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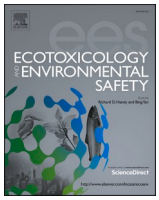

Implications of a combined perinatal exposure to BPA and BP-3 for offspring folliculogenesis and ovarian function in mice

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ABSTRACT

Endocrine disrupting chemicals (EDCs), like bisphenol A (BPA) and benzophenone-3 (BP-3), can interfere with hormone systems, posing risks to fertility and reproduction. Exposure to EDCs is unavoidable making it a relevant environmental health topic, however the impact of real-life EDC mixtures is largely unknown. This study explored the effects of a combined BPA and BP-3 exposure at tolerable intake levels for humans during pregnancy and early life on ovarian development and function in an established mouse model. Mice were daily exposed to concentrations of 4 μg/kg BPA orally, 50 mg/kg BP-3 dermally, and the combination of BPA+BP-3 through gestation and lactation, a susceptible developmental period. Female offspring of BPA and BP-3 exposed mice exhibited increased birth weight and elevated bodyweight by postnatal day 7. By day 30, after hormonal stimulation to induce ovulation, exposed offspring showed disrupted ovarian follicle maturation and altered ovarian response to stimulation with exogenous gonadotropins. Moreover, the number of NK cells rose in the ovaries, and genes linked to hormone signaling, hormone synthesis, and ovarian tissue remodeling were altered relative to unexposed controls. These findings suggest that early life exposure to BPA and BP-3 at environmentally relevant doses impairs ovarian development and function in mice indicating that immune cells and hormonal signaling in the ovaries are targets of endocrine disruptors at relevant concentrations. Such endocrine disruption may be compromising fertility and reproductive health in later life. Our research underscores the importance of investigating the impact of combined EDC exposure on the reproductive system.

1. Introduction

An estimated number of 350,000 chemicals and mixtures of chemicals are currently available or registered for use and a large part of these anthropogenic chemicals shows concerning endocrine activity (Naidu et al., 2021; Wang et al., 2020). Endocrine disrupting chemicals (EDCs) form an important group of substances found increasingly in the environment, in food items and consumer products. Humans and wildlife are exposed orally, dermally, and by inhalation to EDCs and EDC mixtures, which consequently can be detected in human serum and tissue samples in large parts of the population in different countries (Thépaut et al., 2021; Calafat et al., 2008; Karrer et al., 2020). Bisphenol A (BPA) and benzophenone-3 (BP-3) are two widespread proxies of ubiquitous EDCs that have been investigated in human populations, mothers and children alike, and in animal models of toxicological studies (Karrer et al., 2020; Arya et al., 2020; Santamariaía et al., 2017; Santamaria et al., 2020). Both BPA and BP-3 have been found in maternal blood, urine and breast milk as well as in fetal tissue and body fluids demonstrating that these

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chemicals cross the materno-fetal barrier and placenta (Lee et al., 2018; Molins-Delgado et al., 2018; Vela-Soria et al., 2014; Krause et al., 2018). The cumulative amount of bisphenols and predominantly BPA that humans are exposed to from food items, packaging and other consumer products can reach approximately 0.8 μg/kg bodyweight per day (d) in worst-case scenarios (Karrer et al., 2020). BP-3 is found as sunscreen ingredient and ultraviolet (UV) light blocker in cosmetics, cloths and other consumer products (Morrison et al., 2017) with concentrations in human urine, serum and tissue ranging from $< 10^{-9}$ to 10^{-5} g/mL (Calafat et al., 2008). Direct sunscreen application containing BP-3 can lead to an exposure of 50 mg/kg bodyweight or more in humans (Janjua et al., 2008). As a consequence, the Scientific Committee on Consumer Safety (SCCS) recommended to limit BP-3 concentrations in body creams to 2.2 % and in cosmetics for hand, face and lipstick to 6 % (Safety, 2021). Nonetheless, BPA and BP-3 are still used and many people, including women and children at sensitive periods in life, are still exposed frequently to both chemicals.

A comprehensive review published in 2015 summarized that certain bisphenols, phthalates and other EDCs exert toxic and disrupting effects on female reproductive health, promoting subfertility, obesity development and increase the risk for certain types of cancer (Gore et al., 2015). Moreover, the combined knowledge on hormone actions and EDC effects recently led to the development of ten key characteristics for EDCs in an expert consensus statement among which are hormone receptor binding, hormone synthesis and epigenetic alterations, all of which are potentially relevant for reproductive health (La Merrill et al., 2020). EDCs can affect hypothalamic-pituitary-gonadal (HPG) axis function in vertebrates that is the major regulator of gonadal cell function, steroidogenesis and the ovulation process in females (Plunk and Richards, 2020). The HPG axis also provides a link between endocrine organs and fertility by influencing the aforementioned gonadal processes as well as hormone production (Xie et al., 2022). Innate and adaptive immune cells fulfill important roles in the development of the gonads, folliculogenesis and key reproductive events. Furthermore, EDCs have been shown to disturb innate and adaptive immune cells and affect autoimmune disease onset by influencing endocrine systems (Popescu et al., 2021; Dai et al., 2023). It is not surprising that gonadal cells and immune cells are effective targets of EDC since both these cell types possess hormone receptors, such as estrogen and progesterone receptors (Popescu et al., 2021; Jensen et al., 2010; Henderson et al., 2003; Xu et al., 2022; Bloom et al., 2016). Immune cells, such as NK cells, T cells and macrophages play a significant role during folliculogenesis and ovulation, and these cells have been observed in follicular fluid as well (Dai et al., 2023). Depending on the ovarian or uterine microenvironment and external stimuli, NK cells and macrophages change secretion of cytokines and inflammatory factors which may influence follicle maturation and ovulation. However, other cells, including dendritic cells, have also been suggested to play a role in follicle rupture and ovulation, and hormonal changes and ovarian pathologies are associated with immunologic changes in this tissue (Yang et al., 2019). To which extent EDCs can affect different ovarian immune cells, their function and the immune microenvironment in ovaries is still a matter of debate.

Important inherent threats of EDC exposure are low dose effects and nonmonotonic dose responses together with impacts on the endocrine system of offspring when exposure occurs during the gestational and early postnatal or perinatal period (Rolfo et al., 2020; Braun, 2017; Schug et al., 2016). Recent data from cohort studies underline the impact of EDC mixtures at environmentally relevant concentrations on human health when exposure occurs during pregnancy and the perinatal period (Caporale et al., 2022). Nonetheless, there is still a considerable knowledge gap on the effects of perinatal EDC and mixture exposure on health and fertility development in exposed offspring.

In rodents, BPA was shown to affect folliculogenesis and corpus luteum (CL) development and caused changes in expression of ovarian genes relevant for hormone synthesis upon perinatal exposure at environmentally relevant doses (López-Rodríguez et al., 2019; Santamaría et al., 2016). However, it appears that exposure to BPA in the perinatal period may lead to more persistent effects on folliculogenesis than exposure during adult life which indicates programming effects depending on the time point of exposure (López-Rodríguez et al., 2019). The effects of BP-3 on fertility and ovarian function are not as well explored compared to BPA. Human studies found associations between urinary BP-3 and reproductive hormone levels, such as follicle-stimulating hormone and luteinizing hormone, age of menarche and onset of puberty (Pollack et al., 2018; Binder et al., 2018; Wolff et al., 2015). In follicular fluid of women recruited at a fertility clinic, BP-3 and mixtures containing BP-3 and other EDCs were found, however the impact of BP-3 in follicular fluid on fertility remains elusive (Beck et al., 2023). In rodent models, BP-3 showed in vitro effects on folliculogenesis at environmentally relevant concentrations (Santamaría et al., 2019a) and in zebra fish, BP-3 exposure at the embryo stage caused alterations in the ovaries and in egg production of exposed offspring with consequences for fertility of fish (Tao et al., 2023). The limited number of studies investigating BP-3 related effects after perinatal exposure indicates a considerable knowledge gap on the consequences of perinatal BP-3 exposure at environmentally relevant levels for female offspring ovarian development, folliculogenesis and reproductive health. Additionally, the reproductive health effects of EDC mixtures containing BPA and BP-3 are not well studied, which hampers implementing safety regulations and protection policies to limit EDC exposure. The potentially severe and long-lasting reproductive effects of exposure to BPA, BP-3 and mixtures, especially during sensitive developmental periods, demand increased scientific attention also to enhance policy making for reproductive health and environmental protection.

Due to the considerable knowledge gap on mixture effects of environmental chemical exposure in the perinatal period on offspring gonadal function, we aimed to evaluate the effects of single and combined perinatal exposure to BPA and BP-3 at environmentally relevant concentrations on female offspring gonadal development and function. The impact of perinatal exposure to EDCs on offspring gonadal development and function can only be studied in vivo. Mouse models are adequate for our scientific question as pregnancy outcomes are validated for humans, hemochorial placentation in mice resembles human placentation and the immune system composition is also similar (Georgiades et al., 2002; Sojka et al., 2019). Using an established perinatal exposure mouse model, we generated BPA and BP-3 exposed female offspring for superovulation treatment with exogenous gonadotropins to induce folliculogenesis, ovulation and CL development. We analyzed ovarian tissue of female offspring to determine follicle count and CL development, ovulation after PMSG and hCG treatment, ovarian immune cell count and the gene expression of several ovarian differentiation and extracellular matrix markers.

2. Materials and methods

2.1. Animal housing

All procedures of animal housing, treatment and experiments for this study were conducted after approval by the German local authorities (Landesdirektion Sachsen, Germany, TVV21/21). Only authorized individuals were involved in animal handling, treatment and experiments according to the Guide for Care and Use of Animals in Agriculture Research and Teaching. Eight-week-old C57BL/6 female and BALB/c male mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and were acclimatized to the facility for at least one week before mating. Animals were kept at 22 ± 1 °C on a 12 h (6 a.m./6 p.m.) light/dark cycle under specific pathogen-free (SPF) conditions in IVC cages (Tecniplast, Italy) at the animal facility of the Saxon Incubator for Clinical Translation, of the University of Leipzig. Access to water and a standard chow (#V1534–300, sniff, Germany) or a breeding chow (#V1185–300, sniff, Germany) from gestation day (GD) 12 onwards was

allowed ad libitum. To achieve an allogeneic pregnancy, BALB/c males were mated with C57BL/6 females in a one-to-one ratio. The day of detection of a vaginal plug, which was checked for every morning and late afternoon, was considered GD0 and subsequently female mice were assigned randomly to one of the following 4 treatment groups: vehicle, BPA, BP-3 or BPA+BP-3. Then, the dams were kept in groups of 3–4 animals until GD16. From GD16 onwards, dams were separated into individual cages to allow for a calm environment during parturition and until the end of lactation on postnatal day 21 (P21).

2.2. Animal treatment

Dams included in this study received either a control substance (vehicle), single EDC or a combination of two common EDCs (combined exposure) during gestation and lactation, i. e. the perinatal period (Fig. 1A). In this study, dams were exposed to the tolerable daily intake (TDI) of BPA, set by the European Food Safety Authority (EFSA) at 4 µg/kg bodyweight (bw) per day (d) in 2015 (P. P. P. European Food Safety Authority, Italy et al., 2015) which is the same dose that we applied in our previous study (Fischer et al., 2024). Stock solutions of 4 mg/mL (w/v) BPA (#239658, Sigma Aldrich, Germany) dissolved in ethanol were prepared and diluted to a working solution of 400 ng/mL in 0.01 % ethanol (Chemsolute, Germany). A suspension of 10 mg/mL (w/v) BP-3 (#H36206, Sigma Aldrich, Germany) in commercial olive oil (Ja!, REWE Group, Greece) was prepared. BPA was applied by oral gavage using a flexible feeding tube (Instech Laboratories, USA) at 10 µL/g bodyweight (bw), i. e. 4 µg/kg bw per day (d) to dams of the BPA (n = 11) and BPA+BP-3 (n = 13) treatment groups to ensure weight-adapted exposure. Moreover, BP-3 was administered by topical application at 5 µL/g bw, i. e. a dose of 50 mg/kg/d, which is a comparable dose to a single human whole-body topical application of sunscreen products containing BP-3 (Janjua et al., 2008). We have previously used the same BP-3 topical application model and showed that BP-3 was present in maternal serum and in amniotic fluid of pregnant dams making our model suitable to study potential effects of transdermal BP-3 exposure (Santamaria et al., 2020; Safety, 2021). In this study, the BP-3 suspension was administered to dams of the BP-3 (n = 14) and BPA+BP-3 group on an approximately 4 cm^2 large shaved spot on the upper back to achieve total absorption by the skin to mimic human transdermal exposure. Dams of the vehicle group (n = 11) were treated by topical application with pure olive oil (5 µL/g/d) and oral gavage with 0.01 % (v/v) ethanol (10 µL/g/d). To prevent other dams or offspring from grooming during BP-3 or olive oil absorption, dams were housed individually for 5–10 min until oil and BP-3 were visually totally absorbed. Treatment was performed daily at the same time on all dams from GD0 until P21 to mimic EDC exposure during pregnancy and lactation (perinatal exposure). After birth, offspring were weighed and sexed according to the anogenital distance on P1, P7, P14 and P21. On P21, all offspring were weaned and mother dams were sacrificed by cervical dislocation to retrieve blood and tissue samples from organs of the abdomen. Offspring were housed in same-sex groups of 2 – 5 animals per cage and received a standard chow.

2.3. Superovulation of female offspring

Female offspring from dams of the 4 treatment groups were assigned to superovulation treatment on P28 performed with exogenous gonadotropins pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) according to previous studies (Santamaría et al., 2017; Luo et al., 2011; Amorim et al., 2021; Wigger et al., 2023) and protocols from our lab. In brief, female offspring received 5 IU PMSG (RP1782721000, BioVendor, USA) dissolved in 100 µL sterile H_2O via intraperitoneal injection 2 – 3 h before beginning of the dark cycle (6 pm) to induce folliculogenesis. In total, 45 vehicle, 45 BPA, 42 BP-3 and 44 BPA+BP-3 exposed offspring were injected with PMSG. After 48 h, female offspring were weighed and 23 vehicle, 25 BPA, 22

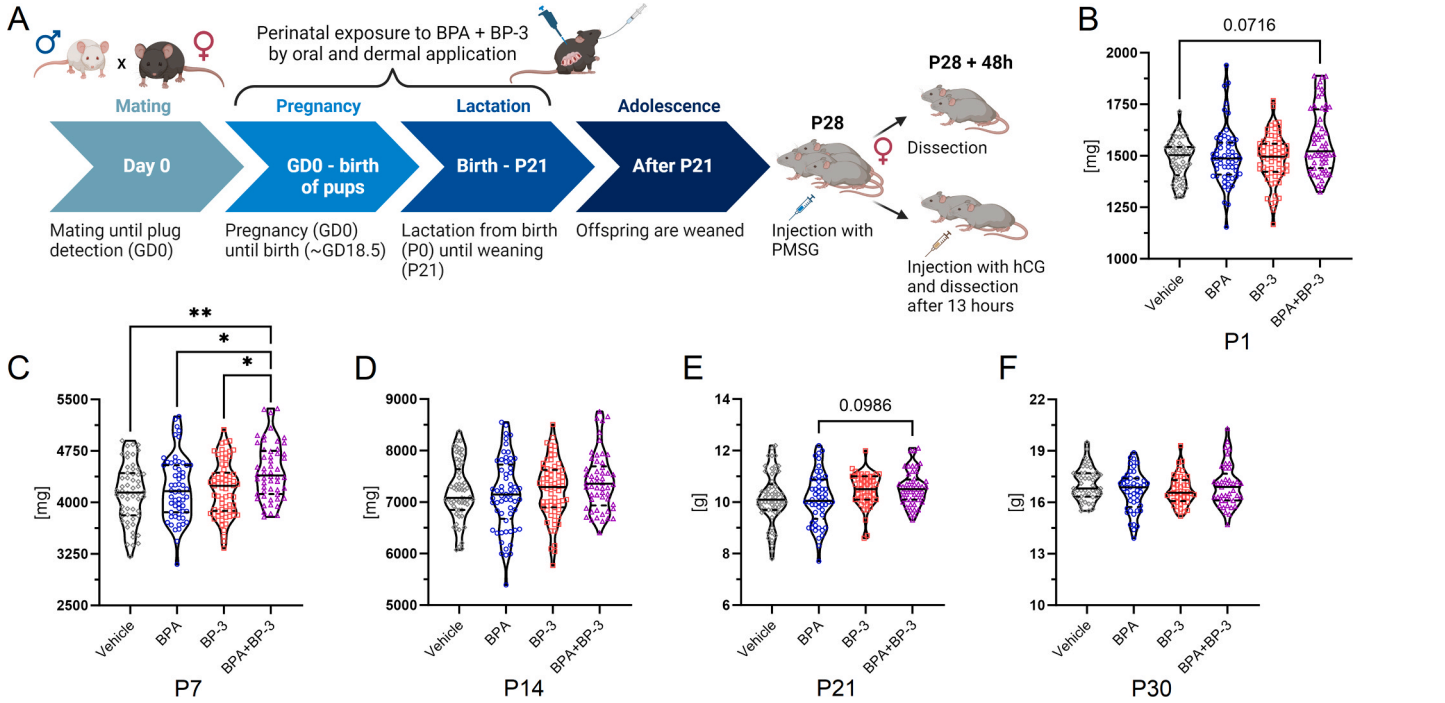

Fig. 1. Experimental setup for perinatal BPA and BP-3 exposure, female offspring superovulation treatment, dissection and bodyweight development. (A) Experimental setup of the perinatal exposure of dams with BPA and BP-3, and female offspring treatment with PMSG and hCG. (B) Violin plots show female offspring bodyweight on P1 ($n = 52$ (vehicle), 59 (BPA), 65 (BP-3), 55 (BPA+BP-3)), (C) on P7 ($n = 56$ (vehicle), 59 (BPA), 66 (BP-3), 54 (BPA+BP-3)), (D) P14 ($n = 57$ (vehicle), 60 (BPA), 66 (BP-3), 54 (BPA+BP-3)), (E) on P21 ($n = 57$ (vehicle), 52 (BPA), 51 (BP-3), 48 (BPA+BP-3)) and (F) on P30 (48 h after PMSG injection, ($n = 45$ (vehicle), 45 (BPA), 42 (BP-3), 41 (BPA+BP-3)). Median is indicated in plots as black line, quartiles as dashed line. Data was tested by Kruskal-Wallis test and differences were considered statistically significant for *P < 0.05 and **P < 0.01.

BP-3 and 24 BPA+BP-3 exposed offspring of the PMSG injected mice received 5 IU of hCG (Ovitrelle®, Merck Serono GmbH, Germany) dissolved in 100 µL sterile H_2O via intraperitoneal injection to induce superovulation. The offspring, which received only PMSG, were sacrificed for blood and tissue retrieval for analysis. All animals were sacrificed by cervical dislocation before collecting blood from the heart after opening the chest, and then collecting both ovaries and other organs of the abdomen. When dissecting the ovaries of only PMSG-treated female offspring, both ovaries were removed, weighed and used for cell isolation for flow cytometry. Additionally, one ovary from another female offspring group treated only with PMSG was used for formalin fixation and paraffin embedding (FFPE), while the other ovary was used for RNA isolation. Female offspring that received PMSG+hCG for superovulation were sacrificed 13 h after hCG injection for serum, oocyte and tissue retrieval for analysis (Fig. 1). In superovulated animals, the cumulus oocyte complex (COC) was located and obtained from the ampulla of both oviducts by careful removal with forceps scissors. COC was then transferred into a petri dish under a binocular at 3 – 5-fold magnification and carefully dissected to release oocytes. Oocytes were counted and documented from each ovaries' COC and the mean number of oocytes per ovary was calculated. Additionally, one ovary from another superovulated female offspring group was weighed and used for FFPE while the second ovary was weighed and used for RNA isolation.

2.4. Histology of ovarian tissue

To explore whether perinatal exposure to BPA and BP-3 can interfere with folliculogenesis and CL development, histological analysis of the ovaries was performed. For this purpose, two sections of each FFPE ovary were analyzed. Briefly, ovarian tissue was fixated overnight in 10 % natural-buffered formalin (Histofix®, Carl Roth, Germany), then transferred into 70 % ethanol (Carl Roth, Germany) and paraffin-embedded after dehydration in increasing concentrations of ethanol and acetic acid n-butylester (Carl Roth, Germany). FFPE ovaries were sectioned on a rotary microtome at a thickness of 5 µm per section and transferred to poly-lysin coated slides (Superfrost, Thermo Fisher Scientific, Germany) and air-dried at 40 °C overnight. From each ovary, two sections were taken for analysis with a distance of 500 µm to ensure that different large antral follicles and CL could be identified on each of the two sections avoiding double analysis of the same large follicle or CL. Resulting sections were dewaxed in Histoclear® (Carl Roth, Germany) and rehydrated before staining with hematoxylin and eosin (H/E, Carl Roth, Germany) following a standard protocol. Stained sections were imaged using a Keyence BX800 with 20-fold magnification objectives and resulting images were stitched together using the Keyence microscope software. The follicles were classified into primordial, primary, secondary, preantral and antral follicles (Santamaría et al., 2016; Kezele and Skinner, 2003; Myers et al., 2004). Atretic follicles and multioocyte follicles (MOF) containing more than one oocyte enclosed within the granulosa cell layer were also considered. In brief, a primordial follicle was defined as an oocyte encapsulated with a single-layered, flat epithelium. A primary follicle is surrounded by a single-layered cubic granulosa epithelium. In a secondary follicle the epithelium comprises two to five rows of granulosa cells. The antral follicles contain more than five rows of epithelium. A subdivision in this category into smaller preantral and preovulatory antral follicles was realized to ensure better comparability. The preantral follicles are recognizable by their multiple layers and showing a follicular cavity depending on the incision. The antral follicles are characterized by a significantly larger overall diameter, a pronounced follicular cavity or a cumulus oophorus. Atretic follicles were defined by fragmented oocyte remnants, wrinkling of the basement membrane, loss of granulosa cells and ingrowth of connective tissue. Each recognizable follicle was assigned to a category, even if no oocyte was incised, counted and measured. CL were counted and measured, and their luteinized cells are larger and show a light-colored cytoplasm. All areas and diameters were measured using the program Image J. From measurements, the diameters were calculated in Microsoft Excel. For better comparability, the follicles counted per development stage of folliculogenesis were standardized to the total number per section, resulting in percentage figures. The investigator performed a blinded image analysis.

2.5. Cell isolation and flow cytometry

To study immune cell infiltration into ovaries during folliculogenesis, whole ovaries were collected and analyzed. Dissected ovaries from only PMSG treated female offspring were washed with PBS (Thermo Fisher Scientific, Germany), cut into small pieces, collected in digestion solution in C tubes (Miltenyi Biotec, Germany) and processed by gentleMACS Octo Dissociator (Miltenyi Biotec, Germany) for obtaining a single cell suspension with the Multi Tissue Dissociation Kit 1 (#130–110–201, Miltenyi Biotec, Germany) according to manufacturer's instructions. After digestion, cells were briefly centrifuged (1 min, 4 °C at 300 rcf) and run over a 30 µm cell strainer followed by 2 washes with RPMI 1640 medium (Life Technologies Limited, UK). Cell suspensions were centrifuged again (10 min, 4 °C at 300 rcf) and resulting pellets were resuspended in PBS + 1 % fetal calf serum (FCS, PAN Biotech GmbH, Germany) and counted using a Neubauer chamber before surface staining with Fixable Viability Dye eFluor™ 506 (#65–0866–14, Thermo Fisher Scientific, Germany) and fluorophore labeled antibodies (Table 1) for 20 min at 4 °C in the dark. Next, cells were washed in PBS/FCS and fixated for 20 – 30 min by using Foxp3 Fixation/Permeabilization concentrate and diluent (#00–5523–00, Thermo Fisher Scientific, Germany). Following FcR blocking with FcR Blocking reagent (#130–092–575, Miltenyi Biotec, Germany), cells were stained intracellularly with fluorophore labeled antibodies (Table 1) for 20 min at 4 °C in the dark. After washing once with PBS/FCS, flow cytometric analysis was performed on an Attune NxT flow cytometer (Thermo Fisher Scientific, Germany). Using FCS Express 7 Research Edition (De Novo Software), the investigator performed a blinded data analysis, and the gating strategy can be found in the Supplementary Material (Figure S1).

2.6. Quantitative RT-PCR of ovarian tissue

Ovarian tissue from only PMSG treated and PMSG+hCG treated female offspring for RT-PCR analysis was snap frozen in liquid nitrogen after dissection and stored at −80 °C before RNA isolation to study changes in gene expression related to folliculogenesis, ovulation and CL development. After thawing, 1 mL of TRIzol Reagent (Invitrogen, Carlsbad, California, USA) was added to each ovarian sample before homogenization in a tissue lyser (TL) 2-times for 3 min, 50 Hz, using 2 stainless steel beads (Qiagen, USA) in each vial. Total RNA isolation from TRIzol solution was performed according to manufacturer's instructions using peqGold PhaseTrap tubes (peqlab, Erlangen Germany). The RNA concentration was measured on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Germany) and cDNA was synthesized

Table 1
Fluorescent dyes and fluorophore labeled antibodies used in this study.

Antibody	Fluorophore	Clone	Cat. Nr.	Company
l/d	eFluor 506	-	65–0866–14	ebioscience
CD45	AF700	30-F11	56–0451–82	invitrogen
CD3e	PerCP-Vio700	REA606	130–119–656	Miltenyi
CD19	APC-Vio770	REA749	130–112–038	Miltenyi
F4/80	PE-Vio615	REA126	130–123–913	Miltenyi
NK1.1	Vio-Bright B515	REA1162	130–120–512	Miltenyi
CD122	PE	REA1015	130–117–009	Miltenyi
CD11c	PE-Vio 770	REA754	130–110–840	Miltenyi
iNOS§	APC	REA982	130–116–423	Miltenyi
CD206§	BV711	C068C2	141727	BioLegend
§Intracellular staining antibody				

from 1 μg RNA following the instructions of the cDNA synthesis with the RevertAid™ H Minus Reverse Transcriptase kit (Thermo Fisher Scientific, Germany). The semi-quantitative PCR was performed on the Biomark HD system (Standard BioTools, San Francisco, CA, USA) using EvaGreen DNA binding Dye with BioMark™ 48.48 Dynamic Array Integrated Fluidic Circuits according to the manufactures recommendations. All reactions were run in duplicates. Sequences of exon-spanning primers of target genes are listed below (Table 2). To quantify the relative expression, the expression of the genes of interest was normalized to the mean of the reference gene glucuronidase beta (Gusb). The relative change in gene expression was calculated using the 2(-Delta-Delta C(T)) method (Livak and Schmittgen, 2001).

2.7. Serum ELISA for estrogens

The ELISA kit (ab285291, BioVision, an Abcam company, Cambridge, UK) was used according to manufacturer's instructions. Briefly, serum samples stored at −80°C were thawed on ice, centrifuged and 50 μL of supernatant was applied in duplicate into pre-coated wells of the ELISA plate. After incubation and washing, tetramethylbenzidine incubation was stopped with stop solution and the plate was read at 450 nm on a Synergy H1 (BioTek, USA).

2.8. Statistical analysis of data

All data were analyzed with GraphPad Prism 10. Statistical outliers were determined by Grubbs' test (alpha 0.05) and were removed from the dataset. Normal distribution was tested by Kolmogorov-Smirnov test for datasets with $>$ 50 samples or by Shaprio-Wilk test for datasets with $\leq$ 50 samples. Significance was calculated for normally distributed data with equal variance by using ordinary one-way ANOVA followed by Sídák´s multiple comparison test for four groups' comparison (Vehicle vs BPA, Vehicle vs BP-3, Vehicle vs BPA+BP-3, BPA vs BPA+BP-3 and BP-3 vs BPA+BP-3; Vehicle+hCG vs BPA+hCG, Vehicle+hCG vs BP-3 +hCG, Vehicle+hCG vs BPA+BP-3 +hCG, BPA+hCG vs BPA+BP-3 +hCG and BP-3 +hCG vs BPA+BP-3 +hCG) and unpaired t test for two groups' comparison (Vehicle vs Vehicle+hCG, BPA vs BPA+hCG, BP-3 vs BP-3 +hCG and BPA+BP-3 vs BPA+BP-3 +hCG). Significance of non-normally distributed data or without equal variance was tested by Kruskal-Wallis test followed by Dunn's multiple comparison test or Fisher's exact test for categorical (discrete) variables for four groups' comparison (Vehicle vs BPA, Vehicle vs BP-3, Vehicle vs BPA+BP-3, BPA vs BPA+BP-3 and BP-3 vs BPA+BP-3; Vehicle+hCG vs BPA+hCG, Vehicle+hCG vs BP-3 +hCG, Vehicle+hCG vs BPA+BP-3 +hCG, BPA+hCG vs BPA+BP-3 +hCG and BP-3 +hCG vs BPA+BP-3 +hCG) and Mann-Whitney test for two groups' comparison (Vehicle vs Vehicle+hCG, BPA vs BPA+hCG, BP-3 vs BP-3 +hCG and BPA+BP-3 vs BPA+BP-3 +hCG). A P-value below 0.05 was considered statistically significant. Graphical abstract and schematics were created with BioRender.com online tool.

Table 2
Primers used in this study for RT-PCR of ovarian tissue.

Gene	Forward Primer	Reverse Primer
Actb	GACGGCCAGGTCATCACTAT	CTTCTGCATCCTGTCAGCAA
Ahr	CAATGCACGCTTGATTTACAG	TCGTCCTTCTTCATCCGTC
Ar	AATGAGTACCGCATGCACAA	ATTTTTCAGCCCATCCACTG
Cyp11a1	CTGGGCACTTTGGAGTCAGT	CGATTCGGTCTTTCTTCCAG
Cyp17a1	ACCAGCCAGATCGGTTTATG	AGGGCAGCTGTTTGTCATCT
Cyp19a1	ATGAACGATCCGTCAAGGAC	ACAGAGTGACCGACATGGTG
Eomes	GGCAAAGCGGACAATAACAT	AGCCTCGGTTGGTATTTGTG
Esr1	TTACGAAGTGGGCATGATGA	CCTGAAGCACCCATTTCATT
Esr2	CTACAGTGTTCCCAGCAGCA	AGTAACAGGGCTGGCACAAC
Fshr	GTGCATTCAACGGAACCCAG	GAAGTTCAGAGGTTTGCCGC
Gusb	CACGGCGATGGACCCAAGAT	CCCATTCACCCACACAACTGC
Hsd3b2	AGGAGATCAGGGTCCTGGAC	CTGGCACACTGGCTTGGATA
Klrb1c	GACACAGCAAGTATCTACCTCGG	TCAGAGCCAACCTGTGTGAACG
Klrk1	CCTATCACTGGATGGGACTGGT	GCTTGAGCCATAGACAGCACAG
Lhcgr	GGGACGACGCTAATCTCG	CCTGGAAGGTGCCACTGT
Pgr	AGGTCTACCCGCCATACCTT	GTTATGCTGCCCTTCCATTG

3. Results

3.1. Perinatal BPA and BP-3 exposure affected pregnancy outcome and female offspring weight

Following perinatal exposure of dams to BPA and BP-3, pregnancy characteristics and female offspring weight development were analyzed. Pregnancy length, gestational weight gain (GWG), the number of alive offspring per litter and the sex ratio were not significantly different in vehicle, BPA, BP-3 and BPA+BP-3 exposed animals (Table 3). Median weight of female offspring on P1 was not significantly different between BPA, BP-3 and BPA+BP-3 exposed and vehicle animals, however BPA exposed female offspring showed the lowest median and highest range in P1 weight and BPA+BP-3 exposed offspring had the highest median weight on P1. We found that perinatal exposure to BPA+BP-3 significantly impacted on gestational weight development compared to vehicle, BPA, and BP-3 exposure (Table 3). The percentage of large-for-

Table 3
Phenotype of pregnant dams, female offspring weight on P1 and gestational growth following perinatal BPA and BP-3 exposure.

Variable§	Vehicle	BPA	BP-3	BPA+BP-3
Animals (*n*)	11 dams 57 ♀ offspring	11 dams 60 ♀ offspring	14 dams 66 ♀ offspring	13 dams 55 ♀ offspring
Median gestation length (d), [Min - Max]	18.5 [18–19]	18.5 [17.5–19]	18.5 [18–19]	19 [18–19]
Median GWG (g), [Min - Max]	18.0 [16.1–19.8]	18.0 [16.6–19.3]	18.4 [15.1–20.8]	17.5 [15.1–20.7]
Median alive born offspring, [Min - Max]	8 [7–10]	9 [7–10]	8 [7–10]	9 [6–10]
Sex ratio (% ♀ offspring), [Min - Max]	62.5 [44.4–77.8]	66.67 [33.3–88.9]	52. 8 [28.6–87.5]	62.5 [11.1–71.4]
Median ♀ offspring weight on P1 (mg), [Min - Max]	1504 [1298–1716]	1487 [1154–1940]	1496 [1167–1768]	1522 [1325–1888]
Kruskal-Wallis test	NS	NS	NS	NS
AGA [%]	76.9	78	70.8	61.8
LGA [%]	11.5	15.3	18.5	36.4
SGA [%]	11.5	6.8	10.8	1.8
FGR [%]	5.8	5.1	10.8	1.8
Fisher's exact test	NS	NS	NS	$^{****}P < 0.0001$ vs vehicle, BPA, and BP−3

§ Median gestation length in days, Median GWG in gram, Median female offspring weight on P1 in milligram; Min = minimum, Max = maximum, GWG = gestational weight gain, AGA = appropriate for gestational age (between 10th and 90th weight percentile), LGA = large for gestational age (>90th weight percentile), SGA = small for gestational age (<10th weight percentile), FGR = fetal growth restriction (<5th weight percentile). Fisher's exact test for discrete variables (AGA, LGA, SGA and FGR) of Vehicle vs BPA, Vehicle vs BP-3, Vehicle vs BPA+BP-3, BPA vs BPA+BP-3, and BP-3 vs BPA+BP-3. Differences were considered statistically significant for $^{*}P < 0.05$ or NS = non-significant.

gestational-age (LGA, weight above the 90th percentile of controls) was more than 3-fold higher in BPA+BP-3 exposed female offspring compared to vehicle. Moreover, merely 1.8 % of female offspring were small-for-gestational-age (SGA, below the 10th percentile following BPA+BP-3 compared to vehicle exposure. However, the percentage of fetal growth restriction (FGR, weight below the 5th percentile of controls) was approximately 2-fold higher in BP-3 exposed offspring and a higher than expected ratio of LGA in BPA exposed offspring compared to vehicle, however, differences between single exposure to BPA or BP-3 were not statistically significant compared to vehicle.

The weight of female offspring was followed up during the lactation period and was significantly higher on P7 in BPA+BP-3 exposed offspring compared to vehicle, BPA and BP-3 exposed offspring. On P14, P21 and on day of dissection, female offspring bodyweight was not significantly different among exposed and vehicle groups, however BPA+BP-3 exposed offspring still had the highest median of all groups until dissection (Fig. 1). These data indicate that perinatal BP-3 and BPA+BP-3 exposure can affect intrauterine growth and birth weight of female offspring, and in case of BPA+BP-3 exposure may lead to an increase in bodyweight during the lactation period.

3.2. Perinatal BPA and BP-3 exposure affects folliculogenesis

To initiate folliculogenesis and ovulation, animals were treated with PMSG (folliculogenesis) or PMSG+hCG (superovulation). Animals exposed to vehicle or EDC and treated with PMSG are referred to as "vehicle, BPA, BP-3 or BPA+BP-3" while animals exposed to vehicle or EDC and treated with PMSG+hCG are referred to as "vehicle+hCG, BPA+hCG, BP-3 +hCG or BPA+BP-3 +hCG". The follicle development stages were categorized into primordial, primary, secondary, preantral and antral follicles, and atretic follicles were also noted (Fig. 2).

Follicle count, diameter of differently matured follicles, CL count and CL diameter were not significantly altered following perinatal BPA, BP-3 or BPA+BP-3 exposure compared to controls (Fig. 2A – D). However, the CL appeared slightly larger in hCG-treated animals in all exposure groups without reaching statistical significance. We observed a small increase in follicle count in BPA+hCG treated females compared to BPA, and in CL count in BPA+BP-3 +hCG treated females compared to BPA+hCG. We found a small decrease in the percentages of primary follicles in the BPA, BP-3 and BPA+BP-3 females compared to vehicle, which was not statistically significant (Fig. 2E). However, we discovered significantly fewer secondary follicles in the BP-3 +hCG and BPA+BP-3 +hCG groups compared to the vehicle+hCG group (Fig. 2F). These results may indicate a delayed development from early to advanced stages of folliculogenesis. Besides healthy follicles, atretic follicles were analyzed and we found an increase of atresia in animals treated with PMSG+hCG in BP-3 ($P < 0.05$) and BPA+BP-3 ($P = 0.0502$) exposed female offspring compared to the vehicle group (Fig. 2G). To see whether hCG has an impact on follicular atresia (Fig. 2H, viii), a comparison between PMSG and PMSG+hCG treated offspring was also performed. We found a slight increase of atresia in all hCG treated groups, which reached significance only in BP-3 exposed offspring. In

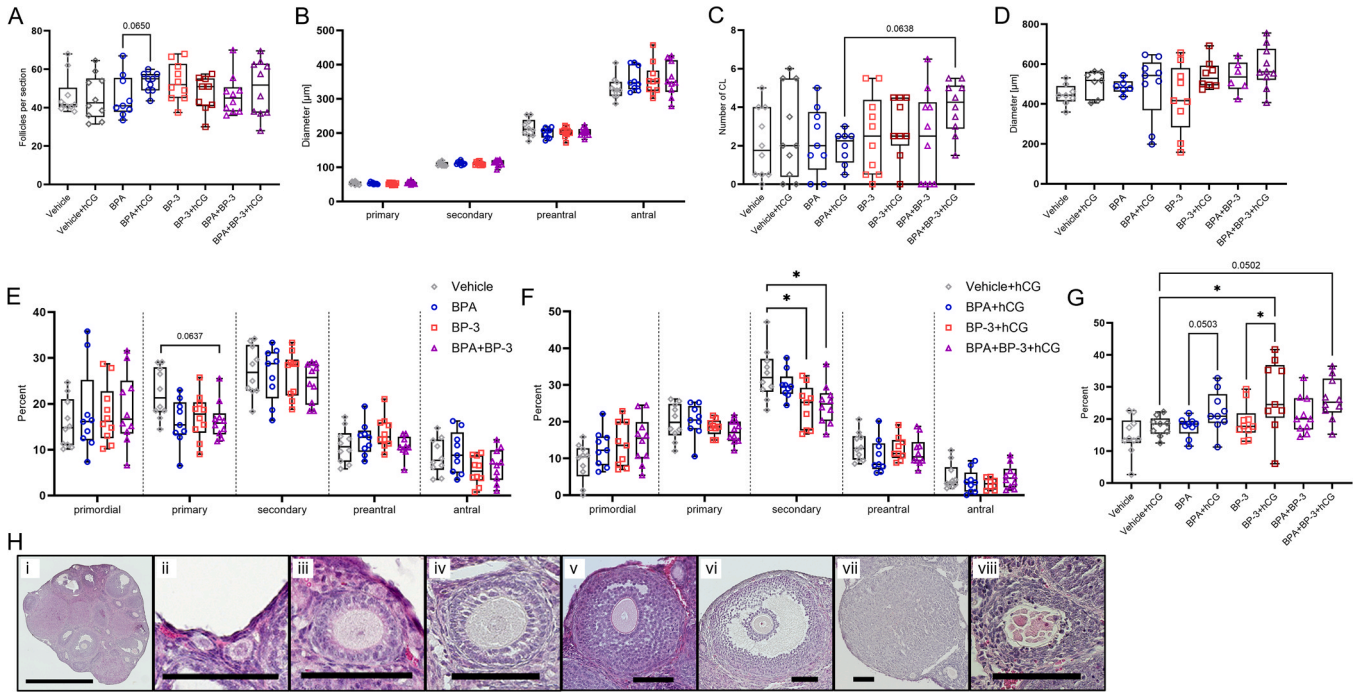

Fig. 2. Folliculogenesis and CL development following perinatal BPA and BP-3 exposure. (A) Box plots showing follicle count per histological section from EDC exposed offspring treated with PMSG and PMSG+hCG. (B) Box plots showing the diameter of follicles in different stages of development from offspring treated with PMSG. (C) Box plots showing the number of CL per histological section from offspring treated with PMSG and PMSG+hCG. (D) Box plots showing the maximum diameters of CL from offspring treated with PMSG and PMSG+hCG. (E) Box plots showing the percent follicles in primordial, primary, secondary, preantral and antral stage from offspring treated with PMSG. (F) Box plots showing the percent follicles in primordial, primary, secondary, preantral and antral stage from offspring treated with PMSG+hCG. (G) Box plots showing the percent atretic follicles from offspring treated with PMSG and PMSG+hCG. (H) Representative images of follicles in different developmental stages from EDC exposed offspring treated with PMSG. (i) A whole ovary dissected from an offspring exposed to BP-3. (ii) Two primordial follicles with flat, single-layered epithelium after BPA+BP-3 exposure. (iii) A primary follicle with cubic granulosa cells from a BPA exposed female. (iv) A two cell-layered secondary follicle following BP-3 exposure. (v) A preantral follicle with developed follicular cavity after vehicle exposure. (vi) Antral follicle showing a cumulus oophorus after exposure to BP-3. (vii) CL with luteinized cells after BPA exposure. (viii) Atretic follicle with characteristic features, i. e. fragmented oocyte remnants, wrinkling of the basement membrane, loss of granulosa cells, following BPA+BP-3 exposure. Magnification (i) 4x, scale bar = 1 mm, (ii)-(viii) 20x, scale bar = 100 µm. Box plots show one data point per ovary from n = 10 (Vehicle), 9 (BPA), 10 (BP-3), 10 (BPA+BP-3), 10 (Vehicle+hCG), 9 (BPA+hCG), 9 (BP-3 +hCG) and 10 (BPA+BP-3 +hCG) animals, median with min-to-max bars after removal of statistical outliers. Differences were considered statistically significant for *$P < 0.05$.

summary, these results suggest that perinatal exposure to BPA and BP-3 could influence folliculogenesis and follicular atresia while CL development was not significantly affected by EDC exposure and hCG treatment.

3.3. Perinatal BPA and BP-3 exposure affects ovarian responsiveness to exogenous gonadotropins

Following the observations of folliculogenesis and CL development, we aimed to determine the effect of perinatal BPA and BP-3 exposure on ovulation and the responsiveness of the ovaries to exogenous gonadotropins PMSG and hCG. We found that following superovulation treatment (PMSG+hCG) and dissection 13 h after hCG injection, the absolute and relative number of COC was altered depending on perinatal BPA and BP-3 exposure (Table 4). The number of ovaries without COC was significantly higher in BPA+hCG (27.59 %) and in BPA+BP-3 +hCG (29.17 %) exposed offspring compared to vehicle (Table 4). However, female offspring exposed to BP-3 +hCG presented no ovaries without COC (0 %), which was not observed in vehicle exposed offspring that showed several ovaries without COC (15.38 %). The mean count of oocytes and mean weight of both ovaries after dissection were not significantly different between vehicle+hCG and BPA+hCG, BP-3 +hCG or BPA+BP-3 +hCG exposed offspring (Figure S2). Taken together, these results suggest that perinatal exposure to BPA and BPA+BP-3 may negatively affect ovarian responsiveness while exposure to BP-3 may enhance the formation of COC following PMSG and hCG treatment.

3.4. Perinatal BPA+BP-3 exposure causes increased ovarian NK cell number

Since various immune cells play important roles in ovarian function, such as folliculogenesis and CL development, and BPA and BP-3 may affect immune cell recruitment to peripheral organs, the number of innate and adaptive immune cells in the ovaries of perinatally exposed offspring was analyzed. Following our gating strategy (Supplementary Figure S1), we found that the relative number of NK cells ($CD45^+CD3^-CD19^-NK1.1^+$) was significantly higher in ovaries of BPA+BP-3 exposed offspring compared to vehicle (Fig. 3D). However, the relative number of alive total immune cells ($CD45^+$), T cells ($CD45^+CD3^+$), B cells ($CD45^+CD19^+$), dendritic cells ($CD45^+CD3^-CD19^-CD11c^+$), total macrophages ($CD45^+CD3^-CD19^-F4/80^+$), pro-inflammatory ($CD45^+CD3^-CD19^-F4/80^+iNOS^+$) and anti-inflammatory ($CD45^+CD3^-CD19^-F4/80^+CD206^+$) macrophages (Fig. 3A-C, E-H) in the ovaries were not significantly different between vehicle, BPA, BP-3 and BPA+BP-3 exposed offspring following PMSG treatment. Interestingly, the lowest frequency of B cells was observed in BP-3 exposed offspring. These results show that perinatal BPA+BP-3 exposure leads to an increased NK cell number in the ovaries during PMSG-induced folliculogenesis.

3.5. Differential gene expression in ovaries following perinatal BPA and BP-3 exposure and superovulation by hCG

To elucidate the effects of perinatal BPA and BP-3 exposure and superovulation by hCG on markers of ovarian hormone biosynthesis, hormone receptor signaling, as well as extracellular matrix remodeling, gene expression was analyzed using RT-PCR. Superovulation by hCG led to a marked decrease in Fshr and Pgr mRNA expression in the ovaries of vehicle+hCG, BPA+hCG, BP-3 +hCG and BPA+BP-3 +hCG exposed offspring compared to controls, i. e. only PMSG-treated offspring of the same exposure or vehicle group (Fig. 4A and E). Lhcgr and Hsd3b gene expression were not significantly altered by perinatal BPA and BP-3 exposure or superovulation by hCG compared to controls (Fig. 4B and G). Downregulation of Esr1 was significant in BPA+BP-3 +hCG exposed offspring, and downregulation of Esr2 was significant in BP-3 +hCG and BPA+BP-3 +hCG exposed offspring compared to the controls (Fig. 4C and D). Ar was significantly downregulated only in BP-3 +hCG exposed offspring compared to controls (Fig. 4F). Moreover, Cyp11a1 mRNA was significantly upregulated in BP-3 +hCG and BPA+BP-3 +hCG exposed offspring, whereas Cyp17a1 and Cyp19a1 were significantly downregulated in vehicle+hCG, BPA+hCG, BP-3 +hCG and BPA+BP-3 +hCG exposed offspring compared to controls (Fig. 4H, I and J). Klrb1c was upregulated in vehicle+hCG, BP-3 +hCG and BPA+BP-3 +hCG, but not BPA+hCG exposed offspring compared to controls (Fig. 4K). Conversely, Klrk1 mRNA expression was not affected by perinatal BPA and BP-3 exposure or superovulation by hCG compared to controls (Fig. 4L). Interestingly, Mmp2 and Timp1 were significantly upregulated in vehicle+hCG, BPA+hCG, BP-3 +hCG and BPA+BP-3 +hCG exposed offspring compared to controls (Fig. 4M and O). The lowest expression of Mmp9 mRNA was observed in BPA+BP-3 +hCG exposed offspring (Fig. 4N). The mRNA expression of Mmp9 and Timp2 was not significantly different between vehicle, BPA and BP-3 exposed offspring, either with or without hCG treatment (Fig. 4N and P). Additional markers of immune cell differentiation and extra cellular matrix remodeling showed no differential gene expression in EDC exposed or control animals (Figure S3). Taken together, our data show that Esr1, Esr2, Ar and Cyp11a1 mRNA expression are markedly affected by BP-3 +hCG and BPA+BP-3 +hCG exposure leading to differential expression which was not observed in vehicle+hCG and BPA+hCG exposed offspring. Moreover, serum estrogen levels measured by ELISA were not significantly different between EDC exposed, PMSG treated offspring and controls (Figure S4) indicating no systemic hormone level differences in those animals exposed to BPA and BP-3 compared to unexposed controls.

4. Discussion

The current study was performed to decipher the effects of single and combined perinatal exposure to BPA and BP-3 at environmentally relevant concentrations on female offspring gonadal development and function. Mice were exposed to a relevant dose of BPA at 4 μg/kg/d, i.e. the TDI of EFSA as of 2015. The TDI was lowered by EFSA at the end of this study in 2023 by a factor of 20,000 to 0.2 ng/kg/d (EFSA Panel on Food Contact Materials et al., 2023). However, due to the high prevalence of BPA in the environment and its use in consumer products from outside the EU, immediate changes in BPA exposure to the level of the new TDI will likely not occur (Karrer et al., 2020). Exposure to BP-3 at a dose of 50 mg/kg/d as used in this study was recently shown to be a valid exposure scenario mimicking daily sunscreen product usage

Table 4
Ovarian responsiveness to stimulation with exogenous gonadotropins PMSG and hCG determined by the number of ovaries without cumulus-oocyte-complex (COC).

Treatment	Animals (*n*)	Number of ovaries	Number of ovaries without COC	Percent of ovaries without COC	P-value§
Vehicle+hCG	13	26	4	15.38 %	
BPA+hCG	15	29	8	27.59 %	*P < 0.05 vs Vehicle
BP−3 +hCG	12	24	0	0.00 %	****P < 0.0001 vs Vehicle, vs BPA+BP−3
BPA+BP−3 +hCG	12	24	7	29.17 %	*P < 0.05 vs Vehicle

§ Fisher's exact test of Vehicle vs BPA, Vehicle vs BP-3, Vehicle vs BPA+BP-3, BPA vs BPA+BP-3, and BP-3 vs BPA+BP-3. Differences were considered statistically significant for *P < 0.05.

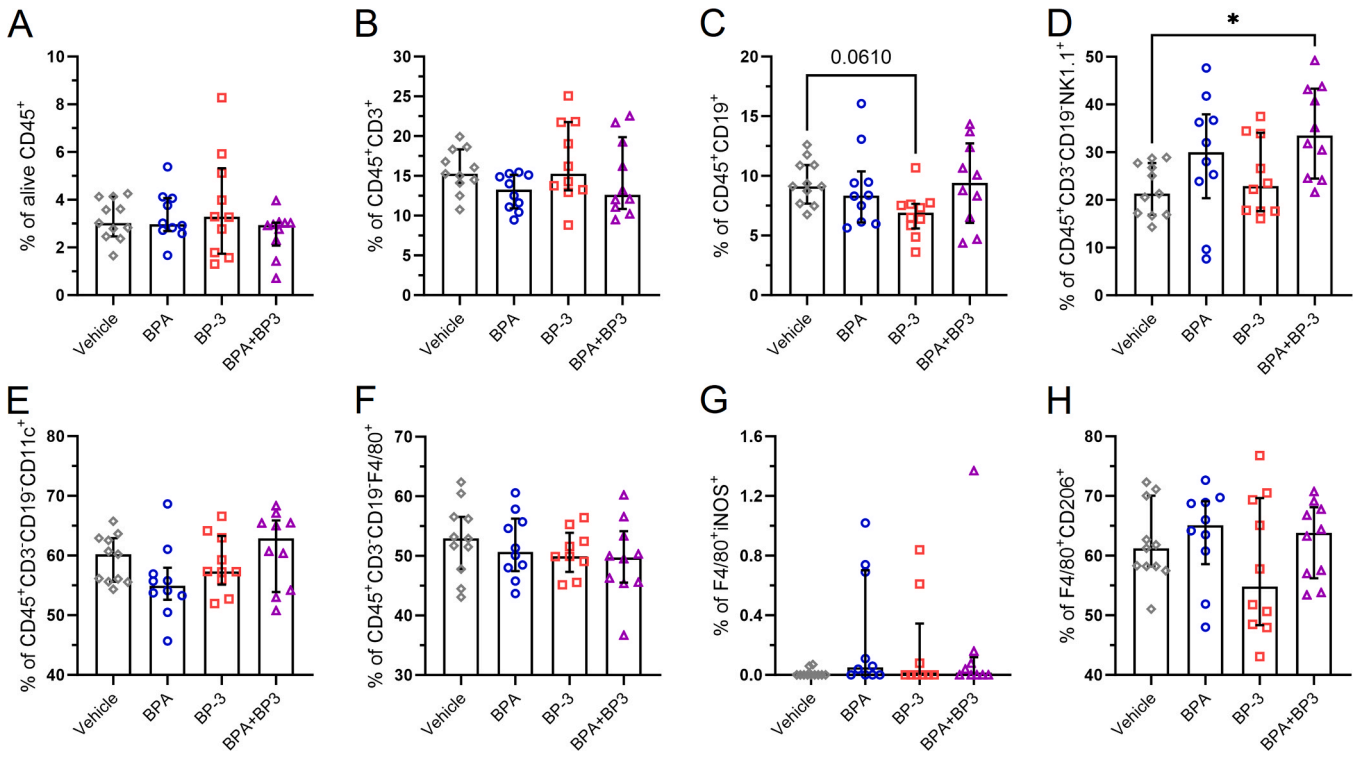

Fig. 3. Flow cytometry of ovarian tissue following perinatal BPA and BP-3 exposure and 48 h after PMSG treatment. (A) The relative number of alive $CD45^+$ immune cells, (B) T cells ($CD45^+CD3^+$), (C) B cells ($CD45^+CD19^+$), (D) NK cells ($CD45^+CD3^-CD19^-NK1.1^+$), (E) dendritic cells ($CD45^+CD3^-CD19^-CD11c^+$), (F) macrophages ($CD45^+CD3^-CD19^-F4/80^+$), (G) pro-inflammatory macrophages ($CD45^+CD3^-CD19^-F4/80^+iNOS^+$) and (H) anti-inflammatory macrophages ($CD45^+CD3^-CD19^-F4/80^+CD206^+$) are shown. Histograms show median with interquartile range with each data point resembling relative cell number from both ovaries from one animal ($n = 11$ (vehicle), 10 (BPA), 10 (BP-3), 10 (BPA+BP-3)). Differences were considered statistically significant for $^*P < 0.05$.

(Safety, 2021). The concentration of BP-3 in sunscreen products is limited in the EU, but the applied dose may vary significantly among individuals without a TDI for BP-3 (Safety, 2021). Through the application of BPA and BP-3 from the first day of pregnancy until weaning, the offspring were perinatally exposed to both EDCs during sensitive periods of pre- and postnatal development (Santamaria et al., 2020). This scenario represents human exposure to EDCs and is a blueprint for combined exposure through different routes. On the one hand, prenatal exposure by materno-fetal transfer of active substances during pregnancy can affect fetal primordial germ cells and gonadal development and therefore may also affect reproductive function later in life (Gore et al., 2015; Vanderhyden, 2002). On the other hand, postnatal exposure by offspring oral uptake of mother's milk during lactation as sub-chronic exposure may further impact on the ovaries, e.g. by increasing follicular atresia and altering follicle growth (Gore et al., 2015; Chen et al., 2016). In perinatal exposure settings, low dose exposure may cause direct adverse effects on cells as well as developmental programming in the ovaries through epigenetic mechanisms elicited by BPA and BP-3, possibly affecting gonadal development, immune cell profiles and other endocrine tissues with long-term consequences for fertility (Robles-Matos et al., 2021).

Interestingly, we found an altered female offspring weight on the day after birth which indicates an adverse pregnancy outcome. On P1, the morning after birth, differences in female newborn weight were observed depending on exposure to EDCs and mixtures showing a 3-fold increase in LGA offspring following BPA+BP-3 exposure compared to controls. Interestingly, using the same EDC exposure model, our previous study showed that in utero exposure to BPA+BP-3 led to an increased prevalence of LGA fetuses on gd12 (Santamaria et al., 2020; Fischer et al., 2024). Thus, the LGA phenotype in BPA+BP-3 exposed offspring may be manifested already at the middle-to-late phase of gestation. The mechanism behind the observed LGA phenotypes could involve the endocrine placenta which can regulate fetal growth by the release of nutrients, hormones or changes in materno-fetal transfer capacity during gestation (Appel et al., 2019; Kretschmer et al., 2020). Alterations in fetal development are known risk factors for disease onset later in life according to the developmental origin of health and disease theory (Barker, 2007). Few studies also suggest that impaired fetal growth can impact on gonadal function and puberty onset in children (van Weissenbruch and Delemarre-van de Waal, 2006; Ibáñez and de Zegher, 2006). Evidence from human and animal studies suggests that overweight and obesity of mothers during gestation and of their offspring lead to reduced fertility and ovarian dysfunction in these offspring (Broughton and Moley, 2017; Snider and Wood, 2019). Similarly, EDCs may play an important role in developmental programming of endocrine and neuroendocrine systems possibly affecting weight gain, gonadal function and fertility in exposed offspring as well (Gore et al., 2015; Sánchez-Garrido et al., 2022).

In this study, we aimed to determine the effects of perinatal EDC exposure on folliculogenesis following PMSG and hCG-induced ovulation. Like LH, hCG completes follicular maturation and triggers ovulation, thereby preventing follicular degeneration. For this reason, it is used during in vitro fertilization (IVF) for final oocyte maturation. We found no decrease in the number of primordial follicles in EDC exposed offspring indicating that the follicle pool, which develops in utero, was not affected. We were surprised to find a rather low number of primordial and primary follicles in the histological analysis. However, previous reports on follicle maturation using PMSG and hCG treatment suggest that in single sections at 12 h post hCG injection, this observation is plausible and may be explained by the maturation of the ovary (Mantri et al., 2024). Since we used 2 sections at a distance of 500 μm to analyze follicle maturation, the absolute number of follicles may be different if more sections or a stereological approach was used. However, we found a significantly lower number of secondary follicles

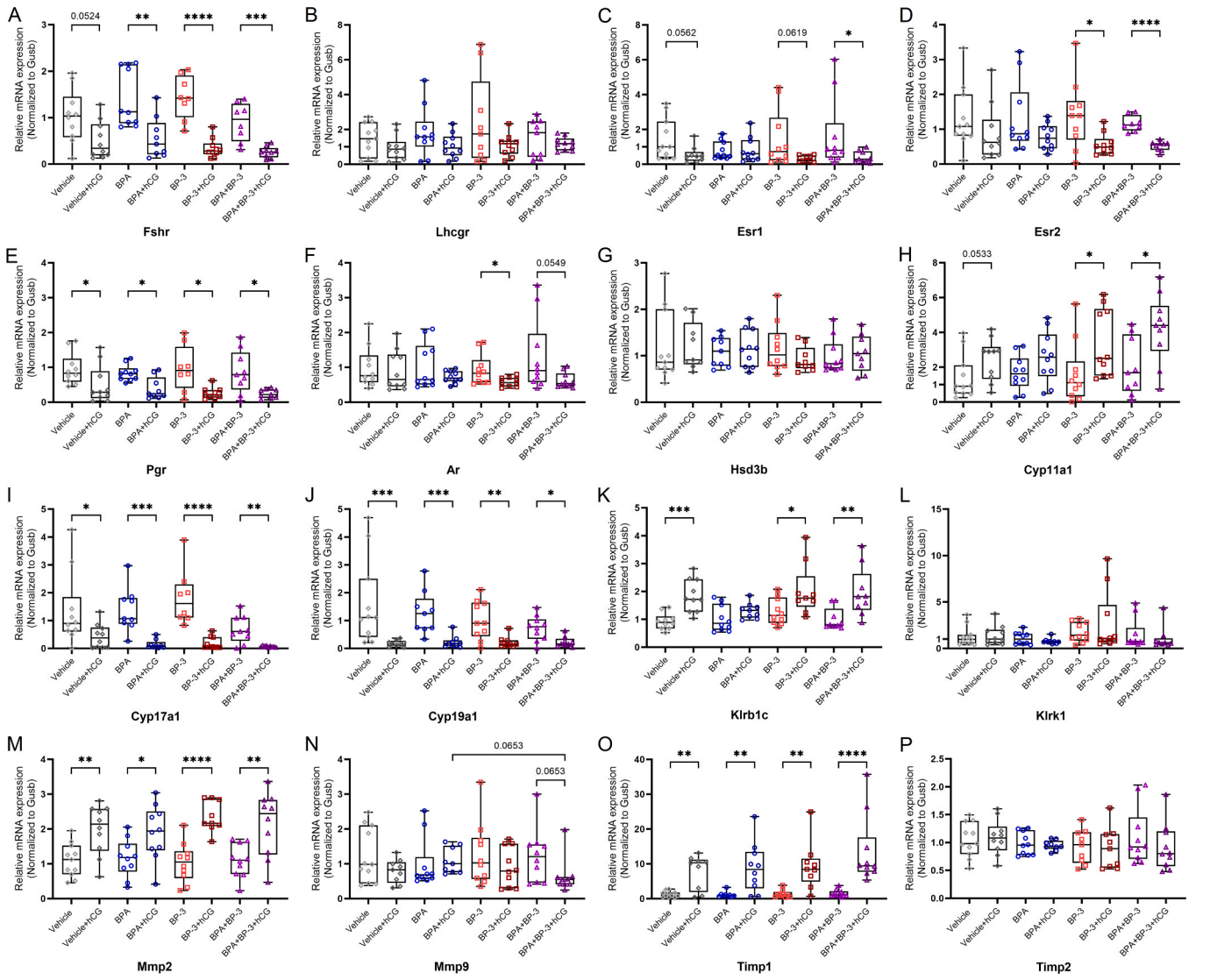

Fig. 4. Quantification of mRNA expression by RT-PCR of ovarian marker genes following perinatal BPA and BP-3 exposure and superovulation by hCG. Relative quantification of mRNA expression evaluated by RT-PCR of hormone receptor genes (A) Fshr, (B) Lhcgr, (C) Esr1, (D) Esr2, (E) Pgr and (F) Ar, (G) the hormone biosynthesis enzymes Hsd3b, (H) Cyp11a1, (I) Cyp17a1 and (J) Cyp19a1, (K) the NK cell genes Klrb1c and (L) Klrk1, and extracellular matrix remodeling genes (M) Mmp2, (N) Mmp9, (O) Timp1 and (P) Timp2 normalized to Gusb (housekeeping gene) and the vehicle control. Ovaries from female offspring: $n = 11$ (vehicle/vehicle+hCG), 10 (BPA/BPA+hCG), 10 (BP-3/BP-3 +hCG), 10 (BPA+BP-3/BPA+BP-3 +hCG), one ovary per offspring, before removal of statistical outliers. Box plots show one data point per ovary, median with min-to-max bars after removal of statistical outliers. Differences were considered statistically significant for *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

following hCG treatment in BP-3 and BPA+BP-3 exposed offspring indicating that perinatal EDC exposure affects follicle maturation. Ovaries with COC in BP-3 +hCG exposed offspring were accompanied by increased atresia in these and BPA+BP-3 +hCG offspring. In addition, atresia was increased in all EDC+hCG offspring compared to EDC+PMSG offspring, but only significantly increased in BP-3 +hCG compared to BP-3 +PMSG exposed offspring, indicating EDC-mediated interference with ovulation and ovarian responsiveness to exogeneous gonadotropins. Atresia is a physiological process that the majority of all follicles undergo to allow the development of a dominant follicle and previous studies have shown that treatment with 5 IU of PMSG and hCG does not significantly increase follicular atresia in mice (Whang et al., 2021; Özbilgin et al., 2022). However, Özbilgin et al. have shown that a high dose gonadotropin treatment with 20 IU leads to a significant increase in atresia and this may impact the outcome of IVF (Özbilgin et al., 2022). In our study, treatment with 5 IU PMSG+hCG showed an increase in follicular atresia in EDC exposed offspring, and since EDCs can interfere with the endocrine system, it is likely that they play a role in the increase of follicular atresia and depletion of the follicular reserve (Land et al., 2022). Therefore, low dose hCG together with EDC exposure may lead to similar effects on atresia as a high dose gonadotropin treatment. As hCG is used in IVF, we suspect that prolonged environmental EDC exposure of women can have a negative impact on the success of IVF treatment. The precise mechanism of EDC-mediated follicular atresia, e. g. apoptosis, necrosis or autophagy, need further investigation. It is conceivable that determining individual EDC exposure of patients undergoing IVF could lead to an improvement in therapy, but this needs to be further investigated in a human cohort.

Regarding CL development, we could not observe significant changes in EDC exposed offspring following PMSG or hCG treatment. However, the largest median CL diameter and median CL count per ovary were observed in BPA+BP-3 +hCG exposed offspring. Previous studies with perinatal BPA exposure at doses of 0.5 and 50 μg/kg yielded complementary results showing reduced primary follicle count and increased CL number per ovary for both doses (Santamaría et al., 2016). Moreover, in vitro studies have shown that BP-3 treatment affected folliculogenesis

in rat whole ovarian culture in a concentration-dependent manner (Santamaría et al., 2019b). In contrast, in vivo studies with EDC mixture exposure containing low dose BPA during the perinatal period showed significant changes in folliculogenesis only in F2 and F3 female offspring (López-Rodríguez et al., 2021). Concerning ovarian function and responsiveness to stimulation with hormones, effects seemed to differ depending on perinatal EDC exposure and BPA+BP-3 appeared to have stronger effects than single EDC exposure. Nonetheless, differences in dosing, experimental design, choice of animal model and other aspects complicate the agreement between in vivo studies and demand further research.

Ovarian immune cells fulfill pivotal functions in folliculogenesis and CL development and thus are important for the fertility cycle of females (Land et al., 2022). We speculated that EDC exposure would affect different immune cell populations that play an important role in the ovaries during folliculogenesis, and we found an increase in NK cell number in the ovaries of BPA+BP-3 exposed offspring next to an altered gene expression of NK cell associated gene Klrbc1 of BPA exposed offspring. NK cells increase during the ovarian cycle and become the most prominent cell type at the end of the luteal phase and in early pregnancy in the endometrium (Richman and Naftolin, 2006). NK cells in the ovarian fluid can secrete cytokines and play a role in follicle development, and some reports suggest adverse effects on ovarian function with increased “cytotoxic” NK cell counts (Yang et al., 2019). NK cells also possess Esr1 and Esr2, and respond to hormones of the estrogen family (Curran et al., 2001), which may cause endocrine disruption by EDC exposure and alterations in NK cell activity. Thus, the increase in NK cells in the ovaries observed in this study in BPA+BP-3 exposed offspring may be related to an unfavorable ovarian phenotype that attracts NK cells and may alter NK cell activity during folliculogenesis. The precise role of ovarian NK cell infiltration and activity of NK cells in the context of folliculogenesis and ovulation in this model of perinatal EDC exposure demand further mechanistic studies.

Ovarian hormone biosynthesis, hormone receptor signaling, and extracellular matrix remodeling are important for ovarian function during the ovarian cycle (Vanderhyden, 2002; Goldman, 2004). Gene expression of markers of hormone biosynthesis and receptor signaling, and extracellular matrix remodeling were affected in most offspring following perinatal BPA and BP-3 exposure in this study. Differential gene expression of Fhsr, Esr1, Esr2 and Ar predominantly in BP-3 and BPA+BP-3 exposed offspring following hCG treatment indicates that ovarian hormone regulation is most strongly affected by perinatal BP-3 and BPA+BP-3 mixture exposure. However, it must be noticed that regulation of ovarian function and development is also under the direct influence of central regulators such as the HPG axis and involves Esr1 and Esr2 in both central and peripheral tissues (Hamilton et al., 2017). Our study is limited by the evaluation of changes following EDC exposure in the ovaries of perinatally exposed offspring, and central effects of EDCs on hormone regulation via Esr1 and Esr2 may balance or exaggerate ovarian function. In the periphery, Esr2 plays a role in primordial follicle reserve and lack of Esr2 may affect granulosa cells, follicle maturation and oocytes (Hamilton et al., 2017; Chakravarthi et al., 2020) indicating a role in folliculogenesis. Other studies also suggest that both Esr1 and Esr2 play pivotal roles in the ovaries for hormonal signaling, ovulation and age-associated changes in ovarian reserve and function (Hamilton et al., 2017). In addition, Cyp11a1 was upregulated significantly in BP-3 +hCG and BPA+BP-3 +hCG exposed offspring compared to PMSG treated controls, but not in BPA+hCG compared to BPA+PMSG exposed offspring. Cyp11a1 is responsible for the conversion of cholesterol to pregnenolone (Guo et al., 2007) and its expression rises in theca and granulosa cells with ovulatory LH surge and shows continued expression during pregnancy (King, 2012). These results give reason to speculate that hCG treatment increases Cyp11a1 gene expression to supraphysiological levels only in ovaries of BP-3 and BPA+BP-3 exposed offspring. At older age, mice may have altered hormone levels, lower ovarian reserves and higher risk of ovarian dysfunction due to altered genetic regulation observed at pubertal age. However, additional studies are required to investigate such possible effects and the consequences of perinatal EDC exposure for hormone physiology and ovarian function at advanced reproductive age also in humans. In this study, serum estrogen levels were not significantly different between EDC exposed and unexposed females following PMSG treatment.

Our study is the first to describe the in vivo effects of single and combined perinatal exposure to environmentally relevant doses of BPA and BP-3 on folliculogenesis, CL development and ovarian function suggesting adverse effects for the offspring. Furthermore, differences in newborn bodyweight, gene expression of ovarian markers and NK cell number following perinatal BPA+BP-3 exposure suggest long-term effects for endocrine and reproductive health of the offspring. More studies are needed to decipher the precise molecular mechanisms behind altered folliculogenesis, ovulation, NK cell infiltration and gonadal hormone regulation following perinatal EDC exposure. Several effects observed on the gene expression level in this study may be the consequence of epigenetic changes caused by perinatal EDC exposure leading to differential gene activity. Following studies will concentrate on understanding the consequences of developmental programming by EDCs for superovulation treatment at advanced reproductive age, especially in conjunction with other risk and lifestyle factors, which may be relevant for women undergoing fertility treatment and artificial reproductive techniques. In order to increase reproductive health and environmental safety, EDC monitoring and legislation may need additional refinement.

CRediT authorship contribution statement

Elisabeth Krieger: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Julia Howanski:** Supervision, Investigation, Formal analysis. **Florence Fischer:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Federica Romanelli:** Methodology, Investigation. **Marita Wagner:** Methodology, Investigation. **Mario Bauer:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Beate Fink:** Methodology, Investigation, Formal analysis. **Tobias Kretschmer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anne Schumacher:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. **Ana C. Zenclussen:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.118750.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

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