The Open Biotechnology Journal

Effects of Endocrine-Disrupting Chemicals on Female Reproductive Health

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The Open Biotechnology Journal, Volume 9, 2015

ISSN: 1874-0707/15
DOI: 10.2174/1874070720151112E024

Article Type: Review Article

Received: May 28, 2014
Revised: May 10, 2015
Accepted: June 05, 2015

Provisional PDF Publication Date: November 17, 2015

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Keywords: endocrine-disrupting chemicals (EDCs), xenoestrogen, ovary, uterus, female reproductive health, developmental reprogramming, epigenetic, diethylstilbestrol, bisphenol a, genistein, methoxychlor, phthalate

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Conflict of Interest: None

Abstract

Endocrine-disrupting chemicals (EDCs) are increasingly prevalent in the environment and the evidence demonstrates that they affect reproductive health, has been accumulating for the last few decades. In this review of recent literature, we present evidence of the effects of estrogen mimicking EDCs on female reproductive health especially the ovaries and uteri. As representative EDCs, data from studies with a pharmaceutical estrogen, diethylstilbestrol (DES), an organochlorine pesticide methoxychlor (MXC), a phytoestrogen (genistein), and a chemical used in plastics, bisphenol a (BPA) have been presented. We also discuss the effects of a commonly found plasticizer in the environment, a phthalate (DEHP), even though it is not a typical estrogenic EDC. Collectively, these studies show that exposures during fetal and neonatal periods cause developmental reprogramming leading to adult reproductive disease. Puberty, estrous cyclicity, ovarian follicular development, and uterine functions are all affected by exposure to these EDCs. Evidence that epigenetic modifications are involved in the progression to adult disease is also presented.

1. Introduction

It is well known that toxic contaminants in air, water, and agricultural produced has contributed to exposure to mutagens that cause numerous health problems including cancers [1-3]. Large numbers of these xenobiotics are endocrine-disrupting chemicals (EDCs) that are, in general, not mutagenic but can cause more subtle effects: they cause disruption in hormone synthesis and signaling. While many hormone responsive organs are sensitive to EDCs, the ovaries and uteri are most sensitive to the EDCs that mimic estrogen, the female steroid hormone.

The direct consequences of the detrimental effects of EDCs on female reproductive health are impaired reproductive organ function, infertility and/or cancer. However ovarian dysfunction can lead to reduced serum estradiol levels, which are associated with increased risk of cardiovascular diseases [4], loss of bone density [5, 6], and sexual dysfunction [7]. In addition, the effects on the female germ cells, the oocytes, can potentially cause multigenerational effects. Therefore, EDCs that disrupt female reproductive health have long-term and widespread effects. Furthermore, the ubiquitous expression of estrogen receptors (ERs) in multiple tissues make the actions of myriad xenoestrogens possible.

It has been shown in numerous epidemiological studies that women's reproductive health is severely affected by exposure to estrogenic EDCs in the form of pharmaceuticals, pesticides, industrial products such as plasticizers, and phytoestrogens [8-13]. The impaired fecundity rate in the U.S. increased from 11% to 15% between 1982 and 2002 [14, 15]. Although various confounding factors such as lifestyle changes could have contributed to this decline, the role of EDCs cannot be discounted. The incidence of female reproductive disorders such as early puberty, premature ovarian failure, impaired fertility as well as breast, ovarian, and uterine cancers [16] have been documented in animal studies with estrogenic EDCs and have been substantiated by a large body of epidemiological evidence from humans and wildlife as well [17-23].

Exposures to EDCs in adulthood cause severe reproductive disorders as mentioned above but the most severe and long-lasting ovarian and uterine diseases that occur by adulthood are caused by exposures in fetal and neonatal periods [24, 25]. Embryonic epigenetic programming is fine-tuned during differentiation and development of the organs [26]. Developmental reprogramming of the organs involves disruption in the epigenetic reprogramming and has been considered to be a mechanism by which the developmental trajectory of these organs is altered [26-28]. The somatic components of the ovary develop during mid-to-late gestation and are modified throughout postnatal folliculogenesis [29]. Similarly, it is proposed that epigenetic reprogramming of the uterine epithelium occurs as the early developmental, tissue organizational events take place in the first week after birth in rodents [30]. Therefore any disruption in the ovarian and uterine epigenomes at this stage could lead to altered gene expression by adulthood [24, 31-33]. In addition, germ cells undergo their own epigenetic programming: the germ cell epigenome that is methylated early in embryonic stage is demethylated in mid-gestation, and remethylated in a sex-specific manner at tissue-specific developmental stages [27, 28], Specifically, female germ cell remethylation is initiated during the early postnatal period, during follicular assembly and initial recruitment, and continues throughout oocyte growth until the antral follicle stage specifically in rodents [34]. Recent observations in mouse and bird embryos have shown that the precursors of oocytes (primordial germ cells) express functional estrogen receptors, namely ESR-1 and GPR-30, respectively, which may be able to activate non-genomic signaling in such cells via the PI3K/AKT signaling pathway [35, 36]. These germ cell processes can also be a target for EDCs, suggesting that EDCs might affect germ cell development during a crucial period of their nuclear reprogramming [37].

There are numerous lines of evidence emerging that suggest that the exposure to estradiol or estrogenic EDCs can cause epigenetic alterations in sensitive developmental windows that might have long-term effects by adulthood [25, 38]. DNA (CpG) methylation and histone

94 modifications are necessary for tissue-specific gene regulation. Usually, an increase in DNA
95 methylation at a locus is associated with the interference of transcription factor (TF) binding,
96 resulting in down-regulation of gene expression, and *vice versa* [39-42]. Post-translational
97 modifications on histone proteins of the nucleosomes such as acetylation, methylation, and
98 phosphorylation at specific amino acid residues (lysine, arginine, serine, or threonine) contribute
99 either to euchromatin or the silencing of loci (heterochromatin). This silencing can be reversible
100 or irreversible, depending on further modification [43-46]. For example, some histone
101 methylation events (e.g., H3K9me3) work in conjunction with DNA methylation to stably silence
102 genes [47].
103
104 **2. Critical ovarian and uterine developmental stages sensitive to estrogenic EDCs.**
105 A female's reproductive lifespan depends on the size and health of the initial pool of
106 primordial follicles and their progression and maturation into primary, secondary, antral, and
107 eventually ovulatory follicles. Complex bidirectional communication occurs between the oocyte
108 and its surrounding somatic cells involving stimulatory inputs from local paracrine factors as well
109 as steroid hormones [48-50]. The gonadotropins, follicle stimulating hormone (FSH) and
110 luteinizing hormone (LH), have a significant role in the selection and maturation of the follicles
111 via stimulation of IGF-1 and estrogen signaling pathways among others [51]. Once an oocyte is
112 fertilized, the implantation of the embryo into the uterus and successful pregnancy and parturition
113 are dependent on healthy uterine function. Critical uterine developmental windows overlap with
114 those of the ovary in the first two weeks after birth, with the development of luminal epithelium
115 and the stromal glandular epithelium. These processes are regulated by the WNT and HOX gene
116 families and are responsive to IGF-1 and estrogen signaling as well [52-54]. Therefore, estrogenic
117 EDC exposures during early ovarian and uterine development are a major threat and have the
118 potential to reprogram ovarian and uterine functions.
119

120 **2.a. Primordial follicle development and transition to primary follicles in ovaries**

121 Oocytes are arrested at the early diplotene phase of meiotic prophase I and enclosed in
122 nests surrounded by somatic pregranulosa cells. Starting at E16.5, in mice and rats, most oocytes
123 are eliminated via apoptosis [55-57]. The remaining oocytes are surrounded by a single layer of
124 flattened pregranulosa cells and form the primordial follicles, a process that is almost complete by
125 PND 3-4. Most of the primordial follicles remain quiescent, but some begin growing and
126 transition to the next stage, primary follicles. Both of these early processes, primordial follicle
127 formation and primordial to primary follicle transition (the initial recruitment), are tightly
128 regulated by interactions between paracrine factors, transcription factors, and steroid hormones
129 while being independent of gonadotropins. However, since these processes determine the success
130 of female reproduction, endocrine disruption can lead to early depletion of follicles and therefore
131 may result in early reproductive senescence [50].

132 In mouse and rat models, estradiol and progesterone have been shown to inhibit
133 primordial follicle formation by inhibiting apoptosis [48, 49] a process that could be reversed by
134 pro-apototic TNFα [58, 59]. The actions of estradiol and estrogenic EDCs (e.g., DES) may
135 involve the inhibition of pro-apoptotic molecules, such as Fas ligand [60] leading to multioocyte
136 follicles (MOFs) that in the long-term, do not progress to healthy ovulations [48]. On the other
137 hand, activins have a stimulatory role in primordial follicle formation. Neonatal activin treatment
138 increased the number of postnatal primordial follicle by 30% in mice. However, the excessive
139 number of follicles was not maintained at puberty or beyond [61]. Thus, there is an interplay
140 between the inhibitory activity of estradiol and the stimulatory role of activins in follicular
141 formation. Neonatal estradiol or DES exposures induce MOFs but also inhibit activin levels in the
142 ovary [62]. These results suggest that the paracrine systems that control the primordial follicle
143 formation process can be influenced by estrogenic EDCs.

144 The oocyte-derived FOXO3 is a major suppressor of primordial to primary follicle
145 transition [63]. When it is deleted in mice, although the initial primordial follicle pool is
146 established normally, primordial follicles are activated en masse, leading to early elimination of
147 follicular reserve and reproductive senescence. Androgens inhibit FOXO3 activity [64], and also
148 suppress the expression of growth differentiation factor-9 (GDF9), a well-known stimulator of
149 follicle development beyond the primary stage. As a result, exposure to androgens causes an
150 accumulation of preantral-stage follicles. Overall, estrogens may inhibit the initial recruitment by
151 stimulating inhibitory paracrine factors (e.g., AMH) while androgens may stimulate the initial
152 recruitment by inhibiting suppressive factors (e.g., FOXO3).

153

154 **2.b. Follicle selection, antral follicle development, and ovulation**

155 A single follicle or multiple follicles, in monoovulators versus polyovulators, within a
156 recruited cohort is/are selected at the antral stage to complete folliculogenesis and achieve
157 ovulation. An important criterion (among several) for the selection is that the follicle secretes
158 high levels of estradiol. Local growth factors such as insulin-like growth factors (IGFs), activins,
159 transforming growth factor (TGF) α and β, hepatocyte growth factor, and FGF7 are also required
160 for this process [65].

161

162 In addition, IGFs are considered to be critical for follicular maturation since they
163 stimulate cell proliferation and steroidogenesis in granulosa cells of various species [66, 67]. In
164 contrast, IGF binding proteins (IGFBPs) can suppress FSH-induced follicular growth and
165 differentiation by sequestering IGF-I protein and inhibiting its activity that leads to atresia [68,
166 69]. Prior to maturation, estradiol production is markedly elevated in the selected antral follicles,
167 which exerts a positive-feedback effect on gonadotropin secretion. The rise in FSH and LH
168 supports further increase in steroidogenesis initiates luteinization, whereby granulosa cells switch

169 from an almost exclusive production of estradiol to the production of both estradiol and
170 progesterone. The feedback dynamics within the HPG axis continue and culminate with the
171 preovulatory LH that stimulates ovulation [70]. Multiple factors play roles in ovulation, including
172 ESR2, progesterone receptor, proteases, epidermal growth factor-like proteins, and prostaglandin
173 synthase-2 (see [70] for review). Following ovulation, the remnants of the ovulated follicle are
174 stimulated by LH to terminally differentiate into the corpus luteum (CL). The CL, as a primary
175 source of progesterone, is essential for enabling the initiation and maintenance of pregnancy
176 (reviewed in [71, 72]).
177
178 A salient point to be noted regarding these processes is that the ovary is a hormone-
179 responsive tissue and contains follicles at every stage of development that are highly dynamic and
180 require temporal- and cell-specific and stage-dependent regulation of numerous genes, which
181 could be controlled by epigenetic mechanisms. Therefore they can be affected by developmental
182 exposures to EDCs thus making the ovary a unique target for EDCs for epigenetic modulation.
183
184 **2.c. Critical steps in prenatal and postnatal uterine organogenesis**
185 The female reproductive tract (FRT) - oviducts, uteri, cervix, and vagina - develops from
186 the Mullerian ducts (MD) in females [73]. The development of the FRT has been described in
187 detail previously [74]. For the purpose of understanding the most severe effects of EDCs on the
188 FRT, uterine development is most pertinent to this review. The uterus has varied roles depending
189 on the reproductive stage: implantation, maintenance of pregnancy, and parturition. Prenatal
190 uterine organogenesis involves the regression of the Wolffian ducts in the absence of MIS and
191 testosterone and the development of the MD after the sexual differentiation of the XX gonad.
192 Fusion of the MDs and formation of the uterus is complete by E16. Mice and rats have a duplex
193 uterus while humans have a single uterus, however the histological architecture has similarities.

194 The uterus consists of the endometrium, whose structure actively alters during the
195 estrous/menstrual cycle, and the myometrium that has a smooth muscle layer that sorrounds the
196 endometrium. The luminal epithelium (LE) of the endometrium is composed of simple and
197 columnar epithelial cells and is surrounded by uterine/endometrial glands. Uterine functional
198 development occur postnatally in rodents, starting at PND 1-3 [75], which is equivalent to human
199 uterine development at around gestational week 14 [76]. At birth, in rodents, the uterus does not
200 have endometrial glands but their rudiments develop by about PND 5 and becomes apparent by
201 PND 7-9. Subsequently the glands extend into the stroma and myometrium and become
202 organized into bundles and are fully developed by the second week after birth [77-79]. Numerous
203 signaling pathways such as the WNT and HOX pathways are involved in the uterine formation,
204 patterning and organogenesis [80-82]. However postnatal uterine function is dependent on the
205 coordinated induction of several growth factors, cytokines and their receptors (e.g., IGF-1, FGF,
206 activin/follistatin signaling) in addition to estrogen signaling [83-85]. In fact, these signaling
207 pathways are highly responsive to and augment the estrogen signaling. Thus developmental and
208 functional uterine stages are prone to EDCs' actions.
209
210 **2.d. Expression patterns and roles of ERs in the ovaries and uteri**
211 Most estrogenic EDCs have been shown to activate genomic or non-genomic estrogen
212 signaling. These actions are mediated via the endogenous ERs (ESR1 and ESR2) in the ovary and
213 uterus. Thus the ubiquitous expression of ERs in multiple reproductive tissues make them prone
214 to the actions of EDCs. ESR1 and ESR2 are expressed in early folliculogenesis in a cell- and
215 stage- specific manner in several species, including primates, cattle, rats, and mice [25, 86-89].
216 ESR1 is expressed primarily in theca cells, and ESR2 is expressed in granulosa cells and essential
217 for FSH-directed granulosa cell differentiation as well as for LH responsiveness [90, 91]. ESR2
218 also facilitates mechanisms that promote follicle maturation from the early antral to the
219 preovulatory stages [92, 93]. In addition it may play a major role in primordial follicle formation

in the ovary [93-97]. In contrast, although ESR1 plays a role in the regulation of theca cell steroidogenesis in the ovary, its main function is to mediate estrogen-regulated feedback in the hypothalamus and pituitary [98, 99]. On the other hand, very little is known about non-genomic estrogen signaling that is mediated by membrane bound ESR1 (mESR1) in the ovary but recent evidence has demonstrated a role for PI3K/AKT signaling downstream of potential mESR1 activation in the ovary [25, 100].

In the uterus, ERs are actively expressed during Mullerian duct development and are seen as early as E13 in the mesenchyme, while the uterine epithelium expresses ERs soon after birth [101]. Interestingly, uterine development is estrogen-independent during neonatal development. However the presence of ERs makes the uterus susceptible to the actions of EDCs. The predominant ER receptor in the uterus is ESR1; using KO studies, it has been demonstrated that ESR1 disruption causes hypoplastic uteri [90, 102]. It is expressed in both the luminal and glandular epithelial compartments.

3. Epidemiological evidence from humans supporting involvement of EDCs in female reproductive disease and *in vivo* studies with EDC exposures in rodent models

3.e. Diethylstilbestrol (DES)

For about 30 years between the 1940s and the 1970s, DES, a nonsteroidal synthetic estrogen was prescribed at doses of 5-150 mg/day, to pregnant women at risk of miscarriage. The most convincing human evidence that estrogenic EDC exposure during development can permanently affect female reproduction, comes from the reports that followed [103]. Numerous abnormalities in the reproductive, cardiovascular, and immune systems have since been reported in both male and female offspring of women treated with DES, and similar effects have been

demonstrated in animal models (reviewed in [104]). These effects are being observed in the granddaughters of DES-treated women as well [105, 106]. While DES caused vaginal clear cell adenocarcinoma in only 0.1% of the female offspring, over 95% reported reproductive tract dysfunction and poor pregnancy outcomes [107, 108]. There is evidence of multi-generational effects and epigenetic mechanisms have been implicated [109-112].

3.f. DES *in vivo* studies

Mice injected with a single dose of 10 µg/kg DES on E15 and examined at 7 months of age had no CL and numerous atretic follicles [113]. They were also found to have vacuolated interstitial tissue with lipid droplet inclusions. Other studies with vary doses of DES (5 µg/kg to 100 µg/kg) administered either *in utero* (E9-E16) [114], or neonatally (PND 1-5) [115], demonstrated that adult DES ovaries developed similar hypertrophy and vacuolation of interstitial tissue, hemorrhagic cysts and lack of corpora lutea. These animals also had high levels of testosterone [114]. There was a dose-dependent reduction in the number of the litters as well as the number of oocytes ovulated after stimulation with exogenous gonadotropins [116]. The oocytes derived from such treated ovaries and used in IVF showed lower levels of fertilizability, suggesting reduced oocyte quality [117-119]. However, 5 µg/day DES-treated ovaries transplanted into untreated ovariectomized host mice were able to give rise to normal female offspring that in turn gave birth to normal size litters and had normal uterine morphology, suggesting that the DES treatment effects were not mediated via germ cells [120].

DES can bind to both ERs with many fold higher affinity than estradiol [94]. Multiple studies from Iguchi and colleagues showed that *in utero* (E15-18) and neonatally (PND 1-5) DES-treated mice had ovaries containing excessive number of MOFs by adulthood [121, 122]. MOFs were also observed in ovaries that were treated *in vitro* at PND 1-5, following their transplantation to untreated mice, suggesting a direct effect of DES in the ovary [122]. Recent studies showed that

neonatal exposure to 3 µg/kg DES induced MOFs, a process mediated by ESR2 and not ESR1 [97]. DES exposure was shown to reduce oocyte apoptosis (potentially suppressing oocyte nest breakdown) via ESR2 signaling mechanisms. Furthermore, it was hypothesized that such alterations in the germ cell and somatic cell populations may affect the invasion of pregranulosa cells and basement membrane remodeling during primordial follicle formation [60]. Interestingly, the incidence of MOFs has been reported with other EDC exposures as well (see below, [96]).

It is well known that DES caused T-shaped uteri and clear cell adenocarcinoma of the uterus, cervix, and vagina in women whose mothers were exposed to DES during pregnancy [123]. Such observations have been replicated in the progeny of DES-treated mice that show malformations of the uterus, squamous metaplasia of the luminal and glandular epithelium, endometrial hyperplasia and leiomyomas, and oviductal proliferative lesions [124, 125]. Ovariectomized animals when supplemented with estradiol are able to respond by a transient increase in gene expression and concomitant uterine proliferation and growth [126-128]. When such a stimulus is removed, the uterus returns to its unstimulated state. However, when DES or estradiol is administered during neonatal development, expression of immediate early genes such as *lactoferrin*, *EGF*, and proto-oncogenes such as *c-fos*, *c-jun*, and *c-myc* is upregulated even into adulthood [126, 129, 130]. Inversely, expression of genes that are necessary for uterine development, such as the *Abdominal B (AbdB) Hox* gene, *Hoxa-10*, (known to be controlled by estradiol and progesterone, [131]), *Wnt7a* as well as *Msx2* are repressed leading to structural abnormalities of the reproductive tract [132-135]. Numerous studies have been conducted to assess the methylation patterns of promoters of several of these estrogen-responsive genes associated with uterine development.

Neonatal DES exposure in mice caused ~ 90% incidence of epithelial cancers of the uterus by 18 months of age [136]. Furthermore, the promoter region of the *lactoferrin* gene was

found to be hypomethylated in the adult uterus. However, if the animals were exposed for the same length of time during adulthood, no such DNA methylation or expression defects were observed [137]. Subsequently, it was also found that *exon 4* of the *c-fos* gene was extensively hypomethylated while the promoter region and intron 1 was unaffected, thereby potentially allowing for the upregulation of *c-fos* expression [138]. QPCR studies performed by Sato and colleagues examining the expression of *Dnmts* in neonatally DES exposed C57BL/6 mice, revealed that expression of *Dnmt1* and *Dnmt3b* was decreased at PND5 in DES-treated mice, and the pattern continued until PND14 [139]. Interestingly, it was found that human leiomyoma samples had alterations in the levels of *Dnmts* as well, with concomitant global hypomethylation [140].

DES down-regulates *Hoxa* gene expression akin to the effects associated with uterine abnormalities found in *Hoxa* KO mice. The predominant phenotype is the loss of boundary between the oviduct and uterus. It has been shown that the anterior to posterior specific pattern of *Hoxa-9* is essential for the normal development and function of the uterus and that DES causes a posterior shift of *Hoxa-9* and *Hoxa-10* expression and homeotic anterior transformations [132]. A recent report by Bromer and colleagues has shown that after *in utero* (E9-16) exposure to 10 μg/kg DES, there is hypermethylation in the promoter and *intron 1* regions of *Hoxa-10* gene, in the caudal part of the uterus with a concomitant increase in the *Hoxa-10* expression in the same region [141]. Recent reports have suggested that cell fate decisions are altered due to exposure DES.

Interesting new studies have now provided a link between mESR1 signaling and regulation of histone modifications. It was found that rapid PI3K/AKT signaling downstream of membrane-associated ER, in response to estradiol as well as DES, caused reduction in trimethylation of H3K27, a repressive histone mark. More interestingly, activation of this

nongenomic signaling caused reprogramming of the uterine gene expression profile [46, 142]. It has also been found that neonatal DES exposure temporarily alters expression of multiple chromatin-modifying proteins and persistently alters epigenetic marks in the adult uterus at the *sine oculis homeobox 1* locus which along with lactoferrin (see above) is an estrogen responsive gene whose expression is persistently upregulated [38].

3.g. Methoxychlor (MXC)

Methoxychlor is a well-studied organochlorine pesticide that is used as a replacement for DDT. It is an estrogenic compound that demonstrates low-affinity binding for estrogen receptors [143]. The major MXC metabolites, HPTE and mono-OH MXC, can function as estrogenic, anti-estrogenic, or anti-androgenic compounds [144], and therefore it is used as a model compound [145]. Epidemiological studies have shown that there is a strong association between developmental exposure to organochlorine pesticides and underdeveloped fetuses and subsequent female fertility problems [146]. For example, presence of *p'p'*-DDT in the mothers' serum 1-3 days after their daughters' birth is associated with a longer time of pregnancy (TPP) as well as with a reduced probability of pregnancy and high infertility [147]. A two to threefold increase in risk of prolonged time-to-pregnancy and spontaneous abortion, among female greenhouse workers [13, 148] and increased infertility in women with agricultural work histories has also been noted [149].

3.h. Methoxychlor *in vivo* studies

Adverse effects that were observed in these association studies are similar to the effects observed in experimental animals exposed to MXC during adulthood. Exposure to MXC (2500 or 5000 ppm) interfered with the normal estrous cycle, reduced mating rate and litter size [150]. However, when the exposure was withdrawn, these animals reverted to regular estrous cycles. In

general, this observation applies to most other estrogenic EDCs as well. Further studies demonstrated that adult mice or rats that were exposed to MXC showed persistent vaginal estrus [151], direct inhibition of embryonic growth, implantation failure [152], pregnancy loss [153], and ovarian atrophy due to inhibition of folliculogenesis leading to atretic follicles and reduced ovulation and decreased numbers of CL [151, 154, 155]. It was shown that exposure to MXC in adult mice selectively affects the antral follicles and induces atresia using the Bcl2/Bax signaling pathway, without affecting the HPG axis [156].

In contrast, when the exposure periods included *in utero* and early postnatal development period, the effects lasted into adulthood with more severe outcomes on reproductive parameters in rats. These included acceleration of the vaginal opening (sign of puberty), acceleration of the onset of the first estrus, irregular cycles with persistent vaginal estrus, reduced pregnancy rate and litter size despite apparent mating, and early reproductive senescence [157-159]. Serum estradiol and progesterone levels were altered with increased FSH levels [158]. The effects on the ovary were dramatic, with both folliculogenesis and ovulation being inhibited.

In a more recent study, female rats were treated during fetal and neonatal development (E19-PND 7) with a dose of MXC that is comparable to the dose used in the above studies (100 mg/kg/day) the exposed females displayed similar abnormalities in reproductive parameters as well as in ovarian morphology by adulthood [160]. A close examination of follicle composition showed that developmental MXC treatment did not affect the total number of follicles or follicles at primary and secondary stages in adult females. However, the number of preantral and early antral follicles was increased and the number of CL was reduced, with numerous large cystic follicles. Immunohistochemical staining and quantification of expression patterns of important regulators of ovarian functions revealed that while LHR, CYP11A1, and CYP19A1 levels were

reduced, levels of AMH and AR were increased, and levels of StAR and ESR1 were unchanged [160]. Especially noteworthy was that ESR2 level was unchanged in primary and secondary follicles, yet decreased dramatically in peri-antral stage follicles, which are responsive to gonadotropins. These observations suggest that hormone-responsive follicles are most affected by EDC exposure.

Epigenetic analyses using bisulfite-sequencing PCR and methylation-specific PCR showed that MXC caused hypermethylation in multiple CpGs in two CPG islands in ESR2 promoter sequences while it had no effect on DNA methylation levels in the ESR1 promoter at PND 60 [24]. This finding correlates with the lack of significant effects on the levels of ESR1 protein in the adult ovary [24, 160]. Further analysis has shown that the DNA methylation levels in the promoter regions of these genes were unchanged in neonatal ovaries (PND 7) immediately after the exposure (Zama, 2013). These data demonstrate the age-dependence/hormone responsiveness of the epigenetic changes, which has also been shown in other tissues (e.g., uteri) with other compounds (e.g., DES, genistein) [161]. The global DNA methylation analysis using AP-PCR showed that there were multiple loci that were hypermethylated in MXC-treated ovaries [24]. The majority of candidates were those encoding transcription factors or ribosomal proteins. One candidate that was shown to be hypermethylated in multiple MXC-treated samples was an endopeptidase encoded by *PAPP-A* locus [24]. Reduced PAPP-A activity due to increased methylation could limit its availability in follicles and thus increase IGFBP content and sequester IGF-1. This could lead to the observed defect in follicle selection and maturation [160]. Interestingly, in the same set of studies, exposure to a low dose of MXC (20 μg/kg/day) caused a significant increase in the expression of AMH [160] and multiple methylation events both in the ESR2 promoter sequences and the PAPP-A locus [24]. There was a significant upregulation in ESR2 expression in the granulosa cells of multiple stages of follicles at PND 7, similar to high-

dose MXC-treated follicles. While these epigenetic alterations did not cause any functional defects in the low dose-MXC treated females, the high dose-MXC treated animals had the characteristic ovarian dysfunction. A more recent targeted genome-wide methylation array study has revealed that members of essential signaling pathways are hypermethylated and their gene expression down-regulated in MXC-treated ovaries. IGF-1 signaling was the most significantly affected pathway wherein several members of the family – *Igf1r*, *insulin receptor* (*Insr)*, *Pik3r1*, *Hras,* and *Foxo3* – were hypermethylated [25]. These data suggested that the initial DNA methylation patterns were representative of the gene expression patterns responsive to the EDC exposure and not the adult hypermethylation events. Furthermore, the long-lasting effects observed by PND 60 could be due to histone modifications. Unpublished data from our laboratory has shown that histone trimethylation, H3K9me3, an inhibitory histone mark, is increased in antral follicles of MXC-treated ovaries suggesting suppression of stage-specific gene expression thus disallowing antral follicle progression to ovulation.

Uterotrophic effects of MXC are well established [162]. MXC increases uterine wet weight, proliferation and protein secretion [163, 164]; these effects have been attributed to its estrogenic actions [152, 165-169]. In some cases, MXC can interfere with or differ from the actions of estradiol [151]; this was also reported in other experimental systems [22, 170, 171]. More recently, it was shown that *in vivo,* neonatal MXC exposure inhibits *Hoxa-10* expression in the adult uterus in mice and interferes with the binding of estradiol to ERE of *Hoxa-10* [172]. Although a potential epigenetic mechanism was suggested, confirmation of this possibility awaits future studies [53].

3.i. Genistein

The use and consumption of soy products is ubiquitous. However the isoflavonoid phytoestrogen, genistein, derived from soy products has been shown to have endocrine-disrupting potential in domestic species: newborn lambs born to ewes fed clover had reproductive abnormalities (in the late 1940s [173]). United States FDA has approved 25g/day soy consumption, approximately equivalent to 75 mg of isoflavones/day (1 mg/kg/day), as being beneficial against coronary artery disease (FDA, 1999). However, a cause for concern is that babies who are fed soy formula consume on average of 6-9 mg/kg body weight, which would result in babies being exposed to 4-7 times higher amounts of soy as compared to adults that are on a soy-rich diet or as per FDA guidelines [174, 175]. Early life exposure to soy formula is associated with a greater risk of uterine fibroids in adulthood among other conditions [176, 177].

3.j. Genistein *in vivo* studies

Neonatal administration of 0.5-50 mg/kg genistein (PND1-PND5) caused an increase in ano-genital distance (masculinization), accelerated puberty, and irregular estrous cycles in adult CD-1 mice [178]. In this context, genistein-treated (50 mg/kg/d) mice exhibited defects in the ovary such as the MOF phenotype, which correlated with a reduction in the number of apoptotic oocytes, previously shown to involve ESR2 mediated actions [48, 95, 96]. This was also associated with fewer pups born to these females over their shortened reproductive lifespan [179, 180]. Genistein and other phytoestrogens have been shown to readily cross the placenta [181] and exposure *in utero* between E15 and E19 has shown similar effects as mentioned above [182]. A most recent report on the oral administration of genistin (the glycosylated form of genistein) revealed that exposure between PND1-5 also resulted in ovaries with MOFs, delayed puberty,

irregular estrous cycles and reduced litter sizes [183]. It has been demonstrated that the estrogenic action of genistein is mediated via ER mediated pathways [93, 184].

Numerous uterine defects have been documented in CD-1 mice that were neonatally exposed (PND1-5) to genistein (50 mg/kg/day) [178, 185, 186] supporting epidemiological data from women who were soy-fed as babies that had irregular menstrual cycle lengths and pain during cycles or uterine fibroids [176, 187]. A recent paper showed that the oocytes are themselves competent for fertilization and early embryonic development, but the uteri are unable to produce viable implantations: the sites were smaller and fewer in number [188]. Another study has shown that genistein induces fluid accumulation in the uterus in ovariectomized rats via ER signaling and the cystic fibrosis transmembrane regulator [189]. These results not only confirm the effect of genistein as an EDC but also shed light on the mechanism of fluid retention, in this case, as a therapy for menopausal conditions.

Tang and colleagues recently investigated whether neonatal DES/genistein exposure could cause epigenetic changes and alter gene expression in adult uteri and whether there are interactions between adult ovarian hormones and such epigenetic reprogramming. CD-1 mice were exposed to DES (1 μg and 1000 μg/kg) or genistein (50 mg/kg) from PND1-5. Subsequently, some animals were sacrificed at PND19 while others were aged to 6 and 18 months with or without ovariectomies. Genome-wide methylation analysis was conducted with MSRF and candidate genes were identified. Of interest was the *nucleosomal binding protein 1* (*Nsbp1*), which was shown to be hypomethylated at PND19 and hypermethylated by puberty, in the control. Low-dose DES- and genistein- treated vs high-dose DES-treated animals had opposing methylation patterns. Furthermore, it was shown that in the aged animals, both DES and genistein caused hypermethylation in the ovariectomized animals but remained hypomethylated in non-ovariectomized animals. These data suggest that *Nsbp1* is hypermethylated in intact mice

with age and that DES and genistein have opposing effects on the methylation patterns in intact vs ovariectomized aging animals (hypomethylation vs hypermethylation), respectively. These studies highlighted the age-dependent aspect of epigenetic reprogramming and also its interaction with steroid hormones [161].

3.k. Bisphenol A (BPA)

Bisphenol A is a high-volume plasticizer whose total worldwide production exceeds 6 million tons per year [190]. Used in the manufacture of polycarbonate plastics and epoxy resins, exposure can occur via plastic food containers (especially when heated or microwaved), food and drink cans, baby bottles, and carbonless paper (reviewed in [191, 192]). As a result, 95% of adults who were tested have detectable levels of BPA in their urine [193].

Infants in neonatal intensive care units have particularly high exposure to BPA, presumably from its use in medical devices and from the migration of BPA into infant formula from the container. It has also been found in detectable amounts in dust [193-196]. Urine BPA levels of women undergoing infertility treatment is negatively correlated with the number and quality of eggs retrieved, and with serum E_2 levels [197, 198]. BPA has been shown to have estrogenic properties and that it can be transferred both lactationally and transplacentally [190, 199]. BPA has a lower binding affinity to ERs than estradiol or DES [94, 200]. A major concern is that the "safe" exposure limit for BPA is 50 μg/kg/day but studies with lower doses than the "safe" dose demonstrated numerous detrimental defects in the female reproductive system [190].

3.l. BPA *in vivo* studies

Perinatal exposure to low environmentally relevant BPA doses (25-250 ng/kg) caused accelerated puberty, altered estrous cyclicity and disrupted ovarian morphology associated with changes in body weight and LH levels [201-203]. An increased occurrence of ovarian cysts with blood filled bursae, abnormal numbers of antral follicles, and decreased CL was found in aged mice that were neonatally exposed to a 100 μg/kg dose of BPA [204]. Another study

498 demonstrated that exposure of rats to 50 μg/kg and 50 mg/kg doses during the period of
499 hypothalamic neuronal establishment (PND0-3), resulted in a reduction in CL and increase in
500 MOF and hemorrhagic follicles confirming that BPA has direct effects on the ovary that are
501 independent of GnRH neuronal activity[205]. MOFs were also observed in studies with neonatal
502 BPA exposure (150 μg/kg dose), in mice [206].

503 Another effect of BPA is exerted at the level of oogenesis and is of very high concern
504 [197]. Studies from Hunt and colleagues demonstrated that BPA released from damaged animal
505 cages and water bottles, which were inadvertently treated with harsh alkaline detergent, induced
506 defects in the meiotic prophase stage of oocyte development in mice: oocytes had increased levels
507 of meiotic aneuploidy due to congression failure. This effect was mimicked when cages were
508 intentionally damaged, or when 20 to 22 day old mice were exposed to a similar dose of BPA (20
509 ng/g body weight) for as few as 7 days [207]. Further studies demonstrated that BPA caused
510 defects in synapsis and recombination in the homologous chromosomes in the fetal ovary.
511 Interestingly, βERKO animals exhibited very similar meiotic defects in the pachytene oocytes of
512 their fetal gonads. *In utero* treatment of βERKO females with low doses of BPA did not enhance
513 the oocyte defects, suggesting that BPA could act via the ESR2 signaling pathway alongside
514 other non-genomic mechanisms [208]. In ArKO mice that were given BPA (0.1 or 1.0% w/w in
515 chow), the ovarian expression of IGF-I, IGF-I receptor, GDF9, and BMP-15 were increased to
516 normal levels, an effect resembling that of ArKO mice given estradiol replacement [209]. These
517 authors further reported that BPA exerted "little effect" within ovarian and other estradiol-
518 dependent tissues of wild-type mice.

519 In the uterus, neonatal BPA exposure has been shown to cause long-term adverse effects,
520 including cystic endometrial hyperplasia, as well as the occurrence of more serious uterine
521 pathologies such as adenomyosis, leiomyomas (fibroids), atypical hyperplasia, and stromal
522 polyps [204]. Furthermore, paraovarian cysts, progressive proliferative lesions of the oviduct, and

cystic mesonephric (Wolffian) duct remnants in the uterus were found in the BPA-treated mice after *in utero* exposure [210]. Similar defects were shown in *in utero* BPA-exposed mice (25 to 250 ng/kg), using Alzet osmotic pumps [203]. Vaginal wet weight was decreased and lamina propria of the endometrium was decreased as well, with concomitant increase in glandular epithelial proliferation at 3 months of age. BPA caused an increase in ESR1 and PR expression in the lumina typifying a hyper-estrogenic response of the uterus. It would be of interest to examine if hypomethylation is associated with such an increase in gene expression. A recent study by Varayoud and colleagues showed that in an ovarectomized, neonatally BPA or DES exposed mouse model, progesterone priming followed by estradiol treatment caused an impaired proliferative response and altered PR and ESR1 expression in the sub-epithelial stroma of the uterus suggesting that the uteri were unable to respond to ovarian steroids [211]. In addition, *Hoxa-10* expression was decreased even though methylation of its promoter was unaffected. Furthermore, an abnormal overexpression of the corepressor, silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), was found in the same stromal cells in which *Hoxa-10* expression was reduced. Other epigenetic analyses on BPA-treated uteri from 2-6 week old mice after E9-E16 exposure to 5 mg/kg BPA were performed by Bromer *et al*. They demonstrated a decrease in DNA methylation of the promoter and intron regions of *Hoxa-10*. This group also found that the hypomethylation allowed for increased ESR1 binding to the EREs present in the *Hoxa-10* promoter thereby allowing the uteri to become hyper-responsive to estrogen/BPA signaling [212].

3.m. Di-ethylhexyl phthalate (DEHP)

Phthalate esters are ubiquitous in our environment and used as plasticizers to give flexibility to PVC-derived plastics [213]. Di-ethylhexyl phthalate is one of the most widely used phthalate ester [214] and present in medical bags and tubings, packaging, and food containers. It is non-covalently bound to plastics, and can leach out of these products, resulting in potential

549 daily human exposure in the range of 3-30 μg/kg/day {Shelby, 2006 #35}. In fact DEHP and its
550 metabolites have been found in breast milk, serum, amniotic fluids and sweat [215, 216] and
551 recently in urine samples from mothers and infants [217]. One the most vulnerable populations
552 are infants in neonatal intensive care units or NICUs, whose daily exposure reaches 22.6 mg/kg
553 [213]. The developmental exposure to DEHP is of special concern. In humans, *in utero* DEHP
554 exposures were associated with shorter pregnancy duration [218] and a shortened anogenital
555 distance (AGD) and index in boys [219, 220]. Increased incidences of miscarriage were reported
556 in women occupationally exposure to high dose of phthalates [221]. Danish girls with high
557 urinary concentration of phthalate metabolites, including DEHP show delayed puberty [222].
558
559 **3.n. DEHP and *in vivo* studies**
560 Animals that are exposed to DEHP during adulthood and peripubertal periods show adverse
561 effects in multiple reproductive parameters, such as estrous cyclicity, pubertal age, litter size, and
562 alterations in serum hormone levels and ovarian morphology [223-225]. Transient daily oral
563 exposures to 2 g/kg of DEHP in female rats result in prolonged estrous cycles, and delay or
564 suppression in natural ovulation time resulting in reduced number of ovulations and hence
565 absence of CL. Suppressed serum levels of estradiol, progesterone, and LH were also found. The
566 primary cause of these disruptions appears to be the low levels of estradiol, insufficient to induce
567 preovulatory LH surge [226, 227]. Studies with cultured ovarian follicles suggest that DEHP acts
568 via its more active metabolite MEHP and inhibits FSH-stimulated cAMP production, thereby
569 preventing activation of the enzymes for progesterone production, and suppresses levels of
570 *Cyp19a1* via activation of PPARs. Prolonged exposures to a lower dose (0.05mg/kg/day) of
571 DEHP resulted in reduced expression of *Cyp17a1*, *Cyp19a1, progesterone receptor (Pgr)*, *Lhcgr*
572 and *Fshr* in the adult ovary (PND41) of the CD-1 mice, all which may affect ovarian
573 steroidogenesis [228]. Besides suppressed ovarian steroid production, multiple studies have
574 reported altered follicular dynamics as one of the major consequences of DEHP exposure. These

575 alterations include accelerated follicular recruitment and failure in follicular maturation and
576 ovulation. Early postnatal (PND 5-20) exposure in mice to relatively low levels of DEHP depletes
577 primordial follicles while increasing the number secondary and antral follicles [229], which is
578 associated with altered pattern of imprinted genes and increased metaphase II spindle
579 abnormalities. Follicular dynamics were similarly altered in adult mice that were transiently (10-
580 30 days) exposed to DEHP (200 µg/kg to 700 mg/kg), which was associated with dysregulation
581 of PI3K signaling pathway {Hannon, 2014 #9}. Studies have also suggested that DEHP exposure
582 inhibits follicular maturation which may be a result of the inhibition of antral follicle growth due
583 to increased oxidative stress leading to increased apoptosis [230]. Most of the studies described
584 above have employed extended exposure periods and larger doses. Therefore, studies with
585 environmentally relevant doses of DEHP specifically targeting the fetal and neonatal ovarian
586 development are needed.
587
588 In the uteri, exposures to DEHP during early pregnancy lead to adverse outcomes. Rats
589 that were exposed to oral DEHP (313 and 573 mg/kg/day) between E0-20 had reduced number of
590 pups in their litters as well as decreased mean pups weights. Similarly mice that were exposed to
591 DEHP (0, 44. 91, 191, and 293 mg/kg/day) between E0-17 showed a dose-dependent increase in
592 number of embryonic resorptions as well as other major malformations, including cardiovascular
593 malformation and skeletal defects with the two highest doses [231]. More recently, a shorter
594 exposure to DEHP (0, 250, 500, and 1000 mg/kg/day) during first 4 to 6 days of pregnancy, in
595 mice, showed that the highest dose leads to extensive embryonic resorption at the end of exposure
596 period, due to reduced endometrial receptivity (characterized by insufficient decidualization),
597 which is associated with an increase in ESR1, PR, and E-cadherin and inhibition of MAPK and
598 Nf-κB signaling pathways [232]. Interestingly, the DEHP exposure (405 mg/kg/day) between E6
599 and PND 21, that resulted in increased antral follicular atresia, did not affect uterine luminal
600 epithelial height [233]. The exact mechanisms of the adverse effects of DEHP on the uterus and

601 embryo are not known, and require further investigations. In addition, it is worth noting that the
602 effects of DEHP are not likely mediated by estrogen receptor as DEHP shows little or no
603 uterotrophic effects *in vivo*, although DEHP binds to estrogen receptor.
604
605 **4. Conclusions**
606 There is a large amount of evidence that demonstrates the adverse effects of EDCs on female
607 reproductive health. Exposures in early ovarian and uterine developmental stages have
608 irreversible, long-term effects on the reproductive function proving that developmental
609 reprogramming occurs after EDC exposures. Epigenetic mechanisms mediate some of these EDC
610 actions and comprehensive genome-wide studies are necessary to deduce the details.
611
612

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