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Impact of endocrine disruptors on ovarian steroidogenesis

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Production of steroid hormones by the ovary plays a key role in the female phenotype maintenance, as well as is critical for regular ovarian processes, including follicle growth, oocyte maturation and ovulation. Thus, optimal ovarian steroid synthesis is an indispensable requisite for the female reproductive health. In the past decades, along with an increased incidence of female reproductive disorders, an increasing concern for the potential reproductive impact of exogenous factors, particularly of environmental pollutants with endocrine disrupting properties, has risen. The scientific studies report that ovarian steroid hormone production is being recognized as an important target for the action of endocrine disrupting chemicals (EDCs). The fact that these chemicals have been detected in the biological samples of general population, and even directly in the follicular fluid of women, emphasizes the demands for testing the influence of EDCs on ovarian steroidogenesis. For these purposes, different methodological approaches have been employed, from *in vivo* studies on female rodents to *in vitro* experimental procedures using steroidogenically active follicular cells. In the present review, the effects of selected EDCs (pesticides, phthalate and phenol derivatives, and halogenated arylhydrocarbons) on the processes of ovarian steroidogenesis are summarized, and possible mechanisms of action of these agents are outlined.

Key words: steroidogenesis, ovary, CYP enzymes, progesterone, estradiol, endocrine disruption, pesticides, phthalates, bisphenol A, dioxin, polychlorinated biphenyls, polybrominated diphenyl ethers

Introduction

Production of steroid hormones by the ovary plays a key role in the female phenotype maintenance as well as is critical for regular ovarian processes, including follicle growth, oocyte maturation, and ovulation. Thus, optimal ovarian steroid synthesis is an indispensable requisite for the female reproductive health. However, in the past years, along with an increased incidence of female reproductive disorders, an increasing concern for the potential reproductive effects of exogenous factors, including agents from specific lifestyle habits or environmental pollutants, has risen. This concern results from the fact that detection of such agents has been evidenced in the biological samples of general population (Younglai et al. 2004; Calafat et al. 2008; Sprague et al. 2013), and even directly in the follicular fluid of women (Ikezuki et al. 2002; De Felip et al. 2004; Meeker et al. 2009).

Published data indicate that exposure to exogenous chemicals may cause alterations in reproductive behavior and contribute to sub-fecundity, infertility, or ovarian failure. The direct effects of exogenous chemicals on steroid hormone homeostasis have been evidenced by many studies. One way, through which endocrine disrupting chemicals (EDCs) can interfere with the role of natural hormones, is by binding to hormone receptors thus "mimicking" the natural hormones. Even without receptor binding, EDCs may interfere with the synthesis, secretion, elimination or action of natural hormones (Gregoraszczuk 2002). Alternatively, reproductive toxicants can have indirect effects by af-

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fecting factors involved in steroid hormone production (Lovekamp-Swan and Davis 2003; Kwintkiewicz and Giudice 2009). The impact of EDCs on reproductive parameters may be deleterious in different ways, e.g. chemicals that interfere with estradiol production in the follicles can act as ovulatory disruptors, while interfering with progesterone production by luteal cells they can act as abortifacients (Gregoraszczuk 2002). The scientific studies suggest that ovarian steroid hormone production is being recognized as an important target for the action of various xenobiotics. In the present review, the effects of several environmental chemicals with endocrine disrupting properties on the processes of ovarian steroidogenesis are summarized, and possible mechanisms of action of these agents are outlined.

Ovary and ovarian steroidogenesis

Fertility of female mammals vitally consists in the ability of the ovaries to release fertilizable oocytes at midcycle what requires optimal function of Graafian follicles. The ovary belongs among the most dynamic and plastic tissues in the body, with the cycle of follicular maturation, ovulation and (in the absence of pregnancy) resorption of the corpus luteum, in order to perform two coherent roles: delivery of female gametes (oocytes) and production of sex hormones. The follicle is the functional unit of the ovary and comprises the oocyte surrounded by layers of somatic cells. In the mature preovulatory (Graafian) follicle, several layers can be defined (Tsafriri et al. 1993): (a) surface epithelium, (b) connective tissue, (c) theca interna, a well-vascularized layer active in steroidogenesis, (d) granulosa cells enveloping the follicular fluid-filled antrum, (e) oocyte-cumulus complex, which occupies an eccentric position within the follicle. Progression through successive stages of folliculogenesis is a multistage process, which requires the appropriately-timed endocrine signals, notably pituitary gonadotropins and metabolic hormones, which act on receptors on two somatic cell types and interact with local autocrine/paracrine signaling pathways, and also depend on effective communication between oocyte and granulosa cells and between granulosa and theca cells (Knight and Glister 2003).

The ovary is capable to synthesize a batch of steroid hormones, which control and maintain female sexual development, behavior and pregnancy, as well as have important local effects within the ovary.

Cholesterol, the starting material for the formation of steroids, is generally supplied for steroidogenesis by following routes: 1) *de novo* synthesis, 2) hydrolysis of stored cholesterol esters (CEs) by neutral cholesterol esterase, 3) hydrolysis of CEs from plasma high density lipoprotein by neutral cholesterol esterase, and 4) hydrolysis of CEs from plasma low density lipoprotein by acid cholesterol esterase (Vahouny et al. 1984-1985; Spady and Dietschy 1985; Latendresse et al. 1993; Nishizato et al. 2014). In the follicle, luteinizing hormone (LH) binds to the LH receptors in theca cells and stimulates uptake of cholesterol via induction of cAMP/PKA signaling (Brannian et al. 1992), concomitantly with Src/Ras/MAPK signaling (Jamnongjit and Hammes 2006). Cholesterol is then transported to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR), a rate-limiting regulator of steroid production (Clark et al. 1994). StAR protein expression and activity is regulated by many factors in steroidogenic tissues, starting with activation of the LH receptors. In the mitochondria, CYP11A1 enzyme (P450 side chain cleavage enzyme) then catalyzes the cholesterol side chain cleavage and conversion to pregnenolone (Boyd et al. 1975; Hanukoglu 1992). *CYP11A1* mRNA is expressed primarily in the theca interna cells, and its expression increases in corpora lutea (Goldring et al. 1987). From the mitochondria, pregnenolone diffuses into the smooth endoplasmic reticulum where it can be converted by oxidization and izomerization to progesterone by 3β-hydroxysteroid dehydrogenase (HSD3B) or after 17α-hydroxylation and 17,20-lyase activity of 17α-hydroxylase-17,20-lyase (CYP17A1; CYP45017α) to dehydroepiandrosterone (DHEA). Both HSD3B and CYP17A1 catalyze the conversion of DHEA and progesterone to androstenedione, respectively (Hanukoglu 1992). Within the theca cells, androstenedione can be converted to testosterone by 17β-hydroxysteroid dehydrogenase (HSD17B) or, in response to LH, androstendione synthesized in theca cells diffuses into granulosa cells. There, following follicle-stimulating hormone (FSH) stimulation, CYP19A1 (aromatase) can convert androstenedione to estrone and testosterone to 17β-estradiol by aromatization of the A-ring of androgens, according to the "*two-cell, two-gonadotropin*" theory (Fowler et al. 1978; Goldring and Orly 1985). Estradiol is the most potent form of estrogens in female reproduction. CYP19A1 expression is regulated by FSH through multiple signaling pathways including cAMP-dependent regulatory events. The expression of *CYP19A1* mRNA seen in mature follicles is consistent with the marked rise in estrogen biosynthesis before ovulation (Steinkampf et al. 1987). Moreover, granulosa cells can convert estrone to estradiol through the ac-

tion of HSD17B. Nevertheless, following its synthesis, estradiol can be metabolized into 2-hydroxyestradiol by CYP1A1/2 and CYP3A4, or to 4-hydroxyestradiol by CYP1B1. These modifications lead to inactivation of estradiol (Hayes et al. 1996; Tsuchiya et al. 2005).

Taken together, LH receptors and the CYP17A1 enzymes are expressed primarily in theca cells (Voutilainen et al. 1986; Hedin et al. 1987), while FSH receptors and CYP19A1 are expressed mainly in granulosa cells. Notably, LH receptors are not exclusively found in theca cells, as their expression levels in mural granulosa cells have been noted to increase in response to FSH just prior to the LH surge (Jamnongjit and Hammes 2006). Furthermore, steroidogenic factor-1 (SF-1 or NR5A1), a nuclear receptor found in both fetal and adult steroidogenic cells (Giguere 1999; Parker et al. 2002), plays a role as a positive regulator of the transcription of genes encoding many steroidogenesis pathway enzymes, e.g., *CYP11A1* (Clemens et al. 1994), *CYP17A1* (Bakke and Lund 1995), *HSD3B* (Leers-Sucheta et al. 1997), *CYP19A1* (Michael et al. 1995), and *STAR* (Sugawara et al. 2000). Therefore, SF-1 is sometimes described as a pivotal regulator of steroidogenesis processes (Mlynarczuk et al. 2014).

The principal pathways of ovarian steroid hormone biosynthesis are shown in Fig. 1.

Experimental models used for assessment of endocrine (steroidogenesis) disruption

The primary way through which EDCs could interfere with the role of natural hormones has been assumed to binding to steroid hormone receptors and "mimicking" the endogenous hormones (Sonnenschein and Soto 1998). However, numerous studies suggest that different EDCs may have other actions apart from those on the steroid receptors – they may interfere with the synthesis, secretion or elimination of natural hormones (Gregoraszczuk 2002).

The scientific studies indicate that ovarian steroid hormone production is being recognized as an important target for the action of EDCs, which can interfere with the pathway of steroidogenesis, e.g. by modulation the expression and catalytic activity of steroidogenic enzymes. However, for complete understanding the mechanisms by which EDCs can affect these processes and to what extent are their effects relevant to humans and wildlife, in-depth investigation is needed and different methodological approaches as well as different species have to be employed.

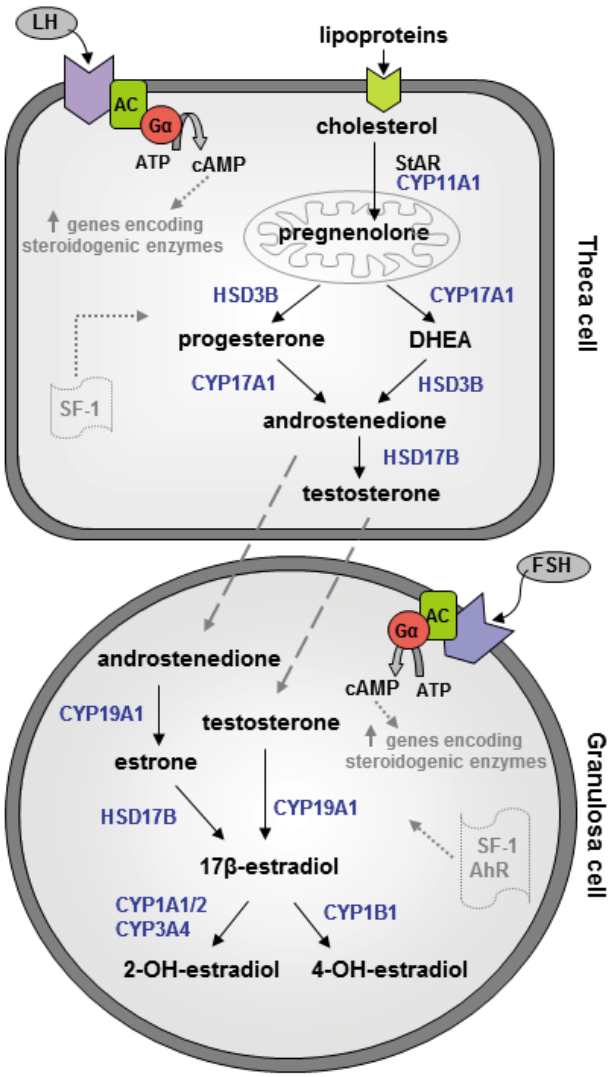

Figure 1. Principle pathways of steroid hormone biosynthesis in the ovary.
LH - luteinizing hormone; StAR - steroidogenic acute regulatory protein; CYP11A1 - cytochrome P450 11A1 (cholesterol side-chain cleavage enzyme); HSD3B - 3β-hydroxysteroid dehydrogenase; CYP17A1 - cytochrome P450 17A1 (17α-hydroxylase-17,20-lyase); DHEA - dehydroepiandrosterone; HSD17B - 17β-hydroxysteroid dehydrogenase; FSH - follicle-stimulating hormone; CYP19A1 - cytochrome P450 19A1 (aromatase); SF-1 - steroidogenic factor 1; AhR - arylhydrocarbon receptor

Most standardized *in vivo* tests developed to study EDC toxicity involve rodents and inquire relevant integrated toxicity endpoints, such as impact on fertility (Stokes 2004). However, even when alterations of serum steroid levels are observed after particular EDC administration, it is difficult to determine whether these rise

from the direct action on ovarian steroidogenesis, since due to the complexity of the endocrine system, *in vivo* approach naturally integrates higher regulatory units, feedback loops, and hormone metabolism.

Valuable biologically relevant bioassays to test various EDCs utilize *in vitro* techniques for culturing ovarian tissue or isolated follicles. Whole ovaries, mostly from rodents, are used to understand follicular development, ovulation, and steroidogenesis under EDCs treatment. Adaptations of this technique include incubation of ovaries in a chamber perfused with medium or perfusion of medium through the intact vasculature (Devine et al. 2002). Another approach is to culture individual follicles isolated by enzymatic or mechanical dissociation. Along with assessment of follicular growth, differentiation and oocyte growth and maturation, measurements of steroid secretion by the follicles allow to confirm findings from *in vivo* animal studies. The applications for these cultures thus include complex studies related to the impact of endocrine disruptors on ovarian function, and further investigations into follicle activation and development. These *in vitro* systems contribute to reduced animal use by performing synchronous experiments on large numbers of ovaries/follicles, and are informative on multiple fertility-related endpoints (Lenie and Smitz 2009).

Cell culture models further allow mechanistic examination of EDC actions on steroidogenesis. The primary cultures of isolated theca or granulosa cells are convenient *in vitro* models for studying the effects of different chemicals on the functional characteristics of steroidogenically active cells (Mlynarcikova et al. 2007). Cultured cells are able to respond to the action of gonadotropins or various endogenously produced factors as well as are sensitive to the action of exogenous chemical compounds. Their steroidogenic function can be maintained by providing the appropriate culture medium with adequate nutrition supplementation. These culture models are even less time- and cost-consuming, spare animal lives (Charles 2004), enable to elucidate toxic mechanisms in a simple isolated system along with testing of a broad range of EDCs concentrations or mixtures. Moreover, when *in vitro* tests are performed on human cells, they avoid difficult interspecies transpositions.

Illustrative examples of EDCs actions throughout the ovarian steroidogenic pathway observed in *in vitro* models are presented in Table 1.

In addition, computational models able to predict ovarian steroid level modulations by EDCs from *in vitro* data are being developed currently. For a reliable quantitative *in vitro* to *in vivo* extrapolation (QIVIVE), both characterization and quantification of toxicity mechanisms are necessary (Holme and Dybing 2002). In order to improve QIVIVE for endocrine toxicity, dynamic systems biology models of steroidogenesis are parametrized into mathematical models. This includes calibration of *in vitro* experimental data obtained from primary ovarian cell cultures, transposition to an *in vivo* context, and comparison of predictions with *in vivo* hormone dosage data obtained in control animals. The predictive capacity of the mathematical models is still limited; however, they may have potential applications for improved evaluation of endocrine disruption properties, in particular for low levels and mixtures of pollutants (Quignot and Bois 2013).

Selected endocrine disruptors and their impact on ovarian steroidogenesis in different models

Pesticides

Since pesticides are found ubiquitously in the environment and in food, their endocrine disrupting potential is of significant health concern.

Dichlorodiphenyltrichloroethane (DDT) was widely used as a broad-spectrum insecticide in the middle of the 20th century. However, after recognizing its persistence in soils and aquatic sediments and the potential to bioaccumulate in birds and mammals, the use in Western countries was banned (Crellin et al. 1999). In mammals, DDT is mainly metabolized by dechlorination and dehydrochlorination to stable 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene, referred to as DDE (Gold and Brunk 1982). Dietary exposure to *p,p′*-DDE has resulted in contamination of human blood (range: 1.08-1.68 mg/ml), adipose tissue, and ovarian follicular fluid (0.01-1.11 ng/ml) (Morgan et al. 1971; De Felip et al. 2004; Meeker et al. 2009). Both DDT and DDE have been described as EDCs with estrogenic effects on the female reproductive system (Sonnenschein and Soto 1998). The presence of *p,p′*-DDE in follicular fluid is of concern because of the intimate contact with follicular cells including oocyte. Notably, both DDT and DDE have been found to be associated with altered ovarian function and steroid hormone production (Wojtowicz et al. 2007). In rats administered with *p,p′*-DDT at dietary levels of 5, 50 or 350 ppm, changes in serum estradiol (decrease) and progesterone (elevation) levels were noted in the higher dose groups in a dose-dependent fashion, suggesting alterations of endogenous endo-

Table 1

Examples of EDCs actions throughout the ovarian steroidogenic pathway observed in *in vitro* models.

Step in the steroidogenic pathway		Effect	Chemical	Species & model system	Reference
LH signaling	LH receptor expression	↓	TCDD	Rat granulosa cells	Minegishi et al. 2004
FSH signaling	cAMP synthesis	↓	DDE	Porcine granulosa cells	Chedrese and Feyles 2001
	CREB and AKT phosphorylation	↑	ATR	Rat granulosa cells	Pogrmic-Majkic et al. 2014
SF-1	action	↑	PCB153	Bovine granulosa cells	Mlynarczuk et al. 2013
StAR	mRNA/protein expression	↓	MXC	Mouse antral follicles	Basavarajappa et al. 2011
		↑	BPA	Rat theca cells	Zhou et al. 2008
CYP11A1	mRNA/protein expression	↓	BPA	Mouse antral follicles	Peretz et al. 2011
		↑	MXC	Porcine granulosa cell line	Crellin et al. 2001
	activity	↓	HPTE	Rat theca cells	Akgul et al. 2008
HSD3B	mRNA/protein expression	↓	MXC	Mouse antral follicles	Basavarajappa et al. 2011
	activity	↑	BDE-99	Porcine luteal cells	Gregoraszczuk et al. 2012
CYP17A1	mRNA/protein expression	↓	Mono-OH	Mouse antral follicles	Craig et al. 2010
		↑	BPA	Rat theca cells	Zhou et al. 2008
	activity	↓	TCDD	Human luteinized granulosa cells	Moran et al. 2003
		↑	BDE-100	Porcine antral follicles	Karpeta et al. 2011
HSD17B1	mRNA/protein expression	↓	MXC	Mouse antral follicles	Basavarajappa et al. 2011
		↑	ATR	Rat granulosa cells	Quignot and Bois 2013
	activity	↑	BDE-47	Porcine antral follicles	Karpeta et al. 2011
CYP19A1	mRNA/protein expression	↓	BPA	Human luteinized granulosa cells	Kwintkiewicz et al. 2010
		↑	ATR	Rat granulosa cells	Quignot and Bois 2013
	activity	↓	MEHP	Human luteinized granulosa cells	Reinsberg et al. 2009
		↑	DDT, DDE	Porcine granulosa cells	Wojtowicz et al. 2007
CYP1B1	mRNA/protein expression	↑	TCDD	Human luteinized granulosa cells	Vidal et al. 2005

ATR – atrazine; BPA - bisphenol A; BDE - brominated diphenylether; DDE - 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene; DDT - dichlorodiphenyl trichloroethane; HPTE - 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane; MEHP - mono(2-ethylhexyl) phthalate; mono-OH - monohydroxy methoxychlor; MXC – methoxychlor; PCB - polychlorinated biphenyl; TCDD - 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

crine functions. However, the changes observed did not result in substantial reproductive disorders (Hojo et al. 2006). Female rabbits treated with *p,p'*-DDT (3 mg/kg b.w.) showed a significantly reduced ovulation rate due to low levels of progesterone (Lindenau et al. 1994). In Asian women, higher DDE concentration was associated with consistent progesterone metabolite levels decrease during the luteal phase of the cycle (Windham et al. 2005).

Further studies using porcine granulosa cells have confirmed that DDT and DDE can alter progesterone synthesis. At lower concentrations (10 ng/ml), DDE was able to increased progesterone synthesis stimulated by protein kinase A activators (8-Br-cAMP, cholera toxin), in a stable porcine granulosa cell line JC-410 as well as in primary porcine and bovine granulosa cells (Crellin et al. 1999; Mlynarczuk et al. 2013). However, higher concentrations of DDE (0.3-320 μg/ml) were shown to in-

hibit FSH-stimulated progesterone synthesis in porcine granulosa cells (Chedrese and Feyles 2001; Crellin et al. 2001). Moreover, the modulation of steroidogenesis may be isomer-specific, since Wojtowicz et al. (2007) found that progesterone secretion was decreased by addition of *p,p*'- and *o,p*'-DDT isomers and *p,p*'-DDE (4 μg/ml), but not by *o,p*'- DDE. In addition, *p,p*'-DDT (0.4-4 μg/ml) was shown to decrease estradiol production, while *o,p*′-DDT and the DDE isomers at doses ranging from 0.004 to 4 μg/ml increased it (Wojtowicz et al. 2007).

The alterations in steroid synthesis by DDT and DDE seem to occur at several stages of steroidogenic process. After low concentration treatment of porcine granulosa cells (1 and 10 ng/ml of DDE), neither basal nor cholera toxin-stimulated cAMP levels were changed, while cholera toxin-stimulated *CYP11A1* (P450scc) mRNA levels were increased by DDE action (Crellin et al. 1999). However, high doses of DDE (3-32 μg/ml) lead to decreased cAMP synthesis (Chedrese and Feyles 2001), along with decreased expression of *CYP11A1* (Crellin et al. 2001). Further, treatment with DDT or DDE (4 μg/ml) resulted in increased CYP19A1 (aromatase) activity in porcine follicular cells (Wojtowicz et al. 2007) as well as in human granulosa cells (100 ng/ml) (Younglai et al. 2004). Recently, it was shown that the disruptive mechanisms of DDT/DDE seem to include also the action on SF-1 as one of the steroidogenesis regulators, since addition of SF-1 antagonist F0160 abolished the stimulatory effect of DDE, and partially of DDT, on progesterone production by bovine granulosa cells (Mlynarczuk et al. 2013). Thus, DDT action may target gonadotropin receptor signaling (cAMP accumulation), local steroidogenesis regulation (SF-1), and may also directly alter enzymatic activity (CYP19A1). However, the mechanism by which different doses of DDT and/or isomers cause opposite effects (e.g. increased/decreased progesterone) is not well understood yet. Perhaps, this can be attributed to differences in affinity for receptors and/or enzymes due to different chemical structures, doses administered and length of exposure (Craig et al. 2011).

Methoxychlor (MXC; 1,1,1-trichloro-2,2-bis-(*p*-methoxyphenyl)ethane) is an organochlorine pesticide introduced to agriculture use as a more labile and readily degradable alternative to DDT. However, numerous studies have demonstrated that MXC has also endocrine disrupting properties and negatively affects reproduction (Tiemann 2008). Several research groups in various experimental models have provided evidence for the ability of MXC to alter levels of ovarian hormones. *In vivo* exposure of rats to MXC (250-500 mg/kg) decreased serum progesterone levels and disrupted female reproductive parameters and ovarian morphology (Gray et al. 1989; Chapin et al. 1997), and inhibited *ex vivo* production of estradiol and testosterone (Cummings and Laskey 1993).

In vitro experiments on bovine granulosa cells showed a decrease in both progesterone and estradiol production after higher doses (above 10 μg/ml) of MXC (Tiemann et al. 1996). Studies on porcine granulosa cells treated with lower MXC doses (0.1-3.5 ng/ml) have also observed reduced progesterone production, either basal or FSH- and cholera toxin-stimulated (Chedrese and Feyles 2001; Crellin et al. 2001), and increased levels of *CYP11A1* transcript without alterations in cAMP levels (Crellin et al. 2001). This indicated that MXC may affect steroid hormone levels by interference with steroid synthesis pathway downstream of generation of cAMP, but upstream of conversion of cholesterol to pregnenolone (Crellin et al. 2001). Moreover, the fact that MXC has also been shown to increase intracellular calcium concentrations at low doses (0.01-0.5 μg/ml) suggests that MXC may interact with membrane receptors and parallel pathways that modulate ovarian steroidogenesis (Wu et al. 2006; Craig et al. 2011). Steroidogenesis-disrupting effects of MXC were proved also in cultured mouse antral follicles where MXC (1-100 μg/ml) decreased expression of mRNAs encoding *Star* and the main steroidogenic enzymes (*Cyp11a1, Hsd3b1, Cyp17a1, Hsd17b1*, and *Cyp19a1*) and increased mRNA level of metabolic enzyme *Cyp1b1*. These changes resulted in decreased testosterone, androstenedione, progesterone, and estradiol production (Basavarajappa et al. 2011).

Following absorption in the body, MXC molecule is demethylated to form a mono-hydroxylated metabolite known as mono-OH and a bis-hydroxylated metabolite known as HPTE (Kapoor et al. 1970), which in fact may largely contribute to the observed MXC effects (Craig et al. 2011). In cultured mouse antral follicles, mono-OH (10 μmol/l) decreased synthesis of androstenedione, testosterone, progesterone, and estradiol. While expression of *Star*, *Hsd3b1*, *Hsd17b1* was not altered, the reduced steroid production may have been caused by down-regulation of expression of *Cyp11a1*, *Cyp17a1*, and *Cyp19a1* which was observed (Craig et al. 2010). Further study of this group found that pregnenolone co-treatment partially restored steroidogenesis (except for estradiol levels), but did not prevent growth inhibition and atresia in mouse ovarian antral follicles treated with mono-OH (Craig et al. 2013).

HPTE (1-10 μmol/l) was shown to inhibit FSH-stimulated synthesis of progesterone and estradiol in cultured rat granulosa cells, and this associated with decreased FSH-induced expression of *Cyp11a1* and *Cyp19a1* in the presence of HPTE (Zachow and Uzumcu 2006). Furthermore, HPTE (50-100 nmol/l) has been shown to directly inhibit the catalytic activity of CYP11A1 in cultured rat theca-interstitial cells (Akgul et al. 2008). Similarly, in rat granulosa cells *in vitro*, Harvey et al. (2009) reported decrease in estradiol production by HPTE (5-10 μmol/l), and their microarray transcriptome analysis revealed the broad influence of HPTE on FSH-signaling pathway, e.g. decreased expression of *Cyp11a1*, *Hsd3b1*, and *Cyp19a1*. In addition, down-regulated expression of mRNAs for ovarian paracrine signaling factors, such as LH/hCG and progesterone receptors, kit ligand or insulin-like growth factor 1, caused by 10 μmol/l HPTE suggests possible paracrine regulation disruption (Harvey et al. 2009).

Additional mechanism contributing to decrease in steroid levels by MXC may be induction of metabolic enzymes involved in their catabolism, as it was shown in isolated mouse antral follicles where MXC (100 μg/ml) induced *Cyp1b1* expression (Basavarajappa et al. 2011). Thus, both decreased synthesis of sex steroids and increase in their catabolism by modulating the expression of the respective enzymes may contribute to alteration of steroid hormone levels caused by MXC.

Atrazine (ATR; 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a broadly applied agricultural pesticide for crop production worldwide. Although it is banned in the EU countries, it is still a contaminant e.g. in the USA or India (levels of 0.003 mg/l in drinking water) (Pogrmic-Majkic et al. 2014). Epidemiologic and laboratory evidence suggests that ATR can disrupt reproductive health and hormone secretion. Study of Cragin et al. (2011) indicated an association of higher ATR intake from tap water in the particular U.S. regions with increased menstrual cycle irregularity, longer follicular phases, and decreased levels of menstrual cycle endocrine biomarkers (estradiol and progesterone metabolites) of infertile ovulatory cycles. Scientific studies clearly show that steroidogenesis is an important target for ATR action in the ovary. In animal experiments, treatment with ATR (2 mg/kg) impaired reproductive physiology of female pigs, caused multiple cysts in the ovaries and persistence of corpus luteum, while lowered plasma estradiol concentrations were measured (Gojmerac et al. 1996; Gojmerac et al. 1999). In female rats, ATR (300 mg/kg) significantly increased serum progesterone production, and at both the gene and the protein levels up-regulated the steroidogenic factors StAR, CYP11A1, and HSD3B while down-regulated the luteolytic gene, *Cyp17a1* in the newly formed corpora lutea (Taketa et al. 2011). Using rat primary granulosa cell culture, treatment with ATR (20 μmol/l) resulted in the marked enhancement of progesterone production by FSH-stimulated cells, in the over-expression of luteal markers *Star* and *Cyp11a1*, and in disbalance in progesterone/estradiol ratio. In the last effect, diminished estradiol production due to decreased *Cyp19a1* and *Lhr* expression by ATR action were involved (Fa et al. 2013). On the opposite, treatment with ATR (10 and 30 μmol/l) elevated estradiol production by rat granulosa cells (Tinfo et al. 2011), and aromatase activity was increased in rat granulosa as well as in human granulosa-lutein cells (Holloway et al. 2008). The mechanism of ATR action on progesterone synthesis seems to rise from the amplification of FSH signal transduction by prolonging the FSH-induced CREB and AKT phosphorylation and elevation of ERK1/2-dependent transcriptional factor CCATT/enhancer-binding protein beta level in granulosa cells (Pogrmic-Majkic et al. 2014). Taken together, ATR can act as a hormone sensitizer and by over-activation of the signaling pathways and enhancement of the FSH-induced transcriptional activity, resulting in an inappropriate stimulation of progesterone production what leads to the premature luteinization phenotype in granulosa cells (Pogrmic-Majkic et al. 2014).

Importantly, it has been revealed that also several other currently used pesticides possess endocrine disrupting potential. Administration of dairy cows with the organophosphate pesticide **malathion** (1 mg/kg) at the onset of induced estrus did not alter the plasma FSH or estradiol concentrations but significantly inhibited plasma progesterone concentration with subsequent poor conception (Prakash et al. 1992). In rats treated with the pesticide mixture (50 mg/kg) containing malathion (with propiconazole, cypermethrin, bitertanole, terbuthylazine), despite no changes in progesterone levels, serum estradiol was reduced indicating modulation of CYP19A1 activity (Taxvig et al. 2013). A synthetic pyrethroid **cypermethrin** induced degenerative changes in the ovaries of female rats when given orally for 4 weeks at a dose of 50 mg/kg, with decreased HSD3B protein expression (Sangha et al. 2013).

Plasticizers

Phthalates (phthalic acid esters), with **di(2-ethylhexyl) phthalate** (DEHP) as the most abundantly used, are synthetic chemicals employed as a plasticizers of

PVC in the manufacture of a variety of consumer products (medical devices: blood storage bags, catheters; food packaging, floors, etc.). Importantly, phthalates are not chemically bound to PVC, thus they can leach out of plastics in contact with lipophilic contents and enter the environment. DEHP is rapidly hydrolyzed to produce its major metabolite **mono(2-ethylhexyl) phthalate** (MEHP), thus the ovary is targeted by DEHP/MEHP. In human serum, DEHP concentration of 0.6-1.15 μg/ml has been measured (Latini et al. 2003), and besides other fluids, DEHP metabolites were detected in human follicular fluid, with the levels up to 50 nmol/l (Krotz et al. 2012). Phthalate esters have been found to exert toxic effects on female reproductive system *in vivo.* In treated rats, DEHP (e.g. 500 mg/kg) has been shown to alter the levels of LH and FSH, to delay ovulation, and impair steroidogenesis resulting in decreased serum progesterone levels (Davis et al. 1994; Ma et al. 2006; Svechnikova et al. 2007). In injected rats, DEHP (doses above 300 mg/kg) significantly reduced levels of estradiol along with a decrease in *Cyp19a1* mRNA and protein (Xu et al. 2010). However, a different study showed that inhaled DEHP (5 and 25 mg/m^3) increased levels of estradiol in serum of prepubertal rats, and increased ovarian *Cyp19a1* expression (Ma et al. 2006).

Similarly, alteration of steroid levels by DEHP/MEHP has been found in culture of isolated rodent ovarian follicles. In mouse (10-200 μmol/l) and rat (100 μg/ml) follicles, MEHP treatment resulted in increased inactivation of estradiol to estrone and a precocious increase in progesterone synthesis (Lenie and Smitz 2009; Inada et al. 2012). DEHP (1-100 μg/ml) and MEHP (0.1-10 μg/ml) inhibited mouse antral follicle growth, reduced estradiol production, and decreased *Cyp19a1* expression. Nevertheless, phthalate-induced inhibition of follicle growth and down-regulation of *Cyp19a1* mRNA levels were prevented with estradiol co-treatment (1-10 nmol/l) (Gupta et al. 2010). In porcine oocyte-cumulus complexes, DEHP increased progesterone production when given at 1 μmol/l (Gunnarsson et al. 2008; Mlynarcikova et al. 2009). The mechanism for progesterone modulation by DEHP/MEHP is not elucidated since MEHP was found to increase progesterone without altering neither cAMP levels, StAR protein expression by the granulosa tumor cell line KK-1 (Gunnarsson et al. 2008) nor the levels of steroidogenic enzyme *Cyp11a1* in rat granulosa cells (Lovekamp and Davis 2001). However, through observed up-regulation of hormone-sensitive lipase and 3-hydroxy-3-methylglutaryl coenzyme A reductase, MEHP could increase the amount of cholesterol available for steroidogenesis (Gunnarsson et al. 2008).

In vitro culture of human granulosa-lutein cells with MEHP (0.6-500 μmol/ml) resulted in suppression of basal, FSH- and 8-Br-cAMP-stimulated estradiol production, and decrease in *CYP19A1* mRNA and activity. In contrast, MEHP did not alter the production of progesterone up to a concentration of 167 μmol/l (Reinsberg et al. 2009). Study in the human granulosa cell line KGN showed in agreement that exposure to DEHP decreased estradiol synthesis and reduced the mRNA expression of *CYP19A1* and *FSHR*, and in addition, DEHP induced the expression of the peroxisome proliferator-associated receptors (PPARα and γ) and aryl hydrocarbon receptor (AhR). Thus, the action of EDCs may have additional mechanisms due to a transactivation via PPARs, inducing subsequent transcriptional changes with a broad range of effects on granulosa cell function (Lovekamp-Swan and Davis 2003; Ernst et al. 2014). Moreover, together with decreased *Cyp19a1* mRNA and protein in MEHP-treated (50 and 100 μmol/l) rat granulosa cells (Lovekamp and Davis 2001), it induced the expression of *Hsd17b4* and *Cyp1b1* (Lovekamp-Swan et al. 2003). The data indicate that MEHP is a specific inhibitor of estradiol production and may alter estradiol levels by its disbalanced synthesis and catabolism.

Moreover, although the majority of studies aimed at ovarian toxicity have focused on DEHP/MEHP, several other phthalate esters are currently used as plasticizers in consumer products, therefore their effects should also be noticed. Previously, we demonstrated that **diisodecyl phthalate** and **diisononyl phthalate** possess endocrine disrupting abilities since they increased progesterone while decreased estradiol production by cultured porcine granulosa cells (Mlynarcikova et al. 2007). However, the effects may depend on the specific phtahalate structure, on species or cell type, since **dibutyl phthalate** and **benzyl butylphthalate** were able to reduce both basal and hCG-stimulated progesterone release in cultured luteal cells isolated from human corpora lutea (Romani et al. 2014). Nevertheless, these findings indicate that phthalates, other than DEHP, may also alter ovarian processes and merit further investigation.

Bisphenol A (BPA) (2,2-bis(4-hydroxyphenyl) propane) is a widely used chemical which has raised a significant concern due to its multiple endocrine disrupting properties. It is employed in the manufacture of polycarbonate plastics and epoxy resins, and thus it is present in multitude of products: interior coating of food and beverages cans, milk containers, baby formula

bottles, dental materials, etc. Depolymerization (due to increased temperature/sterilization, pH, and storage in warehouses) causes leaching of BPA into products, and data from biomonitoring studies clearly indicate that the general population is exposed to BPA ubiquitously (Vandenberg et al. 2010). BPA has been detected in 70-90% of population samples including urine, breast tissue, and follicular fluid (1-2 ng/ml) (Ikezuki et al. 2002; Fernandez et al. 2007), with the concentrations in human serum usually in the range 0.5-7.1 ng/ml (Ikezuki et al. 2002; Cobellis et al. 2009; Sprague et al. 2013).

Numerous *in vivo* and *in vitro* studies have therfore been performed to investigate BPA actions. The disrupting effects of BPA (100 mg/kg) on the onset of puberty, regularity of estrus cyclicity, and development of polycystic ovaries have been observed in exposed mice (Markey et al. 2002; Kato et al. 2003; Nah et al. 2011). Despite the well-known estrogenic properties of BPA (Olea et al. 1996), the female reproduction-altering effects of this compound seem not to be exclusively estrogen receptor-related. At the hypothalamic-pituitary level, perinatal BPA administration resulted in the upregulation of the expression levels of *Kiss1*, *Gnrh* and *Fsh* mRNA in female mouse pups. Exposure to BPA resulted in a greater CYP19A1 expression level and the synthesis of estrogen in the female pups (Xi et al. 2011). Thus, it was hypothesized that the effects of BPA on reproductive dysfunction may partly result from its actions on gonadal steroidogenesis and subsequent anomalous releases of endogenous steroid hormones. This non-ER-mediated effect was potent in affecting the feedback regulatory circuits in the hypothalamic-pituitary-gonadal axis (Xi et al. 2011). Similarly, postnatal BPA exposure (50 mg/kg) of rats was reported to increase serum estradiol levels while progesterone levels were diminished (Fernandez et al. 2010). The BPA effects, however, may largely depend on the exposure time and doses tested. Several studies using *in utero* exposed rats (0.33, 3.3, 33 ppm) (Kobayashi et al. 2012), neonatally exposed rats (0.05 mg/kg and 20 mg/kg) (Varayoud et al. 2011) or lambs (Rivera et al. 2011) did not prove BPA effects on steroidogenesis. In contrast, another study found that although low-dose BPA (10 mg/animal) did not alter estradiol levels, it decreased progesterone levels in adult mice during early pregnancy (Berger et al. 2008). In addition, in adult rats, BPA in a dose as low as 0.001 mg/kg/day) reduced testosterone and estradiol, along with decreased *Star* and *Cyp19a1* expression (Lee et al. 2013). In addition, in adult mice, low-dose BPA (10 mg/animal) decreased expression of estrogen and progesterone receptors but did not modulate hormone levels (Berger et al. 2010).

In vitro exposure of intact murine antral follicles to BPA (44 and 440 μmol/l, 96 h) inhibited their growth and reduced progesterone, DHEA, androstenedione, testosterone, estradiol, and estrone production preceded by decreased *StAR*, *Cyp11a1,* and *Hsd3b1* transcript expression (Peretz et al. 2011; Ziv-Gal et al. 2013). BPA effects were opposite in isolated rat theca-interstitial cells, where in concentrations 0.1-100 μmol/l, an increase in testosterone synthesis and *Cyp17a1*, *Cyp11a1*, and *StAR* expression was observed (Zhou et al. 2008). In isolated granulosa cells from the same study, 0.1-10 μmol/l BPA increased, while 100 μmol/l BPA decreased progesterone synthesis despite increased *Star* expression, and a significant concentration-dependent inhibitory effect of BPA (1 to 100 μmol/l) on estradiol levels and the expression of *Cyp19a1* mRNA was observed (Zhou et al. 2008). In porcine granulosa cells, 0.1 μmol/l BPA increased estradiol levels, whereas higher doses (1 and 10 μmol/l) decreased estradiol levels but all testedμμ BPA concentrations reduced progesterone production (Grasselli et al. 2010). Similarly, our group has previously shown that, depending on the dose given, BPA (1 and 10 μmol/l) increased basal and FSH-induced progesterone production, respectively, while it inhibited (1-100 μmol/l) FSH-induced estradiol production by cultured porcine granulosa cells (Mlynarcikova et al. 2005). In addition, 100 μmol/l BPA was able to decrease progesterone production, and inhibit expansion of cumulus cells and oocyte maturation in porcine oocyte-cumulus complexes (Mlynarcikova et al. 2009). Importantly, BPA treatment (40-100 μmol/l) reduced estradiol production by primary human granulosa cells and a human ovarian granulosa-like tumor cell line probably caused by the observed decrease of *CYP19A1* mRNA and protein expression (Kwintkiewicz et al. 2010; Watanabe et al. 2012). The studies thus indicate that BPA could alter steroidogenesis *in vitro* depending on the concentration and the model system used (intact follicles or isolated cells).

Association between BPA levels, ovarian steroid hormone production and/or metabolism, and pathophysiology of some fertility disorders in women has been suggested previously. The study of Takeuchi et al. (2004) showed that serum BPA concentrations were significantly higher in both non-obese and obese women with polycystic ovary syndrome (PCOS; 1.05±0.10 ng/ml, 1.17±0.16 ng/ml) and obese normal women

(1.04±0.09 ng/ml) compared with those in non-obese normal women (0.71±0.09 ng/ml). There were significant positive correlations between serum BPA and androgen (total and free testosterone, androstenedione, and DHEA) concentrations in all subjects (Takeuchi et al. 2004). Similar association of BPA with increased testosterone and androstenedione levels in women with PCOS was found in the case-control study of Kandaraki et al. (2011). Analysis of samples obtained from women undergoing in vitro fertilization (IVF) have depicted that BPA exposure was associated with a decrease in peak serum estradiol levels prior to oocyte retrieval, along with reduced oocyte yield, MII oocyte counts, and number of fertilized oocytes (Mok-Lin et al. 2010; Bloom et al. 2011; Ehrlich et al. 2012). However, the intent to use an IVF cohort and samples collected at the time of oocyte retrieval as an *in vivo* human model to study the underlying mechanism by which BPA decreases serum peak estradiol resulted to observation of no clear dose-response association between urinary BPA and granulosa cell *CYP19A1* gene expression, and suggested the possibility of a non-monotonic dose response (Ehrlich et al. 2013). On the other hand, in a prospective, population-based study of adults (Chianti region, Italy), BPA was not associated with estradiol or testosterone levels in women (Galloway et al. 2010). Given that most existing studies on BPA exposure and steroid levels were conducted in IVF populations, definite conclusions on actual BPA actions on steroidogenesis cannot yet be made, and it is critical to examine the association between BPA exposure and steroidogenesis in women from the general population (Peretz et al. 2014).

Alkylphenols, such as **4-octylphenol** (OP), **4-*tert*-octylphenol** (tOP) and **4-nonylphenol** (NP), have also received increased attention due to their widespread presence in the environment, and have raised concern as estrogen-mimicking compounds previously (White et al. 1994; Soto et al. 1995). Alkylphenols are mainly used in the production of plastics, pulp and paper, pesticides, and surfactants (alkylphenol polyethoxylates), which can degrade back to basic short-chain alkylphenols (Soares et al. 2008; Xie et al. 2013). Exposure to alkylphenols can thus occur through direct contact with materials or by consumption of food and drinking water (Guenther et al. 2002; Shao et al. 2005). Detectable alkylphenol levels were measured in serum (app. 0.5-1 ng/g serum) and urine (0.1 ng/ml) of more than 50% subjected participants in the studies (Calafat et al. 2008; Gyllenhammar et al. 2012). Importantly, the recent study of Gyllenhammar et al. (2012) showed the correlation of detectable blood NP levels with higher consumption of fruits, vegetables and cereals in Swedish women what refers to the use of alkylphenols as additives in pesticides and subsequent accumulation in the treated plants (Gyllenhammar et al. 2012).

Alkylphenols were shown to interfere particularly with the development of reproductive system, and their effects on steroid production seem to depend on the particular compound and model used. Treatment of rats during the early postnatal period with tOP (50 mg/kg) prevented ovulation, and the ovaries had a decreased number of corpora lutea and an increased number of preantral follicles (Katsuda et al. 2000; Willoughby et al. 2005). After tOP exposure (100 mg/kg) of immature rats, plasma FSH dropped and progesterone increased significantly, while ovarian StAR protein content and *ex vivo* hormone and cAMP production were decreased (Myllymaki et al. 2005a). The study in female rats demonstrated that NP (100 μg/kg/day) stimulated *ex vivo* progesterone release by granulosa cells via activation of StAR protein expression (Yu et al. 2011).

In vitro, OP stimulated basal and FSH-induced progesterone production at doses 10^{-7}-10^{-5} mol/l in rat granulosa-luteal (Nejaty et al. 2001). In porcine granulosa cells, however, OP in similar concentrations did not change FSH-stimulated progesterone production while it inhibited estradiol synthesis (Mlynarcikova et.al. 1997). Using the isolated rat ovarian follicles, tOP (even at 10^{-8} mol/l) was shown to decrease estradiol and testosterone secretion in a time-dependent manner, with no effect on aromatase activity but decreased forskolin-induced cAMP levels (Myllymaki et al. 2005b). In the human model, NP impaired luteal cell steroidogenic function *in vitro* (Romani et al. 2013).

Halogenated aryl hydrocarbons

The organic compounds substituted with halogens are widely found in the environment due to broad range of their industrial use (Hombach-Klonisch et al. 2005; Kotwica et al. 2006). **Polychlorinated dibenzo-*p*-dioxins** are a class of environmental chemicals exemplified by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). This toxicant is produced from combustion in the presence of chlorine and as an indirect by-product in the manufacture of pesticides and disinfectants. Waste incineration contributes most significantly to anthropogenic point-source production of dioxins while forest fires and volcanic eruptions comprise the greatest natural sources of TCDD (Ho et al. 2006). Due

to its lipophilicity, TCDD concentrates in the ecosystem and the food chain. Traces of TCDD are found in wildlife animals and humans (in serum, adipose tissue, milk) depending on region (Mannetje et al. 2012; Eskenazi et al. 2014). TCDD has been classified as a carcinogen by International Agency for Research on Cancer (Davis et al. 2000), and experimentally is considered a prototype ligand of AhR which is involved in the action of xenobiotics. Persistence and ubiquity in the environment make it difficult to ascertain the impact of TCDD on the organisms, although the toxic effects of TCDD certainly include a decrease in reproductive functions in many animals, in addition to its teratogenic, immunosuppressive, tumor promoting, and estrogenic actions (Baba et al. 2005). Importantly, a balanced activity of the AhR is necessary for normal ovarian function, since AhR is involved in folliculogenesis, gonadotropin receptor expression, proliferation of granulosa cells and intraovarian estrogen signaling. Highly potent, non-physiological ligands, such as the TCDD, can thus potentially target the AhR pathway leading to subsequent dysfunction of granulosa cells (Wojtowicz et al. 2005; Horling et al. 2011).

In vivo studies have shown that TCDD exposure reduced follicle development, fecundity and ovulatory rate in rats (Heimler et al. 1998). It was suggested that TCDD endocrine disrupting effects are, at least in part, caused by its direct action at the ovary. However, not all studies have found the similar pattern in the steroid hormone alteration. For example, while TCDD (20 μg/kg) blocked ovulation and reduced ovarian weight in the treated rats, concentrations of progesterone, androstenedione, and estradiol in sera and ovaries were not altered (Son et al. 1999). After maternal exposure of rats to TCDD (1 μg/kg), decreased expression of LH in the pituitaries (Takeda et al. 2009), increased levels of FSH, unaltered progesterone but decreased estradiol levels (Myllymaki et al. 2005c), and decreased *Star*, *Cyp17a1* and *Cyp19a1* mRNAs in the ovaries of the offspring were observed (Myllymaki et al. 2005c; Takeda et al. 2009). Unlike, maternally introduced TCDD (1 μg/kg) increased serum progesterone in the offspring while FSH and estradiol levels were not changed (Pesonen et al. 2006).

The effects of TCDD have been extensively investigated in various culture models *in vitro*, and numerous studies have revealed modulation of ovarian steroid production by TCDD though the mechanisms behind appear to be multilevel, and time-, dose-, and even cell-specific. The results of studies measuring effects of TCDD on progesterone production are inconsistent. TCDD treatment did not alter progesterone accumulation by human luteinized granulosa cells (Heimler et al. 1998). Also at 0.1 nmol/l, TCDD did not affect steroid hormone production or enzyme activity in granulosa cells isolated from medium and large porcine ovarian follicles (Piasecka-Srader et al. 2014) but in another study, the same dose stimulated progesterone secretion by the cells from preovulatory (≥ 8 mm) porcine follicles (Jablonska et al. 2014). On the opposite, the inhibition of progesterone secretion in the presence of the same and higher doses of TCDD (0.1-10 nmol/l, 100 nmol/l) by porcine granulosa cells isolated from medium (3-6 mm) follicles, cultured alone and in co-culture with theca cells, was reported (Grochowalski et al. 2001; Gregoraszczuk 2002; Jablonska et al. 2014). TCDD also caused a decrease in progesterone production by rat granulosa cells, and the mechanism of inhibition seems to involve diminished basal and FSH-stimulated CYP11A1 activities after TCDD treatment (Dasmahapatra et al. 2000). The different results are presumably attributable to differences in the models and species, making comparison among studies difficult.

Nevertheless, TCDD acts as an estrogen-modulating agent in many paradigms. Estradiol secretion by human luteinized granulosa cells or rat granulosa cells was decreased when cultured with TCDD (3.1 pmol/l, 3.1 nmol/l, and 3.1 μmol/l; 10 nmol/l) for 8, 12, and 24 h but increased above controls after 36 and 48 h (3.1 μmol/l) (Heimler et al. 1998; Moran et al. 2000; Dasmahapatra et al. 2000). In porcine granulosa cells, the increase in estradiol secretion was observed only after 48 h (10 nmol/l TCDD), while a long term exposure to TCDD (0.1 nmol/l; 144 h) caused a decrease of estradiol secretion (Pieklo et al. 2000). Also, the higher dose of TCDD (100 nmol/l) significantly stimulated estradiol production by luteinized granulosa cells isolated from medium (3-6 mm) porcine follicles while the lower dose of TCDD (0.1 nmol/l) significantly inhibited estradiol secretion by the cells isolated from preovulatory (≥ 8 mm) porcine follicles (Jablonska et al. 2014). Further, addition of aromatizable precursor substrate androstenedione abolished the initial inhibition by TCDD of estradiol secretion. The addition of DHEA (a CYP17A1/17,20-lyase product) or androgen precursors (17α-hydroxypregnenolone or 17α-hydroxyprogesterone) to the human luteinized granulosa cells culture prevented the TCDD-caused decrease in estrogen secretion indicating normal aromatase activity (Moran et al. 2003). This suggests that a likely

target of TCDD in human cells is CYP17A1/17,20-lyase activity of the CYP17A1 enzyme complex, resulting in insufficient provision of androgens for aromatization (Moran et al. 2000, 2003). TCDD thereby exacts its effects on the enzymes of the ovarian steroidogenic pathway. Its effect may be attributable to AhR interaction with dioxin-responsive elements present in the genes encoding these enzymes (Dasmahapatra et al. 2000). As it was shown, AhR plays a crucial role in female reproduction by regulating the expression of ovarian CYP19A1, a key enzyme in estrogen synthesis (Baba et al. 2005). Indeed, exposure of human granulosa cell line KGN to TCDD has shown, besides the obviously decreased FSH-stimulated estradiol synthesis, reduced the mRNA expression of *CYP19A1* (Ernst et al. 2014). Furthermore, in whole rat ovary microarray studies, chronic TCDD exposure (200 ng/kg/week) down-regulated *Cyp17a1* and growth differentiation factor 9 (GDF-9), a paracrine factor involved in folliculogenesis, and induced *Cyp1b1* (Valdez et al. 2009).

However, TCDD seems to affect the overall regulation of steroid production and release by altering the reproductive axis generally. A potential site for TCDD effect (at doses as low as pmol/l) involved impaired inhibin A production by human granulosa cells after TCDD treatment, which can then inhibit FSH secretion centrally, reducing estrogen peripherally (Ho et al. 2006). Moreover, the endocrine disrupting effects of TCDD are, at least in part, caused by the direct action on the LH receptor expression in (rat) granulosa cells, as 10 pmol/l TCDD significantly decreases FSH-induced LH receptor mRNA expression (Minegishi et al. 2004). In addition, although in the study of Jablonska et al. (2011) TCDD itself did not affect progesterone secretion by luteinized granulosa and theca cells from porcine preovulatory follicles, it abolished the stimulatory effect of prolactin in the follicular cells what suggests for TCDD perturbation of local steroidogenesis regulation (Jablonska et al. 2011).

In addition, as for metabolism of estrogens, TCDD dose-dependently induced a significant increase of *CYP1B1* in human luteinized granulosa cells. The increase in the oxidative metabolism of estrogens by CYP1B1 and potential generation of DNA adducts in the ovary may have significant consequences for oocyte quality, corpus luteum function, and ovarian carcinogenesis (Vidal et al. 2005).

Polychlorinated biphenyls (PCBs) are a large group of organic chemicals (congeners) with a pair of benzene rings as a common structure varying in their degree of chlorination and benzene rings position. Formerly, PCBs have been used in dielectric fluids such as those in transformers, as pesticides or plasticizers, resulting in their direct or indirect release and persistence into the environment (ATSDR 2000; Abass et al. 2013). Their presence is still being confirmed in human serum (Abass et al. 2013; Bastos et al. 2013; Wohlfahrt-Veje et al. 2014), and higher blood levels of persistent organic pollutants including PCBs were noted in females with earlier menarche, shortened menstrual cycle and prolonged menstrual bleeding (Dudarev and Chupakhin 2014). The studies also reported levels of various congeners in ovarian follicle fluid of women (total PCB 0.37 ng/g wet weight) (De Felip et al. 2004). They have been found to be able to accumulate in the wall of porcine ovarian follicles (Gregoraszczuk et al. 2003). Nevertheless, as the group of PCBs present in the environment consists more than 100 congeners, it is difficult to cover all their effects.

Results of experimental studies indicate that PCBs may disrupt follicular steroidogenesis either by mimicking natural hormones as agonist or antagonist, altering the pattern of hormone synthesis, or by altering enzymes involved in hormone secretion. Moreover, their actions may be cell- and congener-specific (Gregoraszczuk and Ptak 2013). In bovine luteal cells, PCBs 126, 77, and 153 (1-100 ng/ml) were able to decrease LH-stimulated secretion of progesterone in the luteal phase (Mlynarczuk and Kotwica 2006). A study testing the effects of PCBs 153, 118, 180, and 138 showed that congeners 153 (8 μg/ml), 118 (3 μg/ml), and 180 (3 μg/ml) increased estradiol secretion, while congener 138 (8 μg/ml) increased testosterone secretion by bovine granulosa cells (Mlynarczuk and Kotwica 2005). In an *in vitro* culture of porcine follicular cells, a decrease of testosterone produced by the theca cells after by both tested PCB congeners 126 and 153 was measured. PCB153 decreased estradiol secretion and increased progesterone secretion suggesting luteinization and disruption of aromatization process in granulosa cells while PCB126 increased concentrations of estradiol and progesterone in the highest dose (100 pg/ml) (Wojtowicz et al. 2000). It has been shown that action on estradiol secretion was correlated with the action on aromatase activity (Wojtowicz et al. 2001; Gregoraszczuk et al. 2003). The alterations in intraovarian processes including steroidogenesis may also partially be caused by the disruption of gonadotropin-induced intracellular signaling since it was found that individual PCBs (153, 77) and their mixture (Aroclor 1248) sig-

nificantly decreased the effect of FSH on intracellular calcium mobilization in porcine granulosa and luteal cells (Mlynarczuk and Kowalik 2013). In addition, it was suggested that disrupting effects of PCB153 on the synthesis and secretion of progesterone from porcine granulosa cells and the consequent development and maturation of ovarian follicles, might take place via SF-1 (Mlynarczuk et al. 2013).

Polybrominated diphenyl ethers (PBDE) are a class of widely used flame-retardants that have been incorporated into many consumer textiles, electronics and furniture (Birnbaum and Staskal 2004). These compounds typically are not chemically bound within the products, may migrate into the environment and have been identified as a human health concern (Birnbaum and Cohen Hubal 2006; Miller et al. 2012). Like among PCBs, there exists a lot of PBDE congeners (varying in bromine atom number in the molecule) and since they are usually used as mixtures, this makes difficulties to consistently summarize the effects of the whole group. PBDEs presence is detected in human blood samples, with BDE-47 and BDE-153 as the major congeners (mean levels of 2.06±1.80 ng/g lipid weight (lw) and 1.39±0.97 ng/g lw, respectively), and the mean sum PBDE levels was up to 4.5 ng/g lw (Johnson et al. 2012; Chovancova et al. 2012; Brasseur et al. 2014). Importantly, PBDEs are detectable in follicular fluid of women undergoing IVF (Johnson et al. 2012). Several human studies have hypothesized that PBDE might interfere with female reproductive functions resulting in shortening of menstrual cycle length (Chao et al. 2007) or even in reduced fecundability in terms of longer time to pregnancy (Harley et al. 2010) and failure in embryo implantation (BDE-153) (Johnson et al. 2012).

Several *in vivo* studies have shown that PBDEs could alter reproductive homeostasis in treated animals. In rats, gestational exposure to BDE-47 (700 μg/kg) decreased circulating levels of estradiol and the number of ovarian follicles in offspring (Talsness et al. 2008); also BDE-99 affected the number of ovarian follicles (Lilienthal et al. 2006). *In vitro* studies using either isolated follicles or individual follicular cell types have observed that PBDEs were able to disrupt steroidogenesis, although the mechanisms were not clearly stated. After exposure of porcine theca and granulosa cells co-culture to BDE-47 (1000 ng/ml), BDE-99 (500 ng/ml), BDE-100 (250 ng/ml), and BDE-209 (10 ng/ml), respectively, all investigated congeners increased both testosterone and estradiol secretion. Additive stimulation of both testosterone and estradiol levels with BDE-47+BDE-100 was observed but subsequent abrogation of this effect when BDE-99 and BDE-209 were included in the mixture (Gregoraszczuk et al. 2008). The effects were different in the whole porcine follicles exposed to BDE-47, BDE-99 or BDE-100 (doses dependent on congener from 0.1 to 50 ng/ml) for 24 h. BDE-99 and BDE-100 stimulated progesterone production, and all of the congeners increased testosterone secretion but did not alter estradiol production. However, in the case of BDE-47 due to activation of HSD17B and BDE-100 due to activation of CYP17A1, a corresponding failure to activate CYP19A1 expression and inhibition of CYP19A1 activity was seen. The lack of an effect of BDE-99 on the expression and activity of all of the investigated enzymes indicated possible action on steps before progesterone secretion (StAR, HSD3B) activity (Karpeta et al. 2011). Culture of porcine luteal cells with PBDE-47, -99, and -100 (doses of 50, 250, and 500 ng/ml) resulted in an increase in progesterone after 24 h while after 48 h, no changes in progesterone production but activation of caspases were observed. The increase in progesterone secretion was the result of the stimulatory action of all PBDEs on HSD3B protein expression and activity (PBDE 99). Thus, despite the initial stimulatory effect on the secretion of progesterone, PBDEs are also an executor of apoptosis what could lead to premature dysfunction of the corpus luteum (Gregoraszczuk et al. 2012).

The situation is even more complicated when the metabolism of PBDEs is taken into consideration. The published data showed fast activation of CYP2B1/2, and late activation of catechol-O-methyl transferase in porcine follicles by BDE-47 what suggests for a possible action of locally produced hydroxylated metabolites prior to their detoxification (Karpeta et al. 2012). It has been shown that 5-OH-BDE-47 and 6-OH-BDE-47 have a different mechanism of action from their parent compound and lead to an increase in estradiol secretion. Thus while the parent compound increases androgen production and stimulates HSD17B protein expression and activity, the metabolites stimulate CYP19A1 expression and activity in the follicles (Karpeta et al. 2013).

Other EDCs

Besides the EDCs, which effects are described afore in details; there is a myriad of different chemicals known to interfere with ovarian steroid hormone production. Further examples of steroidogenesis-impairing environmental pollutants include **polycyclic aromatic hydro-**

carbons (benzo[a]pyrene, dimethylbenz[a]anthracene) formed during the combustion of carbon-containing substances (wood, coal, tobacco) (Craig et al. 2011), or **cadmium** as a pollutant from a variety of sources (mining, smelting, fossil fuel combustion, batteries, paints, tobacco smoke) (Belani et al. 2014). In addition, several natural substances, which can be ingested through food in considerable amounts or on a regular basis, e.g. soy **isoflavones** (genistein, daidzein, biochanin A), are also able to affect steroid synthesis by the follicular cells (Nynca et al. 2013).

Several remarks on studying the EDCs effects

The results of numerous scientific studies provide valuable evidence of negative EDCs impact on the ovarian steroidogenic pathway, and consequent adverse effects for the female reproductive health. Nevertheless, there are several items to take into account when drawing conclusions on definite impact of the substances considered having perturbative effects on the processes of steroidogenesis.

a) The concentrations used in *in vitro* cultures or doses administered in *in vivo* studies are often excessively high in comparison to those detected in human (or affected farm/wildlife animals) biological samples including follicular fluid. Thus, the decisive conclusion should arise from the studies using relevant concentrations of the respective EDC.

b) For testing the EDCs action, many studies use the rodent (mouse, rat) or other animal (pig, cow) *in vivo* or *in vitro* models, which are convenient for practical and ethical reasons. However, due to possible inter-species differences in the metabolism of the particular EDC (Ito et al. 2005), and/or in the steroidogenic processes (Chaffin and Vandevoort 2013), it cannot be certainly defined to what extent the results would apply to other species including humans.

c) Although a lot of EDCs are molecules with complicated chemical structures implying their lipophilicity and accumulation, the majority of EDCs undergoes metabolic transformation upon absorption in the organism, at least to some extent. The effects observed may thus be partially ascribed to their metabolites, and may or may not be identical with those of the parent compounds (Karpeta et al. 2013). Moreover, the amounts and actions of the metabolites may vary depending on various biological conditions, and thus are difficult to be tested precisely.

d) What is of great importance for the real-life situations, organisms are exposed to a myriad of chemical substances within their environment, either of industrial or natural origin. Given that their actions on various physiological processes including steroidogenesis can be additive, synergistic, or on the other hand antagonistic (Gregoraszczuk et al. 2008); it is evident that the exposure to such undefined mixtures may result in a great variability of the effects. The definite EDCs action on steroid hormone production may thus be enhanced or diminished after co-exposure to several substances.

Conclusions

Majority of EDCs can obviously affect several possible endpoints in female reproductive system, from the regulatory units, hypothalamus and pituitary, to the physiology of the ovary. Evidence obtained from numerous studies from both *in vivo* and *in vitro* models points out that steroidogenic pathways within the ovarian follicle represent an important site of action for various EDCs, and alterations of steroid hormone production can significantly contribute to negative reproductive effects observed in the presence of EDCs. However, for complete understanding of the mechanisms, by which particular chemical agents from the environment can affect steroidogenesis, and to what extent are their effects relevant to humans and wildlife, a lot of further investigation is needed.

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