (Pg),
13 estradiol (E2), and prolactin (PRL) in the pregnant rats and their offspring
14 on PND 21 were detected via ELISA (enzyme-linked immunosorbent
15 assay). Changes in mammary glands of pregnant rats, their fetuses, and
16 PND 21 offspring were assessed by haematoxylin and eosin (H&E)
17 staining. The development of mammary gland tissue structures in fetuses
18 and PND 21 offspring was evaluated using whole gland staining.
19 Immunohistochemical staining was used to assess STAT5 expression in the
20 mammary glands of pregnant rats and Ki67 expression in the mammary
21 glands of fetuses and PND 21 offspring.

22 **Results:** In pregnant rats, exposure to HFPO-DA significantly increased

the PRL levels. The expression of STAT5 was also significantly elevated in mammary epithelial cells. The lobular and alveolar areas expanded dose-dependently, and milk secretion was observed in the high-dose group. In fetuses and PND 21 offspring exposed to HFPO-DA, we observed significant growth of secondary mammary ducts, a substantial increase in ductal coverage area, ductal buds, and primary duct length, as well as an increase in Ki67 expression in mammary epithelial cells.

Conclusions: Collectively, these findings suggest that HFPO-DA exposure elevates serum PRL levels in pregnant rats, promotes lactation, and stimulates early-stage mammary gland development in offspring.

Keywords: Hexafluoropropylene oxide dimer acid, rat, mammary gland, hormones, Ki67

1 Background

Perfluoroalkyl substances have been widely used in various life domains, with perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) emerging as the predominant choices (Buck et al., 2011). Even though, because of their environmental persistence, bioaccumulation, and biotoxicity, PFOA was included in the list of persistent organic Pollutants (POPs) under the Stockholm Convention in 2019 (Wang et al., 2019),

leading to restrictions on their use and production. As an alternative to PFOA, hexafluoropropylene oxide dimer acid [(HFPO-DA), GenX] was adopted to act as processing aids in the production of fluoropolymer resins since 2009. In Europe, the annual output of HFPO-DA ranges from 10 to 100 tons following its production and utilization (Pan et al., 2017). Although HFPO-DA production volumes in China have not been reported, the riverine discharge of HFPOs (HFPO-DA and HFPO-TA) alone reached relatively high levels of 2.4 tons in 2018 (Feng et al., 2021).

The increasing applications of HFPO-DA have raised concerns regarding its safety. Investigations have tested HFPO-DA in diverse environmental media, including soil, air, leaves, and surface water (Brandsma, Koekkoek, van Velzen, & de Boer, 2019). Human exposure to HFPO-DA can occur through various pathways, like potable water, food, dust, and air, with traces of HFPO-DA found in blood, hair, and urine (Feng et al., 2021; Galloway et al., 2020; C. Xu et al., 2021). Moreover, researches in animals showed that HFPO-DA is related to growth and developmental toxicity, hepatotoxicity, endocrine disorders, and cardiotoxicity, potentially leading to substance metabolism diseases (Conley et al., 2021; Conley et al., 2019; Guo et al., 2022; Martin et al., 2007; Xie et al., 2021; X. Xu et al., 2021).

To our knowledge, no information has been reported regarding the potential effects of HFPO-DA on mammary toxicity.

Over time, there have been worrisome trends with regard to the decreasing

age at onset of breast cancer, also the increasing incidence of mammary carcinoma itself (Zhu JW, et al.2023). Population studies have shown that breast cancer etiology involves multiple pathways beginning early in life, particularly during the in utero, perinatal, and pubertal stages of the individual—referred to as "windows of susceptibility" (Trichopoulos et al. 2005; Bodicoat et al. 2014) . The risk of developing mammary carcinoma is linked with the number of mammary tissue-specific stem cells established during these early periods, especially in utero or immediately after birth (Trichopoulos et al. 2005) . A large cohort study in the UK revealed that earlier breast developments (thelarche) were related to a higher risk of mammary carcinoma, with hazard ratios of 1.23 for ≤ 10 years, 1 for 11-12 years, and 0.80 for ≥ 13 years (Bodicoat et al. 2014). Moreover, experimental studies suggest that numerous environmental pollutants commonly disrupt hormone regulation, thereby altering the progression of mammary gland development and ultimately increasing the risk of cancer development (Kay et al. 2022). Research has demonstrated that the mammary gland is sensitive to PFOA exposure.

Several human studies have linked high levels of PFOA in maternal serum to shorter breastfeeding durations and an increased likelihood of premature cessation (Hoadley et al. 2023; Rokoff et al. 2023; Timmermann et al. 2023). Moreover, animal studies have revealed that exposure to PFOA results in a delay in early epithelial outgrowth and branching, resulting in

a tendency for the terminal end buds (TEDs) numbers to decrease, thereby inhibiting mammary gland differentiation, milk production, even across generations (Tucker et al. 2015; White et al. 2011; White et al. 2009). These effects may be associated with the overexpression of genes in the oestrogen signalling pathway (Su et al. 2022), the modulation of gene expression associated with milk protein, along with the peroxisome proliferator-activated receptor alpha (PPAR α) activation (White et al. 2007, Yang et al. 2006). Prolactin (PRL) and downstream STAT5 signalling are directly involved in mediating mammary gland survival and are crucial for the development of normal mammary glands, as are the proliferation and differentiation of mammary alveoli during pregnancy (Lee et al. 2016). Epidemiological investigations have also revealed a potential correlation between PFOA exposure and mammary carcinoma risk (V.C. Chang et al. 2023; Jiang et al. 2022; Li et al. 2022), although there are also negative reports (C.J. Chang et al. 2024). In vitro experiments, exposure to PFOA was shown to accelerate breast cancer cell proliferation via both the Akt/mTORC1 pathway (Charazac et al. 2022) and PPAR α activation (Sakai et al. 2022). Moreover, it enhances the migration and invasion of mammary epithelial cells, disrupting normal acinar maturation. These effects are mediated through PPAR α rather than oestrogen receptors (Conley et al. 2019; Pierozan, Jerneren, and Karlsson 2018; Halsne et al. 2016).

111 These findings prompted us to explore the effect of HFPO-DA on rat
112 mammary gland development, particularly during sensitive periods (in
113 utero, perinatal, and pubertal stages). Therefore, we studied the direct
114 impact of HFPO-DA exposure during maternal pregnancy on mammary
115 tissue development, and its impact on mammary gland development in
116 offspring at birth, with postnatal day 21 designated as the developmental
117 toxicity endpoint. These results could provide novel evidence regarding the
118 toxicity effects of HFPO-DA on the mammary glands of SD rats during
119 development and potential pathways of influence.

121 **2 Methods**

122 **2.1 Animals**

123 We obtained ten-week-old female and male specific pathogen-free (SPF)
124 Sprague-Dawley (SD) rats from Jinan Pengyue Experimental Animal
125 Breeding Co., Ltd., Shandong Province, China. The rats were housed in the
126 animal laboratory of the Qingdao Municipal Hospital for one week, in one
127 cage per four, and kept at room temperature at 20-22°C, with 45-55%
128 humidity, and a light cycle of 12:12(17:00 lights off). The rats were fed a
129 standard laboratory animal diet, and the bedding used was sterilized wood
130 shavings. Adequate deionized water and food were provided ad libitum
131 throughout the study.

132 **2.2 Chemicals**

The HFPO-DA (C₆H₁₁O₃, CAS: 13252-13-6, purity≥97%) used in this study was purchased from Shanghai Macklin Biochemical Co., Ltd. The stock solutions were diluted to the target concentration with deionized water (DI water) (1, 10, and 100 mg/kg). Our dose selection for exposure was based on previous studies and preliminary results. For more details, please refer to our previously published article (Lv et al. 2024).

2.3 Experimental Design

After one week of feeding, cage mating was conducted at a 1:1 female:male ratio for two days. We monitored the vaginal port of each female rat every morning to determine the presence of the mating plug. If positive, we defined this day as Gestational Day 0.5 (GD 0.5). And then the rats were randomly separated into 4 groups (each group n=12), subsequently exposed to different doses of HFPO-DA via oral gavage, with control (DI water), 1, 10, and 100 mg/kg, through GD 0.5 to 19.5. On GD19.5, half of the pregnant rats (n=6) were selected from each group for caesarean section, defined as the caesarean section group (n=24). The remaining six pregnant rats in each group, defined as the spontaneous delivery group (n=24), were allowed to give birth naturally. In the caesarean section group, we assessed the serum hormone levels, mammary gland pathology, and ovarian pathology of pregnant rats. Additionally, we assessed mammary gland pathology in fetal rats and recorded the number of alive, dead, and resorbed fetuses, also any developmental abnormalities. To control for litter effects,

one pup was randomly selected from each litter. In the spontaneous delivery group, we assessed serum hormone levels, mammary gland pathology, and ovarian pathology of PND21 offspring. The survival rate, body weight, body length, and tail length of the offspring were recorded from PND1 to PND20. We weighed pregnant rats in each group daily and recorded their survival rates, activity, and abnormal performance.

2.4 Blood and tissue collections

Under deep anesthesia, cardiac blood was collected. Fetuses were humanely euthanized by swift decapitation. In the caesarean section group, pregnant rats were euthanized to collect cardiac blood 2 hours after HFPO-DA exposure on GD19.5. Offspring from the spontaneous delivery group were euthanized on PND21 using the same method as for pregnant rats. The blood was stored in procoagulant tubes and centrifuged at 2500 revolutions per minute for 15 minutes. The supernatants were reserved in -80°C to complete subsequent serological analysis.

For mammary tissue collection from pregnant rats and PND21 female offspring, the abdominal hair was removed to expose the mammary glands. Starting from the chest, the nipples of the 4th and 5th pairs of mammary glands, along with 1 cm of the surrounding skin and subcutaneous tissue, were excised to obtain intact mammary gland tissue. The excised tissue was immersed in paraformaldehyde, and prepared for H&E staining, whole tissue staining, and immunohistochemical staining. Owing to the thickness

of the mammary tissue in pregnant rats, whole tissue staining was not performed. Since the mammary glands of PND21 male offspring did not undergo nipple formation, the surrounding tissue was dissected along the groin, armpit, and outer edges of the ribs to remove the entire peritoneum, which was then fixed in formalin, confirming the absence of nipple formation in the male rats. A portion of the peritoneum was embedded in paraffin for H&E staining and immunohistochemistry, while the remaining peritoneum was bisected along the midline for whole tissue staining.

For fetal rat mammary tissue, one randomly selected complete fetal rat per litter was fixed in paraformaldehyde for H&E and immunohistochemical staining. The entire peritoneum of the remaining fetal rats ($n \geq 7$ per dose group) was removed, and the peritoneum was immersed in paraformaldehyde for whole tissue staining.

The bilateral ovaries of the pregnant rats and PND21 female offspring were also collected, fixed in paraformaldehyde, and preserved for H&E staining.

2.5 Serum sex hormone measurement

Serum from pregnant and PND21 rats was stored at -80°C and thawed at room temperature. Serum levels of sex hormone (progesterone, estradiol, prolactin) were measured by enzyme-linked immunosorbent assay (ELISA) using commercial kits (purchased from Elabscience Co., Wuhan, China).

2.6 Histopathological analysis

The mammary glands and ovarian tissues were removed from the pregnant rats and their offspring and subjected to fix in 10% paraformaldehyde, embedded in paraffin wax, and stained with haematoxylin and eosin (H&E). Histopathological changes in the mammary glands and ovaries were observed under a microscope. HE-stained sections from the pregnant rats were photographed, and ImageJ software was used to measure the lobular and alveolar areas. Six pregnant rats were used for each group. For each pregnant rat, ten low-power fields (10×) were selected to measure the area of complete lobules within these fields. Additionally, ten high-power fields (40×) were selected for each pregnant rat to measure the area of complete acini within these fields (Figure S1). In each litter of pregnant rats, one fetal rat or one male and one female rat at PND21 were randomly selected for HE staining. For each sample, the number of primary and secondary ducts in five low-power fields (10×) was counted.

2.7 Whole mammary gland staining

The mammary glands of fetal and PND21 rats were placed on glass slides and flattened to their natural surface area. Mammary glands were fixed in Carnoy's solution, stained with carmine alum, and then degreased with xylene. We imaged the entire mammary gland via a microscope. All of the data were measured using Image J. The measurements included the covered area of the mammary gland duct, the primary duct length, the

number of duct branches (the number of branches on the longest duct), and the number of ductal buds.

2.8 Immunohistochemistry

Immunohistochemistry (IHC) was used to detect the levels of STAT5 and Ki67 in breast tissue from pregnant, fetal, and PND21 rats as formerly described by Klara Matouskova (Matouskova et al. 2020). 4 μ m sections were evaluated with immunohistochemistry for STAT5 and Ki67. The commercial antibodies used were specifically mouse anti-STAT5 (Santa Cruz Biotechnology, Cat# sc-74442, diluted 1:500). To enhance the IHC signal, mouse IgG Fc binding protein (m-IgG Fc BP-HRP) (Santa Cruz Biotechnology, Cat# sc-525409, diluted 1:100) was used instead of the anti-mouse IgG secondary antibody, followed by streptavidin peroxidase complex (Abcam, Cat# ab64269) and diaminobenzidine chromogen (Abcam, Cat# ab64238) for visualization. Samples were counterstained with hematoxylin and coverslipped with permanent mounting media.

For Ki67, the commercial antibodies used were rabbit anti-Ki67 (Fisher Scientific, Cat# RM-9106-S1, diluted 1:1000) and goat anti-rabbit secondary antibodies (Abcam, Cat# ab64256). For the purpose of calculate the ratio of positive cells, 10 fields (40 $\times$) were randomly detected every slide, subsequently scored by two pathologists. The levels of Ki67 were measured semiquantitatively using a visual grading system based on the extent of staining. The results were exported, and a ratio was calculated

using the number of strongly positive pixels divided by the total number (positive plus negative) of pixels. This ratio was converted to a percentage.

2.9 Statistical analysis

Experimental data were obtained and entered into Microsoft Excel, followed by statistical analysis with SPSS 25.0 software. We used GraphPad Prism 6 to create all figures and the one-way ANOVA for comparisons between multiple groups. Following the one-way ANOVA, Dunnett's tests were applied for analyzing the difference between each experimental group and the control group. $P < 0.05$ was considered to indicate a statistically significant difference.

3 Results

3.1 Effect of HFPO-DA on hormone levels in serum

After treatment with HFPO-DA during GD0.5-GD19.5, we measured the maternal serum concentrations of progesterone (Pg), estradiol (E2), and prolactin (PRL) in all pregnant rats on GD19.5 and PND21 rats. The results showed that the PRL concentrations were 3.11 ± 0.54 ng/ml, 4.16 ± 0.81 ng/ml, 4.58 ± 1.13 ng/ml, and 5.66 ± 1.32 ng/ml after exposure to 0, 1, 10, and 100 mg/kg/d, respectively (Table 1). In all treatment groups, PRL serum levels were higher compared to the control, in the mean time we found that the increases were dose-dependent. The level of Pg was slightly higher, conversely, the E2 were somewhat lower in the control group, however the differences were not significant. In PND21 rats, there were no significant

changes and no gender differences in the levels of PRL, Pg, and E2 when compared with the control group (Table 1).

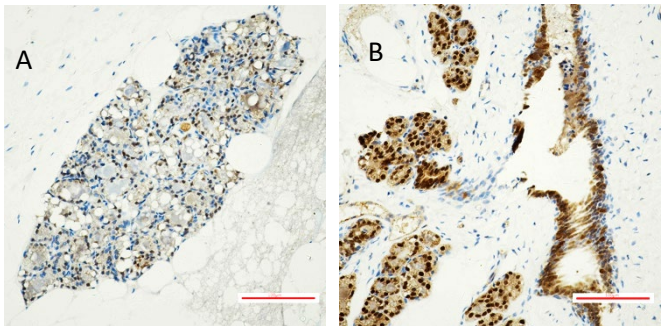
Table 1. The levels of E2, Pg, and PRL in serum.

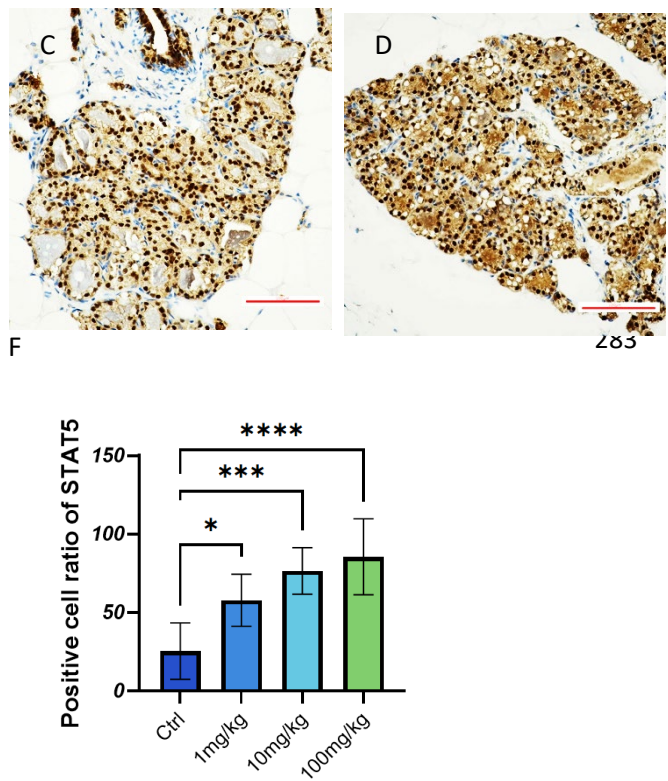
Dose of HFPO- DA (mg/kg)	Pregnant rats			PND21 female rat			PND21 male rat		
	PRL (ng/ml)	Pg (ng/ml)	E2 (ng/ml)	PRL (ng/ml)	Pg (pg/ml)	E2 (pg/ml)	PRL (ng/ml)	Pg (pg/ml)	E2 (pg/ml)
0	3.11±0.54	0.47±0.06	0.11±0.05	1.16±0.17	22.45±1.60	64.13±1.21	1.3±0.06	21.80±1.66	61.34±0.93
1	4.16±0.81	0.42±0.05	0.11±0.01	1.25±0.49	19.75±1.57	60.22±2.59	1.23±0.10	19.07±1.30	59.56±1.55
10	4.58±0.81	0.44±0.06	0.13±0.02	1.34±0.16	19.20±1.54	61.14±1.17	1.29±0.06	19.39±1.11	60.41±0.79
100	5.67±1.32*	0.42±0.09	0.12±0.01	0.93±0.36	22.85±2.10	66.57±4.69	1.17±0.04	21.87±1.78	61.97±1.38

* Indicate P<0.05 and ** indicate P<0.01

3.2 The expression of STAT5 in mammary gland of pregnant rats

Immunohistochemical results indicated that, compared to the control group, the expression of STAT5 in the epithelial cells of pregnant rats' mammary glands significantly increased in the 1,10 and 100 mg/kg/d group in a dose-dependent manner (Figure 1, Table S1).





284
285 Figure 1. The expression of STAT5 (brown cells) in pregnant rats' mammary glands
286 (400X, Scale bar =100 μ m). A: control group; B: 1mg/kg group; C: 10mg/kg group;
287 D: 100mg/kg group. F: The positive cell ratio of STAT5 expression in pregnant rats.
288

289 3.3 Effects of HFPO-DA on the histopathology of maternal, fetal and 290 PND21 rat mammary glands

291 After exposure to HFPO-DA, the analysis of the histological features of
292 the maternal mammary gland showed no significant changes in the glands
293 around the ducts and there was no notable hyperplasia of the ductal
294 epithelial cells (Figure 2). The lobular area increased progressively with
295 higher doses, showing a significant increase in the high-dose group
296 compared to the control. Similarly, the alveolar area expanded with
297 increasing doses of HFPO-DA, particularly in the 10 mg and 100 mg

groups, where the difference was statistically significant compared to the control (Table S2). Milk secretion was observed only in the high-dose group, with the percentage of ducts exhibiting milk secretion as follows: 58.3%, 45.5%, 75%, 60%, 66.7%, and 40.0%. Selected H&E-stained images demonstrating milk secretion are detailed in the supplementary materials (Figure S2).

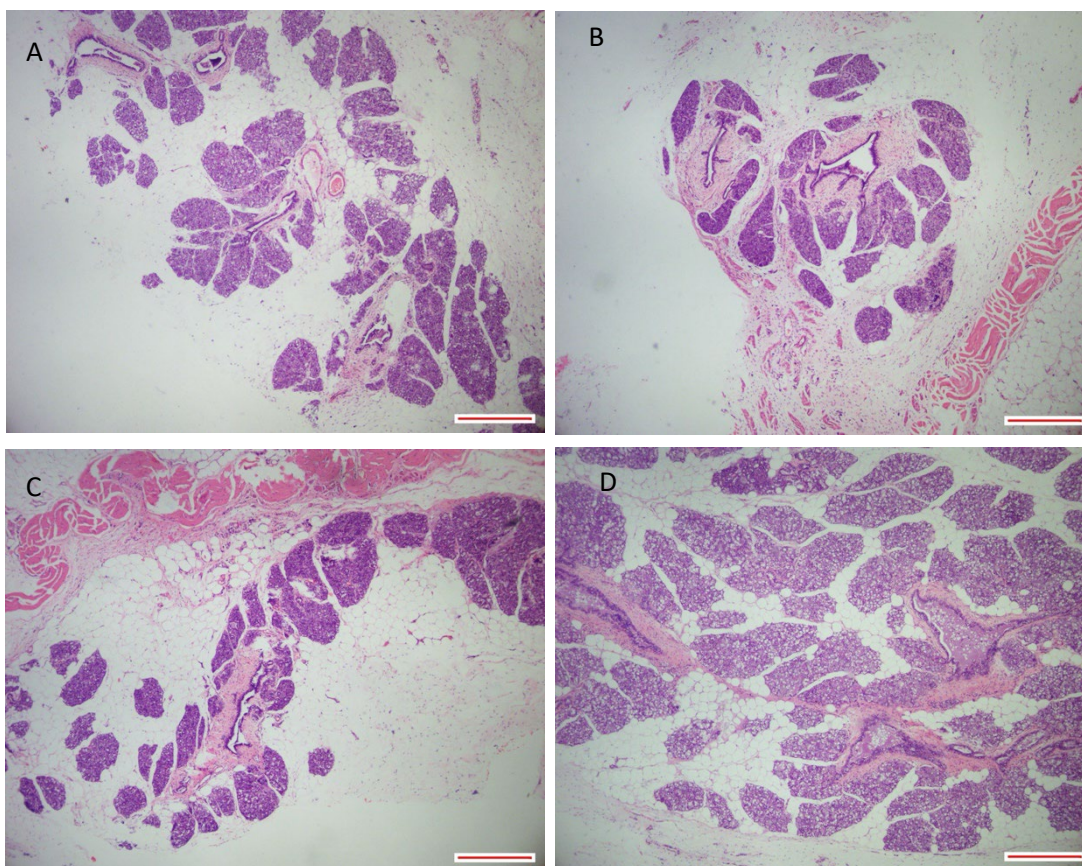


Figure 2. H&E staining of the maternal mammary gland after exposed to HFPO-DA (40X, Scale bar =500 μ m). A: control group; B: 1mg/kg group; C: 10mg/kg group; D: 100mg/kg group.

For H&E staining of fetal mammary glands, primary mammary ducts were observed in each group. In fetal rats, the 10 mg and 100 mg groups exhibited significant increases in secondary ducts. In female rats on PND21,

secondary ducts significantly increased in both the 10 mg and 100 mg groups, while in males, the increase was significant only in the 100 mg group (Figure 3, Table 2).

Control 1mg/kg 10mg/kg 100mg/kg

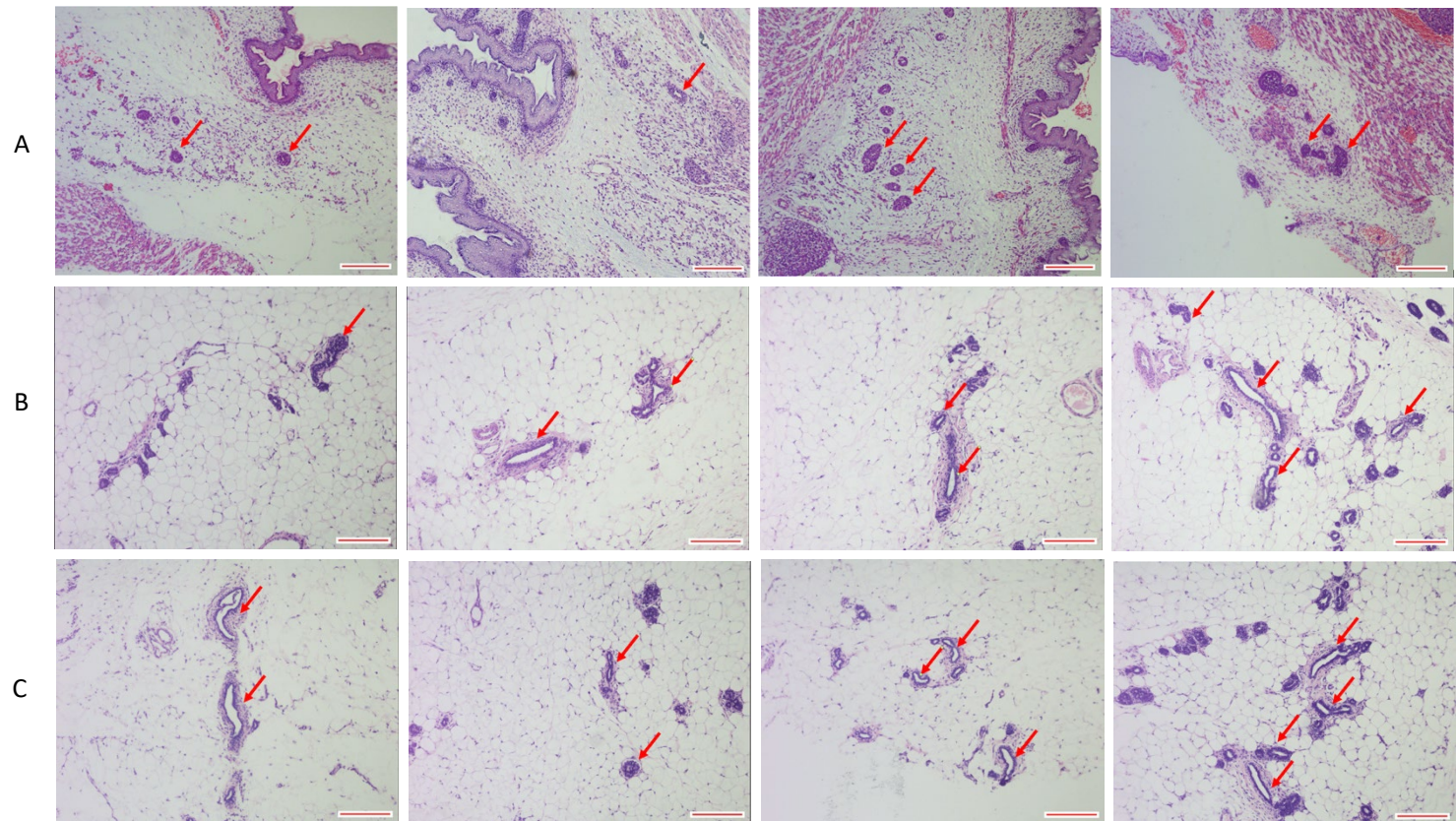


Figure 3. H&E staining of fetal (A) and PND21 female (B) and male (C) rats' Red arrows indicate secondary duct. (A: 100X, Scale bar =200 μ m; B and C: 200X, Scale bar =100 μ m).

Table 2. The number of primary and secondary mammary gland ducts in offsprings.

Dose of HFPO-DA (mg/kg)	Fetal rats		PND21 female rats		PND21 male rats	
	Primary ducts	Secondary ducts	Primary ducts	Secondary ducts	Primary ducts	Secondary ducts
0	2.17 $\pm$ 0.75	1.33 $\pm$ 0.52	1.80 $\pm$ 0.84	3.8 $\pm$ 0.84	1.40 $\pm$ 1.51	4.00 $\pm$ 0.1.58
1	2.33 $\pm$ 0.82	2.17 $\pm$ 0.75	2.20 $\pm$ 1.09	5.00 $\pm$ 1.58	4.20 $\pm$ 0.71	4.20 $\pm$ 1.92
10	3.00 $\pm$ 1.09	3.17 $\pm$ 1.17*	3.20 $\pm$ 1.30	8.00 $\pm$ 2.23*	2.40 $\pm$ 0.55	6.75 $\pm$ 2.50
100	3.05 $\pm$ 0.63	4.83 $\pm$ 1.47**	2.00 $\pm$ 1.78	11.67 $\pm$ 2.65**	1.00 $\pm$ 0.71	11.80 $\pm$ 1.30**

* Indicate P<0.05 and ** indicate P<0.01, P<0.05.

3.4 Effects of HFPO-DA on mammary gland development in fetal and PND21 rats

The results of whole mammary gland staining in fetal rats are displayed in Figure 4. Compared to the control group, the ductal coverage area increased by 10.5%, 63.4%, and 69.2% in the 1, 10, and 100 mg/kg groups, respectively. There was a significant increase in the number of mammary ductal buds and invasion length of ducts in the 10 and 100 mg/kg groups. Although the number of the ductal branches seemed slightly higher in the 100 mg/kg group, the difference was not significant (Table S3 and Figure S3.)

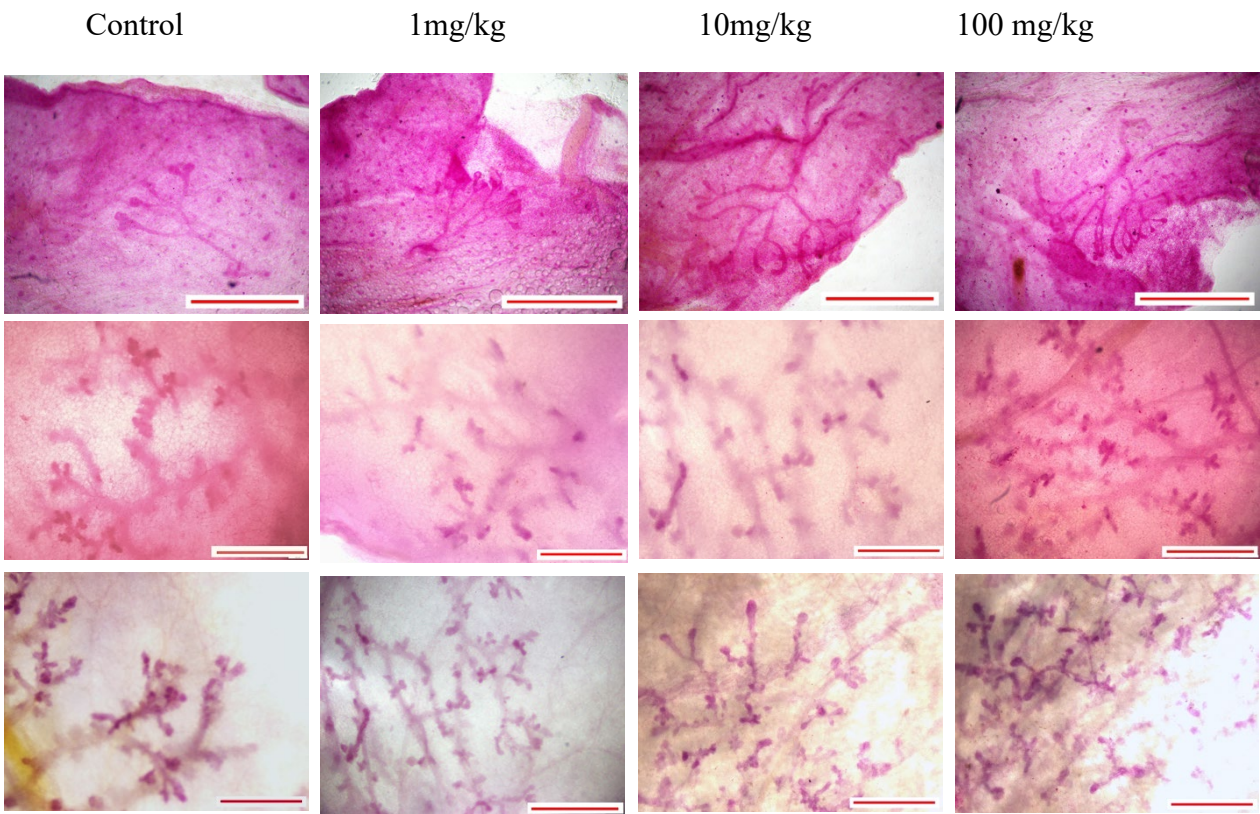


Figure 4. Staining of the whole mammary gland in fetal rats (A), PND21 female (B) and male (C) rats (A: 4X, Scale bar =500 μ m; B and C:10X, Scale bar =1000 μ m).

The results of whole mammary gland staining in PND21 rats are presented in Figure 4B and 4C. Given that whole mammary gland staining of PND21 rats was incomplete under the lowest magnification microscope, we randomly selected three mammary ducts from each group for the analysis of their length and branches (Table S4 and Figure S4). The results revealed no significant differences in the number of ductal branches in PND21 female and male rats across all groups. However, compared with that in the control groups, the ductal length in PND21 female and male rats was significantly higher in the 1, 10, and 100 mg/kg groups ($P < 0.05$).

3.5 The effect of HFPO-DA on the proliferation of fetal and PND21 mammary ductal epithelial cells

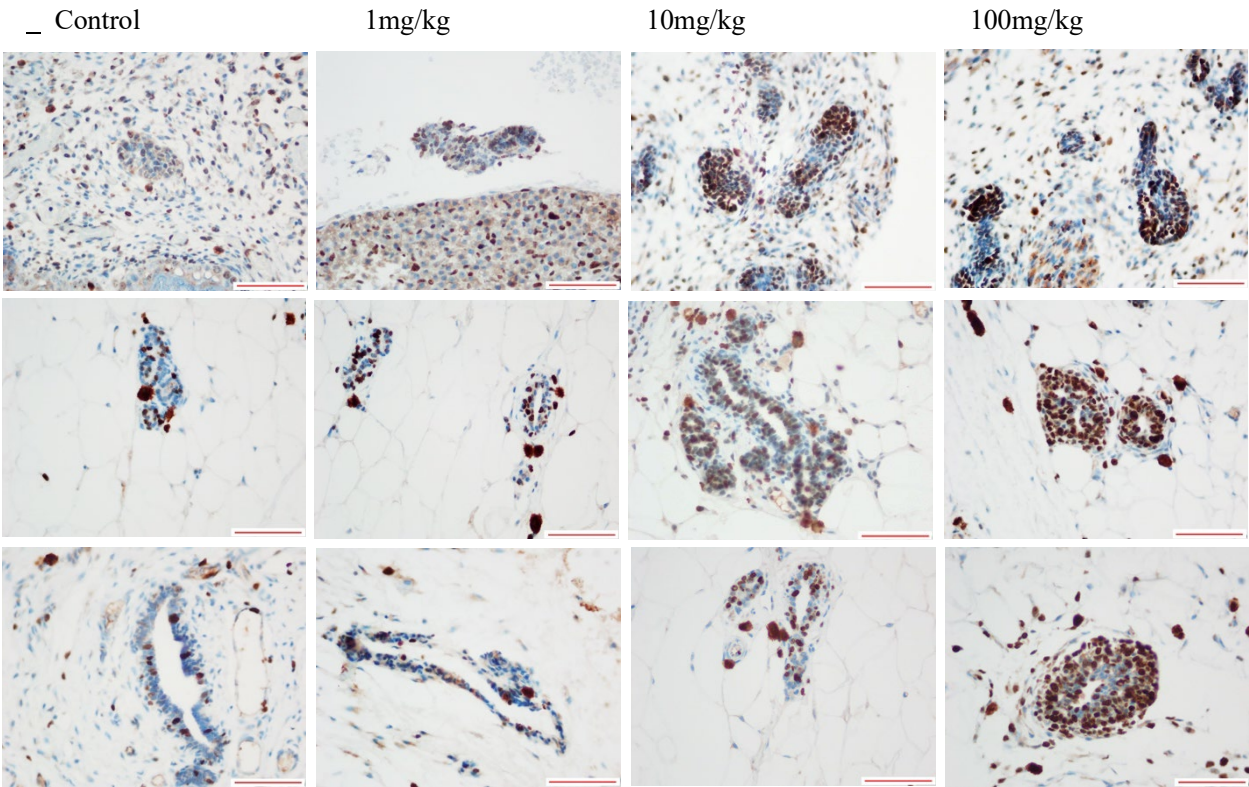
Compared with those in the control group, the levels of Ki67 in the mammary glands of the fetal and PND21 rats were significantly increased in the 100 mg/kg/d group, and the increase followed a dose-dependent pattern (Figure 5). Next, we measured average optical density (AOD) and found that these values were significantly higher in the 1,10 and 100 mg/kg groups of fetal and PND21 rats than in the control groups of fetal and PND21 rats (Table S5, $P < 0.05$). By quantify the percentage of positive cells, positive cell ratio in the epithelial cells was significantly increased in 10 mg and 100 mg/kg groups in fetal rats and in all PND21 experimental group. The expression of Ki67 in stromal cells was very low, and positive cell ratio in the stromal cells was significantly increased in 10mg and 100

Table 3. The positive cell ratio of Ki67 expression in offspring rats.

Dose of HFPO-DA (mg/kg)	Fetal rats		PND21 female rats		PND21 male rats	
	Epithelial cell	Stromal cells	Epithelial cell	Stromal cells	Epithelial cell	Stromal cells
0	16.35±4.45	21.64±7.63	13.77±5.79	8.33±2.89	27.51±9.57	8.44±4.79
1	33.75±14.93	37.44±6.45	43.19±1.31**	15.83±4.92	61.67±8.17**	15.12±4.08
10	59.11±6.15**	54.63±9.16**	62.72±11.26**	20.02±10.11	67.53±11.91**	11.25±6.29
100	71.33±10.81**	57.51±6.32**	76.01±6.15**	26.68±5.77*	87.58±22.82**	38.75±20.16*

* Indicate P<0.05 and ** indicate P<0.01.

mg/kg groups in fetal rats. However, the increase was only observed in the 100 mg/kg groups in PND21 male and female rats (Table 3).



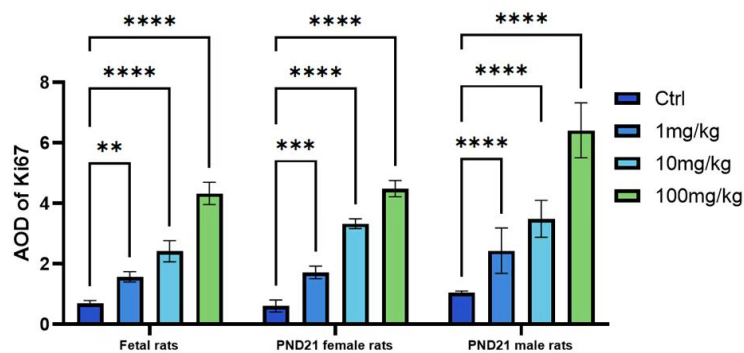


Figure 5. The expression of Ki67 (brown cells) in fetal (A) mammary glands and PND21 female (B) and male (C) rats (400X, Scale bar =100 μ m). D: AOD of Ki67 in mammary gland immunohistochemical in fetal and PND21 female and male rats.

3.6 Effects of HFPO-DA on the ovaries of pregnant rat and PND21 female rats.

Histological examination of the ovaries revealed distinct morphological profiles between the pregnant and PND21 rats, which were consistent with their respective physiological states (Figure 6A, 6B). In pregnant rats, the ovaries were characterized by a predominance of persistent corpora lutea and a notable suppression of follicular development. In contrast, PND21 rat ovaries exhibited active folliculogenesis, presenting multiple follicles ranging from mature to post-ovulatory stages, with only occasional corpora lutea. Importantly, H&E staining analysis detected no significant differences in corpus luteum structure or follicle morphology between the HFPO-DA-exposed groups and the control group, either in pregnant rats or in PND21 offspring.

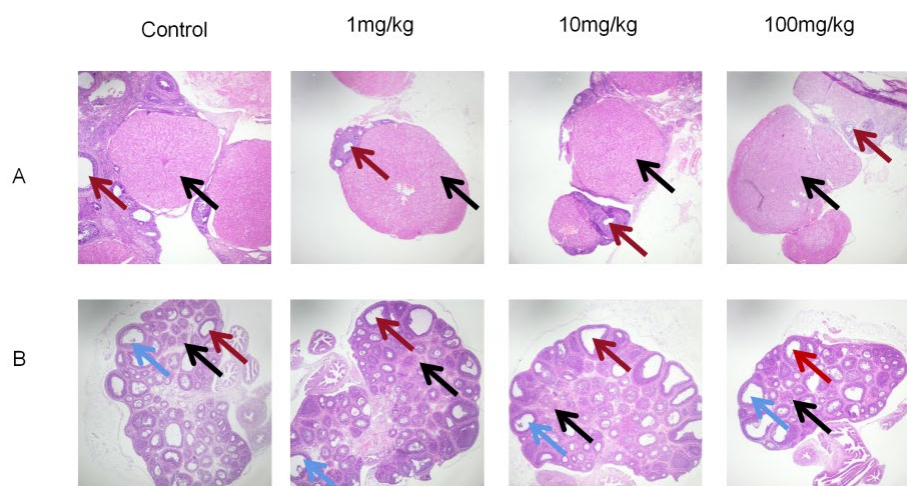


Figure 6. H&E staining of ovaries (40X). A: Pregnant rats. B: PND21 female rats. The black arrows indicate corpus luteus, the blue arrows indicate mature follicles, and the brown arrows indicate ovulated follicles.

4 Discussion

In this study, we found that HFPO-DA exposure during pregnancy could induce milk secretion in pregnant rats, which might associate with the secretion of PRL.

The outcomes of the previous two reports were generally consistent with our findings that in increase in PFASs exposure induces higher levels of PRL. In Pierre Blévin *et al.*'s study, PFOS, PFNA and perfluoroundecanoate (PFUnA) were observed to be positively related to PRL in the serum from black-legged kittiwake from Kongsfjord, Svalbard in the Arctic (Blevin et al., 2020). Additionally, Suyun Zhang *et al.* examined the levels of PFASs and sex hormones in a primary ovarian insufficiency (POI) population, and reported meaningful correlations among the concentration of PFOA or PFOS, and that of PRL(Zhang et al., 2018). This experimental results revealed that the difference of E2 level

was not significant. Furthermore, the focus of several previous studies was on the interfering effect of PFASs on E2 levels, but the results were inconsistent. In their study of the PFASs concentrations in maternal serum and oestradiol in cord blood in a birth cohort from LaiZhouWan, China (Yao et al., 2021), Qian Yao et al. The result also showed that the PFOA concentrations from maternal serum were significantly related to E2 levels from umbilical cord blood. This result was in agreement with the study of Hongxiu Liu. They quantified six PFAS and three estrogens in cord serum in 942 newborns in the Wuhan birth cohort in China between 2013 and 2014. And the result showed a correlation between concentrations of PFASs (including PFOS, PFOA, and F-53B) and higher E2 levels (Liu et al., 2021). Guohui Shi *et al.* revealed that exposure to F-53B in zebrafish increased the levels of E2 (Shi et al., 2018). However, Kai Luo *et al.* selected blood samples from ordinary men in Shanghai, China, and they found that the level of E2 was negatively correlated with PFASs (Luo et al., 2021). Yang *et al.* measured serum concentrations of PFASs (early pregnancy) and estrogen levels (early, middle, and late pregnancy) in a pregnant cohort in Tangshan, Hebei, China. The results showed that PFASs were negatively correlated with E2 levels in the third trimester (Yang et al., 2022). Additionally, our research revealed that the Pg level decreased in the experimental group. However, a study by Md Nur Alam *et al.* showed that chronic low-level PFOS exposure in rats could increase the

concentration of Pg (Alam et al., 2021). Our comparison between female and male offspring at this stage revealed a specific sex difference: in the 100 mg/kg group, E2 levels were significantly higher in females than in males, whereas Pg, PRL, and mammary gland development showed no significant sex-based divergence. Overall, the effects on PRL and Pg secretion were consistent, but the results of PFASs exposure on E2 levels are still controversial. The ovary is the main source of estrogen and progesterone required for mammary gland development. Therefore, the development of ovaries was assessed via H&E staining. And the outcomes indicated that no obvious differences were detected among luteal structure or development of the ovaries of pregnant or PND21 female rats among the groups. Accordingly, in our study, we speculated that HFPO-DA promotes the development of mammary gland development may not be related to ovarian function, but further researches are still needed to confirm these findings.

PRL can combine with steroid hormones during pregnancy to affect mammary gland development and lactation by promoting ductal elongation, bifurcation, and lobular acini formation (Jahn et al., 1991). Our findings were consistent with previously reported data. In the 100 mg/kg/d group, a trend of increasing prolactin (PRL) levels was observed, accompanied by notable milk secretion in the mammary glands of pregnant rats in the mammary glands, as evidenced by H&E staining. Additionally, the number,

length, and area of mammary duct branches in fetal rats were notably increased. Prolactin regulates the proliferation, differentiation, and milk production of mammary cells by activating the PRLR-JAK-STAT5 axis. This study shows that STAT5 expression was significantly increased in the experimental groups, suggesting that the PRL pathway has a significant impact in HFPO-DA's boost of mammary gland development and lactation in pregnant rats (Lee et al., 2016). However, to date, direct causal evidence from further experimental studies (e.g., mechanistic in vitro or pathway-focused in vivo work) is still lacking. The increase in Ki67 levels suggested that the development of mammary ductal epithelial cells in 10 and 100 mg/kg/d. However, two studies (Tucker et al., 2015; White et al., 2011) showed that exposure to PFOA was able to inhibit mammary gland development by reducing the mammary gland development score, the ductal buds numbers, branches and ductal area. These findings are contrary with our results. It should be noted that while Ki67 is a well-validated marker for proliferating cells in mammary tissue, the use of complementary proliferation markers (e.g., PCNA, BrdU) in future studies could help to further characterize the dynamics and phases of epithelial cell proliferation in response to HFPO-DA exposure. The ligand-activated nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ) plays an essential role in regulating inflammatory responses, cell proliferation, and differentiation. PFOA is an agonist of PPAR γ . A previous study revealed

that PFOA can form more hydrogen bonds with PPAR- γ than HFPO-DA does, which indicates that PFOA is better able to activate PPAR γ (Li, Ren, & Guo, 2019). In addition, another study showed that PFOA was much more influenced to the estrogen receptor than HFPO-DA was (Xin, Ren, Wan, & Guo, 2019). Therefore, we speculate that the effects of HFPO-DA and PFOA exposure in mammary glands might relate to different targets and differences in binding to related receptors. Given the novelty of HFPO-DA in mammary toxicology, further studies are needed to investigate more mechanisms, such as oxidative stress and epigenetic regulation.

Notably, the mammary gland development in fetal rats continued following HFPO-DA exposure, even after the exposure in pregnant rats was stopped immediately after vaginal delivery. Previous studies have shown that the half-life of HFPO-DA is 3 h in female, while 8 h in male rats. Because HFPO-DA has a short half-life and fast metabolism, in this study we thought that pregnant rats were no longer exposed to HFPO-DA after GD19.5, the amount of HFPO-DA in their mammary milk was negligible, and PND 1-PND 20 rats were not exposed to HFPO-DA. Interestingly, the results of our study also found that the development of mammary glands in 1,10 and 100 mg/kg/d groups was still advanced. This immunohistochemical staining results showed a significant proliferation of mammary ductal epithelial cells in the 100mg/kg/d group. The size of the animal is also an important factor influencing prepubertal mammary gland

development, with a positive correlation between body weight and mammary development. However, our previous research found that offspring rats showed a decreasing trend in body weight as the HFPO-DA dose increased, suggesting that body weight is not the driving factor for mammary gland development in this case. However, after exposure to HFPO-DA, there were no meaningful changes in sex hormones levels in PND21 rats. In recent years, “the Origin of Health and Disease Development Theory” has suggested (Barker, 2007) that in the early stage of life, exposing the fetus and newborn to an adverse environment may lead to persistent changes in the cells, tissues, organs, and overall level, and have critical effects on near and long-term health after birth and in adulthood. For example, exposure to HFPO-DA reduces neonatal birth weight and increases neonatal mortality (Conley et al., 2021). Intrauterine exposure to PFOA reduces birth weight, delays eye-opening and hair growth time, and decreases body weight during lactation. Therefore, the regulation of sex hormones cannot reverse the effects of HFPO-DA on mammary development (Wolf et al., 2007). Future longitudinal studies are essential to evaluate whether early-life HFPO-DA exposure predisposes to adult mammary dysfunction or cancer. Furthermore, when considering the translational implications of such studies, our rodent findings require cautious human extrapolation due to interspecies differences. Future human-relevant models or epidemiological studies are therefore essential

for direct risk assessment. Notably, the United States Environmental Protection Agency (USEPA) has established guidelines for prenatal developmental toxicity studies, and a DuPont (2010) report identified a NOAEL of 10 mg/kg per day (DuPont, 2010). In light of our findings, the lowest daily dose of 1 mg/kg suggests a potential impact of HFPO-DA on mammary gland development, highlighting the need for further toxicological investigation to assess its effects at lower, environmentally relevant doses.

Conclusions

The strength of our study lies in providing the first evidence of mammary gland developmental toxicity and potential interference with sex hormone secretion by HFPO-DA. We selected the period of pregnancy which sensitive to HFPO-DA exposure as the exposure window. We not only found the promoting effect of intrauterine HFPO-DA exposure in fetal rats, but also found that the promotion effect of HFPO-DA in offspring rats persisted to pre-puberty after HFPO-DA exposure had stopped. These results may provide new experimental evidence and research ideas for further study on the toxicity of HFPO-DA. Since this study focused only on histopathological evaluations and hormone level measurements, without further in vitro experiments such as competitive fluorescence binding assays and immunohistochemistry, the underlying mechanism of

HFPO-DA's effects on mammary gland development remains to be explored.

Declarations

Ethics approval and consent to participate

The experiment followed the institutional and national guidelines for the Care and Use of Laboratory Animals and was approved by the Ethics Committee of the Qingdao Municipal Hospital.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing of interest

The authors declare that they have no competing interests.

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Appendices

Not applicable.

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Effects of prenatal exposure to hexafluoropropylene oxide dimer acid on rat and offspring mammary gland development and associated hormone levels

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