

THE EFFECTS OF ENDOCRINE DISRUPTING CHEMICALS ON THE OVARY

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1. ABSTRACT

Endocrine disrupting chemicals (EDCs) are natural or synthetic chemicals that mimic, enhance, or inhibit endogenous hormones. In this article, we review possible targets of EDCs within the ovary and explore whether EDCs may be acting as estrogen mimics, interfering with apoptosis, altering cell signaling pathways, or affecting estrogen metabolism. Though the study of EDCs has remained controversial, it is important to study them because our society continues to release large amounts of industrial chemicals into our environment and uncovering the mechanisms of action may lead to treatments of any potential adverse effects. In addition,

studying how EDCs affect the ovary may lead to serendipitous discoveries about ovarian function and dysfunction. Finally, understanding the science behind endocrine disruption may influence the political and regulatory handling of EDCs.

2. INTRODUCTION

Endocrine disrupting chemicals (EDCs) are natural or synthetic chemicals that mimic, enhance, or inhibit endogenous hormones (1). In wildlife, exposure to EDCs has been associated with diminished fertility,

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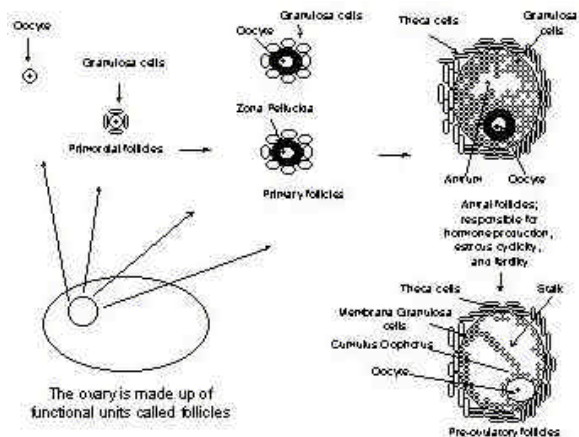


Figure 1. Stages of Ovarian Follicle Growth. This schematic shows the normal stages of ovarian follicles beginning with the oocyte and primordial follicles and the growth of these follicles to the pre-ovulatory stage.

defeminization, and demasculinization (2). An example of an EDC that was eventually banned because of its apparent toxicity to wildlife is the organochlorine pesticide 1,1,1-trichloro-2,2-bis-(4-chlorophenyl)ethane (DDT). DDT and its metabolites were banned in 1973 partly because of their association with eggshell thinning and subsequent population decline in some avian species (3,4). Although DDT was banned, there are other structurally similar organochlorine pesticides that are commercially available such as 1,1,1-trichloro-2,2-bis-(4-methoxyphenyl)ethane, or methoxychlor (MXC). MXC is less persistent and more biodegradable than DDT, however, it is still regarded as a potent EDC (5). Pesticides are not the only chemicals categorized as possible EDCs; the list includes phytoestrogens, polychlorinated biphenyls (PCBs), polyhalogenated aromatic hydrocarbons (PHAHs), alkylphenols and phthalate esters (6).

The study of EDCs and their possible effects on reproduction is important because reproduction is necessary for species survival. The possible effects on the ovary are particularly important because the ovary is central to female reproductive function and viability. In order to understand the targets of ovarian toxicity, it is helpful to review the basic physiology and function of the normal ovary (see below).

3. NORMAL OVARIAN FUNCTION

The follicle is the functional unit of the ovary and contains the female germ cell and the somatic cells that surround it. The different stages of follicular development are illustrated in Figure 1. The earliest stage of follicular development is the primordial follicle (7). During this stage, the follicle contains an oocyte surrounded by approximately four fusiform granulosa cells. The next stage is the primary follicle stage, which is characterized by a slightly larger oocyte surrounded by 6-8 cuboidal granulosa cells (7). Early in its growth the oocyte secretes glycoproteins that form an acellular layer called the zona pellucida. The zona pellucida serves to protect the oocyte

against polyspermy and to induce the sperm receptor/acrosome reaction at fertilization (8). Following the primary stage, follicles grow to the pre-antral stage, which is characterized by a larger oocyte surrounded by 2-4 layers of granulosa cells and the beginnings of a thecal cell layer. At the pre-antral stage, an antrum is either nascent or absent. The antrum is a fluid filled cavity that contains steroid binding proteins and high levels of estrogen and progesterone (7). The granulosa and thecal cells serve to maintain the health of the oocyte and to produce hormones such as estrogens, which are required for fertility (7). The pre-antral follicles then grow into antral follicles, which contain the oocyte surrounded by ≥ 5 layers of granulosa cells, an antrum, and two distinct layers of thecal cells (7,8). The final stage of development is the pre-ovulatory stage, in which the follicle contains a ripe oocyte surrounded by several inner layers of granulosa cells (cumulus granulosa cells), a large antral space, outer layers of granulosa cells (membrana granulosa), and two distinct thecal layers. Communication between the cumulus and membrana granulosa is maintained through a thin stalk of cells that connects the two layers (8).

It is important to note however, that the vast majority of follicles never grow to the pre-ovulatory stage. Instead, over 99% die via a process called atresia (7). Atresia has been demonstrated to be an apoptotic process. Zeleznik *et al* demonstrated that rat granulosa and luteal cells possess a calcium/magnesium-dependent endonuclease, the activity of which results in the characteristic DNA ladder that is a biochemical marker for apoptosis (9). In addition, numerous studies have shown that ovarian follicles undergo atresia via apoptosis using a variety of *in vivo* and *in vitro* systems (reviewed in 10). Because over 99% of ovarian follicles undergo atresia, any chemical that hastens this process could have potentially devastating effects on fertility.

Female fertility depends on the maintenance of a constant stream of growing follicles (11). This "continuum" of growth may help to ensure that only an appropriate number of follicles mature to the antral stage (11). A given EDC may target ovarian follicles at one particular stage of development, whereas another EDC may affect a different stage. The overall damage to the ovary and its implications for reproductive health depends on the type of follicle affected by the EDC. For example, administration of a single dose of a toxicant that destroys primordial follicles would eventually result in permanent sterility because the primordial follicle pool is finite; although normal cycling might continue until the remaining larger follicles have been depleted through ovulation or atresia. Administration of a single dose of a toxicant that targets primary follicles may result in temporary infertility followed by normal cycling, assuming the primordial follicles are unaffected and a new crop of primordial follicles grows and takes the place of the damaged primary follicles. Administration of a toxicant that targets pre-antral, antral or pre-ovulatory follicles may result in temporary infertility followed by normal cycling once new primordial and primary follicles grow to an appropriate size and normal cycling commences (11). These scenarios

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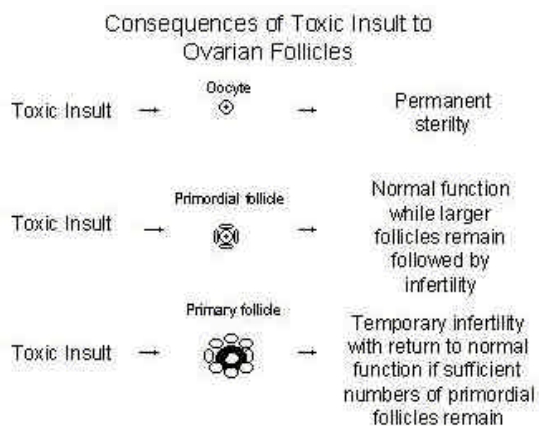


Figure 2. Consequences of Toxic Insult to Small Follicles. This schematic shows the possible consequences of toxic insult to oocytes, primordial, and primary follicles.

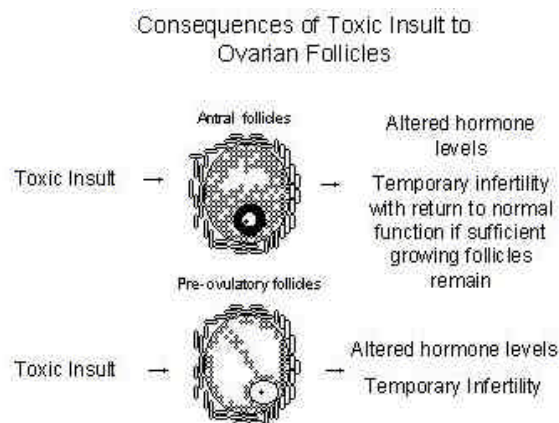


Figure 3. Consequences of Toxic Insult to Large Follicles. This schematic shows the possible consequences of toxic insult to antral and pre-ovulatory follicles.

assume that exposure to the toxicant is removed, and that the small follicles retain developmental competency, otherwise permanent infertility would result in all of the cases. Figures 2 and 3 illustrate these principles.

Although EDCs can target different follicle types and induce toxicity, the mechanisms by which this occurs are unclear. Some of the potential mechanisms of EDC-induced ovarian toxicity include estrogen mimicry, induction of apoptosis, and perturbations of signal transduction pathways. Each of these possible mechanisms is described below

4. EDCS AS ESTROGEN MIMICS

Just as hormones can act on target organs at distant sites, EDCs are suspected to affect different organs; a quality that often makes it difficult to discern their mechanism(s) of action. Estrogen mimicry is one of the most commonly reported effects of EDCs (2) so it is understandable that many of the studies looking at EDCs focus on estrogen sensitive tissues in the female reproductive system. These studies are complicated by the

fact that: 1) there are two known types of ER, ER α and ER β , 2) ER α and ER β are distributed differently throughout the reproductive system, and 3) different chemicals can have different affinities for each receptor (12-14). Jefferson *et al* demonstrated that ER α is highly expressed in the oviduct and uterus of mice, whereas ER β is more strongly expressed in the ovary, particularly in the differentiating granulosa cells (15). If EDCs show different affinities for different ER subtypes, and different ER subtypes are distributed differently throughout the reproductive tract, then it is possible that different EDCs may not act on the same target tissues or via the same mechanisms.

When estrogen or EDCs bind to the ER, the ER changes its shape, allowing it to interact with estrogen responsive genes and to modulate transcription (16). The relative binding affinity of many suspected EDCs for either ER α or ER β is quite low when compared to 17 β -estradiol (16). However, the weak estrogenic activity of putative EDCs does not necessarily mean they do not present a threat because an enormous amount of these chemicals are introduced into the environment each year (17). Therefore, the sheer amount of EDCs that we are exposed to in the environment may compensate for their weak estrogenicity. Furthermore, while hormones such as estrogen are bound to plasma proteins to keep them from acting, we cannot assume EDCs are inactivated in a similar fashion (6).

Detecting the estrogenicity of a compound can be done either *in vivo* or *in vitro*, but both of these approaches have their limitations. While *in vivo* assays can reveal effects of chemicals in the whole animal, taking into account absorption (depending on route of exposure and/or administration of the test chemical), distribution, metabolism and excretion, the sheer complexity of the animal may limit our ability to discern some mechanisms. On the other hand, *in vitro* assays can provide insights into mechanisms, but without the whole animal, we cannot be sure whether a given result represents a true physiological finding or an artifact of experimental design.

An *in vivo* study done in rats by Gray *et al* revealed that an EDC capable of mimicking estrogen might affect reproductive behavior (18). The pesticide and putative EDC, methoxychlor, was chosen as a test chemical and running wheel activity (RWA), a behavior the authors characterized as increased by estrogen and reduced by progesterone, was used as the reproductive endpoint. The investigators determined that RWA was increased in MXC- and estrogen-treated rats and then reduced in both of these cases following progesterone treatment. The similarity of the effect of MXC to estrogen on RWA and the fact that like estrogen, its effect could be diminished by progesterone, led the authors to conclude that MXC mimicked estrogen (18). This study is certainly suggestive that MXC may mimic estrogen and work through the ER to produce its effects. However, an *in vivo* study by Ghosh *et al* concluded that MXC may be acting in an ER-independent fashion (19). The authors treated ER α knockout mice with estrogen or MXC and found that while estrogen did little to induce the estrogen responsive genes,

lactoferrin and glucose-6-phosphate dehydrogenase, MXC did induce these genes (19). Additionally, anti-estrogen pretreatment did not block induction of these genes by MXC, thereby suggesting that MXC was not acting through ER β to produce estrogenic effects (19). Presumably if MXC were acting through an ER α - or ER β -mediated pathway, the pure anti-estrogen would block its effects. Gaido *et al* observed that the potent MXC metabolite, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), acts as an ER α agonist and an ER β antagonist in human hepatoma cells (20). Hodges *et al* found that HPTE mediated growth of uterine leiomyoma cells through the ER by inducing transcription via the activation function of the ER (21), which suggests that this MXC metabolite may be binding to the ER in leiomyoma cells and acting as an agonist. In addition, the authors found that HPTE was capable of inducing the progesterone receptor, which is normally induced by estrogen, thereby suggesting further that it may be acting through the ER to produce estrogenic effects. It is still unclear, however, whether MXC also might be mimicking estrogen through an ER-mediated pathway or whether it could simply have more than one mode of action. These are questions that need to be further addressed, particularly in the ovary.

In addition to acting as estrogens, EDCs may be acting as anti-androgens, thus producing an “estrogenic environment” (14). Sohoni and Sumpter prepared yeast-based assays that expressed either human ER or human androgen receptor (AR) and measured estrogenic and/or androgenic activity by the ability of test chemicals to stimulate β -galactosidase synthesis in the yeast (14). Anti-estrogenic and/or anti-androgenic activity was assessed by the ability of test chemicals to inhibit the ability of the natural ligands (17 β -estradiol and dihydrotestosterone, respectively) to stimulate β -galactosidase synthesis (14). The authors found that while the EDCs tested did produce estrogenic activity as expected, the test chemicals, hydroxytamoxifen, flutamide, bisphenol A, butyl benzyl phthalate, nonylphenol and vinclozolin all produced anti-androgenic activity. This finding demonstrates that in addition to their ability of acting as steroid hormone receptor agonists, EDCs may also act as receptor antagonists.

5. EDCS AND APOPTOSIS

Estrogen mimicry is not the only possible mechanism for endocrine disruption. Another possibility is the alteration of apoptotic pathways. Atresia is a natural process by which follicles in the ovary degenerate, and it is thought to occur due to massive apoptosis in granulosa cells or the oocyte, depending on follicle stage (22). The apoptotic pathways provide many opportunities for an EDC to alter function and ultimately drive ovarian cells towards inappropriate cell death (i.e. too much or too little death). The intracellular pathways regulating cell death in ovarian cells are largely the same as those in other cell types, however, their involvement and regulation vary among the different ovarian cell types and according to the developmental status of the follicle (23).

There are two discrete periods of time during which large amounts of apoptosis occur in the ovary. The first occurs late in gestation, at which time a large percentage of germ cells undergo attrition (24,25). In the mouse, this first period of apoptosis is detectable by embryonic day 15 (E15) and continues through the first few days of postnatal life. This wave of germ cell apoptosis results in the depletion of the germ cell store to less than a third of the peak number seen before meiotic arrest (24,26). The second period of ovarian apoptosis is a result of follicular atresia and begins following the onset of puberty, at which time cohorts of primordial follicles begin to be recruited into the pool of growing follicles.

Typical morphological and biochemical changes that occur during apoptosis include active synthesis of apoptosis-related proteins, chromatin condensation, DNA fragmentation, membrane blebbing followed by shrinkage, cytosolic fragmentation, packaging of fragments into membrane bound vesicles called apoptotic bodies, and engulfment by neighboring cells (27). There are many factors that regulate apoptosis, some of which are part of a decision step, in which a cell's fate is determined, and some of which are part of an execution step, in which either pro- or anti-apoptotic proteins are activated (28). The mechanisms involved in regulating apoptosis are conserved among cell types, with two families of proteins being central to this regulation. The first is the B cell lymphoma 2 (Bcl-2) family and the other is the cysteine aspartate protease (caspase) family. The Bcl-2 family consists of both anti-apoptotic members (e.g. Bcl-2, Bcl-xl, Boo) and pro-apoptotic members (e.g. Bax, Bok, Bim, Bid) (27). It is thought that the relative ratio between pro- and anti-apoptotic proteins can determine the cell's ultimate fate, i.e. a greater ratio of pro- to anti-apoptotic proteins would result in the drive towards apoptosis (28).

There are many functions of Bcl-2 family members, but a primary role is to regulate the release of cytochrome c from the mitochondrial intramembrane space, a process that is a decisive step in the induction of apoptosis (reviewed in 29). Pro-apoptotic Bcl-2 members such as Bax, Bak and Bid cause the release of cytochrome c by pore formation in the outer mitochondrial membrane or by opening the permeability transition pore (PTP) following mitochondrial permeability transition, while anti-apoptotic Bcl-2 family members counteract these actions. Cytochrome c interacts with apoptotic protease activating factor-1 (Apaf-1) and procaspase 9 to form the apoptosome, resulting in the activation of caspase 9, and subsequent activation of downstream caspases, which in turn results in DNA fragmentation. The proteolytic activities of the caspases also serve to activate a variety of proteins, including other caspases and Bcl-2 family members, and to degrade cellular proteins (30).

Apoptosis can be induced by a variety of stimuli, including paracrine growth factors (e.g. transforming growth factor (TGF) α and TGF β) and intracellular stress (reviewed in 27,29). These stimuli can induce apoptosis by altering the relative levels or activity of Bcl-2 family members, altering the activity of caspases, inducing

mitochondrial permeability transition, opening the PTP, and releasing cytochrome c or other pro-apoptotic factors from the mitochondrial intramembrane space (e.g. apoptosis inducing factor (AIF) (reviewed in 29,30).

Induction of apoptosis within the various ovarian cell types (e.g. the oocyte, granulosa cells, or thecal cells) leads to death of the follicle, an end to its steroidogenic capacity, and loss of the oocyte. If a sufficiently large number of follicles are lost via atresia, hormonal imbalance and infertility could result. Therefore, the ability of EDCs to induce apoptosis and follicular atresia is of great concern, as it may affect both the physiology and the fertility of the organism.

There is evidence that environmental toxicants induce ovarian atresia in animals. For example, Swartz *et al* found abnormalities indicative of atresia in the antral follicles of MXC-treated mice, such as thinned thecal cell layers and the presence of pyknotic cells (31). Adult mice exposed to MXC in utero have been shown to have a higher number of atretic follicles compared to mice exposed to vehicle (32). Borgeest *et al* demonstrated an increase in the percentage of atretic follicles in CD-1 mice treated with MXC as compared to controls (33). Increased follicular apoptosis has been found to occur in wild fish populations exposed to paper mill effluent (34). It also has been shown to occur in juvenile catfish treated with the polyaromatic hydrocarbons, β -naphthoflavone and dimethylbenz[a]anthracene (DMBA) (35). Similarly, DMBA has been shown to induce Bax expression and apoptosis in mouse oocytes (36), and the bioflavonoid quercetin has been shown to increase the number of atretic follicles and the number of apoptotic ovarian somatic cells in Japanese medaka (37).

While EDCs have been shown to induce atresia and/or apoptosis in various experimental systems, little is known about the mechanisms by which this induction occurs. Possible mechanisms may involve production of reactive oxygen species (ROS) and/or induction of mitochondrial damage (27). The mechanisms by which chemicals exert their effects often depend on their concentration, duration of exposure, and the experimental system (27,38). An example of this involves tributyltin (TBT), an EDC used in herbicides and in antifouling paints for ships (38). In one study, this chemical induced apoptosis in Jurkat cells at low concentrations by inducing cytochrome c release from mitochondria and by activating the caspase cascade (reviewed in 27). In another study, low levels of this chemical inhibited apoptosis in PC12 cells by causing a downregulation of Bax protein levels (38). These data reveal that testing EDCs in different experimental systems can produce different results. Although the results are divergent, the consistent theme is that TBT interacts with apoptotic pathways to influence cellular viability. The data highlighted above clearly demonstrate that interaction with the pathways involved in programmed cell death is a mechanism of EDC toxicity. The precise role of each EDC in terms of whether it is pro-apoptotic, anti-apoptotic, or has no effect on apoptosis appears to be dependent on the experimental system and the target organ or cell line under investigation.

6. EDCS AND CELL SIGNALING PATHWAYS

In addition to estrogen mimicry and alterations of apoptotic pathways, there are signaling pathways in the ovary that might be the target of some EDCs. Cell signaling pathways serve to convert extracellular signals into one or more intracellular events that contribute to growth, differentiation and function in cells. G-protein coupled receptors (GPCRs) and mitogen activated protein kinases (MAPKs) are examples of cell signaling pathways employed by all eukaryotic cells including ovarian cells. GPCRs are known to activate or inactivate plasma membrane-bound enzymes or ion channels (39). Briefly, some GPCRs may activate or inactivate adenylate cyclase and alter the levels of cAMP. In addition, other GPCRs may act through phospholipase C and generate two intracellular messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 increases the intracellular concentration of Ca^{2+} and intracellular Ca^{2+} along with DAG activate protein kinase c. In turn, protein kinase c activates phosphorylation of various proteins. An example of cell signaling through the GPCRs in the ovary is the model for the two gonadotropin-two cell concept of follicular estradiol production (40). Briefly, luteinizing hormone (LH) binds to its receptor (LHR) on the thecal cells of preantral and antral follicles. LHR is a GPCR that stimulates adenylate cyclase production of cAMP. In turn, cAMP activates protein kinase A (PKA), which stimulates the cytochrome P450 enzymes to convert cholesterol to progesterone and then androgen. Androgen diffuses out of the thecal cells into the granulosa cells. Meanwhile, follicle stimulating hormone (FSH) binds to its receptor FSHR, which is also a GPCR, on granulosa cells of the preantral and antral follicle, and sets off similar cell signaling cascades that result in the conversion of androgen to estrogen via the cytochrome P450 aromatase enzyme. Estrogen diffuses into the follicular fluid and into circulation and then travels to target tissues, where it regulates cellular growth, differentiation, and death. In addition, estrogen negatively feeds back to the hypothalamus and anterior pituitary to stop releasing gonadotropin (40). In addition, estrogen can interact with MAPKs to regulate growth, proliferation, and death of cells (41).

As suggested in the section on estrogen mimicry, some EDCs may interact with estrogen receptors in the same manner as steroid hormones. Although interaction of EDCs with nuclear steroid receptors has been repeatedly offered as the basis for their reproductive toxicity, recent studies suggest that EDCs may interfere with other cell signaling pathways (42-43). Estrogens may instantly trigger the release of Ca^{2+} in chicken and pig ovarian granulosa cells by an IP_3 -mediated mechanism (42). Estrogens also may activate adenylate cyclase and increase the concentration of cAMP in cultured uterine cells (43). The implications of these observations may be significant since Ca^{2+} and cAMP affect the behavior of other proteins and they modulate the expression of different genes. Although studies report that estrogens stimulate increased intracellular levels of Ca^{2+} and cAMP and the exact mechanism of estrogen action remains to be determined,

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several studies suggest the existence of specific membrane recognition sites for estrogen in granulosa cells (42), adenohipophyseal cells (44), uterine cells (45) and liver cells (46). Furthermore, some studies indicate that the MAPK signaling pathway is known to directly interact with the estrogen receptor (41,47). If estrogens are capable of interacting with cell signaling pathways, it is conceivable that EDCs also interfere with cell signaling pathways.

Chedrese *et al* examined the effects of two putative EDCs, dichlorodiphenyldichloroethylene (DDE) and MXC, on steroidogenesis in porcine ovarian cells and Chinese hamster ovary (CHO) cells (48). The authors suggested that DDE may inhibit the generation of cAMP while MXC may act through a mechanism distal to cAMP generation in the ovarian cells. Juberg *et al* observed that the chlorinated insecticide p,p'-DDD, a well known DDT isomer, significantly increased the concentration of intracellular free calcium in cultured rat myometrial smooth muscle cells (49). Collectively, these data indicate that EDCs may modulate the expression of cAMP-regulated genes in the ovary by affecting the cAMP-signaling pathway and strongly suggest that non-genomic components of EDCs must be considered in evaluating their reproductive toxicity.

EDCs also may exert their effects through peptide growth factor signaling pathways (50). Peptide growth factors include epidermal growth factor (EGF), insulin-like growth factor (IGF), TGF α , and TGF β . Ignar-Trowbridge *et al* demonstrated that there is cross-talk between peptide growth factors and estrogen receptor signaling systems (50). The authors observed that epidermal growth factor (EGF) interacted with uterine ER in vivo and that EGF may be involved with regulation of ER-dependent activation of an estrogen response element. Although their experiments did not test EDCs, there is the possibility of EDCs interacting with ER and causing changes in peptide growth factor signaling pathways.

7. COULD EDCS INTERFERE WITH ESTROGEN METABOLISM?

The antral follicles of the ovary are major producers of estrogen in the body and the targets of some EDCs. Thus, it is possible that EDCs might interfere with estrogen metabolism in antral follicles (51,52). Kester *et al* found that the hydroxylated metabolites of polyhalogenated aromatic hydrocarbons (PHAH-OHs) may inhibit an enzyme important for estradiol inactivation, thereby leading to increased levels of estradiol (51). Bradlow *et al* demonstrated that exposure of MCF-7 cells to organochlorine pesticides significantly increased production of the estrogen metabolite 16 α -hydroxyestrone, a potent compound that is thought to be tumorigenic and genotoxic (52, 53). In this case, the EDC itself is not mimicking estrogen per se, but rather it is altering the levels of endogenous estrogens by inhibiting the breakdown and inactivation of estrogen or promoting the production of genotoxic estrogen metabolites.

8. CONCLUSION

The study of EDCs has remained controversial because of the difficulty in obtaining consistent results.

However, a possible explanation for lack of consensus may be the use of different experimental systems, as well as the fact that any number of mechanisms could be acting together, or perhaps even canceling each other out. Despite the difficulties involved with studying EDCs, they are important to study because we release large amounts of industrial chemicals into our environment (17). It seems likely that we will continue to introduce new chemicals into our environment, therefore, it is important to attempt to uncover the mechanisms of action of these chemicals if we ever hope to combat any potential adverse effects of EDCs. An added bonus in the study of endocrine disruption is serendipitous discoveries that may lead to a better understanding of basic physiology of reproduction. Finally, it is important to establish whether the more subtle effects of EDCs present a true reproductive health risk to wildlife and humans so that we can reassess the regulation of these chemicals. In short, understanding the science behind endocrine disruption may influence the political and regulatory handling of EDCs.

9. ACKNOWLEDGEMENTS

The authors thank Ms. Lynn Lewis for her help with the preparation of this manuscript. This work was supported by NIH grant R01AG18400, NIH grant R01HD38955, and a grant from the Women's Health Research Group at the University of Maryland.

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Key Words: Endocrine Disruptor, Ovary, Estrogen, Apoptosis, Review

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