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Perinatal exposure to known endocrine disrupters alters ovarian development and systemic steroid hormone profile in rats

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ABSTRACT

Disrupted ovarian development induced by chemical exposure may impair fertility later in life. Since androgens are essential for early ovarian development, we speculated that perinatal exposure to a binary mixture of the known anti-androgens DEHP and procymidone could alter steroid synthesis, disrupt ovarian follicle recruitment and ultimately maturation in female rat offspring. Wistar rat dams were exposed by oral gavage from gestation day 7 to postnatatal day 22 to two mixture doses known to alter reproductive development in male offspring (low: 10 mg/kg bw/day of procymidone and 30 mg/kg bw/day of DEHP; high: 20 mg/kg bw/day of procymidone and 60 mg/kg bw/day of DEHP). The Effects on plasma steroid hormones, ovarian follicle distribution and expression of markers related to steroid synthesis were examined in female offspring. In prepubertal offspring, we observed an increased number of newly recruited (primary) follicles in exposed animals compared to controls, and the plasma steroid hormone profile was altered by exposure: levels of progesterone, corticosterone and estrone were dose dependently elevated, whereas androgen levels were unaffected. In adulthood, a trend towards a smaller number of early-stage follicles may point to accelerated loss of follicle reserves, which is disconcerting. The changes in follicle distribution in exposed ovaries may reflect the combined influence of androgen receptor antagonism and altered ovarian steroid synthesis. This study adds to a growing body of evidence showing altered ovarian development following exposure to human relevant chemicals with possible severe consequences for female fertility.

1. Introduction

Fertility problems in women have increased in recent decades. The causes are not well known, but exposure to endocrine disrupting chemicals (EDCs) is proposed to contribute to the disease burden (Buck Louis et al., 2011; Johansson et al., 2017). EDCs can disrupt the endocrine system and lead to a wide range of health problems that may contribute to reproductive disorders later in life (Schug et al., 2011; Isling et al., 2014; Gore et al., 2015), including altered ovarian follicle development and recruitment (Karavan and Pepling, 2012; Moyer and Hixon, 2012; Johansson et al., 2017). This is of great concern since girls are presumed to be born with a set number of oocytes, a reserve that is meant to last throughout their reproductive lifespan. Compromised ovarian development and establishment of the oocyte reserve may therefore impair fertility, possibly contributing to disease such as premature ovarian insufficiency (McGee and Hsueh, 2000).

In a previous study, we suggested that perinatal exposure to chemical mixtures can shorten the reproductive lifespan in female rat offspring by advancing reproductive senescence (Johansson et al., 2016). We saw indications of altered ovarian development as early as the pre-pubertal stages, with a reduced pool of primordial follicles. The effect was particularly pronounced in the group exposed to anti-androgenic chemicals, which suggests that impaired androgen signaling could be an underlying cause of these effects (Johansson et al., 2016). Androgens are essential for early ovarian development, as they induce the expression of follicle stimulating hormone receptor (FSHR), which supports follicle stimulating hormone (FSH)-driven follicular growth and maturation (Dewailly et al., 2016; Lebbe and Woodruff, 2013).

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Androgen receptor (AR) knockout mice (ARKO) develop an ovarian phenotype resembling premature ovarian insufficiency (Walters et al., 2007; Shiina et al., 2006). The same phenotype is seen in granulosa cell-specific AR knockout mice (GCARKO), manifesting as reduced growth rate among secondary follicles and a higher rate of atresia in the ovaries (Sen and Hammes, 2010; Walters et al., 2012; Cheng et al., 2013). GCARKO ovaries display a significant reduction in large secondary and small antral follicle numbers at three months of age. In general, these studies suggest that a loss of AR signaling in granulosa cells leads to premature decline in follicle populations. Little is known about the impact of impaired androgen signaling in immature females, however, which is problematic when assessing potential impacts of anti-androgens on female reproductive health.

Based on current knowledge, it is plausible that chemicals with a capacity to inhibit AR activation, or reduction in androgen synthesis, could alter follicle recruitment during ovarian development. In turn, this could have long-term consequences in females and lead to early reproductive senescence. To investigate this further, we designed a rat study using a mixture of two well-characterized anti-androgens, diethylhexyl phthalate (DEHP) and procymidone. These compounds have both been studied extensively for their anti-androgenic effects in males, but are less studied in females. DEHP, an abundantly used plasticizer, interferes with steroid synthesis in males, but can also affect ovarian development; DEHP alters follicle assembly and accelerates follicle recruitment both *in vitro* and *in vivo* (Zhang et al., 2015; Mu et al., 2015; Zhang et al., 2013; Hannon et al., 2015). Procymidone is a pesticide with AR antagonistic properties *in vitro* and displays clear adverse anti-androgenic effects in males (Ostby et al., 1999; Christiansen et al., 2008). Potential effects on female reproductive development are not well characterized.

Since we have already shown that a binary mixture of DEHP and procymidone has anti-androgenic effects in male rats – likely including reduced androgen synthesis (Christiansen et al., 2020) – we speculated that circulating androgen levels would be lower in female rat offspring, and that follicle recruitment and maturation would be disrupted.

2. Methods

2.1. Chemicals and mixtures

Procymidone (purity 99.9 %, CAS no. 32809-16-8, from Fluka no. 36640-250mg-R) was purchased from Sigma-Aldrich (Brøndby, Denmark). DEHP (purity 99 %, CAS no. 117-81-7, from Merck no. 8.2174.1000) was purchased from VWR- Bie & Berntsen (Herlev, Denmark). Corn oil purchased in glass bottles from Sigma-Aldrich (Brøndby, Denmark) was used as both control and vehicle. The mixture solutions used for dosing were stored in glass bottles at room temperature, protected from light, and continuously stirred during the dosing period.

2.2. Animals and exposure

Animal experiments were carried out at the National Food Institute, Technical University of Denmark (Mørkhøj, Denmark) facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate under the authorization number 2012-15-2934-00089 C4. The National Food Institutes in-house Animal Welfare Committee for animal care and use oversaw the experiments.

Ovary data presented herein are from a larger animal study described by Christiansen et al. (2020). Briefly, time-mated nulliparous, young adult Wistar rats (weight range 180−220 gram, HanTac:WH, SPF, Taconic Europe, Ejby, Denmark) arrived at our animal facilities at gestation day (GD) 3 of pregnancy. The day when a vaginal plug was detected was designated GD 1. Independently of the actual day of delivery, the expected day of delivery, GD 23, was designated pup day (PD) 1. Upon arrival, the females were randomly distributed in pairs and housed under standard conditions (Christiansen et al., 2020) with 12 h reverse light–dark cycles with light starting at 9 p.m. Polysulfone bottles and cages, as well as aspen wood shelters (instead of plastic), were used to reduce the risk of migration of plastic components that potentially could confound the study results.

Mated females (n = 18) were exposed by oral gavage each morning from GD 7 – PD 22 and the offspring were examined for effect outcomes. Female offspring from two dose groups (low: 10 mg/kg bw/day of procymidone and 30 mg/kg bw/day of DEHP; high: 20 mg/kg bw/day of procymidone and 60 mg/kg bw/day of DEHP) and the control group were included in this study (Fig. 1). As not all mated females were pregnant, n = 15–16 litters were available. Low mixture doses were selected as individual compound doses reducing male anogenital distance (AGD) by 5% in previous studies on single chemical exposures (Christiansen et al., 2010; Hass et al., 2012, 2007).

On PD 17, one female pup per litter (N = 13–15 per exposure group) was weighed and decapitated. The ovaries were excised, weighed and snap frozen in N_2. On PD 22, both ovaries from one female pup per litter were excised, trimmed, weighed, placed in formalin overnight and processed for paraffin embedding. Pups were weaned on PD22 and kept to adulthood (PD 90), N = 15–17, 1–2 females from each litter. At around PD 90, females were humanly killed on the day when a vaginal smear showed signs of estrous. Both ovaries were excised, trimmed, weighed, placed in formalin overnight, and processed for paraffin embedding. At all ages investigated, blood was collected in heparinized plastic tubes for plasma preparation and hormone analysis.

2.3. Follicle count

One whole ovary from each female (n = 12) was sectioned at 5 μm thickness. Every 20th section (PD 22) or every 40th section (PD 90) were stained with haematoxylin & eosin (H&E) and used for follicle count. Only follicles with a visibly defined oocyte were counted. The follicles were classified by five different stages according to the definitions (OECD, 2009 and Picut et al., 2015): primordial follicles = oocyte surrounded by one layer of squamous granulosa cells; primary follicles = oocyte surrounded by one layer of cuboidal granulosa cells; secondary follicles = oocyte surrounded by more than one layer of cuboidal granulosa cells and sometimes with multiple fluid-filled spaces within the zona granulosa; antral follicles = an oocyte surrounded by more than one layer of cuboidal granulosa cells and with a large central cavity in zona granulosa. Corpora Lutea (PD 90 only) were defined as large eosinophilic structures consisting of luteal cells. Some follicles were in a transition state between primordial and primary, thus having both squamous and cuboidal granulosa cells. In these cases, the distinction was made by the predominating granulosa type as described in Myers et al. (2004). An average of 11–14 sections per animal were evaluated, and for each of the five stages of follicle maturation the sum of the counts in all sections is presented in Fig. 2. For each follicle stage, this number is expected to be proportional to the total number of follicles in the ovary. Follicle counting was done by an examiner blinded with respect to treatment groups using a Leica DMR microscope (Leica microsystems A/S, Ballerup, Denmark).

2.4. Hormone analysis PD 17

Steroid hormone levels in plasma samples (n = 6, randomly selected) were analysed by LC–MS/MS as previously described (Draskau et al., 2019). Of the 15 hormones included in the assay, seven were detected: (pregnenolone, progesterone, corticosterone, androstenedione, testosterone, estrone and estradiol), while eight were not (epitestosterone, aldosterone, dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), cortisol, hydroxycortisol, deoxycortisol, 17α-OH-progesterone). Androstenedione, pregnenonlone and estradiol levels were below the level of quantification or undetectable in several samples.

Limit of quantification (LOQ) was estimated as the concentration corresponding to 10 times the signal to noise ratio of plasma samples

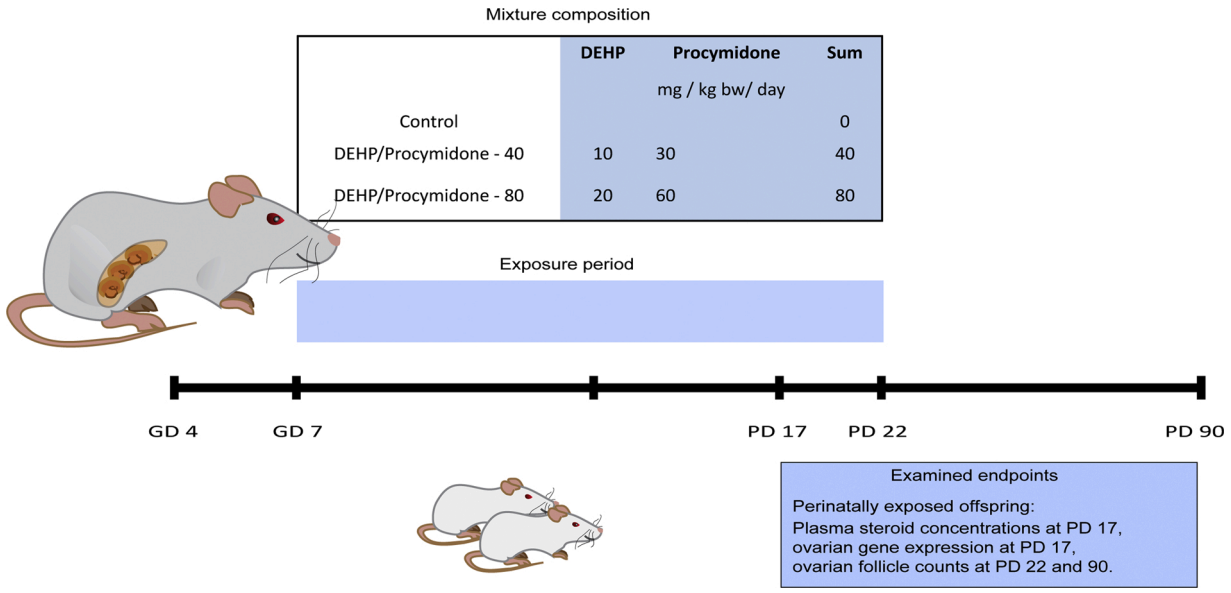

Fig. 1. Study design. Wistar rats were exposed to a binary mixture of DEHP and procymidone from gestation day (GD) 7 to pup day (PD) 22. In each dose group, n = 18 time-mated females were included, and as not all mated females were pregnant, n = 15-16 litters were available for further analysis. Plasma steroid hormones and ovarian gene expression were examined at PD 17 (n = 6-10), and ovarian follicle counts were determined at PD 22 and 90 (n = 12).

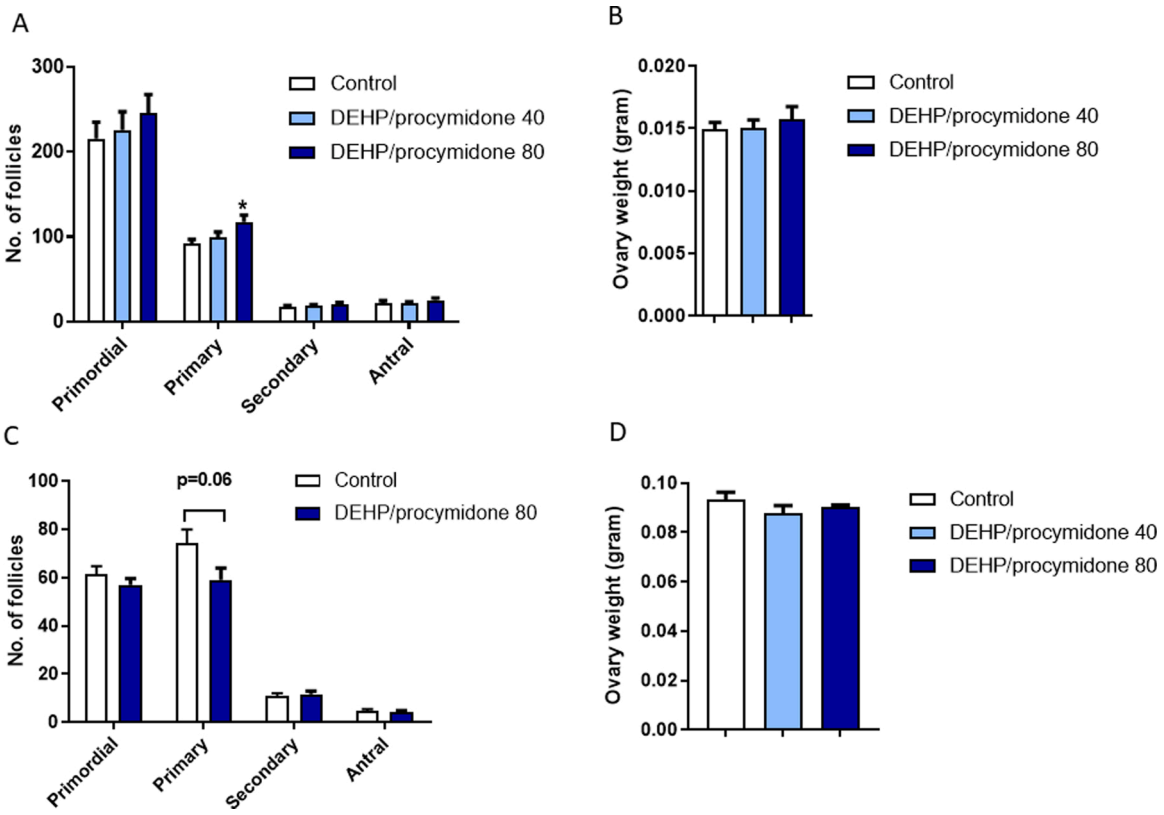

Fig. 2. Ovarian follicle distribution and weight in PD 22 and 90 rats perinatally exposed to a DEHP/procymidone mixture. (A) An increased number of primary follicles was seen in PD 22 ovaries after perinatal exposure to DEHP/procymidone-80. (B) No changes in ovary weights were seen at PD 22. (C) There was no effect on follicle distribution on PD 90, except for a slight decrease in the number of primary follicles (p = 0.06) (D) No changes in ovary weights were seen at PD 90. Follicles were counted in every 20th section at PD 22 and every 40th section at PD 90. Graphs show mean + SEM for the number of follicles counted per animal. Asterisk * indicates $p < 0.05$ in ANOVA. N = 12 for follicle counts, n = 13-17 for ovary weights.

spiked with analyte. LOQs were estimated to be 0.1 ng/mL for androstenedione, progesterone, testosterone, estrone and estradiol, 0.3 ng/mL for corticosterone and aldosterone, and 2.0 ng/mL for pregnenolone. For quantification, external calibration standards were run before and after the samples at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 ng/mL, with 2.0 ng/mL internal standard. For quality control, blank plasma samples were spiked with analyte at three concentration levels: 0.0 (blank sample), 0.5, and 2.0 ng/mL and analysed. The mass

spectrometer was an EVOQ Elite Triple Quadropole Instrument from Bruker (Bremen, Germany) and the UPLC system was an Ultimate 3000 system with a DGP-3600RS dual-gradient pump. For data handling MS Workstation v. 8.2.1 software was used.

Plasma LH measurement was carried out by a sensitive LH sandwich ELISA (Steyn et al., 2013; López-Rodríguez et al., 2019). Briefly, a 96-well high-affinity binding microplate (Corning Life Sciences, Corning, NY) was coated overnight with 50 µl of the monoclonal antibody, anti-bovine LHβ subunit (518B7, provided by L. Sibley, University of California, UC Davis) at a dilution of 1:1000 (in 0.015 M Na2CO3 and 0.035 M NaCO3 coating buffer, pH 9.6). The following day, each well was incubated with 200 µl of a blocking buffer (5% milk powder in PBS-Tween 0.05 %) for 2 h. Then, 50 µl aliquots of the diluted plasma samples (1:10) or the LH standard curve (from rLH-RP3) were incubated for 2 h. After incubation, a rabbit polyclonal primary antibody for LH (1:5,000 ; AFP-240580Rb; provided by NIDDK-NHPP), a polyclonal goat anti-rabbit IgG secondary antibody (1:2000; Dako, Glostrop, Denmark), and 1-Step™ ultra tetramethylbenzidine ELISA substrate (Thermo Fisher Scientific, Waltham, MA) were added to each well. The plate was read at a wavelength of 450 nm (and at 620 nm to detect background). LH concentrations were calculated by interpolating the OD values of unknown samples against a nonlinear regression of the LH standard curve The sensitivity of this assay was 0.06 ng/mL and intra- and inter-assay coefficients of variation were 6.3 % and 10 %, respectively.

Plasma FSH levels were measured by a double Ab method and the rat FSH RIA kit supplied by the National Institutes of Health (Dr. A. F. Parlow, NIDDK-NHPP, Torrance, CA). The primary antibody (NIDDK anti– rFSH-S-11, AB_2687903) was used at a dilution factor of 1/31,250. Rat FSH antigen (NIDDK rFSH-I, AFP-5178B) was labeled with ^{125}I by the chloramine-T method, and the hormone concentration was calculated using the rat FSH reference preparation (NIDDK rFSH-RP-2, AFP-4621B) as standard. The intra-assay and inter-assay coefficients of variation were 7 % and 10 %.The sensitivity of the assay was 0.125 ng/100 µl.

2.5. Gene expression analysis

Protocols were essentially as described previously (Svingen et al., 2015). In short, total RNA was extracted from PD 17 ovaries (n = 10 per dose group) using an RNeasy Micro kit (Qiagen). 300 or 500 ng of total RNA was used for cDNA synthesis using an Omniscript RT kit (Qiagen) and Random Primer Mix (New England Biolabs). Each RT-qPCR reaction contained 3 µl of diluted (1:20) cDNA and TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG and TaqMan™ Gene Expressions Assay (Life Technologies). RT-qPCR assays were run on 384-well plates using the QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems). Gene specific TaqMan assays (Life Technologies Europe BV, Nærum, Denmark), and in-house assays used in this project are presented in Supplementary Material S1. Relative gene expression was calculated using the comparative-CT method using the geometric means of reference genes *Rps18* and *Sdha*.

2.6. Immunohistochemistry

5-µm-thick sections from the middle of PD 22 ovaries were used for immunohistochemistry (IHC) experiments. Following microwave pretreatment for 2 × 5 min in either citrate or Tris-EDTA buffer, sections were blocked for endogenous peroxidase activity in 3% H2O2 in PBS, and blocked in 1% bovine serum albumin in PBS. Sections were incubated over night at 4 °C with antibodies Anti-Mülllerian Hormone (AMH; 1:6400, MIS (C-20) sc-6886, Santa Cruz Biotechnology, Santa Cruz, CA), P450scc (1:30.000, Chemicon AB1244, Temecula, CA), or Steroidogenic Acute Regulatory protein (StAR; 1:2000, Affinity Bio Reagents, PA1-560, Golden, CO). Sections were then incubated for 30 min with secondary antibody (EnVision + System-HRP, Anti-rabbit: K4000, Dako, Denmark), stained in diaminobenzidine (DAB + Substrate Chromogen System, DAKO, Glostrup, Denmark), and counterstained in Meyer's hematoxylin. For AMH, an additional step using a secondary antibody (1:2000, rabbit anti-goat, A4174; Sigma-Aldrich) was included before the EnVision + step.

2.7. Statistical analysis

Data were analysed in GraphPad Prism 5 (GraphPad Software, San Diego, California, USA). Data sets were tested for normal distribution (D'Agostino & Paerson omnibus test and the Shapiro-Wilk test) and analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's post-test and a test for trend. Follicle count data from PD90 animals were evaluated by *t*-test as only controls and one dose group were included for follicle count. A p-value ≤ 0.05 was considered statistically significant.

3. Results and discussion

Our hypothesis was that exposure to a binary mixture of DEHP and procymidone during development would alter follicle recruitment and maturation. This was surmised from the fact that DEHP is known to reduce testosterone levels *in vivo* and that procymidone can antagonize the AR as well as impair steroid hormone production. As expected, the binary mixture did affect ovarian follicle dynamics in exposed animals. Unexpectedly, the mixture increased rather than decreased serum steroid hormone levels. In male offspring exposed to the same mixture, we see clear effects on male reproductive development that are likely related to an anti-androgenic influence (Christiansen et al., 2020), and we speculate that other modes of action may be prominent in females, as discussed below.

3.1. Signs of altered follicle recruitment

In a previous study, we saw a reduced ovarian reserve on PD 22 after perinatal exposure to a chemical mixture of eight substances with anti-androgenic properties, including DEHP and procymidone (Johansson et al., 2016). Here, we wanted to analyze if exposure to a mixture of only DEHP and procymidone as anti-androgenic "model compounds" could affect the primordial follicle pool. When assessing follicle distribution on PD 22, no effects were seen on primordial follicle numbers, but an increase in the number of newly recruited (primary) follicles in the high-dosed offspring. No effects were seen on secondary or tertiary follicle numbers (Fig. 2A). The increased number of primary follicles could suggest an increased recruitment from the primordial to the primary stage. This transition is stimulated by activation of the PI3K/Akt/FOXO3 pathway and is histologically identified by a change in granulosa cell shape, followed by granulosa cell proliferation (Lebbe and Woodruff, 2013). In line with our results, the DEHP metabolite mono-ethylhexyl phthalate (MEHP) accelerates early folliculogenesis in cultured mouse ovaries, most likely through over-activation of PI3K signaling (Hannon et al., 2014, 2015), which could indicate that the same pathway is activated by our binary mixture. Alternatively, the increased number of primary follicles could be due to impaired growth and maturation from primary to secondary and antral follicle stages. As androgens play an important role in secondary follicle growth (Lebbe and Woodruff, 2013; Walters et al., 2010), impaired androgen signaling exerted by DEHP and procymidone could explain a buildup of primary follicles incapable of transitioning to the secondary and antral stages. This has been seen *in vitro* where the AR antagonistic pharmaceutical flutamide inhibits androgen-stimulated primary to secondary follicle transition (Yang and Fortune, 2006).

As we did not see effects on the number of secondary and antral follicles, nor a reduced ovary weight (Fig. 2A and B) that would be expected if secondary and antral follicle numbers were reduced, it is unlikely that the observations are due to impaired transitioning to secondary and antral stages. The observations thus better reflect

findings described for DEHP metabolite MEHP than for AR antagonist flutamide. Postnatal injection of mice with DEHP induces a marked reduction in primordial follicle numbers and an increase in secondary and antral follicle numbers at postnatal days 15 and 21, indicating accelerated follicle recruitment and growth (Zhang et al., 2013). This phenotype mirrors what we observed with regard to accelerated recruitment from the follicle pool, but differs with regard to increased follicle growth. It is possible that DEHP in the binary mixture did accelerate primordial to primary follicle transition, but that the presence of procymidone to some extent counteracted the stimulatory effect DEHP may exert on growth of secondary and antral follicles, as observed by others (Zhang et al., 2013).

We also investigated the follicle distribution in adult animals (PD 90) where we observed a trend towards lower follicle counts on PD 90, mainly for primary follicles ($p = 0.06$). The number of secondary and antral follicles, however, was unaltered (Fig. 2C). If this trend reflects a persistent change in follicle dynamics from prepubertal age to adulthood, there is a concern for early depletion of follicle reserves. In mice

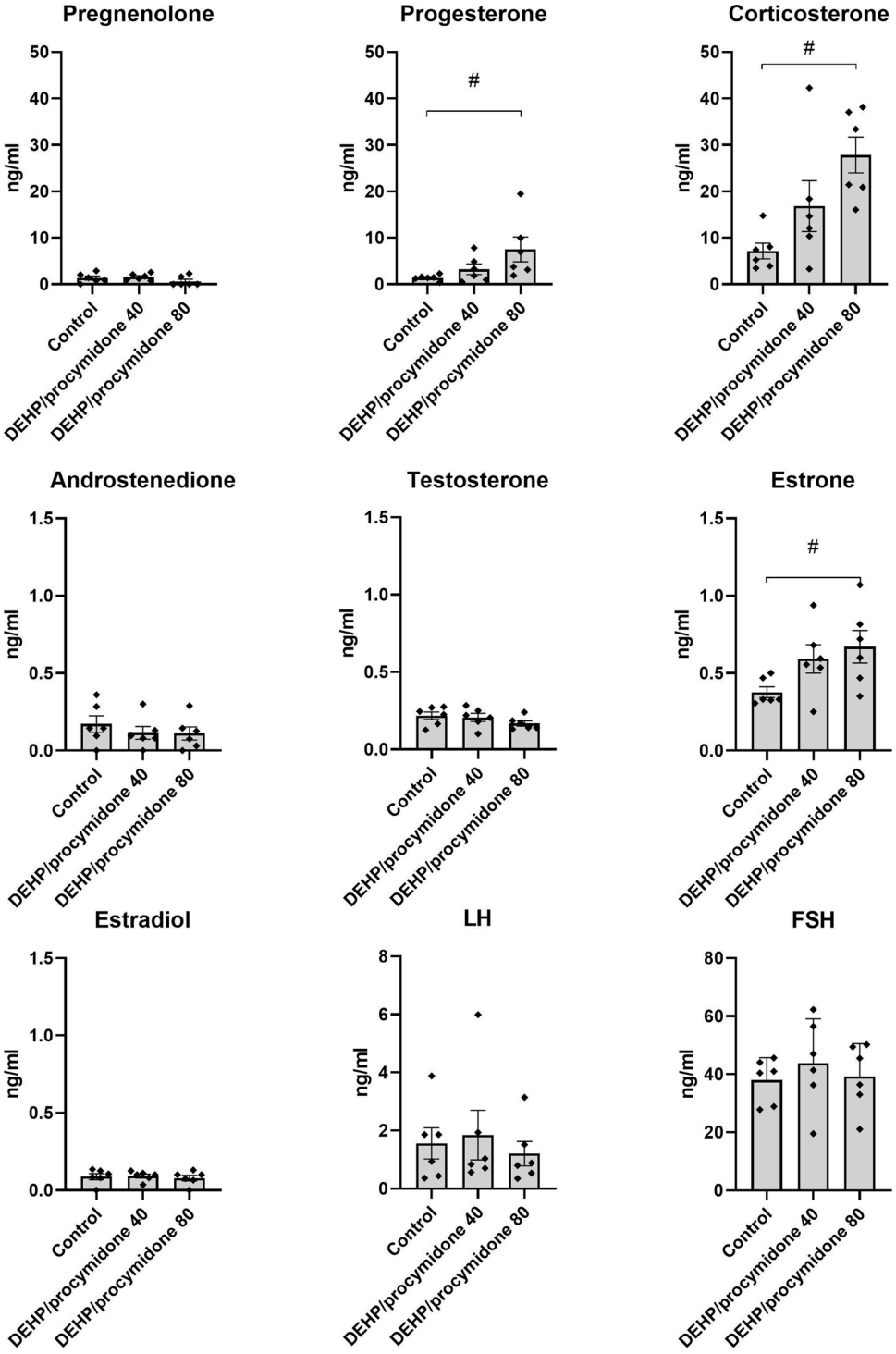

Fig. 3. Plasma steroid hormone and Luteinizing hormone (LH) levels in PD 17 rats perinatally exposed to a DEHP/procymidone mixture. Plasma levels of progesterone, corticosterone and estrone were significantly higher in the DEHP/procymidone 80 group compared to controls ($p < 0.05$ in ANOVA, indicated by #). Mean + SEM, n = 6.

exposed postnatally to DEHP, pre-pubertal reduction in primordial follicle numbers persists into adulthood (Zhang et al., 2013). This is also seen in mice exposed perinatally to DEHP, where exposure causes a reduced number of primordial follicles and an increased number of preantral follicles in adulthood (Pocar et al., 2012). In other words, a change in follicle dynamics with accelerated follicle recruitment can deplete the follicle reserve and perhaps increase the risk of early reproductive senescence.

3.2. Altered steroid hormone profiles in plasma, with unchanged expression of steroidogenesis-related factors in the ovaries

We hypothesized that the binary mixture would impair synthesis of androgens and other steroid hormones in female offspring, as previously reported in a study on fetal rats exposed to DEHP (Borch et al., 2004), and that this would be measurable in the offspring's plasma. However, plasma androgen levels were unaffected at PD 17 whereas levels of progesterone, corticosterone and estrone were dose-dependently elevated. This was statistically significant in the highest dose group (Fig. 3A). We speculated that this could be due to excess stimulation by luteinizing hormone (LH) and FSH, but no change in LH or FSH levels were seen at this age (Fig. 3B). The examined period marks a stage of transition from an inefficient androgen dominated control of gonadotropin release to the onset of more efficient estrogen negative feedback after PND 15 (Andrews and Ojeda, 1981), but nevertheless there was poor correlation between plasma LH level and steroid hormone levels in all dose groups (data not shown).

Since estrone synthesis takes place in the granulosa cells, we explored whether increased estrone levels in the high dose animals could be due to an increased expression of aromatase in ovaries in exposed offspring. At the mRNA level, aromatase (*Cyp19a1*) was unchanged at PD17, as were other genes involved in ovarian steroid synthesis: *StAR, Cyp11A1/P450scc, P450 c17,* and *Hsd3β* (Fig. 4). Analyses of aromatase, STAR and P450SCC at the protein level by immunohistochemistry (PD22) did not reveal any obvious changes in the localization or intensity of staining in ovaries (Fig. 5). Notably, these observations do not exclude the possibility that enzyme activity was affected.

Since altered estrone levels might affect expression of estrogen receptors or the expression of gonadotropin receptors, we also examined expression of other genes of interest. Again, we observed no changes in *Esr1, Esr2*, *Lhr*, or *Fshr expression,* nor did we observe any changes to expression of *Ar*, the oocyte marker (*Ddx4*), proliferation markers (*Ki67, Pcna*) or the apoptosis markers *Bax, Bcl2* (data not shown). Notably, we saw a lower expression of *Amh* in the low dose group but without any obvious change to AMH localization by IHC analysis. AMH is a key regulator of follicle recruitment and growth, as well as steroidogenesis (Dewailly et al., 2016; Lebbe and Woodruff, 2013; Nilsson et al., 2011), so indication that AMH might be disrupted by exposure to anti-androgenic chemicals is of interest. However, the effect on AMH expression was minor and only observed at the mRNA level in one dose group, so any general conclusions cannot be drawn from these data. Taken together, the general lack of changes to any of the steroidogenesis- or growth-related factors suggests that the exposed ovaries were relatively unaffected beyond the observed alterations in follicle dynamics and serum hormone levels. There is also the potential issue with changes to overall cellularity in multi-cellular tissues such as the ovaries, so that changes to gene expression can be confused with changes to overall cell populations, although the overall results do not suggest this to be the case in this instance

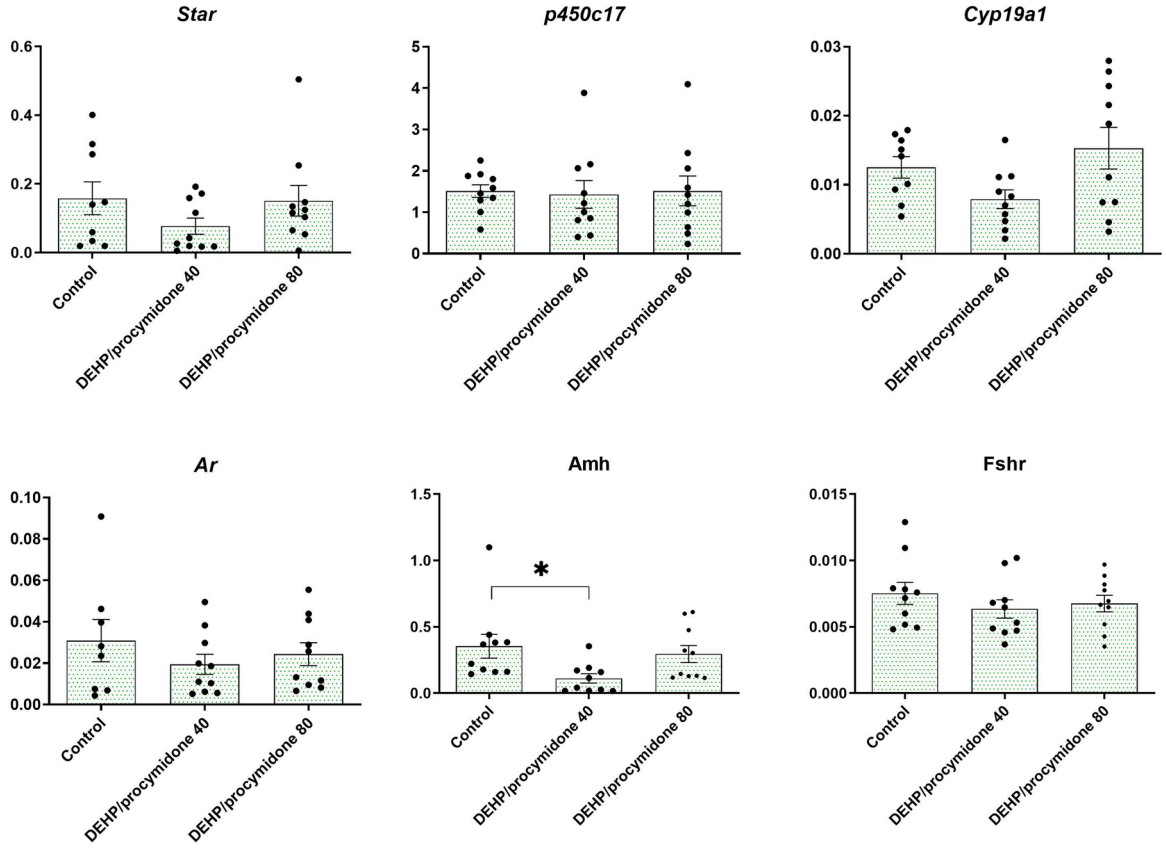

Fig. 4. Relative mRNA expression in ovaries of PD 17 rats perinatally exposed to a DEHP/procymidone mixture. *Amh* expression was significantly lower in the DEHP/Procymidone-40 group, but not in the DEHP/Procymidone-80 group. No changes were observed for genes related to steroidogenesis, steroid receptors or factors regulating follicle recruitment and growth. Mean + SEM, n = 10, * indicates $p < 0.05$.

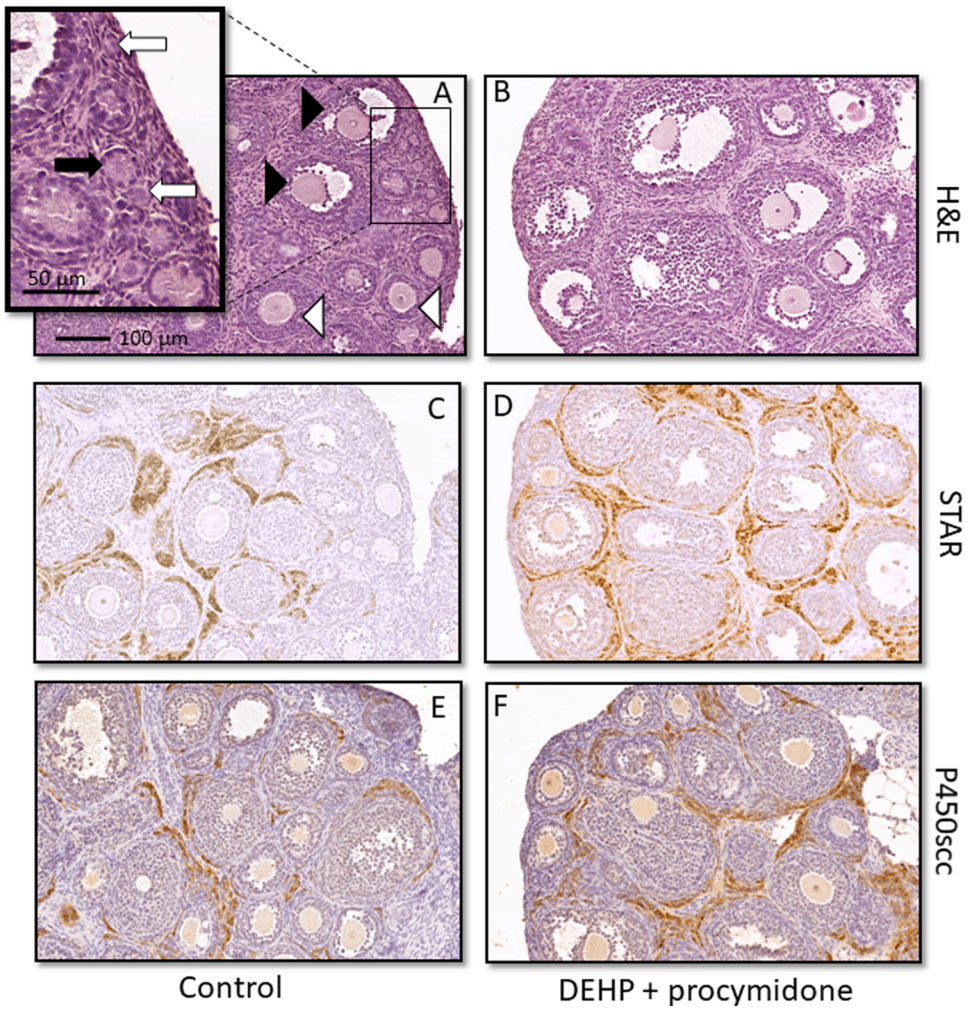

Fig. 5. Ovary histology in PD 22 rats perinatally exposed to a DEHP/procymidone mixture. A, C, E: control group, B, D, F: DEHP/procymidone-80 group. Fig. A (H&E staining) shows follicle types quantified (as described for Fig. 2); white arrows indicate primordial follicles, black arrow indicates a primary follicle; white arrowheads are secondary follicles and black arrowheads are tertiary/antral follicle. No differences were seen in the localization or intensity of immunostaining for STAR (Fig. C and D) or P450SCC (Fig. E and F).

The reduction in corticosterone levels in exposed rats points to adrenal glands being a potential target of this chemical mixture in the prepubertal period. Pregnenolone and progesterone are produced by both adrenals and ovaries, and we cannot determine which organ is responsible for the observed changes in plasma hormone levels as no tissue samples were available for steroid analysis. It is possible, however, that the higher estrone levels in high dose animals indirectly reflects the higher availability of progesterone as a precursor of estrone production, but direct effects on ovarian steroidogenesis could also contribute.

Investigation of potential influences on ovarian steroidogenesis earlier in life would be relevant, but blood or ovaries from before PD 17 were not available for further examination. In the developing ovary, the role of androgens is not as well characterized as it is in cycling adult animals (Franks and Hardy, 2018; Lebbe and Woodruff, 2013). Future studies should investigate effects within specific time windows of ovarian development after exposure to single chemicals acting as anti-androgens by either AR antagonism or steroid synthesis inhibition.

3.3. Conclusions

Combined exposure to the two environmental chemicals DEHP and procymidone during development affects ovarian follicle composition and steroid hormone levels in female rat offspring. Changes to follicle distribution in exposed ovaries may reflect the combined influence of AR antagonism and altered ovarian steroid synthesis. This study adds to a growing body of evidence suggesting negative effects on ovarian development by anti-androgenic chemicals. These effects can have severe consequences for female fertility and reproductive health and thus warrant further studies. New studies aimed specifically to delineate the relationship between observed effect outcomes and exposure to compounds acting as either AR antagonists or steroid synthesis inhibitors could help establish adverse outcome pathways related to endocrine disrupting chemicals.

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Declaration of Competing Interest

All authors declare no conflict of interest. After completing this work JHC has been employed by Novo Nordisk A/S and declares that this does not implicate any conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, at doi:https://doi.org/10.1016/j.tox.2021.152821.

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