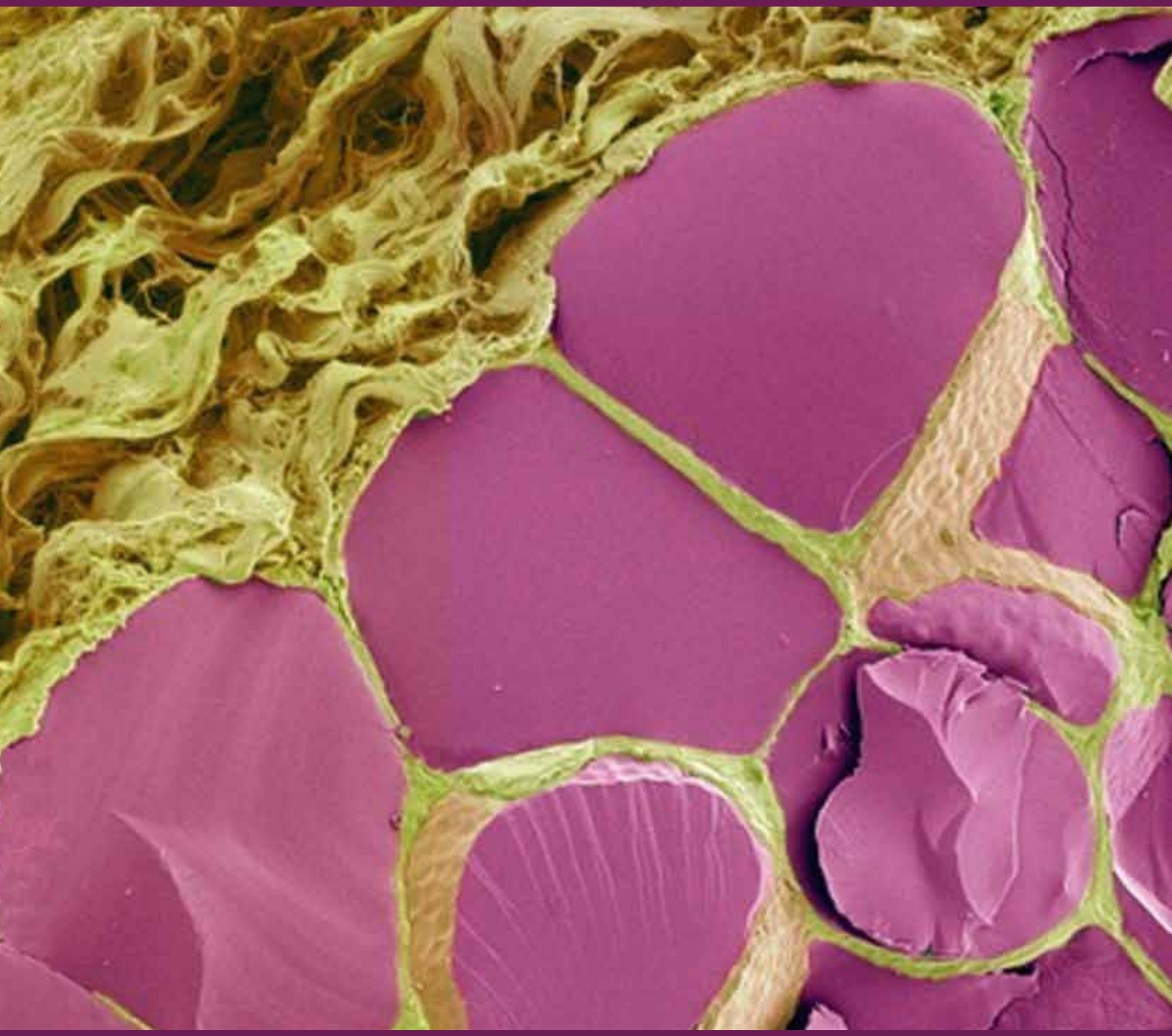
International Journal of Endocrinology

The Impact of Endocrine Disruptors on Endocrine Targets

Guest Editors: Ewa L. Gregoraszczuk and Radmila Kovacevic



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Editorial Board

Contents

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 340453, 2 pages
http://dx.doi.org/10.1155/2013/340453

Editorial

The Impact of Endocrine Disruptors on Endocrine Targets

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Received 18 June 2013; Accepted 18 June 2013

The potential effect of the so-called endocrine disruptors (EDCs) or xenoestrogens on human health and the proven effect on wildlife have got considerable attention in the scientific community. Endocrine disruption represents one of the most controversial environmental issues despite the fact that many substances, both natural and artificial, have been recognized to interfere with endocrine signaling pathways. Such interactions have been documented both in laboratory animal studies as well as *in vitro*. However, in humans there is limited evidence of endocrine disruption caused by EDCs. EDCs are a large group of persistent organic pollutants (POPs), such as polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and polybrominated ethers (PBDEs), chloronaphtalenes (PCNs), and bisphenol A (BPA), stable, lipophilic pollutants that affect fertility and cause serious reproductive problems. Xenoestrogens are synthetic compounds, but there are also numerous natural molecules in food that exhibit estrogen-mimetic activities. These natural molecules are mainly phytoestrogens isoflavones, and the most consumed are genistein and daidzein. Additionally, certain mushrooms or fungi can contain estrogen-like compounds called mycoestrogens.

Xenoestrogens activities in oocyte, ovary, placenta, and mammary gland and in the consequent serious reproductive problems, including ototoxic action, lack of ovulation, premature ovarian failure (POF), or polycystic ovarian syndrome (PCOS) are discussed in the review E. Gregoraszczuk and A. Ptak "*Endocrine-disrupting chemicals: some actions of POPs on female reproduction.*"

G. Kerdivel et al., in their paper "*Assessment and molecular actions of endocrine-disrupting chemicals that interfere with estrogen receptor pathways*" discuss different molecular actions of some of the major xenoestrogens found in food or the environment and summarize the current models used to evaluate environmental estrogens. This paper is accompanied by clinical study of Caserta et al. "*Correlation of endocrine disrupting chemicals serum levels and white blood cells gene expression of nuclear receptors in a population of infertile women*" compares the internal exposure to bisphenol A (BPA), perfluorooctane sulphonate (PFOS), perfluorooctanoic acid (PFOA), monoethylhexyl phthalate (MEHP), and di(2-ethylhexyl) phthalate (DEHP) in serum samples of 111 infertile women and 44 fertile women and analyses levels of gene expression of nuclear receptors (ERα, ERβ, AR, AhR, PXR, and PPARγ) as biomarkers of effective dose.

Two of the papers deal with aspects of alkylphenols action as endocrine disruptors.

The paper by B. Yi et al. "*Association between endocrine disrupting phenols in colostrums and maternal and infant health*" showed that most neonates who are exposed to BPA rather than NP or OP via colostrum are recommended continuous biomonitoring of the phenols to clarify their suspected health risk on neonates and pregnant or gestation mothers. Furthermore, A. Hejmej in their paper "*Photoperiod-dependent effects of 4-tert-octylphenol on adherens and gap junction proteins in bank vole seminiferous tubules*" evaluating *in vivo* and *in vitro* effects of 4-tert-octylphenol (OP) on the expression and distribution of adherens and gap junction proteins, N-cadherin, β-catenin, and connexin 43 (Cx43), in testes showed that long-term

treatment with OP resulted in the reduction of junction proteins expressions independent of FSH indicating that OP acts directly on adherens and gap junction proteins in the testes.

I. Wocławek-Potocka et al. in their paper "*Diverse effects of phytoestrogens on the reproductive performance: cow as a model*" review how exposure of soybean-derived phytoestrogens can have adverse effects on reproductive performance in female adults. Authors suggest that these findings should be specially taken into consideration when recommendations are made regarding dietary or therapeutic phytoestrogen intake in humans. Particularly that they are commonly recognized as therapeutic compounds.

Polychlorinated naphthalenes (PCNs) are new player as endocrine disruptors. Data concerning their potency and action on ovarian function are scarce. Dr J. Barc et al. in their paper "*Action of halowax 1051 on enzymes of phase I (CYP1A1) and phase II (SULT1A and COMT) metabolism in the pig ovary*" describe local ovarian metabolism of PCNs in ovarian tissue and suggest that fast activation of phase I enzymes with simultaneous inhibition of phase II enzymes indicates that androgenic action of PCNs on follicular steroidogenesis may partially result from metabolite action occurring locally in ovarian follicles.

To complete the issue, B. Ayala-García et al. revise current knowledge about the epigenetic mechanisms that underlie the effects of EDs on phenotypic variability and plasticity to stress the value of using the information derived from experiments with EDs to unveil the mechanisms that underlie phenotypic variability and speciation through epigenetic phenotypic plasticity.

Taking into account that in this special issue have been published review articles, research articles and clinical studies, we hope that the information published in this special issue enriches the knowledge of our readers and scholars interested in the influence of xenobiotics on human health.

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 828532, 9 pages
http://dx.doi.org/10.1155/2013/828532

Review Article

Endocrine-Disrupting Chemicals: Some Actions of POPs on Female Reproduction

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Received 7 January 2013; Accepted 26 April 2013

Academic Editor: Radmila Kovacevic

Persistent organic pollutants (POPs), such as polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and polybrominated ethers (PBDEs), chloronaftalens (PCNs), and bisphenol A (BPA), are stable, lipophilic pollutants that affect fertility and cause serious reproductive problems, including ovotoxic action, lack of ovulation, premature ovarian failure (POF), or polycystic ovarian syndrome (PCOS). Most of the representatives of POPs influence the activation of transcription factors, not only activation of aromatic hydrocarbon receptor (AhR), but also the steroid hormone receptors. This minireview will focus on a variety of PAH activities in oocyte, ovary, placenta, and mammary gland. The complexity and diversity of factors belonging to POPs and disorders of the reproductive function of women indicate that the impact of environmental pollution as an important determinant factor in fertility should not be minimize.

1. Introduction

For the past decade, scientists, institutions, governments, and policymakers have warned the general public about serious health hazards associated with chemicals known as endocrine disruptors (EDs). EDs are exogenous compounds that interfere with the synthesis, secretion, transport, metabolism, and/or action of endogenous hormones that are responsible for normal homeostasis, reproduction, and development. Chemicals with hormonal activity can be divided into three main groups: (i) synthetic compounds used in industry and agriculture as well as in consumer products, (ii) synthetic compounds used in pharmaceutical drugs, and (iii) natural compounds present in the food chain (i.e., phytoestrogens, compounds that are structurally similar to estrogen (E2)). Only (i) synthetic compounds used in industry and agriculture and consumer products will be discussed in presented paper. Within this class, compounds can be further subcategorized into those that are persistent in all elements of the environment, bio-accumulative, transportable over long distances, and capable of adversely affecting life forms that reside within short and long distances from the site of contamination. These compounds include dichlorodiphenyltrichloroethane (DDT, a pesticide), polychlorinated biphenyls (PCBs), polychlorinated naphthalenes (PCNs), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polybrominated diphenyl ethers (PBDEs) (Figure 1).

Because xenobiotics can accumulate in the body for an extremely long period of time (i.e., decades), they can be detrimental to human health even at very low doses. For instance, pesticides and other synthetic compounds used in the 1950s have polluted the air that we breathe, the water that we drink, and the soil that we grow our food in. In addition, many of these compounds are nonbiodegradable. Studies have shown the presence of EDS both in adipose tissue and other organs, in almost all humans and many animals. Human exposure to environmental toxicants is mediated via food and water chains, breathing, and dermal absorption, with approximately 90% of the exposure coming from food. Moreover, several mammalian organs (e.g., ovary, breast, uterus, cervix, bone, muscle, and skin) rely on sex steroids for normal function, and these organs are especially vulnerable to endocrine disruption by environmental toxicants. This

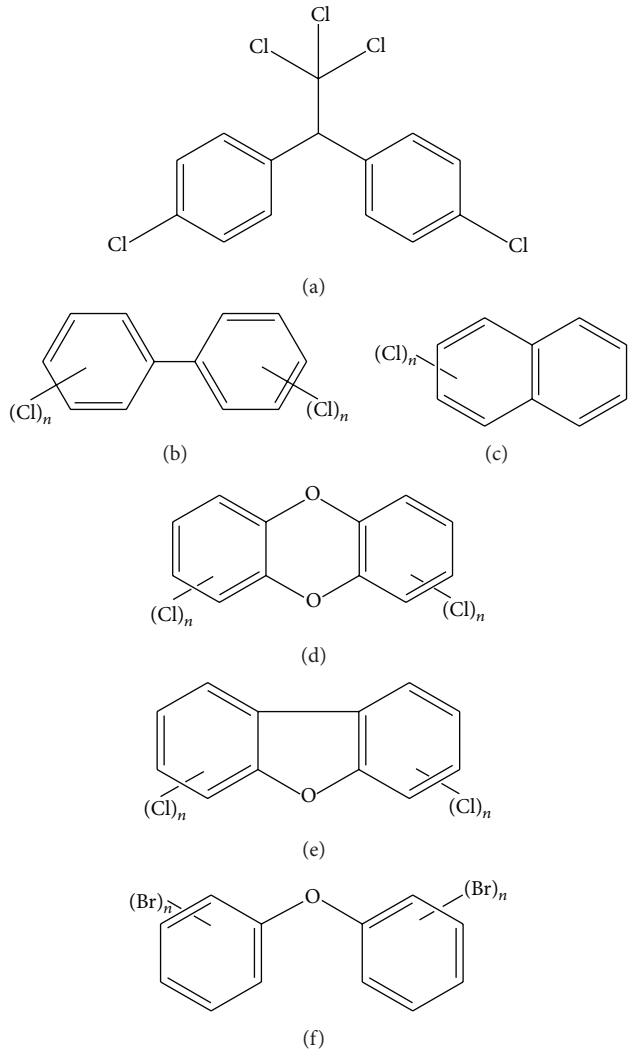

Figure 1: Generic structure of the (a) DDT, (b) PCBs, (c) PCNs, (d) PCDDs, (e) PCDFs, and (f) PBDEs.

chapter will focus only on selected actions or abnormalities. Much of the information on the impact of EDs on human health has come from *in vivo* studies where animals were exposed to a single compound, usually at an acute (i.e., pharmacological) dose; however, humans are exposed to several compounds at unknown concentrations for unknown durations. For example, hospital patients receive an average of six drugs daily (i.e., aspirin, antihistamines, antibiotics, anti-cough syrup, etc.). Food and water may also contain low levels of organic and inorganic compounds (e.g., pesticides and heavy metals) and solvents (e.g., benzene, toluene, and chloroform), and air is filled with hundreds of chemicals (e.g., industrial pollutants, smoke, gasoline vapors, etc.). The ability of these environmental toxicants to affect human health depends on several factors, including interactions between compounds; absorption, metabolism, accumulation, and excretion of compounds; and the ability of some compounds bind to cell receptors which can affect hormone action. Unfortunately, little is known about how these environmental toxicants interact with each other existing in a mixture. It is known that such xenobiotic acts in different ways depending on whether their action is observed on the fetus, newborn babies, or adult individuals, depending on the period during whose body has been exposed to these factors. In many cases, the effect of their actions may be undetected until sexual maturity, especially when exposure occurs during a period of embryonic development or shortly after birth. The female reproductive cycle is a complex process comprised of gametogenesis, embryogenesis, menstruation, ovulation, possible pregnancy, endometrial, and mammary gland changes. Development of the female reproductive system during fetal life determines reproductive success. Women are born with all the oocytes they will ever have through their life, and therefore oocytes are more vulnerable to toxic chemicals than germ cells in men who continue to make more germ cells.

2. Actions on the Oocyte

Oocyte maturation, the final step of germ cell differentiation, determines reproductive capacity. Follicles are especially susceptible to the adverse effects of environmental toxicants, and developing oocytes may be damaged directly or indirectly by action on follicular cells (i.e., granulosa cells) that may also be vulnerable to endocrine disruption, thereby affecting oocyte function indirectly. A previous study illustrated that human oocytes harvested from follicles with elevated levels of polycyclic aromatic hydrocarbons (PCAHs) had fewer cell divisions after *in vitro* fertilization [1]. Chloroorganic mixtures such as PCBs and DDT, as well as its metabolites, have also been reported to affect puberty, development, and oocyte viability adversely [2]. Some of these studies point to unpredictable changes of translational regulation within the oocyte under the influence of PCBs [3]. Polyspermia (secretion of an excessive amount of semen) has also been demonstrated in cattle exposed to an environmentally relevant mixture of more than 15 organochlorines [4]. Presently, additional research is needed to better understand the molecular mechanisms behind mammalian ovotoxicity caused by exposure to environmental toxicants.

Ovarian function is controlled by the hypothalamus, pituitary, and autoparacrine factors. Hormone-mimicking compounds can bind to cell receptors, interfere with hormone action, and affect ovarian function. How EDs affect ovarian function is not yet clear, but a disruption in gonadotropin (i.e., follicle stimulating hormone (FSH) and luteinizing hormone (LH)) secretion and feedback mechanisms involving E2 and progesterone (P4) may be involved. Alternatively, EDs may affect ovarian hormone production and oocyte maturation. Damaged oocytes can affect overall hormone production and follicular function, resulting in an endocrinological imbalance (i.e., a decrease in E2 and P4, but an increase in FSH and LH) and ovarian failure.

3. Actions on the Ovary

3.1. Disruption of Ovarian Function. Fertility in sexually mature women depends largely on the maintenance of healthy follicles, and their steady production ensures that

an adequate number of follicles reach the antral stage. The stage of development at which the follicles are destroyed determines the influence of these factors on the fertility of women. Complete depletion of the follicle reserve results in irreversible infertility; partial depletion of the follicle reserve results in moderate effects on periodicity. Damage to large follicles can also lead to reversible acyclic disorders that affect hormone production and ovulation. Nevertheless, the effects of POPs on the hypothalamic-pituitary-ovarian (HPO) axis are generally reversible because they do not permanently affect the follicle reserve.

3.1.1. TCDD. TCDD (2, 3, 7, 8-teterachlorodibenzo-para-dioxin) is an isomer of PCDD, and one of the most toxic man-made compounds to pollute our environment. Studies on the mechanism of TCDD action during ovulation indicate a dysfunction in the HPO axis. Misregulation of FSH and LH secretion before ovulation was noted in rats exposed to TCDD. Moreover, gonadotropin-releasing hormone (GnRH) inhibited the surge in FSH and LH secretion, thereby restoring ovulation [5, 6]. TCDD also inhibited FSH secretion and downregulated FSH receptor gene expression [7]. TCDD affected the levels of other hormones as well. For instance, Gore [8] showed methoxychlor to affect GnRH expression in hypothalamic GT1-7 cells. In a study from our laboratory, TCDD inhibited E2 secretion by follicular cells and P4 secretion by luteal cells dose-dependently. These adverse effects on hormone production were mediated in part by enzymes involved in steroidogenic biosynthesis. In luteal cells, TCDD action was independent of E2 receptor stimulation, instead involving the aryl hydrocarbon receptor (AhR) [9]. On a final note, other environmental toxicants (i.e., PCDFs, biphenyls, DDT, and methoxychlor) can also block the surge in LH and FSH secretion during the female reproductive cycle [10, 11].

3.1.2. PCBs. Polychlorinated biphenyls (PCBs) are the man-made chemicals that may disrupt follicular steroidogenesis either by mimicking natural hormones as agonist or antagonist, altering the pattern of hormone synthesis, modulating hormone receptor affinities or numbers, or by altering enzymes involved in hormone secretion. In our previous study, we showed that the orthosubstituted PCB 153 congener accumulated preferentially in the follicular wall when compared to the nonorthosubstituted PCB 126 congener. 71%, 71.4%, and 30.4% of the total exposure for PCB 153 were in small, medium, and large follicles, respectively (Figure 1). Interestingly, about 70% of PCB153 accumulated in early antral and antral follicles and only 30% in preovultory follicles. The consequence was a reduction in estradiol secretion by early antral and antral follicles and lack of influence on estradiol secretion by preovulatory follicles. Moreover, Moreover, it has been showed that action on estradiol secretion was correlated with action on aromatase activity [12–14]. A similar dose-responsive relationship was reported after exposure of follicular cells to a mixture of organic pollutants [15] or a mixture of PBDEs [16].

3.1.3. PBDEs. Polybrominated dibenzoethers (PBDEs) are persistent and ubiquitous environmental toxicants found at increasing levels in humans and animals. Despite recent bans by the European Union [17], United States, and China on the production of penta- and octa-BDE as well as on the diminished use of PBDE in Japan [18], 2,2′,4,4′-tetra-BDE (BDE-47), 2,2′,4,4′,5-penta-BDE (BDE-99), and 2,2′,4,4′,6-penta-BDE (BDE-100) are the major PBDE congeners present in humans and animals [19]. The literary data related to the effects of PBDE mixture are limited predominantly to commercial mixture DE-71 which contains mainly penta-BDEs (BDE-99 and BDE-100) and tetra-BDE (BDE-47). Zhou et al. [20] and Stoker et al. [21] indicated that DE-71 (a mixture of BDE-99, BDE-100, and tetra-BDE) affected the production of thyroid and sex steroids and the development of reproductive organs [20, 21]. Results from our laboratory have shown an increase in the P4/testosterone (T) ratio but a decrease in the T/E2 ratio, in ovarian follicles suggesting premature luteinization of antral follicles. Removal of the PBDE mixture from cell cultures did not reverse adverse effects [22]. In a follow-up study, we reported changes in the levels of steroidogenic enzymes (e.g., 17β-hydroxysteroid dehydrogenase (17β-HSD), cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17), and aromatase (CYP19)) by PBDE congeners 47, 99, and 100 [23]. Last published data showed fast activation of CYP2B1/2, and late activation of COMT (with a very low basal SULT1A activity) in ovarian follicles by BDE-47 indicates a possible action of locally produced hydroxylated metabolites prior to their detoxification [24]. Additionally, it have been showed that 5-OH-BDE-47 and 6-OH-BDE-47 have a different mechanism of action in ovarian follicles from their parent compound and lead to an increase in estradiol secretion. The metabolites stimulate aromatase expression and activity, while the parent compound increases androgen production and stimulation of 17β-HSD protein expression and activity [25].

3.1.4. PCNs. Polychlorinated naphthalenes (PCNs) are members of a large and diverse group of compounds with several industrial applications. PCNs occur as mixtures of congeners sold under various trade names (e.g., Halowax, Nibren Wax, and Seekay Wax) [26]. The toxicological characteristics of PCNs are similar to those caused by PCBs, PCDDs, and PCDFs [27, 28]. Presently, there are little data on the toxicity of PCNs in experimental animals, and our recently published data was the first showed direct action on ovarian function. We showed an increase in basal testosterone secretion in all doses used, with the highest stimulatory action of the smallest dose, which was accompanied by a parallel decrease in basal estradiol secretion, induced by Halowax 1051 suggesting androgenic properties of Halowax 1051. As a mechanism we propose direct stimulatory action on 17β-HSD activity and protein expression, enzyme responsible for testosterone synthesis and inhibitory action on CYP19 activity, and enzyme responsible for conversion testosterone to estradiol [29]. It should be taken into consideration that as in the case of BDE-47 [24], the effects of exposure to PCNs may be due to the side effects of PCN metabolites. Examining the effects of the Halowax 1051 on phase I (CYP1A1) and phase II (SULT1A and COMT) enzyme activities and expression in cultured ovarian follicles we showed fast activation of enzymes involved in

phase I and concurrent inhibition of enzymes involved in phase II metabolism confirming our suggestion that the observed effects of Halowax 1051 are partially result from the action of metabolites formed locally in ovarian follicles [30].

3.1.5. HCBz. Data on the effects of hexachlorobenzene (HCBz) on reproduction are scant. While HCBz is toxic to humans [31, 32] and animals [33], information on the effects of HCBz in ovarian steroidogenesis is limited despite data that showed an increase in the P4 serum level in superovulated rats exposed to HCBz [34]. Treatment of Cynomolgus monkeys with HCBz, on the other hand, decreased the P4 serum level and unaltered the E2 serum level during the luteal phase. In a recently published study, we determined *in vitro* accumulation of hexachlorobenzene (HCBz) and pentachlorobenzene (PeCBz) in porcine ovarian follicles, the effect on steroidogenesis, and the expression of enzymes responsible for steroid synthesis [35]. We showed that sixty percent of the HCBz and almost 100% of the PeCBz that was added to the culture medium accumulated in ovarian tissue, and only 1% of each was found in the medium. Moreover, we showed inhibitory HCBz effect and stimulatory PeCBz effect on testosterone and estradiol secretion. As a conclusion we mentioned that the greater exposure to an estrogenic action of PeCBz than antiestrogenic HCBz would be a consequence of the preferential accumulation of PeCBz in the ovarian follicles. Another situation was observed in human placental tissue. Both HCBz and PeCBz did not accumulate in placental tissue. We showed that HCBz by fast activation of I and II phase metabolism is probably metabolized to PeCBz in placental tissue (Gregoraszczuk et al., unpublished data).

3.1.6. A Mixture of Environmental Toxicants. Toxicants usually occur in a mixture of several toxicants, making it difficult to predict adverse effects on human health. Younglai et al. [36] demonstrated human follicular fluid obtained from women to contain 1, 1-dichloro-2, 2-bis (p-chlorophenyl)-ethylene (p, p′-DDE), mirex (a pesticide), hexachloro-ethane 1, 2, 4-trichlorobenzene, and numerous PCBs (i.e., PCBs 49, 153, and 180). Of these, p, p′-DDE was the most frequently detected ED in follicular fluid and serum. Several environmental toxicants are ER agonists, and they include estrogenic steroids (natural and synthetic), phyto- and mycoestrogens, and xenoestrogens (e.g., pesticides, plasticizers, and alkylphenols). In addition to simple additive effects [37], interactions between different chemicals in a mixture may result in either a weaker (antagonistic) [38] or stronger (synergistic, potentiated) combined effect than would be expected from knowledge about the toxicity and mode of action of each individual compound. These interactions may occur during toxicant uptake, distribution, metabolism, and/or excretion (i.e., toxicokinetic phase), or during toxicant binding to receptors and cellular targets (i.e., toxicodynamic phase) [39, 40]. A mixture of xenoestrogens may alter the way a cell responds to endogenous estrogens. For instance, xenoantiestrogens found within mixtures can inhibit endogenous E2. Moreover, some estrogenic compounds exert their effects, not by binding to ER but rather by binding to estrogen plasma transport proteins, resulting in an increase in free endogenous E2. Nevertheless, mixtures containing both xenoestrogens and endoantiestrogens may have no adverse effects [41]. In our previously published data, to determine which compounds within a PCB-DDT-DDE mixture stimulated E2 secretion, we exposed cells to PCBs 118, 138, 153, and 180, DDT, and DDE alone or in different combinations. Interestingly, DDT and DDE affected E2 secretion [42], and these results are in agreement with another series of experiments that used mixtures of PBDEs [43]. In the next experiments, using Western blot analysis indicated that PCBs mixture ("Marine mix") is an inducer of AhR and mixed-type CYP inducer (CYP1A1 and CYP2B1) while PBDEs mixture ("Mjosa mix") is an inducer of ERβ and CYP2B [44].

3.2. Pathology of the Ovary

3.2.1. Early Puberty. Early puberty is a recent growing concern as there are reports of many girls reaching their first menstruation and developing breasts earlier in life than was the case 40 years ago [45]. Early puberty associates with polycystic ovarian syndrome (PCOS), obesity, breast cancer, depression, and a number of social challenges such as experimentation with sex, alcohol, or drugs at a younger age. Moreover, earlier menarche and thelarche ages have been reported in girls after exposure to PCBs, PBBs, DDT, and/or phthalate esters [46, 47], and precocious puberty has been observed following exposure to DDT metabolites [48].

3.2.2. Polycystic Ovarian Syndrome (PCOS). The acyclicity of the syndrome is linked with the hyperfunctioning of theca and hypofunctioning of granulosa cells. PCOS also associates with other processes (e.g., neuroendocrine function and ovarian steroidogenesis) and diseases (e.g., insulin resistance, and obesity) that are regulated by hormonal and metabolic factors. Thus, exposure to environmental toxicants may indeed contribute to the pathogenesis of PCOS. For instance, women with PCOS have higher levels of bisphenol A (BPA, a plastics additive) [49] and testosterone which is consistent with the decreased clearance of BPA that is often observed [50]. Although a cause-and-effect relationship has not been established, the role of environmental toxicants in the pathogenesis of PCOS is worthy of further consideration.

3.2.3. Premature Ovarian Failure (POF). POF, the cessation of normal ovarian function before the age of 40, occurs in approximately 1% of women of reproductive age [51]. The underlying causes of POF are largely known in most cases, and any factor that can decrease the ovarian reserve can result in POF. For instance, disruption of germ cell migration from the genital ridge to the developing gonad results in ovarian dysgenesis and POF. In addition, adult and *in utero* exposure of mice to BPA resulted in oocyte damage [52, 53], whereas exposure of women to cigarette smoke decreased fertility, *in vitro* fertilization (IVF) success rates, and the ovarian reserve resulted in earlier menopause and increased miscarriage rate [54]. In another study, exposure of rats to TCDD *in utero* and throughout reproductive life resulted in premature reproductive senescence [55]. Endocrine disruption caused by acute exposure to environmental toxicants such as the

AhR agonist TCDD suggests that AhR-mediated apoptosis of oocytes may be involved.

4. Hormone-Dependent Cancer

4.1. Breast Cancer. Breast cancer is the most frequent neoplasm affecting women residing in Western countries and is the second leading cause of death [56]. The general population is exposed to several hormonally active compounds on a daily basis. The majority of these compounds are xenoestrogens (e.g., PCAHs, pesticides, PCBs, PBDEs, DDT, selected drugs, fungicides, phytoestrogens, mycotoxins, BPA, and phthalates), and they can possess estrogenic action, affect estrogen levels, and/or bind to ERs [57].

4.1.1. Recent Findings on PCBs. The role of PCBs in breast cancer has been investigated intensively. Data suggest that a correlation may exist between high levels of PCBs in mammary tissues or sera and breast cancer risk [58], while another study reported no association [59]. High PCB levels upregulated CYP expression [60], suggesting that PCB metabolites may be critical for the pathogenesis of breast cancer. Likewise, Pang and colleagues [61] reported that exposure of MCF-7 human breast cancer cells to PCBs 81, 126, and 39 increased CYP1A1 and CYP1B1 mRNA levels, resulting in the formation of E2 metabolites. We demonstrated PCB 3 to induce and to be a substrate for CYP1A1in MCF-7 cells [62]. On the other hand, others have shown PCBs 52 and 77 to induce oxidative damage (i.e., DNA strand breaks) in ERα (−)/MDA breast cancer cells but not in ERα (+)/MCF-7 cells [63], suggesting that the ERα receptor may play a protective role in breast cancer. Moreover, PCBs can interfere with the balance between proliferation and apoptosis. Radice et al. [64] demonstrated those PCB congeners such as PCBs 101, 118, 138, 153, and 180 increased proliferations of MCF-7 cells. Our published data demonstrated that from investigated congeners (118, 138, 153, and 180), PCB138 and 153 had the highest stimulatory effects on basal MCF-7 cell proliferation as well as the highest inhibitory actions on basal caspase-9 activity. Moreover, we showed that PCBs 138 and 153 contribute to the action of endogenous 17β-estradiol on cell proliferation and apoptosis in the breast cancer cell line MCF-7 [65]. *In vivo* experiments have also shown PCBs to increase metastasis by triggering the production of reactive oxygen species (ROS), thereby activating the Rho-associated protein kinase (ROCK) signaling pathway [66] or vascular endothelial growth factor (VEGF) overexpression that stimulates endothelial hyperpermeability and transendothelial migration of cancer cells [67].

4.1.2. Recent Findings on PBDEs. Data on the effects of PBDEs on breast cancer are scant. PBDEs trigger micronucleus formation and proliferation in MCF-7 cells [68]. An increase in MCF-7 cell proliferation by DE-71 (a mixture of BDE-47, -99, -100, -153, and -154) was also reported by Mercado-Feliciano and Bigsby [69]. PBDE-209 also induced MCF-7 cell proliferation by affecting critical steps of the cell cycle [70]. At the molecular level, PBDE-209 triggered protein kinase C α (PKCα) and ERK1/2 phosphorylation. On the other hand, Kwiecińska et al. [71] have shown no changes in MCF-7 cell proliferation following exposure to BDE-47, -99, -100, and -209; however, apoptosis was inhibited by decreasing caspase-9 activity in these cells [71]. Resistance to apoptosis associates with tumorigenesis as it enables tumorigenic cells to expand even in a stressful environment. Thus, additional studies are needed to determine if exposure to PBDEs can indeed cause breast cancer.

4.1.3. Recent Findings on Bisphenol A (BPA). Laboratory studies in rodents suggest a link between BPA exposure and breast cancer incidence. When rodents were exposed to BPA early in life, there were changes in mammary gland morphogenesis and tumor susceptibility [72–74]. These findings are supported by *in vitro* data which demonstrated BPA to induce transformation of MCF-10F human breast cancer cells. These cells formed tubule-like structures when cultured in 3D collagen matrix, but spherical masses were noted after BPA treatment [75], leading the authors to conclude that BPA produces adducts or ROS which can inadvertently introduce a variety of DNA modifications and cause breast cancer. Others have shown BPA to stimulate proliferation but to inhibit apoptosis in MCF-7 cells [76–78]. BPA may also induce breast cancer cell proliferation by upregulating cell cycle genes and downregulating antiproliferative genes, especially genes that control the G1/S transition via ERα signaling [79].

4.2. Ovarian Cancer. Ovarian cancer is the most prevalent type of gynecological cancer affecting women residing in Western countries. As more than 60% of tumors are diagnosed at stage III and certain forms of cancer are very aggressive, ovarian cancers are associated with a high mortality. While most cells undergo neoplastic transformation, including germ cells, granulose, and stromal cells, approximately 90% of tumors are derived from the ovarian surface epithelium (OSE). Similar to breast cancer, hormonal factors such as estrogen and xenoestrogens have been linked to ovarian cancer [80, 81]; however, the role of environmental toxicants in ovarian cancer requires further study.

4.2.1. Recent Findings on PCBs and PBDEs. The role of PCBs in the initiation and progression of ovarian cancer is unknown. Studies in adult C57BL mice showed that orally administered PBDEs-47, -85, and -99 to accumulate in the liver, adrenal cortex, and ovary in adult C57BL mice [82] suggest a possible carcinogenic activity, especially in light of research showing that exposure of CHO and OVCAR-3 cells to PBDE-209 initiated S and G2/M phases of the cell cycle, respectively [83].

4.2.2. Recent Findings on BPA. Several *in vitro* studies have shown BPA to induce chromosomal aberrations in CHO cells [84, 85], a common genetic alteration in cancer. Moreover, ovarian cyst formation was observed in mice treated neonatally with BPA [86]. Ovarian cancer may stem from incessant ovulation, which may be linked to the formation of cysts that are frequently found in perimenopausal women. In a study from our laboratory, we demonstrated an increase in proliferation in OVCAR- 3 cells treated with BPA. Specifically, we showed BPA to promote the cell cycle by upregulating

the expression of cyclin D1, CDK4, E2F1, E2F3, and PCNA (a mediator of G1 to S-phase progression) and cyclin A (a mediator of G2-phase progression to mitosis), but by down-regulating the expression of p21WAF1/CIP1, Weel-1, and GADD45α [87]. Additionally, we demonstrated a decrease in the expression of proapoptotic genes (i.e., FAS, FADD, RAIDD, caspase-8, -10, -3, -6, and 7, CAD, Bax, Bak, Bok, and Apaf-1) but an increase in the expression of prosurvival genes (i.e., Bcl-x and Mcl-1). BPA also activates a caspases-independent apoptotic pathway by inducing endonuclease G gene expression. Also, Hwang et al. [88] using microarray analysis increased mRNA levels of E2-responsive genes in ER-positive ovarian cancer BG-1 cells under the influence of BPA. In a subsequent study, we showed BPA to trigger phosphorylation of Stat3, ERK1/2, and Akt in OVCAR-3 cells [89]. Cited results of research indicated that BPA acting as a mitogen as well as an antiapoptotic factor may be an additional factor responsible for ovarian cancer.

5. Conclusions

In recent years there has been a growing evidence that exposure to chemicals in the environment poses a serious threat to human and animals reproduction via disrupting effects on endocrine function. Despite the fact that these substances are persistent, they may be metabolized into more toxic compounds than the parent molecule in endocrine organs. This endocrine disrupting chemicals (EDCs) adversely affect health and reproduction even at very low concentrations and may exert their effects on the embryo and fetus. The complexity and diversity of factors belonging to EDCs, its direct action on the ovary, and disorders of the reproductive function of women indicate that the impact of environmental pollution as an important determinant factor in fertility should not be minimize. Current estimates of cancer risk in humans do not account properly for transplacental and environmental (including occupational) exposure to xenoestrogens. It is important to reevaluate the role of xenoestrogens in cancer development using new approaches that better reflect the complexity of carcinogenesis. Testing new compounds before they are allowed to use should be expanded to determine their effect on the endocrine system, in order to assess the hormonal activity. In addition, attention should be directed towards dose-response relationships in environmental toxicology. Such studies can provide useful information that might have a significant impact on the strategies for risk assessment of toxic substances.

Acknowledgment

This research is supported by K/ZDS/002592 Jagiellonian University in Cracow, Poland.

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 862739, 8 pages
http://dx.doi.org/10.1155/2013/862739

Review Article

Speciation, Phenotypic Variation and Plasticity: What Can Endocrine Disruptors Tell Us?

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Received 21 February 2013; Accepted 28 March 2013

Academic Editor: Ewa Gregoraszczuk

Phenotype variability, phenotypic plasticity, and the inheritance of phenotypic traits constitute the fundamental ground of processes such as individuation, individual and species adaptation and ultimately speciation. Even though traditional evolutionary thinking relies on genetic mutations as the main source of intra- and interspecies phenotypic variability, recent studies suggest that the epigenetic modulation of gene transcription and translation, epigenetic memory, and epigenetic inheritance are by far the most frequent reliable sources of transgenerational variability among viable individuals within and across organismal species. Therefore, individuation and speciation should be considered as nonmutational epigenetic phenomena.

1. Introduction

Phenotypic variability among individuals within different organismal species is essential for them to prosper. Indeed, by expanding each species phenotypic repertoire, the possibility of organism populations to overcome environmental contingencies increases. Phenotypic variability is not only important at the species level to improve the chances of assuring their continuity over time; it is also at the heart of the emergence of new species during the process of evolution. For decades, evolutionary thought has claimed that individual or group phenotypic variation and speciation primarily arise from genetic mutations and gene allelic polymorphisms (i.e., genetic drift) combined with natural selection, geographic and sexual isolation, and the interruption of gene flow between parental and emerging species. Even though this view is still going strong and favored by traditional evolutionary biologists, recent discoveries support that phenotypic variability also results from shifts of gene expression controlled by epigenetic mechanisms during ontogenesis and during adult gametogenesis. Clearly, this new information makes it possible to conceive within and across species phenotype variation and speciation as epigenetic phenomena, having no need to look for mutations as the main source of variability or to make natural selection, geographic and sexual isolation and gene flow discontinuities the forces leading to speciation (also see [1]). Variation is never made by natural selection. Natural selection acts only after the phenotypic repertoire for each species unfolds generation after generation. In the epigenetic context, phenotypic variability is an intrinsic property of individuals and arises from decisions made by the developing organism after processing and integrating the information extracted from the environment, the genome, and the metabolic state. Where and how cell decisions are made is yet unknown, but once taken they are likely to deviate ontogenetic trajectories enough to promote either the emergence of new phenotypic traits or even new species. Hence, unraveling the mechanisms underlying the epigenetic modulation of gene expression becomes central in

order to understand phenotypic variation within and among species, as well as the evolutionary process of life.

As briefly mentioned before, individual phenotypic variation is commonly "constructed" during embryonic and fetal life. It is during early ontogenetic stages when somatic cells may redirect their ontogenetic trajectories in response to epigenetic information. Commonly, this circumstance gives rise to unique, variable individuals that preserve or not, in different degrees, phenotypic features specific to the species. Hence, one of the mechanisms leading to contingent phenotypic variation is ontogenetic phenotypic plasticity of somatic cells (also called developmental phenotypic plasticity) [2].

Other critical processes that lead to ontogenetic phenotypic variability involve the epigenetic reprogramming of the genome of precursor cells that originate oocytes and spermatozoa. It is known that the first reprogramming event occurs in the gonocyte's genome (i.e., gamete primordial precursor) while colonizing the embryo's urogenital crest [3–5]. We believe this event imprints an epigenetic memory on the gonocyte's genome that "depicts" the environmental circumstances under which these cells were committed to the gamete lineage. Surely these early epigenetic memories not only influence future gamete differentiation, but the development and maturation of the organism as a whole after fertilization. A second episode of gamete epigenetic reprogramming takes place during the process of differentiation that gives rise to spermatozoa in sexually mature males. We think that by constantly reediting epigenetic memories in spermatogonial populations, this process allows spermatozoa to inherit an updated epigenome that fits current environmental circumstances. This would permit spermatozoa of different generations to provide fresh information about the environment during consecutive episodes of fertilization and to inherit this information to the offspring. Finally, a last event of epigenetic reprogramming occurs soon after fertilization. From our point of view, by mixing prenatal (mainly provided by the oocyte) and postnatal (principally provided by the spermatozoa) memories and reediting them again, based on actual environmental conditions, the zygote has a chance to create an updated epigenetic/genetic framework based on which somatic cells will take decisions to adjust the ontogenetic trajectories during prenatal and postnatal life. Hence, studying at different ages the details of the cellular and molecular underpinnings underlying epigenetic phenotypic plasticity, whether somatic or gametic, is mandatory to fully understand individuation and speciation. In doing so, the establishment of experimental models through which such details may be reasonably explored becomes critical to the field. Here is where endocrine disruptors (EDs) enter into the scene.

EDs are a broad class of chemicals that, after modifying early or late development and maturation, promote the expression of alternative phenotypes in the exposed organisms or in their offspring. In some cases, such phenotypes are incompatible with life. In many others, however, EDs-exposed organisms display alternative adult phenotypes with variable reproductive fitness and disease susceptibility [6–8]. Even though EDs may induce mutations [9, 10], a great deal of their effects on the phenotype result from their ability to interfere with endocrine communication and/or through directly inducing epigenetic changes [9–13]. Thus, designing experiments involving the prenatal and postnatal exposure to EDs may help us understand the epigenetic bases of phenotypic variability and plasticity between individuals, across species, and throughout evolution.

In this text, we revise current knowledge about the epigenetic mechanisms that underlie the effects of EDs on phenotypic variability and plasticity. Because previous reviews have already deeply discussed EDs' epigenetic and transgenerational effects on human biology and disease, here we intend to stress the value of using the information derived from experiments with EDs to unveil the mechanisms that underlie phenotypic variability and speciation through epigenetic phenotypic plasticity.

2. Endocrine Disruptors: Their Chemical Nature

EDs constitute a heterogeneous group of natural and synthetic chemicals that mimic, block, or disrupt the synthesis, transport, or elimination of natural chemical messengers such as classic hormones, cytokines, and neurotransmitters [14–17]. When their active forms are released to the environment, they are absorbed by organisms through epithelial linings. Based on their physiological actions, EDs may be classified as antiandrogenic, androgenic, estrogenic, arylhydrocarbon receptor agonists, inhibitors of steroid hormone synthesis, antithyroid substances, and retinoid acid agonists [9]. Chemically, pesticides (DDT, demeton-S-methyl, dimethoate, permethrin, diazinon, and chlorfenvinphos), fungicides (vinclozolin, maneb, and metam sodium), herbicides (atrazine, simazine, linuron, diuron, and 2,4-D), industrial products (pentachlorophenol, polychlorinated biphenyls, phthalate plasticisers, alkylphenol ethoxylates, and bisphenol A), pharmaceuticals (diethylstilbestrol), nutriceuticals, and synthetic hormones used for elaborating contraceptive pills or for designing hormone replacement therapeutic schemes are among the most important EDs so far described. Also, plant and animal derived natural hormones such as phytoestrogens, 17β-oestradiol and testosterone may disrupt the endocrine milieu of organisms exposed to them in nature. In addition to the natural and synthetic compounds mentioned previously, it has been demonstrated that chronic hypoxia associated to organic pollution and eutrophication also exert disrupting effects on the endocrine system [18, 19].

3. Endocrine Disruptors and Phenotypic Plasticity

A number of studies conducted both in wild and in laboratory settings have convincingly shown that the prenatal exposure to EDs induces early and late onset phenotypic plasticity. For instance, prenatal exposure to the synthetic estrogen diethylstilbestrol increases the short-term risk of acquiring testicular abnormalities in men [20] and the long-term risk of developing cervical and vaginal cancer in adult women, reviewed by Rubin, [21]. Phenotypic plasticity associated with EDs exposure is not restricted, nonetheless, to prenatal

Table 1: Enzymes involved in chromatin epigenetic tagging/untagging [2–4].

Epigenetic modification (tagging/untagging)	Enzymes
DNA modifications:	*DNA modifying enzymes*
Methylation	DNA methyltransferases (DNMTs)
Histone modifications:	*Histone modifying enzymes*
Acetylation of specific lysine residues/Deacetylation	Histone acetyltransferases (HATs)/Histone deacetylases (HDACs)
Methylation of specific lysine or arginine residues/Demethylation	Histone methyltransferases (HKMTs and HRMTs)/Lysine-specific demethylase (LSD1); arginine-deiminases
Phosphorylation of serine or threonine groups/dephosphorylation	Histone Kinases (HKs)/Phosphatases (PPTases)
Ubiquitinylation/removal of ubiquitin	Ubiquitinases/"deubiquitinases" or ubiquitin hydrolases (Ubps)
Sumoylation	SUMO E3 ligase
Poly(ADP-ribosyl)ation/removal of Poly(ADP-ribose) units	PAR polymerases/poly(ADP-ribose)glycohydrolases (PARGs)

developmental stages. Indeed, adult women exposed to bis4-chlorophenyl-1,1,1-trichloroethane or bis4-chlorophenyl-1,1-dichloroethene reduce or increase their fertility and develop longer or shorter than normal pregnancies, respectively [22]. Similarly, numerous cases of infertility have been reported among adult men exposed to 1,2-dibromo-3-cloropropane while working for a pesticide factory. Azoos- and oligospermia as well as increased levels of follicle-stimulating and luteinizing hormones were common findings among these men [23]. In addition, vertebrates different from humans are also affected by EDs exposure. Indeed, pregnant rats exposed to vinclozolin (an antiandrogenic compound) or methoxychlor (an estrogenic compound) during the last stages of embryonic development give rise to offspring with decreased spermatogenic capacity (cell number and viability) and increased incidence of male infertility [24]. *Xenopus laevis* larvae exposed to atrazine, a commonly used herbicide, display hermaphroditism, demasculinization, and reduced testosterone plasmatic levels at adult age [25, 26]. Finally, fish exposed to oxidative stress show impaired migration of primordial germ cells [18]. Even though significant anatomical and functional differences are observed in the reproductive system of EDs-exposed organisms when compared to nonexposed ones, the emergence of alternative phenotypes is not restricted to the reproductive sphere. EDs exposure redirects the trajectory of embryonic morphogenesis and modifies also thyroid gland, immune, and neural functions during postnatal life [14, 25–29].

At this point, a consideration of the biological meaning of EDs-induced alternative phenotypes is worth doing. Although these phenotypes might be considered as "abnormalities", from an ecological and evolutionary perspective, reducing fertility, debilitating the immune response, increasing disease susceptibility, or modifying to the organism's behavior is, however, advantageous to the species by decreasing the fitness of individuals exposed to EDs at any age. It would not make sense, for example, to permit the reproduction of exposed organisms, given their greater possibility to sire offspring that will circumstantially display maladaptive phenotypes. This is particularly significant under the light of the evidence showing that genetic expression of germ cells may be primed permanently and trans-generationally by epigenetic information during periods in which these cells undergo epigenetic programming [9, 24, 30–32]. Furthermore, recent discoveries have shown that adults exposed to EDs prenatally are less attractive to nonexposed mates [33]. Hence, at worst, these modified phenotypes must be considered as circumstantially maladaptive but never abnormal. EDs-exposed organisms might then choose from their ontogenetic alternatives the traits that better cope with EDs exposure. Therefore, the emergence of epigenetically generated seemingly maladaptive, alternative phenotypes may be a fundamental process that allows natural selection "to pick the fittest organism" at the population level under specific circumstances.

4. EDs Induce Phenotypic Variability through Epigenesis

As mentioned before, EDs may act as hormonal agonists or antagonists or modify the synthesis, transport, or elimination of hormones. Hence, by changing hormone functional availability, EDs promote the expression of alternative phenotypes in developing and adult organisms. Since many of them do not induce mutations, their actions are likely translated through epigenetic mechanisms. But what does epigenesis mean? Epigenesis may be conceived as a series of cellular and molecular processes that "print out" (or encode) on to the genetic library the information extracted from the environment. This environmentally driven code restricts or facilitates the cell's access to distinct shelves of its genetic library, thus guiding the cell's search for genetic information. Once the best genetic files from the available repertoire are picked, the cell makes decisions on what ontogenetic trajectories are necessary to construct to provide a proper phenotypic response. Such processes do not involve mutations of DNA. Epigenetic information coding takes place in the genome by differentially tagging or untagging histones with acetyl, methyl, phosphoryl, ubiquitin, sumo and ADP-rybosil groups at particular amino-acid residues or the DNA with methyl groups at specific cytosine-guanine dinucleotide locations. The process of epigenetic tagging or untagging is catalyzed by enzymes (Table 1) whose activity may be modulated by different signaling cascades following the activation of receptors by their specific ligands (reviewed by Arzate-Mejía et al., [34]). The transcription of genes coding for "chromatin remodeling" enzymes may be also regulated by environmental factors [35, 36].

Chromatin epigenetic tags are either transient/removable or permanent/likely heritable. Commonly, transient/removable epigenetic tags allow the organism's cells to make moment-to-moment adjustments of their gene expression state, their metabolic status, and hence of their phenotype. Permanent epigenetic tags, in contrast, give rise to an epigenetic memory that, once posted, primes and channels each cell's adjustable genetic and metabolic responses for the rest of the organism's life. Interestingly, when permanent chromatin epigenetic tags occur in gametes, stem cells and/or amplifying precursor cells, they are inherited by their progeny both at the cellular and at the organismal level. Hence permanent epigenetic tags [37, 38], and thus past and relatively present environmental conditions, are transgenerationally heritable. Thus, the phenotype expressed by a given animal at a particular time point of life and the lifespan plasticity that such phenotype might display in response to prevailing, but changing, environmental conditions are facilitated by a highly dynamic process of epigenetic tagging channeled by the epigenetic memory.

But how can the shifts of epigenetic tags prime and channel gene expression and metabolism in a constant and permanent manner? The trick in part lies in the stereochemistry of chromatin, whose three-dimensional structure is modified by addition and/or removal of functional chemical groups to histones and/or DNA. Chromatin relaxation or compaction lead, respectively, to the differential formation of transcriptomically active or inactive gene expression domains along the chromosomes. Also, chromatin tagging/untagging (i.e., remodeling) adjust chromosomes' nuclear topology, a circumstance that modifies gene expression by changing chromosome-chromosome spatial interactions and the spatial relationship of genes with the transcriptional factories in the cell nucleus.

Other modes of modifying gene transcription and translation through epigenetic processes have been recently uncovered. Indeed, the insertion of histone variants, the coupling of ATP-dependent remodeling complexes and/or noncoding RNAs [39–41] also lead to chromatin remodeling. Nuclear transcription and protein synthesis may also be modified by shifting the availability of nuclear and cytoplasmic small, noncoding RNAs. Finally, genome transposable elements (e.g., transposons or retrotransposons) are now known to be regulated through epigenetic mechanisms that involve DNA methylation, interference RNAs, and hence chromatin condensation [42].

Based on the information commented, we believe EDs might use several, if not all, of the epigenetic mechanisms described to induce phenotypic plasticity. This is supported by data showing that diethylstilbestrol decreases methylation of protooncogenes and lactoferrin in mouse reproductive tissues by reducing the activity of the DNA methyl transferase-1, a condition that decreases CpG methylation [43–45]. Also, mice treated with bisphenol A either pre- or neonatally show greater body mass, modified reproductive function, increased cancer risk, and reduced DNA methylation [38, 46–48]. Similar observations have been reported in mice exposed to genistein (an estrogen-like polyphenol) [48–50], vinclozolin (a fungicide) [51], or methoxychlor (a pesticide) [24]. In fact, in the last case, alternative phenotypes may be expressed by individuals belonging to subsequent generations [24]. In rats, developmental exposure to exogenous estradiol and bisphenol A also produces permanent changes in DNA methylation levels of multiple cell signaling genes important for proper prostate development and function [47]. Another endocrine disrupting compound, the insecticide methoxychlor, was found to modify DNA methylation patterns of the rat germ cell line when administered during development. It also decreases sperm number and viability and causes infertility across generations [24]. In male rats, vinclozoline modifies both the testis transcriptome and epigenome transgenerationally through modifying DNA methylation during development [7, 30, 51]. Vinclozoline also shifts sperm methylation levels of at least six known imprinted genes throughout three generations [52]. Using a reporter gene H19, it was recently found that the pesticide chlorpyrifos affects DNA methylation patterns in male mice primordial germ and liver cells [53]. A very recent study in mice has revealed that gestational exposure with the dioxin 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin shifts interference RNA availability and DNA methylation patterns in the offspring of exposed females [54]. More recent studies have shown that the exposure of steroidogenic tissues to gonadotropins in male and female mice induces the expression of VL30 retrotransposons [55]. Also, benzo(a)pyrene exposure induces the trimethylation of the lysine 4 residue and the acetylation of the lysine 9 residue of histone 3 leading to the downregulation of the expression of the DNA methyltransferase 1 locus and the upregulation of the LINE-1 retrotransposon site [56]. Finally, pregnant mice exposed to bisphenol A show hypomethylation of an intracisternal A-particle retrotransposon located upstream of the Agouti gene. This effect was counteracted by supplementing maternal diets with methyl donors [38]. These last results support that EDs may also exert their action on phenotypic plasticity by promoting mobilizations of these elements. Transposons and retrotransposons are replicative DNA sequences that can move across chromosomes [57, 58]. The transposition of these elements among chomosomes is achieved after having them cleaved, transcribed, or retrotranscribed. Transcription, retrotranscription, cleavage, and transposition are all mediated by distinct families of enzymes and a host of interference RNAs that work in an orchestrated fashion [58, 59].

5. EDs as Tools to Explore the Evolution of Life

Based on what we have written so far, we hope that the reader concurs with the idea that the variations of the phenotype within and across species achieved through epigenetic phenotypic plasticity might be a driving force of phenotypic variability and perhaps of speciation. Although many may argue against the value of using EDs exposure to understand speciation since they promote the emergence of seemingly maladaptive phenotypes with reduced fitness, we must remember that events of speciation (e.g., adaptive radiation) may occur following massive extinctions induced by climatic catastrophes [60]. Such circumstances surely expose

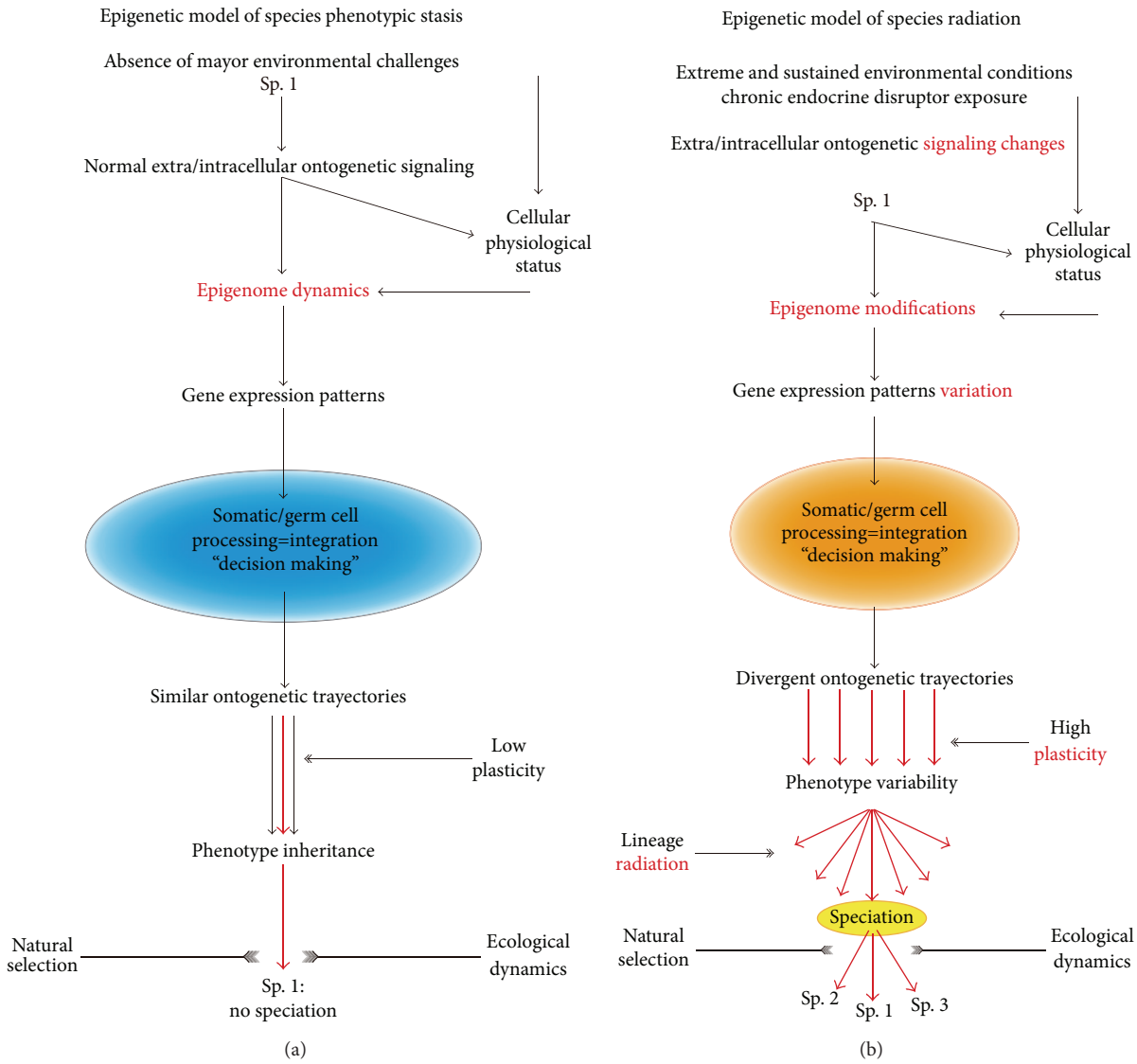

FIGURE 1: Diagrams that illustrate the hypothetical models by which the phenotype of a given animal species may be preserved under normal environmental conditions (a) or diversified under extreme environmental conditions (b). Phenotypic stasis (a) or speciation (b), respectively, are the end results of these models. Sp., Species.

the surviving organisms to extreme environmental conditions that likely force them to develop extreme phenotypic plasticity to thrive over time. EDs exposed organisms might display phenotypes that resemble those shown by organisms exposed to extreme climatic challenges [61]. It is known that highly stressful conditions impair somewhat reproductive fitness and may lead to phenotypic "abnormalities." However, these "abnormalities" might be the raw substrate upon which extreme phenotypic plasticity may be built up giving rise to new species traits. In this scenario, EDs could help us understand how epigenomes are configured under such extreme circumstances and how they influence the decisions taken by developing organisms to select their ontogenetic trajectories. In this process, the mobility and overexpression of transposable elements induced by EDs exposure may be particularly important since they could modify developmental processes as important as body patterning [62], see also [63]. Because such actions result from the interpretation of the epigenomic code by the somatic and germ cells of the developing system, such changes may be transgenerationally inherited. How far these changes may last is unclear, but given the dynamic nature of epigenomes, they may be perpetuated or reedited based on the environmental conditions as they evolve. Hence, EDs could generate a phenotypic variation by reconfiguring the epigenome that could lead to divergent phenotypes based upon changes of gene expression patterns. In addition, EDs could also enhance phenotypic plasticity by promoting novel DNA recombination events after increasing the mobility of transposable elements through epigenetic modulation. We believe that both processes might lead in the long run to *epigenetic species radiation* without point mutations (Figure 1). Our arguments fully concord with the philosophical framework posed by the emerging field of environmental epigenetics [1].

Authors' Contribution

Marta López-Santibáñez Guevara and Lluvia I. Marcos-Camacho contributed equally to the present work.

Acknowledgments

Authors thank Ivonne Mora, Jesús Ramirez Santos, Raymundo Reyes, and Edel Pineda López for the technical and administrative assistance. This work was partly financed by CONACyT (Grant no. 94312 to GGO, No. 82879 to GGO and 180762 to EMH), CIC-UMSNH (Grant no. 8.37), and PAPIIT-UNAM (Grant no. IN203912).

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 134589, 11 pages
http://dx.doi.org/10.1155/2013/134589

Research Article

Photoperiod-Dependent Effects of 4-*tert*-Octylphenol on Adherens and Gap Junction Proteins in Bank Vole Seminiferous Tubules

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Received 10 December 2012; Revised 2 April 2013; Accepted 5 April 2013

Academic Editor: Radmila Kovacevic

In the present study we evaluated *in vivo* and *in vitro* effects of 4-*tert*-octylphenol (OP) on the expression and distribution of adherens and gap junction proteins, N-cadherin, β-catenin, and connexin 43 (Cx43), in testes of seasonally breeding rodents, bank voles. We found that in bank vole testes expression and distribution of N-cadherin, β-catenin, and Cx43 were photoperiod dependent. Long-term treatment with OP (200 mg/kg b.w.) resulted in the reduction of junction proteins expressions ($P < 0.05$, $P < 0.01$) and their delocalization in the testes of males kept in long photoperiod, whereas in short-day animals slight increase of Cx43 ($P < 0.05$), N-cadherin, and β-catenin (statistically nonsignificant) levels was observed. Effects of OP appeared to be independent of FSH and were maintained during *in vitro* organ culture, indicating that OP acts directly on adherens and gap junction proteins in the testes. An experiment performed using an antiestrogen ICI 182,780 demonstrated that the biological effects of OP on β-catenin and Cx43 involve an estrogen receptor-mediated response. Taken together, in bank vole organization of adherens and gap junctions and their susceptibility to OP are related to the length of photoperiod. Alterations in cadherin/catenin and Cx43-based junction may partially result from activation of estrogen receptor α and/or β signaling pathway.

1. Introduction

Alkylphenols (e.g., 4-*tert*-octylphenol, OP; 4-*tert*-nonylphenol, NP) and alkylphenol ethoxylates are a group of endocrine-disrupting chemicals that accumulate at high concentrations in air, soil and aquatic environment from the use of detergents, paints, pesticides, and plastic manufacturing [1]. They are also found in the fluids and body fat of animals and humans [2]. These compounds are classified as xenoestrogens, since they are able to induce inappropriate estrogenic action or interfere with the actions of endogenous estrogens affecting reproductive development and functions of laboratory and wild animals, and most likely humans [3]. Alkylphenols bind to the nuclear estrogen receptors (ERs), but their binding affinity and ability to activate ER-mediated functions is 100–10,000 times less potent than that of 17β-estradiol [4]. However, they exhibit quite robust nongenomic activity [5]. Since 2000, European Union restricted use of alkylphenols as priority hazardous substances by Directive 2000/60/EC [6]. Nevertheless, they are still detected world widely in the environment and organisms [7]. Recent analyses indicate that although alkylphenol ethoxylates levels have been decreasing in the last years, Europe is much more contaminated than North America and developing countries [1].

Spermatogenesis, a process by which spermatogonia undergo a series of divisions and differentiation to become spermatozoa, is tightly controlled by Sertoli cells. Direct interactions between Sertoli cells and between Sertoli and germ cells are mediated by proteins that form specialized cell junctions. N-cadherin and β-catenin are constituent proteins of testicular adherens junctions. They form complexes localized between Sertoli cells at the site of blood-testis barrier (BTB) and between Sertoli cells and elongated spermatids, in the apical ectoplasmic specializations. Cadherin/catenin complexes play a determining role in stabilizing cell-cell

contacts and their restructuring during movement of preleptotene spermatocytes across the BTB. It was reported that the altered expression or loss of the protein-protein interactions between N-cadherin and β-catenin induces germ cell detachment from the seminiferous epithelium [8, 9]. Connexin 43 (Cx43), the predominant gap junction protein in seminiferous epithelium, is of absolute requirement for normal testicular development and spermatogenesis [10]. In adult testis Cx43 is a component of the junctional complex enabling direct communication and exchange of small molecules between adjacent Sertoli cells, Sertoli and germ cells, and between Leydig cells. Recent studies provided clear evidence that Cx43 can also control spermatogenesis through regulation of tight and anchoring junctions that are closely intermingled with each other at the site of the BTB [11].

Recent papers have reported that xenoestrogens can affect spermatogenesis by perturbing direct cell-cell interactions in the seminiferous epithelium. Extensively studied xenoestrogen bisphenol A was demonstrated to cause a dose-dependent reduction in several junction proteins (including N-cadherin, β-catenin, and Cx43) in Sertoli cells *in vitro* and to disrupt the BTB when administered to immature rats [12, 13]. The estrogenic actions of alkylphenols on the testis are less studied when compared with bisphenol A. Although it was demonstrated that exposure to OP or NP induces changes in multiple gene transcription, cell proliferation, and hormone production *in vitro* and *in vivo*, scarce data are available on their influence on junction proteins in the testes [14–16].

In our previous study bank voles, seasonally breeding rodents, were used to investigate the effects of OP on male reproductive organs, depending on the length of exposure and reproductive status of animals [17]. We found that long-term exposure of adult males affected expressions of 3β-hydroxysteroid dehydrogenase, aromatase, estrogen receptor α, and androgen receptor as well as sex steroids levels in the testes and seminal vesicles. Concomitantly, increased apoptosis and, occasionally, germ cell sloughing were found. Interestingly, a subtle difference in the sensitivity to OP between voles kept in different light conditions was noted [17]. To better understand the mechanism of OP-induced alterations in the testis, the present study was aimed to examine changes in the distribution and expression of cell junction proteins, N-cadherin, β-catenin, and connexin 43, in bank vole following OP exposure *in vivo*. Importantly, to our knowledge this is the first *in vivo* study on the effect of OP on adherens and gap junction proteins in the testes. In addition, to evaluate potential direct effects of OP on junction protein expressions in seminiferous epithelium and to examine whether these effects are mediated through binding OP to ERs, organ culture model was used.

2. Material and Methods

2.1. Animals. Bank voles (*Clethrionomys glareolus*, Schreber) were obtained from our own colony (Department of Endocrinology, Institute of Zoology, Jagiellonian University, Krakow, Poland) which have been reared under long light cycles (18 h light and 6 h darkness; 18L : 6D) or short light cycles (6 h light and 18 h darkness; 6L : 18D). The animal rooms were maintained at a temperature of 18°C and a relative humidity of 55 ± 5%. Voles were housed in polyethylene cages ($42 \times 27 \times 18$ cm^3) furnished with sawdust and wood shavings for bedding. A standard pelleted diet (LSM diet, Agropol, Motycz, Poland; total isoflavone content below 450 mg/kg diet) supplemented with seeds of wheat or oat, red beet, apples, and water was provided *ad libitum*.

2.2. Exposure In Vivo. Twenty-four mature male bank voles (60–70-day-old) kept in long light cycles (18L : 6D, $n = 12$) and short light cycles (6L : 18D, $n = 12$) were dosed orally with 4-*tert*-octylphenol (OP, 200 mg/kg body weight; Sigma-Aldrich, St Louis, MO, USA) every Monday, Wednesday, and Friday for 30 (OP30) or 60 days (OP60). A total of four experimental groups were formed ($n = 6$/each group). 4-*tert*-octylphenol was dissolved in a minimum amount of absolute ethanol and then a measured amount of sesame oil (Sigma-Aldrich, St Louis, MO, USA) was added in 1 : 14 (v/v), as previously described in detail [17]. A dose of exposure to OP was based on the literature [15, 18] and it was finally selected upon our preliminary study in which lower doses (50 and 100 mg/kg body weight) had no effects on bank vole reproductive organs weight and morphology.

The control animals ($n = 24$) for each experimental group ($n = 6$/each group) were given a vehicle only (ethanol + sesame oil). After the animals were sacrificed by cervical dislocation, testes were immediately excised and serum was collected and frozen in −20°C.

2.3. Ethics of Experimentation. Experiments were performed in accordance with Polish legal requirements, under the license given by the Local Ethics Committee at the Jagiellonian University, Krakow, Poland (No. 88/IV/2010).

2.4. Tissue Preparation. For immunohistochemical analysis one testis from each animal was immersed immediately in 4% formaldehyde and embedded in paraplast (Sigma-Aldrich, St Louis, MO, USA). Sections of 5 μm in thickness were mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St Louis, MO, USA), deparaffinized, and rehydrated through decreasing alcoholic solutions. For Western blot and ELISA analysis, contralateral testis from each animal was frozen and stored in −80°C.

2.5. Organ Culture and Exposure In Vitro. Twenty-four untreated male bank voles (60–70-day-old) reared under long light cycles or short light cycles were sacrificed and testes were immediately removed and trimmed free of excess fat and connective tissue. After the capsule had been removed, the testes were cut into small pieces of 2-3 mm in diameter and were placed on Millipore filters (pore size 0.4 μm) (Millipore Corporation, Billerica, MA, USA). The filters were floated on 1.5 mL culture Waymouth's media (Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA) and L-glutamine containing 50 U/mL penicillin, 50 μg/mL streptomycin in tissue culture dishes and incubated at 37°C, in a humidified atmosphere

containing 95% air: 5% CO_2, for 24 h. 4-*tert*-octylphenol and ICI 182,780 (Sigma-Aldrich, St Louis, MO, USA) were dissolved in absolute ethanol and the final concentration of the solvent in culture medium was 0.1% (v/v).

In the first experiment the response to OP was measured by comparing the testes from 18L : 6D and 6L : 18D males cultured in medium containing OP (at doses 10, 100, and 500 mg/L) with the testes cultured in medium containing solvent (control). In the second experiment (to examine the mechanism of OP action) testes were cultured in medium containing OP (at doses 10, 100, and 500 mg/L) or ICI (6 mg/L), or combination of each dose of OP plus 6 mg/L ICI. As a control, testes were exposed to medium containing solvent.

Doses of exposure were based on the literature [19]. Lower doses of OP (0.1, 1 mg/L) and ICI (2 mg/L) were tested in preliminary study. Since they appeared to have no effect on junction proteins, the data were not presented herein. After culture, the media and testes were collected and stored at −20°C and −80°C, respectively.

2.6. Immunohistochemistry. To optimize immunohistochemical staining, slices were immersed for 4 min in 10 mM citrate buffer (pH 6.0) and heated in a microwave oven (600 W). The whole procedure has been described in detail elsewhere [20]. In short, nonspecific staining was blocked twice, first with 3% H_2O_2 in methanol for 15 min, to inhibit endogenous peroxidase activity, and second with 10% normal goat or horse serum (Sigma-Aldrich, St Louis, MO, USA) for 30 min at room temperature to block nonspecific binding sites. Thereafter, sections were incubated overnight at 4°C in a humidified chamber in the presence of primary antibodies: (1) a mouse monoclonal antibody against N-cadherin (1 : 100; Invitrogen, Eugene, OR, USA); (2) a rabbit polyclonal antibody against β-catenin (1 : 300; Invitrogen, Eugene, OR, USA); (3) a rabbit polyclonal antibody against Cx43 (1 : 2,000; Sigma-Aldrich, St Louis, MO, USA). Next, biotinylated secondary antibody, goat anti-rabbit IgG or horse anti-mouse IgG, respectively (1 : 400; Vector Labs., Burlingame, CA, USA) was applied for 1 h. Finally, avidin-biotinylated horseradish peroxidase complex (Vectastain ABC Kit; 1 : 100; Vector Labs., Burlingame, CA, USA) for a further 30 min was used. After each step in these procedures, sections were carefully rinsed with Tris-buffered saline (TBS; 0.05 M Tris-HCl plus 0.15 M NaCl, pH 7.6). Bound antibody was visualized with TBS containing 0.05% 3,3′-diaminobenzidine (Sigma-Aldrich, St Louis, MO, USA) and 0.07% imidazole (Sigma-Aldrich, St Louis, MO, USA) for 3-4 min. The slides were processed immunohistochemically at the same time with the same treatment so that the staining intensities among testicular cells could be compared. Experiments were repeated three times on serial sections per animal. Control sections included omission of the primary antibody. The sections were examined with a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany).

2.7. Western Blot Analysis. Tissues were homogenized on ice with a cold RIPA buffer (Sigma-Aldrich, St Louis, MO, USA), sonicated, and centrifuged at 15,000 g for 20 min at 4°C. The protein concentration for each sample was estimated using Bradford dye-binding procedure with bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) as a standard. Homogenates containing 100 μg of protein were solubilized in a sample buffer (Bio-Rad Labs. GmbH, München, Germany) and boiled for 3 min. After denaturation the samples were subjected to electrophoresis on 10% polyacrylamide gels. Separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA, USA) using a wet blotter in the Genie Transfer Buffer (pH 8.4) for 90 min at a constant voltage of 135 V. Then blots were blocked with 5% nonfat dry milk in TBS, 0.1% Tween 20 for 1 h with shaking, followed by an incubation with appropriate primary antibody (as for immunohistochemistry; anti-N-cadherin, dilution 1 : 250; anti-β-catenin, dilution 1 : 800; anti-Cx43, dilution 1 : 10,000) overnight at 4°C. The membranes were washed and incubated with the horseradish-peroxidase labeled goat anti-rabbit IgG or horse anti-mouse IgG (Vector Labs., Burlingame, CA, USA) at a dilution 1 : 3,000, for 1 h at room temperature. Immunoreactive proteins were detected by chemiluminescence with Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and images were captured with a ChemiDoc XRS+ System (Bio-Rad Labs. GmbH, München, Germany). All immunoblots were stripped with stripping buffer containing 62.5 mM Tris-HCL, 100 mM 2-mercaptoethanol and 2% SDS (wt/vol) (pH 6.7) at 50°C for 30 min, and incubated in a rabbit polyclonal antibody against β-actin (dilution, 1 : 3,000; Sigma-Aldrich) which served as a loading control. Each data point was normalized against its corresponding β-actin data point. Molecular masses were estimated by reference to standard proteins (Fermentas, GmbH, St. Leon-Rot, Germany). To obtain quantitative results, immunoblots were scanned using Image Lab 2.0 (Bio-Rad Labs. GmbH, München, Germany).

2.8. ELISA Assay. Plasma FSH concentrations were measured with commercially available Mouse Follitropin subunit beta ELISA Kit (EIAab, Wuhan, China) according to the manufacturer's instructions. The sensitivity of the assay was 0.01 mIU/mL. The measurements were performed in duplicate.

2.9. Statistical Analysis. All statistical analyses were performed using one-way analysis of variance (ANOVA). Tukey multiple comparison test was used to determine which values differed significantly from controls ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Data were presented as mean ± SD.

3. Results

3.1. In Vivo Experiment. In control males expression and distribution of N-cadherin, β-catenin, and Cx43 were dependent on the length of the photoperiod. In seminiferous tubules of 18L : 6D animals strong, linear staining for N-cadherin and β-catenin was localized at the region of BTB, running parallel to the basement membrane. Additionally,

discrete punctuate staining was observed in the adluminal compartment, mainly at Sertoli cell-elongated spermatid interface (Figures 1(a) and 1(e)). Cx43 was detected predominantly at the base of the tubules, between Sertoli cells and spermatogonia or pachytene spermatocytes as well as in the cytoplasm of some Sertoli cells. A moderate staining was also seen at Sertoli cell-spermatid junctions (Figure 1(i)). The localization and intensity of N-cadherin, β-catenin, and Cx43 immunostaining displayed some evidence of stage-specificity. In the Leydig cells, a very strong linear Cx43 signal on the plasma membrane was found as the prevalent staining pattern (Figure 1(i)).

In tubules of 6L : 18D animals the intensity of N-cadherin and β-catenin staining in the basal region of the epithelium was clearly reduced when compared to 18L : 6D voles; weak, punctuate signal extended through much of the epithelium (Figures 1(c) and 1(g)). Similarly as in 18L : 6D animals, Cx43 was present predominantly in the basal compartment, however its distribution at the region of BTB was irregular and discontinuous. In the adluminal compartment weak staining was frequently dispersed in the cytoplasm of Sertoli or germ cells (Figure 1(k)). Cytoplasmic staining was also detected in Leydig cells of 6L : 18D males (insert in Figure 1(k)).

Exposure to OP for 30 days did not alter the distribution of N-cadherin, β-catenin, and Cx43 neither in 18L : 6D nor 6L : 18D males (not shown). In males treated with OP for 60 days distribution pattern of these proteins was changed in tubules with altered spermatogenesis, as well as in some morphologically normal tubules. N-cadherin and Cx43 were frequently localized in the form of irregular lines or distinct foci between the cells (Figures 1(b) and 1(j)), in the cytoplasm of Sertoli cells (Figures 1(b), 1(d), and 1(j)), or at the entire surfaces of Sertoli and germ cells (Figure 1(l)). β-catenin reactivity remained at the region of BTB, whereas loss of the staining was detected in the apical compartment of seminiferous epithelium (Figures 1(f) and 1(h)). In 18L : 6D group signal intensities of all studied proteins were diminished following OP treatment when compared to the respective controls. In Leydig cells of OP60 males cytoplasmic staining for Cx43 was found (Figure 1(j)-insert, l). No staining was detected when the antibody against N-cadherin, β-catenin, or Cx43 was omitted (inserts in Figures 1(c), 1(g), and 1(l)).

Immunodetectable N-cadherin, β-catenin, and Cx43 proteins were observed as single bands near the 127, 92, and 43 kDa positions of the SDS gel, respectively, in testicular homogenates of the control voles and those treated with OP (Figure 2). In the testes of 18L : 6D males expression levels of N-cadherin and β-catenin were significantly decreased only in OP60 animals when compared to the respective controls (Figures 2(a) and 2(b)), whereas Cx43 was reduced in both OP30 and OP60 groups (Figure 2(c)). In 6L : 18D group an increase in the expression levels of all studied proteins was found. However, the increase was statistically significant only in case of Cx43 (Figure 2(c)).

Serum FSH concentration was higher in 18L : 6D animals when compared with 6L : 18D males. No significant influence of OP was seen on FSH concentrations in both 18L : 6D and 6L : 18D groups (Figure 3).

3.2. Organ Culture. When OP was present in the culture medium at concentrations of 10, 100, and 500 mg/L, in homogenates of testicular pieces from 18L : 6D animals a progressive decline in N-cadherin and Cx43 expression levels was obtained at all doses after 24 h of culture (Figures 4(b) and 4(d)); however only higher concentrations (100 and 500 mg/L) elicited statistically significant effect. Expression of β-catenin decreased following exposure to 500 mg/L OP, whereas lower doses had no effect. In testis explants of 6L : 18D males protein expression levels were increased, but a relatively large variation in the expressions of N-cadherin and β-catenin between the individual testes exposed to OP meant the differences were not statistically significant (Figures 4(b)–4(d)).

An inhibitory experiment was performed using pure antiestrogen, ICI 182,780. Explants from 18L : 6D males were tested with 6 mg/L ICI alone for 24 h; 100 and 500 mg/L OP alone for 24 h; or combination of each dose of OP plus 6 mg/L ICI. Since ICI showed no discernible effect on proteins' expression, solvent-treated explants served as controls in this experiment. Coadministration of ICI did not block the effects of OP on N-cadherin expression (Figure 5(a)). On the other hand, ICI partially reversed the OP-induced decrease in β-catenin and Cx43 in testicular fragments cultured *in vitro* (Figures 5(b) and 5(c)).

4. Discussion

To assess the effects of OP on junction proteins expression in bank vole testis *in vivo* and *in vitro* methods were used. *In vivo* experiments are required for understanding the action of tested chemical in the internal environment of the organism, where it undergoes various biochemical transformations, affecting their bioavailability and activity. It was reported that in mammals OP is metabolized by the liver to two glucuronide conjugates [21]. Glucuronidation of OP eliminates its estrogen-like activity; however glucuronides may be hydrolysed back to active compounds [22]. At doses that exceed the metabolization level, OP accumulates in various organs, particularly, in adipose tissue, liver, muscles, and brain [23, 24]. Therefore, the effects of OP action are noted following administration of high doses or after chronic exposure, as it was demonstrated by data obtained in our and other laboratories [17, 25]. In addition, the influence of OP on endogenous estrogens production might contribute to the effects observed *in vivo* [17].

In contrast, during *in vitro* organ culture of the testis the influence of extratesticular factors is avoided, while relationship among the cells remains intact and the interactions between the cells can be accurately evaluated [26]. Therefore, short-term organ culture was used to examine whether OP could exert direct and immediate effects on junction proteins expression in the cells of the seminiferous tubule.

The results presented herein demonstrate that exposure of male bank voles to OP has a potential to induce adverse effects on junction proteins in the testes. Interestingly, alterations in the expressions and localizations of these proteins appeared to be dependent on the length of the photoperiod. Our

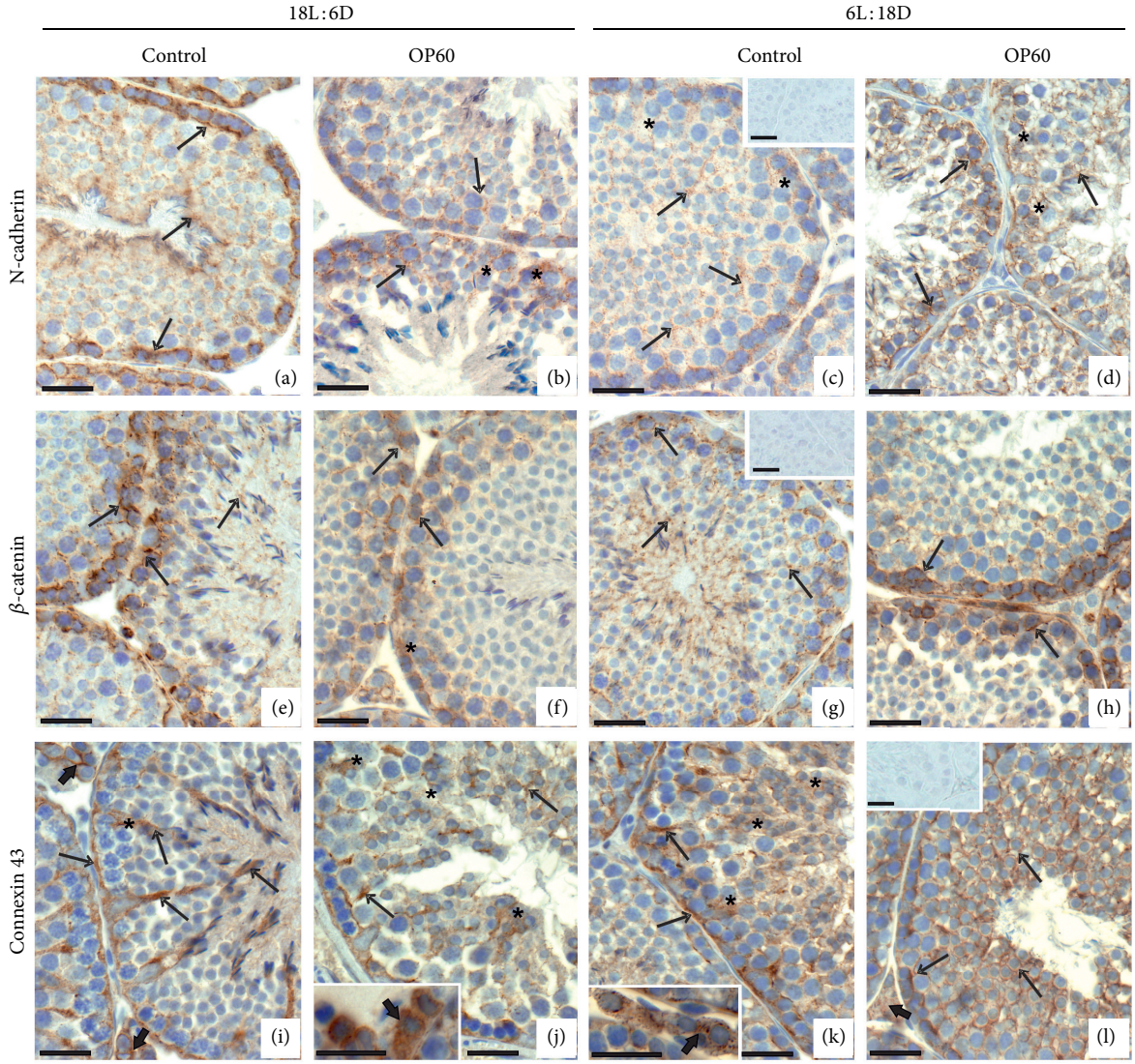

Figure 1: Immunohistochemical localization of N-cadherin (a)–(d), β-catenin (e)–(h), and connexin 43 (Cx43) (i)–(l) in the testes of bank voles kept in long (18L : 6D) or short (6L : 18D) photoperiod treated with vehicle (control) or 4-*tert*-octylphenol for 60 days (OP60). Scale bars represent 20 μm. (a)–(d) Immunohistochemical localization of N-cadherin. (a) In control 18L : 6D males strong, linear staining at the region of blood-testis barrier and discrete punctuate staining in the adluminal compartment is observed (arrows). (b) Reduced intensity and delocalization of the staining following OP60 treatment (arrows). (c) In control 6L : 18D voles weak staining extends through much of the epithelium (arrows). (d) In OP60 tubules enhanced staining is visible (arrows). No signal was detected when anti-N-cadherin antibody was omitted (insert in (c)). (e)–(h) Immunohistochemical localization of β-catenin. (e) Typical distribution of β-catenin signal is seen in the basal compartment of seminiferous epithelium and at the regions of membrane apposition of adjacent Sertoli cell and elongated spermatids (arrows) in the testes of control 18L : 6D males. (f) Note decreased intensity of the staining at the blood-testis barrier site (arrows) and loss of the signal in the adluminal compartment in OP60 voles. (g) In the tubules of 6L : 18D animals weak staining is dispersed in the seminiferous epithelium (arrows). (h) Increased staining intensity in the basal compartment in OP60 males (arrows). No signal was detected when anti-β-catenin antibody was omitted (insert in (g)). (i)–(l) Immunohistochemical localization of connexin 43 (Cx43). (i) In the tubules of control 18L : 6D males Cx43 is seen predominantly between Sertoli cells and spermatogonia or pachytene spermatocytes and in the cytoplasm of some Sertoli cells as well as at Sertoli cell-spermatid junctions (arrows). Note strong linear staining between Leydig cells (small arrows). (j) In OP60 males Cx43 signal is localized in the form of irregular lines or distinct foci between the cells (arrows) and sometimes in the cytoplasm of Sertoli cells (asterisks). Cytoplasmic staining is present in most Leydig cells (small arrow; insert). (k) In the tubules of 6L : 18D voles irregular and discontinuous signal is visible (arrows). Frequently, the staining is confined to the cytoplasm of the cells (asterisks). In Leydig cells, staining of moderate intensity is detected in the cytoplasm (small arrow; insert). (l) Note altered staining pattern in OP60 animals; signal visible at the entire surfaces of Sertoli and germ cells (arrows). Very week staining in Leydig cell cytoplasm (small arrow). No signal was detected when anti-Cx43 antibody was omitted (insert in (l)).

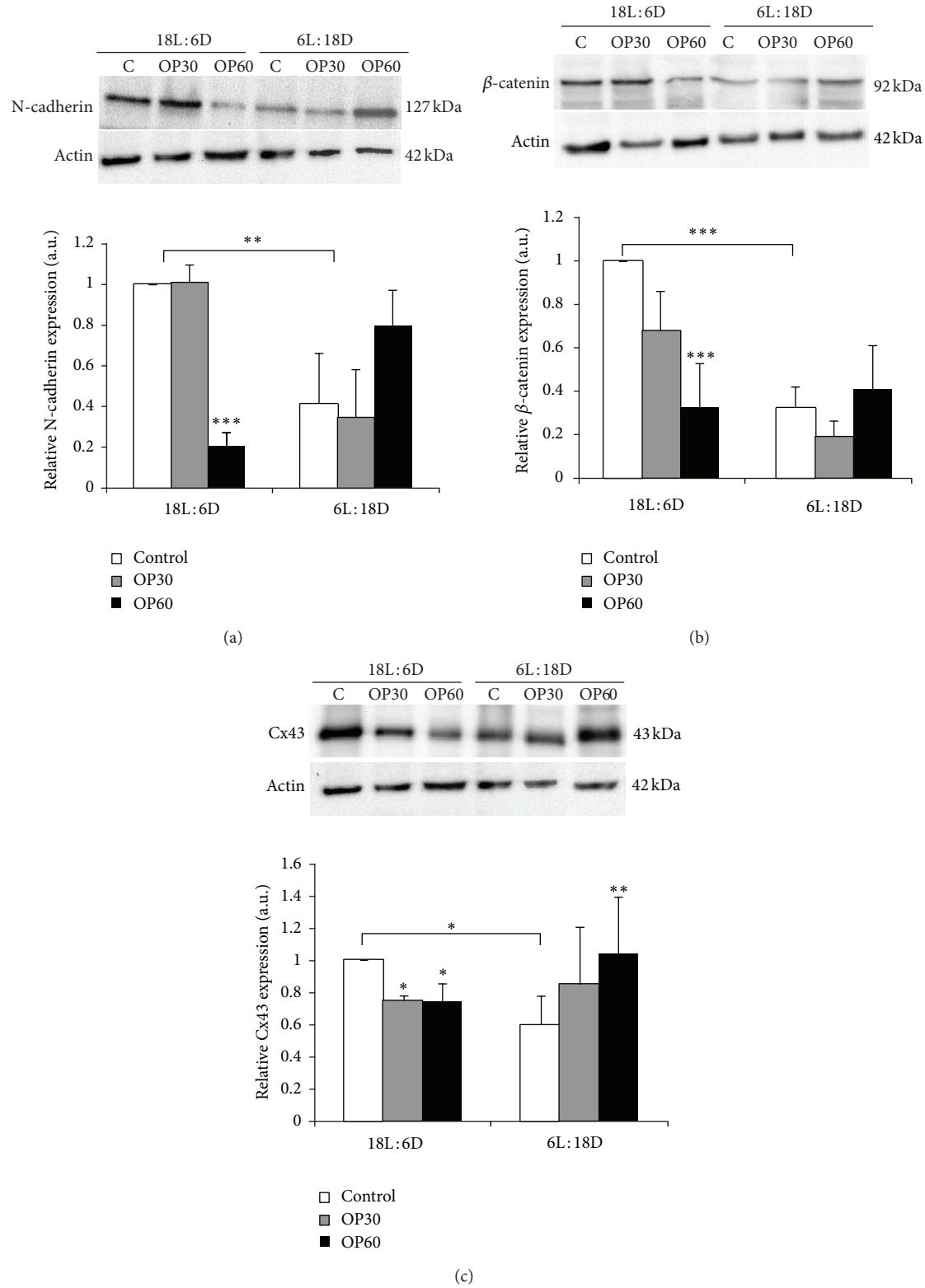

Figure 2: Representative Western blot analyses and relative N-cadherin (a), β-catenin (b), and connexin 43 (c) protein expression levels in testicular homogenates of bank voles kept in either long light cycles (18L : 6D) or short light cycles (6L : 18D) treated with vehicle ($n = 6$) or with 4-*tert*-octylphenol for 30 and 60 days (OP30, $n = 6$; OP60, $n = 6$). The relative amount of N-cadherin, β-catenin, and connexin 43 proteins normalized to β-actin. Data obtained from three separate analyses is expressed as mean ± SD. Significant differences from control values are denoted as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

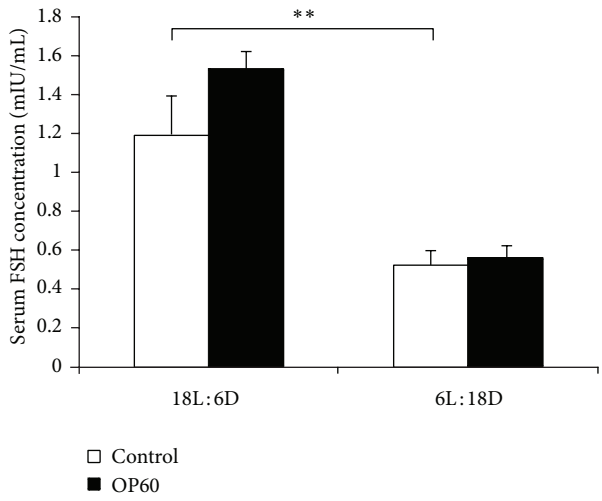

FIGURE 3: The effect of 4-*tert*-octylphenol treatment on FSH levels in the testes of 18L : 6D and 6L : 18D bank voles. Values are expressed as mean ± SD. Asterisks indicate significant differences between control animals (control, $n = 6$/each group) and males exposed to OP for 60 days (OP60, $n = 6$/each group). Statistical significance: $^{**}P < 0.01$.

results are the first reported on the influence of the length of the photoperiod on N-cadherin and β-catenin proteins in the testis of seasonally breeding rodents. It should be noted that bank voles kept under long photoperiod (18L : 6D) show similar reproductive characteristics as those observed in the wild in reproductively active males during spring and summer, whereas animals exposed to short light regime (6L : 18D) show regressive phase of reproduction that occurs in the wild voles during late autumn and winter. In the testes of males kept in short light cycles most of seminiferous tubules exhibit small or no lumen with not fully differentiated germ cells. Decrease in the level of plasma gonadotropins during the short photoperiod results in reduced testosterone production decreased expression and activity of steroidogenic enzymes and reduced expression of androgen receptor when compared to long-day animals [17, 27, 28]. The present study provides clear evidence that in bank voles also direct cell-cell interactions are regulated by length of the light cycle, since the expression and localization of protein markers of adherens and gap junctions are different in animals reared under different light conditions. We noticed that organization of N-cadherin/β-catenin and Cx43-based junctions in the seminiferous epithelium of 18L : 6D males was consistent with the organization of morphologically mature junctions in the adult testes of continual breeders such as mice and rats [11, 13, 29, 30]. On the contrary, in 6L : 18D animals distribution of junction proteins was similar to those observed in immature or prepubertal males, in which weaker, punctuate, or cytoplasmic staining was dispersed in the basal and adluminal compartment of the epithelium [13, 31]. This is in line with the results of Tarulli et al. [32], who reported that in short-day males of seasonally breeding Djungarian hamster Sertoli cells have characteristics of both adult and immature phenotypes, suggesting that they take on an intermediate or transitional state. It should be mentioned that early study by Pelletier [33] demonstrated the localization of Cx43 in another seasonal breeder, mink. In contrast to our observations, the author reported that during winter testicular regression immunoexpression of Cx43 is present only in the basal third of the epithelium. Such a result was presumably a consequence of performing less sensitive immunohistochemical method with the use of secondary antibody conjugated directly to peroxidase.

Disorganization and decreased expressions of N-cadherin, β-catenin, and Cx43 proteins in 6L : 18D bank vole males were accompanied with reduced FSH level in these animals.

There is evidence that FSH stimulates the formation of extensive inter-Sertoli cell adherens junctions; in the absence of FSH adherens junction puncta were observed in rat Sertoli cells *in vitro*, whereas the addition of FSH induced the reorganization of these puncta into adherens junction belts [34]. Also gap junction coupling and organization of Cx43 gap junction plaques between Sertoli cells *in vitro* appeared to be regulated by FSH and cAMP [35, 36]. Moreover, recent data on Djungarian hamsters indicate that gonadotropin suppression induced by short photoperiod disrupted distribution and expressions of tight junction proteins, and FSH replacement led to a rapid reorganization of these proteins [32]. Based on these data and our results we believe that in bank voles FSH could be a factor controlling adherens and gap junctions arrangement in the seminiferous epithelium during transition from reproductive quiescence to reproductive activity.

Despite numerous studies on testis histopathology of males treated with OP, very little is known about the alterations induced by this xenoestrogen on cell–cell junction molecules. In the only paper reporting the effects of OP on Sertoli cell junction proteins, Fiorini et al. [37] observed reduction in the levels of occludin, N-cadherin, and Cx43 in the SerW3 Sertoli cell line treated with 0.2 μM OP for 24 h. In addition to its effect on protein levels, OP was able to delocalize the proteins from the membrane to the cytoplasmic compartment [37]. In the present study, it was shown that long-term *in vivo* OP treatment of adult bank voles also resulted in the reduction of N-cadherin, β-catenin, and Cx43 proteins expressions and their delocalization, but only in the testes of males kept in long photoperiod. Surprisingly, in short-day animals slight increase of Cx43 ($P < 0.05$), N-cadherin, and β-catenin (statistically nonsignificant) levels was found. These differences appeared to be independent of FSH, since OP treatment did not change FSH concentrations neither in long-day nor in short-day animals. Thus, we hypothesize that OP affects junction proteins expression acting directly on the testis. To test this hypothesis we used organ culture model. We found that in testis explants cultured with OP for 24 h expression levels of all studied proteins were reduced in long-day voles and slightly elevated in short-day animals. Photoperiod-dependent effect of OP on junction proteins in bank vole testes was therefore maintained in *in vitro* conditions, indicating that hypothalamic-pituitary axis is not involved in this effect.

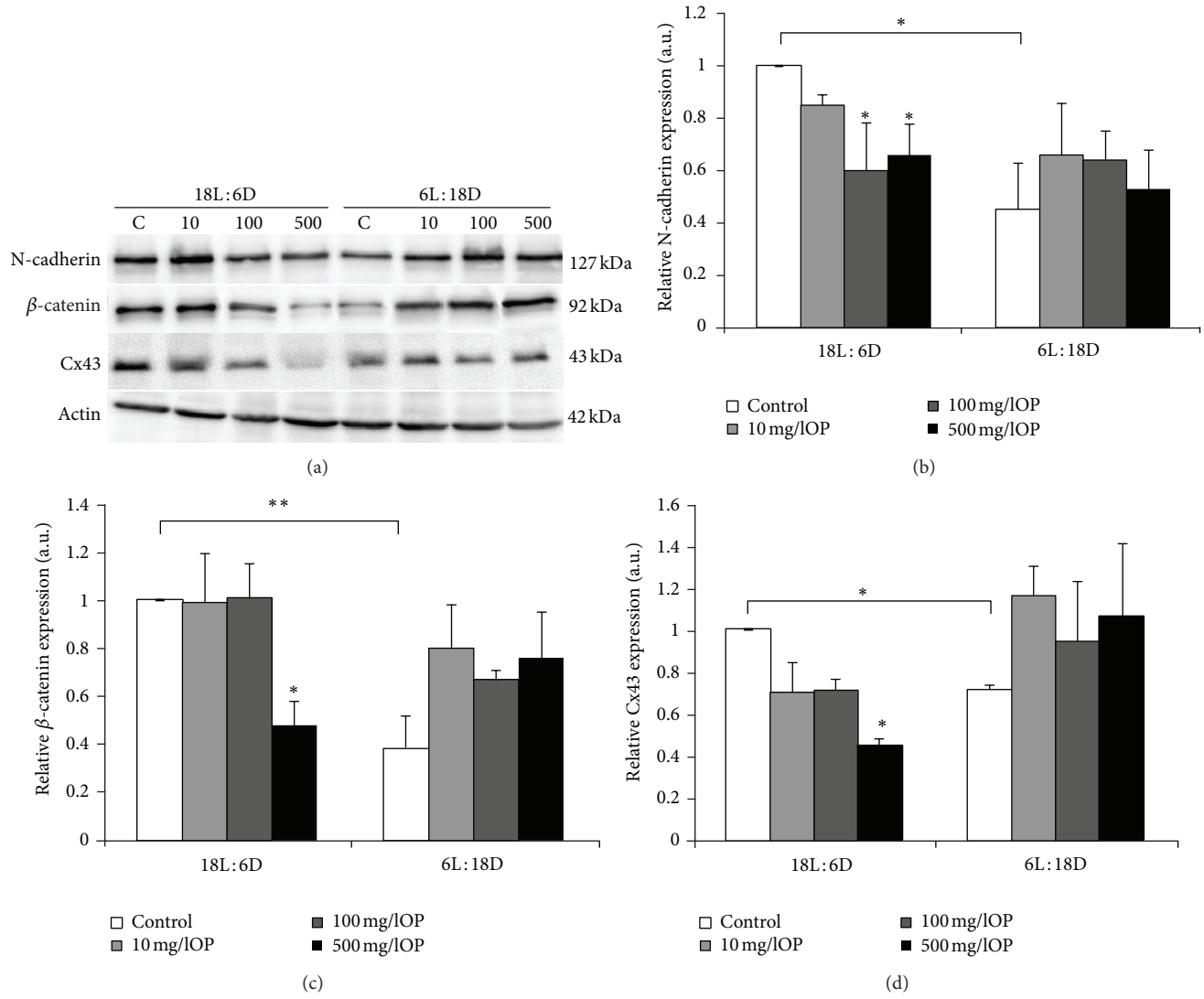

Figure 4: (a) Representative Western blot analysis of N-cadherin, β-catenin, and connexin 43 protein expression levels in homogenates of testis explants from bank voles kept in either long light cycles (18L : 6D; $n = 8$) or short light cycles (6L : 18D; $n = 8$). Explants were cultured in control medium (c) or in media containing 10, 100, or 500 mg/L 4-*tert*-octylphenol for 24 h. Anti-β-actin labeling served as an internal protein loading control. (b)–(d) The relative amount of N-cadherin (b), β-catenin (c), and connexin 43 (d) proteins normalized to β-actin. Data obtained from three separate analyses is expressed as mean ± SD. Significant differences from control values are denoted as $^{*}P < 0.05$ and $^{**}P < 0.01$.

The reason for diverse responses of males kept in different light conditions to OP remains to be elucidated. Nevertheless, since in our previous studies on bank vole testis we found elevated estradiol concentrations and aromatase expression following OP exposure [17], it is possible that in short-day voles OP directly acting (as estrogen-like compound), or more likely through induction of local estradiol production, restores the expression levels of junction proteins. These hypothesis is based on our earlier observations that exposure to low dose of exogenous estradiol induced acceleration of the onset of spermatogenesis in voles kept under short light cycle conditions and on the finding that estrogens have FSH-independent stimulatory effect on spermatogenesis in photoregressed Siberian hamster [38, 39].

On the other hand, in long-day males, OP exerts negative effect on the expression of junction proteins, presumably by inducing supraphysiological estrogen level or action. Indeed, our previous studies revealed that in bank vole males treated with a high dose of estradiol disruption of testicular structure and tubular atrophy occurred [38]. Moreover, in rats exogenous estrogens appeared to affect cell-cell junctions in the testis; 17β-estradiol was shown to be disruptive to the BTB integrity, while 17α-ethinylestradiol altered intercellular communication by disrupting gap junction functionality and Cx43 trafficking in cultured Sertoli cells as well as in isolated rat seminiferous tubules [12, 40].

It should be noted that in our study OP had the most rapid effects on Cx43 protein expression in the testes of 18L : 6D

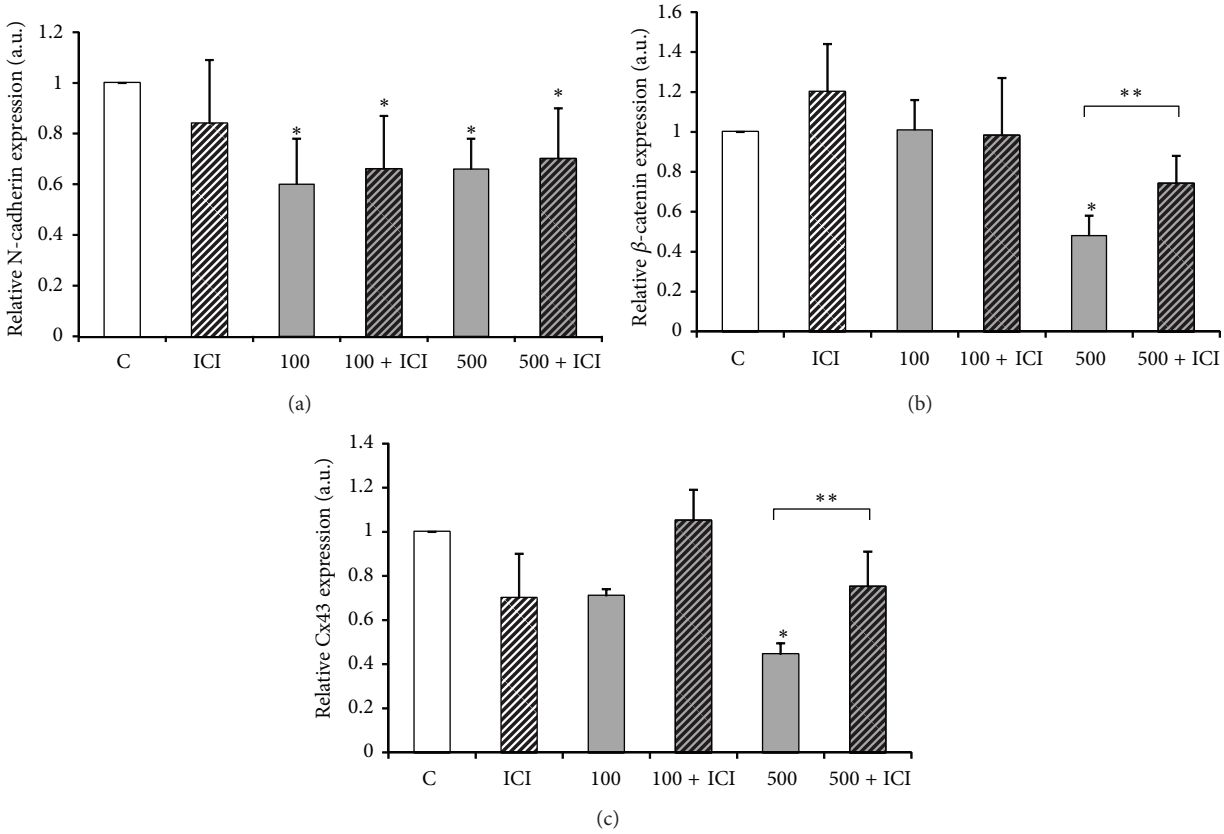

FIGURE 5: The effects of ICI 182,780 on octylphenol-induced decrease of N-cadherin (a), β-catenin (b) and connexin 43 (c) protein expression in homogenates of testis explants from bank voles kept in long light cycles ($n = 8$). Explants were cultured in control medium (c) or in media containing 6 mg/L ICI 182,780 (ICI); 100 or 500 mg/mL 4-*tert*-octylphenol (100, 500); 100 or 500 mg/mL 4-*tert*-octylphenol and 6 mg/L ICI 182,780 (100+ICI; 500+ICI) for 24 h. Graphs represent the relative amount of N-cadherin (b), β-catenin (c), and connexin 43 (d) protein normalized to β-actin. Data obtained from three separate analyses is expressed as mean ± SD. Significant differences from control values are denoted as $^{*}P < 0.05$ and $^{**}P < 0.01$.

males (visible just after 30-day exposure). This observation argues for the recent hypothesis that Cx43 is one of the main targets for endocrine disruptors and that impaired Cx43 expression affects other proteins of the BTB [41].

The question arises as to the mechanism of OP action on junction proteins in the seminiferous tubules. To determine whether this actions was mediated through binding to ERα or ERβ, testicular explants from 18L : 6D males were treated concomitantly with a pure estrogen antagonist, ICI 187 780 (6 mg/L), and increasing OP concentrations (10 to 500 mg/L). We found that β-catenin and Cx43 OP-induced decrease was partially blocked by ICI, suggesting that the biological effects of OP on the expression of these proteins involve an ER-mediated response. On the other hand, the influence of OP on N-cadherin does not appear to be mediated through the classic nuclear ERs, since ICI did not alter the effects of OP. Several earlier papers reported that also some other effects of OP were not mediated through the ER, possibly involving interactions with membrane components or receptors. For example, Murono et al. [42, 43] described the effects of OP on the conversion of progesterone to testosterone and hCG-stimulated testosterone biosynthesis as independent of ERα and ERβ.

It cannot be, however, excluded that after the relatively short incubation period (24 h) some amounts of the proteins are not degraded and still exist in the cultured tissue, even if the mRNA level was downregulated. Therefore, further studies including sequencing of bank vole genes and the application of RT-PCR method are needed to verify data regarding mechanisms of OP action on junction proteins expression in the bank vole testes.

5. Conclusion

In this study we demonstrated that in seasonally breeding bank vole organization of adherens and gap junctions as well as the expression of junction proteins is related to the length of photoperiod and, in consequence, to the reproductive status of the animal. In addition, we found that reproductive status may determine response to OP. Finally, we found that

alterations in cadherin/catenin and Cx43-based junction in the testes following OP exposure may partially result from activation of ERα and/or ERβ signaling pathway.

Conflicts of Interests

The authors declare that there is no conflicts of interests.

Acknowledgment

This work was financially supported by the Ministry of Science and Higher Education, a Grant no. N N303816640.

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 282381, 7 pages
http://dx.doi.org/10.1155/2013/282381

Research Article

Association between Endocrine Disrupting Phenols in Colostrums and Maternal and Infant Health

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Received 31 December 2012; Accepted 28 March 2013

Academic Editor: Ewa Gregoraszczuk

Bisphenol A (BPA) and alkylphenols (APs) are well-known endocrine disrupting chemicals (EDCs) which may threat the next generations' health. We performed biomonitoring of these phenols in colostrums to assess risk of the phenols in breast-fed neonates. Study subjects were the lactating mothers who delivered babies within 2 weeks ($N = 325$; 30.67 ± 3.45 years) and their neonates ($N = 326$; embryonic period, 39.1 ± 1.5 weeks). BPA, nonylphenol (NP), and octylphenol (OP) in colostrums were quantified with LC/MS/MS. Information for environmental exposure sources of the phenols was obtained by questionnaires. As results, median level of BPA in colostrums was 7.8 ng/mL, while most NP or OP was not detected. Regarding health risks of phenols, levels of total NP in colostrums were elevated among sick mothers with toxemia, thyroid disorders, gastritis, and so forth than health mothers (3.51 ± 4.98 versus 2.04 ± 3.71 ng/mL, $P = 0.02$). Dairy products intake and detergents use were positively correlated with total BPA levels ($Ps < 0.05$). In conclusion, we estimate most neonates who are exposed to BPA rather than NP or OP via colostrums and recommend continuous biomonitoring of the phenols to clarify their suspected health risk on neonates and pregnant or gestation mothers.

1. Introduction

Effects of endocrine disrupting chemicals (EDCs) on human health and wildlife are receiving growing attention for the next generation's health and have been known to interfere with endocrine systems by mimicking, blocking, and triggering actions of hormones and implicated with toxic effects, for example, disorders in development and reproduction [1]. Among EDCs, bisphenol A [BPA, 2,2-bis (4-hydroxyphenyl) propane] is widely used for a variety of applications, for example, baby feeding bottles, food-can lining, and sealants in dentistry. In addition, 4-tertiary-octylphenol (OP) and 4-nonylphenol (NP) of alkylphenols (APs) have been used to make alkylphenol ethoxylates, nonionic surfactants applied as emulsifying, wetting, or stabilizing agents in industries, and various consumer products including detergents and pesticide formulations [2]. Due to the wide uses of these phenols, it has been speculated that human exposures to environmental phenols may be widespread [3–6]. Thus, concerns about various adverse health effects caused by EDCs are increasing, and rigid risk assessment for EDCs throughout valid biomonitoring studies has been called for. Particularly, considering low-body weights and susceptibility, we suspect that body burden or real exposure level of infants or children to BPA or APs is expected to be heavier than those of adults. In the view of susceptibility, the exposures to environmental phenols in infants and children have got the public attention because EDCs treat the second generation's health, for example, genital malformations, testicular abnormalities, impairment in fertility or sexual functions, and neonates are considered to be a vulnerable subgroup to xenobiotics [7, 8]. Therefore, environmental phenols including BPA and APs

should be continuously monitored for achievements of public heath, particularly for infants and children.

Contamination of ECDs in colostrums raises concerns gravely because neonates, who are solely dependent on colostrums, are considered to be a high susceptible to EDCs. Detoxifying enzymes of neonates would not be fully developed at this early time point and exposure to EDCs during the critical periods of developments could cause morphologic and functional alterations by influencing growth, reproduction, and development [7, 8]. Considering the characteristics of EDCs that affect the second generation's health, we need biomonitoring of EDCs in colostrum, which is the main route of exposure to EDCs for breast-fed infants. A number of investigations have reported the occurrence of several environmental chemicals such as persistent organic pollutants (POP), polychlorinated dibenzo-dioxins (PCDDs), and organochlorines (OCs) in breast milk [9–11]. BPA, OP, and NP have the potency to partition into breast milk, since they are lipophilic compounds, which have octanol-water partition coefficient value (log P or Kow) around 3-4 [12, 13].

Concerning the phenol exposure sources, we have studied various environmental sources; however, we could not find crucial exposure sources, yet [14, 15]. In a case of NP, dairy products and sea food were suspected as its exposure sources [16, 17]. In addition, ethoxylation products of APs have been used for cosmetics or surfactants [18]. Thus, we focused on the consumption of dairy products and sea food or the use of cosmetics or surfactants to find phenol exposure routes in this study. In addition, we established a sensitive analytical method for BPA, OP, and NP in human colostrums and performed biomonitoring of these phenols among Korean lactating women's colostrums to assess risk of BPA and APs for breast-fed neonates.

2. Materials and Methods

2.1. Study Subjects. Study subjects were 325 lactating mothers, who stayed in postpartum care centers in Seoul, Republic of Korea. All subjects consented to participate in this study and donated their colostrums (≈10 mL). Colostrums were collected in dark glass tubes with glass caps and stored −20°C prior to analyses. We also obtained their questionnaires addressing physical characteristics, lifestyle patterns including dietary habits, and health and pregnancy-related properties [14, 19]. All of the above procedures were approved by the institutional review boards of Inje University, Paek Hospital (Seoul, Republic of Korea).

2.2. Analysis of Phenols in Milk. Throughout the entire procedure, plastic wares were replaced with glassware in order to avoid possible phenol contamination. We analyzed total forms (conjugated and free phenols) and free forms of BPA and APs for each colostrum sample with/without enzyme hydrolysis, respectively. The concentration of conjugated phenols was calculated after subtracting the amount of the free form of phenols from that of total phenols.

To determine total forms of the three phenols levels in colostrum, we established an optimal method with modification of the BPA analysis. In brief, 100 uL of internal standard (125 ng/mL of bisphenol B (BPB), Tokyo Kasei Chemical, Tokyo, Japan), 120 uL of 2.0 M sodium acetate, and 48 uL of β-glucuronidase (2,784 U) (Sigma, St. Louis, MO) were added to 2 mL of milk samples. The mixture was incubated at 37°C for 5 hours; 4 mL of 2-propanol was added and mixed thoroughly. Thereafter, the mixture was centrifuged (3,000 g, 20 min), and 3 mL of supernatant was transferred to new glass tubes. This extraction was repeated, and total transferred 6 mL of supernatant was evaporated. After the evaporation, the residue was dissolved with 250 uL of 60% acetonitrile, and this solution was centrifuged (14,000 rpm, 10 min). Thereafter, 200 uL of the supernatant was transferred to a vial, and 50 uL of the supernatant was injected for LC/MS/MS analysis. The LC/MS/MS system was composed with Waters alliance 2695 XELC/MS/MS (Waters, Watford, UK) and Zobax SB-C18 (5 μm, 4.6 × 250 mm, Agilent, USA). Separation was accomplished with a gradient mode: mobile phase A, water: mobile phase B, acetonitrile: flow rate 0.3 mL/min, and ratio of A to B: 0–3 min, 70 : 30; 3-4 min, from 70 : 30 to 95 : 5; 4–6 min, from 95 : 5 to 100 : 0; 6–25 min, 100 : 0; 25–30 min, from 100 : 0 to 70 : 30. The Waters alliance 2695 Quattro Premier XE was used in negative ion ESI mode. The electrospray ionization (ESI) settings were as follows: capillary voltage, 3.5 kV; cone voltage, 40 V; desolvation gas-flow (Argon gas), 800 L/hr; cone gas-flow, 20 L/hr; collision energy, 20 V. To determine concentrations of the free forms of phenols, we followed the procedures described above except enzyme hydrolysis.

2.3. Statistical Analyses. Distribution normality of the phenols levels was tested with Shapiro-Wilk W test. If their probability (W) is <0.05, their distribution is considered not to follow normal distribution.

"Nondetectable" was assigned a value of half of the minimum value of detected each phenol level for further statistical analyses. Considering each value's characteristic (normality and nominal, continuous, or ordered values), Wilcoxon test, Fisher's exact test, Spearman's rho, and regression analysis were used for the study analyses. $P < 0.05$ was considered to be statistically significant. The statistical package of JMP Version 4 (SAS Institute, Cary, NC, USA) was used for all analyses.

3. Results

3.1. Characteristics of Study Subjects. The characteristics of the subjects are summarized in Table 1. Mothers were 30.67 ± 3.45 years old, and, as expected, their body mass indices (BMIs) were increased during the pregnancy (median of before and after: 20.19 and 23.53 kg/m^2, resp.). Based upon education level, occupation, and monthly household income, participants reflected typical middle class in the Republic of Korea. Approximately, 11% ($N = 35$) of the mother have clinical disease(s), such as toxemia ($N = 22$), thyroid disorders ($N = 6$), and gastritis ($N = 1$). Similar number of

Table 1: Characteristics of subjects.

(a) Mothers

Items	% (N = 325)
Health status	
Normal	89.2
Disease[a]	10.8
Education	
≤9 yrs	0.3
>9 yrs and ≤12 yrs	22.4
>12 yrs and ≤16 yrs	69.4
>16 yrs	7.9
Job	
House wife	43.0
Office workers	21.7
Teachers	10.8
Others	24.5
Monthly income	
<$2,000	17.1
$2,000~$4,000	55.7
>$4,000	27.2
Food intake	
Vegetable preferred	16.9
Meat preferred	14.7
Fish preferred	14.3
Evenly	53.9
Consumption of dairy products[b]	
1~2 times/month	11.3
Once/week	12.0
2~3 times/week	40.8
Every day	35.9
Use of detergents for food	
Rare	1.0
2~3 times/week	11.2
Every time	87.8
Use of cosmetics	
1-2 times/month	24.1
Once/week	20.6
2~3 times/week	27.3
Every day	28.0
Alcohol drinking[c]	
Never	29.1
1-2 times/month	47.7
Once/week	46.2
≥2 times/week	7.0
Tobacco smoking	
Never smoker	88.07
Ex-smoker[d]	11.58
Smoker	0.35

[a]Toxemia, thyroid disorders, gastritis, and so forth.

[b]Milk, cheese, and so forth.

[c]Before pregnant.

[d]Quitted smoking over 1 year.

(b) Infants (N = 326)

Items	
Embryonic period (wks)	39.1 ± 1.5
Body weight (kg)	3.2 ± 0.4
Stature (cm)	50.2 ± 2.3
Health status (%)	
Normal	88.7
Disease[a]	5.0
Recovered[b]	6.3

[a]Jaundice (N = 6), intestinal obstruction (N = 2), congenital malformation (N = 2), enteritis (N = 1), cyanosis (N = 1), and so forth.

[b]Jaundice (N = 7), respiratory distress (N = 5), conjunctivitis (N = 1), dyspepsia (N = 1), acute enteritis (N = 1), and so forth.

the infants were sick at birth (N = 34), and most of the sick babies were recovered within 2 weeks after birth or on the therapy except two infants who were born with a congenital malformation. There was no association of disease presence between the mothers and their infants (P = 0.66).

3.2. Exposure Levels of the Three Phenols in Milk. We established a sensitive analytical method to measure BPA and APs in colostrum samples with LC/MS/MS. Limit of detection (LOD) and limit of quantification (LOQ) were calculated with signal to noise ratio 10 and 30, respectively. The calibration curve for simultaneous analyses of the three phenols was obtained within 5 different concentrations in phenols-spiked milk. The recoveries of BPA, NP, and OP were over 80%.

The distributions of the three phenols were left skewed near to zero, that is, nondetectable values and did not follow normal distributions (Ps < 0.01 by Shapiro-Wilk W test for nonnormality). Ranges of the three phenols' levels in colostrum were shown in Table 2. OP and NP were not detected in most colostrum samples as free forms without any metabolism. In addition, there was a significant positive correlation between total OP and NP levels (β = 0.37, P < 0.01 by regression analysis) and somewhat positive association between total OP and BPA levels (β = 0.06, P = 0.09).

Finally, we could estimate the daily exposure to the phenols in the infants via their mothers' milk and determined that the current exposure levels to BPA and NP were lower than their tolerable daily intakes (TDIs) (Table 2).

3.3. Exposure Routes of the Phenols in Mothers. To identify the potential routes of phenols exposure from environment, we investigated associations between phenol levels in colostrum and exposure sources. We observed that dairy products intake and detergents use were positively correlated with total BPA levels (P < 0.05) (Table 3). However, we did not find any statistically significant association between phenol levels and other exposure (Table 3) including air pollution at resident area or use of plastic wrap.

3.4. Effects of Phenol Exposures on Mother and Infant Health. Considering no associations between mothers' and infants' disease presence, we separately analyzed effects of phenol exposure on mothers or infants. At first, we analyzed effects of

Table 2: Comparison between exposure levels and regulation levels of phenols.

	Phenols	Detection of all samples (%)	Median (ng/mL)	Range (ng/mL)	Estimated daily exposure in neonates[a,b] (μg/kg)	TDI (μg/kg)[c]
Free	BPA	39.8	<LOD	<LOD–54.2		
	NP	0.0	<LOD	<LOD		
	OP	2.9	<LOD	<LOD–14.1		
Total	BPA	70.6	7.8	<LOD–57.3	1.20	50
	NP	15.9	<LOD	<LOD–23.4	0.12	5–15[d]
	OP	27.5	<LOD	<LOD–30.9	0.02	Unknown
Conjugated	BPA	70.6	4.2	0.0–30.2		
	NP	15.9	<LOD	0.0–23.4		
	OP	27.5	0.0	0.0–30.7		

[a]Nondetectible values were designated to half of the lowest value of each phenol.
[b]Daily intake volume of milk in neonates, 500 mL; mean body weight of the infants, 3.24 kg; calculated from median level of each phenol × 500/3.24/1000.
[c][3, 14].
[d]NOAEL (no observable adverse effect level), 15 mg/kg; uncertainty factor, 1000 (WHO) and 3000 (Danish Institute of Safe and Toxicology).

Table 3: Correlations between total phenol levels and candidates of exposure sources.

	BPA		OP		NP	
	Correlation coefficient[a]	P	Correlation coefficient	P	Correlation coefficient	P
Consumption of dairy products	0.36	0.02*	0.04	0.55	−0.08	0.19
Use of detergents	0.12	0.04*	0.07	0.29	−0.06	0.32
Use of cosmetics	−0.05	0.39	−0.05	0.43	−0.04	0.48
Fish preference	−0.09	0.11	−0.00	0.96	−0.02	0.76
Alcohol drinking	0.04	0.46	−0.01	0.82	−0.05	0.45
Tobacco smoking	−0.04	0.46	−0.02	0.78	−0.03	0.67

[a]Spearman's *Rho* by pair wise correlation analyses.
*Statistically significant.

phenols exposure on physiological characteristics. Mothers' age was not associated any phenol levels; however, embryonic period in neonates showed somewhat negative association with NP levels ($\beta = -0.25$, $P = 0.09$). Concerning phenols disposition at adipose sites, we studied effects of phenol exposure on BMI. As result, we found some positive associations between levels of mothers BMI (before and after delivery) and NP ($\beta = 0.09$, $P = 0.01$ and $\beta = 0.04$, $P = 0.19$, resp.) or OP ($\beta = 0.07$, $P = 0.07$ and $\beta = 0.03$, $P = 0.34$, resp.). However, there were some negative associations between mothers BMI (before and after delivery) and levels of BPA ($\beta = -0.11$, $P = 0.07$ and $\beta = -0.09$, $P = 0.12$, resp.).

Secondly, we found that levels of total NP in mothers or infants with diseases were higher than those in health subjects (Table 4). In the case of mothers' toxemia, a major mother disease ($N = 22$), we found more strong association between the disease and NP exposure ($P < 0.01$). For neonates' major disease, jaundice ($N = 13$ including recovered cases), we could not find any significant association between the disease and the 3 phenol levels ($Ps > 0.05$). In addition, neonates with congenital malformation ($N = 2$) showed somewhat high levels of BPA, NP, and OP rather than others ($P = 0.07$, 0.11, and 0.27, resp.). In detail, the colostrum of the infant with congenital malformation of uvulas showed quite high levels of total NP and OP (medians, 21.5 and 18.1 ng/mL, resp.) compared to others' levels (medians <LOD, i.e., <0.5 and <0.3 ng/mL, resp.).

4. Discussion

Considering physical complexity as a matrix of colostrum, proper preparations of colostrums specimens are required. Recently, many researchers have used solid phase extraction (SPE) for this purpose [6]. Considering broad use of BPA in plastic, we avoided SPE approach. Therefore, we performed liquid-liquid extraction; even this preparation was time-consuming and labor intensive. In a case of biomonitoring environmental phenols, especially BPA, a general problem is background contamination during the analyses, which interfere with quantification at low concentrations of BPA. Therefore, we used glassware throughout the entire analytical procedure in order to avoid possible contamination of BPA. Blank tests, which were conducted with water instead of colostrum, were also performed at every daily experiment to confirm an absence of environmental phenol contamination in the whole of our experimental process. In addition, we tried to our best to avoid a drawback of LC/MS/MS analyses, for example, over estimation in low phenol-contained samples [20]. Thus, we established an optimal condition to

Table 4: Association between health status and total phenol levels[a].

	BPA		NP		OP	
	mean ± std (ng/mL)	P	mean ± std (ng/mL)	P	mean ± std (ng/mL)	P
Mothers (N)						
Healthy (290)	7.75 ± 7.36	0.57	2.04 ± 3.71	0.02*	2.24 ± 4.24	0.38
Sick (35)	7.99 ± 7.35		3.51 ± 4.98		2.59 ± 3.66	
Infants[b]						
Healthy (312)[c]	7.83 ± 7.50	0.76	1.86 ± 3.29	0.09	2.20 ± 4.13	0.31
Sick (14)	6.65 ± 7.06		3.95 ± 6.35		3.91 ± 6.06	

[a] Wilcoxon's test.
[b] Data show phenol levels in their mothers' breast milk.
[c] It includes recovered subjects.
* Statistically significant between healthy and sick subjects, $P < 0.05$.

measure BPA, OP, and NP in the colostrum samples. We also performed analyses of HPLC/FLD to confirm LC/MS/MS results in low phenols samples (<medians of each phenol: $N = 50$) and obtained high reproducibility between two analyses (CVs < 15%).

Among the present 325 colostrum samples, 70.6% of them have detectable levels of BPA in colostrum (Table 2). Mendonca et al. recently reported similar detection frequency of BPA in USA—breast milk for 3–15 months infants, that is, 75% [21]. However, Völkel et al. detected BPA in 42% of infant urine [22]. Considering the relatively high frequency of BPA detection in colostrum samples, we suggest a high potential for exposure of breast-fed infants to BPA via colostrum. In addition, median level of total BPA (7.8 ng/mL) in the present colostrum study is somewhat higher than those in other biomonitoring studies with breast milk samples [12, 23, 24]. For example, another study with 101 colostrum samples detected BPA at a range of 1–7 ng/mL and a mean level of 3.41 ng/mL [25]. However, they used convenient ELISA methods and detected BPA in all samples without confirmation of BPA free system for analyses, for example, blank tests. Mendonca et al. also reported quite low levels of total BPA (median, 0.8 ng/mL) in small number of infant breast milk samples ($N = 23$) [21].

On the basis of three phenol levels in colostrum, volume of daily intakes of milk (500 mL), and mean body weight of the baby subjects (3.24 ± 0.46 kg), we estimated the daily exposure of infants to BPA, OP, and NP (Table 3). The estimated exposure levels of BPA and NP via colostrum in Korean neonates appear to be safe, compared to their TDIs [3, 8]. In a case of OP, we cannot determine whether the present exposure status of OP is acceptable or not at this point because there is no reference dose data of OP. When we compared the daily exposure levels in the present neonate to those in adults, they may be highly exposed to phenols due to body burden. For example, the neonates may be ≈10-fold highly exposed to BPA than adults [1.2 ug/kg (Table 3)] versus ≈0.13 ug/kg, which was estimated from Korean adult urines [14].

Concerning EDCs' disposition, many researchers have analyzed EDCs at adiposities. As lipophilic characteristics of EDCs can induce chronic diseases even with buffering of acute toxicity, this issue has been emphasized. A recent Spanish study showed that the most obese woman had the highest levels of NP and PCBs in adipose tissue [26]. However, it is not clear, yet, whether body fat contents affect phenol accumulation in the body. In the present study, we found positive associations between levels of mothers BMI before delivery and NP or OP levels. However, BPA levels showed opposite trend, that is, negative association with mothers BMIs. For the reason of the opposite trend, we consider physiochemical difference in octanol-water partition coefficient value ($\log P$ or Kow) between NP and BPA (4.48 and 3.32, resp.) [27]. In addition, people are simultaneously exposed to multiple EDCs; thus, we screened combined effects of BPA, NP, and OP on the present mothers' and neonates' health. However, the simple sum of total BPA, NP, and OP levels did not show any health risk. In order to study future and real combination effects of phenols or EDCs, their weight for risks or reliable simulation of multiple exposures should be considered.

Referring pharmacokinetics of phenols in human or rhesus monkeys [28, 29], which may be mainly metabolized into urine as conjugated forms with glucuronyl or sulfonyl groups, we have used conjugated BPA for biomonitoring of BPA in urine or blood samples [14, 15, 30]. Detoxification enzymes which metabolize free phenols into conjugated phenols are known to decrease in pregnant women [31]. As most conjugated BPA was analyzed in urine, we analyzed BPA in some urines among the present mothers who donated urine ($N = 21$) to confirm conjugation capacity in the present mothers. Their conjugated BPA levels in urine were approximately half of those in colostrum (mean ± std, 0.98 ± 1.96 ug/L versus 2.44 ± 3.68 ug/L, mean difference, −1.46 ug/L.) When we compared the conjugated BPA levels in the present lactating mothers' urine samples to those in other adults' [14], they were quite lower than the others (median, <0.6 ng/mL versus 7.86 ng/mL). It may support pregnant women' loss of detoxification enzyme activity, even though we consider the decline of BPA exposure due to years [32].

We also studied exposure of routes of the phenols in mothers and found association between BPA levels and

consumption of dairy products (Table 3). Dairy food always needs its storage, packing, or some manufacturing. The real parts, which people intake, directly contact to containers or packing materials rather than other food. Like PCBs accumulation in perennial fish [3], accumulation of BPA in cow can be thought via food chain. Interestingly, we also found a positive association between BPA levels and use of detergents for food. A recent report [33] concerning the "effect of detergents in the release of bisphenol A from polycarbonate baby bottles" can support our result. Referring reports of AP-related food [16, 17], we studied more other sources for APs, but we could not find any similar source, for example, fish, fruit, or fish oil.

Considering health risks of phenols, we found negative association between embryonic period in neonates and their colostrum NP levels and suspect NP induces embryonic instability. NP showed some embryotoxicity in crustaceans and oysters, for example, low survival rates and poor embryonic and larval development [34, 35]. However, experimental studies in mammals to clarify effects of NP on embryonic period have been thoroughly performed, yet. Thus, future enlarged epidemiological studies or experimental studies are required to confirm risks of NP on embryonic stability. Secondly, we found that levels of total NP in sick mothers, particularly, toxemia-patients, were higher than those in health subjects (Table 4). In addition, we found that the infant with congenital malformation of uvulas showed quite high levels of total APs in compared to others' levels. Thus, our finding should be further confirmed in enlarged studies, even though the present subjects well represent the Korean women in childbed from similar proportion of toxemia or gestational complications [36].

In conclusion, we found that most of neonates are exposed to BPA rather than NP or OP via colostrums. Although current exposure levels of the phenols are safe based upon their TDIs, we suggest continuous biomonitoring of them to clarify their unclear health risk on neonates and pregnant or gestation mothers.

Acknowledgment

This work was supported by the SRC Research Center for Women's Diseases of Sookmyung Women's University (2010).

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 501851, 14 pages
http://dx.doi.org/10.1155/2013/501851

Review Article

Assessment and Molecular Actions of Endocrine-Disrupting Chemicals That Interfere with Estrogen Receptor Pathways

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Received 8 January 2013; Revised 26 March 2013; Accepted 28 March 2013

Academic Editor: Ewa Gregoraszczuk

In all vertebrate species, estrogens play a crucial role in the development, growth, and function of reproductive and nonreproductive tissues. A large number of natural or synthetic chemicals present in the environment and diet can interfere with estrogen signaling; these chemicals are called endocrine disrupting chemicals (EDCs) or xenoestrogens. Some of these compounds have been shown to induce adverse effects on human and animal health, and some compounds are suspected to contribute to diverse disease development. Because xenoestrogens have varying sources and structures and could act in additive or synergistic effects when combined, they have multiple mechanisms of action. Consequently, an important panel of *in vivo* and *in vitro* bioassays and chemical analytical tools was used to screen, evaluate, and characterize the potential impacts of these compounds on humans and animals. In this paper, we discuss different molecular actions of some of the major xenoestrogens found in food or the environment, and we summarize the current models used to evaluate environmental estrogens.

1. Introduction

Xenoestrogens are natural or industrial compounds found in the diet and environment that are capable of mimicking part of the effects of endogenous estrogens or interfering with estrogen signaling pathways [1]. Xenoestrogens are considered endocrine disruptors, also called endocrine disrupting chemicals (EDCs). The notion of endocrine disruptors appeared at the end of the 20th century, and these chemicals were defined as exogenous compounds that interfere with the signaling pathways of endogenous hormones at the level of their synthesis, storage, metabolism, transport, elimination, and binding to their specific receptors [2]. Additionally, EDCs are characterized by their ability to have deleterious effects on the health of living organisms and their descendants. EDCs can have numerous origins, various chemical structures, and act on various targets at the molecular level (Figure 1 and Table 1, see also [3, 4]).

Xenoestrogens, such as phthalates, can be extremely persistent in the environment. Some EDCs, for example, polychlorinated biphenyls (PCBs), are able to bioaccumulate in the food chain or in several biological matrices (as fats) and often exhibit effects at weak concentrations or in combination [5]. Among the numerous sources of exposure, the ingestion of water or contaminated food, cosmetics, pharmaceuticals, industrial exposure, and contact via professional activities (e.g., pesticides) are the most common. It is important to underline that the exposure to these compounds can have particularly critical effects at the fetal and postnatal stages [6]. Indeed, the development of the nervous system and the reproductive organs can be severely disrupted at these stages, as numerous tissues are particularly sensitive to hormonal regulation.

Many xenoestrogens are synthetic estrogens stemming from human activity, which, due to their use, can enter in contact with living organisms or be released into the environment. For instance, workers in the production of contraceptive pills were exposed to the potent estrogen ethinyl-estradiol (EE2) which is capable of being absorbed by the skin [7]. A correlation was also established between the massive exposure to pesticide DDT (dichlorodiphenyltrichloroethane) by farm laborers and the risks of oligospermia [8]. There is also the notorious example of diethylstilbestrol (DES), considered at its discovery as a miracle pill to

Table 1: Illustration of the structural diversity of estrogenic compounds from diverse origins.

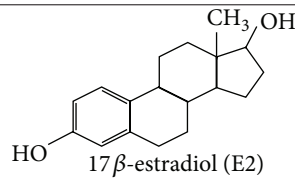
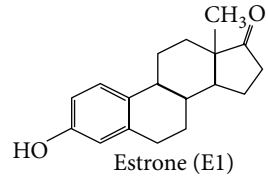
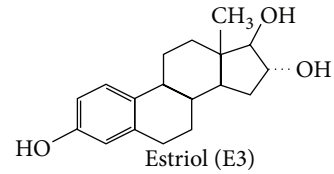
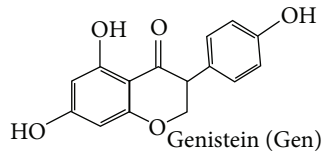
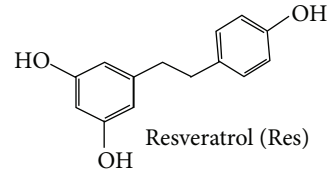
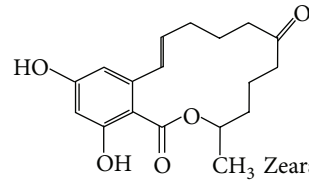
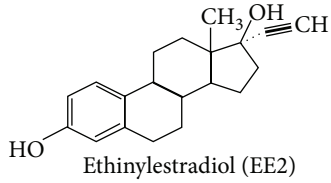
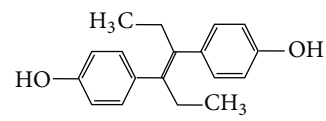
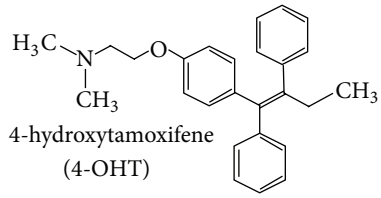
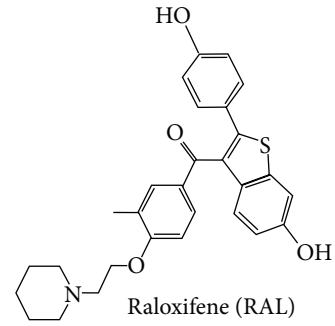
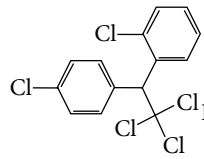
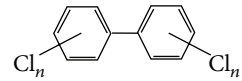
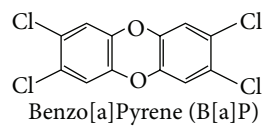
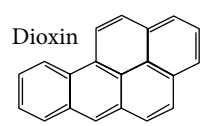
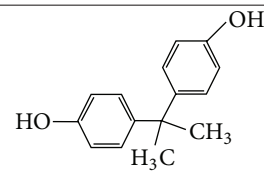
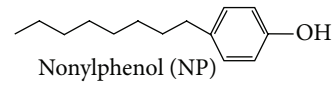
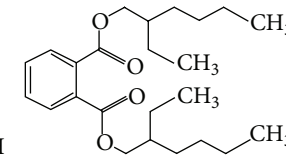
Familly	Compounds
Natural estrogens	 17β-estradiol (E2)  Estrone (E1)  Estriol (E3)
Phytoestrogens	 Genistein (Gen)  Resveratrol (Res)
Mycoestrogens	 Zearalenone
Drugs	 Ethinylestradiol (EE2)  Diethylstilbestrol (DES)  4-hydroxytamoxifene (4-OHT)  Raloxifene (RAL)
Xenoestrogens	
Insecticides	 Dichlorodiphenyltrichloroethane (DDT)
Flame retardant	 Polychlorobiphenyls (PCBs)
Product of incomplete combustion	 Benzo[a]Pyrene (B[a]P)  Dioxin

Table 1: Continued.

Familly	Compounds
Plastic derivatives	 Bisphenol A (BPA)  Nonylphenol (NP)  Diethylhexylphthalate (DEHP)

fight against miscarriages and widely prescribed to pregnant women in the 70's in France. Exposure to this chemical *in utero* induced serious deformities and disorders in the reproductive system of male and female children [9]. More recently, an epidemiological study performed in the French West Indies analyzed the relationship between exposure to chlordecone and the risk of prostate cancer [10]. Chlordecone is an insecticide which exhibits estrogenic-like activity, that was used extensively (from 1973 to 1993) to control the banana root borer, thus contaminating the foodstuffs and population for several years. Interestingly, this study showed a significant increase in the risk of prostate cancer as the plasma chlordecone concentration increased [10]. Of course, these molecules are only a few of the numerous molecules whom estrogenic activities have been demonstrated or are suspected.

As above mentioned, xenoestrogens are not only synthetic compounds, but there are also numerous natural molecules in food that exhibit estrogen-mimetic activities. These natural molecules are mainly phytoestrogens isoflavones, and the most consumed are genistein and daidzein; in particular, these two xenoestrogens are contained in the subproducts of soy and some legumes, fruits, and nuts. Other groups of phytoestrogens such as flavones, coumestans, and lignans were also found [11]. Additionally, certain mushrooms, mosses, or fungi can contain estrogen-like compounds called mycoestrogens, such as zearalenone [11].

2. Estrogen Receptor Pathways

The physicochemical characteristics of estrogens, in particular their liposolubility, allow them to passively enter the cell through the plasma membrane. The majority of estrogen effects are mediated by their binding, in the cytoplasm or directly in nucleus, to estrogen receptors (ERs) which are expressed in numerous cell types. Two ERs, ERα (ESR1, NR3A1), and ERβ (ESR2, NR3A2) were identified in mammals, although numerous splice variants exist (Figure 2(a)).

2.1. Characteristics of ERs. ERs are members of the nuclear receptor superfamily which also includes the glucocorticoid receptor (GR), progesterone receptor (PGR), and androgen receptor (AR). The ability to act as a transcription factor whose activity depends on ligand binding is a common characteristic of most nuclear receptors. ERs are modular proteins that consist of distinct structural and functional domains. The A/B domain contains the ligand-independent transactivation function AF-1. The C domain contains the conserved zinc finger DNA-binding domain (DBD). The D domain contains nuclear localization signals (NLSs), and, finally, the E/F domain carries the ligand-dependent transactivation function AF-2 and the ligand-binding domain (LBD) [12, 13]. Mostly, the estrogen effects mediated by ER occur at the transcriptional level of a large number of estrogen-dependent genes [14–16]. These effects are called "genomic" actions as opposed to the nongenomic actions of estrogens that involve cytoplasmic signaling pathways (Figure 2(b)). These nongenomic effects are rapid effects of estrogens, of the order of a second or of a minute, which result in the activation of several intracellular signaling pathways such as MAPK or PI3K [17]. In addition, numerous studies have described the cross-talk between the genomic and nongenomic actions of ER, allowing a fine regulation of several target genes and increasing the complexity of the estrogenic signalization [18, 19]

2.2. Mechanisms of ER Actions. E2 mediates multiple phenotypic changes in cells by binding to its receptors, ERs that mediate E2 effects through diverse transcriptional mechanisms. Indeed, ERs modulate the expression of E2-target genes by directly binding to the chromatin at a consensus DNA sequence, the estrogen response element (ERE), within the promoter of target genes. This ER-DNA interaction induces the mobilization of the coregulators necessary for transcription (Figure 2(b)). This represents the classical pathway, but numerous E2-sensitive genes do not contain the ERE. ERs thus regulate transcription by interacting with other transcription factors, such as stimulating protein 1 (Sp1) or activator protein 1 (AP1), which are already bound to the promoter [20]. Ligand binding to the receptor induces ER conformational changes. The precise positioning of the helix H12, dependent on the nature of the bound-ligand, is essential for the interaction with coregulators and transcriptional activity of the ER. Thus, the expression of ER-target genes and ER-mediated cellular functions is dependent on both the promoter context and the nature of the estrogenic ligands [21, 22].

2.3. Tissue-Specific ER Expression. ERs are coded by two different genes localized on two different chromosomes, chromosome 6 in the locus 6q25.1 for ERα and chromosome

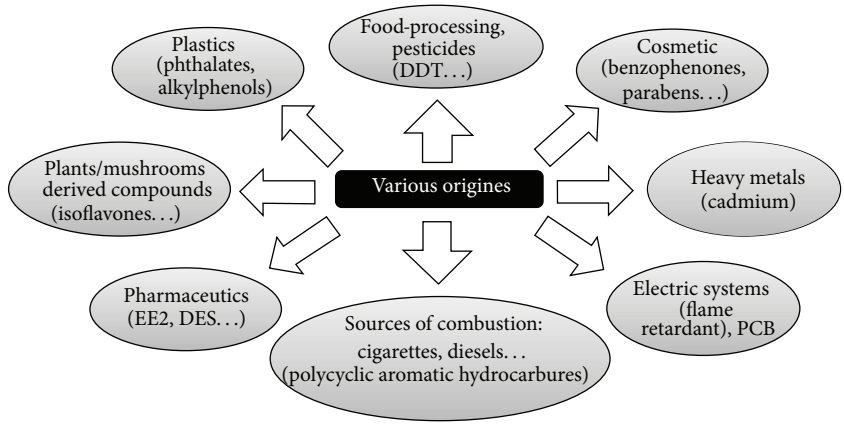


Figure 1: Sources of exposure to xenoestrogens. Various natural or synthetic molecules that enter in contact with humans by alimentation or during professional activities can interfere with estrogenic signaling pathways, explaining the great diversity of origins of the so-called xenoestrogens, illustrated here.

14 in the connection between loci 14q11.1 and 14q11.2 for ERβ [23, 24]. The utilization of different promoters results in multiple variants that code for the same protein, 66 kDa for ERα and 55 kDa for ERβ, but the use of various promoters allows a fine tissue-specific regulation of ER expression, allowing for the modulation of transcript synthesis and regulating their stability and translational efficiency [25, 26]. In addition, several splice variants were identified. Among them, ERα-46 and the ERα-36 are the best-characterized forms. Due to the use of an alternative promoter resulting in the direct splicing to exon 2 of the ERα transcript, ERα-46 is deleted from the N-terminal part of the protein and lacks the AF-1 function. The ERα-46 isoform can inhibit the transcriptional activity of ERα-66 in various cell types [27, 28]. ERα-36 was discovered more recently and lacks both the N- and C-terminal domains, resulting in a form that lacks the two transactivation functions [29]. ERα-36 is capable of acting as a dominant negative form of ERα-66 and is also found anchored at the plasma membrane where it can modulate the activation of intracellular signaling pathways, such as the PI3K/Akt or MAPK signaling pathways.

As a result, various tissues that express the ERs present very variable expression profiles of both ER subtypes. Thus, a strong expression of ERα is observed in tissues related to female reproduction (ovary, womb, mammary gland); ERα is also strongly expressed in men and is the most expressed ER subtype in the testicle (Leydig cells). ERβ is also abundantly expressed in ovaries but expressed a little in the mammary gland. In men, ERβ is expressed in the prostate, germinal cells, and epididymis. In both sexes, lung, hepatic, fat, osseous, nervous tissues, and endothelial cells express both receptors with variable expression levels [30, 31].

2.4. ER Expression during Development. In addition to the diverse roles of estrogens in different target tissues, they have also multiple functions during development, particularly during the development of reproductive tissues such as the ovaries, uterus, and gonads. Estrogens play roles in the development of the brain, as they contribute to neuronal growth and differentiation [32]. ER knockout in mice demonstrated key roles for both ERα and ERβ in gametogenesis. Interestingly, ERα-deficient mice exhibit significantly elevated levels of testicular testosterone secretion compared with wild-type fetal mice [32, 33]. The appearance of ERs appears to be under a spatial-temporally control during development [32]. For instance, the expression of ERα has been detected in the developing uterus as early as fetal day 15 in mesenchymal cells, whereas it appears in the epithelial cells at later fetal stages and increases during the neonatal period. In the rodent cerebral cortex, the expression of ERα is higher in postnatal life and decreases considerably during puberty [34]. However, ERβ distribution in the developing brain of mice showed that ERβ appears mainly in the midbrain and hypothalamus at E12.5, and its expression increased at E15.5 and E16.5. Interestingly, the expression of ERβ appears strongly and widely throughout the brain including the cerebellum and striatum at E18.5, while very few positive cells could be detected in the ventricular region [35].

3. Mechanisms of Xenoestrogen Actions

Xenoestrogens can affect the endocrine system at every level. First, they can disrupt the action of the enzymes involved in steroidogenesis. For example, a perturbation of aromatase activity can modify the estrogen/androgen balance and thus alter the development or the function of reproductive organs, as was observed with the tributyltin and some other pesticides [36]. Other enzymes of the steroidogenesis can be impacted (mostly inhibited), as can the enzymes involved in metabolism of estrogens (Figure 3(a)). For instance, some PCB metabolites inhibit sulfotransferase, resulting in an increase of circulating estradiol rates [37]. The transport of hormones can also be used as the target of certain compounds capable of interacting with the binding sites of SHBG (sex hormone binding globulin), thus competing with endogenous estrogens (Figure 3(b)) [38].

The most studied mode of actions of xenoestrogens is focusing the ability of these chemicals to bind and activate

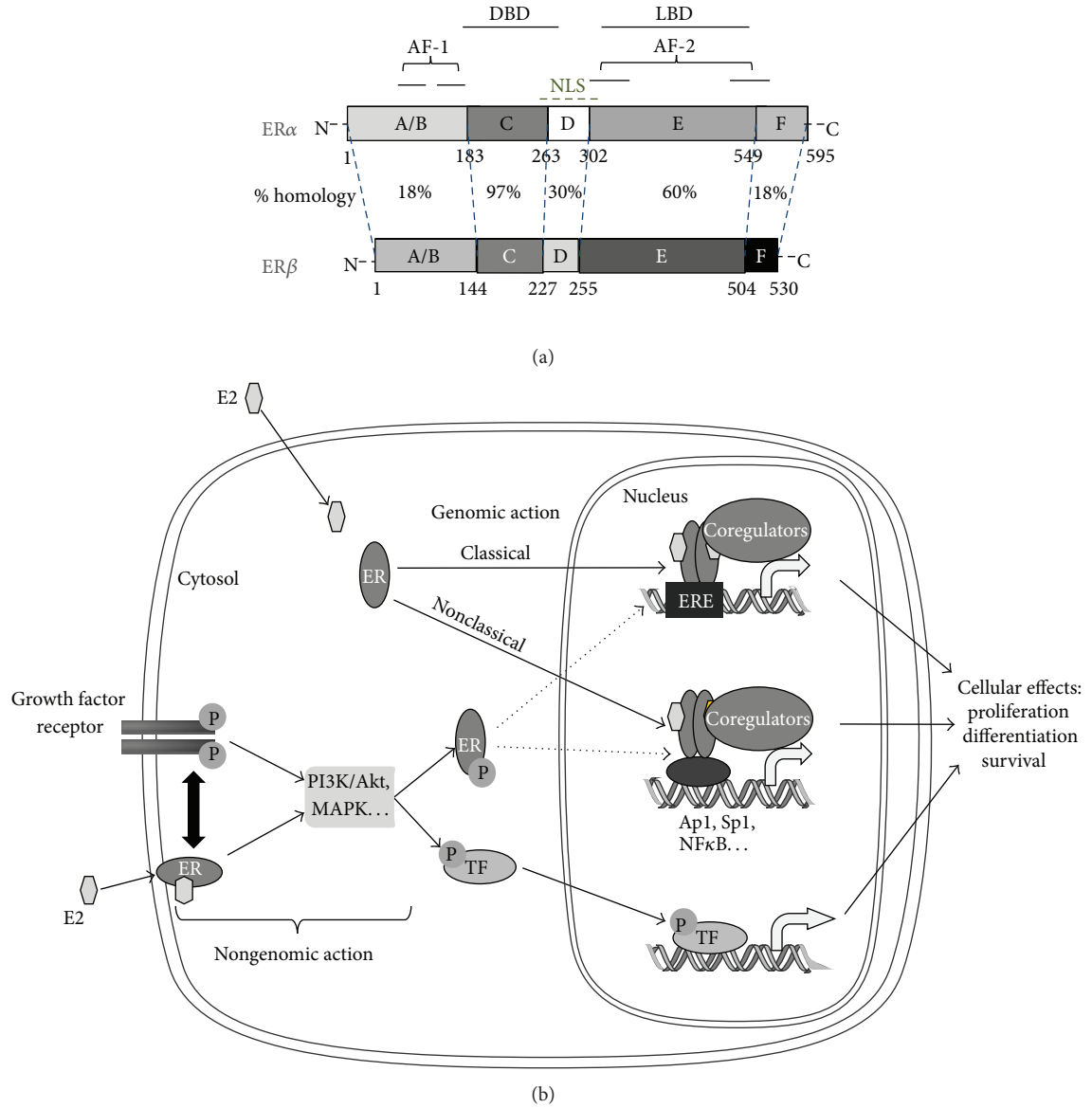


Figure 2: Structure and mechanisms of action of estrogen receptors. (a) ERα and ERβ have an evolutionary conserved modular structure. The percentages of homology between the two forms are presented. The localizations of the ligand-binding domain (LBD) within the E domain and the DNA-binding domain (DBD) within the C domain are also presented. ERs possess two transactivation functions (AF-1 and AF-2), each divided into two subdomains which regulate the expression of target genes and contain a nuclear localization signal (NLS). (b) Due to its lipophilic properties, estradiol (E2) can passively enter the cell, through the lipid membranes. E2 can then bind ERs in the cytoplasm or the nucleus. ER dimers bind to the chromatin to modulate target gene expression. This mechanism corresponds to the genomic action of ERs, but ERs can also exercise nongenomic action, fast, directly in the cytoplasm. Indeed, the cytoplasmic or membrane-bound fraction of ER can induce, after E2-binding, the activation of intracellular signaling pathways independently or in association with the growth factor pathways.

the ERs in target tissues [21]. However, it is of note that the two ERs mediate distinct biological effects in many tissues such as the mammary glands, bone, brain, and vascular system in both males and females. Therefore, because ERα and ERβ show partially different tissue distribution and distinct physiological functions, xenoestrogens could display agonist or antagonist activity in a tissue-selective manner or during development. Considering the significant differences between ER subtypes in structural features and relative ligand binding affinity, xenoestrogens can induce distinct conformational changes in the tertiary structure of the ERs, affecting the recruitment of cofactors differently. These interactions between ERs and coactivators/corepressors are critical steps in ER-mediated transcriptional regulation and consequently the modulation of the expression of ER-target genes. For example, the phytoestrogen genistein exhibits an affinity for ERβ that is 20-fold superior to its affinity for ERα [39]. Moreover, the genistein effect is often tissue specific because it depends on numerous factors such as the expression of specific cofactors, the ERα/ERβ ratio, and the

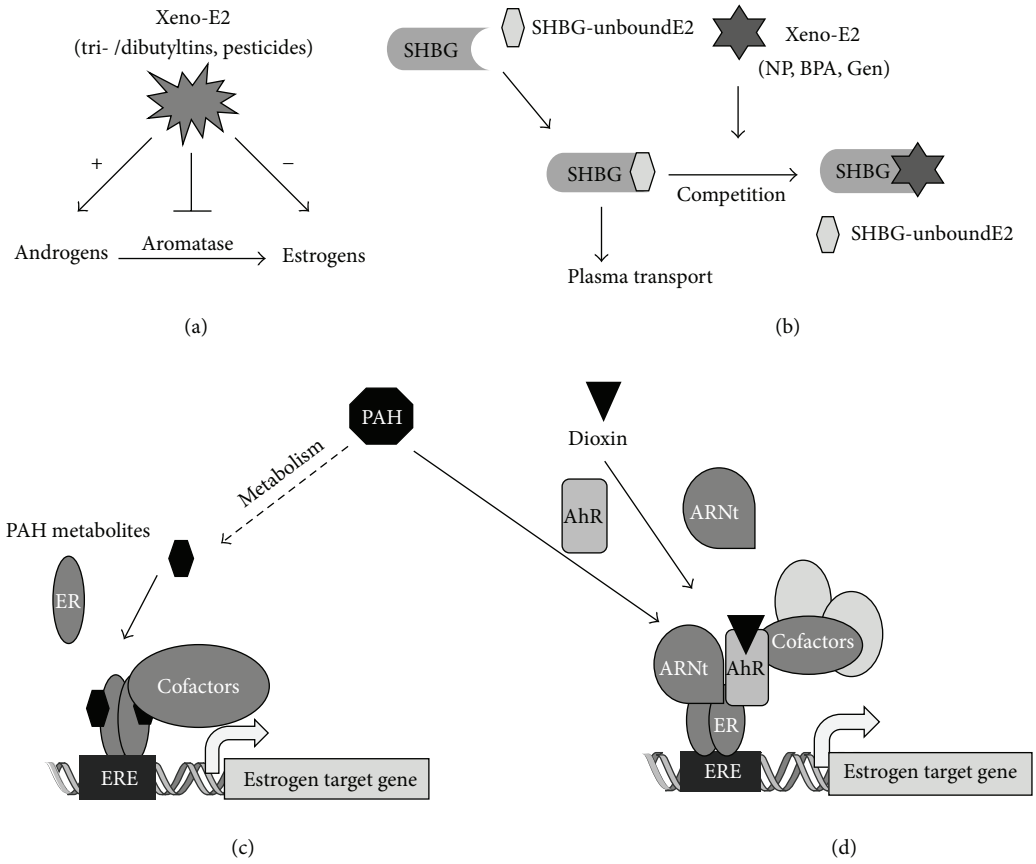


Figure 3: Examples of interaction between xenoestrogens and estrogen signaling pathways. (a) Some xenoestrogens, such as tributyltins, can inhibit aromatase, the enzyme responsible for the conversion of androgens in estrogens, resulting in the perturbation of the androgen/estrogen balance. (b) Other groups of compounds can interfere with estrogenic signaling by competing with natural estrogens for binding to sex hormone binding globulin (SHBG), resulting in defects in E2 plasma transport. ((c) and (d)) Interaction between polycyclic aromatic hydrocarbons (PAHs) and ERE-dependent E2-target gene transcription. (c) Some PAH metabolites can bind ER, resulting in the recruitment of ERs at the ERE and, subsequently, in the recruitment of coregulators that modulate the expression of E2 target genes. (d) Some PAH metabolites or dioxin are also capable of binding to the aryl hydrocarbon receptor (AhR), resulting in heterodimerization with aryl hydrocarbon nuclear translocator (Arnt). This transcriptionally active complex can then interact with ligand-unbound ER at the ERE site and modulate E2 target gene expression.

level of expression of certain intracellular kinases, including cytoplasmic tyrosine kinases. Genistein has been reported to have proliferative and antiproliferative effects in cancer cells [40].

Xenoestrogens generally act in 100–1000 folds greater concentrations than estradiol but can have additive or synergic effects with endogenous estradiol or when they are present in combination [5]. Furthermore, the ability of some xenoestrogens to act as agonists in certain tissues and as antagonists in the others leads to the development and use of selective ER modulators (SERMs), in particular for antihormonal treatments, such as tamoxifen and raloxifene.

Some xenoestrogens can also affect the ER nongenomic pathways and induce an endocrine disruption [41]. For instance, a recent study performed on structurally different xenoestrogens showed that at high concentrations, bisphenol A (BPA) and diethylstilbestrol (DES) are able to activate ERs via the activation of MAPK and PI3K in breast cancer cells. In addition, the activation of PKC by some xenoestrogens has been observed [42, 43]. Interestingly, PKC has been reported to modulate ERα transcriptional activity [44]. Therefore, synergic or additive effects between these pathways to combine the activation of ER signaling could be envisaged.

Although the mechanistic studies on the interaction between dioxin and estrogen produced conflicting results, several studies reported that the ligands of AhR, such as polycyclic aromatic hydrocarbons (PAHs), can also affect estrogenic signaling in mammary or uterine cells (Figure 3(c)). Ohtake et al. [45] showed that an AhR agonist, methylcholanthrene (3MC), is able to activate a reporter gene containing an ERE without affecting the expression level of ER. However, when this promoter is activated by estradiol, 3MC has an antagonistic effect. Coimmunoprecipitation assays showed that these functional interactions are correlated with the physical interaction between AhR and ER. The proposed model (Figure 3(d)) suggests that 3MC activates AhR, which dimerizes with Arnt. The AhR/ARNT heterodimer can directly associate with ER that is not ligand

bound to activate estrogen-sensitive gene transcription by recruiting the coactivator p300 [45]. This model is consolidated by *in vivo* experiments performed in the mouse. In fact, the proliferative effect of 3MC on the uterus is observed in ovariectomized mice, but not in AhR knockout mice. These studies suggest an original mechanism of activation of ERα in the absence of estradiol because ligand-activated AhR is able to cooperate with the ER that is not ligand bound to activate transcription. However, some metabolites of AhR ligands, including 3MC, could also behave as partial agonist on estrogen signaling pathways by direct interaction with ERα (Figure 3(a)).

Several mechanisms have also been proposed to describe the antiestrogenicity of AhR ligands [46]. By binding to AhR, these compounds could interfere with transcriptionally active ER/SP1 or ER/AP-1 complexes [47–49]. They can also inhibit the binding of ER to ERE sites by direct association with ERα [45]. The antiestrogenic effects of dioxins could also be mediated by the reduction of ERα protein level through activation of the proteasome [50]. However, AhR-mediated degradation rates may vary according to the specific cellular context [46]. Therefore, these interactions should be taken into account in the interpretations of studies that investigate the estrogenic effects of AhR ligands, particularly in mixtures.

Similarly, both the potent estrogenic and antiestrogenic effects of the heavy metal cadmium (Cd) have been reported *in vitro* in mammary cell lines, recombinant yeast assays, or fish hepatocyte cultures and *in vivo* in the rodent uterus [51–53]. Although the precise mechanisms underlying the effects of Cd as an endocrine disruptor remain unclear [54], two different mechanistic explanations were suggested. Cd could directly interact with the LBD of ERα, inducing a conformational change in LBD that favors the interaction between helix 12 of ERα with transcriptional coactivators [55]. Other studies suggested that the interaction of Cd with the LBD of ERα induces conformational changes in the DBD which could inactivate the DNA binding activity of the receptor, reducing transactivation [51, 53]. However, cadmium is not the sole heavy metal able to interfere with estrogen signaling pathways, even if the precise modes of actions of these metalloestrogens are largely misunderstood [56, 57].

Because EDC can also modulate hormonal signaling indirectly via their metabolites, EDC metabolism should be taken into account in the evaluation and identification of their mechanisms of action. For instance, the insecticide DDT and its metabolite DDE (dichlorodiphenyldichloroethylene) were characterized as weak estrogens in the environment and were suspected to affect reproduction function in several animal species [58]. More recently, DDE, which is highly lipophilic and resistant to biodegradation, was identified as the compound that induced the feminization of alligators (i.e., micropenis and various abnormalities of the testes) from Lake Apopka. These effects are likely mediated by the inhibition of androgen signaling during the critical developmental window [59]. In fact, although DDE shows low affinity to ERs, it is capable of binding to AR and repressing the transcriptional activity of this receptor. This antiandrogenic action of DDE shown in different cell-based assays could clearly cause abnormalities in the steroidogenic cells of rat testis and could disturb the development and function of fetal testis [60–62]

4. Assessment and Quantification Methods, Biosensors, and Bioassays

In environmental monitoring, there are two major questions: what is the quantity of each pollutant in an environmental sample, and what is the molecule's effect on humans and wildlife? To answer these two questions, several methods have been developed. These methods have progressed with the understanding of estrogenic functions at the organismal, organ, cell, and molecular levels. Due to this diversity of EDC actions, evaluation and quantification require physicochemical, biophysical, biochemical, cellular, and whole organism-based methods.

4.1. Analytical Methods. The most widely used methods for the quantification of estrogenic compounds are analytical methods such as high-performance liquid chromatography (HPLC), gas chromatography/mass spectrometry (GC/MS), GC-spectrometry of mass coupled (MS/MS), and liquid-phase chromatography (LC-MS/MS). These methods allow the extensive identification and quantification of compounds with estrogenic properties, within solid or liquid samples [63]. However, these methods are not directly sensitive enough for the direct measurement of the estrogenic compounds contained in an environmental sample. These methods need therefore a preconcentration step to increase the concentration of compounds between 100 and 1000 folds. Moreover, the preconcentration step is aimed to specifically extract the estrogenic compound through liquid-liquid, solid-phase extraction (SPE), solid-phase microextraction (SPME) or stir bar sorptive extraction (SBSE) [64–69]. These extractions methods are determined by the chemical properties of the target molecules. The elution leads to specific concentration of the compound in the adequate solvent for chromatography analysis. For a technical review see Farré et al. [70]. Together, these techniques allow for a precise detection of the compounds with a low limit of detection, but they do not provide information about the estrogenic or antiestrogenic properties of these molecules. Furthermore, these methods target specific molecules which imply that the estrogenic potential of the molecules of interest was already identified. However, using bioassays ranging from *in vitro* receptor binding assays, tissue culture, and cell-based assays, and *in vivo* animal models can overcome most analytical drawbacks.

4.2. In Vivo Methods for Estrogenic Potency Assessment. The use of the whole organism methods presents the advantage of an *in vivo* evaluation of the estrogenic potential of molecules in biological functions or in the expression of markers of hormonal exposure. These approaches have been developed in amphibians, fishes, rats, and mice to estimate the estrogenicity of compounds [71]. The uterotrophic test is based on the strong proliferative effect that estrogens have in the

rodent female genital tract. This test is commonly conducted with measurements of the uterus weight of immature or ovariectomized rodents. This test has widely been used by researchers for estrogenic compound evaluation [72, 73] and has been validated by OECD and the endocrine disrupters testing assessment group (EDTA) [74]. Other *in vivo* tests examine the expression of the vitellogenin of male fishes, by ELISA or Western blot [75]. The induction of vitellogenin after exposure to estrogenic compounds has been demonstrated in several fish species [76, 77]. Transgenic mice and zebrafish that express an easily quantifiable reporter gene were also recently developed. These models allow for the specific expression of estrogen-dependent genes in different cell types [78–81].

4.3. In Vitro Cell-Based Methods for Evaluation and Quantification. *In vitro* cell-based assays using cell lines offer a good sensibility but do not allow the determination of the specific effect of a particular xenoestrogen in an environmental sample containing several compounds. The use of these assays in environmental monitoring gives global information of the estrogenic potency of the sample. Moreover, these *in vitro* bioassays do not elucidate the overall effects of the biotransformation and pharmacokinetics of compounds. However, these assays provide a method to quickly estimate the total estrogenicity of a mixture or given compound and generally require less expensive equipment than the analytical methods.

Several bioassays have been developed for the estrogenic potency assessment. The estrogenic actions evaluated by these methodologies are based on the estrogenic action in cells, for instance, the proliferation of ER-positive breast cancer cell lines (MCF7, T47D), known as E-screen [82], and optimized by several authors to ameliorate detection [82–85]. Other bioassays are based on the capacity of estrogenic compounds to bind and activate ER. These assays that induce an estrogen-regulated gene were previously reported as ER-CALUX [86], YES assay in yeast [87–89] and various reporter gene assays [90, 91]. These assays target ERE, SP1, and AP1 regulated genes [22, 92]. Other methods use the differentiation of ER-positive cell lines to evaluate the estrogenic potency of EDC [93]. Together, these methods permit the evaluation of estrogenic potency of compounds and, in some cases when test is sensitive enough, the environmental quantification of estrogenic compounds [83, 85, 94].

4.4. Biosensor. The term biosensor appeared in 1962 when the first method was developed for the detection of glucose concentration in blood sample thanks to amperometric method [95]. A biosensor consists of two parts, the biological recognition element and the transducer. The biological recognition element is able to interact specifically with the target, while the transducer is able to convert the biological recognition event into an electrical signal because of physical property changes (Table 2). Several strategies have been established to optimize the couple biological transducer [96, 97].

Table 2: Biological recognition elements and transducers usually used in the development of biosensors.

Biological recognition elements	Transducers
(i) Cells	(i) Optical
(ii) Membranes	(a) Fluorescence (BRET, FRET,
(iii) Nucleic acids	fluorescence anisotropy ⋯)
(iv) Protein:	(b) Colorimetry
(a) Antibodies	(c) Surface plasmon resonance (SPR)
(b) Enzymes	(d) Reflectometric interference
(c) Membrane	spectroscopy (RfIS)
receptors	(ii) Electrochemical
(d) Nuclear	(a) Amperometric
receptors	(b) Conductimetry
(e) Peptides	(c) Potentiometry
	(iii) Piezoelectric
	(a) Quartz crystal microbalance (QCM)
	(iv) Thermal
	(a) Differential scanning
	microcalorimetry (DSC)
	(b) Isothermal microcalorimetry (ITC).

As detailed in Table 2, there are many possibilities in the combination of biological recognition element and transducer. However, in the development of methods for the evaluation and quantification of estrogenic compounds, the main biological recognition elements used are as follows: antibodies against estradiol [98], estrogen receptor (complete ER protein [99, 100], LBD [101, 102] or recombinant and genetically modified ER [103–105]), ER dimerization [106], DNA binding [107–111], and finally the ER interaction with cofactors. The transducers usually used are the following: fluorescence anisotropy [112–114], surface plasmon resonance (SPR) [108–111], reflectometric interference spectroscopy (RfIS) [102], fluorescence resonance energy transfer (FRET) [115], and bioluminescence resonance energy transfer (BRET) [103, 104]. However, the high diversity of the biological recognition elements and transducers that are usually used in biosensor methods for the evaluation of estrogenic compounds makes comparison between methods difficult. While the time of responsiveness from these methods is generally shorter than with the cellular methods, it is currently not sensitive enough to use them for environmental detection. Therefore, for environmental monitoring purposes, a preconcentration step is currently needed.

5. Conclusion

The origin and the exposure sources of xenoestrogens are multiple. They can come from food, products of combustion, and agricultural and industrial chemicals. Because xenoestrogens have varying structural complexity and produce a great number of metabolites or biodegradation products in the environment, they exhibit various mechanisms of action. Moreover, these mechanisms could differ depending on the

cellular and tissue context. For instance, it has been reported that the widespread environmental contaminants PAHs have both estrogenic and antiestrogenic activity [116]. Similarly, both potent estrogenic and antiestrogenic effects and an androgen-like effect of Cd have been shown *in vivo* and *in vitro* [51–53].

In addition to the direct actions of xenoestrogens in primarily exposed organisms which usually result in the modulation of gene expression and potentially in phenotype alterations, there is increasing evidence to suggest that EDC can also act across generations. For instance, a study in a mouse model showed an increase in uterine adenocarcinoma in the female descendants (lineage F2) of mice exposed developmentally to diethylstilbestrol [117]. Parental exposure to environmental contaminants could thus induce epigenetic modifications and gene expression alterations that can pass from one generation to the next, resulting in physiological changes in their offspring [118]. It has been reported that the exposure during development to the fungicide vinclozolin induces a reduction of fertility in treated male animals that is transmitted through four generations without further exposure to vinclozolin [119–121]. This pesticide has been characterized as an antiandrogenic compound, and some of its metabolites could interact with other steroid receptors including the receptors for progesterone, glucocorticoids, and mineralocorticoids. Thus, vinclozolin could interfere with hormone signaling pathways during development, but it is currently not known whether the effects of vinclozolin are mediated by its interference with hormonal signaling during development [122–125].

These studies exemplify the diversity and complexity of xenoestrogen effects and the need for the further understanding of the diversity of their molecular actions. In particular, the effort concerning xenoestrogen effects in epigenetic modifications at the DNA sequences and chromatin-associated proteins should be a priority research.

It is important to emphasize that xenoestrogens do not necessarily mediate their effects by binding to specific nuclear ERs. Indirect effects can thus be considered. For instance, modifications on the expression or activity of their associated protein kinases, enzymes, or transcription factors necessary for the activity of the specific ER subtypes (DNA binding, phosphorylation, transactivation, degradation, and subcellular translocation) [37, 40].

However, identifying the G-protein-coupled receptor homologue GPR30 as the plasma membrane receptor for estrogens provides a higher level of complexity to the mechanisms of action of these hormones [126]. GPR30 is able to bind 17β-estradiol and allows fast nongenomic responses of estrogens such as the stimulation of MAPK pathways, adenylyl cyclase, or c-fos expression in the breast cancer cell line SKBR3 which does not express the classical ERs [127–129]. Notably, several phytoestrogens, such as genistein and quercetin, or other xenoestrogens such as bisphenol A, zearalenone, and nonylphenol, have been shown to bind to this membrane estrogen receptor [130, 131]. Because GPR30 is expressed in a wide number of cell types, it could potentially mimic environmental estrogen effects in a great number of tissues. The further characterization of cellular and tissue distribution and the mode of action of GPR30 and other plasma membrane receptors for steroid hormones will likely contribute to a better comprehension of the xenoestrogen actions in relation to the important number of physiological roles played by estrogens.

The assessment of environmental estrogens has greatly increased in the past decade in different areas such as the development of biomarkers, cell- and animal-based bioassays, bioinformatics, and bioanalytical and biosensor technology [51, 78, 82, 86, 110]. To elucidate the estrogenic or antiestrogenic properties of suspected compounds, several *in vivo* screening approaches, which generally cover the kinetics and potential degradation of compounds, were developed. A transgenic mouse model expressing an estrogen-dependent green Fluorescent Protein (GFP)-based reporter gene constitutes a powerful animal model because it provides a method to determine the *in vivo* delivery, stability, and tissue specificity of the compounds within the mammalian body [79]. More recently, a similar method was adapted to nonmammalian vertebrates such as zebrafish [78, 80, 132]. Transgenic zebrafishes constitute an interesting animal model because of their rapid and ex-utero development, the transparency of their embryos, and their small size. While the assessment of xenoestrogens by *in vitro* assays does not fully take into account metabolism and pharmacokinetics, some of these assays are notably valuable tools for (i) the high specificity of responsiveness, (ii) the high throughput screening of large numbers of chemicals, and (iii) the determination of molecular and cellular actions of the environmental contaminants and identification of their signalization pathways and cofactor and ER selectivity. Therefore, the combination of *in vivo* and *in vitro* approaches is necessary to obtain a better understanding of the molecular actions of xenoestrogens.

Acknowledgments

This work was supported by fellowships from the Brittany Region (to G. Kerdivel) and funds from the French Ministry of Ecology, Energy and Sustainable Development (PNRPE); the INSERM and CNRS; La Ligue Contre le Cancer (Grand-Ouest, comités 35 et 56), IFR140 Biosit, the University of Rennes 1; and the European University of Brittany.

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 650984, 15 pages
http://dx.doi.org/10.1155/2013/650984

Review Article

Diverse Effects of Phytoestrogens on the Reproductive Performance: Cow as a Model

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Received 14 January 2013; Accepted 4 March 2013

Academic Editor: Ewa Gregoraszczuk

Phytoestrogens, polyphenolic compounds derived from plants, are more and more common constituents of human and animal diets. In most of the cases, these chemicals are much less potent than endogenous estrogens but exert their biological effects via similar mechanisms of action. The most common source of phytoestrogen exposure to humans as well as ruminants is soybean-derived foods that are rich in the isoflavones genistein and daidzein being metabolized in the digestive tract to even more potent metabolites—*para*-ethyl-phenol and equol. Phytoestrogens have recently come into considerable interest due to the increasing information on their adverse effects in human and animal reproduction, increasing the number of people substituting animal proteins with plant-derived proteins. Finally, the soybean becomes the main source of protein in animal fodder because of an absolute prohibition of bone meal use for animal feeding in 1995 in Europe. The review describes how exposure of soybean-derived phytoestrogens can have adverse effects on reproductive performance in female adults.

1. Introduction

The present paper focuses particularly on soybean-derived isoflavones and summarizes recent knowledge on their biological impact on ruminant and human reproduction. Phytoestrogens belong to a heterogenous group of herbal substances with their structure similar to estradiol-17β (E_2). They are called estrogen-like molecules or nonsteroidal estrogens structurally similar to E_2. Phytoestrogens are diphenolic as well as nonsteroidal compounds.

Systematically, the group of phytoestrogens includes over 100 molecules, divided according to their chemical structure into: isoflavones (genistein, daidzein, glycitein, and formononetin), flavones (luteolin), coumestans (coumestrol), stilbenes (resveratrol), and lignans (secoisolariciresinol, matairesinol, pinoresinol, and lariciresinol) [1] (Figure 1). Isoflavones are found at high concentrations in soybean products whereas lignans are found in flax seed, coumestans are found in clover, and stilbenes are found in cocoa- and grape-containing products, particularly red wine.

Phytoestrogens have recently come into considerable interest due to the following facts: first increasing information on their adverse effects in human and animal reproduction, second an increasing number of people substituting animal proteins with plant-derived proteins. Finally, the soybean becomes the main source of protein in animal (especially, dairy cows, pigs, and poultry species) fodder because of an absolute prohibition of bone meal use for animal feeding in 1995 in Europe.

There is some evidence that consumption of soy diets containing phytoestrogens has some positive effects on human and animal health. Phytoestrogens as potent antioxidants [2] are thought to reduce the risk of mammary cancer [3, 4], prevent cardiovascular disease [5], stop the progression of

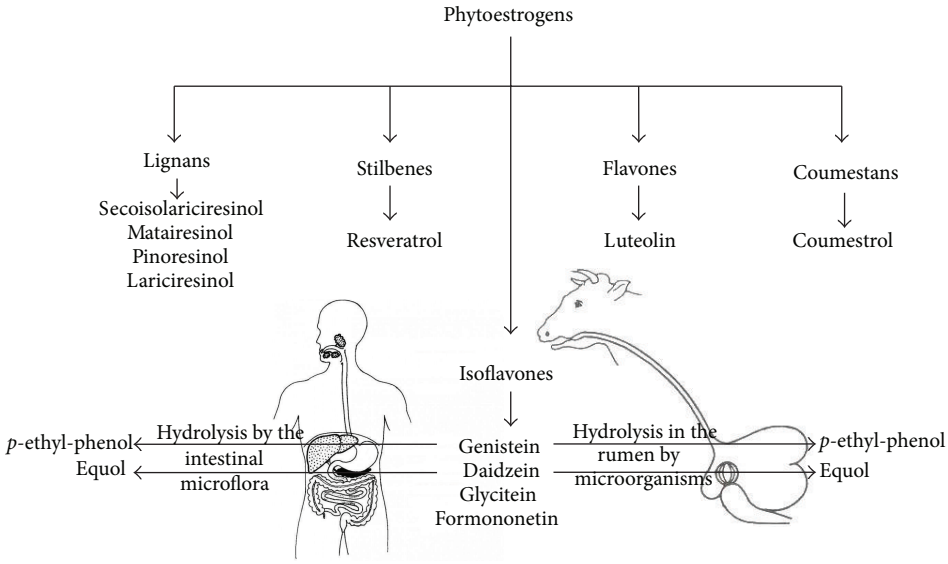

Figure 1: Classification and metabolism of phytoestrogens.

atherosclerosis [6], or have positive effects on hot flushes, vaginal symptoms, cognitive function, or dementia in postmenopausal women [7]. On the other hand, these substances also have some hazardous effects, especially in animals fed with pasture rich in phytoestrogens [8, 9]. The earliest evidence that naturally occurring phytoestrogens could cause reproductive disturbances in mammals was reported in 1946 by Bennetts et al. [10] indicating that ingestion of clover pasture rich in plant estrogens caused infertility in sheep. About 20 years later, a similar observations had been noted in cows that had fertility disturbances resulting from periods of feeding with red clover [11, 12]. Similarly, abnormalities in reproductive health due to high intake of soy products have been reported in women [13–16]. These observations demonstrate that dietary phytoestrogens can have adverse effects on reproductive performance in female adults.

2. Mechanism of Isoflavone Action

Environmental estrogens exert their effects through classical, genomic, or nongenomic pathways (Figure 2). Due to their similarity with the endogenous hormones, these compounds can bind to nuclear receptors. Their affinities for ERα and ERβ are relatively weak compared to endogenous E_2; thus, they can have agonist or antagonist activity depending on the presence of E_2 [17]. It has been proved that some isoflavones are selective estrogen receptor modulators that have higher affinity to ERβ than ERα [18, 19]. Environmental estrogens have much lower (up to 100 fold) affinity for nuclear receptors compared to the endogenous ligands (E_2). Thus, even low concentrations of environmental estrogens can trigger an altered response of the biological systems. This interference is often achieved by the activation of nongenomic pathways. There are numerous nongenomic pathways affected by isoflavones, such as nongenomic signaling mediated by oxidative stress pathways, tyrosine kinases, nuclear factor-kappaB, and extracellular-signal-regulated kinases [20, 21]. In addition to classical ERs, isoflavones serve as ligands for peroxisome-proliferator-activated receptors, the nonclassical estrogen receptor GPER1, the estrogen-related receptors, and the aryl hydrocarbon receptor [20, 22–24]. Besides these direct actions to modulate signaling pathways, isoflavones can alter epigenetic marks by altering activities of DNA and histone methyltransferases, NAD-dependent histone deacetylases, and other modifiers of chromatin structure [25–27]. The last, described in the literature, way of isoflavone action in the cells is the competitive inhibition of the production of endogenous E_2 by aromatase [27, 28]. The action of isoflavones in the human or animal body is even more complex since these substances are usually present *in vivo* as mixtures of several dietary components that can affect various signaling pathways or affect the same pathways in opposing directions.

3. Adverse Effects of Isoflavones on the Reproductive Performance in Ruminants

3.1. Metabolism and Bioavailability of Phytoestrogens. In the late 80s and early 90s, there were a lot of studies on feeding dairy cows with synthetic fodder containing phytoestrogens. The fodder commonly used for feeding dairy cattle contains phytoestrogens, such as genistein, daidzein, formonentin, and biochanin A [29]. Lundh et al. [30] showed that in cows and ewes daidzein and genistein present in the fodder are immediately converted in the rumen to equol and *p*-ethyl-phenol, respectively (Figure 1). The concentration of daidzein and genistein decreases within one hour after feeding, whereas equol and *p*-ethyl-phenol are present in

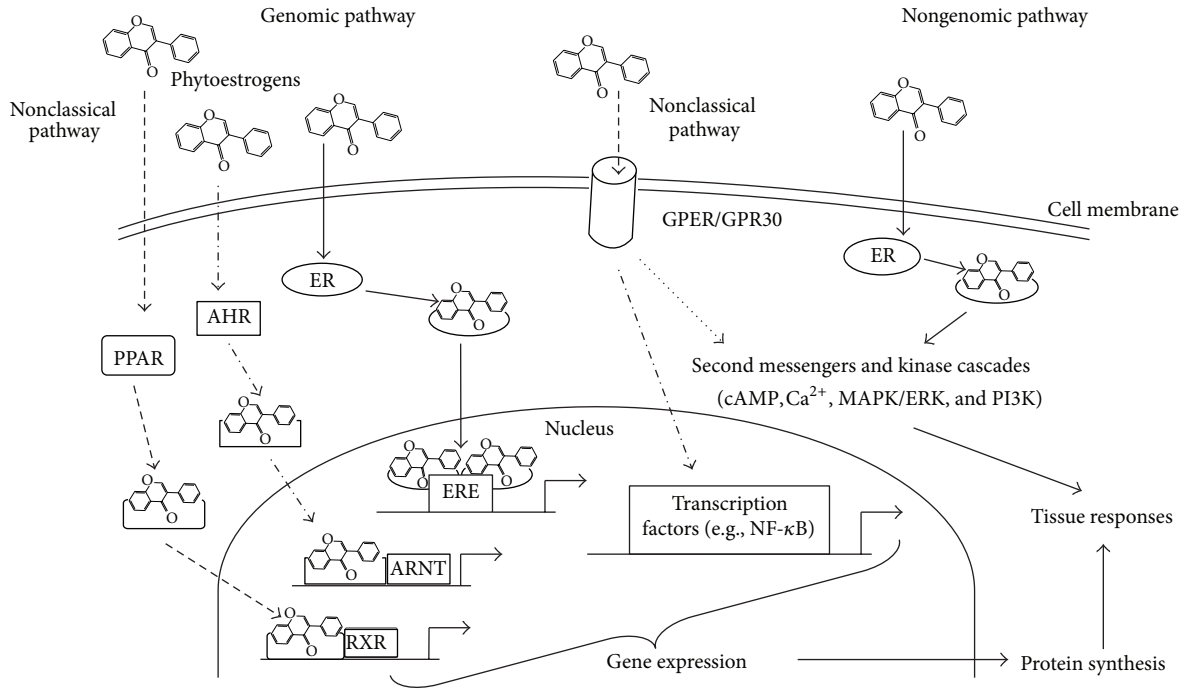

Figure 2: Schematic model illustrating the possible mechanisms of phytoestrogen action (the abbreviations on the figure stand for: AHR—aryl hydrocarbon receptor; ARNT—AHR nuclear translocator; ER—estrogen receptor; ERE—estrogen response element; cAMP—cyclic adenosine monophosphate; Ca^{2+}—calcium ions; GPER/GPR30—G protein-coupled estrogen receptor 1; MAPK/ERK—mitogen-activated protein kinases/extracellular-signal-regulated kinases; NF-κB—nuclear factor kappa-light-chain-enhancer of activated B cells; PI3 K—phosphatidylinositide 3-kinases; PPAR—peroxisome-proliferator-activated receptor; RXR—retinoid X receptor).

the blood of cows for many hours after feeding [30]. The metabolism of phytoestrogens from synthetically prepared fodder, rich in phytoestrogens was thoroughly investigated by Lundh et al. [29, 30]. However, we were the first to study the effects of feeding cattle with fodder rich in phytoestrogens derived from natural soybean [31, 32]. At the beginning, we established which metabolites of phytoestrogens are present in the blood of cows fed a diet rich in soybean. We found large amounts of daidzein and genistein in the soybean commonly used for feeding dairy cattle [31]. These phytoestrogens occur in plants as glycosides and are hydrolysed in the rumen by microorganisms [33]. Daidzein is metabolized in the rumen to equol, whereas genistein is metabolized to *p*-ethyl-phenol [30, 33]. We found high concentrations of both of these metabolites in blood plasma and urine of the cows fed with high-soybean-based diet [31].

We have also used a cow model to compare metabolism of phytoestrogens in cyclic versus early-pregnant and late-pregnant heifers [32]. In this study, we found that in the blood plasma of the early- and late-pregnant heifers, there were lower concentrations of daidzein and genistein compared with control heifers at the mid luteal stage of the estrous cycle (Figure 3). In the blood plasma of the early-pregnant heifers, we noticed the decreases in isoflavone concentrations beginning at 3 h after soybean feeding, which was explained by acceleration of their metabolism leading to increases in the concentrations of their active metabolites, equol and *para*-ethyl-phenol [32] (Figure 3). In the late-pregnant heifers, we did not notice any increase in isoflavone metabolite concentrations after soybean feeding compared with the cyclic animals [32] (Figure 3). Taking other studies and above data into consideration, isoflavone absorption, biotransformation, metabolism, and bioavailability depend on various factors such as differences in digestive conditions, differences in the hormonal status of the animal during early and late pregnancy, and perhaps the most important factor, differences in immunological conditions connected with the phase of pregnancy [32, 34]. We also found out that during early pregnancy different isoflavone metabolism resulted from β-glucuronidase activation because of prompt changes in the immune system leading in turn to release of active forms of isoflavones into the blood plasma [32]. β-Glucuronidase is the enzyme responsible for isoflavone metabolism and biotransformation. It activates the release of free active forms of isoflavones from inactive conjugated with sulphuric and glucuronic acid forms. We have shown that isoflavone absorption and the concentrations of their metabolites in the blood plasma of late- or early-pregnant animals are completely different from those of animals during the estrous cycle [32] (Figure 3). Therefore, it could be assumed that there is some hormonal mechanisms that may lead to a decrease of soy-derived phytoestrogen absorption and deceleration of their metabolism, resulting in a lower active phytoestrogen metabolite concentration/accumulation in the blood plasma during late pregnancy in cows [32]. In fact, physiological status (cyclicity or pregnancy) of the female influenced

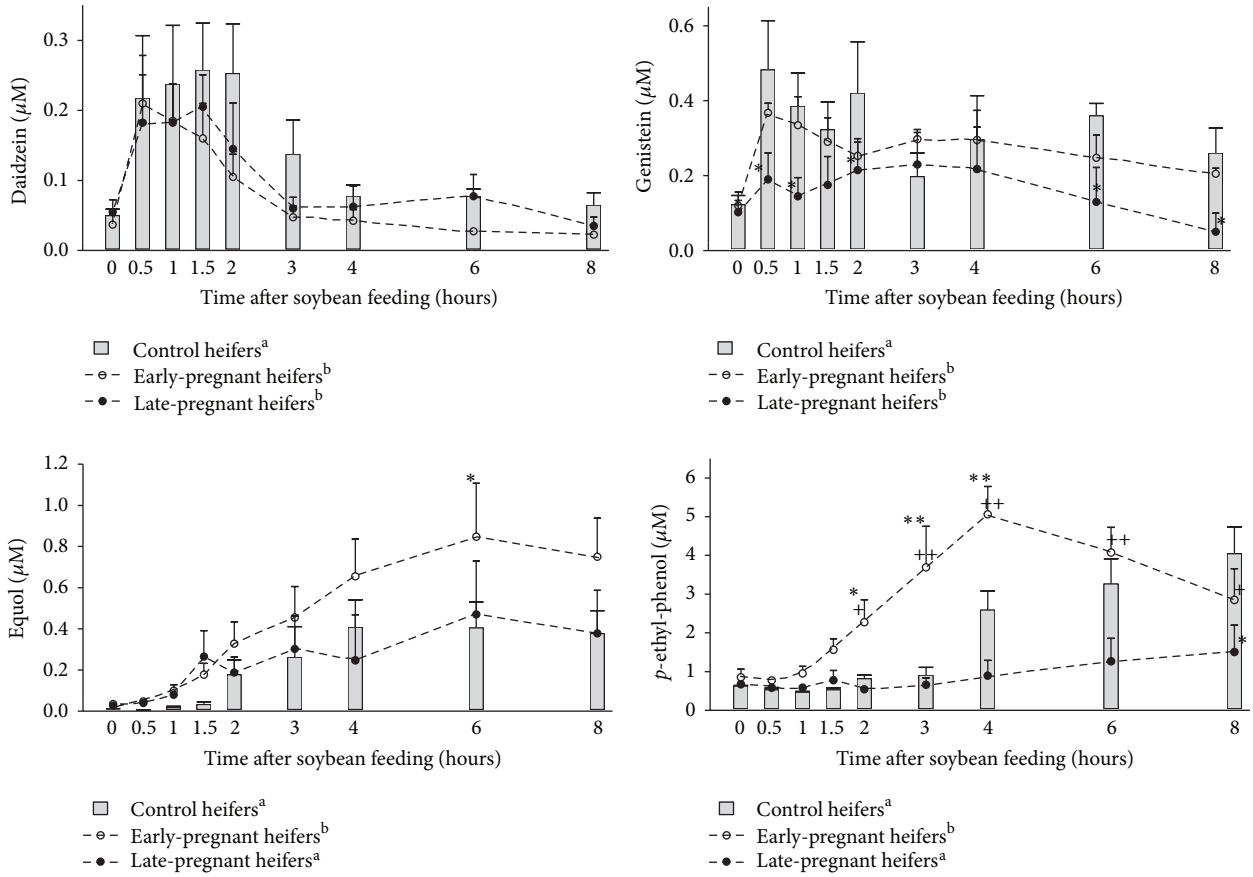

Figure 3: Time-dependent effect of soybean feeding on the concentrations of daidzein, genistein, equol, and *para*-ethyl-phenol in the blood plasma of the control, early-pregnant, and late-pregnant heifers (adapted from [32]).

the concentration/accumulation of isoflavone metabolites in the blood plasma of the heifers. Pregnancy had different effects on isoflavone absorption, biotransformation, and metabolism that resulted in higher concentrations of active metabolites of isoflavones during early pregnancy compared with lower concentrations during late pregnancy. Therefore, we surmised that early-pregnant heifers were more sensitive to hazardous active isoflavone metabolite actions than cyclic or late-pregnant heifers, and this in turn suggests that there are some other mechanisms preventing hazardous increases of the active metabolites of phytoestrogens in the blood plasma during late pregnancy [32]. Moreover, Kindahl et al. [35] documented that endogenous steroid metabolism changes during pregnancy due to various metabolic changes are connected with the conceptus. The data of Kindahl et al. [35] and our own [32] prove that exogenous estrogen metabolism changes during early pregnancy.

In humans, isoflavone absorption and bioavailability depend also on intestinal bacteria [36], gut transit time, fecal digestion rates, and the fiber content in the diet [37]. It has recently been reported in humans that within different physiological and pathological statuses, especially those connected with immune system mobilization, there is acute activation of β-glucuronidase activity leading to the release of active isoflavones into the blood plasma [38]. It has also been reported that this type of physiological immune system mobilization takes place during early pregnancy [34]. On the other hand, it has been known for a long time that the immune signals related to new embryo development are not only local, but spread very quickly throughout the entire female organism [39].

We also were the first to use the cow model to study isoflavone absorption and the concentrations of their metabolites in the blood plasma of the cows with the inflammation (experimentally induced *mastitis* and *metritis*) in comparison to healthy animals [40]. We found that the decrease in genistein concentration in the blood plasma of the cows with experimentally induced *metritis*, can be explained by acceleration of its metabolism leading to an increase in the concentration of its active metabolite, *para*-ethyl-phenol [40]. Kowalczyk-Zieba et al. [40] also documented higher β-glucuronidase activation during experimentally induced *metritis* connected with different isoflavone metabolism. Thus, the metabolism of isoflavones derived from the soybean (daidzein and genistein) was slower in the control and *mastitis* groups of cows compared to the cows with induced *metritis* [40]. The authors explained higher equol and *para*-ethyl-phenol concentrations in the blood plasma of cows with induced *mastitis* compared to control group due to the slight increase of β-glucuronidase activity in these cows

compared to control animals [40]. Thus, during experimentally induced inflammations—*mastitis* or *metritis,* there is higher concentration of free, unconjugated phytoestrogen metabolites which may in turn influence on the immune system. In conclusion, Kowalczyk-Zieba et al. [40] found that *mastitis* and *metritis* in the cows influenced the accumulation of isoflavone metabolites in the blood plasma. Therefore, the authors suggested that cows with induced *mastitis* and *metritis* were more exposed to active isoflavone metabolite actions than healthy cows. We expected that during such inflammatory processes phytoestrogens can easier disturb reproductive processes including, modulation the hypothalamic-pituitary-ovarian axis or inhibition of gonadotropin secretion and [41, 42]. This caused a decrease of progesterone production which in turn led to high abortion rate [43]. Moreover, we hypothesized that at the time of *mastitis* and *metritis* phytoestrogens may disturb estrous and ovulation through their effects on the central nervous system [40].

3.2. Phytoestrogen Exposure Influences Reproductive Performance on Various Regulatory Levels. Phytoestrogens can disturb reproductive processes on different regulatory levels [44]. Many studies have been conducted on a ruminant model to define the direct effect of phytoestrogens within the central nervous system (CNS; pituitary gland and hypothalamus). Mathieson and Kitts [45] studied the binding of phytoestrogens to the estradiol receptor in the pituitary gland and hypothalamus. These authors indicated that phytoestrogens could interfere with the estradiol feedback mechanism to release luteinizing hormone (LH) in the ewe [45]. However, the effect of dietary exposure to phytoestrogens on LH secretion seemed to be dependent on the type of phytoestrogen and reproductive status and seasonality. In ovariectomized ewes, an increased concentration of coumestrol in the diet significantly reduced the amplitude of LH pulses during the breeding, but not during the anestrous season [46]. Furthermore, Romanowicz et al. [47] investigated whether genistein was capable of evoking effective changes in LH and prolactin (PRL) secretion in ovariectomized ewes during seasonal anoestrus. After several hours of genistein infusion into the third ventricle, plasma LH concentrations and the frequency of LH pulses decreased. Moreover, plasma PRL concentrations during and after genistein infusion were also significantly higher than the control. These data demonstrated that genistein may effectively modulate LH and PRL secretion in ovariectomized ewes by acting within the CNS [47].

Polkowska et al. [48] found that genistein infused to the third ventricle of the brain changed the endocrine activity strictly of LH-producing cells in the pituitary glands of ewes during the anoestral season. However, the infusion of genistein did not affect the expression of genes encoding FSHβ and the storage of the β-subunit in the FSH-producing cells. The authors observed that genistein decreased the percentage and density of immunoreactivity of the LHβ-positive cells, nevertheless stimulated the percentage and integral density of LHβ mRNA-expressing cells. Furthermore, the increase in LHβ mRNA in LH-positive cells of the treated animals was accompanied by an increased expression of ERα after genistein infusion. These results suggest that probably a rapid release of the hormone together with an enhanced synthesis of LH is possibly mediated by ERα. Data obtained by Polkowska et al. [48] implicated that genistein stimulated the expression of ERα in the LHβ-expressing cells, decreased the pool of secretory granules stored in the LH-producing cells, and augmented the synthesis of β subunit for LH. Misztal et al. [49] analysed the effect of intracerebroventricularly genistein administration on growth hormone (GH) secretion in ewes. During the genistein infusion into the third ventricle of the brain, GH plasma concentration increased. Furthermore, several hours later, with the immunohistochemistry method the cited authors observed measurable diminished storage of GH in the pituitary somatotropes. The authors suggested that this plant-derived isoflavone, as 17β-estradiol [50], can be a stimulator of GH secretion in ewes and may exert its effect at the level of the CNS.

The decrease of fertility can also be attributed to the local—direct effect of phytoestrogens on reproductive tract. Phytoestrogens can inhibit endogenous estrogen production in the ovary leading to disturbances in immune system regulation as well as in follicle development and lack of estrous [14]. High concentrations of active metabolites of phytoestrogens have been found in the CL tissues collected from heifers receiving soy diet compared to animals fed with standard fodder [42] (Figure 4). These high concentrations of phytoestrogen metabolites in heifers were associated with lower concentrations of P_4 compared to heifers fed standard diet [42] (Figure 4). The authors of this study suggested that high concentrations of active metabolites of phytoestrogens present in the CL, directly disrupt its function by inhibiting P_4 secretion [42]. Corpus luteum produces P_4 required for the establishment and maintenance of pregnancy [51]. Therefore, active metabolites of phytoestrogens inhibiting P_4 secretion may disrupt CL function and induce various disturbances during early pregnancy including the early embryo mortality [52]. On the other hand, it has been documented before that pituitary LH and luteal and/or ovarian PGE_2 stimulate P_4 production and output from bovine CL [53]. Piotrowska et al. [42] documented that LH and PGE_2 stimulated P_4 secretion in CL tissues collected from cows fed with standard diet in contrast to cows fed with soybean diet (Figure 5). These authors also found that in microdialyzed *in vitro* CLs, equol and *para*-ethyl-phenol inhibited LH-stimulated P_4 secretion in comparison to the saline treated group. However, active metabolites of phytoestrogens did not influence basal P_4 production *in vitro* [42]. Additionally, the experiments conducted on the bovine steroidogenic CL cells isolated from the late-luteal phase of the estrous cycle demonstrated that active phytoestrogen metabolites stimulated only luteolytic substance production—$PGF_{2\alpha}$ and T in the cells [54]. It was well documented before that in the cow, P4 is the main luteotropic hormone of CL origin [51], whereas $PGF_{2\alpha}$, E_2, and T are the primary factors responsible for cessation of luteal P4 production and steroidogenic cell involution [55]. Therefore, any phytoestrogen-dependent increase in the $PGF_{2\alpha}$ secretion, and consequently elevation of E_2 and

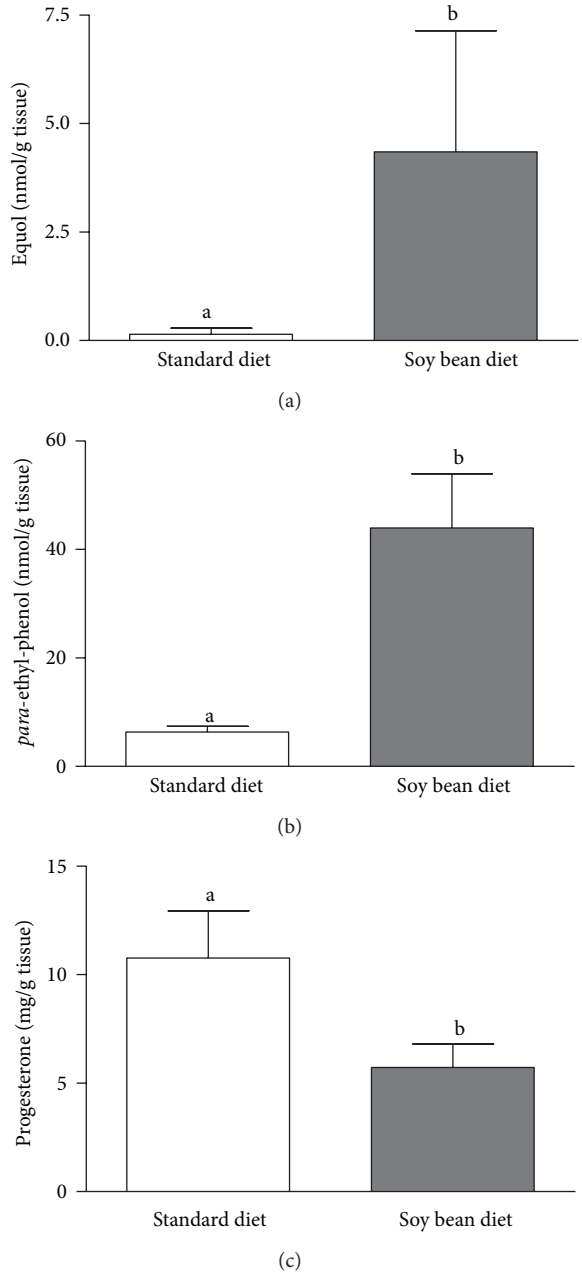

Figure 4: Concentrations of equol (a), *para*-ethyl-phenol (b), and progesterone (c) in the corpus luteum tissue of cows fed with soy diet (grey bars; 2.5 kg soy bean/animal/day) or with standard diet (white bars) (adapted from Piotrowska et al., 2008).

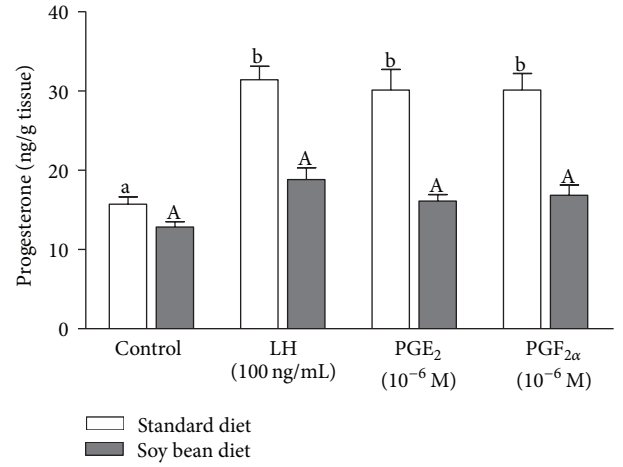

Figure 5: The effect of soybean diet on the LH-, PGE_2-, and $PGF_{2\alpha}$-stimulated *in vitro* progesterone secretion by the bovine CL (adapted from Piotrowska et al., 2008).

T production at the late luteal phase, may lead to the termination of CL function and even abortion in case of early pregnancy [56]. Phytoestrogen-dependent stimulation of luteolytic $PGF_{2\alpha}$ and T in the steroidogenic CL cells at the luteal phase of the estrous cycle [54] agree with our previous *in vivo* studies, which proved that high soy diet significantly increased PGFM concentration in the serum of soy-fed animals causing the decrease of the rate of successful pregnancies and the increase of the mean insemination rate [31]. The influence of phytoestrogens and their active metabolites on P_4 secretion is indirect, since it depends on the ability of phytoestrogens to inhibit LH and PGE_2-stimulated P_4 production. Feeding cows with high soybean diet may be the reason for disorders in the estrous cycle and several ovarian dysfunction during early pregnancy [31, 42, 54] (Figure 6).

In the series of *in vitro* experiments, we also studied local effects of phytoestrogens on the secretory function of the bovine endometrium [31, 57–59]. In these *in vitro* experiments, phytoestrogen metabolites (equol and *p*-ethyl-phenol) turned out to be much more potent disruptors than the original phytoestrogens themselves. We found that the stronger effects of the metabolites were due to their higher affinities for estrogen receptors than original phytoestrogens [31, 57, 58]. This hypothesis is supported by findings of other authors [44, 60] who showed that phytoestrogen metabolites are about 100–150% more active than environmental estrogens. We studied the influence of phytoestrogens derived from soybean and their metabolites on $PGF_{2\alpha}$ and PGE_2 production in the cultured bovine endometrium at different stages of the estrous cycle [31]. Prostaglandins E_2 and $PGF_{2\alpha}$ are crucial for proper development and maintenance of the CL. On the other hand, the maintenance of CL and P4 production is regulated by several luteotropic factors, including PGE_2 [60]. However, the most important for the maternal recognition of pregnancy, maintaining the function of CL, embryo implantation and development is proper $PGF_{2\alpha}/PGE_2$ ratio [56, 61] (Figure 6). Phytoestrogens and their metabolites greatly increased $PGF_{2\alpha}$ production and moderately but significantly increased PGE_2 production during the luteal phase of the estrous cycle [31]. In case of pregnancy establishment, the $PGF_{2\alpha}/PGE_2$ ratio should decrease. This relaxes the blood vessels and increases blood flow in the uterus, which prepares it for the embryo implantation [62]. The decreased $PGF_{2\alpha}/PGE_2$ ratio also stimulates P4 synthesis [63]. Soybean phytoestrogens preferentially stimulated $PGF_{2\alpha}$ during the luteal phase of the estrous cycle (Wocławek-Potocka et al. [31]). Because

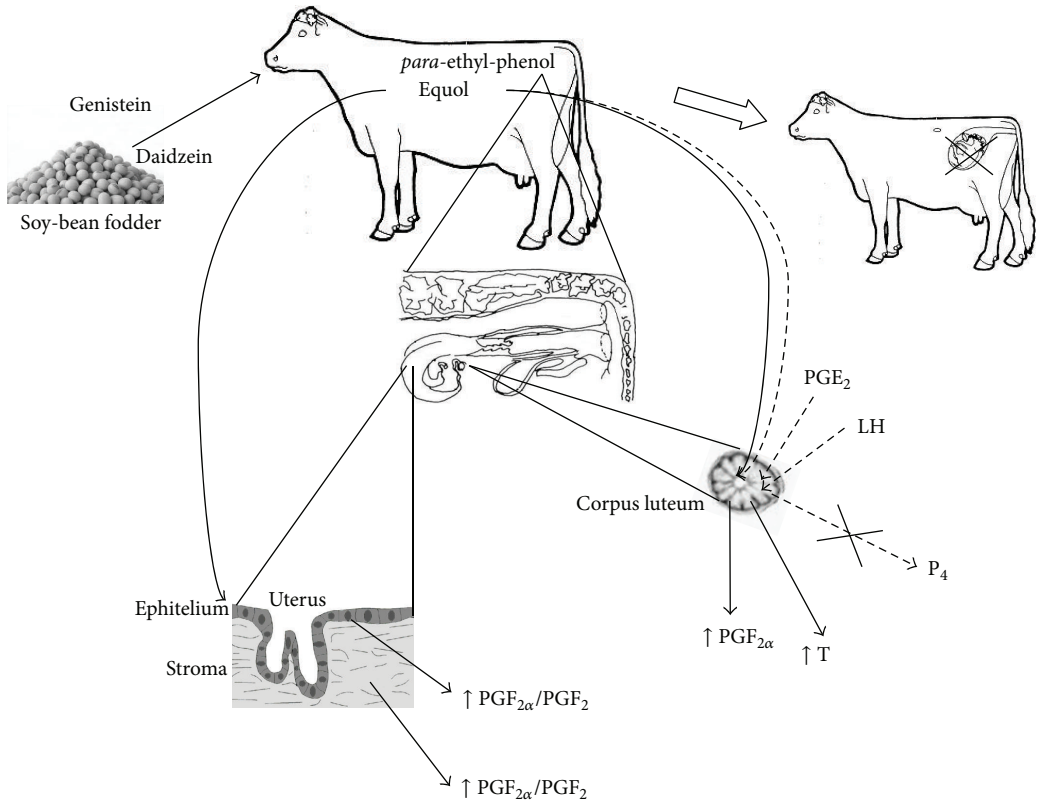

Figure 6: Possible influence of phytoestrogen action in the cow (the abbreviations on the figure stand for: LH—luteinizing hormone; P_4—progesterone; PGE_2—prostaglandin E_2; $PGF_{2\alpha}$—prostaglandin $F_{2\alpha}$; T—testosterone).

$PGF_{2\alpha}$ has a direct and negative effect on bovine embryo development *in vitro* [64], the strong stimulation of $PGF_{2\alpha}$ production compared to PGE_2 production that was observed in the bovine endometrial tissue may be one of the reasons of the early embryo mortality or abortion [31] (Figure 6).

However, when animals are not pregnant, during the estrous cycle (especially during late luteal and follicular phase of the cycle), this preferential $PGF_{2\alpha}$ stimulation can have positive effects on mechanisms responsible for luteolysis and returning the animals to cyclicity and ovulation [31]. During luteolysis, stimulation of $PGF_{2\alpha}$ secretion by estrogenic-like substances accelerates the positive feedback loop between $PGF_{2\alpha}$ and other regulators of luteolysis, such as, for example, oxytocin (OT) [56, 65] or TNFα [66, 67]. It was proved before that E_2 increases OT-stimulated $PGF_{2\alpha}$ production in cultured bovine endometrial cells [68], as well as amplifies the stimulatory effect of OT on endometrial $PGF_{2\alpha}$ synthesis [69]. Additionally, gonadal steroids upregulate OT gene expression in the hypothalamus and upregulate OT receptors in the uterus; thus, they can alter the frequency of the central OT pulse generator, leading to the pulsatile $PGF_{2\alpha}$ output from the endometrium during luteolysis in ruminants [61, 70]. Therefore, the data obtained by Wocławek-Potocka et al. [31] proves that in this case if phytoestrogens and their metabolites act like endogenous estrogens, at the time of luteolysis and ovulation, they may amplify the mechanisms that return the cow to cyclicity after labor.

3.3. Intracellular and Enzymatic Mechanisms of Phytoestrogen Actions in Reproductive Tract. There are even more obstacles to overcome to study the intracellular and enzymatic mechanisms of phytoestrogen actions. The cow is also a relevant model for such kind of studies. Phytoestrogens and their metabolites differentially modulate PG synthesis in a cell-specific manner, increasing both PG without altering $PGF_{2\alpha}/PGE_2$ ratio in stromal cells and directing the biosynthetic pathway toward $PGF_{2\alpha}$ in epithelial cells via stimulation of PGFS expression [31, 58].

It has been documented before that phytoestrogens inhibited the binding of (H^3)-E_2 or (H^3)-Organon to their respective receptors, but the relative affinities of (H^3)-E_2 and (H^3)-Organon were lower than those of E_2 [14, 18, 71]. The affinities of phytoestrogens for estrogen receptors are only 0.1% to 1% of those of circulating estrogens (E_2 or estrone) both in humans and cows [72]. Thus, the many biological effects attributed to phytoestrogens may be due to their relatively high concentrations. We found more than a thousand times greater concentrations of *p*-ethyl-phenol and equol ($1.6 \pm 0.31\,\mu$M and $1.2 \pm 0.28\,\mu$M, resp.) in plasma of cows fed with soybean [31] than the concentrations of endogenous E_2 (1–10 nM) [73]. These high concentrations may compensate much weaker affinity of phytoestrogens for estrogen receptors [18]. It has been previously shown that the concentrations of phytoestrogens in plasma of pregnant women consuming soybeans are over 1000 times higher

than E_2 concentrations and 10000 to 100000 higher than E_2 concentrations during the menstrual cycle [9, 72, 74].

As mentioned before, estrogens in target tissues and cells exert their physiological effects by genomic [75] and nongenomic pathways [76] (Figure 2). However, we documented that phytoestrogens stimulate both $PGF_{2\alpha}$ and PGE_2 in epithelial and stromal cells of bovine endometrium as well as $PGF_{2\alpha}$ production in the steroidogenic CL cells via only an estrogen-receptor-dependent, genomic pathway [31, 54]. Phytoestrogens and their active metabolites may compete with endogenous E_2, thus disturbing the processes influenced by E_2.

In the nongenomic pathway of estrogen action, PKA and PLC are the most important compounds of the intracellular second messenger system. Dubey et al. [77] found that genistein inhibited MAP kinase activity and PLD activity [78] as well as PLC-dependent intracellular calcium release [79]. However, in our previous study, neither the PKA inhibitor nor the PLC inhibitor (inhibitors of nongenomic pathways and second messengers), inhibited equol- and *para*-ethyl-phenol-mediated stimulation of $PGF_{2\alpha}$ synthesis in epithelial and stromal cells [57] or $PGF_{2\alpha}$ production in the steroidogenic CL [54], suggesting the lack of nongenomic mechanism of phytoestrogen metabolites action on the PG synthesis in bovine endometrium and CL, in contrast to endogenous E_2.

Diverse effects on phytoestrogens on reproductive processes may depend not only on different intracellular and receptors pathways activation, but also on activation of various enzymes involved in arachidonic acid metabolism [58]. Although, phytoestrogens did not affect on either gene expression or protein level of prostaglandin-endoperoxide synthase-2 (PTGS-2; COX-2) and PGE_2 synthase (PGES) in bovine endometrial stromal and epithelial cells, the stimulative effects of equol and *para*-ethyl-phenol on $PGF_{2\alpha}$ synthase-like 2 (PGFSL2) gene expression and protein level were observed in epithelial cells [58]. These results explain on enzymatic level why phytoestrogens can increase ratio of luteolytic $PGF_{2\alpha}$ to luteotropic PGE_2 in bovine uterus [58] (Figure 6). The effect of estrogens and phytoestrogens on the viability of various types of cells was also studied in the literature. Phytoestrogens and their metabolites decreased the viability of bovine endometrial epithelial and stromal cells [58]. Similarly, Asselin et al. [68] and Nilsson et al. [80] also demonstrated that endogenous estrogens inhibit proliferation of epithelial cells and vascular endothelial cells in several organs. On the other hand, estrogens have been also reported to stimulate epithelial and endothelial cell growth and proliferation in the female reproductive tract of many animal species [81].

4. Relevance of a Cow Model to Human Reproductive Performance

Perfectly designed studies to examine the effects of isoflavones on humans should be done in human subjects. However, this situation is very hard to be accomplished. We have to take into account that in that kind of studies there are a lot of obstacles to overcome. Citing the group of Verkasalo et al. [82], there is usually wide variation in human exposures, these exposures are difficult to measure accurately, and the exposures are inherently difficult to control effectively. There is also extensive variability in isoflavone content of many dietary sources over time, whether standard food products or commercial botanical extracts are sold as dietary supplements [83]. What is more, the metabolism of isoflavones is not the same in all humans since there is different activity of metabolizing enzymes and also varies the influence of gut microflora on phytoestrogen bioavailability [84]. Summarizing, there are a lot of complications in the design and interpretation of human studies, combined with the ethical issues regarding experimentation in humans, that continuously increases interest in studies that utilize animal models. The relevance to human health of studies performed in animal models has been questioned many times in the literature, since in many of the animal studies exposure to phytoestrogens was by a nonoral route, whereas most human phytoestrogen exposure is from dietary intake [82]. This kind of exposures is usually chosen for rodent models of phytoestrogen exposure. Taking above arguments into consideration, it has been well documented that the cow can be a relevant animal model for studies of human reproduction because ovarian physiology and many aspects of embryo development, pregnancy and assisted reproductive techniques are similar between these two single-ovulating species [85, 86]. This model has broad applicability and may be used to extend investigations to different physiologic/pathologic states and to other species including humans. Moreover, the bovine model has the potential to be used as a sensitive *in vivo* bioassay to study the influences of xenoestrogens factors, including phytoestrogens on reproductive performance because of similar basic phytoestrogen metabolisms (genistein and daidzein) in both species (Figure 1).

Therefore, we believe that a cow model is far better since the main, natural exposure in this animal is also oral that does not vary from human exposure. The bovine model ensures a greater availability of biological material compared to studies in human. More importantly, the possibility to conduct *in vivo* studies represents a powerful tool that could possibly clarify the conflicting data obtained in different human studies. Altogether, these arguments support the use of studies in the cow in modeling exposure of humans to phytoestrogens.

5. Adverse Effects of Isoflavones on the Reproductive Performance in Human

The most common plant-derived proteins belong to soybean-based products. Isoflavones commonly enter the human body through the food chain. As the Oriental diet contains many soy-based products, isoflavone levels are high in the blood plasma of people living in the Oriental countries [87]. However, isoflavones are becoming more and more common in Western countries as well. This situation results from the increasing presence on the market of soy-derived dietary

supplements, that represent ergonergic products for sportive people [88]. As a result, an increasing number of people in reproductive age assumes these phytoestrogens. Although these products are perceived as by the consumers "safe" because of being "natural", in fact there is limited control on their safety [88].

Even though isoflavones are metabolized and excreted quite rapidly, their effects on human health can be remarkable. There are contradictory data in the literature on the isoflavone effect on human health. In this aspect, both beneficial and adverse effects of these natural estrogens are reported. Isoflavones, such as genistein and daidzein, have been addressed as preventive factors for cancer risk and cardiovascular diseases, and as antiobesity, neuroprotective, and osteoprotective agents [87, 89–91]. However, data on phytoestrogen action of estrogen sensitive tumors are contradictory [91, 92]. On one hand, epidemiological studies encounter a reduction in cancer incidence in populations consuming a soy-based diet, and on the other hand, some *in vitro* studies reveal some contradictory data [93–95]. It has been shown that phytoestrogens such as genistein, daidzein, and equol are able to mediate the proliferation of breast cancer cell lines [96, 97]. In particular, the modulation exerted by isoflavones on cancer cell lines seems dose dependent, with some doses promoting and other doses diminishing cell proliferation [98, 99].

Discussing diverse effects of phytoestrogens on human health differences and similarities about isoflavone metabolism in humans and ruminants should be taken into account. Similar to the cow, in some humans, daidzein—the main soy-derived isoflavone, can be transformed to equol by the intestinal flora [30, 100]. This metabolite is more bioactive than its parental compound in both human and other animals [10, 31, 101]. However, unlike ruminants, not all humans are able to produce equol. The ability to convert daidzein into equol derives from the different intestinal floras [102]. As equol shows much higher estrogenicity than its parent compounds, the effects exerted by isoflavones on human health should be more remarkable in "equol producers." On the other hand, it has been demonstrated that, upon long-term exposure to isoflavones, "nonequol producers" can develop the ability to metabolize equol [103]. Thus, the differentiation between "equol producers" and "nonequol producers" depends mostly on the type of diet, and not on constitutive differences between individuals.

Another explanation for these contrasting data resides in the time frame in which the phytoestrogens exposure takes place, being the developmental window (i.e., pre- and early postnatal exposure), one of the most sensitive periods of human life. In fact, a big concern is arising from the use of soy-based infant formulas because of the delicate life period in which they are administered [104]. The exposure to phytoestrogens during prenatal and early postnatal life represents a matter of concern. Prenatal exposure can occur due to the life style of the mothers (e.g., vegetarian diet, dietary supplements intake, and soy milk intake) [87, 104]. Postnatal exposure often occurs because of soy-based infant formulas and soy milk intake.

Isoflavones cross the placental barrier and reach the fetal circulation [105, 106]. Many animal models have been applied for the study of intrauterine and perinatal exposure to hormones mimicking compounds of plant origin [107–111]. These studies demonstrated how intrauterine exposure to isoflavones can have consequences on the reproductive system in adulthood [108, 112, 113]. Unlikely for the exposure in adult life, the exposure in pre- or perinatal life seems to lead to irreversible alterations of the reproductive system. Such an effect might be due to epigenetic modifications that persist though the rest of life [113–115]. Male children exposed to isoflavones in utero showed hypospadias [116]. In this perspective, isoflavones can be encountered within the contributors, together with other hormone-mimicking compounds, to the decreasing efficiency in male reproduction registered in the last decades [8, 117]. Even though not registering significant differences, a study on infants fed with soy-based infant formulas appears worth of mention [118]. This study evaluated the differences in hormone-sensitive organs in infants fed with soy-based, milk infant formula, or with breast milk. Interestingly, a trend towards diminished testicular development was found in infants fed with soy-based or milk formulas. Exposure to genistein altered the male reproductive features not only in human [88, 119–122] but also in animal models [112, 123] and is not reviewed in this paper.

In utero exposure to isoflavones can also impair the reproductive system of female descendants. The evidence of such interferences comes mainly from animal studies. Isoflavones exposure in the womb resulted in a decreased sensitivity to the estrogen by the mammary gland [124]. If such finding reveals a possible cancer-preventive activity of isoflavones, on the other hand it raises concern for other possible health outcomes. In particular, isoflavones exposure during fetal life alters the estrogen receptor ratios, thus impairing the physiological action of estrogens. Surprisingly, genistein administration during fetal life resulted in an increased risk of uterine cancer and in a promotion of leiomyoma [125]. Perinatal exposure to isoflavones resulted in alterations in the uterus and ovaries of female pups [126, 127].

On the other hand, consumption of isoflavones in women reproductive age has been linked to dysmenorrhea, endometriosis, and secondary infertility [16, 128]. A high intake of phytoestrogens resulted in dysmenorrhea and persistent sex arousal syndrome in one case-study reported by Amsterdam et al. [15]. In this study, like in the one reported by Chandrareddy et al. [16], withdrawal of soy intake from the diet resulted in the lessening or in the complete disappearance of the symptoms. Remarkably, in the studies of Chandrareddy et al. [16] one patient was able to conceive after isoflavone withdrawal from her diet. Keeping in mind that these adverse effects have been encountered only in a restricted number of cases, it still appears advisable to handle the phytoestrogens' intake with care. *In vitro* studies strengthen the observation that isoflavones can directly modulate endometrial physiology [99, 129]. Interestingly, genistein was able to modulate the proliferation of Ishikawa

cells, an epithelial cell line derived from adenocarcinoma, in a dose-dependent fashion, being the low doses an inhibitor factor for proliferation, that was instead promoted by high doses [99]. At similar doses, genistein promoted the proliferation of leiomyoma cells [129]. These findings raise concern for the beneficial effects of isoflavones. Surprisingly, genistein revealed to cure endometrial hyperplasia in a clinical trial [130].

Isoflavones can exert their effect not only on the uterus level. Other estrogen-sensitive organs such as ovaries can be affected by these natural estrogens. In order to guarantee a normal ovarian function, estrogen circulating levels must oscillate during the cycle. Low estrogen levels stimulate FSH release by the hypothalamus/pituitary, thus leading to follicle growth. The presence of isoflavones can nullify the required low levels of endogenous estrogen. This could lead to irregular cycle, and even to reproductive impairment [131]. Following soy intake, cases of altered steroid hormones levels and trends for increased cycle length have been reported [131–133]. Moreover, soy supplementation to women in reproductive age resulted in decreased LH and FSH levels during the periovulatory phase [133]. If such alterations can be sufficient to impair the ovarian cycle is still argument of debate. Moreover, there are conflicting results on the effects of isoflavones on the hypothalamus-pituitary-gonads axis [132, 133]. Thus, it is not possible to evaluate if the effects exerted by isoflavones on human reproduction are due to a local or a systemic action. Interestingly, animal studies demonstrated that genistein is able to impair ovarian differentiation in mice [134, 135]. In this light, the results collected among women in reproductive age raise great concern for the effects of isoflavones' exposure [16, 134]. Fortunately, the effects exerted by isoflavones in adult life appear reversible once dietary intake is ceased [131].

Women in menopause represent another important category of people exposed to high concentrations of isoflavones [136, 137]. However, there are many reports showing that dietary supplements containing genistein seem to lessen menopausal symptoms [137]. While phytoestrogens seem to exert a positive effect on postmenopausal women, their effect could be deleterious in women in reproductive age. Isoflavones lessen menopausal symptoms and do not seem to show the contraindications of the estrogen replacement therapy, even though some exceptions have been registered [136–140]. In particular, genistein is able to promote estrogen synthesis in an extragonadal pathway, thus exerting a positive effect in menopausal women [141].

To summarize, the data reported above clearly indicate that phytoestrogens are able to modulate important processes of human physiology. The conflicting results encountered in the literature do not allow us to draw conclusions on whether phytoestrogens exert a positive or a negative effect on human reproductive health. The often opposite effects registered in the available literature can be generated by the different genders, ethnics, and, more importantly, at different timeframe of exposure considered. Thus, the effect of isoflavones on reproductive efficiency in humans should be investigated on a relevant animal model.

6. Conclusions

There is overwhelming evidence in many studies using a ruminant model that phytoestrogen exposure can have significant consequences for reproductive health. The effects of phytoestrogens depend on many various conditions such as dose and route of exposure because these parameters impact the final serum level of the bioactive compound. Moreover, the timing of exposure is critical in determining the phytoestrogen-induced effects and different tissues have species-specific windows of sensitivity to morphological and functional disruption. However, the most important issue connected with phytoestrogens is the fact that they are more and more commonly recognized as therapeutic compounds. Therefore, it is crucial to examine carefully the effects of these chemicals on reproductive outcomes using animal models that replicate human exposure levels.

In spite of many limitations in conducting well-designed human studies, information gathered from already published ones combined with the large number of animal studies already available, clearly demonstrate that phytoestrogens have the ability to influence the reproductive performance of an adult. These findings should be specially taken into consideration when recommendations are made regarding dietary or therapeutic phytoestrogen intake in humans.

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 510703, 7 pages
http://dx.doi.org/10.1155/2013/510703

Clinical Study

Correlation of Endocrine Disrupting Chemicals Serum Levels and White Blood Cells Gene Expression of Nuclear Receptors in a Population of Infertile Women

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Received 18 December 2012; Revised 2 April 2013; Accepted 3 April 2013

Academic Editor: Ewa Gregoraszczuk

Significant evidence supports that many endocrine disrupting chemicals could affect female reproductive health. Aim of this study was to compare the internal exposure to bisphenol A (BPA), perfluorooctane sulphonate (PFOS), perfluorooctanoic acid (PFOA), monoethylhexyl phthalate (MEHP), and di(2-ethylhexyl) phthalate (DEHP) in serum samples of 111 infertile women and 44 fertile women. Levels of gene expression of nuclear receptors (ERα, ERβ, AR, AhR, PXR, and PPARγ) were also analyzed as biomarkers of effective dose. The percentage of women with BPA concentrations above the limit of detection was significantly higher in infertile women than in controls. No statistically significant difference was found with regard to PFOS, PFOA, MEHP and DEHP. Infertile patients showed gene expression levels of ERα, ERβ, AR, and PXR significantly higher than controls. In infertile women, a positive association was found between BPA and MEHP levels and ERα, ERβ, AR, AhR, and PXR expression. PFOS concentration positively correlated with AR and PXR expression. PFOA levels negatively correlated with AhR expression. No correlation was found between DEHP levels and all evaluated nuclear receptors. This study underlines the need to provide special attention to substances that are still widely present in the environment and to integrate exposure measurements with relevant indicators of biological effects.

1. Introduction

An endocrine disrupting compound (EDC) is defined as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" [1].

The homeostasis of sex steroids and the thyroid are the main targets of EDC effects; hence, reproductive health, considered as a continuum from gamete production and fertilization through to intrauterine and postnatal development

of progeny, is recognized as being especially vulnerable to endocrine disruption [2].

This study stems from the PREVIENI project (http://www.iss.it/prvn/), founded by the Italian Environment Ministry. Aim of the project is to integrate biomarkers of chemical exposure, biologically effective dose biomarkers, and clinical findings, in order to assess the relationship between reproductive health and emerging EDC that are still incompletely considered in the environment and health surveillance.

This study presents the data on internal EDC exposure levels in infertile women from three different IVF units as well as the expression of nuclear receptors (NRs) in peripheral blood mononuclear cells (PBMCs), as biomarkers of effective dose.

Molecular targets were bisphenol A (BPA), perfluorooctane sulphonate (PFOS), perfluorooctanoic acid (PFOA), monoethylhexyl phthalate (MEHP), and di(2-ethylhexyl) phthalate (DEHP).

Bisphenol A (BPA) is primarily used in the manufacture of polycarbonate plastic sheets and epoxy resins present in various consumer products, such as food contact materials [3]. BPA migration from the plastic polymers leads to contamination of both food and environment.

Perfluooctane sulphonate (PFOS) and perfluorooctanoic acid (PFOA) are widely present in consumer's products, including textiles, films, and electric materials. Dietary exposure may be also considerable, in particular through food of animal origin, such as fish [4].

Di(2-ethylhexyl)-phthalate (DEHP) is a major representative of phthalates, widely used plasticizer used also in cosmetics, personal care products, and food packaging materials [5]. Mono(2-ethylhexyl)-phthalate (MEHP) is the major toxicologically relevant metabolite of DEHP [6]. Human exposure to phthalates may occur through diet or indoor environment.

The panel of NRs includes estrogen receptor alpha (ERα) and beta (Erβ), androgen receptor (AR), pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR), and peroxisome proliferator-activated receptor gamma (PPARγ).

2. Materials and Methods

From January 2009 to April 2010, 111 women aged 18–40 years and affected by primary infertility were enrolled in the study. They were examined and admitted to three IVF units:

n = 50: Department of Women Health and Territory's Medicine of "Sapienza," "S. Andrea" Hospital, University of Rome.

n = 38: Department of Biomedical Sciences and Advanced Therapies, Section of Obstetrics and Gynaecology, University of Ferrara.

n = 33: Infertility Center S.T.S. of Sora.

Forty-four fertile women aged 18–40 years with regular menstrual cycle who obtained a spontaneous pregnancy in the last year and stopped breastfeeding at least six months before were enrolled as control group. Women were asked to fill out a questionnaire about their life habits (alcohol, smoke, and diet), age, parity, work, residence, and previous diseases. Patients reporting smoking habit, vegetarian diet, occupational exposure to EDC, body mass index (BMI) > 30, inflammatory or infectious diseases, and diagnosis of male infertility factor were excluded from the study.

This work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Approval from the ethical committee of Department of Woman Health and Territory's Medicine, University of Rome "Sapienza" had been obtained before the beginning of this study, and all patients subscribed an informed consensus.

2.1. Collection and Storage of Samples. A 20 mL sample of venous blood was collected from each woman. All samples obtained from infertile women were collected before hormonal stimulation. Each blood sample was divided into three parts: two 5 mL aliquots of heparin-treated whole blood and a 10 mL aliquot centrifuged to obtain serum. Serum and a 5 mL aliquot of heparin-treated whole blood were then frozen and sent to the Environmental Sciences Department "G. Sarfatti" of University of Siena for the chemical analyses to detect EDC levels. Heparin-treated whole blood from IVF Rome and whole blood collected by PAXgene tubes from IVF Ferrara and Sora were sent to the Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità in Rome to assess NR expression levels.

2.2. Chemical Analyses. BPA, PFOS, PFOA, DEHP, and MEHP were extracted using a liquid-liquid separation procedure and measured through a high-performance liquid chromatography (HPLC) with electrospray ionization (ESI) tandem mass spectrometry. For each substance, the limit of detection (LOD) corresponded to the respective measurement value in blanks + 3DS.

The extraction procedure of BPA follows the procedure previously described by Prins et al. [7]. Samples were defrosted, and 2 μL/mL of the hydrolytic enzyme glucuronidase were added to each 0.5 mL of serum. The sample was mixed and incubated at 37°C for 12 hours. Three mL of diethyl ether were then added, and the sample was mixed up for 30 minutes and centrifuged for 5 minutes at 4000 rpm. The fluid part was collected in a 15 mL BPA-free vial. This procedure was repeated three times. Solvent evaporated at room temperature with a light nitrogen flow, and the deposit was reconstituted with 0.5 mL of acetonitrile and then filtered through a nylon filter with 0.2 μm pores; 0.5 mL of acetonitrile were added, and the sample was then collected in autosampling vials. Analytical separation was performed according to Coughlin et al. [8]: 20 μL were injected, by autosampling, in the HPLC column (C18 Betasil C18, 50 mm × 2.1 mm Ø, 5 μm of inside thickness film) with a 250 μL/minutes flow. The LOD for BPA was 0.5 ng/mL.

The analytical procedure for PFOS and PFOA follows Governini et al. [9]. The blood sample was defrosted, and then 0.5 mL of serum or whole blood, 1 mL of tetrabutylammonium hydrogen sulfonate 0.5 MpH 10, and 2 mL of sodium carbonate buffer 0.25 M were added in a 15 mL polypropylene

tube. After mixing, 5 mL of methyl-tert-butyl ether (MTBE) were added and mixed again for 20 minutes. The sample was centrifuged at 3500 rpm for 25 minutes in order to separate the organic part (ether) from the fluid part. A volume of 4 mL of the organic part was collected in a 15 mL tube. The fluid part was treated with 4 mL MTBE, mixed up for 20 minutes, and centrifuged for 25 minutes at 3500 rpm in order to separate organic part by the remaining fluid part. Solvent evaporated at room temperature with a light nitrogen flow, and the deposit was reconstituted with 0.5 mL of methanol. The sample was then mixed by vortexing for 30 seconds, filtered through a nylon filter pores of 0.22 μm diameter, and collected in autosampling vials. Analytical separation was performed by Finnigan LTQ Thermo Electron Corporation. Twenty μL were injected, by autosampling, in the HPLC column (C18 Betasil C18, 50 mm × 2.1 mm Ø, 5 μm of inside thickness film); column temperature was kept at constant temperature (30°C), and the substances of interest were separated by a mobile phase of ammonium/acetate/methanol 2 mM with a 300 μL/minutes flow. After 10 minutes chromatographic running, HPLC was interfaced, through an electrospray ionization (ESI) source, working in negative mode, to a mass spectrometer at triple linear quadrupoles. Ions used for identifying PFOS and PFOA were 412,8 > 168.8, 218.8, and 498.8 > 368.9, respectively. For the quantitative analysis, a four-point calibration curve, obtained by progressive methanol dilution of a standard solution with relevant analytes (Chiron, Trondheim, Norway), was used. The LOD for PFOS and PFOA was 0.4 ng/mL.

The extraction procedure of DEHP and MEHP follows the protocol described by Takatori et al. [10]. After thawing, 4 mL of acetone were added to 0.5 mL of serum, mixed up for 5 minutes, and centrifuged at 3000 rpm for 20 minutes. The fluid part was collected in a 15 mL tube, treated with 1 mL of acetone, mixed up for 5 minutes, and centrifuged for 20 minutes at 3000 rpm in order to complete the separation of the organic part from the fluid part. Solvent evaporated at room temperature with a light incomplete nitrogen flow, and the deposit was reconstituted with 0.5 mL of acetonitrile. The sample was filtered through a nylon filter pores of 0.22 μm diameter and collected in autosampling vials. Twenty μL were injected in the HPLC and analyzed using an ODS-2 Hypersil 150 × 2.1 mm column with a flow of 200 μL/mL. The LOD was 2 ng/g for MEHP and 10 ng/g for DEHP.

Glass vials were used in order to avoid possible release of EDC from plastic materials. In addition, to avoid contamination, all materials were rinsed with methanol. Data quality assurance and quality control protocols included matrix spikes, analyses of laboratory blanks, and continuous verification of the calibration. Blanks were analyzed with a set of six samples as a check for possible laboratory contamination and interferences.

2.3. Expression of Nuclear Receptors. NR gene expression was assessed on PBMC. PBMCs were separated from whole blood by Ficoll-Hypaque density centrifugation from heparin-treated samples from IVF Rome and extracted for their total RNA content by the RNeasy Mini Kit (QIAGEN). Samples from IVF Ferrara and Sora were extracted for their total RNA content by the PAXgene Blood RNA Kit (PreAnalytiX GmbH, 8634 Hombrechtikon, CH). RNA samples were quantified by NanoDrop. One μg of total RNA from each sample was retrotranscribed to cDNA by the cDNA Synthesis Kit (Quantace). Gene expression analysis was performed by quantitative real-time PCR using the SensiMix SYBR Kit (Quantace). GAPDH was used as reference gene. Specific primers for the selected NR and GAPDH were designed using the Primer-BLAST web application and purchased by Life Technologies. Real-time PCR reactions were run on a Stratagene MP3005P Thermocycler. Gene expression levels were reported as 2exp(ΔCt).

2.4. Statistical Analysis. Analysis of data was performed using the Statistical Package for Social Sciences (SPSS) version 16.0 for Windows (SPSS, Chicago, USA). Normally distributed data were analyzed by the Student's t-test. The Mann-Whitney U test for continuous non-parametric variables was used to evaluate group differences in NR. χ^2 and Fisher test were used for comparison of rates and proportions. Pearson's test was used to demonstrate correlations between EDC levels and nuclear receptors expression and between age and BMI with EDC and nuclear receptors expression. P values <0.05 were defined as statistically significant.

3. Results

The mean age of infertile women group was 35.3 ± 0.4 years and was comparable to the mean age of the control group (34.8 ± 4.6 years). Mean BMI of the infertile group did not significantly differ from the control group (23.44 ± 0.4 and 23.24 ± 0.7 kg/m^2, resp.). The following infertility factors were diagnosed in the study group: infertility sine causa (n = 34, 31.5%), tubal infertility (n = 23, 20.7%), immunological infertility (n = 17, 15.3%), thyroid dysfunction (n = 15, 13.5%), endometriosis (n = 11, 10%), polycystic ovarian syndrome (n = 7, 6.4%), and reduced ovarian reserve (n = 4, 3.6%). In the control group, 26 women had their first pregnancy in the last year, whereas 12 had their second pregnancy and 6 their third pregnancy before enrollment.

Table 1 shows the distribution of frequency of blood samples with EDC levels above LOD in both infertile and fertile groups. The percentage of women with BPA serum levels above the LOD was significantly higher in the infertile group (50/111, 45%, range 0.5–133.5 ng/mL) than in the control group (10/44, 22%, range 0.5–60.9 ng/mL), (OR 2.79, CI 95% 1.25–6.19, and P < 0.01). No statistically significant difference was found considering PFOS, PFOA, MEHP, and DEHP serum levels above the LOD between the two groups. A slightly, but not significant, difference between study group and control group was demonstrated for PFOS (32.4% in cases versus 18.1% in controls) and PFOA levels (43.2% in cases versus 18.1% in controls); noticeably, the upper range of PFOS levels in both cases and control groups was about one magnitude order higher than that of PFOA levels. The percentage of subjects with DEHP concentrations above the LOD was low in both groups (7.2% and 2.2% in cases and controls, resp.).

Table 1: Proportion of samples with EDC levels > LOD in both infertile and fertile groups.

Endocrine disruptor	Infertile group ($n = 111$)	Fertile group ($n = 44$)	P value	OR (CI 95%)
BPA	50 (45)	10 (22.7)	0.01	2.79 (1.25–6.19)
PFOS	36 (32.4)	8 (18.2)	0.11	2.16 (0.91–5.12)
PFOA	48 (43.2)	16 (36.4)	0.47	1.33 (0.64–2.65)
MEHP	73 (65.8)	27 (61.4)	0.71	1.21 (0.59–2.49)
DEHP	8 (7.2)	1 (2.3)	0.45	1.26 (0.40–27.5)

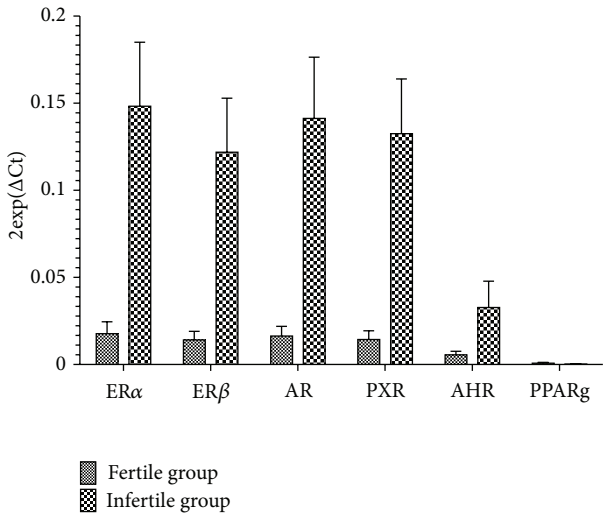

Figure 1: Nuclear receptors expression in infertile and fertile women.

Figure 1 and Table 2 show the cellular gene expression level of nuclear receptors in infertile and fertile women. All investigated NRs were expressed in PBMCs of both infertile and fertile women, with marked interindividual variability in both groups. Infertile patients showed gene expression levels of ERα, ERβ, AR, and PXR significantly higher than the control group. The expression of AhR and PPARγ did not show significant difference between the two groups.

Tables 3 and 4 point out the correlation between biomarkers of exposure and biomarkers of effects. A strong correlation was found between serum BPA concentration and ERα ($r = 0.467$; $P < 0.0005$), ERβ ($r = 0.474$; $P < 0.0005$), and AR ($r = 0.444$; $P < 0.0005$) expression in infertile women. A positive correlation was also found in the control group (ERα $r = 0.415$, $P < 0.05$; ERβ $r = 0.371$; $P < 0.05$; AR $r = 0.378$; $P < 0.05$). A positive association between BPA levels and AhR ($r = 0.335$; $P < 0.005$) and PXR expression ($r = 0.429$; $P < 0.0005$) was also demonstrated among infertile women, but not in controls. PFOS concentration positively correlated with AR ($r = 0.236$; $P < 0.05$) and PXR expression ($r = 0.239$; $P < 0.05$) in infertile women, while no association between PFOS exposure levels and NR expression was found in the control group. Only PFOA levels showed negative correlations with AhR ($r = -2.242$; $P < 0.05$) in infertile women. A positive correlation was found also between MEHP and ERα ($r = 0.388$; $P < 0.005$), ERβ ($r = 0.398$, $P < 0.005$), AR ($r = 0.366$; $P < 0.005$), AhR ($r = 0.291$; $P < 0.05$), and PXR expression ($r = 0.364$; $P < 0.005$) in the infertile group, but not in the control group. No correlation was found between DEHP levels and the expression of all considered nuclear receptors.

Table 2: Cellular gene expression level of nuclear receptors in infertile and fertile women.

NR	Infertile group ($n = 111$)	Fertile group ($n = 44$)	P value
ERα	0.15 ± 0.31	0.02 ± 0.04	0.02
ERβ	0.12 ± 0.27	0.01 ± 0.03	0.02
AR	0.14 ± 0.30	0.02 ± 0.03	0.02
PXR	0.13 ± 0.27	0.014 ± 0.03	0.01
AhR	0.03 ± 0.13	0.005 ± 0.01	0.23
PPARγ	0.0002 ± 0.0004	0.0007 ± 0.0003	0.15

Table 3: Correlation between ECD and receptor expression in infertile group.

	ERα	ERβ	AR	PXR	AHR	PPARγ
BPA	0.467*	0.474*	0.444*	0.429*	0.335*	0.091
PFOS	0.214	0.209	0.236*	0.239*	0.090	0.122
PFOA	−0.203	−0.203	−0.203	−0.191	−0.242*	−0.150
MEHP	0.388*	0.398*	0.366*	0.364*	0.291*	0.082
DEHP	−0.101	−0.179	−0.134	−0.145	−0.157	−0.151

*$P < 0.05$.

Table 4: Correlation between ECD and receptor expression in fertile group.

	ERα	ERβ	AR	PXR	AHR	PPARγ
BPA	0.415*	0.371*	0.378*	0.331	0.300	−0.155
PFOS	−0.086	0.007	−0.031	−0.209	−0.086	−0.059
PFOA	−0.259	−0.185	−0.176	−0.364*	−0.310	0.089
MEHP	0.342	0.157	0.170	0.230	0.239	0.007
DEHP	0.240	0.240	0.240	0.222	0.257	0.186

*$P < 0.05$.

4. Discussion

Our study underlines the relationship between environmental exposure and female infertility and the need to integrate internal exposure measurements with relevant indicators of

biological and clinical effects [11]. Our results show how infertile women have higher levels of BPA when compared to healthy fertile women. The infertile group showed also significantly higher expression in PBMC of several NRs (ERα, ERβ, AR, and PXR) that regulate endocrine pathways and are also potential EDC targets. BPA serum concentration showed a positive correlation with ERα, ERβ, and AR in both fertile and infertile women, although the strength of this association appeared higher in the infertile group; moreover, in infertile women, BPA showed a specific, positive correlation with AhR and PXR expression. PFOS concentration showed a positive correlation with the expression of AR and PXR only in the infertile group. We investigated EDC that is still in use, widespread in living environment, and still receiving limited attention in environment and health surveillance.

BPA is a ubiquitous contaminant but is also considered a nonpersistent compound in our environment [12, 13]. Many studies on BPA focus on urinary excretion and in some cases make a distinction between BPA and its excreted metabolite BPA-glucuronide [13]. We decided to consider the serum level of total BPA as an indicator of BPA presence in the organism, as a result of repeated and prolonged uptake of the compound from the living environment. In 2010, the European Food Safety Authority has comprehensively reviewed the toxic effects of BPA, underlining how further data are necessary to clarify its association with human reproductive disorders [13]. BPA is considered as an agonist of estrogen receptors alpha (ERα) and beta (ERβ) [14], but it could also interact with androgen receptors (ARs) [15]. Our data support the hypothesis that BPA may influence the expression of different NR involved in hormone response pathways and/or in steroid biosynthesis. This association is supported by recent experimental studies showing that BPA may alter ovarian steroidogenesis [16, 17], early events of uterine implantation, oocyte quality, and estradiol response to gonadotropin stimulation [18].

The increased PFOS exposure rate of infertile subjects did not reach statistical significance in our study; nevertheless, PFOS concentration showed a positive correlation with the expression of AR and PXR only in the infertile group. Both NRs are significantly upregulated in our infertile group, and a contribution of PFOS to such upregulation should not be ruled out. The percentage of subjects with PFOS concentration > LOD was found to be higher than the one reported by the study of Kannan et al. [19] and Ericson et al. [20] but comparable to those found by Yeung et al. [21], underlining the high variability of PFOS internal exposure, possibly related to factors influencing the persistent PFOS binding to plasma proteins [4, 19]. Toxicological and epidemiological evidence suggests a possible link between PFOS exposure and reproductive problems [22], including reduced oocyte fertilization capacity [9] and prolonged time to pregnancy [23].

Our study showed a widespread internal exposure of the women enrolled in the study to MEHP, as demonstrated by the fact that it is the only EDC consistently detected also in women of the control group. This result is consistent with previous biomonitoring studies [24]. However, a positive correlation between MEHP exposure levels and NR expression was found only in the infertile group. The emerging literature has demonstrated that exposure to DEHP and MEHP is associated with smaller preovulatory follicles, anovulation or delayed ovulation, longer estrous cycles, decreased synthesis of estradiol, decreased serum progesterone levels, and increased serum follicle-stimulating hormone (FSH) levels [25]. Beyond their well-described reprotoxic effects, phthalates are also suspected to interact with different members of the nuclear receptor (NR) superfamily or to act via their pathways by modulating expression of some nuclear receptors and their targets in various organ models [26].

The significantly increased expression of ERα, ERβ, AR, and PXR in infertile women may be a direct consequence of endocrine-related reproductive disorders. Estrogens, whose functions are mediated by ERα and ERβ, play a major role in steroidogenesis, follicular growth, ovulation, and endometrial cycle. AR is expressed in granulosa cells, oocytes, and in theca-insterstitial cells [27]. Its function is essential in optimizing follicular growth, final follicle development, and ovulation [28]. PXR indirectly mediates steroid hormone functions, playing an important role in their metabolism [29]. Therefore, a condition of altered ovarian function and/or endocrine signaling between the ovary and other reproductive tissues may be connected to NR dysregulation. Any change in NR expression may represent a relevant and plausible biomarker of effective systemic exposure to EDC [11].

Our study showed a strong positive correlation between BPA serum concentration and ERα, Erβ, and AR expression both in infertile and fertile groups. Melzer et al. provided the first report regarding the relationship between BPA exposure and NR gene expression in humans, finding higher expression of ERα and ERβ gene in PBMC of men associated with higher urinary BPA levels [30]. To date, our study is the first one investigating the expression of a comprehensive panel of NR in relation to internal exposure to BPA, as well as other EDCs, showing an NR upregulation. Infertile women showed a specific positive correlation between BPA and AhR and PXR expression. AhR is known as the "dioxin receptor," but it may also play an important role in female reproduction. Studies on mice revealed that it is expressed in oocytes, granulose, and theca cells and that it is involved in ovarian follicle growth and estradiol biosynthesis [31]. Most importantly, AhR is involved in the cross-regulation of other NRs, in particular ERs [32]. PXR expression emerges as a potential biomarker related to EDC exposure and infertility, since it is enhanced in infertile women in association to either BPA and PFOS exposure. The upregulation of PXR is biologically plausible, since this "sensor" NR is involved in the metabolism of xenobiotics and endogenous compounds, including steroids [29]. It may be noteworthy that PFOA behaved differently than PFOS, as its internal levels are negatively correlated with PXR in fertile women and with AhR in infertile patients. Indeed, Kraugerud et al. [33] suggest that the toxicological patterns of PFOS and PFOA might not overlap.

Finally, the expression of PPARγ in PBMC was low and did not show any meaningful change, indicating that it is not a reliable biomarker for EDC in women at childbearing age.

We are aware of the potential limitations of our study related to the presence of potential confounding factors,

the sample size, and the cross-sectional design. We checked the presence of potential confounding factors, such as age, smoking, BMI, metabolic disease conditions, or special dietary habits (celiac disease, vegetarianism). No significant differences between infertile and control fertile women were found. Our sample size (both infertile and control groups) has limited the statistical power of the study in detecting small differences, assuming that EDC could have multiple targets and can affect woman reproduction in more than one way [2, 34].

To date, our data cannot completely state the effective role of EDC in the onset of reproductive disorders but point out how specific NRs could be relevant markers to be used in EDC risk assessment. Even if the currently available studies are still insufficient to support a full risk characterization of EDC, evidence prompts to further investigation to address knowledge gaps and precautionary actions against excess exposure to specific compounds. It is really important to consider human exposure to multiple compounds as a real-life scenario to provide special attention to substances that are still widely present in consumer products and to integrate exposure measurements with relevant indicators of biological effects. Such evidence should be seen as sufficient grounds to take precautionary action to substitute potential endocrine disruptors as well as endocrine disruptors.

Conflict of Interests

The authors report no conflict of interests.

Acknowledgment

This study is founded by the Italian Environment Ministry (Project no. Q75): study in model areas on the environmental and health impact of some emerging chemical contaminants (endocrine disrupters), living environment, reproductive outcomes, and repercussions in childhood (PREVIENI).

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 590261, 7 pages
http://dx.doi.org/10.1155/2013/590261

Research Article

Action of Halowax 1051 on Enzymes of Phase I (CYP1A1) and Phase II (SULT1A and COMT) Metabolism in the Pig Ovary

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Received 4 January 2013; Accepted 21 March 2013

Academic Editor: Radmila Kovacevic

Polychlorinated naphthalenes (PCNs) are a group of organochlorinated compounds exhibiting dioxin-like properties. Previously published data showed the direct action of PCN-rich Halowax 1051 on ovarian follicular steroidogenesis. Taking into consideration that the observed biological effects of PCNs may be frequently side effects of metabolites generated by their detoxification, the aim of this study was to determine the activity and expression of enzymes involved in phase I (cytochrome P450, family 1 (CYP1A1)) and phase II (sulfotransferase (SULT1A) and catechol-O-methyltransferase (COMT)) detoxification metabolism. Cocultures of granulosa and theca interna cells collected from sexually mature pigs were exposed to 1 pg/mL to 10 ng/mL of Halowax 1051 for 1 to 48 hours, after which levels and activities of CYP1A1, SULT1A, and COMT were measured. Dose-dependent increases of CYP1A1 activity and expression were observed. High doses of Halowax 1051 were inhibitory to COMT and SULT1A activity and reduced their protein levels. In conclusion, fast activation of phase I enzymes with simultaneous inhibition of phase II enzymes indicates that the previously observed effect of Halowax 1051 on follicular steroidogenesis may partially result from metabolite action occurring locally in ovarian follicles.

1. Introduction

Polychlorinated naphthalenes (PCNs) are chlorinated organic compounds that have been used in various industries as capacitor impregnates, electrical insulating compounds, flame-resistant seals for condensers, gauge fluids, and lubricants because of their beneficial properties [1]. Until the 1980s, PCNs as mixtures of congeners were synthesized in North America (Halowax) and Europe (e.g., Nibren Wax, Basileum, and Seekay Wax) [2]. Currently, there is no commercial use for PCNs [1]. Production of PCNs has ceased due to its substitution by less toxic chemicals [3]. Even though the production of PCNs has ended, humans are still exposed to PCNs via food consumption [4–7].

As with other polychlorinated diaromatic hydrocarbons, PCNs are lipophilic compounds that persist in the environment and bioaccumulate in biological tissues [8]. There are reports indicating the presence of PCNs in adipose tissue and body fluids of people exposed to these agents [6, 9, 10].

In spite of significant research into the presence of PCNs in various samples and their dioxin-like properties, data concerning their action as endocrine disruptors are scarce. Akerblom et al. [11] showed that differences in oocyte maturation exist between control and PCNs-exposed ovaries. Further, published data from our laboratory showed the direct action of Halowax 1051 on ovarian follicular steroidogenesis [12]. Together, these reports indicate that PCNs can disrupt the endocrine system, thereby leading to reproduction defects.

It should be taken into consideration that the effects of exposure to PCNs may be due to the side effects of PCN metabolites. Environmental chemicals such as PCNs may be metabolized to more polar compounds in living organisms. For instance, PCNs can be transformed into hydroxylated metabolites [3]. Cytochrome P450 proteins (CYPs) play key roles in the metabolism and elimination of exogenous substances. Enzymes belonging to the CYP family are called phase I enzymes; they monooxygenate, reduce, and hydrolyze various substances such as lipids, steroidal hormones, and xenobiotics [13]. Enzymes of phase I are expressed mainly in the liver, but they are also present in other tissues such as uterus, adrenal glands, placenta, kidney, brain, and testis

[14]. In previously published papers we showed that three cytochrome P450 (CYP) isoforms, CYP1A1, CYP1A2 and CYP2B, are present in porcine prepubertal ovary cells [15–17].

After phase I reactions, xenobiotics may be further metabolized by conjugation with charged species such as glutathione, sulfate, glycine, or glucuronic acid to form more polar products, which are more efficiently eliminated from the organism. Rabbits exposed to monochloro- or dichloronaphthalenes excreted 70 to 90% of these compounds in four days, mainly as conjugates of glucuronic acid (54–69%) and mercapturic acid (13–18%). Minor amounts of naphthalene sulfates and phenolic conjugates were also excreted [3]. Phase II enzymes are expressed mainly in the liver, but they are also found in the ovary. Catechol-O-methyltransferase (COMT) is expressed in porcine and human granulosa cells [17, 18] and sulfotransferase (SULT1A) is expressed in porcine ovaries [17, 19]. When determining the metabolism of 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) in the porcine ovary, Karpeta et al. [17] showed fast activation of CYP2B1/2, late activation of COMT, and lack of activation of SULT1A. This confirmed the action of phase II enzymes in the ovary and suggested the possible action of locally produced hydroxylated metabolites prior to their detoxification.

In this study, we ought to determine whether PCNs are metabolized in the ovary by examining the effects of the Halowax 1051 on phase I (CYP1A1) and phase II (SULT1A and COMT) enzyme activities and expression in cultured granulosa and theca interna cells.

2. Materials and Methods

2.1. Reagents. Halowax 1051 was obtained from Koppers Co., USA. A stock solution of this compound was dissolved in DMSO. The final concentration of DMSO in the medium was 0.1%. Parker's medium (M199) lacking phenol red, trypan blue, Laemmli lysis-buffer, dimethyl sulfoxide (DMSO), Tris, sodium dodecyl sulfate (SDS), Tween 20, 4-nitrocatechol, SAM, 3,5-diniotrocatechol, PAPS, p-nitrophenyl sulfate, 2-naphthol, and 2,6-dichloro-4-niotrophenol were obtained from Sigma Chemical Co. (Saint Louis, MO, USA). Fetal bovine serum (FBS, heat inactivated), phosphate-buffered saline (PBS), and Trypsin-EDTA and antibiotic, antimycotic solution (penicillin 100 U/mL, streptomycin 100 μg/mL, amphotericin B 0.25 μg/mL) were obtained from PAA Laboratories GmbH (Colbe, Germany).

2.2. Tissue Culture. Porcine prepubertal ovaries were obtained from a local abattoir. Granulosa cells (Gc) and theca interna cells (Tc) obtained from the same follicles were subsequently prepared according to the technique described by Stoklosowa et al. [20, 21]. After isolation, Gc and Tc were collected and suspended in M199 medium supplemented with 10% fetal bovine serum. The viability of the cells was determined before seeding by the trypan blue exclusion test; viability was 60–75% for Gc and 85–90% for Tc. Gc and Tc were inoculated at concentrations similar to those observed *in vivo* (Gc : Tc, 4 : 1). The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h to allow for attachment. Then the cells were cultured for an additional 1, 6, 24, or 48 h with Halowax 1051 doses of 1, 10, and 100 pg/mL and 1 or 10 ng/mL.

2.3. Experimental Procedure. To determine CYP1A1 activity, cells were seeded in 96-well tissue culture plates at concentrations of 1.5×10^5 viable cells per well and exposed to the test compound for 1, 6, 24, or 48 h. At the end of the incubation, media were removed and the cells were washed with cold phosphate buffered saline (PBS) and stored at −70°C.

To determine SULT1A and COMT activity, cells were seeded in 48-well tissue culture plates at concentrations of 2.5×10^5 viable cells per well and exposed to the test compound for 6, 24, or 48 h. At the end of the incubation, media were removed and the cells were washed with cold PBS and stored at −70°C.

To examine the dose- and time-dependent effect of Halowax 1051 on CYP1A1, SULT1A, and COMT protein expression, cells were seeded in 24-well tissue culture plates at concentrations of 5.0×10^5 viable cells per well and exposed to Halowax 1051 for 1, 6, or 48 h. After incubation, media were removed and the cells were washed with cold PBS and then lysed using Laemmli lysis-buffer. Total cell lysates were stored at −70°C.

2.4. CYP1A1 Activity. Frozen cells were lysed when removed from the freezer and allowed to thaw for 10 min. The ethoxyresorufin-O-deethylase assay (EROD), a specific measure of CYP1A activity, was performed as described by Kennedy and Jones [22]. The fluorescence of resorufin generated by the conversion of ethoxyresorufin by CYP1A was measured at 15 min interval for up to 2 h with a fluorescence plate reader (FLx 800, Bio-Tek, USA) using a 530 nm excitation filter and a 590 nm emission filter. After 2 h, the protein concentration in each well was determined by the fluorescamine protein assay (Sigma Chemical Co. MO, USA). Results were calibrated against a resorufin standard curve (0–100 nM) and a BSA standard curve (0–1000 μg).

2.5. COMT Activity. COMT activity was measured using a modified colorimetric assay described by Herblin [23] (which uses methylation of 4-nitrocatecholas as a marker of COMT activity) with the following modifications. Frozen cells were thawed for 10 min. Each reaction well received 200 μL of 25 μM 4-nitrocatechol, 0.01 M of $MgCl_2$, and 1 mM of Tris-HCl buffer (pH 7.0). Plates were preincubated for 10 min at 37°C. Then 0.2 mM of S-adenosyl methionine was added and incubated for an additional 60 min. The reaction was terminated by the addition of 1 M NaOH, and absorbance was measured at a wavelength of 520 nm using a micro-ELISA plate reader (Bio-Tek Instruments).

2.6. SULT1A Activity. SULT1A activity was measured using a modified colorimetric assay developed by Frame et al. [24]. This method is based on the release of p-nitrophenol from a 3′-phosphoadenosine-5′-phosphosulfate (PAPS) regenerating system. Frozen cells were thawed for 10 min. Each reaction well contained 50 mM of potassium phosphate

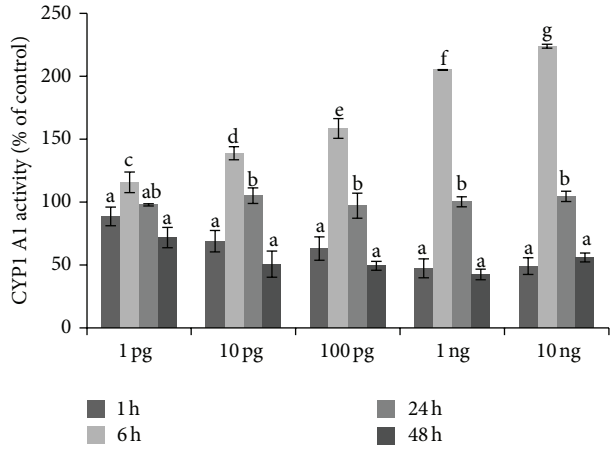

Figure 1: Time- and dose-dependent action of Halowax 1051 on CYP1 A1 activity showed as a percentage of control. Cocultures of granulose and theca cells were exposed to 1, 10, and 100 pg/mL and 1 or 10 ng/mL of Halowax 1051. Each treatment was repeated three times ($n = 3$). Statistically significant differences between points in graph are indicated with different letters; the same letters indicating no significant differences, with a < b < c < d < e < f < g.

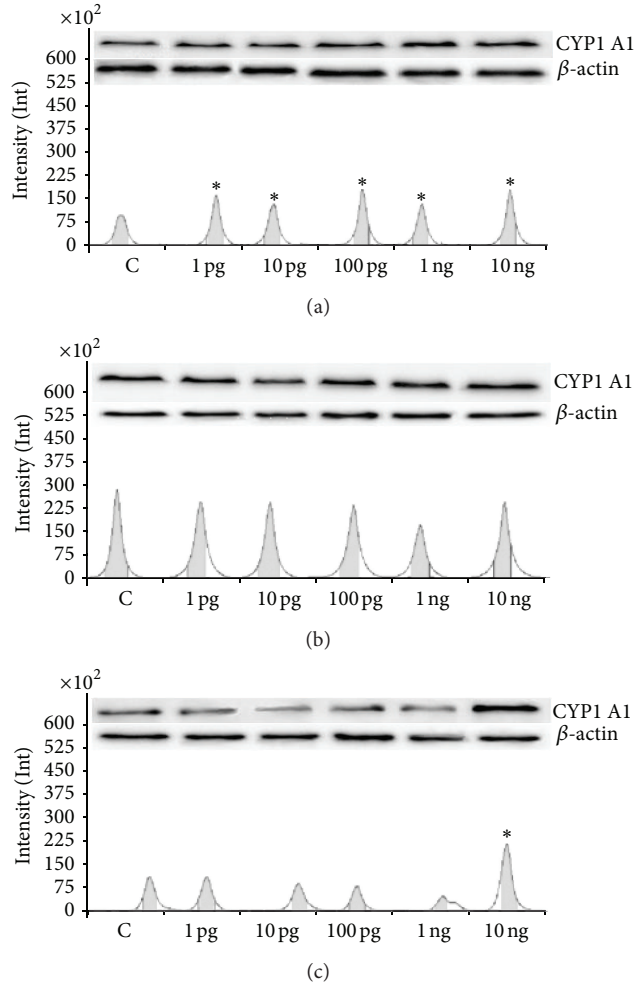

Figure 2: (a) Protein expression (immunoblot) of CYP1 A1 after 1 (a), 6 (b), and 48 (c) hours of incubation with Halowax 1051. The amount of protein in each sample was checked using an anti-β-actin antibody. All means marked with ($^*P < 0.05$) are significantly different from the control.

buffer, 5 mM of $MgCl_2$, 20 μM PAPS, 5 mM p-nitrophenyl sulfate, and 0.1 mM 2-naphthol in a total volume of 250 μL. As a negative control, cells were incubated for 48 h with the selective inhibitor of SULT1A, 2,6-dichloro-4-nitrophenol, at a dose of 0.5 μM. The reactions were incubated at 37°C for 60 min and then terminated by the addition of 0.25 M of Tris-HCl buffer (pH 8.7). Absorbance was measured at a wavelength of 405 nm using a micro-ELISA plate reader (Bio-Tek Instruments).

2.7. Western Blot Analysis. Equal sample volumes were separated by SDS-PAGE and electrophoresed onto PVDF membranes using a Bio-Rad Mini-Protean 3 apparatus (Bio-Rad Laboratories, Inc., USA) according to the manufacturer instructions. Blots were incubated overnight with 1 : 200 dilutions of antibodies specific to CYP1A1 (sc-9828), SULT1 A (sc-27980), and COMT (sc-25844) (all from Santa Cruz Biotechnology Inc., CA, USA) and with 1 : 2000 dilution of antibodies specific to β-actin (A5316) (Sigma Chemical Co., MO, USA). An anti-β-actin antibody was used as a loading control. Primary antibodies were detected by a horseradish peroxidase-conjugated secondary antibody: P0447 (Dako-Cytomation, Denmark) for β-actin diluted 1 : 5000; sc-2020 (Santa Cruz Biotechnology Inc., CA, USA) for CYP1A1 and SULT1 diluted 1 : 2000; and sc-2004 for COMT diluted 1 : 2000, essentially according to the manufacturer's guidelines. Signals were detected by enhanced chemiluminescence using the Western Blotting Luminol Reagent (sc-2048, Santa Cruz Biotechnology) and were visualized using the ChemidocTM XRS+ System (Bio-Rad Laboratories). Data visualized by chemiluminescence were quantified by using Image LabTM 2.0 Software (Bio-Rad Laboratories).

2.8. Statistical Analysis. Each treatment was repeated three times ($n = 3$) in quadruplicate. Statistical analysis was performed using GraphPad Prism 5. Statistically significant differences between groups are indicated with different letters; the same letters indicating no significant differences, with a < b < c < d < e < f; statistically significant differences between control and treated groups were marked with $^*P < 0.05$. All data ($n = 12$) are expressed as the mean ± the standard error of the mean.

3. Results and Discussion

3.1. Phase I Metabolism. CYP1A1 enzyme activity was assayed using the ethoxyresorufin-O-deethylase assay. Basal CYP1A1 activity was the highest in 6 h of incubation (with values: 26.27 ± 1.05; 101.08 ± 2.13; 16.36 ± 0.58; and 31.86 ± 0.93 pmol per 100 μg protein min^{-1} after 1, 6, 24, and 48 h of culture, resp.). This is in accordance with our last published data [17] showing also the high basal CYP1A1 activity in ovarian follicles in 6 h of culture. A stimulatory effect on

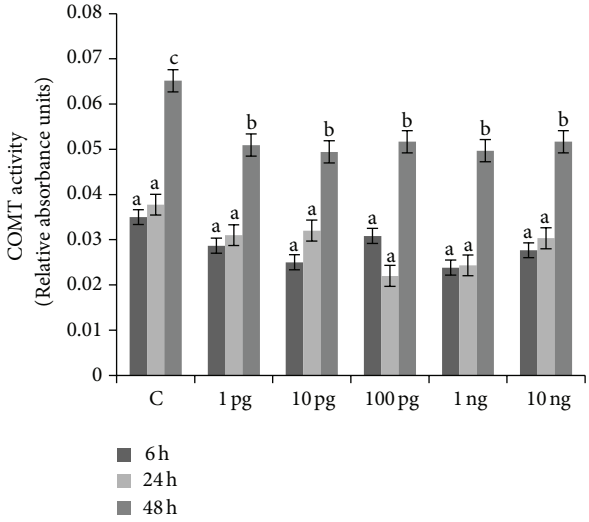

Figure 3: Time- and dose-dependent action of Halowax 1051 on COMT activity. Cocultures of granulose and theca cells were exposed to 1, 10, and 100 pg/mL and 1 or 10 ng/mL of Halowax 1051. Statistically significant differences between points in graph are indicated with different letters; the same letters indicating no significant differences, with a < b.

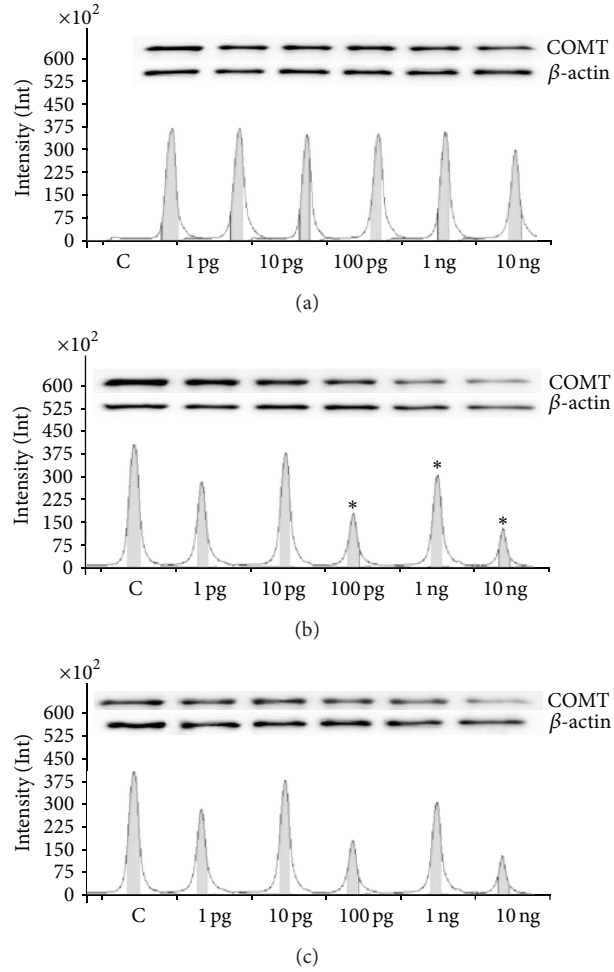

Figure 4: Protein expression (immunoblot) of COMT after 1 (a), 6 (b), and 48 (c) hours of incubation with Halowax 1051. The amount of protein in each sample was checked using an anti-β-actin antibody. All means marked with ($^{*}P < 0.05$) are significantly different from the control.

CYP1A1 activity was observed after 6 h of exposure to all doses of Halowax 1051 used (Figure 1). Basal CYP1A1 protein expression increased from 1 h to 24 h of culture and then decreased at the 48 h time point. Halowax 1051 after 1 h of exposure to all doses had a stimulatory effect on CYP1A1 protein expression. In addition, only for dose of 10 ng/mL activation of the CYP1A1 protein expression maintained for 48 h (Figure 2). Our previous studies have shown compound-dependent differences in the time of CYP1A1 activation: faster induction of the CYP1A1 protein by PCB3 (from 1 to 48 h) than by 17-β estradiol (from 6 to 48 h) [25].

The observed rapid activation of CYP1A1 under the influence of Halowax 1051 is probably associated with dioxin-like properties of PCN while longer-lasting activation under the influence of PCB-3 or 17-β estradiol with nondioxin-like properties of tested compounds. This suggests that the activation time depends not only on doses but also on type of used reagent.

Similarly, increased EROD activity in the livers of juvenile Baltic salmon, *Salmo salar*, exposed to a mixture of Halowax 1001, 1014, and 1051 [11] and in liver of rat exposed to PCNs [26] was demonstrated. Previously, Villeneuve et al. [27] estimated that relative potencies of individual PCNs in relation to a 2,3,7,8-tetrachlorodibenzodioxin standard generally increased with increasing chlorine substitution and were the highest for the most chlorinated compounds. Highly chlorinated PCNs are present in Halowax 1051. To our knowledge, this report is the first to show the impact of Halowax 1051 on microsomal enzymes in the ovary, suggesting that metabolites of PCNs are formed locally in this organ.

3.2. Phase II Metabolism. The second, and probably most important, finding was the inhibitory effect of Halowax 1051 on the activities and expression of COMT and SULT1A. The second phase of detoxification metabolism is particularly important because it leads to the formation of compounds that are removed via the urinary system. In the existing literature, no data show the detoxification of any PCNs by phase II enzymes in the ovaries. A very early study (by Cornish and Block) [28] showed that metabolites of hepta- and octachloronaphthalenes (main compounds of Halowax 1051) were absent from the urine of rabbits after the oral administration of PCNs, suggesting that the metabolism of these compounds proceeded only partially.

Basal COMT activities were 0.035 ± 0.003, 0.038 ± 0.004, and 0.065 ± 0.004 relative absorbance units after 6, 24, and 48 h, respectively. The inhibitory action of each dose of Halowax 1051 on COMT activity was noted after 48 h of exposure (Figure 3). A high level of COMT protein expression was observed in the control after 6 h of culture. COMT protein expression was decreased relative to the control after 6 h of

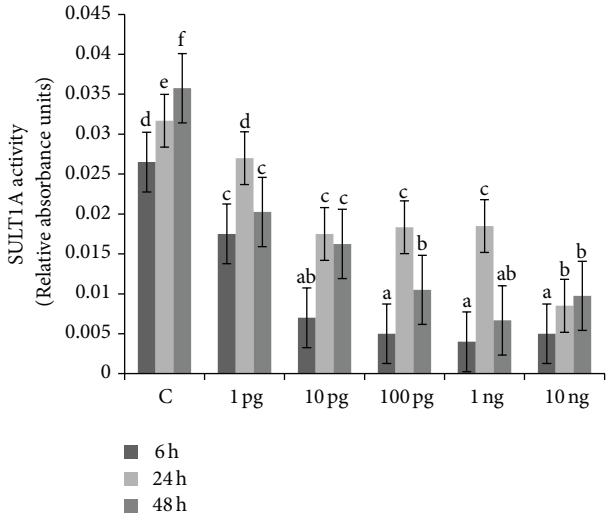

Figure 5: Time- and dose-dependent action of Halowax 1051 on SULT1A activity. Cocultures of granulose and theca cells were exposed to 1, 10, and 100 pg/mL and 1 or 10 ng/mL of Halowax 1051. Statistically significant differences between points in graph are indicated with different letters; the same letters indicating no significant differences, with a < b < c < d < e < f.

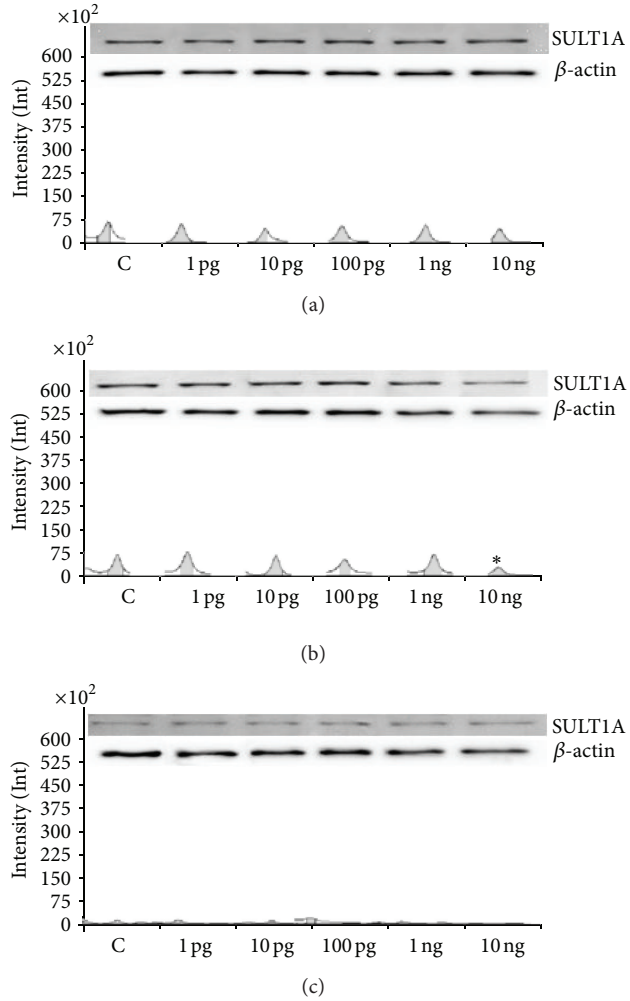

Figure 6: Protein expression (immunoblot) of SULT1A after 1 (a), 6 (b), and 48 (b) hours of incubation with Halowax 1051. The amount of protein in each sample was checked using an anti-β-actin antibody. All means marked with ($^{*}P < 0.05$) are significantly different from the control.

exposure to 100 pg/mL, 1 ng/mL and 10 ng/mL, and after 48 h of exposure to 10 ng/mL of Halowax 1051 (Figure 4).

COMT is widely distributed throughout the animal kingdom and is primarily associated with the cytosolic fraction of many tissues including porcine granulosa cells [17, 29]. Substrates of COMT include xenobiotic catechols, catecholamines, and catechol estrogens. It has been shown that exposure to 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) increases COMT activity after 24 and 48 h with no effect on protein expression, as measured by immunoblot and ELISA analyses. This suggests that hydrophilic methoxylated polybrominated diphenyl ethers may be formed locally in the ovary. There are no previous data on the effects of Halowax 1051 on COMT activity in the ovary; however, Hernandez et al. [30] showed that COMT inhibition in pregnant rats produces arterial hypertension and endothelial dysfunction due to reduced nitric oxide bioavailability. Further, la Merril et al. [31] showed that exposure to 2,3,7,8-tetrachlorodibenzodioxin lowers COMT expression in mouse mammary glands, which are also hormone-dependent tissues. Therefore, the results in this paper are consistent with those of previous reports and indicate that hydrophilic methoxylated PCNs are not formed in the ovary.

Basal SULT1A activities were 0.026 ± 0.007, 0.031 ± 0.006, and 0.035 ± 0.008 relative absorbance units after 6, 24, and 48 h, respectively. The inhibitory action of Halowax 1051 on SULT1A activity was noted at each dose and time point. The strongest effect of the mixture was observed after 6 h of exposure to doses ranging from 10 pg/mL to 10 ng/mL (Figure 5). Basal SULT1A protein expression was indistinguishable between 1 and 6 h of culture but decreased after 48 h of culture. SULT1A protein expression decreased after only 6 h of incubation with 10 ng/mL Halowax 1051 (Figure 6).

SULT1A is the most abundant sulfotransferase; it has a broad substrate specificity and a wide tissue distribution [32]. Several SULT1A subfamily members contribute to sulfate conjugation of endogenous substrates, such as the thyroid hormone 17β-estradiol, as well as exogenous compounds including xenobiotics [33, 34]. Sulfate conjugation generally results in a decrease of biological activity and an increase in the hydrophilicity of compounds, which facilitates their excretion. As mentioned earlier, a previous study showed that rabbits could excrete 70 to 90% of ingested monochloro- or dichloronaphthalenes. Unfortunately, there have been no subsequent studies concerning PCN activation of enzymes involved in the second phase of detoxification metabolism. However, some studies have shown that other xenobiotics such as polychlorinated biphenyls may inhibit SULT1A activity [35]. As in the case of COMT activity, there have been no

previous reports of the effects of Halowax 1051 on SULT1A activity in the ovary. Considering that sulfate conjugation of xenobiotics usually decreases their toxicity, we suggest that the inhibition of this pathway may lead to prolonged compound exposure in the ovary and subsequent disruption of ovarian function.

4. Conclusion

The activation of phase I enzymes (CYP1A1) and inhibition of phase II enzymes (SULT1A and COMT) confirm the dioxin-like properties of PCNs. Fast activation of enzymes involved in phase I and concurrent inhibition of enzymes involved in phase II metabolism indicate that the observed effects of Halowax 1051 may partially result from the action of metabolites formed locally in ovarian follicles.

Acknowledgment

This work was supported by K/DSC/000884/2012, Jagiellonian University, Kraków, Poland.

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