DELAYED AND TRANSGENERATIONAL EFFECT OF DEVELOPMENTAL EXPOSURE TO DI-(2-ETHYLHEXYL) PHTHALATE IN THE OVARY AND ON THE FEMALE REPRODUCTIVE FUNCTION IN RATS

By

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ABSTRACT OF THE THESIS

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Di-(2-ethylhexyl) phthalate (DEHP) is present in medical devices, packaging and food containers, from where it leaches into the external environment and causes reproductive toxicity among others in humans and animals. DEHP is an endocrine-disrupting chemical (EDC) and *in utero* and lactational exposure of DEHP causes testicular dysgenesis syndrome in males. Studies in females, suggest that the ovary is the target of DEHP toxicity, however, its mechanism of action remains unknown. Therefore in our study, we exposed timed-pregnant Fischer CDF rats (F0) to DEHP (500 mg/kg/day) from embryonic day (E) 11 to 21 and their F1 pups from postnatal day (PND) 0 to PND 7, a developmental window that includes female gonadal differentiation, critical events in ovarian folliculogenesis and epigenetic reprogramming in females, to specifically study the effect of developmental exposure of DEHP on ovarian function in the F1 offspring. The present study also investigated a transgenerational effect, if any on the female reproductive function, specifically through either the maternal germline (maternal cross; MC) or both maternal and paternal germlines (double cross; DC). Various reproductive parameters such as puberty, estrous cyclicity, pregnancy rate, and litter size were examined. The results showed a significant delay in the onset of puberty, as monitored by vaginal opening, only

in the DEHP-exposed F1 females but not in the F3 females. No effect on the regularity of the estrus cycle was seen despite reduced serum estradiol levels in the F1 offspring. Follicular composition was analyzed in adult ovaries and expressed as a percentage of total follicles. Significantly large number of growing follicles which seem to progress towards atresia were observed in the DEHP exposed F1 animals at young adulthood (PND 50-85). Therefore the data is suggestive of 'premature ovarian failure'. Our study showed that altered follicular dynamics were accompanied by increased Mullerian inhibiting substance (MIS) and androgen receptor (AR) expression in growing follicles in F1 generation. Decreased estradiol levels, up-regulated AR and MIS expression inhibits progression of the early antral follicles, resulting in increased follicular atresia that reduced the number of total follicles per section in the F1 generation. Decrease in primordial follicles and an increase in early antral follicles accompanied by attenuated MIS production by both primary and antral follicles, reemphasized the possibility of disrupted follicular development in the DC group. Similarly, in MC group, there was an increase in CL without affecting the litter size which may be indicative of premature luteinization. These alterations in follicular dynamics are similar to those in the F1 generation, suggesting developmental DEHP exposure can have a delayed and transgenerational effect on female ovaries.

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I dedicate this thesis to my grandparents, Dinkar Bhurke and Waman Pitale.

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CHAPTER 1:

INTRODUCTION AND BACKGROUND

A. INTRODUCTION

Human epidemiological studies and studies with animals [1-3] indicate that suboptimal environments in the womb and during early neonatal life can alter development and predispose an individual to lifelong health problems. This concept of the developmental origins of health and diseases is well accepted with increasing evidence from animal studies precisely elucidating the effects of specific developmental exposures such as nutrient restriction [4, 5], maternal stress [6, 7] and exposure to endocrine-disrupting chemicals [8, 9].

According to the Environmental Protection Agency, an endocrine-disrupting chemical (EDC) is an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are responsible for homeostasis, reproduction, and developmental processes [10]. Exposure to EDCs, such as bisphenol A or methoxychlor, during prenatal and neonatal development have been linked to a wide variety of effects in the male and female reproductive tracts which include but are not limited to meiotic abnormalities in fetal oocytes [11], abnormal testicular development [12, 13], reduced fertility affecting pregnancy outcomes [14, 15], and alterations in brain sexual differentiation [16-18].

Evidence in animal models suggests that developmental exposure to EDCs may affect not only the exposed individual but also the offspring and subsequent generations. The mechanism of such transmission involves epigenetic modifications of the germ line such as changes in DNA methylation and histone modifications [19]. Studies have shown early life exposure to several other EDCs promotes multigenerational phenotypes [20].

The focus of the current study is one such EDC – di-(2-ethylhexyl) phthalate (DEHP). DEHP is one of the most commonly used phthalate plasticizers [21]. In humans, *in utero* DEHP exposure is associated with shorter pregnancy duration [22] and a shortened anogenital distance (AGD) in boys [23, 24]. Increased incidences of miscarriages were reported in women

occupationally exposed to high doses of phthalates [25]. In rodents, a high dose of DEHP reduces implantations, increases resorptions, and decreases fetal weights of offspring [26]. Many studies investigating these reproductive outcomes suggest that the ovary may be a target of DEHP toxicity. Studies report compromised ovarian steroidogenesis and disrupted ovarian folliculogenesis in DEHP exposed females [27-29]. *In vitro* studies show DEHP exposure causes inhibition of antral follicle growth by inducing production of reactive oxidative species [30] and decreases transcript levels of the enzyme aromatase, reducing estradiol levels [26]. Although these studies demonstrate the effects of DEHP on ovarian function, other factors may also contribute to disrupted ovarian folliculogenesis. Therefore the current study was undertaken to investigate the effect of DEHP exposure during critical ovarian developmental time window, on ovarian function and female fertility in early adulthood. Furthermore, recent studies have reported a transgenerational effect of DEHP in male rodents [31]. Therefore, this thesis presents research studies that investigate both the effect of developmental exposure of DEHP at early adulthood and the potential transgenerational effects due to this exposure, on ovarian function and female fertility.

To further understand these processes the following summarizes the most critical steps in ovarian development.

B. OVARIAN DEVELOPMENT

The functional unit of the ovary is the ovarian follicle composed of somatic cells and the developing oocyte. The somatic cells are the thecal cells and the granulosa cells. The most important functions of the ovary are ovulation and steroidogenesis.

1. Primordial follicle formation

In rodents primordial germ cells migrate, undergo mitosis to colonize the genital ridge at E 10.5 to 11.4 and this migration is completed by E 13.5 [32, 33]. In rats, by E 18.5 mitosis rarely

occurs and most germ cells have entered the leptotene phase of meiosis. Some of them progress through meiosis to be arrested in the diplotene stage of meiosis I and are now referred to as the oocyte, while others undergo atresia [34]. After they enter meiotic arrest, the oocytes form interconnected clusters and associate with somatic cell precursors forming oocyte nests [35, 36]. Prior to birth these nests break down, the somatic cell precursors differentiate into pre-granulosa cells and surround each oocyte forming primordial follicles [37]. Thus in rats, the oocytes undergoes extensive atresia, first at E 18.5 and later, at birth, when they get arrested in the diplotene stage. Thus their number is reduced to 30% of what was present on E 17.5, as they form primordial follicles [34]. In rodents, this assembly of primordial follicles is inhibited by estradiol and occurs when the estradiol levels drop at birth [38]. A recent study shows Kit signaling promotes cyst breakdown in mice [39]. Each primordial follicle consists of a single oocyte surrounded by a morphologically distinct layer of somatic cells, called granulosa cells.

2. Primordial to primary follicle transition

In rats, by PND 5, a finite number of primordial follicles are established, each with its own basement membrane enclosing the pre-granulosa cells and the oocyte. While most of these remain quiescent, some begin to grow. The pre-granulosa cells that are usually crescent-shaped in quiescent primordial follicles become cuboidal granulosa cells as they proliferate [35]. Expression of the factor in germ-line alpha (FIGLA) within the oocyte stimulates production of zona pellucida glycoproteins [40]. All of these events leading to transition from primordial to primary follicle stage are promoted by paracrine factors such as Kit ligand, bone morphogenic protein-4 while being negatively regulated by others such as Mullerian inhibiting substance (MIS) [41].

3. Preantral and antral follicle development

As the number of granulosa cells increases exponentially, the oocyte increases in volume substantially forming the preantral follicle [35]. Once the granulosa cells reach several layers of thickness, an antrum or fluid-filled space develops in the follicle giving rise to the antral follicle. Antral follicles express follicle stimulating hormone (FSH) and luteinizing hormone receptors and development beyond the early antral follicle stage is dependent upon gonadotropins. The formation of a follicular antrum divides the population of granulosa cells into two main groups: cumulus cells associated with the oocyte and mural granulosa cells lining the follicular wall. Most oocytes from the antral follicles are meiotically competent capable of resuming meiosis [43].

4. Follicular recruitment and atresia

The dormant primordial follicles are recruited into the growing follicle pool in a continuous manner- termed 'intial recruitment', whereas increases in circulating FSH during each reproductive cycle recruit a cohort of antral follicles- termed as 'cyclic recruitment'. Initial recruitment is continuous in the beginning at primordial follicle formation and is a gonadotropin independent event. Although primary and secondary follicular development can take place in the absence of gonadotrophins, these follicles are responsive to gonadotropins and optimal development to preantral follicle stage may require these hormones [44]. Cyclic recruitment of follicles beginning at puberty is dependent on gonadotropins, especially FSH. As the circulating FSH levels increase, one (humans) or more (rodents) of the follicles grows faster than the rest of the cohort and produces higher levels of estrogens and inhibins. These dominant follicles become more sensitive to FSH, producing more estradiol which in turn suppresses the pituitary FSH production. Thus the few antral follicles that can survive despite the declining FSH levels are selected against the rest that undergo atresia (reviewed in [44]). Initial and cyclic recruitment leads to two kinds of follicular reserves – pre-established primordial follicle reserve and the gonadotropin-responsive preantral follicle reserve – that are functionally related. The dynamics of both follicular reserves appears to result from the delicate interplay of two main pathways, the

phosphatase and tensin homolog (PTEN)/phosphatidylinositol-3 kinase (PI3K)/3-phosphoinositide-dependent protein kinase-1 (PDPK1)/v-akt murine thymoma viral oncogene homolog 1 (AKT1) and the bone morphogenetic protein (BMP)/MIS/SMAD signaling pathways. The PTEN/PI3K/PDPK1/AKT1 signaling pathways is involved in activation and/or premature loss of the primordial follicles and germ cell growth. This pathway is activated by various hormones, growth factors, and cytokines (IGF, Kit ligand). The formation and development of the follicles is regulated by oocyte derived factors (BMP15, GDF-9) and somatic cell (BMP2, BMP4, MIS, activins) derived factors. All of which are members of TGFβ family that activates SMAD signaling. Among them, activins and BMP are mitogenic and anti-apoptotic factors for granulosa cells, whereas MIS produced by the granulosa cells of the large preantral and the small antral follicles restricts primordial follicle activation and modulates follicle development. During the transition between the reserves, the development of each growing follicle also depends, importantly, on the molecular dialog established between the oocyte that produces the GDF9 and BMP15 and its surrounding granulosa cells that secrete the oocyte growth-promoting factor Kit ligand. Insulin and IGFs, can support the development of the growing follicles by sensitizing their granulosa and thecal cells to FSH and LH, respectively. Furthermore, BMP, the inhibin/activin system, and the IGF system (IGFs and their binding proteins) can, modulate the gonadotropin-dependent differentiation of granulosa and thecal cells, thus determining the fate of each antral follicle (i.e., atresia or development towards ovulation) [43, 45, 46].

5. Ovulation and formation of corpus luteum

Well-developed dominant antral follicles are referred to as preovulatory follicles upon the preovulatory LH surge. These follicles support the increase in steroidogenic factors and upregulate the expression of genes necessary for rapid cellular differentiation, ovulation, oocyte maturation and luteinization for corpus luteum (CL) formation. During the growth of preovulatory follicles, aromatase and the LH receptor are induced in granulosa cells. The LH

surge is triggered by increased levels of estradiol produced by these follicles. The LH surge acts on the granulosa cells of the preovulatory follicle to rapidly upregulate the expression of genes for ovulation such as progesterone receptor and luteinization. In response to the LH surge these follicles ovulate, releasing the mature oocyte and the follicle reorganizes itself into the corpus luteum [47]. IGF-1, estrogen, and FSH mainly regulate the follicular growth while Wnt/Frizzled signaling might possibly be involved in ovulation and luteinization [48].

C. REPRODUCTIVE SENESCENCE

The loss of reproductive function is termed as reproductive senescence. In humans, prior to menopause, there is a prolonged increase in gonadotropin levels which accelerate the recruitment of the remaining primordial follicles pool [37]. The natural phenomenon of reproductive senescence typically begins at 10 to 12 months of age (middle-age) in rats when they begin exhibiting prolonged and irregular estrous cycles. By this age the timing and amplitude of the proestrous and estradiol-induced LH surge is altered. There is a significant decline in the number of activated GnRH neurons, resulting in irregular estrous cycles [49]. More recent studies show that the age-related LH surge dysfunction results, partially due to reduced sensitivity of the kisspeptin neurons in the anteroventral periventricular nucleus to estradiol and hence leading to insufficient kisspeptin neurons activating the GnRH neurons under positive feedback conditions [50]. Beyond 12 months of age rats exhibit persistent estrus before they enter continuous diestrus and anestrus accompanied by fluctuating estradiol levels [51].

Many EDCs act in various ways to alter the timing and the natural course of reproductive senescence. For example, BPA disrupts germ cell breakdown reducing the primordial follicle pool [14] while heavy metal chromium (CrVI) accelerates the primordial to primary follicle transition [52]. Both of these can lead to premature ovarian failure and early reproductive senescence. Current demographics show an increase in the aging population (women in their 30s and early 40s) seeking pregnancy [53]. A nation-wide study showed that the fecundity of women

decreases significantly beginning from 30 years of age, and especially beyond 37 years of age [54]. Considering the two scenarios, coupled with exposure to EDCs these individuals could be at a much greater risk of reproductive failure and early senescence.

D. OVARIAN MARKERS

1. Mullerian inhibiting substance (MIS)

MIS is produced in the primary, secondary follicles, the preantral follicles and antral follicles [55]. MIS is expressed in cumulus granulosa cells of growing follicles- primary, secondary, pre/early antral but not in germ cells or thecal cells [56]. The oocyte regulates this centripetal gradient of granulosa cell MIS expression by mechanisms that are yet not clear [57]. MIS is not expressed in primordial follicles, and has been shown to block primordial follicle development [41, 58]. MIS also inhibits FSH-dependent growth of cultured mouse preantral follicles by attenuating aromatase activity and LH receptor expression [59].

EDC exposure can alter expression of MIS in these growing follicles. An earlier study from our lab had shown that *in utero* and perinatal MXC exposure inhibits ovarian folliculogenesis and stimulates AMH production in prepubertal rats [15, 60]. Increased MIS transcript and protein levels in the granulosa cells of small growing follicles in the ovary at PND 6 in estradiol benzoate-treated rats leads to inhibition of follicular growth [61]. Hence the current study investigated the effect of perinatal DEHP exposure on expression of MIS.

2. Luteinizing Hormone receptor (LHR)

In rat ovaries, the functional LHR begin to appear towards the end of the first week of life, which will eventually (PND 10) get localized to thecal cells [62]. In healthy small antral (200 - 550 µm) follicles, LHR is present in thecal pericytes, but not in cells of the theca interna while in healthy relatively larger antral follicles (550 - 750 µm) it is expressed in the entire thecal cell population. Fresh CL expresses LHR in abundance and the expression decreases as the CL

degenerates [63]. LH acts via the thecal LH receptors to stimulate androgen production, trigger ovulation and to stimulate estrogen and progesterone production. However, progesterone production by granulosa cells independent of LHR has also been reported [64]. LH receptor knockout female mice are infertile. Their follicles are able to reach the antral stage but no preovulatory follicles or corpora lutea are noted [65]. A previous study from our lab showed a down-regulation of LHR as one the consequences of developmental exposure of MXC [15]. Ovaries from mice exposed to 500 mg/kg/day and 1000 mg/kg/day of DEHP during late gestation showed a 50% reduction in LHR transcript levels [66]. The current study analyses expression of LHR protein levels in ovaries of rats exposed to the same (500 mg/kg/day) dose of DEHP, at a developmental time window that is more specific for ovarian development.

3. Androgen receptor (AR)

Androgens serve as precursors for the synthesis of estrogens, alternatively, they may mediate their effect via the androgen receptor (AR). AR is expressed in the nuclei of the granulosa layer of preantral and very early antral follicles at all stages of the estrous cycle, but on the day of proestrus appears to be confined to cumulus-oocyte-complexes and a few antral cells bordering the antrum [67]. The oocyte-secreted factors regulate this centripetal gradient of granulosa cell AR expression [45]. In the absence of ligand (androgens), the inactive AR is predominantly within the cytoplasm. Androgen binding causes the nuclear localization signals of the receptor to be unmasked and to be recognized by the import apparatus of the nuclear pore complex for import into the nucleus to affect transcription of genes promoting granulosa cell proliferation [68, 69]. The activated ARs transcriptionally regulate the expression of a selected group of genes via direct or indirect association with the regulatory regions (enhancer/promoter) upstream of the genes [70]. In whole ovary culture, ARs enhance the expression of the anti-apoptotic microRNA (miR) miR-125b, which likely contributes to androgen-induced follicular survival [71]. Furthermore, locally produced androgens bind to granulosa cell-specific AR to enhance the

actions of FSH, potentially contributing to androgen mediated preantral follicular growth. *In vivo*, in rodents, the administration of subcutaneous implants containing dihydrotestosterone promoted the increased expression of FSH receptor mRNA in preantral follicles, sensitizing follicles [72]. Ovaries from granulosa cell-specific knockout mice contained more preantral and fewer antral follicles, indicating that AR signaling is involved in progression of preantral follicles to early antral follicle stages [73]. When cultured murine follicles were treated with androgen receptor antgonists, hydroxyflutamide or bicalutamide, follicular growth was reduced, the steroidogenic environment was altered, and an arrest in oocyte meiotic maturation in response to human chorionic gonadotropin occurred [74]. DEHP on the other hand is not an androgen receptor antagonist, but acts as an anti-androgenic chemical by suppressing the androgen biosynthesis in males [75]. Since AR mediates the actions of androgens in ovary, we hypothesized that the expression of AR in rats developmentally exposed to DEHP, might be altered.

4. Cytochrome P450 side chain cleavage (P450scc/Cyp11a)

Import of cholesterol into the mitochondria is a critical step in the production of steroids and is regulated by the steroidogenic acute regulatory protein (StAR) [76]. The mitochondrial side chain cleavage enzyme (P450scc), catalysis the cleavage of the cholesterol side chain converting cholesterol to pregnenolone, which is an important step in the biosynthesis of androgens that serve as precursors for estrogen synthesis [77]. The expression of P450scc is detected in thecal cells of preantral and antral follicles [78]. In some cases of endogenous hyperandrogenism upregulation of P450scc in ovarian thecal cells is observed [29]. MEHP, a metabolite of DEHP, has been shown to alter expression of P450scc in testes [79, 80]. Fetal exposure to DEHP suppresses testosterone levels by reducing StAR, P450scc and PPARgamma protein levels in fetal Leydig cells [81].

5. Estrogen receptor alpha (ERα)

Studies in estrogen receptor knockout mice have shown that normal ovarian function requires both estrogen receptors –alpha and -beta. ERβ plays an important role in mediating the stimulatory effects of estrogens on granulosa cell proliferation while ERα is not required for follicle growth under wild type conditions but is indispensable for ovulation [82]. In the ovary ERα is expressed in nuclei of thecal cells and germinal epithelium in rodents [83]. Estrogen acts via ERα to mediate a negative feedback loop on the thecal cells to maintain a proper steroidogenic environment for the growing follicles firstly by inhibiting the expression of *Cyp17a1* to suppress androgen synthesis and secondly by inhibiting the synthesis of the more biological active androgen, testosterone, by repressing the expression of hydroxysteroid (17 β) dehydrogenase type 3 (*Hsd17b3*), a testis-specific steroidogenic enzyme [84].

Some studies show DEHP has a weak estrogenic effect stimulating cell proliferation in human breast cancer MCF-7 cells [85] while other studies using the same [86] and different [87] cell lines claim DEHP does not compete with binding of estradiol to the estrogen receptors.

E. DEVELOPMENTAL EXPOSURE and TRANSGENERATIONAL EFFECTS

Prenatal exposure to environmental stressors (e.g. EDCs) can modify normal cellular and tissue development and alter developmental programming such that the individuals may have a higher risk of reproductive pathologies and metabolic and hormonal disorders later in life. This concept of 'Developmental origin of health and disease (DOHaD)' was first proposed by the late David Barker in 1997 [88]. A developing organism is more vulnerable to the disturbance caused by EDCs because mechanisms such as DNA repair, a competent immune system, detoxifying enzymes, liver metabolism, and the blood/brain barrier that are present in the adult are not fully functional in the fetus or newborn. Additionally, the developing organism has an increased metabolic rate as compared to an adult that, in some cases, may cause increased toxicity [89]. Since each organ system has a different developmental trajectory, and the sensitive window for

exposures to cause toxicity varies with the tissues, the effects of exposures are dependent not only on the dose of the chemical, but also when the exposure occurs. Developmental exposure or exposure during the critical developmental time window of organogenesis can cause changes in gene expression, protein activity, and cell communication which could have life-long implications. For example i*n utero* (E 11 until birth) BPA exposure inhibits germ cell nest breakdown by increasing the expression of selected anti-apoptotic factors and decreasing the expression of selected pro-apoptotic factors which may reduce the number of primordial follicles formed, thus resulting in premature ovarian failure and shorter reproductive life-span [14].

EDC such as diethylstilbestrol [DES], a potent synthetic estrogen, which was prescribed to reduce the risk of pregnancy complications and losses can affect multiple generations. Exposure of a gestating female to DES was found to promote an abnormal reproductive tract and gonadal dysfunction in the F1 generation males and females, as well as abnormal female reproductive tract function in the F2 generation [90]. Furthermore, developmental exposure of EDCs can have transgenerational (TG) [50] effects. When a pregnant female is exposed to EDCs it not only affects her (F0), but also the next two generations (F1 and F2) through the fetus and its germ line. When subsequent generations (F3 and beyond) are affected it can be considered as a TG effect [19]. Male mice (F0) exposed to DEHP from E 7 to 14 exhibit disrupted testicular germ cell organization and spermatogonial stem cell function. This effect is TG and F3 male offspring also demonstrate the same physiology [31].

Studies have shown such physiological effects of *in utero* and/or neonatal exposure could be due to DNA mutations or due to more subtle modifications in gene expression which are independent of mutations (*i.e*., epigenetic effects). Epigenetic modifications include changes in DNA methylation, histone modification and those that are induced by non-coding RNA [91]. Exposures to EDCs can alter these epigenetic mechanisms in various tissues such as the ovaries, testes and other male and female reproductive organs, inducing subtle functional changes leading to disease later in life and in future generations. For example, a fungicide vinclozolin modifies the

germline epigenetically causing kidney disorders and reproductive dysfunctions in the third (F3) generation [18].

F. DEHP as an EDC

1. Environmental abundance and human exposure

Phthalates are ubiquitous in the environment due to widespread use as plasticizers and DEHP is one of the most widely used phthalate esters [21]. DEHP is present in medical bags and tubings, packaging and food containers. The human adult exposure in patients receiving dialysis, blood transfusions, artificial ventilation, and exchange transfusions that is through parenteral route can be >1000 µg/kg/day which exceeds the tolerable intake for DEHP of 600-800 µg/kg/day [92]. A study reported DEHP exposure via intravenous routes in platelet donors can be as high as 31.6 µg/kg body weight/day resulting in urinary concentration of 178 µg MEHP [93]. Needless to say, neonates and infants are among the ones that receive the highest doses of DEHP especially from medical devices [92]. DEHP is non-covalently bound to plastics, and can leach out of these plastic products, resulting in potential daily human exposure to DEHP in the range of 3-30 µg/kg/day [94, 95]. In fact DEHP and its metabolites have been reported to be found in breast milk, serum, amniotic fluids and sweat [96, 97] and recently in urinary samples from mothers and infants [98]. This developmental exposure is especially concerning. In humans, *in utero* DEHP exposure was associated with shorter pregnancy duration [22] and a shortened anogenital distance (AGD) and index in boys [23, 24]. Increased incidences of miscarriages were reported in women occupationally exposed to high doses of phthalates [25]. Danish girls with high urinary concentrations of phthalate metabolites, including DEHP, showed delay in puberty [99].

2. Animal studies investigating effects of DEHP exposure in females

Animal studies show that transient [100], lactational [9] or prepubertal and peripubertal [101] DEHP exposure affects various female reproductive parameters such as estrous cyclicity, pubertal

age and litter size. High doses of 2 g/kg of DEHP result in prolonged estrous cycles with no ovulation and hence absence of corpora luteum. This is because the estradiol levels were insufficient for the LH surge to occur and trigger ovulation [26, 102]. Reduced progesterone and estradiol levels have also been reported with relatively lower dose (500 mg) prepubertal exposure (10 days) of female rats [28] and in follicle culture exposed to 1–100 μg/ml of DEHP [27]. Extensive *in vitro* studies elucidate the molecular basis of the disrupted ovarian steroidogenesis. Results from DEHP treated ovarian follicle cultures show DEHP suppresses estradiol production by granulosa cells via its metabolite MEHP. MEHP acts on the FSH membrane receptor to inhibit FSH-stimulated cAMP production thereby preventing activation of the enzymes for progesterone production. Furthermore, MEHP decreases levels of aromatase enzyme by activating PPARγ pathway. Both progesterone and aromatase are required for estradiol production and decrease in their levels reduces estradiol production [26]. Prolonged exposure to a low-dose (0.05 mg/kg/day) of DEHP resulted in reduced expression of *Cyp17a1*, *aromatase*, *progesterone receptor*, *Lhr* and *Fshr* in the adult ovary (PND 42) of CD-1 mice, all which may affect ovarian steroidogensis [103]. DEHP not only inhibits steroidogenesis in GCs but also enhances pituitary LH production. [28]. However, these results contradict the finding of others that report elevated estradiol levels accompanied by attenuated LH production and decreased transcripts of *lhcgr, aromatase and StAR* [66]. This discrepancy could be due to a difference in the phase of the estrous cycle at the time of ovary collection. A study documented high estradiol levels at diestrus and low estradiol levels at estrus [104]. Some studies report delayed pubertal onset [66] while others have reported precocious puberty accompanied by enhanced LH and estradiol levels [101]. The exact mechanisms by which DEHP affects the hypothalamic pituitary axis, hence puberty, remains unclear [103, 129]. Most studies report disrupted ovarian folliculogenesis as one of the major consequences of DEHP exposure. DEHP inhibits antral follicle growth, *in vitro,* by inducing production of reactive oxidative species and decreasing the expression and activity of superoxide dismutase-1, one of the antioxidant enzymes [30]. Increased atretic follicles were observed in rats

exposed to 405 mg/kg DEHP from E 6 to PND 21 [105]. A prolonged 60-day exposure to 300 and 600 mg/kg/day of DEHP resulted in decreased populations of growing follicles (primary secondary and antral follicles), and increased irregularity of estrous cycles and granulosa cell apoptosis [106]. Low progesterone levels in the DEHP exposed animals also arrests the granulosa cells in G0/G1 phase resulting in their apoptosis [107]. Multiple factors may contribute to follicular atresia in DEHP exposed animals, for example, increased spindle abnormalities in metaphase II oocyte [9]. A recent study showed in vivo DEHP exposure increased PI3K signaling leading to increased recruitment of primordial follicles [108].

Such endocrine disruption may have even more severe physiological consequences such as absence of vaginal orifice in rats treated with 750 and 1000 mg/kg DEHP from E 14 to 17 [109] or complete pregnancy failure in the case of prolonged (from E 0.5 until the end of lactation) treatment of 500 mg/kg/day of DEHP [103]. While high dose (2 g/kg) exposure caused anovulation and reduced estradiol levels [26, 102], prepubertal low dose exposure to DEHP reduced the litter size and body weight of the pups [9]. Interestingly, animals exposed to 1000 mg/kg MEHP from E 17 to 19 exhibited mammary gland hyperplasia at one year of age, often observed with elevated estrogen levels [66].

All of these studies have employed extended exposure periods, high doses or *in vitro* systems. The present study therefore aims to specifically target the fetal and neonatal ovarian development in rats. In our model, we exposed Fisher CDF rats from E 11 until birth and the litters were exposed to DEHP until PND 7. In rodents this is the critical ovarian developmental time window which includes oocyte (cyst) nest breakdown via germ cell apoptosis [110], ovarian follicular assembly, and the initial transition from the primordial to the primary follicle stage [41]. This period also encompasses germ cell epigenetic programming, thus effects are likely to be transgenerational (reviewed in [8]).

G. RATIONALE FOR CURRENT STUDY

Because the ovarian reserve is established at birth and folliculogenesis is an irreversible process, aberrant regulation of folliculogenesis can have adverse reproductive implications. In particular, the accelerated depletion of primordial follicles, either through apoptosis or irregular activation of development, can result in infertility and/or premature ovarian failure, or early onset of menopause. Our study is different from others with respect to 1) the exposure to DEHP which is specific to the critical time window of ovarian development 2) The ovarian markers investigated and 3) TG effect, if any, in the ovaries that were investigated.

H. HYPOTHESIS

1) Fetal and early postnatal exposure of DEHP affects the reproductive function of the F1 generation female rats

2) Fetal and early postnatal exposure of DEHP can affect females transgenerationally

CHAPTER 2:

EFFECT OF DEVELOPMENTAL EXPOSURE TO DEHP IN F1 FEMALE RATS

Introduction

Phthalates are ubiquitous in the environment with their widespread use in plastics and other consumer products. Amongst phthalates, di (2-ethyhexyl) phthalate (DEHP) is the most widely used phthalate ester [21]. DEHP is present in medical bags and tubings, packaging, and food containers. DEHP is non-covalently bound to plastics, and can leach out of the plastic products, resulting in potential daily human exposure to DEHP in the range of 3-30 μg/kg/day, although measurements of DEHP in household dust could be as high as 508 mg/kg dust [94, 95, 111]. In fact DEHP and its metabolites have been found in breast milk, serum and amniotic fluid [96] and recently in urine samples from mothers and infants [98]. This developmental exposure is of special concern.

Extensive human epidemiological studies show that a compromised *in utero* fetal environment increases the propensity of adverse health outcomes in adulthood [2]. Previous studies have indicated that there is a correlation between *in utero* and neonatal exposure to EDCs such as methoxychlor, bisphenol A and reproductive health in adulthood [14, 15]. In humans, *in utero* DEHP exposure was associated with a shorter pregnancy duration [22], increased risk of preterm birth [112] and delayed pubarche in girls [99]. Increased incidences of miscarriage were reported in women occupationally exposed to high dose of phthalates [25]. These studies suggest developmental exposure to DEHP adversely affects the female reproductive health. Animal studies show that transient [100], lactational [9] or prepubertal and peripubertal [101] DEHP exposure affects various female reproductive parameters such as estrous cyclicity, pubertal age, and litter size. In rodents, a high dose of DEHP reduces implantations, increases resorptions, and decreases weights of offspring [26]. Studies investigating these reproductive outcomes suggest that the ovary may be the target of DEHP toxicity [27, 28]. However, the mechanism by which DEHP affects the ovaries remains unknown.

To specifically examine the effects of developmental exposure of DEHP on ovarian function, we designed a study wherein we exposed animals to DEHP during a critical time

window of ovarian development (embryonic day (E) 11- postnatal day (PND) 7). We then investigated various reproductive parameters and ovarian follicular dynamics. Expression of ovarian markers that are known positive or negative regulators of ovarian function such as Müllerian inhibiting substance (MIS), androgen receptor (AR), luteinizing hormone receptor (LHR), estrogen receptor α (ERα), cytochrome P450 side chain cleavage enzyme (P450scc) were examined.

Materials and Methods

Animals

Timed-pregnant Fischer (CDF) inbred rats were obtained from Charles River Laboratories (Wilmington, MA). The inbred Fischer strain was used since it has minimal polymorphisms, which facilitates the detection of treatment effects. The animals were maintained in a room with controlled illumination (lights on 0700 h – 2100 h), temperature (26–28ºC), and humidity (30–70%). The rats were provided soy-reduced scientific diet 5V01 rat chow (Lab Diet manufactured by PMI Nutrition International LLC, Brentwood, MO) and tap water *ad libitum*. The soy-reduced diet was given to minimize background-level exposure to estrogenic compounds [113] while studying the effects of DEHP. All procedures in the present study were carried out in accordance with the guidelines of the Rutgers University Animal Care and Facilities Committee.

Dose and Treatments

Timed-pregnant female rats were randomly divided into treatment (DEHP) and control groups. The females in the treatment group received 500 mg/kg/day of DEHP in 1 ml/kg vehicle, while the control animals only received vehicle (ethanol:oil; 1:9). DEHP (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO) and tocopherol-stripped corn oil (MP Biomedicals, Solon, OH) was used as the vehicle.

To precisely control the dosage, the respective doses were administered by interaperitoneal (ip) injections. Humans are exposed to a wide range of doses of DEHP which can be 31.6 μg/kg/day or >1000 μg/kg/day [92, 93]. 500 mg/kg/day dose of DEHP is known to induce adverse effects in the reproductive system of male offspring rats without causing overt maternal toxicity [114]. It was also the lowest observable adverse effect level (LOAEL) in some ovarian studies [66, 105]. Hence this dose was used.

As shown in Figure 1, the pregnant rats were treated from E 11 until the birth of offspring. The day of birth was designated as PND 0. Both male and female offspring were treated via subcutaneous injection daily from PND 0 –7 (Fig. 1). Offspring of both sexes were treated to eliminate maternal bias towards her offspring. The first injection was within the first 24 h following birth.

Assessment of female reproductive function

Anogenital distance (AGD) was measured at birth (i.e. within 24 hours of birth) for all the males and females pups born. AGD was defined as the distance in millimeters (mm), between the anus and genital tubercle. After weaning (PND 28), two females per dam were separated and maintained for the study. They were monitored for vaginal opening starting from the day of weaning to determine the age at puberty. Estrous cyclicity was determined by performing vaginal smears starting on day of puberty. The classification of proestrus, estrus, and diestrus was based on their respective characteristic vaginal cytology. A vaginal smear on the day of proestrus consisted of nucleated mostly rounded epithelial cells with granulated cytoplasm. Estrus was characterized by the presence of keratinized vaginal epithelial cells while presence of leukocytes dispersed among few epithelial cells with clear cytoplasm was typical of diestrus. Inter-estrus interval of 4-5 days was considered as ‘normal’ estrous cycle. Inter-estrus interval greater than 5 days was categorized as prolonged estrous cycle. Persistent estrus and persistent diestrus was

defined as such if the vaginal cytology typical of estrus or diestrus respectively was demonstrated for five or more consecutive days [15].

For mating, individual females were placed with an untreated/treated male overnight on their third proestrus day. A sperm-positive vaginal smear on the following day was considered a successful mating. Females displaying a vaginal cytology with characteristics of diestrus up to and including the 7^{th} day after mating were considered pregnant, and the pregnancy was subsequently confirmed with a live delivery of the litter. In our rat colony, gestation length is 21 days and delivery normally occurs on the 22^{nd} day (sperm-positive vaginal smear day = E 0). The litter size, sex ratio and anogenital distance were examined on the day of birth (PND 0). To determine the pregnancy rate upto three mating attempts were done. If the female delivered litter/s she was not mated again.

Measurements for all of the reproductive parameters mentioned above (except AGD) were repeated with total of 2 sets of animals to ensure consistency and reproducibility of the data. Set 1 consisted of 15 control and 18 DEHP exposed females. Of the 15 control female rats, 10 rats (each from a different dam) were mated and 5 rats (each from a different dam) were dissected for collection of ovary and serum collection. Of the 18 DEHP exposed female rats, 11 rats (each from a different dam) were mated and 7 rats (each from a different dam) were dissected for collection of ovary and serum collection. Set 2 consisted of 13 control and 16 DEHP exposed females. Of the 13 control female rats, 6 rats (each from a different dam) were mated and 7 rats (each from a different dam) were dissected for collection of ovary and serum collection. Of the 16 DEHP exposed female rats, 8 rats (each from a different dam) were mated and 8 rats (each from a different dam) were dissected for collection of ovary and serum collection. Pubertal age and % normal estrous cycle was measured for 15 control females from set 1, 13 control females from set 2 and 18 DEHP exposed females from set 1, 16 DEHP exposed females from set 2. However, due to time constrains data from only set 1 was analysed for all the parameters mentioned here onwards. AGD was analysed only for set 2 rats.

Serum and ovary collection

While some of the littermates were mated to determine the pregnancy rate and litter size, others were dissected on the day of proestrus between PND 50 and 85. One rat pup per dam was dissected. The blood was collected for measuring serum hormone levels and the ovaries were collected for histology and immunohistochemistry (IHC). The collections were conducted the morning of proestrus between 0900 h and 1200 h.

Ovarian histological analysis

The ovaries were cleared of the surrounding fat tissue and bursa and placed in Bouin's fixative (Sigma-Aldrich) overnight at room temperature. They were then processed (dehydrated), embedded in paraffin, and sectioned at 5µm. Three consecutive sections ~80 to 100 µm apart at the largest cross-sectional area were processed for hematoxylin and eosin (H and E) staining. The H-E sections were imaged using light microscopy to determine size of the ovary and follicle numbers and stages in the ovary. Ovaries from 5 rats from control and 7 rats from the DEHP group were analyzed. Each rat was from a separate dam. The follicles were classified into primordial, primary, secondary, preantral, early antral, mid/late antral and atretic follicles. The number of follicles and corpora lutea (CL) were counted, their percentages of the total follicle number was calculated and averaged. The follicles were essentially classified according to the previously published studies [5] and as described in [60]. Atretic follicles showing oocyte fragmentation and nuclear envelope breakdown, as described by Osman [116] were also counted.

Radioimmunoassay

Trunk blood was collected, kept at 4°C for 2 h. The blood was centrifuged at approximately 400xg for 15 minutes for serum separation and the serum was stored at −80°C until used for measuring the hormone concentrations. Hormones were measured in 100 µl serum

samples using commercially available radioimmunoassay (RIA) kits, according to the manufacturer's instructions. Serum concentrations of progesterone (P4) and estradiol (E2) were measured using Coat-A-Count RIA kits (Siemens Medical Solutions). Radioactivity was measured in counts per minute (cpm) and the standard curve was used to generate serum concentration values nanograms per milliliters (ng/ml) for P4 (sensitivity for the P4 RIA assay was 0.02 ng/ml) and picograms per milliliters (pg/ml) for E2 (sensitivity for the E2 RIA assay was 8 pg/ml).

Immunohistochemistry

The paraffin sections were deparaffinized in Citrisolv (D-limonene, Fisher) and rehydrated in PBS for 10 min. Antigen retrieval was performed by microwaving slides in 0.01 M sodium citrate buffer (pH 6.0) for 5 min (ERα, LHR and AR) and 15 min (MIS and P450scc). The sections were blocked with non-immune serum (goat; 1.5%, donkey; 1.5%) for 30 min and incubated with the primary antibodies at room temperature overnight in a humidified chamber. Primary antibodies included MIS (sc-6886); P450scc (sc-18043); ERα (sc-542); and LHR (sc-25828) from Santa Cruz Biotechnology and AR (Affinity BioReagents: PA1-111A). Biotinylated anti-rabbit secondary antibodies (Santa Cruz) were added at 1:200 dilution and incubated for 60 min at room temperature. Detection was with streptavidin-FITC (Vector Laboratories). All sections were counterstained with ethidium homodimer-2 (EthD-2, Invitrogen; not shown). After three washes in PBS, slides were mounted in Prolong Gold Anti-fade reagent (Invitrogen). Negative controls sections were treated similarly, except primary antibody was replaced with buffer solution. Sections were analyzed and imaged using a Nikon Eclipse E800 microscope with epifluorescence attachments and suitable filters for streptavidin-FITC (green) and EthD-2 detection (red). Images were acquired with a Nikon DXM1200F camera with ACT1 software (Version 2) at equal exposure levels. Minimal and equal adjustments, if any, for only brightness were made on all images with Adobe Photoshop CS and quantified.

Quantification of immunohistochemical staining intensity

Mean staining intensity per unit area of selected structures or the entire section for each marker was determined using ImageJ software (NIH, http://rsb.info.nih.gov/ij/). The polygonal selection tool was used to select the respective structures as described below. Ovaries from 5 rats each from a separate dam (N = 5) for control and N = 6 or 7 rats each from a separate dam, for DEHP group were analyzed. For androgen receptor (control - N = 5, follicles = 88; DEHP - N = 6, follicles = 81) and MIS (control - N = 5, follicles = 46; DEHP - N = 7, follicles = 38) the mean staining intensity was determined for granulosa cells of each follicle, excluding oocyte, antral space, and thecal layer in randomly selected preantral or early antral stage follicles. For MIS additionally the mean staining intensity of the primary follicle (control - N = 5, follicles = 50; DEHP - N = 7, follicles = 51) was also measured. For ERα (control - N = 5, follicles = 102; DEHP - N = 7, follicles = 118) the mean staining intensity per unit area for the thecal layer surrounding randomly selected large antral follicles was determined. For P450scc and LHR, two separate analyses were made. The mean staining intensity per unit area (a) for the thecal cells (P450scc: control - N = 5, follicles = 46; DEHP - N = 7, follicles = 35; LHR: control - N = 5, follicles = 96; DEHP - N = 7, follicles = 77) and (b) for individual CL (P450scc: control - N = 5, follicles = 38; DEHP - N = 7, follicles = 45; LHR: control - N = 5, follicles = 34; DEHP - N = 7, follicles = 46) were determined.

Statistical analysis

Age at puberty, litter size, pregnancy rate, serum hormone levels, ovarian weight, intensity of IHC staining and regularity of estrous cycles were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). The values were expressed as the mean ± SEM. Statistical analysis was performed using unpaired *t* test and 2- way ANOVA (ovarian histological analysis data). A statistically significant difference was confirmed at $P < 0.05$.

Results

Reproductive parameters

To determine whether developmental exposure to DEHP causes changes in reproductive function, various reproductive parameters were measured. The AGD measured at PND 0 was significantly reduced in both males and females (Fig. 2). Control females had AGD of 2.17 ± 0.02 mm (N = 22 pups, from 13 dams) while DEHP-treated females had 2.08 ± 0.03 mm (N = 18 pups from 16 dams; $P < 0.05$). In males the AGD was reduced from 3.97 ± 0.05 mm in controls (N = 18 pups from 13 dams) to 3.59 ± 0.06 mm (N = 17 pups from 16 dams) in DEHP group ($P < 0.001$). The control animals had an average pubertal age of PND 37.13 ± 0.31, (N = 28 pups from 14 dams, 2 from each dam) while it was delayed in DEHP animals (PND 39.18 ± 0.40; N = 34 pups from 17 dams, 2 from each dam; $P < 0.005$; Fig. 3). There was no significant difference in the estrous cyclicity (expressed as % normal cycles) between the control and the treatment animals (Fig. 4). The control females showed 77.34 ± 4.5 % normal estrous cycles (N = 28 from 14 dams, 2 from each dam), while the DEHP females showed 73.35 ± 5.9 % normal estrous cycles (N = 34 pups from 17 dams, 2 from each dam). One female from each dam was mated. All females that were mated in both control and DEHP groups became pregnant and delivered litters, except one from control and one from DEHP group which did not get pregnant/delivered litters. Exposure to DEHP did not cause any significant change in litter size (Fig. 5) and the sex ratio (not shown) at birth, as compared to control animals.

Serum estradiol and progesterone levels

Serum was collected from females on proestrus and P4 and E2 levels were measured using RIA (Fig. 6). DEHP significantly decreased the E2 levels (control; 22.37 ± 4.6 pg/ml; DEHP;10.65 ± 2.5 pg/ml; $P < 0.01$) without affecting the P4 levels (control; 12.97 ± 0.5 ng/ml; DEHP; 7.77 ± 2.0 ng/ml).

Developmental exposure to DEHP altered ovarian follicular dynamics

To investigate the ovarian basis of the alterations in reproductive parameters, we examined ovarian histology between PND 75 and 85. Both the control and the DEHP-treated ovaries contained follicles at all developmental stages (Fig. 7). The DEHP-treated ovaries appeared smaller in size as compared to control ovaries (statistically not significant).

Developmental exposure to DEHP resulted in a significant decrease in the percentage of primordial follicles (control; 44.84 ± 1.0%; DEHP; 39.59 ± 2.0%; $P < 0.05$) and the primary percentage of follicles (control; 27.30 ± 1.3%; DEHP; 21.98 ± 1.3%; $P < 0.05$), but there was no significant change in the percentage of secondary follicles (control; 3.40 ± 0.5%; DEHP; 4.78 ± 0.3%). The percentage of preantral (control; 15.32 ± 1.1% DEHP; 19.0 ± 2.3%) remained unchanged, while the early antral follicles (control; 7.39 ± 1.0%; DEHP; 12.76 ± 1.3%; $P < 0.05$) and atretic follicles (control; 7.89 ± 1.0%; DEHP; 14.28 ± 1.6%; $P < 0.05$) were increased in response to developmental exposure to DEHP. However the percentages of the mid/ late-antral follicles (control; 1.75 ± 0.6%; DEHP 1.98 ± 0.2%) and number of CL (control; 32.10 ± 2.5%; DEHP; 37.21 ± 6.5%) remained unchanged. Furthermore, there was a significant decrease in the total number of follicles per ovarian section (control; 40.87 ± 2.0%; DEHP; 30.83 ± 3.0%; $P < 0.05$).These changes in the follicular dynamics suggest a reduced follicular pool with increased atresia.

Developmental exposure of DEHP affected expression of ovarian markers in adult ovary

To further investigate the effects on ovarian development we examined the relative protein expression levels of MIS, LHR, P450scc, ERα and AR via immunohistochemistry followed by image analysis.

MIS is expressed in cumulus granulosa cells of growing follicles - primary, secondary, pre/early antral but not in germ cells or thecal cells [55, 56]. MIS immunoreactivity was abundant

in the primary, preantral and early antral follicles of the DEHP ovaries (Fig 8). Quantification with ImageJ software showed an increase in staining intensities for MIS in preantral/early antral follicles (control; 56.41 ± 3.4; DEHP; 71.78 ± 5.1; $P < 0.05$), in the DEHP-treated ovaries. However, the intensity of MIS expression was not significant different in primary follicles (control; 63.72 ± 9.7; DEHP; 75.10 ± 8.2).

P450scc plays a critical role in steroidogenesis. The expression of P450scc was detected in thecal cells of preantral and antral follicles and in the CL (Fig. 9) as reported by previous studies [78]. There was no significant alteration in the staining intensities for P450scc in either CL (control; 77.87 ± 4.2; DEHP; 84.56 ± 5.1) or the thecal cells of antral follicles (control; 55.54 ± 3.7; DEHP; 57.54 ± 3.8). The thecal cells surrounding large antral follicles, CL and interstitial cells in control and DEHP ovaries showed strong immunoreactivity for LHR (Fig. 10). Intensity of expression of LHR in CL (control; 25.68 ± 4.9; DEHP; 31.28 ± 5.7) and in thecal cells (control; 22.51 ± 2.8; DEHP; 27.6 ± 4.6) was not significantly altered by DEHP exposure.

AR expression was seen in the nuclei of the granulosa layer of preantral and very early antral follicles (Fig. 11) which was consistent with other studies [67]. When the staining intensity in these follicles was quantified it was found to be significantly increased with DEHP exposure (control; 18.7 ± 1.8; DEHP; 25.92 ± 2.1; $P < 0.05$). In the ovaries ERα was expressed in nuclei of thecal cells, interstitial gland cells and germinal epithelium (Fig. 12), as observed by others [83]. DEHP exposure did not affect the expression of ERα (control; 28.29 ± 1.7; DEHP; 34.05 ± 4.1).

Discussion

In this study we showed that exposure to DEHP during a critical ovarian developmental window affected folliculogenesis and expression of some ovarian markers, delays puberty, without affecting the estrous cyclicity and pregnancy outcome. There were effects on steroidogenesis as well, as demonstrated by the reduced serum estradiol levels. In addition, we

observed reduced AGD in both males and females indicative of anti-androgenic effect of DEHP, which is consistent with studies by others [42, 117].

Developmental exposure to DEHP also caused delay in onset of puberty which is consistent with studies of others [103, 109]. Several factors can alter the pubertal timing. However the mechanisms remain unknown.

One of the essential ovarian processes affected by DEHP is folliculogenesis. Developmental exposure to DEHP causes decrease in primordial and primary follicles. A female is born with a finite number of primordial follicles which dictate the reproductive life span of the females. Various paracrine growth factors such as kit ligand, leukaemia inhibitory factor, bone morphogenic proteins, keratinocyte growth factor and basic fibroblast growth factor regulate the assembly of these finite number of primordial follicles [41, 118]. Furthermore, transition from primordial to primary is also regulated in paracrine manner by MIS, growth differentiating factor 9, while others such as BMP15 are required for progression of primary follicles into latter stage follicles [119]. We investigated expression of MIS in primary follicles, which was not significantly altered due to developmental exposure to DEHP. Therefore, other factors need to be analysed to decipher the cause for reduced primordial and primary follicles, in the current study. A recent study shows, transient exposure to DEHP (10 and 30 days), affects PI3 kinase signaling pathway which accelerates the recruitment of primordial follicles into primary follicles, thereby decreasing the number of primordial follicles while increasing the primary follicles [108]. However, developmental exposure from E 11 to PND 7 reduces both primordial and primary follicles suggesting, that the formation of primordial follicles itself may be affected.

There was an increase in early antral follicles without any significant increase in mid/late antral follicles, CL or litter size, which is indicative of inhibition of progression of the follicles. Furthermore there is increase in atretic follicles, suggesting many of these accumulated are undergoing atresia. Of note, the increase in atresia was accompanied by reduction in number of

total follicles per section. Further investigation of expression of ovarian markers revealed increased expression of AR and MIS by the preantral and early antral follicles.

An increase in expression of AR was observed in the nucleus of the granulosa cells. AR typically resides in the cytoplasm and are activated and translocated to nucleus upon binding to androgen. However, since androgen levels were not measured, at this point we cannot determine if the increased nuclear localization of the AR is due to increase in androgen levels. Nevertheless the increased nuclear localization of the AR is suggestive of increased androgen-AR mediated signaling. This increased signaling may have promoted more of preantral follicles to early antral stages via mechanisms described in previous studies [72]. However persistent increased in expression of AR inhibits progression of early antral follicles, as down-regulation of AR is necessary to promote progression from an FSH-androgen regulated growth to an estrogen dominated and LH stimulated follicular growth that will lead to ovulation. AR down-regulation allows most androgens to be utilized for production of estrogens as well as allows LH to suppress FSH induced gene expression while promoting the expression of genes for ovulation. Hence hyper stimulation of androgen-AR signaling inhibits follicular progression leading to atresia [120, 121]. Thus, persistent expression of AR as is observed in the current study, might have contributed to increased follicular atresia.

In adult females, MIS inhibits FSH-stimulated growth of the follicles by attenuating expression of *aromatase* and *lhr*. [59]. The MIS-positive growing follicles contain oocytes arrested in meiosis suggesting that MIS might also act as a regulator of oocyte maturation [122]. Thus MIS plays a critical role in follicular selection for atresia during cyclic recruitment. Increased production of MIS as is observed in the current study may also be the reason for increased follicular atresia. EDC exposure can alter expression of MIS in these growing follicles. An earlier study from our lab had shown that perinatal MXC exposure stimulates excessive production of MIS which inhibits ovarian folliculogenesis in prepubertal and adult rats [15, 60].

Additionally, over expression of AR and MIS may affect production of estradiol which regulates folliculogenesis, thus indirectly promoting follicular atresia. Thus, low estradiol levels in the females developmentally exposed to DEHP could be because of reduced bioavailability of androgens as precursors for estrogen production due to persistent expression of AR and/or due to increased MIS which may have attenuated expression of *aromatase*, the enzyme that converts androgens to estradiol. Since neither androgen levels nor expression of *aromatase* were analysed, the cause for low estradiol levels can only be speculated. Additionally other factors may have contributed to low estradiol levels such as the expression of key enzymes involved in steroid biosynthesis. The present study measured the expression of one such enzyme - P450scc, which was not significantly altered between the control and the DEHP group. Studies in males showed fetal exposure to DEHP suppresses testosterone levels by reducing protein levels of StAR in fetal Leydig cells [81]. Specifically, MEHP a metabolite of DEHP has been shown to increase the expression of P450scc in testes [79, 80]. However, in our study DEHP did not alter the expression of P450scc in the ovaries. Hence we speculate developmental exposure to DEHP modulates steroid biosynthesis without affecting the expression of P450scc in the ovaries.

In summary, all of these factors collectively cause significant number of follicles to undergo atresia. These results are consistent with other studies. Other studies have reported increased depletion of primordial follicular pool resulting in accumulation of antral follicles [9]. Increase numbers of atretic follicles were observed in rats exposed to 405 mg/kg DEHP from E 6 to PND 21 [105, 123]. Increased granulosa cell apoptosis [106], inhibition of antral follicle growth due to production of reactive oxidative species and decrease in expression and activity of superoxide dismutase-1, one of the antioxidant enzymes are some of the effects of DEHP exposure [30]. Thus DEHP may act at multiple levels to induce follicular atresia.

Interestingly, the attenuated serum estradiol levels and disrupted follicular dynamics did not affect expression of ERα and LHR in the present study. However intra-ovarian estradiol levels were not measured. Granulosa cell-derived estrogens act via ERα to modulate thecal cell

steroidogenesis via a short negative-feedback loop within the follicle. Peri-pubertal and pre-pubertal exposure of DEHP (0.5 mg/kg/day, 5 mg/kg/day) significantly increased the transcripts of *Lhβ* in the pituitary while decreasing the transcript levels of *Fshr* and *Lhr* in the ovaries of mice at PND 21[103]. Similarly, the ovaries analyzed from mice (PND 56) exposed to 500 mg/kg/day and 1000 mg/kg/day of DEHP during late gestation also reported 50% reduction in *lhcgr* transcript levels in ovaries [66]. However in the present study, there was no difference in the expression of LHR protein, CL numbers and litter size, between the control and the DEHP group, suggesting that at young adulthood, developmental exposure to DEHP affects the ovarian dynamics without altering ovulation or pregnancy outcome. However these might be altered in aged animals.

Collectively these data suggests developmental exposure to DEHP may delay puberty and reduce the follicular pool available for the reproductive function, resulting in a shorter reproductive life span.

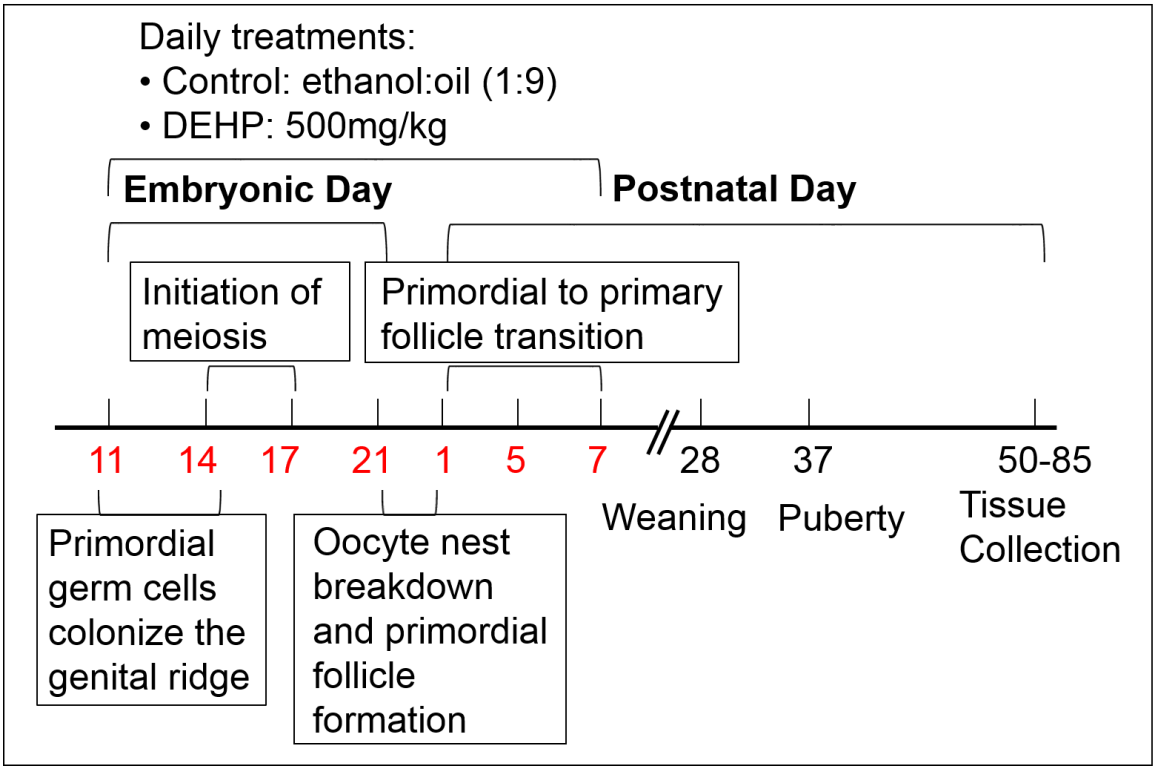


Figure 1. Timeline for treatments and critical ovarian development and experimental design: The numbers designate time points in days. The period of exposure to DEHP is indicated by numbers in red (E 11 – PND 7) which encompasses critical event in ovarian development. Pregnant dams are injected with either the control or the treatment from E 11 until litters are born (PND 21) and then the litters are injected upto PND 7. Upon reaching puberty, their estrous cycle was followed. Some litters were mated and others disected for ovary and serum collection.

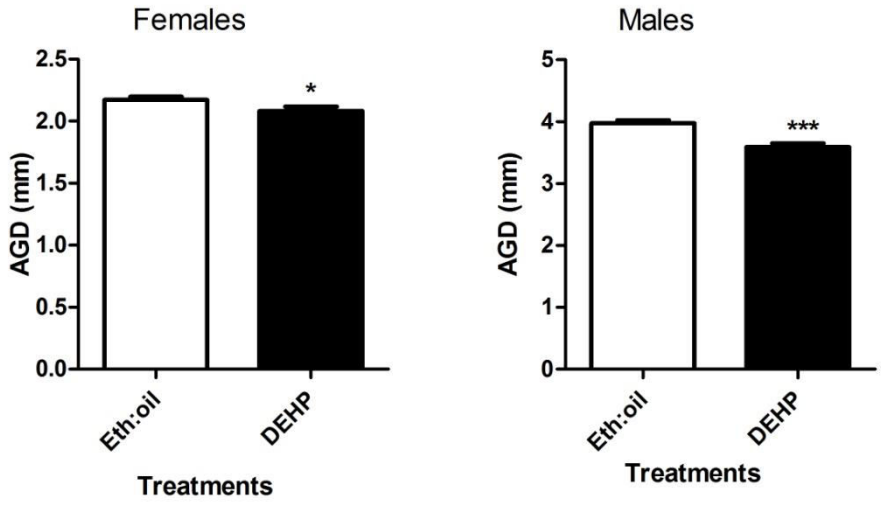


Figure 2. Effect of developmental exposure of DEHP on anogenital distance (AGD): At PND 0 AGD was significantly reduced in males ($P < 0.001$) and females ($P < 0.05$). The AGD was significantly decreased in both DEHP-treated males [3.97 ± 0.05 mm in controls (N = 18 pups from 13 dams) to 3.59 ± 0.06 mm (N = 17 pups from 16 dams) in DEHP group ($P < 0.001$)] and DEHP-treated females [control 2.17 ± 0.02 mm (N = 22 pups, from 13 dams) while DEHP-treated females had 2.08 ± 0.03 mm (N = 18 pups from 16 dams; $P < 0.05$)].

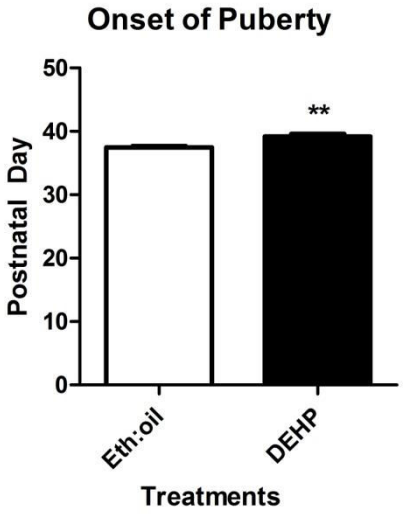


Figure 3. Effect of developmental exposure of DEHP on the onset of puberty. Pubertal onset was determined by the day of vaginal opening. Significant delay in the onset of puberty, in DEHP-treated females (PND 39.18 ± 0.40; N = 34 pups from 17 dams, 2 from each dam) compared to control females (N = 28 pups from 14 dams, 2 from each dam; ***= $P < 0.005$) was observed.

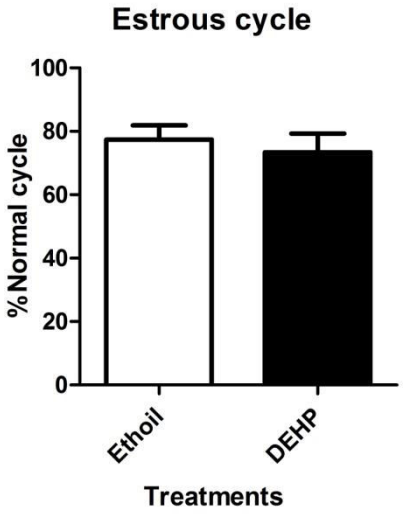


Figure 4. Effect of developmental exposure of DEHP on estrous cyclicity. Stage of estrus was recorded based on vaginal cytology described in materials and methods. Rats typically showed 4-5 day estrous cycle. Percentage of normal cycles (non-persistent estrus/diestrus or non-irregular) was calculated and there was no significant difference in the percentage of normal cycles, between the control and the DEHP group. The control females showed 77.34 ± 4.5 % normal estrous cycles (N = 28 from 14 dams, 2 from each dam), while the DEHP females showed 73.35 ± 5.9 % normal estrous cycles (N = 34 pups from 17 dams, 2 from each dam).

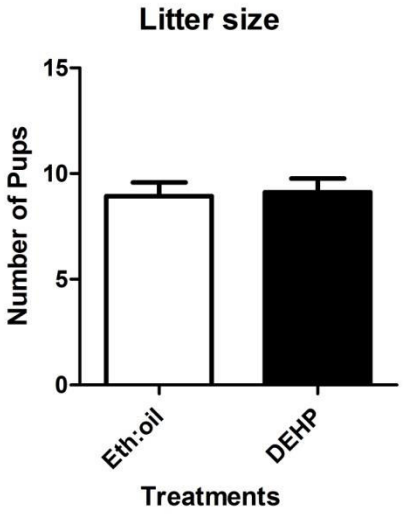


Figure 5. Effect of developmental exposure of DEHP on litter size. No significant change in the number of pups born to F1 females that were developmentally exposed to either the control (control; 9.00 ± 0.9; N = 15, each from a separate dam) or the treatment (DEHP; 9.36 ± 0.81; N = 18, each from a separate dam), was observed.

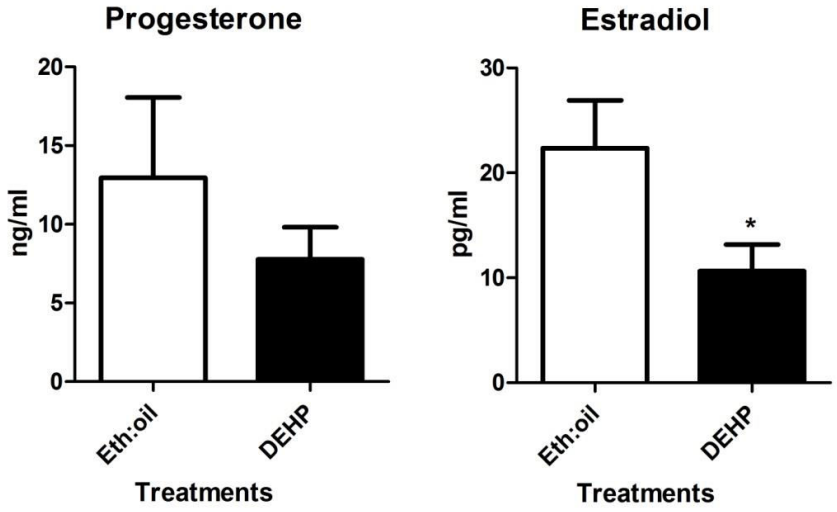


Figure 6. Effect of developmental exposure of DEHP on serum concentration of estradiol and progesterone. Serum was collected on the day of proestrus. Significant decrease in the serum estradiol levels of the DEHP-treated females was noted (control; N = 5; DEHP; N = 7; each from a separate dam; $P < 0.05$). A similar trend was seen for the serum progesterone levels.

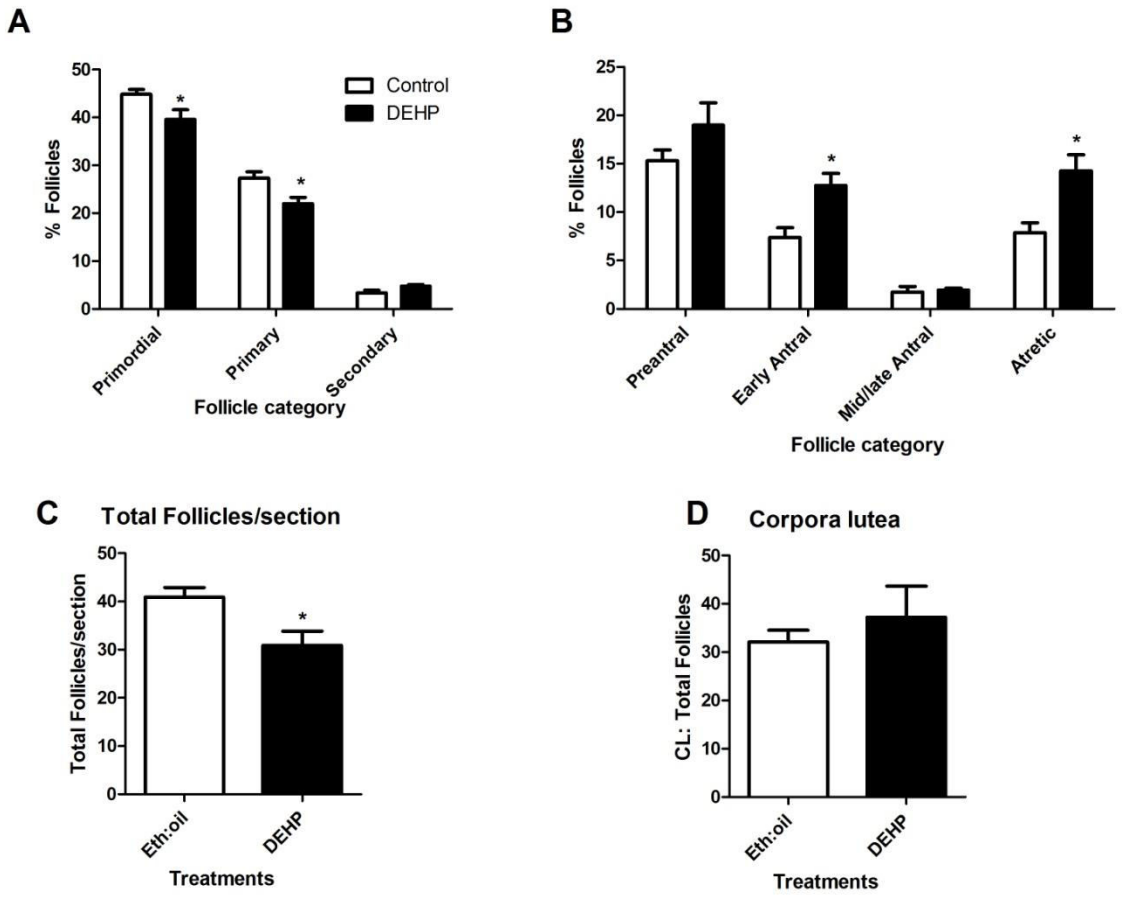


Figure 7. Effect of developmental exposure of DEHP on folliculogenesis. The ovaries were collected between PND 72 and 84 processed for histological evaluation of follicle counts. Percentages of each follicle type were calculated and compared in each treatment group (control; N = 5 and DEHP; N = 7, each from a separate dam). The results show a significant decrease in the number of primordial and primary follicles but no change in in the number of secondary (A).The number of preantral follicles and mid/late antral follicles were not altered, while the number of early antral and atretic follilces were increased (B). Furthermore, there is significant decrease in the number of total follicles (TF) per ovarian section (C). However the number of CL remained unaltered (D) ***=** $P < 0.05$

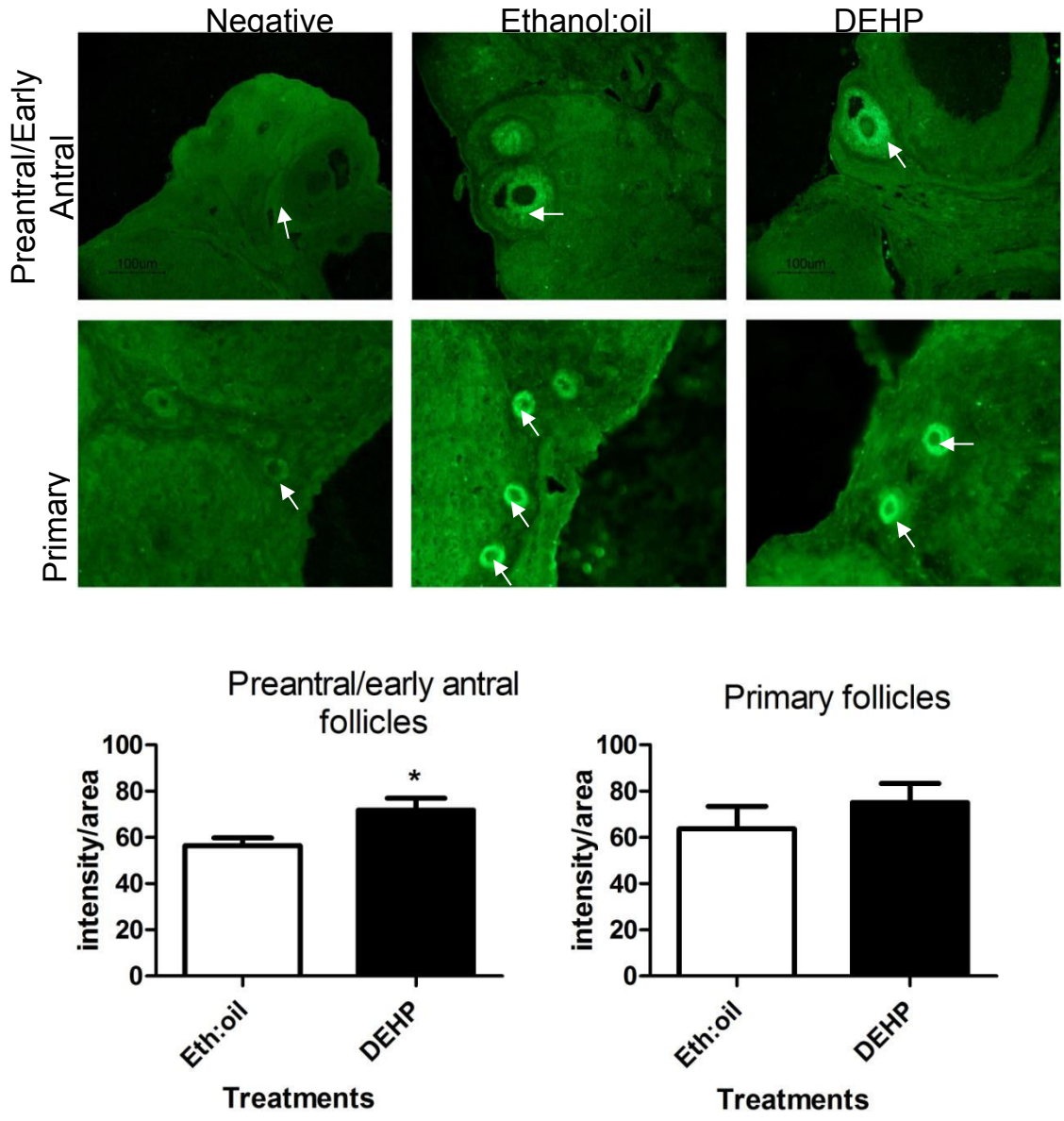


Figure 8. Effect of developmental exposure of DEHP on expression of MIS protein in rat ovaries. Immunolocalisation of MIS was observed in cumulus granulosa cells of growing follicles- primary, secondary, pre/early antral follicles (arrows), from the ovaries of control and DEHP animals. Quantification using ImageJ (NIH) software showed significant increase in expression of MIS (intensity/area) specifically in pre/early antral follicles, while a trend for increase in expression of MIS (intensity/area) in primary follicles (arrows) of DEHP-treated females as compared to control females was observed (control; N = 5 and DEHP; N = 7; each from a separate dam; $P < 0.05$).

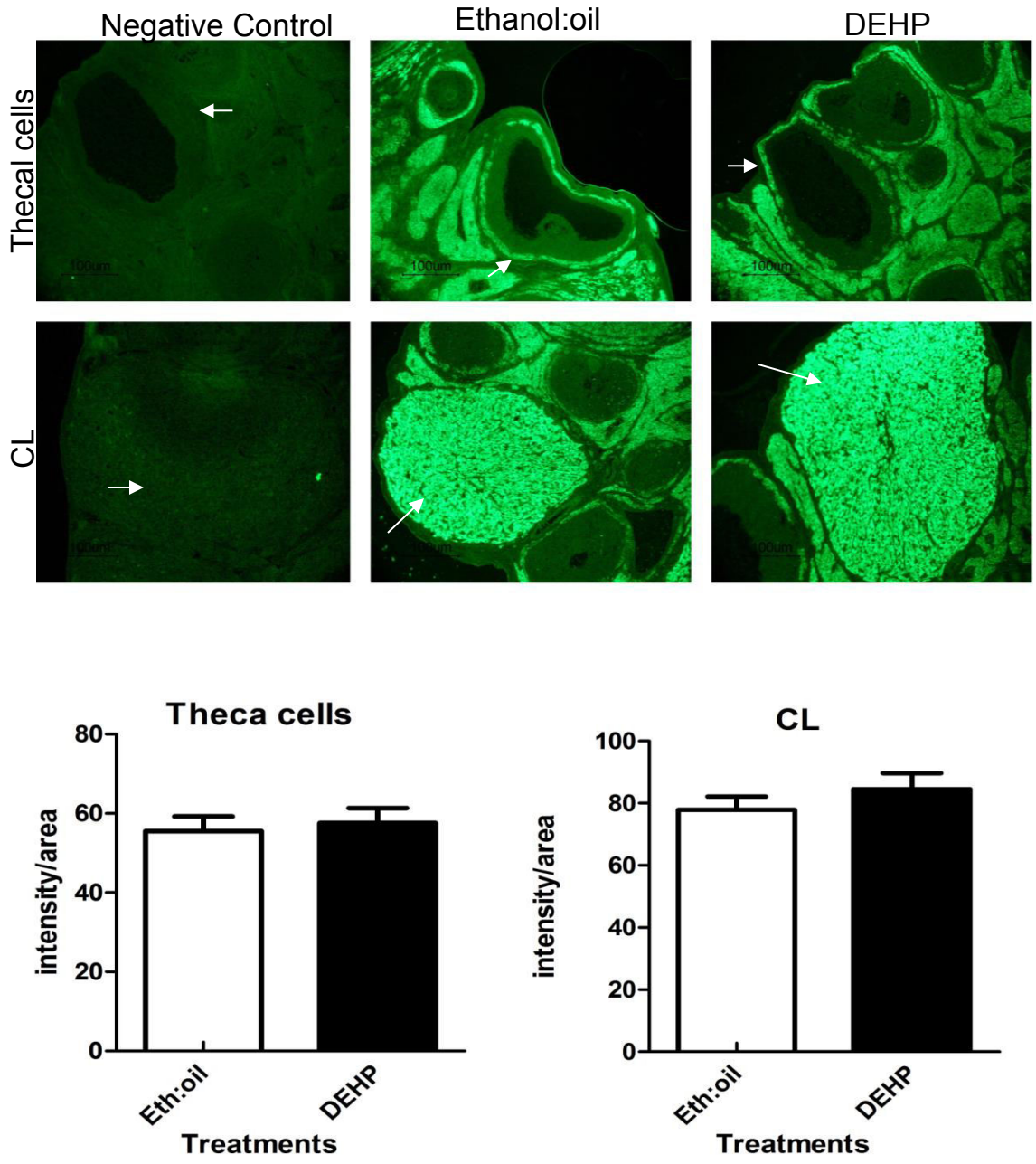


Figure 9. Effect of developmental exposure of DEHP on expression of P450scc in rat ovaries. Immunolocalisation of P450scc was observed in thecal cells (arrows) of the follicles and corpus luteum from the control and DEHP ovaries. There was no significant change in expression of P450scc (intensity/area) in either thecal cells of the ovarian follicles or the corpus luteum, when analyzed using ImageJ (NIH) software (control; N = 5 and DEHP; N = 7; each from a separate dam).

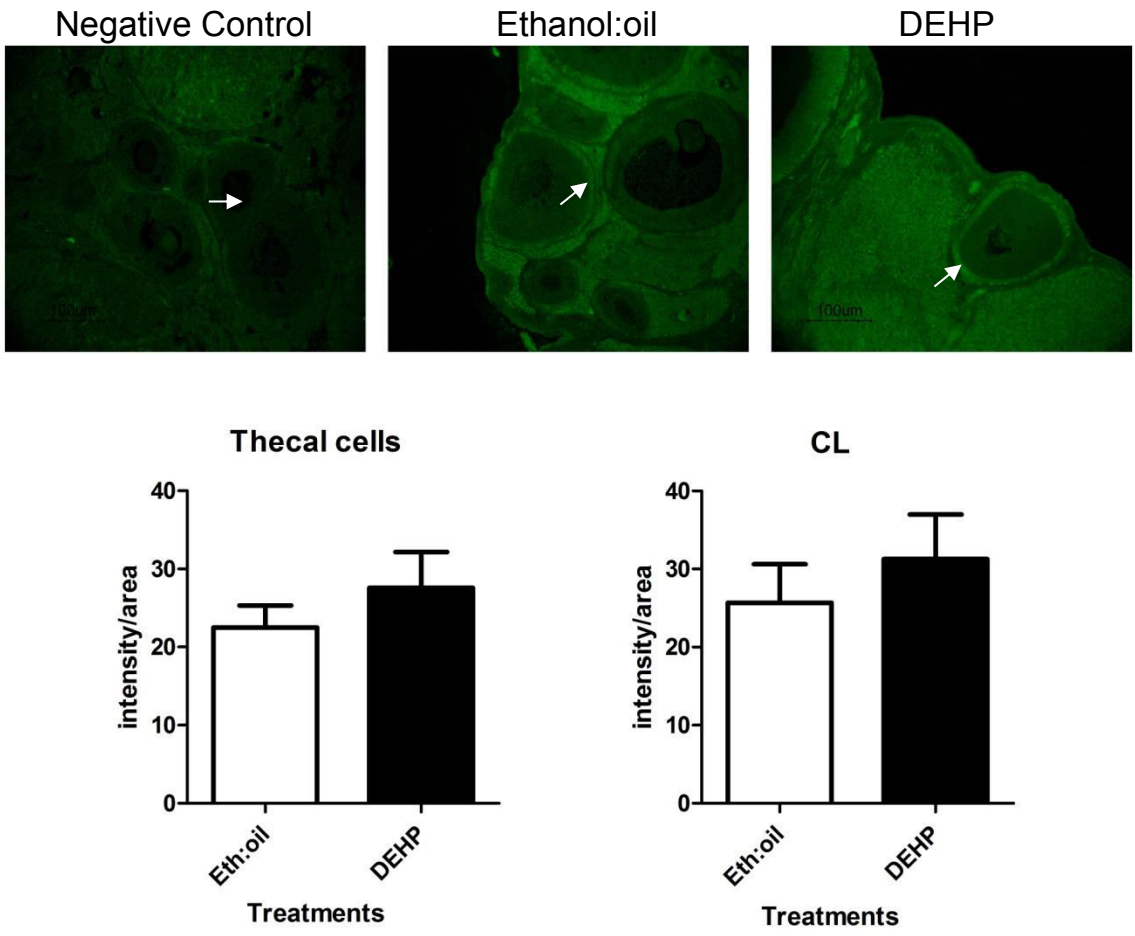


Figure 10. Effect of developmental exposure of DEHP on expression of LHR in rat ovaries. Immunolocalisation of LHR was observed in thecal cells (arrows) surrounding large antral follicles and the interstitial cells in control and DEHP ovaries. There was no significant change in expression of LHR (intensity/area) in thecal cells of the ovarian follicles, when analyzed using ImageJ (NIH) software (control; N = 5 and DEHP; N = 7; each from a separate dam).

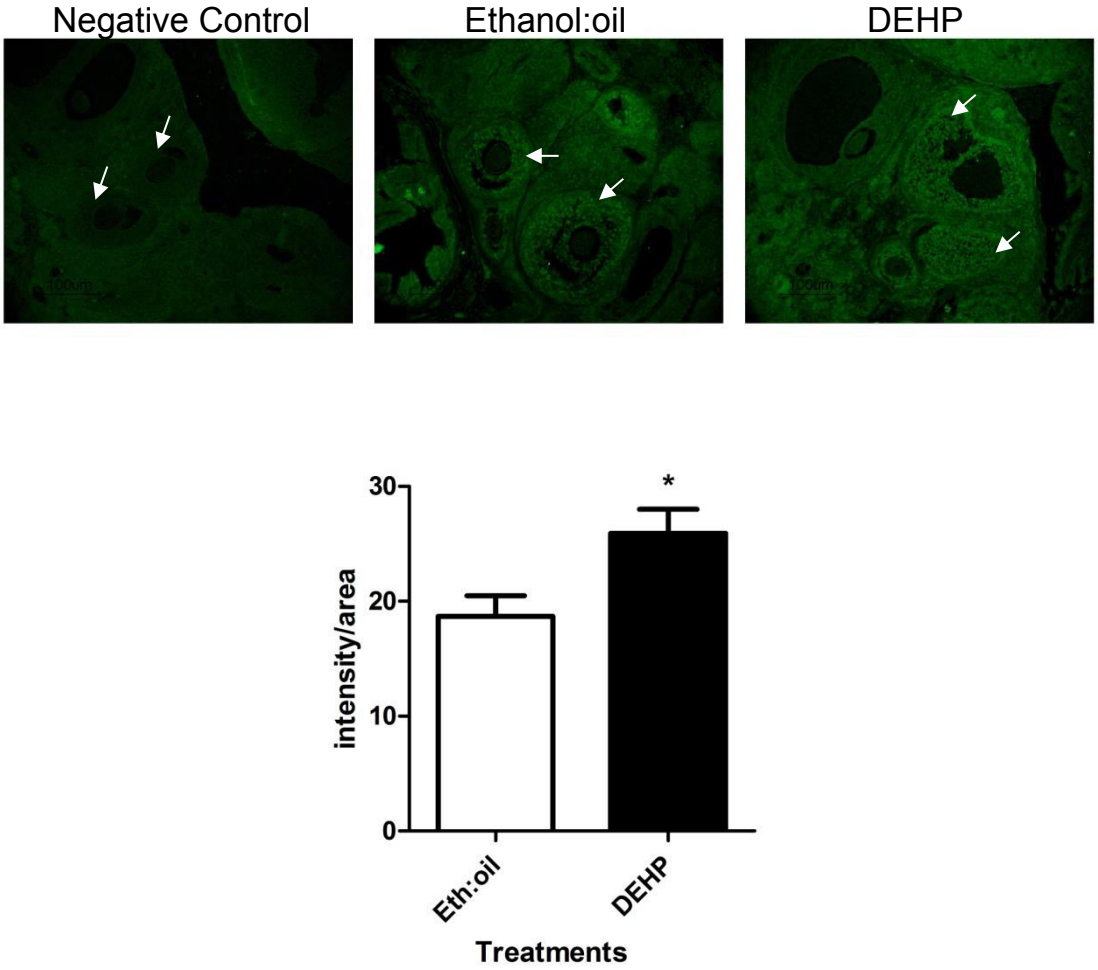


Figure 11. Effect of developmental exposure of DEHP on expression of AR in rat ovaries. Immunolocalisation of AR was noted in the nuclei of the granulosa cells of the growing (preantral/early antral) follicles (arrows). Quantification using ImageJ (NIH) software showed significant increase in expression of AR (intensity/area) in these follicles from DEHP-treated females as compared to control females was observed (control; N = 5 and DEHP; N = 6; each from a separate dam; $P < 0.05$).

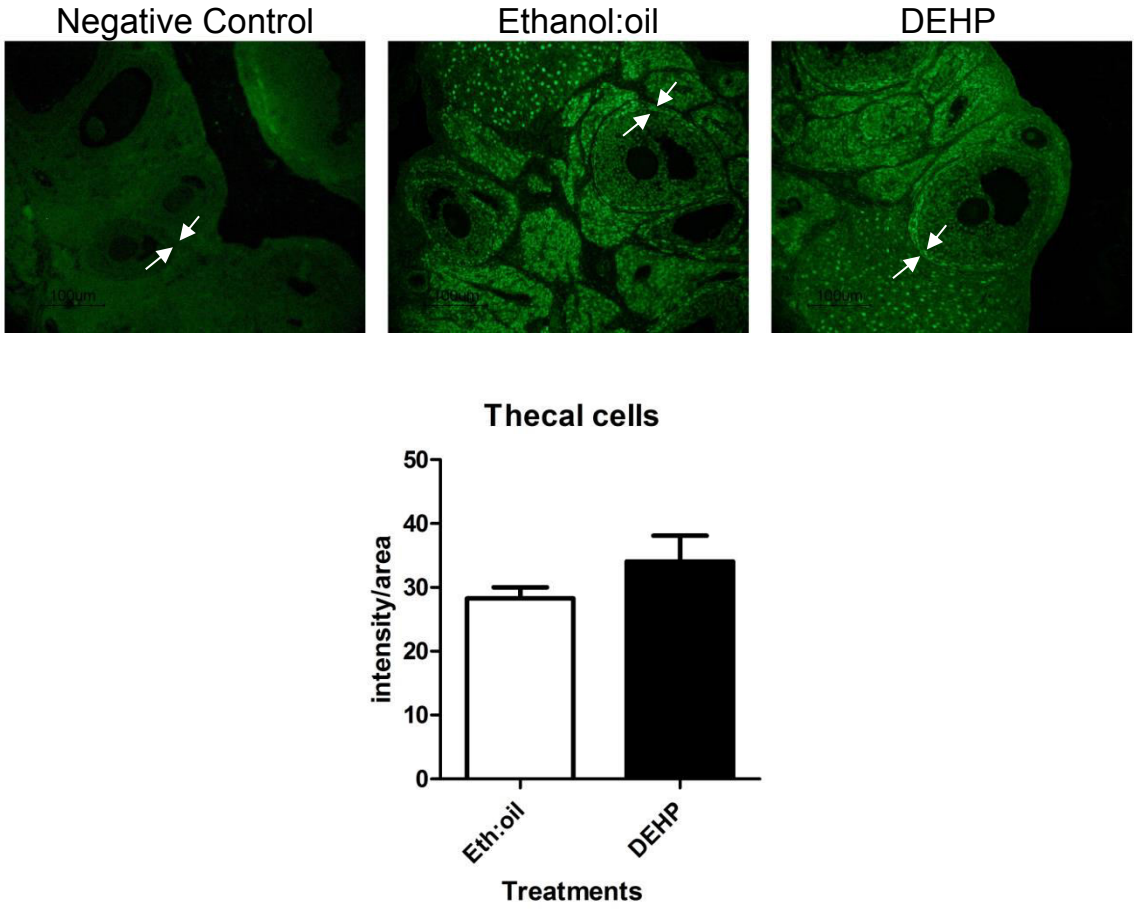


Figure 12. Effect of developmental exposure of DEHP on expression of ERα in rat ovaries. Immunolocalisation of ERα was observed in nuclei of thecal cells (arrows), interstitial gland cells and germinal epithelium in the control and DEHP ovaries. There was no significant change in expression of ERα (intensity/area) in thecal cells of the ovarian follicles, when analyzed using ImageJ (NIH) software (control; N = 5 and DEHP; N = 7; each from a separate dam).

CHAPTER 3:

TRANSGENERATIONAL EFFECTS ON OVARIAN FOLLICULAR DYNAMICS DUE TO DEHP EXPOSURE DURING CRITICAL OVARIAN DEVELOPMENTAL TIME WINDOW

Introduction

Prenatal exposure to environmental stressors such as EDCs can modify normal cellular and tissue development and alter developmental programming such that the individuals may have a higher risk of organ pathologies and metabolic and hormonal disorders later in life. This concept of 'fetal origin of adult disease' is based on the paradigm of "developmental origins of health and disease" (DOHaD) which was first proposed by the late David Barker in 1997 [88]. The developing organism is more vulnerable to the disturbances caused by EDCs because mechanisms such as DNA repair, a competent immune system, detoxifying enzymes, liver metabolism, and the blood/brain barrier that are present in the adult are not fully functional in the fetus or newborn. Additionally, the developing organism has an increased metabolic rate as compared to an adult which, in some cases, may cause increased toxicity [89]. Since each organ system has a different developmental trajectory, and the sensitive window for exposures to cause toxicity varies with the tissues, the effects of exposures are dependent not only on the dose of the chemical, but also when the exposure occurs.

Developmental exposure or exposure during the critical developmental time window of organogenesis can cause changes in gene expression, protein activity, and cell communication which could have life-long implications. For example *in utero* (E11 until birth) BPA exposure inhibits germ cell nest breakdown by increasing the expression of selected anti-apoptotic factors and decreasing the expression of selected pro-apoptotic factors which may lead to premature ovarian failure, reducing female fertility with age [14].

Furthermore, developmental exposure of EDCs can have transgenerational (TG) effects. When a pregnant female is exposed to EDCs or other stressors, it not only affects herself (F0), but also the next two generations (F1 and F2) through the fetus and its germline. When subsequent generations (F3 and beyond) are affected, then, it can be considered as a TG effect [19]. Male mice (F0) exposed to DEHP from embryonic day 7 (E 7) to E 14 exhibit disrupted testicular germ cell organization and spermatogonial stem cell function. This effect is TG and F3 male offspring

also demonstrate the same physiology [31]. Therefore, in the current study we investigated the F3 generation females for any transgenerational effect of DEHP exposure during the critical ovarian developmental time window.

Studies have shown physiological effects of *in utero* and/or neonatal exposure could be due to DNA mutations or due to modifications in gene expression which are independent of mutations (*i.e.*, epigenetic effects). Epigenetic modifications or epimutations include changes in DNA methylation, histone modifications and those that are induced by non-coding RNA [91]. All of these play a critical role in ovarian development. In addition, in the germ cells undergoes specific epigenetic reprogramming during development. In germ cells, DNA demethylation occurs during gonadal differentiation, while remethylation in male germ cells takes place *in utero* around E14 and that in females it occurs postnatally, starting between PND 1 and 5 and continues throughout the oocyte growth until the preantral follicle stage [8, 124]. Exposures to EDCs can alter these epigenetic mechanisms in germ cells resulting in TG pathologies in various tissues such as the ovaries, testes and other organs, inducing functional changes leading to disease later in life and in future generations [18].

Therefore the current study aims to investigate if DEHP exposure during both *in utero* and postnatal development encompassing germ cell epigenetic reprogramming, induces TG epigenetic effects in females and to investigate the role of the female germ line in transmitting the TG effects. The present study was designed specifically to investigate the inheritance of epimutations, if any, specifically through either the maternal germline (maternal cross; MC) or both maternal and paternal germline (double cross; DC). Hence we investigated various reproductive parameters, ovarian follicular dynamics and the expression of ovarian markers in the F1 (as detailed in chapter 1) and in F3 females (current chapter).

Materials and methods

Animals

Timed-pregnant Fischer (CDF) inbred rats were obtained from Charles River Laboratories (Wilmington, MA). The inbred Fischer strain was used since it has minimal polymorphisms, which facilitates the detection of treatment effects. The animals were maintained in a room with controlled illumination (lights on 0700 h – 2100 h), temperature (26–28ºC), and humidity (30–70%). The rats were provided soy-reduced scientific diet 5V01 rat chow (Lab Diet manufactured by PMI Nutrition International LLC, Brentwood, MO) and tap water *ad libitum*. The soy-reduced diet was given to minimize background-level exposure to estrogenic compounds [113] while studying the effects of DEHP. All procedures in the present study were carried out in accordance with the guidelines of the Rutgers University Animal Care and Facilities Committee.

Dose, treatments and mating protocol

The timed-pregnant female rats were randomly divided into treatment (DEHP) and control groups. The females in the treatment group received 500 mg/kg/day of DEHP in 1 ml/kg vehicle, while the control animals only received vehicle (ethanol:oil; 1:9; control). DEHP (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO) and tocopherol-stripped corn oil (MP Biomedicals, Solon, OH) was used as the vehicle.

The rats were treated as shown in Fig. 1 and as described in the materials and methods of Chapter 1. As shown in Fig. 1, the pregnant rats (F0) were treated from E 11 until the birth of offspring (F1). To precisely control the dosage, ip injections of the respective doses were administered. The day of birth was designated as PND 0. The first injection was within the first 24 h following birth. Both male and female F1 offspring were treated via subcutaneous injection daily from PND 0 –7 (Fig. 1). Developmentally treated rats were breed to obtain the subsequent generations as illustrated in Fig. 13. The F1 females were divided into two groups, double cross (DC) and maternal cross (MC). The F1, non-sibling treated males were used for mating with the F1 females in the DC group while age-matched untreated males were used for mating with the F1 females in the MC group. The subsequent generations – F2, F3, were not treated and bred within

their respective groups. For example, F2 females from DC were mated with non-sibling F2 males from DC. Non–sibling mating was practiced to avoid consanguinity.

Assessment of female reproductive function

On the day of weaning (PND 28) two females per F0/F1/F2/F3 dam were separated and maintained for the study. They were monitored for vaginal opening starting from the day of weaning to determine the day of onset of puberty. Estrous cyclicity was determined by performing vaginal smears starting on day of puberty. The classification of proestrus, estrus, and diestrus was based on their respective characteristic vaginal cytology. A vaginal smear on the day of proestrus consisted of nucleated mostly rounded epithelial cells with granulated cytoplasm. Estrus was characterized by the presence of keratinized vaginal epithelial cells while presence of leukocytes dispersed among few epithelial cells with clear cytoplasm was typical of diestrus. Inter-estrus interval of 4-5 days was considered as 'normal' estrous cycle. Inter-estrus interval greater than 5 days was categorized as prolonged estrous cycle. Persistent estrus and persistent diestrus was defined as such if the vaginal cytology typical of estrus or diestrus respectively was demonstrated for five or more consecutive days [15].

For mating individual females were placed with an untreated/treated male overnight on their third proestrus day. A sperm-positive vaginal smear on the following day was considered a successful mating. Females displaying a vaginal cytology with characteristics of diestrus up to and including the 7^{th} day after mating were considered pregnant, and the pregnancy was subsequently confirmed with a live delivery of the litter. In our rat colony, gestation period is ~21 days and delivery normally occurs on 22^{nd} day. The litter size, sex ratio and anogenital distance were examined on the day of birth (PND 0). To determine the pregnancy rate upto three mating attempts were done. If the female delivered litter/s she was not mated again

Serum and ovary collection

While some of the littermates (one female per F1 dam) from F2 and F3 generations were mated to determine the pregnancy rate and litter size, others were dissected on the day of proestrus between PND 50 and 75. The blood was collected for measuring serum hormone levels in the future, and the ovaries were collected for histology and immunohistochemistry (IHC). The collections were conducted the morning of proestrus between 0900 h and 1200 h.

Ovarian histological analysis

The ovaries were cleared of the surrounding fat tissue and bursa and placed in Bouin's fixative (Sigma-Aldrich) overnight at room temperature. In this study ovaries from the F3 generations from both groups were analysed. They were then processed (dehydrated), embedded in paraffin, and sectioned at 5µm. Three consecutive sections ~80 to 100 µm apart at the largest cross-sectional area were processed for hematoxylin and eosin (H and E) staining. The H-E sections were imaged using light microscopy to determine size of the ovary and follicle numbers and stages in the ovary. For MC ovaries from 5 rats (N = 5) from control and 7 rats (N = 7) from the DEHP group were analyzed and for DC ovaries from 5 rats (N = 5) from control and 6 rats (N = 6) from the DEHP group were analyzed. Each F3 rat represented a separate F1 dam. The follicles were classified into primordial, primary, secondary, preantral, early antral, mid/late antral and atretic follicles. The number of follicles and corpora lutea (CL) were counted, their percentages of the total follicle number was calculated and these results were averaged. The follicles were essentially classified according to the previously published studies [125] and as described in [60]. Atretic follicles showing oocyte fragmentation and nuclear envelope breakdown were counted, as described previously [116].

Immunohistochemistry

The paraffin sections were deparaffinized in Citrisolv (D-limonene, Fisher) and rehydrated in PBS for 10 min. Antigen retrieval was performed by microwaving slides in 0.01 M

sodium citrate buffer (pH 6.0) for 5 min (ERα, LHR and androgen receptor) and 15 min (MIS and P450scc). The sections were blocked with serum (goat; 1.5%, donkey; 1%) for 30 min and incubated with the primary antibodies at room temperature overnight in a humidified chamber. Primary antibodies included MIS (sc-6886); P450scc (sc-18043); ERα (sc-542); and LHR (sc-25828) from Santa Cruz Biotechnology and androgen receptor (Affinity BioReagents: PA1-111A). Biotinylated anti-rabbit secondary antibodies (Santa Cruz) were added at 1:200 dilution and incubated for 60 min at room temperature. Detection was with streptavidin-FITC (Vector Laboratories). All sections were counterstained with ethidium homodimer-2 (EthD-2, Invitrogen; not shown). After three washes in PBS, slides were mounted in Prolong Gold Anti-fade reagent (Invitrogen). Negative controls sections were treated identical, except primary antibody was replaced with buffer solution. Sections were analyzed and imaged using a Nikon Eclipse E800 microscope with epifluorescence attachments and suitable filters for streptavidin-FITC (green) and EthD-2 detection (red). Images were acquired with a Nikon DXM1200F camera with ACT1 software (Version 2) at equal exposure levels. Minimal and equal adjustments, if any, for only brightness were made on all images with Adobe Photoshop CS and quantified.

Quantification of immunohistochemical staining intensity

Mean staining intensity per unit area of selected structures of the entire section for each marker was determined using ImageJ software (NIH, http://rsb.info.nih.gov/ij/). For MC ovaries from 5 rats (N = 5) from control and 7 rats (N = 7) from the DEHP group were analyzed and for DC ovaries from 5 rats (N = 5) from control and 6 rats (N = 6) from the DEHP group were analyzed. Each F3 rat represented a separate F1 dam. The polygonal selection tool was used to select the respective structures as described below. For MIS (DC - pre/early antral: control - N = 5, follicles = 67; DEHP - N = 7, follicles = 83; MC - pre/early antral: control - N = 5, follicles = 57; DEHP - N = 7, follicles = 63), the mean staining intensity was determined for granulosa cells of each follicle, excluding oocyte, antral space, and thecal layer in randomly selected preantral or

early antral stage follicles. For MIS additionally the mean staining intensity of the primary follicle (DC – primary: control - N = 5, follicles = 61; DEHP - N = 7, follicles = 81; MC - primary: control - N = 5, follicles = 41; DEHP - N = 7, follicles = 60) was also measured. For ERα (control - N = 5, follicles = 72; DEHP - N = 7, follicles = 84) and the mean staining intensity per unit area for the thecal layer surrounding randomly selected large antral follicles was determined. For P450scc and LHR, two separate analyses were made. The mean staining intensity per unit area (a) for the thecal cells (P450scc: control - N = 5, follicles = 52; DEHP - N = 7, follicles = 47; LHR: control - N = 5, follicles = 86; DEHP - N = 7, follicles = 73) and (b) for individual CL (P450scc: control - N = 5, follicles = 31; DEHP - N = 7, follicles = 38; LHR: control - N = 5, follicles = 34; DEHP - N = 7, follicles = 36) were determined.

Statistical analysis

Age at puberty, litter size, pregnancy rate, serum hormone levels, ovarian weight, intensity of IHC staining and regularity of estrous cycles were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). The values were expressed as the mean ± SEM. Statistical analysis was performed using unpaired *t* test and ANOVA (ovarian histological analysis data). A statistically significant difference was confirmed at $P < 0.05$.

The study was repeated with 2 sets of animals. However, since entire data for F3 animals from set 2 was not available at the time of data analysis, data from only set 1 was analysed for all the parameters (except AGD) mentioned above. AGD was analysed only for set 2 rats. Set 1 DC study consisted of 12 control and 14 DEHP exposed females. Of the 12 control female rats, 6 rats (each from a F2 dam representing a separate F1 dam) were mated and 6 rats (each from a F2 dam representing a separate F1 dam) were dissected for collection of ovary and serum collection. Of the 14 DEHP exposed female rats, 7 rats (each from a F2 dam representing a separate F1 dam) were mated and 7 rats (each from a F2 dam representing a separate F1 dam) were dissected for collection of ovary and serum collection. Set 1 MC study consisted of 14 control and 18 DEHP

exposed females. Of the 14 control female rats, 7 rats (each from a F2 dam representing a separate F1 dam) were mated and 7 rats (each from a F2 dam representing a separate F1 dam) were dissected for collection of ovary and serum collection. Of the 18 DEHP exposed female rats, 10 rats (each from a F2 dam representing a separate F1 dam) were mated and 8 rats (each from a F2 dam representing a separate F1 dam) were dissected for collection of ovary and serum collection.

Results:

Reproductive parameters

To determine the potential TG effects of the developmental exposure to DEHP various reproductive parameters were measured. The AGD measured at PND 0 was not altered in both males and females from the F3 generation from both DC and MC groups. There was no significant difference in the pubertal age and the estrous cyclicity between the control and the treatment animals in either group (Fig. 14).

In the DC group, the control animals had an average pubertal age of 39.00 ± 0.5 days, while DEHP animals reached puberty at PND 39.36 ± 0.7 days (control; N = 12 pups from 6 F2 dams, 2 from each dam; DEHP; N = 14 pups from 7 F2 dams, 2 from each dam) and in MC group, the control animals had an average pubertal age of 37.93 ± 0.4 days, while DEHP animals reached puberty at PND 37.72 ± 0.6 (control; N = 14 pups from 7 F2 dams, 2 from each dam; DEHP; N = 18 pups from 9 F2 dams, 2 from each dam). In the DC group, the control females showed 62.25 ± 9.7 % normal estrous cycles (N = 12 pups from 6 F2 dams, 2 from each dam), while the DEHP females showed 78.36 ± 6.4 % normal estrous cycles (N = 14 pups from 7 F2 dams, 2 from each dam). In the MC group, the control females showed 73.43 ± 6.2 % normal estrous cycles (N = 14 pups from 7 F2 dams, 2 from each dam), while the DEHP females showed 74.72 ± 5.2 % normal estrous cycles (N = 18 pups from 9 F2 dams, 2 from each dam). All females mated in both MC and DC groups became pregnant. Exposure to DEHP did not cause

any significant change in litter size of the DEHP-treated animals when compared to control animals from DC and MC group (DC - control; 9.16 ± 1.3, N = 6; DEHP =11.13 ± 0.4, N = 8; MC - control; 10 ± 0.9, N = 7; DEHP =11.13 ± 0.4, N = 10; Fig. 14).

Developmental exposure to DEHP altered ovarian follicular dynamics

To investigate the morphological basis of the alterations in reproductive parameters, we examined ovarian histology between PND 50 and 75 in the F3 females. In both groups, the control and the DEHP-treated ovaries contained follicles at all developmental stages. In the DC group [Fig. 15 (panel i)], developmental exposure to DEHP resulted in a significant decrease in the percentage of primordial follicles (control; 45.59 ± 2.3%; DEHP; 39.25 ± 0.9%; $P < 0.05$) in the F3 offspring. The percentage of primary follicles (control; 23.57 ± 3.6%; DEHP; 22.42 ± 0.5%) and secondary follicles (control; 5.28 ± 1.0%; DEHP; 5.02 ± 0.6%) were not significantly altered. The percentage of preantral follicles (control; 15.89 ± 1.5%; DEHP; 17.53 ± 1.1%) were not significantly altered, while the early antral follicles (control; 7.39 ± 0.9%; DEHP; 13.26 ± 1.5%; $P < 0.05$) were significantly increased. No significant changes in the percentage of mid/late antral (control; 2.26 ± 0.8%; DEHP 2.4 ± 0.9%), CL (control; 30.46 ± 5.4%; DEHP; 46.15 ± 5.5%), total number of follicles per ovarian section (control; 32.18 ± 4.4%; DEHP; 28.25 ± 2.5%) and the number of atretic follicles (control; 15.69 ± 2.2%; DEHP; 17.19 ± 0.9%) were noted in the DC group.

However, in the MC group [Fig. 15 (panel ii)], no significant changes in the percentage of primordial follicles (control; 31.87 ± 2.1%; DEHP; 31.26 ± 2.5%), primary follicles (control; 35.16 ± 2.6%; DEHP; 33.70 ± 2.0%), secondary follicles (control; 4.52 ± 0.6%; DEHP; 2.88 ± 0.6%), preantral follicles (control; 18.74 ± 1.7%; DEHP; 17.74 ± 1.7%), early antral follicles (control; 6.59 ± 1.3%; DEHP; 8.25 ± 1.5%) and mid/late antral follicles (control; 3.11 ± 1.0%; DEHP; 3.68 ± 1.0%) were observed. But there was a significant increase in CL (control; 36.44 ± 5.7%; DEHP; 52.16 ± 4.2%; $P < 0.05$). The total number of follicles per ovarian section (control;

31.17 ± 5.1%; DEHP; 27.66 ± 4.0%) and the number of atretic follicles (control; 22.96 ± 4.7%; DEHP; 20.02 ± 3.0%) were also not altered in the MC group.

These alterations in the follicular dynamics in the F3 generation, suggest that the ovaries may be transgenerationally affected when the DEHP exposure occurs during the critical ovarian developmental time window.

Developmental exposure of DEHP affected expression of ovarian markers in adult ovary

To further investigate the effects on ovarian development, we examined immunolocalization and relative expression levels of MIS, LHR, P450scc and ERα.

MIS is expressed in cumulus granulosa cells of growing follicles- primary, secondary, pre/early antral but not in germ cells or thecal cells [55, 56]. The MIS immunoreactivity was abundant in the primary, preantral and early antral follicles of the DEHP ovaries. Unlike the F1, where overexpression of MIS was observed (Fig 8), the F3 in DC group (Fig 16 A) showed a statistically significant decrease in staining intensities for MIS in primary follicles (control; 41.37 ± 5.2; DEHP; 25.33 ± 1.8; $P < 0.005$) and in preantral/early antral follicles (control; 30.07 ± 1.8; DEHP; 22.59 ± 2.2; $P < 0.05$). However, there was no change in expression of MIS in primary (control; 26.89 ± 2.3; DEHP; 31.62 ± 4.3) and pre/early antral follicles (control; 26.81 ± 3.3; DEHP; 25.87 ± 2.0) in the MC group (Fig 16 B).

The expression of P450scc was detected in thecal cells of preantral and antral follicles (Fig. 17) in both the groups, as reported by previous studies [78]. In both DC and MC groups, the thecal cells surrounding large antral follicles as well as interstitial cells in control and DEHP ovaries showed strong LHR immunoreactivity (Fig. 18). In the ovaries from DC and MC groups ERα was expressed in nuclei of thecal cells and germinal epithelium (Fig. 19), consistent with studies of others [83]. Quantification of the staining intensities of LHR, P450scc and ERα using ImageJ, showed they were not significantly altered by DEHP exposure.

Discussion

Developmental exposure to DEHP in the F1 generation did not affect the AGD, age at puberty, estrous cyclicity and litter size of the F3 females from either the MC or the DC group. However, the ovarian follicular dynamics were altered in both DC and MC females and the expression of MIS was reduced only in the DC females. Thus, this study shows exposure to DEHP during the critical ovarian developmental window may have a TG effect on the ovarian follicular development, and alter the expression of ovarian markers.

In the DC group, decrease in primordial follicles with concomitant increase in early antral follicles suggests the possibility of accelerated follicular development. Reduced number of primordial follicles may be indicative of a compromised follicular pool. Furthermore a significant decrease in the expression of MIS by the primary follicles from the DC group can cause increased activation of the primordial follicles into the primary follicle stages, reducing primordial follicle pool. With attenuated MIS produced by the preantral/early antral follicles, these extensively recruited primordial follicles progress to the early antral stages and may result in premature progression of the follicles to ovulation. However all of these excess follicles may not result in healthy oocytes available for fertilization and without a concomitant increase litter size, premature luteinization is suspected [130]. Similarly, in MC group, increase in CL, without affecting the litter size may be indicative of premature luteinization. Such alterations in follicular dynamics without affecting the litter size, are similar to those in the F1 (Fig. 7), suggesting DEHP exposure can affect transgenerationally in both MC and DC groups, disrupting ovarian folliculogenesis. Previous studies have reported requirement of both parents' exposure to the EDC for a TG effect to ensue. However, these studies employed an exposure period that may have missed the female germ cell remethylation time window [126]. Hence, by the nature of our study design, we suggest that DEHP exposure can affect females transgenerationally via either the male or the female germ line. Studies have shown DEHP exposure affects the testicular germ cell organization and spermatogonial stem cell function and this effect is TG [31]. Therefore, we

speculate compromised paternal epigenome might be responsible for the altered follicular development in the F3 females from DC group. However, the data from the MC group warrants further investigation with respect to the epigenetic status of the oocyte. Furthermore there were differences in the effects observed in the F1 and the F3 generation such as there was increase in MIS in F1 and a decrease in MIS in F3. This could be due to different mechanisms underlying these effects. In F1 any epigenetic alteration in cell types (either somatic or germ cell) can cause the changes observed in the adulthood, where as in F3 generations these alterations are mainly mediated via germ cells, although context dependent (e.g. fetus growing in unhealthy uterus) TG effect is also possible.

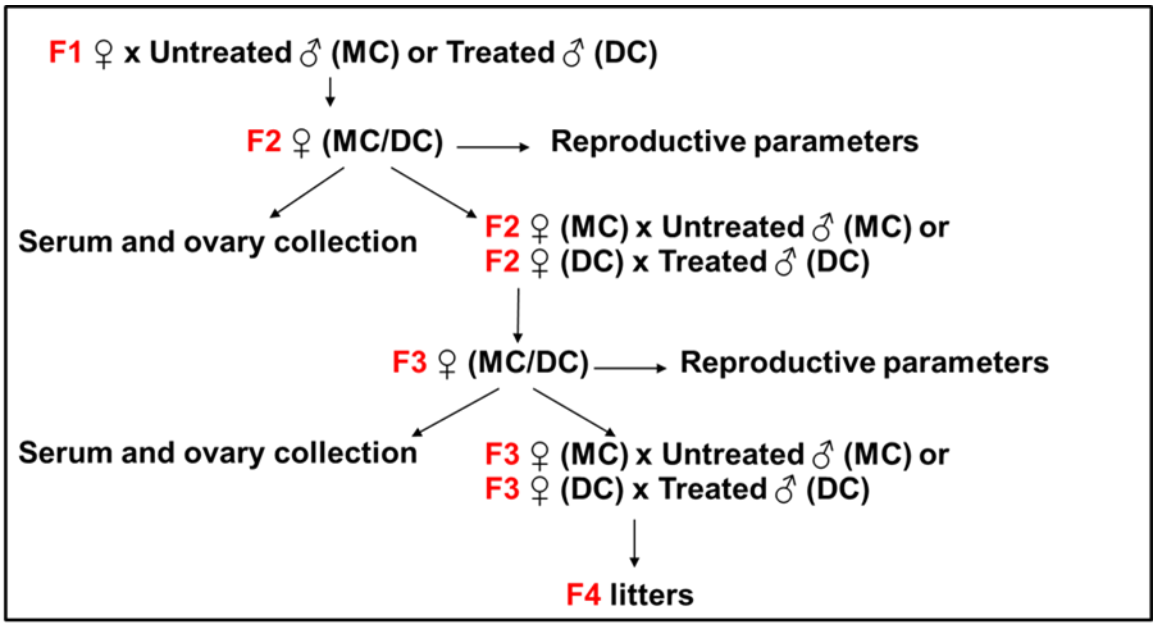
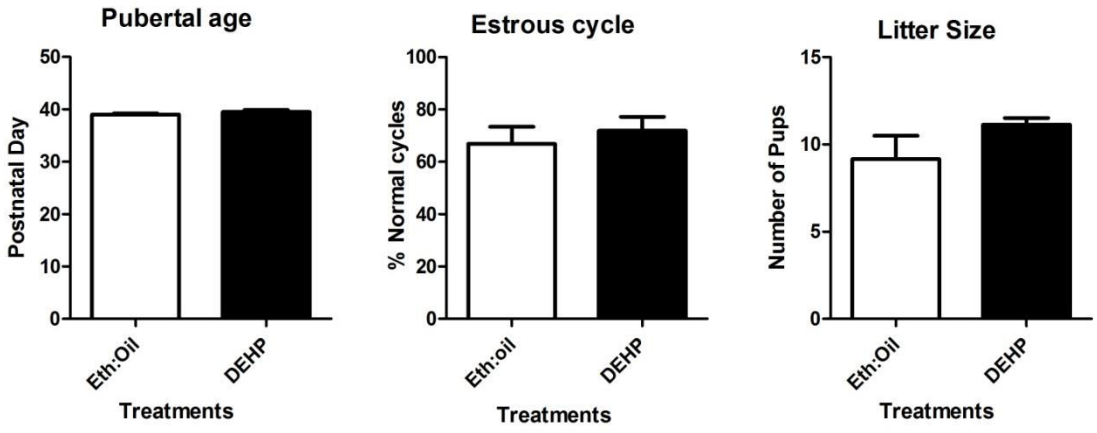


Figure 13. Mating scheme for transgenerational study: Reproductive parameters were analyzed for two females per dam. One female was utilized for breeding the subsequent generation while the other was euthanized between PND 50 and 75 to collect ovaries and serum. The females (F1, F2, F3) were mated to either treated (double cross: DC) or untreated (maternal cross: MC) males to produce the subsequent generations representative of MC or DC group, respectively.

A. DC group



B. MC group

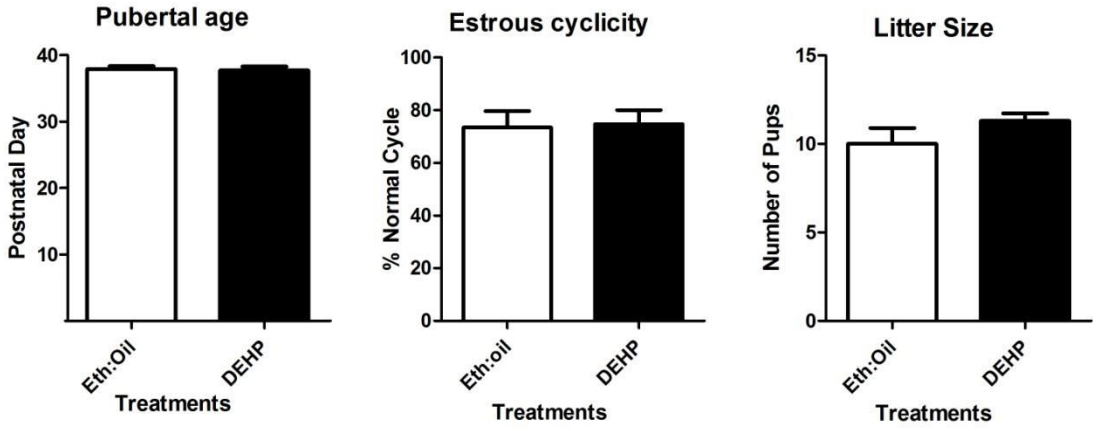
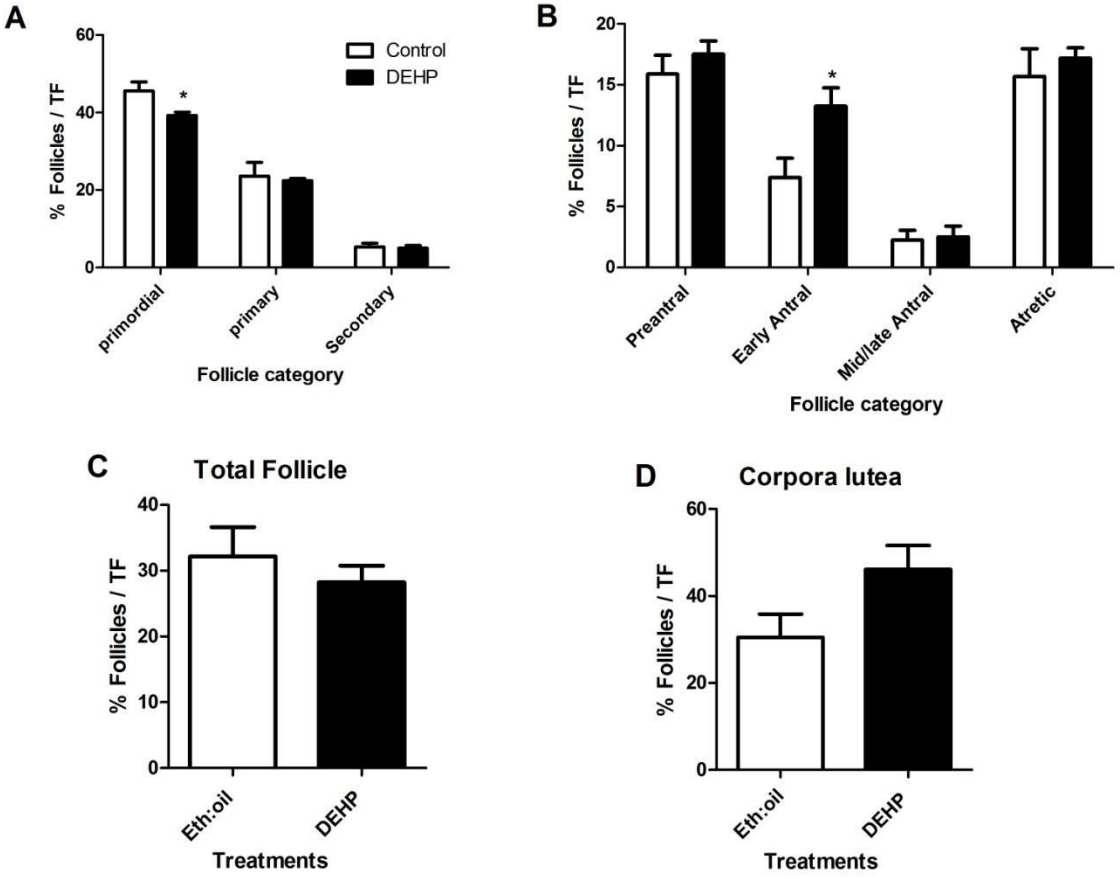


Figure 14. Transgenerational effect of developmental exposure of DEHP on the reproductive parameters. Pubertal age, estrous cyclicity and litter size were determined as described in the materials and methods. No significant difference between the control and the DEHP group was observed in the third generation (F3) of from both double cross (DC; panel A.) and maternal cross (MC; panel B.) groups.

i. DC group


A
% Follicles / TF
Control
DEHP
primordial
primary
Secondary
Follicle category
B
% Follicles / TF
Preantral
Early Antral
Mid/late Antral
Atretic
Follicle category
C
Total Follicle
% Follicles / TF
Eth:oil
DEHP
Treatments
D
Corpora lutea
% Follicles / TF
Eth:oil
DEHP
Treatments

ii. MC group

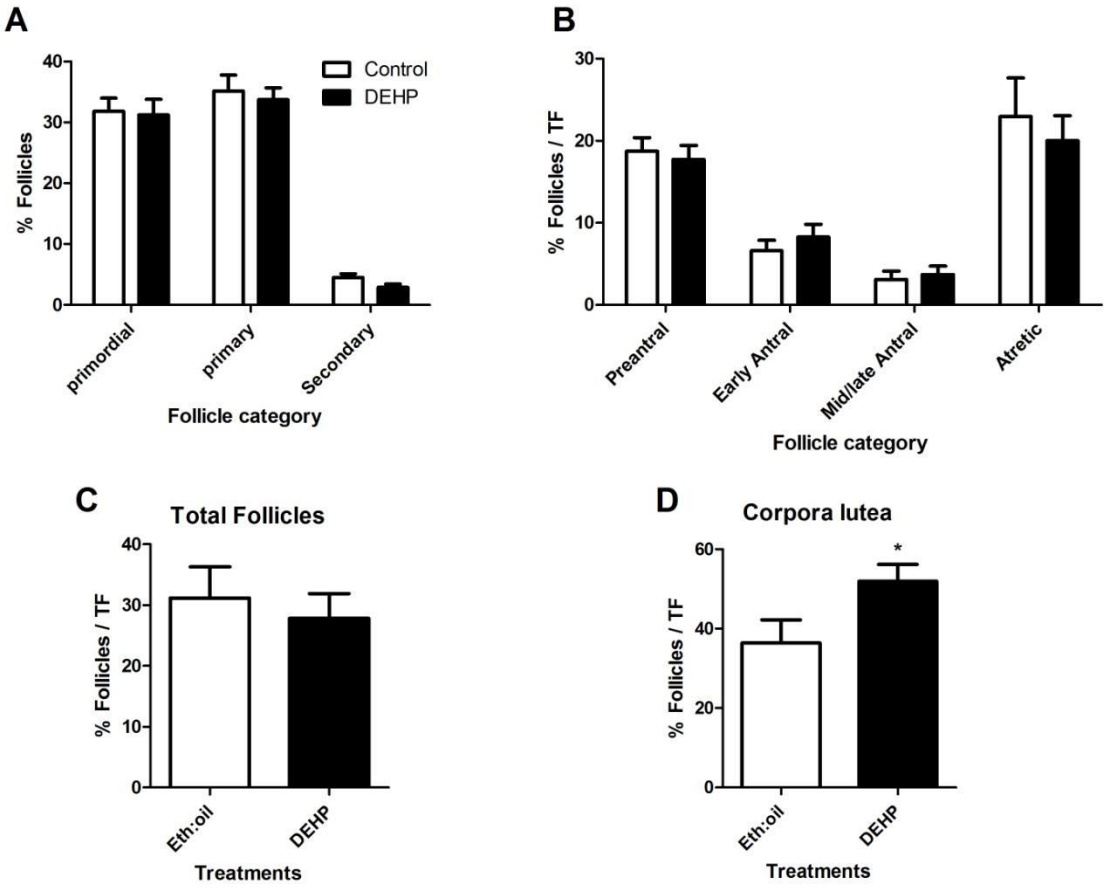
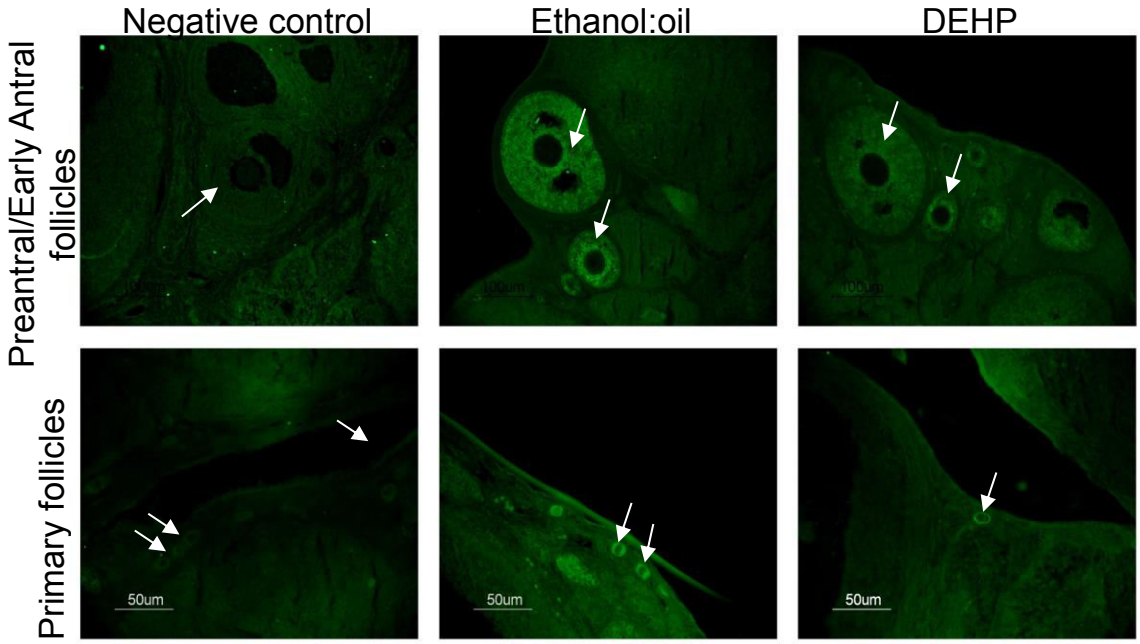
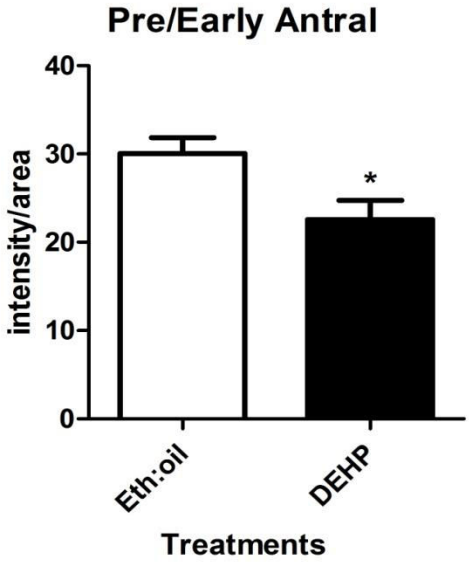
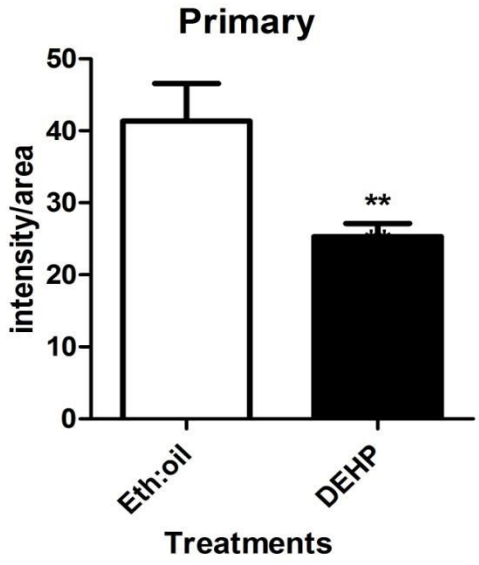


Figure 15. Transgenerational effect of developmental exposure of DEHP on ovarian follicular development. The ovaries were collected between PND 50-75 and processed as described in the materials and methods. There was a significant decrease in the percentage of primordial follicles and an increase in the early antral follicles in DEHP-treated animals from the DC group (panel i). DEHP-treated females from the MC group had increased number of CL (panel ii).

A. DC group


Negative control
Ethanol:oil
DEHP
Preantral/Early Antral follicles
Primary follicles
50um
50um
50um


Pre/Early Antral
intensity/area
40
30
20
10
0
*
Eth:oil
DEHP
Treatments


Primary
intensity/area
50
40
30
20
10
0
**
Eth:oil
DEHP
Treatments

B. MC group

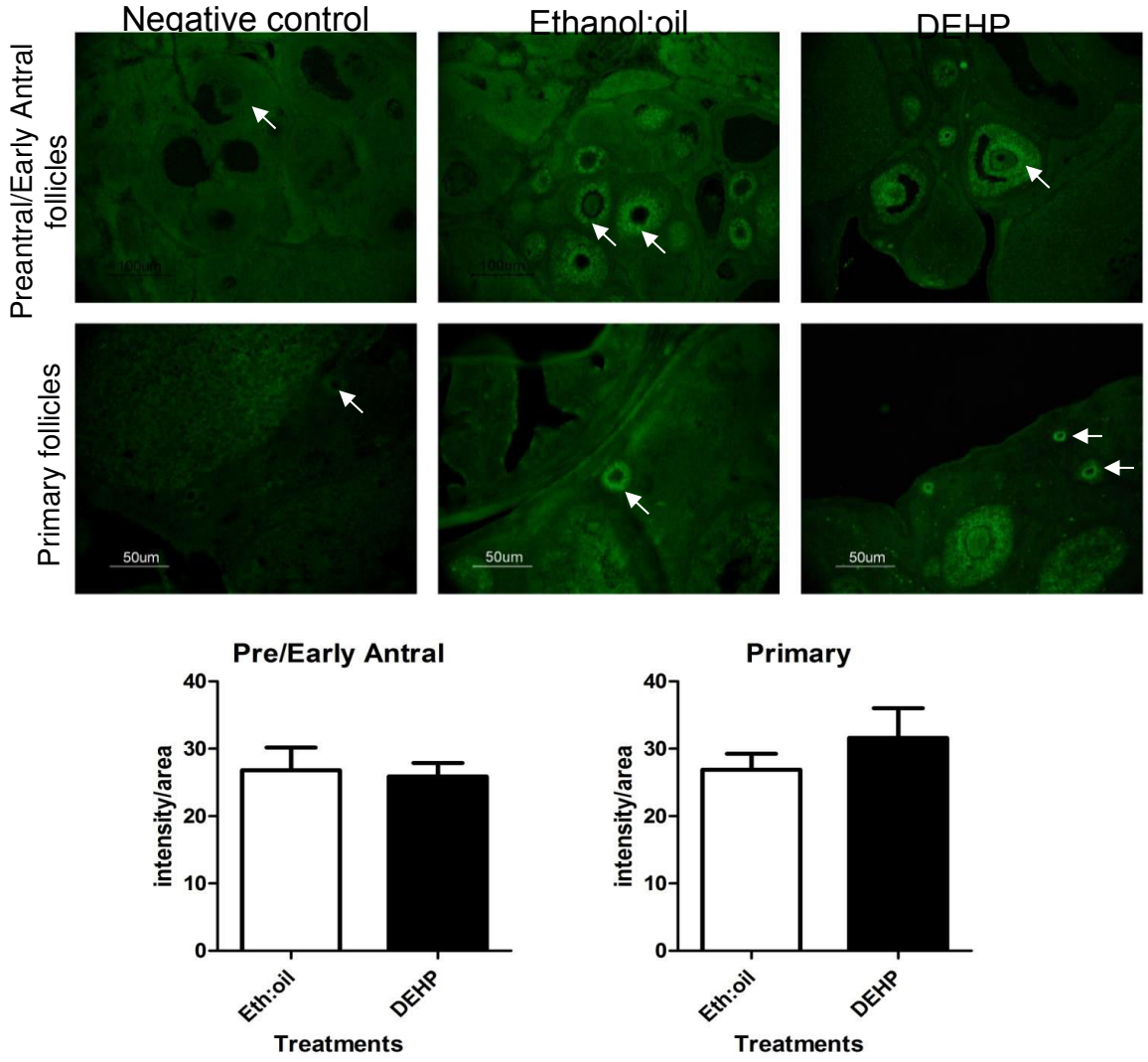


Figure 16. Transgenerational effect of developmental exposure of DEHP on expression of MIS protein in rat ovaries from DC and MC groups. MIS was expressed in the granulosa cells of primary follicles and cumulus granulosa cells of the antral follicles from the ovaries of control and DEHP animals from both DC and MC groups (control; N = 5 and DEHP; N = 7). Analysis using ImageJ (NIH) software showed expression of MIS was decreased in primary and preantral/early antral follicles from the DEHP-treated animals in the DC group (panel A). However there was no change in expression of MIS in the ovaries from MC group (panel B).

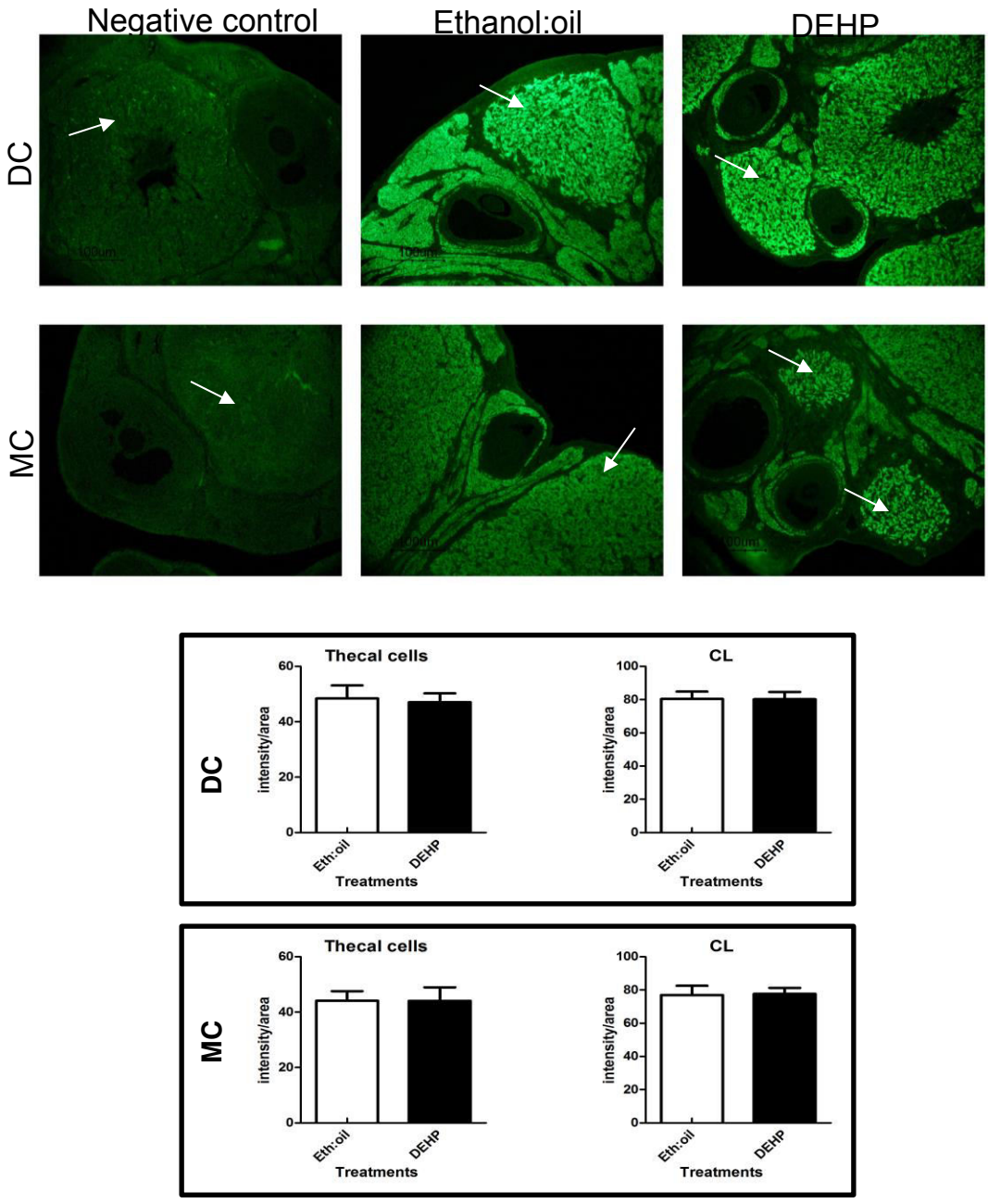


Figure 17. Transgenerational effect of developmental exposure of DEHP on expression of P450scc in rat ovaries from DC and MC groups. Immunolocalisation of P450scc was observed in thecal cells of the follicles and corpus luteum from the control and DEHP ovaries from both DC and MC groups. There was no significant change in expression of P450scc (intensity/area) in either thecal cells of the ovarian follicles or the corpus luteum, when analyzed using ImageJ (NIH) software in either of the groups (control; N = 5 and DEHP; N = 7).

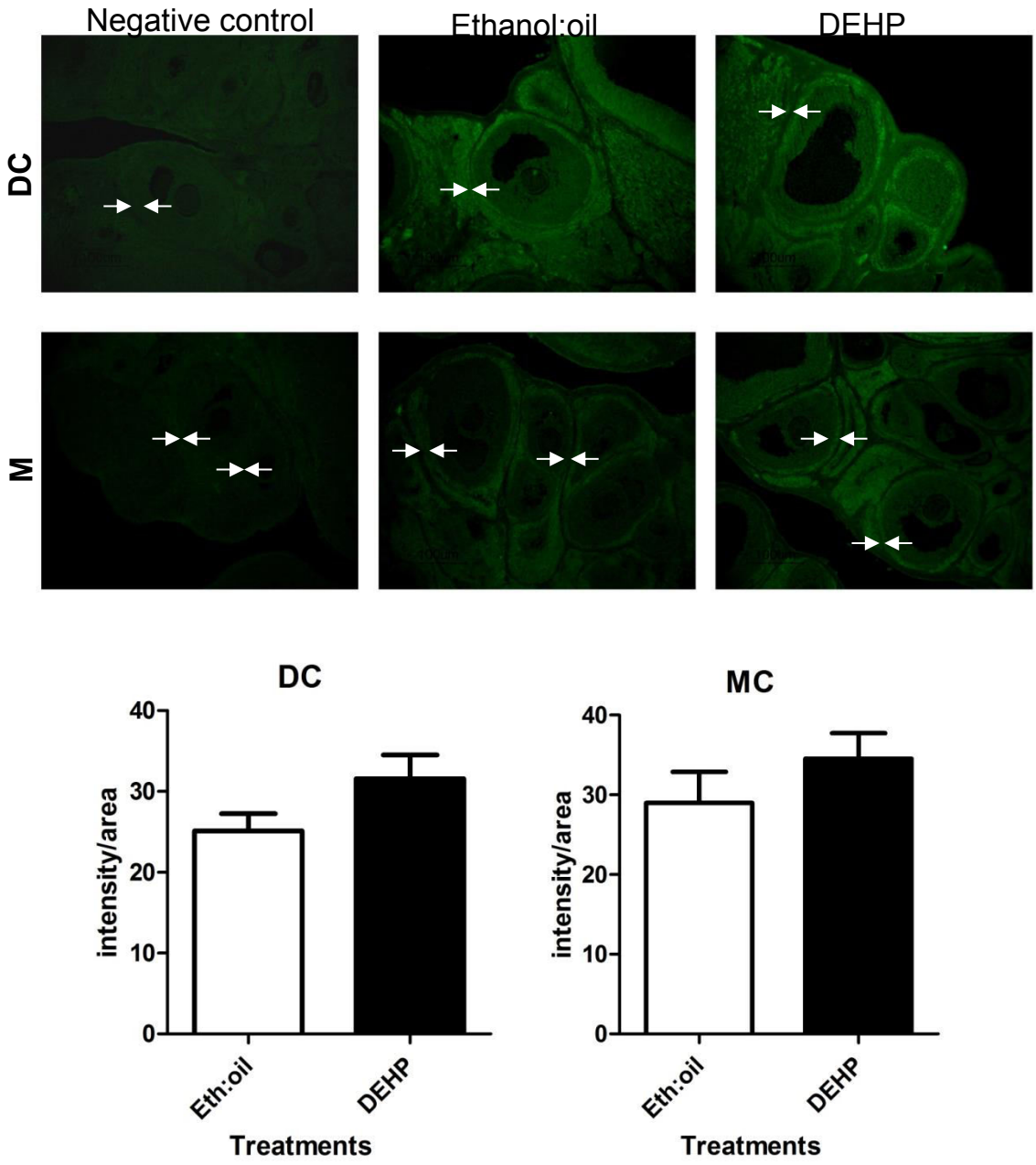


Figure 18. Transgenerational effect of developmental exposure of DEHP on expression of LHR in rat ovaries from DC and MC groups. Immunolocalisation of LHR was observed in thecal cells surrounding large antral follicles and the interstitial cells in control and DEHP ovaries from both DC and MC group. There was no significant change in expression of LHR (intensity/area) in thecall cells of the ovarian follicles, when analyzed using ImageJ (NIH) software (control; N = 5 and DEHP; N = 7).

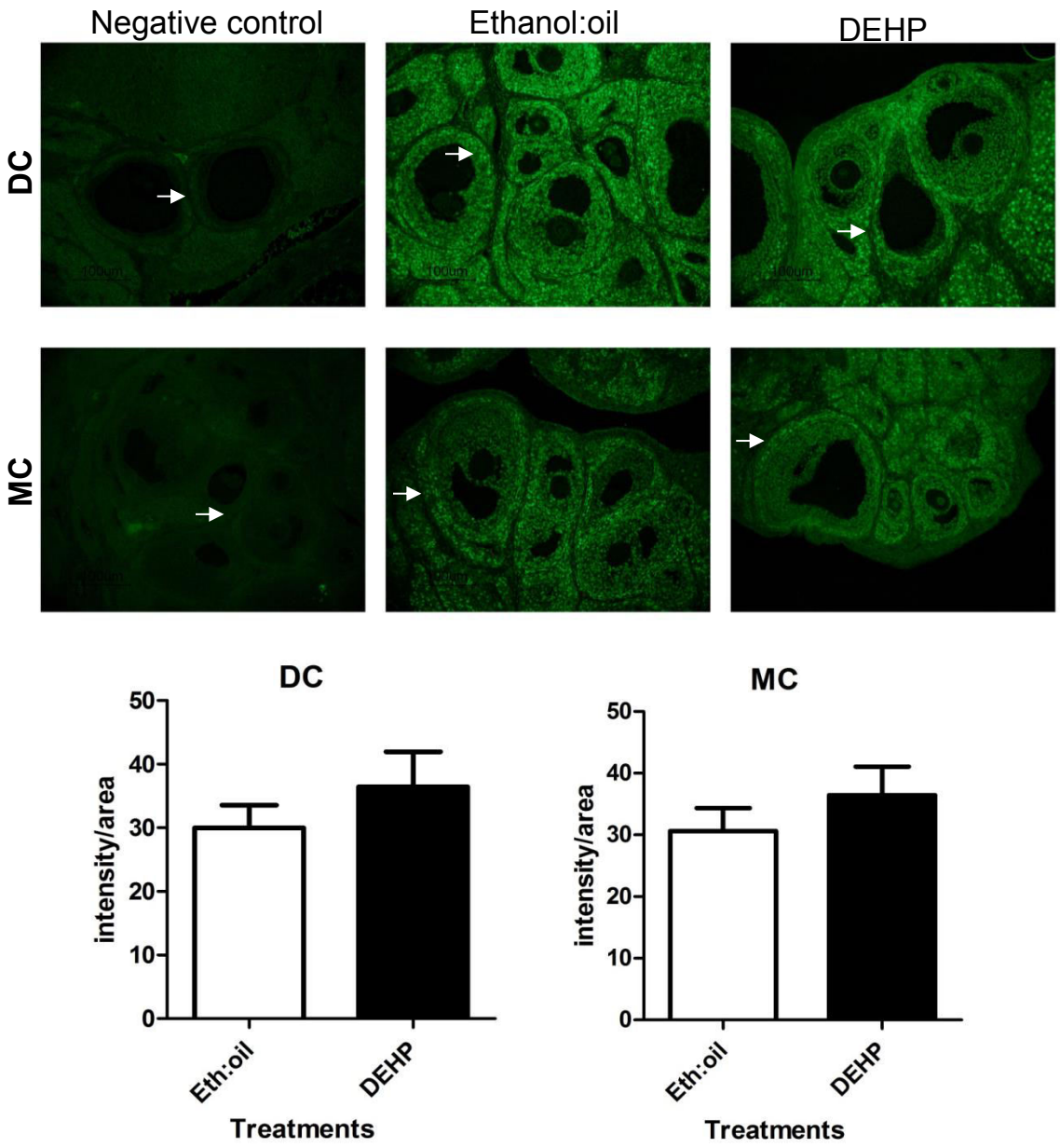


Figure 19. Transgenerational effect of developmental exposure of DEHP on expression of ERα in rat ovaries from DC and MC groups. Immunolocalisation of ERα was observed in nuclei of thecal cells, interstitial gland cells and germinal epithelium in the control and DEHP ovaries from DC and MC group. There was no significant change in expression of ERα (intensity/area) in thecal cells of the ovarian follicles, when analyzed using ImageJ (NIH) software (control; N = 5 and DEHP; N = 7).

CHAPTER 4:

CONCLUSIONS AND FUTURE DIRECTIONS

In utero and early post natal exposure to DEHP delayed puberty, reduced serum estradiol levels and altered ovarian follicular dynamics and the expression of ovarian markers in the F1 females. Some of these effects such as altered ovarian follicular dynamics were also observed in the F3 females from the DC and MC group, suggesting a possible TG effect of the exposure.

Developmental exposure to DEHP resulted in the delay in onset of puberty in the F1 females. Puberty is intricately regulated by hypothalamus gonadal axis. *In* utero and lactational exposure to DEHP has been shown to affect the GnRH level in the hypothalamus [127]. Expression of neuropeptides such as kisspeptin, can be altered by exposure to EDC [109]. Similar mechanisms may be the cause for delay in onset of puberty seen in the present study.

Interestingly, the regularity of estrous cycles was not affected despite reduced serum estradiol levels in the F1. Additionally, the current data does not fully explain the cause for attenuated hormone levels therefore, expression of other enzymes involved in key steps in steroidogenesis such as StAR and aromatase, should be investigated.

The present study did not measure the expression of ERβ. MIS expression is differentially regulated by estradiol depending on the ER. MIS decreases in granulosa cells of growing follicles, which mainly express ERβ [128]. Since MIS is increased in granulosa cells in F1 and decreased in F3, expression of ERβ may be altered in both generations and hence it must be measured.

In the current study we observed significantly large number of growing follicles which seem to progress towards atresia in the DEHP exposed F1 animals at young adulthood (PND 50-85). Similar trends were seen in the F3 generations. However, the present study endpoint limits the confirmation of 'premature ovarian failure' and hence, ovaries from aged animals need to be analysed to confirm early reproductive senescence.

Additionally since the data is suggestive of increase in follicular atresia, expression of markers of follicular atresia such as caspases – 3 may be investigated [21]. Additionally, both granulosa cell apoptosis and inhibition of oocyte maturation have been indicated in DEHP

induced follicular atresia in *in vitro* culture systems. The cause for follicular atresia in the current study needs to be investigated.

One of the essential ovarian processes affected by DEHP is folliculogenesis. Recent studies have shown transient exposure of adult mice to DEHP activates the PI3kinase signaling pathway recruiting more number of primordial follicles, thereby increasing the primary follicles and decreasing the primordial follicles [108]. However in our study involving *in utero* and early postnatal exposure, we observed reduction in primordial follicles and primary follicles, suggesting DEHP exposed animals may have a smaller the follicular pool available for the reproductive function, resulting in a shorter reproductive life span. This discrepancy suggests DEHP may act differentially on the ovaries depending on the exposure period and duration.

The current TG study investigated follicular dynamics in relation to the expression of the ovarian markers, in the F3 ovaries. However the steroid hormone levels in the F3 need to be measured.

Further studies are necessary to elucidate the epigenetic mechanism altering the ovarian follicular dynamics in the F3 generation. A study reported low doses of DEHP reduced the level of imprinted gene methylation and increased metaphase II spindle abnormalities in oocytes matured *in- vitro* [9] Such alteration in the oocyte of the F1 females can adversely affect ovaries of the subsequent F3 females. The data from the both MC and DC group warrants further investigation with respect to the epigenetic status of the oocyte from both the F1 and the F3 generation. Thus, epigenetic studies involving histone modification and DNA methylation status of the oocyte may provide insight into the mechanistic of the TG effect observed.

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