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Phthalates, ovarian function and fertility in adulthood

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Phthalates are a family of high-production volume industrial chemicals used in the manufacture of plastics. Some phthalates are regulated as endocrine disrupting chemicals (EDCs) and reproductive toxicants based on adverse effects in the male. Potential effects in females are less understood although exposure levels can be higher in women compared to men. Here, we review the literature on the effects of phthalate exposures in adulthood on ovarian function and fertility in women. Experimental studies using cell cultures and rodents combined with human evidence from epidemiological studies suggest that phthalates pose a hazard to ovaries. Phthalates can disrupt follicle growth pattern, increase oxidative stress and cause follicle death. These effects could lead to infertility, faster depletion of ovarian reserve, and earlier reproductive senescence. However, more studies using more realistic exposure levels will be needed to properly assess the risks in women.

Abbreviations: benzyl butyl phthalate, BBP; dibutylphthalate, DBP; diisobutyl phthalate, DIBP; di(2-ethylhexyl) phthalate, DEHP; diethylpthalate, DEP; diisononyl phthalate, DINP; dimethylphthalate, DMP; endocrine disrupting chemical, EDC; monobenzyl phthalate, MBzP; mono(2-ethylhexyl) phthalate, MEHP; monoethyl phthalate, MEP.

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Introduction

Phthalate exposure and metabolism

Phthalates are a diverse group of synthetic esters of phthalic acid that vary in length and branching of the alkyl side chains. Long-chain phthalates are often used as plasticizers and short-chain phthalates as solvents. As plasticizers, phthalates enhance the durability and flexibility of the product [1]. In flexible plastics like polyvinyl chloride (PVC), phthalates can comprise up to 80% of the final product weight [1]. The shorter and more volatile phthalates are widely used as solvents and carriers in a variety of personal care products, including lotions, perfumes, shampoos, hair sprays and makeup [2]. Phthalates are also used in soft tubing of medical devices and as excipients in medications [2]. Commonly used phthalates include di (2-ethylhexyl) phthalate (DEHP), diisononyl phthalate (DINP), dibutylphthalate (DBP), dimethylphthalate (DMP), diethylphthalate (DEP). In plastics, phthalates are not covalently bound to the structure, which means that they leach from the products and end up contaminating food, water, soil, air and dust [2]. General populations are exposed to phthalates and their mixtures primarily via intake of contaminated food as well as by inhalation of air-borne phthalates and direct skin contact with phthalate containing products [2].

Upon uptake, phthalates are quickly metabolized in the body to a variety of biologically active metabolites. Phthalates do not bioaccumulate and are excreted from the body via urine, feces and breast milk [3]. Short-chain phthalates can be excreted unchanged from the body, whereas long-chain phthalates are converted to a more water-soluble form for excretion [3]. Phthalates are hydrolyzed to mono-alkyl phthalate esters already upon uptake in the gut epithelium, and extensive continued hydrolysis and oxidation in the body lead to the formation of multiple secondary metabolites [3]. For example, DEHP is first rapidly hydrolyzed to mono (2-ethylhexyl) phthalate (MEHP) under the catalysis of non-specific lipase enzymes. The metabolism then continues with side-chain hydrolysis and oxidation catalyzed by cytochrome P450 enzymes in multiple organs resulting in the formation of dozens of metabolites [3,4]. Metabolism can be affected by the exposure dose and physiological factors like age, body mass index and week of pregnancy [5]. Rapid metabolism and excretion make phthalate exposure assessment in humans challenging. Human exposure to phthalates is often quantified by the presence of metabolites in the urine [3]. However, measuring total phthalate exposure is difficult because analytical methods exist for only some of the metabolites and not all breakdown products are even known. In addition, multiple samples would be needed for reliable estimates [6].

Phthalates have been found in liver, lungs and adipose tissue as well as in serum, urine, ovarian follicular fluid and amniotic fluid [3,5]. The presence of phthalates in ovaries and amniotic fluid means that exposure is life-long starting from germ cell development and fetal life [3]. Estimated exposure levels in humans vary. For example, the daily exposure of the general population to DEHP has been estimated to be between 3 and 30 μg/kg based on urinary excreted metabolites [7]. Women are generally more exposed to phthalates than men, which is attributed to more frequent use of cosmetics and toiletry products containing phthalates [8]. More specifically, significantly higher concentrations of monoethyl phthalate (MEP) and monobenzyl phthalate (MBzP) have been observed in women compared to men [9]. Occupation is another factor leading to higher levels of exposure. For instance, urine phthalate levels are elevated among hairdressers compared to the general population and are associated with the length of their exposure [10]. In this study, the mean urinary concentration of MEHP was almost double in hairdressers compared to the control group (10.23, 5.63 ng/ml) and the maximum measurement was ten times higher (239.70, 23.34 ng/ml).

Phthalates as endocrine disrupting chemicals

Endocrine disrupters are exogenous chemicals or their mixtures that interfere with the body's normal endocrine function, leading to adverse effects on an organism and/or its future generations. Originally, studies on endocrine disruption were mainly focused on mechanisms involving the estrogen and androgen receptors and steroidogenesis [11]. It has become clear over the years that multiple additional modes of action can disrupt the endocrine system including changes in hormone receptor

activation, inactivation or expression, changes in hormone-sensitive cell signaling or the epigenome, and changes in hormone function such as synthesis, distribution and clearance [12].

Currently, DEHP, DBP, benzyl butyl phthalate (BBP) and diisobutyl phthalate (DIBP) have been categorized as substances of very high concern due to their endocrine disruptive and reprotoxic properties in the European Union (REACH, annex XIV). These classifications rely on evidence of adverse effects on male reproductive health. In a guideline three-generation reproductive toxicity study using rats, DEHP caused testicular defects, shorter anogenital distance and reduced sperm count [13]. In humans, exposure to phthalates during fetal development significantly correlates with shorter anogenital distance in baby boys, a marker of anti-androgenic effects that correlates with poorer semen quality and fertility in adult life [14]. These effects are thought to depend on the ability of phthalates to disrupt steroid hormone synthesis in the body, leading to reduced androgen levels. Low androgen levels during development lead to disrupted masculinization of the fetus that manifests as genital tract malformations, susceptibility to testicular cancer and reduced semen counts, collectively labeled as testicular dysgenesis syndrome [15]. Human epidemiological studies have linked phthalates to an array of adverse effects on female reproductive health too. For example, urinary phthalate levels in women have been linked to decreased rates of pregnancy, increased rates of miscarriages, pregnancy complications as well as diminished ovarian reserve [16–18]. Furthermore, a possible link with other conditions has been made, such as endometriosis, uterine leiomyoma and polycystic ovarian syndrome [19–21]. However, the evidence is not considered as robust as that in males and current phthalate regulations are not based on risks these chemicals may pose to women.

Our earlier studies have suggested that endocrine disruptive phthalate mixtures that are defined based on associations with shorter anogenital distance in baby boys can also disrupt fertility in females when tested in mice [22]. When mice were exposed *in utero* to an epidemiologically defined phthalate mixture consisting of DBP, DBzP and DiNP, not only male but also female offspring were affected. In males, shorter anogenital distance, lower testis weight and reduced sperm production were observed as expected. In females, the same exposure levels associated with reduced ovarian weights accompanied by reduced numbers of growing follicles and increased atretic follicles in adulthood. This suggests that prenatal phthalate exposure could prime ovaries for faster reproductive senescence at the same exposure levels where the typical reproductive effects in males are observed. In animal studies, the timing of exposures can be designed to target certain suspectable windows in development, such as fetal development. In humans, this is not the case as everyone faces a life-long exposure. How the persisting exposure to EDCs like phthalates may affect ovaries is an important question to dissect because it can take decades before a woman uses her oocytes to have children.

Ovaries define fertility in women

It takes years to make mature oocytes. The immature oocytes form already *in utero* in the ovaries of female fetuses, and they can remain dormant and arrested in the first meiotic division for decades [23]. Cohorts of non-growing follicles continuously initiate growth embarking on a growth and maturation process (folliculogenesis) that takes at least half a year in humans [24]. Although follicles grow even in childhood, they can only complete the folliculogenesis to release a mature oocyte after puberty in response to gonadotropins. During folliculogenesis, the 30 μm immature follicle grows size via oocyte growth, granulosa cell proliferation, antrum formation and theca cell recruitment to a 2 cm antral follicle containing the mature oocyte [24]. Growing follicles synthesize steroid hormone estradiol that prepares the endometrium for possible pregnancy together with progesterone that will be secreted by the corpus luteum that forms after ovulation. Only *ca* 400 follicles of the millions originally formed will ever reach full maturity. Conversely, the most common fate of a follicle is death through atresia [23,24]. Careful balance in follicle dormancy, death and growth are essential for normal fertility.

Factors that speed up the depletion of ovarian reserve associate with accelerated reproductive aging, premature ovarian failure, and infertility. Such factors include genetic mutations, gonadotoxic medical treatments, and smoking [25,26]. Importantly, fertility markedly declines already well before the onset of menopause due to the deteriorating quality of oocytes [27]. Despite the importance of ovarian reserve in determining the remaining fertility potential of a woman, there are no direct ways to measure the quality or quantity of ovarian reserve. The non-growing follicles are too small for detection

by ultrasound, and they do not secrete any known factors that could be used as biomarkers. Analysis of human ovarian health relies on clinical manifestation of function (menstrual cycle, pregnancy), ultrasound examination (structure, number of large growing follicles) and serum biomarkers such as estradiol, follicle stimulating hormone and anti-Müllerian hormone levels [28]. All these markers reflect the numbers of *growing follicles*. In population studies, endpoints that reflect ovarian function include regularity of menstrual cycles, pregnancy rates and occurrence of infertility, although the latter ones are also affected by the fertility of the partner. In rodent studies, effects on ovaries can be assessed in more detail. For example, guideline assays for reproductive toxicity involve analysis of ovarian weights and histological examination of presence of follicles in different stages of folliculogenesis (*e.g.* OECD test guideline TG 443). Guideline assays do not encompass analysis of follicular atresia or estimation of effects on reproductive aging. Therefore, exposures that lead to gradual changes in ovaries over decades of exposure could be missed in regulatory guideline studies.

Scope of the review

In this review, we focus on literature describing the effects of phthalates on ovarian function and female fertility in adulthood. We choose to focus on adult exposures for multiple reasons: i) full follicle growth and oocyte maturation only take place after puberty in adulthood, ii) the developmental competence of the oocyte is defined during its maturation, iii) phthalate metabolites have been detected in ovarian follicular fluid, and iv) women postpone childbearing leading to longer cumulative exposure of their oocytes to chemicals. We collected literature describing the effects of phthalates or their mixtures on ovaries in adult mice and rats *in vivo*, and on ovarian cells and follicles *in vitro*. In addition, we included epidemiological studies reporting associations between phthalate exposure levels and reproductive outcomes in women.

Effect of phthalates on ovarian cells and follicles *in vitro*

We identified 16 studies where phthalate effects were studied in controlled exposure *in vitro*. Table 1 summarizes the studies conducted in monolayer cell cultures and encompasses both commercial ovarian cell lines and primary human ovarian cells. Table 2 summarizes studies using *in vitro* cultures of antral follicles derived from sexually mature mice. These test systems were exposed to single di-alkyl phthalates and mono-alkyl metabolites, or their mixtures. The most often studied phthalate was DEHP and its metabolite MEHP.

All cell culture studies used short exposure (2–48 h), high concentrations of single phthalates (1–50,000 μM), and focused on cell survival and oxidative stress as primary outcomes (Table 1). Collectively, the studies found decreased viability and increased cell death, and one study also reported increased oxidative stress.

The studies using *in vitro* exposure of isolated mouse antral follicles to phthalates were conducted over a wide range of concentrations (0.065–1000 μg/ml, *ca* 0.1 μM–3 mM) and times varying from 24 h to a week (Table 2). Both mixtures and single phthalates were tested. Collectively, the studies found that exposures led to increased oxidative stress, reduced follicle growth and increased atresia. One study reported a reduction in atresia upon phthalate mixture exposure [29]. The authors attributed the observed reduction in atresia to reduced growth of the follicle. The authors hypothesized that the control follicles acquired apoptotic bodies due to normal granulosa proliferation, whereas the exposed follicles did not due to cell cycle arrest of granulosa cells. They concluded that overall, phthalates adversely affected ovarian follicle health by reducing antral follicle growth, inducing oocyte fragmentation and decreasing hormone production.

Collectively, the *in vitro* studies reported significant adverse effects of phthalate exposures on ovarian cells and follicles. The advantage of the *in vitro* systems is that they can identify direct effects of chemicals on ovarian cells. The disadvantage is that they do not consider the hypothalamus–pituitary–gonadal axis, which is the cornerstone of normal ovarian function. In addition, the tested concentrations were high and, in most cases, clearly over concentrations found in humans. In human ovarian follicular fluid, MEHP levels have been estimated to be between 9.34 ng/ml (*ca* 34 nM) and 239 ng/ml (*ca* 860 nM) [30,31]. These levels are clearly lower than those used in experimental

in vitro studies (Tables 1 and 2). It must be noted that none of the *in vitro* studies controlled the actual exposure levels by analysis of culture medium. This could be important to do in future studies because phthalates are likely metabolized in ovarian cells as suggested by a study in mice showing that ovaries, follicles and oocytes are all capable of metabolizing phthalates [32]. It should further be noted that real-life mixture exposures spanning over several decades are impossible to model in *in vitro* systems, and cell culture studies should be considered as an opportunity to reveal hazards instead of quantifying risks in women. Nevertheless, it will be important to characterize the effects of phthalates on ovarian cells and follicles in low exposure in future studies. Importantly, three of the studies using isolated follicles detected significant changes with MEHP already at 0.1 μg/ml concentration, which can be considered as human relevant [33–35]. This encourages to carry out more low dose studies in the future.

Effects of phthalates on ovarian function and fertility *in vivo*

We identified 17 papers describing controlled oral exposure of sexually mature rats or mice to defined phthalates or their mixtures where ovarian function and fertility were measured as outcomes (Table 3). The most common model used was CD-1 mice, and the most often studied phthalate was DEHP. Only parental di-alkyl phthalates were used. Exposure times varied from 2 to 16 weeks, and doses from 0.1 to 3000 mg/kg/day. Effects on ovaries were assessed by histological examination, immuno-staining for apoptotic markers, and calculation of pregnancy rates. Collectively, the *in vivo* studies showed increased levels of apoptosis, follicular atresia and oxidative stress. Changes in the proportion of follicles at the different stages of maturation, indicative of accelerated follicle development, were also common findings in these studies [36–41]. Reduced fertility measured by fertility index and number and rate of births, was observed in several studies [37,42,43]. When follicle growth acceleration was observed, the developmental competency of the oocytes was not studied, which should also be investigated in studies where no adverse effects on follicle populations upon phthalate exposure were found [44]. Some *in vivo* studies observed effects on follicles long after the exposure ceased, and this gap between exposure and effect needs to be taken into consideration [36,37,40]. As full folliculogenesis takes several weeks in mice and several months in humans, the timing of exposure in relation to observed adverse effects could reveal sensitive time windows during folliculogenesis [24,45].

Table 1
***In vitro* studies using phthalate exposure of monolayer cultures of ovarian cells.** Both commercial cell lines (cl) and primary cell (pc) cultures are included. The responses are classified under categories "survival" and "oxidative stress" as these were the endpoints most often considered. Not assessed endpoints are displayed as NA.

Test system	Compound	Effect Dose	Exposure Duration	Endpoints		Reference
				Survival	Oxidative stress	
Pc - Human luteinized mural granulosa cell culture	DBP	100 μg/ml (estimated: 359 μM)	48 h	No difference in viability Decreased expression of anti-apoptotic gene	NA	Adir et al., 2017 [83]
Cl - Chinese Hamster Ovary cells (CHO)	MEHP	10,000, 50,000 μM	2 h	Decreased viability DNA damage	Increased ROS levels Increased lipid peroxidation	Chang et al., 2017 [84]
Cl - Human ovarian granulosa cell line culture (HO23)	BBP	1 μM	48 h	Increased necrosis/ apoptosis	NA	Chen et al., 2012 [85]
Pc - Human granulosa – lutein cell culture	MEHP	500 μM	48 h	Decreased viability	NA	Reinsberg et al., 2009 [86]
Cl - Chinese Hamster Ovary cells (CHO)	MEHP	700, 1000, 1300 μM	2 h	Cytotoxicity DNA damage	No change in peroxisomes	Phillips et al., 1986 [87]
Cl - Chinese Hamster Ovary cells (CHO)	MEHP	800, 1000, 1250, 1500, 1750 μM	2 h	Decreased viability DNA damage	NA	Phillips et al., 1982 [88]

Table 2
***In vitro* studies using phthalate exposure of isolated adult mouse antral follicles.** The responses are classified under categories "survival", "oxidative stress" and "follicle growth" as these were the endpoints most often considered. Not assessed endpoints are displayed as NA.

Compound	Dose	Exposure Duration	Endpoints			Reference
			Survival	Oxidative stress	Follicle growth	
Phthalate mixture (35% DEP, 21% DEHP, 15% DBP, 15% DiNP, 8% DiBP, 5% BBzP)	1, 10, 100, 500 μg/ml	96 h	NA	NA	Decreased ovulation rate	Land et al., 2021 [89]
Phthalate metabolite mixture (36,7% MEP, 19.4% MEHP, 15.3% MBP, 10.2% MiBP, 10% MiNP, 8.2% MBzP)	0.065, 0.65, 6.5, 65, 325 μg/ml	96 h	Increased expression of pro-apoptotic genes and decreased of anti-apoptotic	Altered gene expression of antioxidant enzymes	Reduced growth	Meling et al., 2020 [90]
DBP	10, 100, 500, 1000 μg/ml	24, 48, 72 h	Increased atresia Increased expression of pro-apoptotic genes and decreased of anti-apoptotic DNA damage	NA	Reduced growth	Rasmussen et al., 2017 [91]
Phthalate mixture (21% DEHP, 35% DEP, 15% DBP, 8% DiBP, 5% BBzP, 15% DiNP)	10, 100, 500 μg/ml	96 h	Reduced atresia Increased oocyte fragmentation	Decreased gene expression of antioxidant enzymes	Reduced growth	Zhou et al., 2016 [29]
DEHP	1, 10, 100 μg/ml	24, 48, 72 h	Increased atresia Increased expression of pro-apoptotic genes and decreased of anti-apoptotic	NA	Reduced growth	Hannon et al., 2015 [92]
MEHP	0.36, 3.6, 36 μM (estimated: 0.1, 1, 10 μg/ml)	48, 96 h	Increased atresia Increased expression of pro-apoptotic genes and decreased of anti-apoptotic	NA	NA	Craig et al., 2014 [33]
DBP	1, 10, 100, 1000 μg/ml	24, 48, 72, 96, 120, 144, 168 h	Increased atresia	NA	Reduced growth	Craig et al., 2013 [93]
MEHP	0.1, 1, 10, and 100 μg/ml	24, 48, 72, 96 h	Increased expression of pro-apoptotic genes and decreased of anti-apoptotic	Increased ROS levels Decreased gene expression of antioxidant enzymes	Reduced growth	Wang et al., 2012 [34]
DEHP	10, 100 μg/ml	24, 48, 72, 96 h	NA	Increased ROS levels Decreased gene expression of antioxidant enzymes Reduced activity of antioxidant enzymes	Reduced growth	Wang et al., 2012 [94]
DEHP, MEHP	1, 10, 100 μg/ml DEHP 0.1, 1, 10 μg/ml MEHP	24, 48, 72, 96 h	NA	NA	Reduced growth	Gupta et al., 2010 [35]

Acceleration of primordial follicle recruitment was observed in response to phthalate exposure, which suggests phthalates might affect follicle dormancy [41]. The same study observed a bigger difference in follicle populations with shorter exposure compared to the longer exposed group. The authors hypothesized that the ovary was able to compensate for the phthalate toxicity by altering metabolism. Another hypothesis was that the excess of primary follicles might undergo atresia in the longer exposed group. This would also explain why no changes were observed in later stages of folliculogenesis. This study highlights possible follicle stage-specific effects of phthalates.

The advantage of *in vivo* studies includes the ability to study ovaries in their natural context as a part of the hypothalamus–pituitary–gonadal axis. Hypothalamus and pituitary gland were not studied as separate organs in any of the mentioned studies, however, most of the studies assessed the levels of gonadotropins or steroid hormones [36–38,40,46–52]. Estrus cycle was also monitored in several of the studies [37,38,40–43,46–48,50–53].

The exposure levels in the *in vivo* rodent studies covered a very wide range, from 0.01 to 3000 mg/kg body weight per day, and were clearly focused on the higher end that is far from typical human exposure levels, even considering mouse to human extrapolation [54]. The geometric mean of the estimated daily intake of DEHP by Swedish women in the SELMA study was 3.9 μg/kg body weight/day, which is in line with other studies suggesting a typical intake range of 3–30 μg/kg per day [7,55]. There is a need for more long exposure studies that dare to test low doses *in vivo,* to better mimic the situation in women.

On the other hand, rats and mice might not be ideal test systems to model effects on fertility in humans. Rodents reach sexual maturity at the age of one month, have short estrus cycles of approximately four days and can ovulate tens of highly developmentally competent oocytes at a time. In stark contrast, women become sexually mature at about 15 years of age, the most fertile women ovulate one oocyte a month, and at best only 30% of human oocytes are developmentally competent [27]. Bovine as long-lived mono-ovulatory species with long estrus cycles has been proposed as a model system that mimics humans more closely [56,57]. Interestingly, the effects of DEHP on ovaries have been tested in cows *in vivo* and *in vitro* [58–60]. Acute three-day oral exposure of cows to DEHP leading to 23 nM MEHP in follicular fluid disrupted follicle development and steroid levels, and a similar low dose exposure of bovine cumulus oocyte complexes to MEHP *in vitro* led to changes in oocyte maturation, competence and gene expression, reinforcing the importance of studying the fertilization potential of follicles [59,60]. Collectively, *in vivo* studies show that phthalate exposures can disrupt ovaries in rodents, and even in cows, and suggest that effects may even take place at levels that can be measured in humans.

Evidence from human cohort studies

We identified eight studies correlating phthalate levels measured in blood or urine with reproductive outcomes in women. In these studies, significant associations between phthalate exposure levels and infertility, longer time-to-pregnancy, earlier menopause, premature ovarian failure and low antral follicle count were found [18,61–67]. We have also identified three original and two meta-analysis studies focused on hairdressers because they are a group with high occupational exposure. Longer time-to-pregnancy and premature ovarian failure were more common among hairdressers compared to women with low occupational exposure [68–72]. All these endpoints connected to higher phthalate exposure could depend on disrupted folliculogenesis and ovarian function. We have also identified studies that failed to find any associations between the levels of phthalates and fertility in women [31,73]. Further, a systematic review on phthalates and female reproductive outcomes found no conclusive evidence connecting phthalates and longer time-to-pregnancy [74].

The majority of the epidemiological studies were based on a limited number of participants (60–938 total participants) leading to low power. The study designs did not reveal any selection biases, and important confounding factors like age, smoking and body mass index were accounted for. In most of the studies, active measures were taken to reduce phthalate cross-contamination during collection, processing and analysis of the samples. However, information bias could be introduced through the choice of test matrix used to measure phthalate levels. Measuring phthalate metabolites in urine is a more reliable biomarker of phthalate exposure than blood [6,75]. It has also been observed in both urine and blood that some metabolites are more accurate exposure biomarkers than others [75,76]. The fast metabolism of phthalates presents a challenge to reliable exposure estimation. Because the levels

Table 3
***In vivo* studies using phthalate exposure in sexually mature rodents.** Both rats and mice are included. The responses are classified under categories "survival", "oxidative stress", "follicle growth" and "fertility" as these were the endpoints most often considered. Not assessed endpoints are displayed as NA.

Strain	Compound	Effect Dose	Exposure Duration	Endpoints				Reference
				Survival	Oxidative stress	Follicle growth	Fertility	
ICR mice	DEHP	500 and 1500 mg/kg	30 d	Increased atresia	Increased lipid peroxidation Decreased levels of antioxidant enzyme in the ovary	NA	NA	Fu et al., 2021 [47]
CD-1 mice	DEHP, DiNP	20, 200 mg/kg DEHP 0,02, 0,1, 20, 200 mg/kg DiNP	10 d	NA	NA	Accelerated folliculogenesis indication	NA	Chiang et al., 2020 [36]
CD-1 mice	DEHP, DiNP	0,02, 0,2, 20, 200 mg/kg DEHP 0,02, 0,1, 20, 200 mg/kg DiNP	10 d	Increased number of atretic/abnormal follicles in low doses and decreased in higher doses	NA	Accelerated folliculogenesis indication	Decreased ability to produce pups Increased pregnancy loss	Chiang et al., 2020 [37]
Wistar rats	DEHP	300, 1000, 3000 mg/kg	4 weeks	Increased atresia Increased granulosa cell apoptosis	NA	NA	NA	Li et al., 2020 [48]
C57 mice	DMP	500, 1000, 2000 mg/kg	20, 40 d	Increased ovarian cell apoptosis	NA	NA	NA	Mei et al., 2019 [49]
CD-1 mice	DBP	0,01, 0,1, 1 mg/kg	30 d	Increased atresia Reduced expression of DNA damage repair genes in the ovary	NA	No statistically significant changes in follicle stage percentages	NA	Liu et al., 2019 [53]
CD-1 mice	DEHP, DiNP	0.02 mg/ml DEHP 0,02, 0,1, 20, 200 mg/ml DiNP	10 d	NA	NA	NA	Decreased fertility index Decreased ability to produce pups	Chiang et al., 2019 [42]
ICR mice	DEHP	0,01, 0,04, 0,08 mg/kg	14 d	Primary follicle degeneration Increased oocyte apoptosis Oocyte DNA and mitochondrial damage	Increased oocyte ROS levels	Reduced oocyte maturation	Reduced oocyte fertilization rate	Lu et al., 2019 [95]
Sprague Dawley rats	DEHP, BBP, DBP	25 mg/kg DEHP 250 mg/kg BBP	6 weeks	NA	NA	Accelerated folliculogenesis indication	NA	Tran et al., 2018 [38]

Table 3 (*continued*)

Strain	Compound	Effect Dose	Exposure Duration	Endpoints				Reference
				Survival	Oxidative stress	Follicle growth	Fertility	
CD-1 mice	DEHP	250 mg/kg DBP 1,33 mg/ml	10 weeks	NA	NA	Accelerated folliculogenesis indication	NA	Cha et al., 2018 [39]
CD-1 mice	DEHP	0,02, 0,2, 20, 200, 500 mg/kg	10 d	Increased antral atresia Decreased total number of follicles Increased expression of pro-apoptotic genes and decreased of anti-apoptotic	NA	Accelerated folliculogenesis indication	No statistically significant changes of breeding outcomes	Hannon et al., 2016 [40]
CD-1 mice	DBP	0.01, 0.1, 1000 mg/kg	10 d	Increased expression of pro-apoptotic genes	NA	Decreased number of antral follicles and corpora lutea	NA	Sen et al., 2015 [50]
CD-1 mice	DEHP	0,02, 0,2, 20, 200, 750 mg/kg (10 d) 0,02, 0,2, 20, 200 mg/kg (30 d)	10, 30 d	NA	NA	Accelerated folliculogenesis indication	NA	Hannon et al., 2014 [41]
ICR mice	DEHP	500, 2000 mg/kg	16 weeks (6 days/week)	Increased granulosa cell apoptosis	NA	Decreased granulosa growth	NA	Li et al., 2012 [51]
Sprague Dawley rats	DEHP	300, 600 mg/kg	60 d (on alternate days)	Increased atresia Increased granulosa cell apoptosis	NA	Decreased number of corpora lutea	NA	Xu et al., 2010 [52]
Sprague Dawley rats	DEHP	1000, 3000 mg/kg	2, 4 weeks	Increased atresia	NA	Decreased number of corpora lutea	Decreased pregnancy rate	Takai et al., 2009 [43]
Sprague Dawley rats	DEHP	2000 mg/kg	3, 5, 6, 7 d	Increased number of apoptotic bodies in preovulatory follicles	NA	Decreased granulosa cell area of preovulatory follicles	NA	Davis et al., 1994 [46]

in body fluids change quickly in response to exposures, reliable estimation of average exposure levels would require multiple urine samples over a long period of time. More than one urine sample was collected in two of the mentioned studies [18,64]. Overall, the evidence from human epidemiological studies on phthalates suggests negative effects on fertility in women but not all studies can detect significant associations and the evidence, therefore, remains inconclusive.

Summary

Data from *in vitro* systems, animal studies and human cohorts suggest that phthalates pose a hazard to ovarian function and may thereby contribute to infertility. It is crucial to better understand the modes of action of individual phthalates in order to avoid regrettable substitution and to be able to

predict mixture effects. A better mechanistic understanding could help interpreting results from different test systems and carry out risk assessment more accurately. Further, identification of the sensitive endpoints in humans will help to design better epidemiological studies.

An important factor to consider in all phthalate studies is cross-contamination with other phthalates and EDCs as these contaminants are omnipresent in the environment, patient samples, and research laboratories. Sources of cross-contamination include leakage from animal housing and laboratory plastic as well as the fetal calf serum used in all culture studies. These cross-contaminations could create mixture effects, thus complicating the interpretation of results even further.

In males, anti-androgenic effects of phthalates have been recognized as most critical but there is a lack of studies investigating the sensitive modes of action in females. One way forward could be more detailed analyses of clinical patient samples. For example, urinary concentrations of phthalates have been associated with altered expression of extracellular vesicle-miRNAs (EV-miRNAs) in human ovarian follicular fluid [77]. EV-miRNAs are associated with cellular communication in the ovarian follicles. Thus, their altered expression indicates that phthalate exposure could dysregulate the ovarian follicle function, potentially affecting fertility.

This review suggests that oxidative stress could be one plausible mechanism of action of phthalate-induced disruption of ovaries. Urinary phthalate levels have been associated with intra-ovarian oxidative stress in women [78], which could accelerate ovarian aging via effects on mitochondria and DNA damage [79–81]. Mitochondrial integrity of oocytes affects their quality and is crucial for folliculogenesis, fertilization and embryo development [82]. Because changes in oxidative stress and antioxidant system have been observed even in low exposure in some experimental studies, this mechanism should be considered in future studies in more detail.

Research agenda

- For a better understanding on phthalate effects on fertility in women, all parts of the female reproductive system need to be considered.
- Mechanisms of action of phthalate-induced disruption of hypothalamus–pituitary–ovary axis need to be identified.
- Most sensitive endpoints need to be identified in different test systems so that holistic evaluation of data available in the literature can be carried out.
- Mechanistic information should be tailored into adverse outcome pathways (AOP) to increase regulatory impact.
- More low dose effect studies are needed to allow for better risk assessment.
- Multiple individual phthalates need to be assessed to reveal if all phthalates pose similar hazards.
- Because phthalates occur in mixtures, mixture effects also need to be studied.

Practise points

- Phthalates are a large group of industrial chemicals used in plastics, personal care products, perfumes and medications.
- Exposure to phthalates is life-long starting from germ cells and fetal development.
- Women can have higher exposure to phthalates compared to men because of lifestyle and occupational choices (use of cosmetics, beauty salon work).
- Some phthalates have been classified as endocrine disruptive chemicals and reproductive toxicants in humans based on data of adverse effects in males.
- Literature suggests that phthalates pose a hazard to female fertility and reproductive health too.
- There is an urgent need to identify modes of action and most sensitive endpoints of phthalate toxicity in women in order to protect populations from harmful effects.

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