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Endocrine Disruptors

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ENDOCRINE DISRUPTORS

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Meet the editor

Dr. Ahmed R.G. received his doctorate degree in Developmental Biology (Developmental endocrinology) from Beni-Suef University, Egypt, and received research training (postdoctoral fellowship) as a visiting scholar at the Catholic University, Belgium. He also has outstanding records of scientific and academic accomplishments with multiple research funding initiatives, numerous publications (books/papers) in highly prestigious journals, and various presentations at both national and international conferences. He is a member of a number of eminent societies, organizations, and schools. He has served as a scientific editor and reviewer for national and international research institutions. He received a Publons Peer Review Award (one of the top 1% of peer reviewers in Science and Research) [Honoring the Sentinels of Science and Research].

Contents

Preface

Endocrine-disrupting chemicals (EDCs), including many agents of chemical or natural origin, are able to imbalance hormone-driven processes in animals and humans (fish-eating populations). Disruption of maternal endocrine hormones during fetal development may result in irreversible consequences in offspring. This book, Endocrine Disruptors, starts with an overview of what endocrine disruptors are, the questions surrounding them, and the basis of these chemicals in the ecosystem. The book covers the mechanism of action of bisphenol S and dibutyl phthalates. This is followed by the biotransformation of bisphenol A and its adverse effects on the next generation. The book also describes the effect of benomyl and its metabolite carbendazim on the reproductive and developmental health and also the role of endocrine disruptors on reproductive toxicity in males. The book offers comprehensive coverage about the communication between the endocrine disruptors and obesity. The final chapter addresses detection of the endocrine-disrupting chemicals using autobioluminescent cellular bioreporters. This book will be of interest to scientists, neuroendocrinologists, neurotoxicologists, physicians, and lay readers wishing to review recent developments in the field of EDCs.

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Chapter 1

Occurrence of Endocrine Disruptor Chemicals in the Urban Water Cycle of Colombia

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Jaime Andrés Lara-Borrero

Additional information is available at the end of the chapter

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Abstract

In developing countries such as Colombia, information on the occurrence of endocrine disruptors is still incipient. Bogotá, the capital of Colombia, has a complexity at an anthropogenic and environmental level that makes it particularly important to determine the possible presence of this type of compounds and the risks associated with its presence in aquatic environments. During the present study, the occurrence of endocrine disruptors, mainly pharmaceuticals, plasticizers, and hormones in different aquatic matrices including wastewater, surface water, runoff water, and drinking water was evaluated; the results show that phthalates present the highest occurrence followed by bisphenol A, with an important participation of carbamazepine (0.68–31.45 μg L^{-1}), the most commonly found compound is bis(2-ethylhexyl) phthalate (BEHP). It was also found in the drinking water, this leads to the conclusion that endocrine disruptors in Colombia and Bogotá are a reality and deserve attention from researchers to deepen their potential sources of generation and control strategies, as well as the provision must start generating policies in this regard.

Keywords: endocrine disruptors, occurrence, water pollution, hazard ratio, emerging contaminants

1. Introduction

The increased interest in the study of endocrine disruptors and other emerging pollutants in aquatic matrices that is evidenced in different scientific publications [1] arises from the evidence of their presence in components such as wastewater and its association with problems and effects on ecosystems. This is also due to the high production and commercialization of chemical

products in the world with different uses that cause them to be in important concentrations in all matrices, including water [2]. However, both the occurrence studies and the ecotoxicological and risk studies have not been conducted with the same frequency throughout the world, with large differences in the amount of research conducted in the United States and Europe relative to that in Latin America or elsewhere in the world [3].

Although there has been a significant increase in research in this field, there is still a marked difference between the quantity of substances produced and the capacity to monitor, control and understand the totality of the transformations and impacts on the ecosystems they generate. Research and policy tools are lacking in many contexts around the world [2, 4]. Research on ECs encompasses multiple concepts and definitions that are related, including the concepts of micropollutants, personal care products, pharmaceuticals, disinfection byproducts, and flame retardants, among others [5].

To address the issue of endocrine disruptors, it is necessary to speak on emerging pollutants in the first instance, an EC or, preferably, a contaminant of emerging concern (CEC) is defined as any naturally occurring substance, chemical or artificial material that has been discovered or suspected to be present in various environmental compartments and whose toxicity or persistence is likely to significantly alter the metabolism of a living being [6]. The classification of ECs has also been the subject of discussion based on the aforementioned definitions; many of the so-called micro-contaminants are ECs, and even so-called nanoparticles are included. Some of the most widespread classifications include drugs, hormones, polymers, pesticides, stimulants, nanoparticles, and nanomaterials [7–10].

Endocrine disruptors (EDs) chemicals are a category within ECs associated with the type of health risk, such as those that are capable of disrupting the normal functioning of the endocrine system, responsible for all hormonal physiology in living beings [11]. There has been considerable research on EDs around the world, predominantly in Europe and the United States [12, p. 37, 13–16]. Studies have also been performed in Asia, specifically in China, where ED concentrations rarely exceed micrograms per liter [17, 18]. The risk and toxicity of the different ECs have been approached from different points of view, including controlled experimental trials, cohort studies, epidemiological studies of cases and controls, and ecotoxicological studies focusing on the chronic risk as EDs [19, 20]; one of the best known cases is that of bisphenol A, a recognized plasticizer of widespread use worldwide [21]. As part of what continues to be found regarding the level of the risk, contaminants appear on various lists around the world according to precautionary principle, and more than 56 were identified in a single sample of surface water [22]. One of the main sources of EDs in the aquatic environment is wastewater, where it is possible to find sufficient concentrations of these compounds to contaminate surface sources or subsequently contaminate soils or food when the compounds are used for irrigation [23]. This situation has even led water for human consumption exhibiting significant amounts of disruptors; according to a study by Plotan et al. [24], even in bottled and flavored water, it is possible to find concentrations of EDs such as β-estradiol (10 ng L^{-1}), testosterone (26 ng L^{-1}), progesterone (123 ng L^{-1}), and hydrocortisone (13.5 ng L^{-1}).

Another aspect that has presented challenges in the monitoring of EDs is the diversity of compounds that can affect the endocrine system and the techniques for measuring them, which are increasingly sensitive and robust; Mol et al. [25] proposed as a technique for the quantification of

EC gas chromatography-mass spectrometry, noting that this technique offers good results with respect to other methodologies used, such as supercritical fluid extraction, derived fluorescence followed by liquid-liquid microextraction, and other techniques of chromatography with mass spectrometry. This in turn has been proven to be a high precision technique with a sensitivity of 4–6 ng L^{-1}, except for hormones, for which the precision is 50–300 ng L^{-1} [26].

At the level of Colombia and specifically in the city of Bogotá, there have been no studies evaluating the occurrence of EDs in aquatic matrices or the associated ecosystem risk; thus, it is important to set a precedent taking into account the problems of domestic and industrial wastewater pollution in the primary cities around the country [27]. The city of Bogotá has chemically assisted coverage for the treatment of domestic wastewater of approximately 30% at the primary level, which implies the arrival of large amounts of organic matter and different compounds in surface waters such as rivers and channels [28]. On the other hand, the evaluation of photocatalytic treatments [29] or natural systems (wetlands) [30] for the treatment of some endocrine disruptors of general interest has been studied.

2. Study area

Colombia is a tropical country located north of South America and whose capital is Bogotá, has around 9 million inhabitants, also constituting the most important commercial and industrial city [31]. The main river that crosses the extension of the capital district is the Bogotá River that in turn receives as main tributaries the Tunjuelo, Fucha, and Arzobispo rivers that flow into the Northwest zone of the city; these present a significant pollution due to the discharge of wastewater and industrialists from all areas of the city.

Figure 1 shows the distribution area of the sampling points that included three points in the Bogotá River, in the mouths of its effluents, in three rainwater channels, three wetlands, the

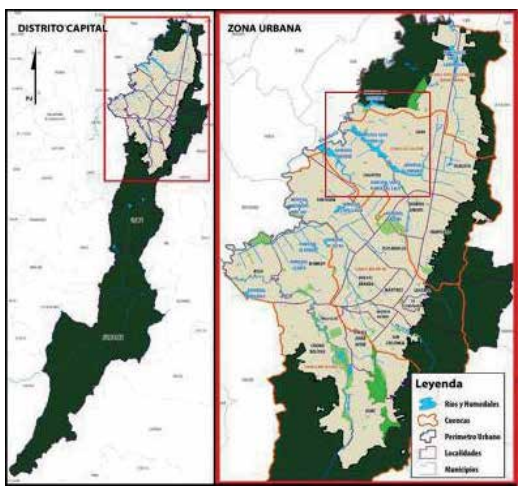

Figure 1. Distribution of monitoring points of EDs in the city of Bogotá.

main sources of supply for the city, and points of the water distribution network; the potable water points were selected according to their distribution from the three current supply systems in the city, including the reservoirs used to supply each system. As for the residual water points, the determination was made at the plant (northwest of the city) and at a sewage pumping station known as the San Benito lifting plant in the southeast.

The selection of the compounds for the monitoring of EDs was based on preliminary and secondary information that allowed us to know the possible sources and quantities generated, groups and commercially available categories, and availability of external standards used in the assembly of the standards and their corresponding calibration curves. These categories are as follows: pharmaceutical compounds (14 compounds), organophosphorus pesticides (20 compounds), hormones and steroids (8 compounds), and phthalates (14 compounds); all high-quality standards were obtained from RESTEK® (Pennsylvania, USA).

3. Sampling

The sampling sites were monitored at selected points according to the criteria of representativeness, access, importance, and physicochemical characteristics. In the points that had current such as rivers, sewers, and channels, composite sampling was carried out, whereas specific samples were taken in the dams and wetlands. **Table 1** shows the number of points and their description and observations.

Samples were taken at the defined points on the dates scheduled for four monitoring days during the periods of August to December 2015 and February to May 2016. The method used

Component	Number of points	Location	Observations
Surface water	3	Tributaries of the Bogotá River (Tunjuelo, Fucha, and Archbishop)	The main contributors of flow and pollutant load
	3	Rio Bogota entrance to Bogota, exit and intermediate point	The purpose is to observe the contribution of the city to the occurrence of EDs
	18	Wetlands (Jaboque, Juan Amarillo and La Conejera)	Within each wetland, samples were taken at different points (6 per wetland)
Drinking water	3	Inputs to the supply systems (Chingaza, Tibitoc, and Sumapaz)	Selected from stratification according to the coverage percentages of the three current supply systems (Wiesner [70%], Tibitoc [20%], and El Dorado [10%])
	49	Points in supply network	29 for Chingaza, 11 for Tibitoc, and 9 for Sumapaz
Sewage water	2	Entry and exit of Salitretreatment plant	Coverage of approximately 40% of the city's wastewater
	1	San Benito lift plant exit	High presence of companies for tanning of skins
Runoff waters	3	South zone, north zone, and center zone	Three channels of rainwater that are part of the city's sewage system.
Total	82		

Table 1. Number and type of points selected for each component of the urban water cycle of the city of Bogotá.

to determine the flow in terms of area by velocity was to take a sample every hour for a period of 8 h, recording the values of flow rates and parameters at the measuring site (pH, conductivity, salinity, and oxygen). In drinking water, it was necessary to seek the assistance of the users of the system, who were trained to take the samples in their homes; these samples were subsequently collected for analysis.

At the exit of the reservoirs used as sources of supply, rainwater channels, sections of the Bogotá River and its tributaries, we used windmills. In the wetlands, samples were taken at different points in the water mirror and in the exits and entries that could be identified, taking into account that the latter are composed of large areas of water and in some cases are fractioned. Two liters of water were taken for each sampling point in each campaign in amber glass bottles of 1 l capacity, refrigerated, and taken as soon as possible to the laboratory for preprocessing.

4. Results and analysis

The spatial occurrence of the pharmaceutical group was determined, in particular, the pharmaceutical compounds are of interest due to their pharmacological activity and their wide use in all contexts; recent studies catalog them as some of the compounds of greater occurrence in aquatic matrices [1, 3, 32]. As expected, the presence of this type of compound exhibits a regular behavior, indicating that these compounds come from sources of continuous contamination, possibly from domestic wastewater containing residues of these compounds that are not fully metabolized [33]. In general terms, the concentrations exhibit similar behaviors for compounds such as fluoxetine but higher in the case of trimethoprim, 860 ng L^{-1}, relative to a range of 10–120 ng L^{-1} [3, 34]. The presence of carbamazepine is striking, as it is known to pose a significant risk to ecosystems and public health [35]. Based on this descriptive information, the highest occurrences are seen for fluoxetine and carbamazepine compounds in runoff waters (12%) and trimethoprim (17%) and carbamazepine (26%) in wastewater and surface waters; these compounds are commonly used as antiepileptics and antidepressants. In the case of carbamazepine, which presents the greatest occurrences, its presence in all type of aquatic matrices has been previously reported [36, 37]; its presence is related to incomplete metabolism in the body, excessive use by people and its persistence in the environment [35, 36].

Figure 2 shows that the points with the highest occurrence of pharmacists are in the western part of the city, specifically in the points located in the Bogota River and the mouths of its tributaries Tunjuelito, Fucha, and Arzobispo; this makes sense since they receive the runoff from city waters and also domestic and industrial wastewater resulting from wrong connections to the sewage or combined sewer system.

On the other hand, it should be noted that the point observed in the eastern part of the city corresponds to the main point of supply of the drinking water network (wiesner about 70% in coverage), this was presented for the carbamazepine compound that was found in two of the samples corresponding to this point. There is no evidence of contamination sources close to this source of supply, but an alert is generated in this regard that should lead to greater monitoring, mainly to one of the reservoirs that supply this system that is known as the

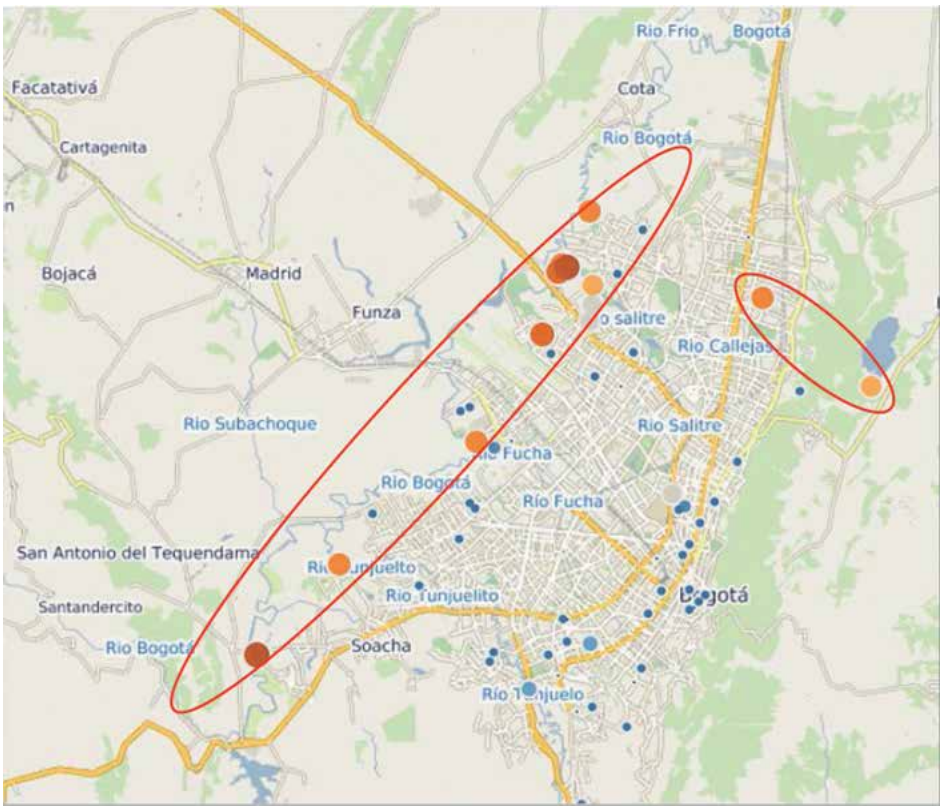

Figure 2. Spatial distribution of occurrence of pharmaceutical compounds.

San Rafael Reservoir, which is very close to inhabited areas and also to lands where agriculture is carried out.

Within the group of phthalates, a much more uniform distribution of these is observed within the city, which implies its distribution not only geographically speaking but in the different matrices evaluated, that is, runoff water, surface water, waste water, and drinking water. Of particular interest is the magnitude of the phthalates in the water supply network of the eastern part of the city, where it can be seen in **Figure 3** that the red color is darker, which represents a higher concentration of the compound bis(2-ethylhexyl) phthalate (BEHP). The area corresponds to the oldest part of the city, where the buildings are the oldest and there are also the oldest internal storage networks.

Aldana and Lopéz [31] ensure that the supply network of Bogotá presents a high complexity due to the variety of diameters, materials, unions, etc., which makes it extremely difficult to associate what is found to a particular factor; however, it is interesting to see the occurrence so high in all the matrices evaluated. Only some points of the drinking water network did not present any of the phthalates, the occurrence of this group of compounds was 100% in wastewater, runoff water, and surface water. The concentrations of the main compounds can be observed in **Figure 4**.

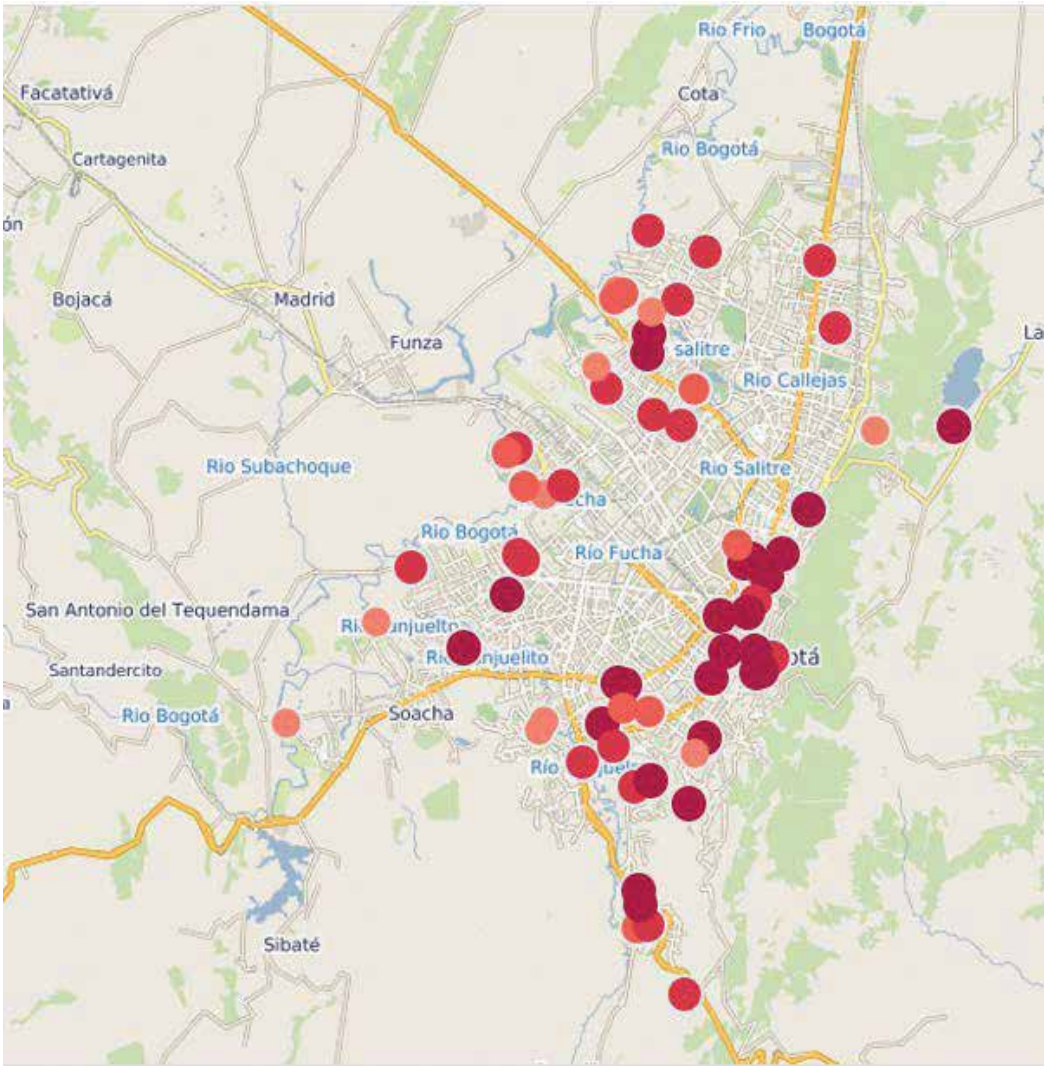

Figure 3. Spatial distribution of occurrence of phthalates.

Phthalates had a much higher occurrence in all evaluated matrices, including potable water points, both at source supply points and network points. Phthalates are widely extended compounds and are therefore found in all spheres of the environment [38]. However, their use as plasticizers in consumer products or industrial activities ensure that in cases such as drinking water, their presence tends to increase, specifically in the case of compounds such as N-diethylhexyl phthalate (BEHP), which is used as an additive in PVC pipes [39, 40]. In total, five compounds were found during the monitoring in all matrices, namely, DEP, DPP, BEHP, DnHP, and BnBP. For DEP, the highest occurrence was found in drinking water (28%); however, the highest occurrences in drinking water were measured for DPP and BEHP, with values higher than 80%. These compounds also showed wide variability in

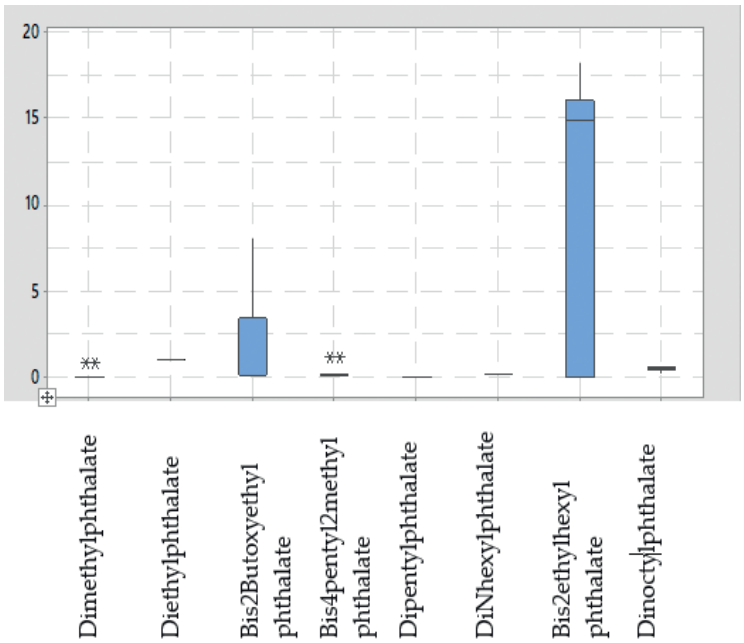

Figure 4. Concentration (μg L^{-1}) of phthalates in wastewater.

concentration, with most being below the concentration considered to have a negative effect on ecosystems and health [41].

It should be noted that the only wastewater treatment plant in the city of Bogotá works with the technology of advanced primary treatment or chemically assisted, this plant does not present high efficiencies for the removal of emerging contaminants, for which it would be required the implementation of other secondary or tertiary treatments such as membrane bioreactors [42] or constructed wetlands [43].

It is not surprising that the behavior observed in the surface waters composed of the Bogotá River and its tributaries (**Figure 5**) is similar to that observed in wastewater, since, as mentioned previously, these are the receptors of most of the city's pollution, including domestic waste water and in some cases industrial waste water.

In the case of the concentrations of pharmaceutical compounds, the presence of this type of compound exhibits a regular behavior, indicating that these compounds come from sources of continuous contamination, possibly from domestic wastewater containing residues of these compounds that are not fully metabolized [33]. In general terms, the concentrations exhibit similar behaviors for compounds such as fluoxetine but higher in the case of trimethoprim, 860 ng L^{-1}, relative to a range of 10–120 ng L^{-1} [3, 34]. The presence of carbamazepine is striking, as it is known to pose a significant risk to ecosystems and public health [35] (see **Figure 6**).

The results by the type of water evaluated show that the highest occurrences are seen for fluoxetine and carbamazepine compounds in runoff waters (12%) and trimethoprim (17%) and carbamazepine (26%) in wastewater and surface waters; these compounds are commonly

used as antiepileptics and antidepressants. In the case of carbamazepine, which presents the greatest occurrences, its presence in all type of aquatic matrices has been previously reported [36, 37]; its presence is related to incomplete metabolism in the body, excessive use by people

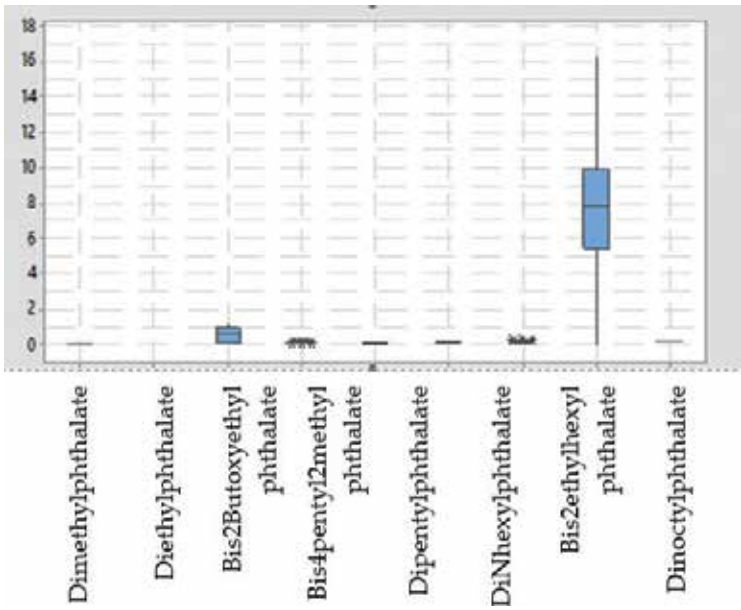

Figure 5. Concentration (μg L^{-1}) of phthalates in surface water.

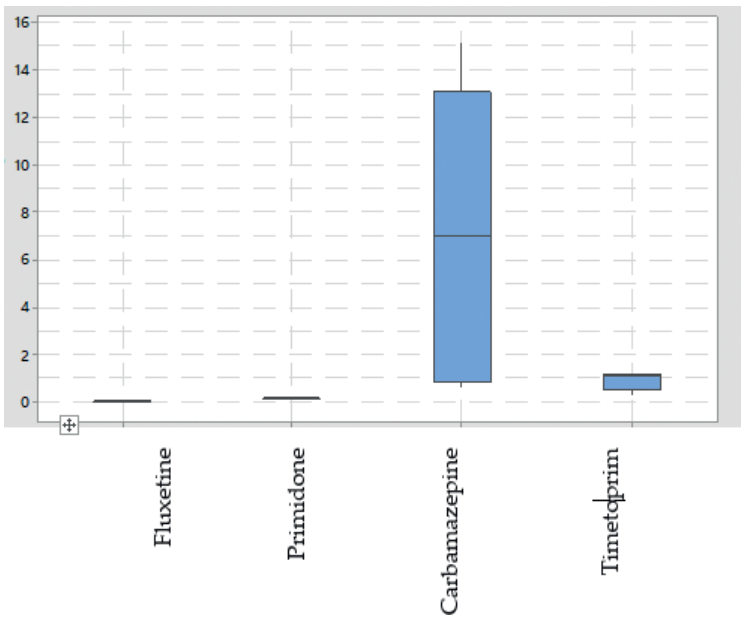

Figure 6. Concentration (μg L^{-1}) of pharmaceuticals in wastewater.

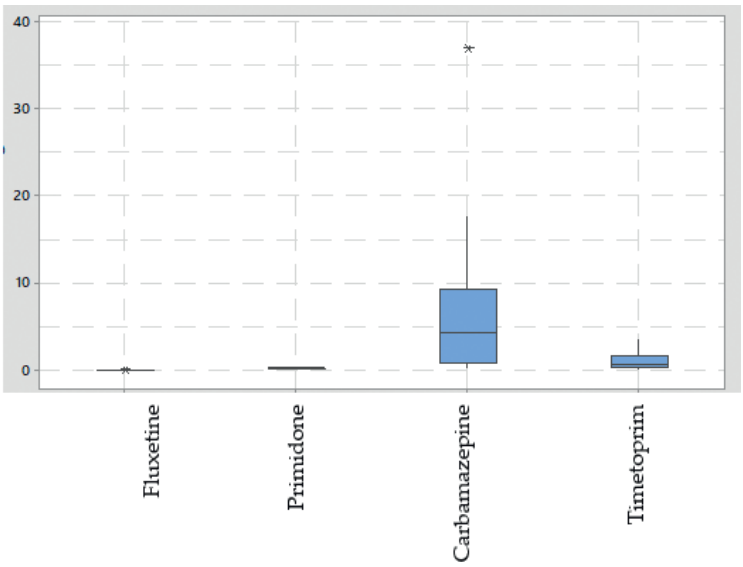

Figure 7. Concentration (μg L^{-1}) of pharmaceuticals in surface water.

and its persistence in the environment [35, 36]. Pharmaceutical compounds again behave similarly in terms of occurrence and concentration in both wastewater and surface water, **Figure 7** shows this behavior.

Studies of emerging pollutants with the potential to act as endocrine disruptors have been carried out in other important cities of Colombia such as Cali [44], which shows the importance of expanding the information and generating new lines of research that allow clarifying the possible effects and impacts of this type of compounds on environmental health.

5. Conclusions

The studies of occurrence imply the first step to be able to dimension a problematic one; in this case of environmental type, for it, it is seen the need to identify that compounds can generate risk as endocrine disruptors, in where and in what magnitude they are. This in order to compare the current situation in countries like Colombia that have little information, with what has already been defined in other contexts and in this way to be able to define better research, control, and regulatory tools.

The study of occurrence in the urban water cycle of the city of Bogotá revealed that endocrine disruptors do represent a problematic to be taken into account at the management level and that compounds such as bis-2(methylhexyl)phatalate and carbamazapine are found in important concentrations and should continue to be investigated in this respect.

Acknowledgements

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Abbreviations

ECs	emerging contaminants
EDs	endocrine disruptors
BPA	bisphenol A
CBZ	carbamazepine
TMP	trimetoprim
P4	progesterone
DMP	dimethylphthalate
DEP	diethylphthalate
BMHP	bis(2-methoxyethyl) phthalate
DIHxP	bis(4-methyl-2-pentyl) phthalate
DPP	dipentylphthalate
DnHP	di-n-hexylphthalate
BBP	benzylbutylphthalate
BnBP	bis(2-n-butoxyethyl)phthalate
BEHP	bis(2ethylhexyl) phthalate
DnOP	di-n-octylphthalate

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Chapter 2

First Approach to Screening Endocrine Disruption Activity in Sediments from the Uruguay River (Uruguay Coast)

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Additional information is available at the end of the chapter

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Abstract

The Uruguay river basin supports intensive agricultural and forest production, and receives municipal sewage discharge and industrial effluent. Therefore, the river receives xenobiotic compounds which can be distributed in sediments, biota, water and particulate matter. There is evidence of the ability of several of these compounds to interfere with the endocrine system and the sediments are an important source. The aim of this study was to determine whether exposure of immature *Cyprinus carpio* to Uruguay river sediments undergo physiological and endocrine alterations. A 30-day semi-static assay was performed using sediments from four sites along the Uruguay river and compared with an unexposed group in dechlorinated water as a negative control. The results showed that plasma vitellogenin levels increased along the river, and significant differences were found in exposed fish. Significant difference in hepatosomatic index was observed in fish exposed to sediment from an industrial site. In the histological analysis, only reproductive stage of males showed differences, where the number of primary spermatocyte accumulations was lower in exposed ones, and some exposed individuals from industrial sites presented with testis-ova. Our results suggest that the Uruguay river sediments are a source of endocrine-disrupting compounds available to the aquatic organisms.

Keywords: endocrine disruptors, sediments, bioassay, *Cyprinus carpio*, Uruguay river

1. Introduction

The endocrine system is the main regulator of important metabolic processes such as nutrition, development and reproduction. It is responsible for the maintenance of homeostatic

mechanisms through regulatory actions positive and negative that allows keeping hormone levels in balance [1].

Several natural and synthetic chemical compounds released into the environment by human activities have the ability to interfere with the normal functioning of the endocrine system [2–4, 7, 8, 12–18]; these compounds are called endocrine disruptors (EDCs). The term endocrine disruptor defines, by both, to a compound or set of exogenous chemical compounds (natural or synthetics) that alter the normal functioning of the endocrine system through alterations in hormonal balance, causing effects on exposed organisms and including about his progeny [1, 5, 6].

A wide variety of EDC sources have been documented, including municipal sewage discharges and industrial effluents (e.g., pulp mills), as well as some pesticides and their metabolites [1, 7, 8]. Some of these compounds are persistent and lipophilic, and their concentrations are higher in sediments than in river water [9]. Growing scientific evidence shows that the EDCs can exert estrogenic, androgenic, antiandrogenic, and antithyroid actions on aquatic organisms and can induce alterations in the functional development and reproduction of fish [10–13]. Several studies have detected high concentrations of EDCs in sediments, suggesting that the sediments could be responsible for the observed alterations; however, bioavailability of EDCs is complex. Sediments could be acting as a sink and reducing EDC bioavailability or rereleasing the chemical compounds into the water and acting as a source. The possible exposure routes to aquatic organisms include direct uptake of free compounds across the gills or skin and ingestion of sediment particles [9]. Several laboratories and field studies have reported that fish exposed to sediments experience significant alterations in endocrine functions [14–19].

Several approaches have been employed to show effects derived from the exposure to chemical compounds; however, those based on the use of biomarkers are the most widely used. These have been defined as "xenobiotically induced variations in cellular or biochemical components, processes, structures or functions that are quantifiable in a biological system" [20]. The first level of action of a compound or mixture of chemical compounds occurs in the biochemical-molecular component, triggering responses that tend to maintain the functioning of the organism within the homeostatic levels. If the exposure concentrations are high or are maintained during periods of prolonged time, the answers may not be enough to counteract the effect. In such a case, the agency triggers in the first instance mechanisms of compensation and then repair [21].

The Uruguay river is the natural border between Uruguay from Argentina and supports intensive agricultural and forest production and receives a variety of municipal sewage discharges and industrial effluents [22–24]. Since 1992, studies carried out by the Administration Commission of the Uruguay River (CARU) show the presence of some EDCs like chlorinated compounds (aldrin, dieldrin, HCH, HCB, DDT and its derivatives) and PCBs in fish [25]. Other works from the lower Uruguay river, detected in water and sediments several chemicals such as resin acids, phytosterols, polychlorinated dibenzo-p-dioxins, and dibenzofurans [26, 27]. At the time of the study was carried out, a large pulp mill was under construction at Fray Bentos along the Uruguay river and was associated with considerable controversy [28]. Pulp mill effluents have been associated with endocrine impacts in Canada as well as in other countries [29]. The main objective of this study was to determine whether exposure of

immature common carp to Uruguay river sediments undergo physiological and endocrine alterations. *Cyprinus carpio* was exposed to sediments collected upstream and downstream of the construction site to provide a baseline prior to the initiation of effluent discharges from the new pulp mill facility. Indicators widely used for monitoring the effect of plasma vitellogenin levels, condition factor, liver and gonad somatic indexes and histology of gonads on the reproductive systems of fish were evaluated.

2. Methodological approach

The study was implemented applying a combination of field activities and laboratory, in order to evaluate the potential of the sediments of the Uruguay river of interfere with the normal functioning of the endocrine system and generate effects to reproductive level in fish.

Sediments have been selected as exposure matrix since that several compounds cataloged as endocrine disruptors have high affinity for them and that they have been identified in previous studies as one of the main sources of persistent estrogenic contaminants [6]. On the other hand, several crop protection compounds recognized as endocrine disruptors have been detected in water and fish in the Uruguay River [25, 30].

A battery of biomarkers was selected for the study, which included early warning signs (plasma vitellogenin levels) and late ones (condition factor, hepato and gonadosomatic indices and histological analysis of gonads). These were evaluated under controlled laboratory conditions by exposing juvenile individuals of *Cyprinus carpio* to sediments from different sectors of the Uruguay river.

The presence of vitellogenin, an estrogen-inducible protein, in plasma indicates a high internal concentration of estrogenic compounds, both of endogenous origin as exogenous (xenoestrogens) [31]. Therefore, the presence of detectable concentrations of vitellogenin in plasma of males or immature individuals has been proposed as a biomarker of exposure to xenoestrogenic compounds [1, 31–34]. Several studies have shown the interference of various compounds or mixture of these on the functioning of the endocrine system. Such is the case, where *Cyprinus carpio* males captured in the effluent channel of a plant of household waste treatment, presented levels of plasma vitellogenin significantly elevated [32]. These effects can lead to alterations in the structure of the trophic webs, causing changes in the transfer flows of matter and energy to and from the aquatic ecosystem. They can also cause a stock reduction or a loss in the quality of commercially exploitable species, by the accumulation of xenobiotics in tissues (bioaccumulation and/or biomagnification) [3, 35].

Gonadosomatic (GSI) and hepatosomatic indices (HSI) reflect the dynamics of the use of energy by organisms. Changes in IGS are directly related to sex, age and reproductive stage [30]. Therefore, an acceleration of the maturation of the gonadal cells by exposure to xenoestrogens will be reflected in an increase in the IGS and vice versa. While the changes in the IHS are linked to alterations in the main functions of the liver as the synthesis and degradation of hormones and detoxification of xenobiotics. In this sense, exposure to xenobiotics can cause an increase in the size of the liver [37].

Although through changes in somatic indices, it is possible to demonstrate the existence of effects, it is not possible to elucidate the mechanism (s) by which changes were generated. In this sense, by analyzing histological sections of the gonads, it is possible to determine if the increase in size is due, for example, to acceleration in the maturation of the reproductive cells induced by exposure to xenoestrogens.

Finally, the condition factor reflects the degree of adaptability of the organism to environment, in terms of an adequate energy balance between physiological needs and the increase in body biomass. Therefore, exposure to natural or artificial stressors will cause changes in the storage and transfer of lipids and proteins tending to counteract the effect of the stressor in detriment of the increase in body weight [38].

Cyprinus carpio "common carp" is a teleost fish belonging to the Cyprinidae family. It is originally from Asia with a great ability to adapt to different media, has been widely introduced worldwide. In our country, the introduction was made for commercial purposes in the 1960s from Brazil [39]. It has wide ranges of temperature tolerances (12–32°C) and acidity (pH 5–10), resists low levels of dissolved oxygen (1–2 mg/l) and high turbidity. It is an omnivorous species, mainly benthophages [36]. About its reproductive characteristics, this species reaches its sexual maturity between 18 months and 2 years of life, depending mainly on the temperature of the water. The gonadal differentiation is within the category of differentiated gonochorist, starting at 50 days post-birth [40–42]. The Organisation for Economic Co-Operation and Development (OECD) and the United States Environmental Protection Agency (EPA) consider *Cyprinus carpio* as a good bioindicator specie for evaluation effects of endocrine disruption [43, 44] and has already been used in several studies [32, 40, 41, 45–48]. This allows the comparison of the results obtained with those generated in other studies.

3. Materials and methods

3.1. Sediment samples

The sediments samples were collected with an Eckman dredge in December 2006, transported at 4°C to the laboratory, the proportion of organic carbon in each sample was determined by calcination and then were stored at −20°C until bioassay. The zones were selected considering representative sectors of the diverse anthropic activities developed in the basin (**Figure 1**):

(a) Paysandu–PY (S. 32°19′ W58°06′): near to the mouth of the Sacra Stream. This stream receives the sewage and industrial effluents of the Paysandu city.

(b) Nuevo Berlin–NB (S 32°59′ W 58°04′): located at North of the Fray Bentos city. This site presents an important agricultural use, especially soybean and forestry. On the other hand, according to hydraulic studies carried out under the installation of a pulp mill in the city of Fray Bentos, this one zone would not be influenced by the plume of the effluent. Therefore, it could be considered as a preimpact point in future monitoring.

(c) Las Cañas–LC (S 33°15′ W 58°16′): located at South of the Fray Bentos city, is a tourist and agricultural area.

(d) Juan Lacaze–JL (S 34°26′ W 57°27′): located in the Río de la Plata river (Colonia Department). It is an urban-industrial area and is directly influenced by discharge form an elemental chlorine bleached Kraft pulp mill (was considered a positive control).

3.2. Experimental design

The assay was performed in January 2007. Immature common carp were obtained from the National Direction of Aquatic Resources Fish Hatchery (DINARA – Villa Constitucion station). They were transported in plastic bags, which were injected with oxygen and placed in ice. Once received in the laboratory, total weight (g), total and standard length (cm) of the totality of the individuals were recorded (mean body length, 7.2 ± 0.9 cm; mean weight, 9.0 ± 3.2 g) The fish were acclimatized for 15 days in an aerated pool (800 L of dechlorinated water, renewed every 2 days). During the acclimation period as well as during the assay, they were fed *ad libitum* with commercial feed (Marplatense S.A., Montevideo, Uruguay) and were kept under controlled temperature conditions (22 +/− 1°C), light: dark cycle (12:12 h) and percentage of dissolved oxygen (89 +/− 1%).

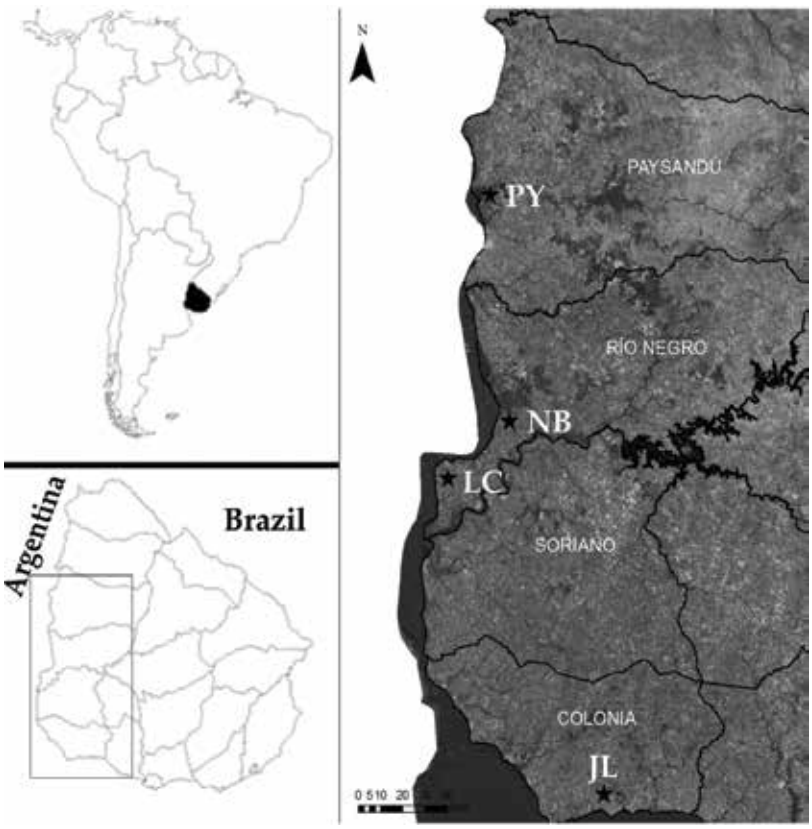

Figure 1. Study area and location of the sediment sampling zones. PY Paysandu, NB Nuevo Berlin, LC Las Cañas, JL Juan Lacaze.

Forty-eight hours prior to the start of the assay, the collected sediments were placed in 30-l tanks in a 1:10 ratio (sediment/water), in order to allow them to decant. Each of the treatments (sectors of the river) was analyzed in triplicate (total 12 fish tanks) and included three tanks with dechlorinated water and no sediment (negative control). Four fishes were placed in each tank randomly and were weighed and measured at the beginning of the assay. The spatial location at de laboratory of the treatments and controls was established randomly by using a table of random numbers. The assay was semi-static, with water replacement every 7 days and an exposure time of 30 days.

3.3. Plasma vitellogenin (VTG) levels

Once the bioassay was completed, blood samples were extracted from the vena caudalis using heparinized syringes for plasma VTG analysis (heparin solution 20 units/ml). Plasma was separated by centrifugation (Universal 32R, Hettich Zentrifugen) at 1500 rpm for 10 min, and plasma VTG was quantified using antibody pre-coated enzyme-linked immunosorbent assay kits (Biosense Laboratory, Bergen, Norway; product no. V01003402) [49]. The microplates were measured at a wavelength of 492 nm in a Biorad 680 microplate reader spectrophotometer (Hercules, CA, USA). The VTG concentration was calculated based on a standard calibration curve and expressed in μg/ml [50, 51].

3.4. Gonadal histology

The individuals were sacrificed by cervical dislocation, and the gonad was removed. Gonads were immediately weighed and fixed in 10% phosphate buffered formalin at pH 7.4. Then the tissue was dehydrated gradually through the passage through alcohol 70°, 96°, 100° and chloroform and embedded in paraffin. The cuts were made using a Reichert-Jung microtome at 5 μm. Finally, they were re-hydrated and stained with Harris's hematoxylin and eosin. The fish were sexed, and the reproductive maturity of the gonad cells was determined according to Smith and Walker in 2004 using an optical microscope with 10×, 20× and 40× eyepieces (Olympus Vanox; Tokyo, Japan) and photographed using a digital camera [36, 52–53].

3.5. Physiological indices

The somatic indices were calculated according to the following morphometric parameters: body weight (g), liver weight (g) and gonad weight (g).

For the condition factor, in first instance, was analyzed the length-weight relationship (log–log curve) from which the slope was obtained (p). This value was the allometric coefficient and was used in the equation Eq. 1.

$$K = \left(\frac{weight}{st.length^{p}}\right) \times 100 \quad (1)$$

The hepatosomatic index (HSI) and gonadosomatic index (GSI) of each fish were determined according to Eqs. (1) and (2)

$$HSI = \left(\frac{liver\ mass}{body\ mass}\right) \times 100 \quad (2)$$

$$GSI = \left(\frac{gonad\ mass}{body\ mass}\right) \times 100 \quad (3)$$

3.6. Statistical analysis

Normality and homogeneity of variance were verified, and a single factor analysis of variance or Kruskal-Wallis test was used to determine differences between the physiological indices and plasma VTG levels. Statistical significance was confirmed by Tukey's post hoc test; $p < 0.05$ was considered significant.

4. Results

4.1. Plasma VTG levels gonadal histology

Plasma VTG levels are presented in **Figure 2**. The values increased along a latitudinal gradient from Paysandu to Juan Lacaze (means: control = 0.360, Paysandú 6.128 and Juan Lacaze = 9989) accompanied of a reduction in the data dispersion. Only significant differences were observed among the sediment of exposed groups with the control (Tukey's HSD, $p < 0.05$); however, no differences were detected among the exposed groups.

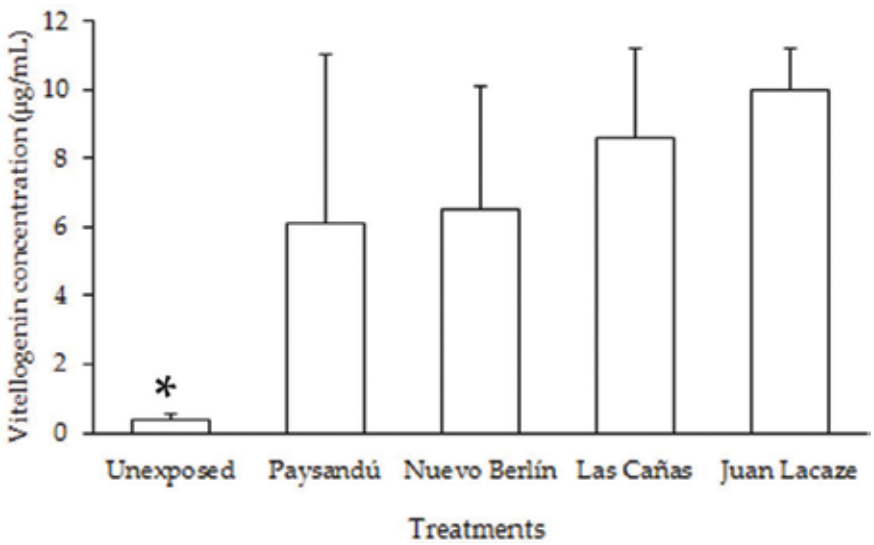

Figure 2. Plasma vitellogenin levels in common carp exposed to sediments from the Uruguay River. Vertical bars indicate the 95% confidence intervals. *Indicate significant differences ($p < 0.05$).

4.2. Gonadal histology

The gonadal histological analysis of females revealed that oocyte stages was not different between exposed and unexposed groups and that all oocytes were in the previtellogenic and perinucleolar stages according to the classification made by Smith and Walker [36] (**Figure 3C**, **D**). No significant differences were observed in relation to the size of the oocytes (control group range: 59–114 μm, Paysandu range: 79–113 μm, Nuevo Berlin range: 66–109 μm, Las Cañas range: 74–116 μm) nor in the amount of them within the displayed field.

Regarding testicular development, this in general does not have a clear differentiation, and it is only possible to observe a delay in maturity of the exposed individuals respect to

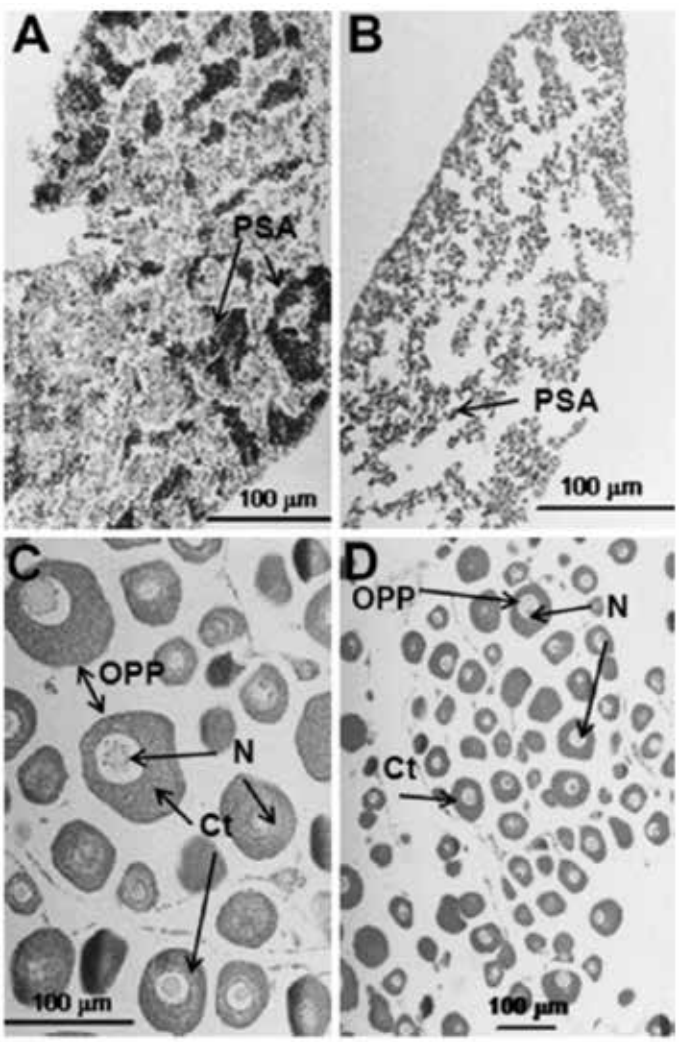

Figure 3. Photographs of gonadal histology. (A) male from control (20×); (B) male from Nuevo Berlin (20×); (C) female from the control (20×); (D) female from Nuevo Berlin (10×). PSA primary spermatocyte accumulation, OPP oocytes in previtellogenic and perinucleolar stage, Ct cytoplasm, N nucleus.

Treatment	Sex distribution (males/females)	Testis-ova (number/males)
Unexposed	9/3	0/9
Paysandu	9/3	1/9
Nuevo Berlin	4/8	0/4
Las Cañas	4/8	0/4
Juan Lacaze	10/2	1/10

Table 1. Sex distribution between treatments and the control and the occurrence of testis-ova.

Treatment	K	GSI	HSI
Unexposed	2.6 ± 0.4 (12)	0.5 ± 0.6 (12)	1.3 ± 0.8 (12)*
Paysandu	2.8 ± 0.3 (12)	0.5 ± 0.6 (12)	1.7 ± 0.6 (12)
Nuevo Berlin	2.6 ± 0.5 (12)	1.3 ± 1.0 (12)	1.8 ± 0.6 (12)
Las Cañas	2.6 ± 0.4 (12)	0.9 ± 0.6 (12)	0.9 ± 0.6 (12)*
Juan Lacaze	2.8 ± 0.4 (12)	0.6 ± 0.8 (12)	2.5 ± 0.4 (12)*

Values are given as mean ± standard deviations for each treatment, and respective n in parentheses. *indicates statistical differences between treatments ($p < 0.05$). K: condition factor, GSI: gonadosomatic index, HSI: hepatosomatic index.

Table 2. Physiological indices in the exposed and unexposed groups.

control. This delay is observed by a decrease in the number of accumulations of primary spermatocytes (**Figure 3A**, **B**). Some individuals exposed to sediments from industrial sites (Paysandu and Juan Lacaze) presented oocytes in testicular tissue (testis-ova) (**Table 1**).

4.3. Physiological indices

Values of the physiological indices in the exposed and unexposed groups are given in **Table 2**. No significant differences were observed among the groups for the condition factor (K) or gonadosomatic index (GSI); however, significant differences in HSI were detected. Post-hoc comparisons revealed that fish exposed to sediment from Juan Lacaze had significantly increased HSIs compared with those in the control ($p = 0.04$) and Las Cañas groups ($p = 0.02$)

5. Discussion

The results obtained in plasma vitellogenin levels indicate a marked effect of the treatments. Several known sources of endocrine disruptors are located in the Uruguay river basin, and previous studies have detected some EDCs in sediments [27] that could be responsible for the observed alterations. However, only significant differences were observed in Las Cañas and Juan Lacaze treatments. In the first one, the increase may be due to the contribution of known estrogenic pesticides such as chlorpyrifos, endosulfan, and cypermethrin

from agricultural surrounding areas [54]. Additionally, phytoestrogens released by crops as a defense strategy may be reaching the river in overland runoff. In particular, soybeans contain high levels of genistein, daidzein, and glycitein, which can elicit alterations in endocrine function in wildlife and humans [55]. Juan Lacaze receive untreated municipal sewage effluent containing a complex cocktail of natural (estrone or 17b-estradiol) and synthetic estrogens used in oral contraceptives as well as surfactants used in soaps and detergents (alkylphenols and alkylphenolpolyethoxylates) Furthermore, the plasma VTG concentrations in fish were highest where deposition processes were predominant at Juan Lacaze and where pulp mill effluent was discharged near the sampling site. In that sense, several works worldwide have documented the increase in vitellogenin plasma in juvenile individuals exposed to effluents from cellulose plants [14, 56, 57]. Elevated levels of VTG in males and immature females were clearly an estrogen-mediated response. It is important to note that VTG levels were not affected by the sex ratio, as shown by similar VTG concentrations in the Las Cañas and Juan Lacaze groups with opposite sex ratios, same when comparing the Paysandu and Nuevo Berlin treatments.

The gonad histology analyses indicated that female fish did not exhibit differences in maturation state. Unlike other works carried out under similar conditions, not differences in the stage of oocyte development were observed [33, 58, 59]. Whereby it could only be a trophoblastic but not protoplasmic growth, this could be checked by increasing the exposure time to see if there are changes in the stages in the individuals with the highest number of cells. However, sediment-exposed males presented delayed testicular maturation than that in the unexposed group. Jobling et al. reported that the induction of VTG in males is negatively correlated with testicular maturation, and Devlin and Nagahama observed retarded gonadal maturation in *C. carpio* males exposed to estrogenic compounds [60, 61]. Changes in sex ratios and intersex individuals have been reported in common carp exposed to EDCs [40, 41, 61]. However, the intersex condition occurs naturally in approximately 5% of the population in this species [7]. Thus, the presence of individuals with testis-ova observed in our study was possibly a natural phenomenon and may not have been caused by exposure to contaminated sediments.

The condition factor showed higher values in all the treatments with respect to the control group; however, the differences are not statistically significant, and this agrees with the results obtained by Orrego et al. [14]. The significant increase in liver mass at Juan Lacaze may have been caused by induction of the hepatic mixed function oxidase system in response to discharge of persistent organic compounds from the pulp mill effluent [62–64]. Increased protein synthesis generates proliferation of endoplasmic reticulum, which can be reflected in increased hepatocyte size [65–69].

6. Conclusions

This study is the first report about endocrine disruption in fish exposed to sediment from the lower Uruguay river. The results can be considered a reference condition for monitoring the impacts of the new ECF bleached Kraft Eucalyptus pulp mill. Nonpoint (soybean-wheat crops) and point sources (municipal sewage and pulp mill effluent) can explain the VTG induction observed in immature fish and suggest the presence and bioavailability of

EDCs in the sediments. The specific agents responsible for the toxic effects were not identified because it was beyond the scope of this study. Future research is needed to identify the causal agents (natural or synthetic) and to determine exposure routes (e.g., grazing on sediments or bioconcentration from the water column). Finally, in relation to the adequacy of the bioassay developed to be applied as a monitoring tool, since Juan Lacaze sediments generated the greatest changes in the analyzed biomarkers, confirming their inclusion as a positive control. Likewise, the selection of negative controls (without sediment exposure) showed the lowest levels of changes as well as the lowest dispersion of values between replicates.

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Conflict of interest

None.

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Chapter 3

Crop Protection Compounds: A Source of Endocrine Disruptors in Uruguay?

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Additional information is available at the end of the chapter

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Abstract

The intensive use of agrochemicals in agriculture has been raised the concern about their potential effects on human health and the environment. In this way, regarding crop protection compounds a complex frameworks and restrictions had been established in several countries, particularly for compounds identified as endocrine disruptors. In Uruguay, the General Direction of Agricultural Services is the agency responsible for registry, but the authorization process does not consider the potential effects on endocrine system. Uruguay has significantly increased the use of crop protection compounds, of which several of them have been identified as endocrine disruptors and the environmental risks associated have not been studied. The aim of this study was to be bridging the gap between registry process and environmental protection policies. An eco-epidemiological analysis of the database of compounds imported in 2017, use guideline, national agricultural census as well as the public endocrine disruptor databases were carried out. Main class of crop protection compounds were ranked according to imported volumes and the top 10 of each class were contrasted with the disruptor databases. In function to recommended doses and geographical localization of the crops was identified the main hot spots associated to the use of agricultural compounds identified as endocrine disruptors.

Keywords: endocrine disruptors, crop protection compounds, summer rain-fed crops, environmental risk assessment

1. Introduction

In the agricultural production, a wide variety of crop protection compounds are used and several of them may interfere with endocrine system functioning [1–15]. According to Kavlock et al. [16], an "endocrine disrupting compound" (EDC) is "an exogenous agent

that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction, development and or behavior."

Throughout the 1990s, the concern about the adverse effects on human and wildlife resulting from interaction between environmental chemicals and endocrine system has been increasing. However, given that hundreds of synthetic compounds have been released into the environment, the possible mechanisms for disruption and their physiological effects are enormous and not well understood. In this way, several regulatory agencies have developed different screening and testing strategies to assess the potential of crop protection compounds to interfere with the endocrine system.

In Uruguay, the registry and use of these chemical compounds are regulated by the General Direction of Agricultural Services (Res DGSA N° 01/2009 y Dec. 294/2004), but the authorization process does not consider the potential to induce adverse effects in humans and wildlife via interaction endocrine system. In this way, some laboratory and field studies have detected masculinization process [17], induction of the synthesis of plasmatic vitellogenin in immature organism and changes in somatic index in fish exposed to potential sources of endocrine disruptors [18, 19].

In order to bridge the gap between authorization process of crop protection compounds and environmental protection policies, the aims of this study were as follows:

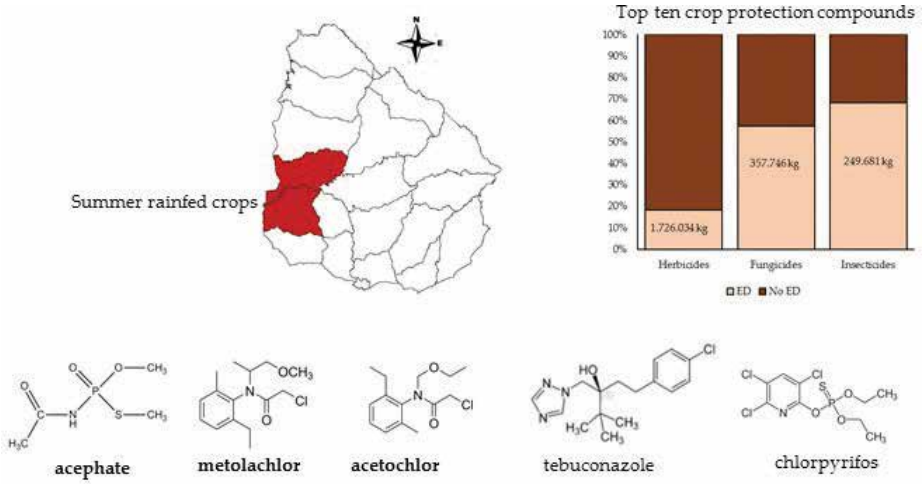
1. to identify the crop protection compounds used in Uruguay that have a documented or presumed effect on endocrine functions,
2. to identify the main geographical areas potentially affected by endocrine disrupting compounds, and
3. to propose a research strategy to guide the effort and identify the potential scope of the problem.

We do this by an eco-epidemiological analysis of the Uruguayan database of crop protection compound imported in 2017 (DGSA database), the use guidelines (SATA Guide 2016) [20], the national agricultural census [21] as well as the public endocrine disruptor databases (PAN Pesticide Database (PANNA) and Pesticide Properties Database (PPDB)). The main class of crop protection compounds (herbicides, insecticides and fungicides) was ranked according to the imported volumes, and the top 10 of each class were contrasted with the endocrine disruptor databases and scientific articles. In function of the use guidelines (recommended doses) and the geographical localization of the main crops, the hot spot areas and the crop protection compound priorities were identified due its potential effects on endocrine system.

2. Survey of crop protection compounds currently used in Uruguay

The agriculture is one of the most important economic activities in Uruguay, and in the past decades, the summer rain-fed crops have experienced an important expansion and

intensification process. In this way, the soybean crop occupied 1,140,000 ha and its exportable volumes exceed 3 million tons/year [21]. This process implied an increase in the use of crop protection compounds, mainly herbicides [22].



The survey of crop protection compounds currently used in Uruguay was conducted based on the active ingredients (AI) imported in 2017 and considered only herbicides, insecticides and fungicides. A total of 175 AI (11,358,732 kg), corresponding to 48% herbicides, 29% insecticides and 23% fungicides, were analyzed [22]. In **Table 1**, top 10 compounds for each class ranked in function to the imported volumes are shown.

Active ingredients	Category	Kg imported
Glyphosate, dimethylammonium salt	Herbicide	2,792,921
Glyphosate, ammonium salt	Herbicide	2,572,365
Glyphosate, isopropylamine salt	Herbicide	1,217,482
Glyphosate, potassium salt	Herbicide	980,073
2,4-D, dimethylamine salt	Herbicide	896,773
Acetochlor	Herbicide	407,484
S-Metolachlor	Herbicide	244,339
Paraquat	Herbicide	134,036
2,4 DB Butyl ester	Herbicide	98,910
Metolachlor	Herbicide	78,528
	Total	9,422,911
Mancozeb	Fungicide	164,400
Sodium metabisulfite	Fungicide	117,600
Chlorothalonil	Fungicide	71,544

Active ingredients	Category	Kg imported
Copper oxide	Fungicide	68,264
Captan	Fungicide	67,115
Folpet	Fungicide	43,008
Sulfur	Fungicide	36,032
Azoxystrobin + cyproconazole	Fungicide	20,275
Tebuconazole	Fungicide	18,812
Mancozeb + metalaxyl	Fungicide	15,600
	Total	622,650
Chlorpyrifos	Insecticide	181,795
Triflumuron	Insecticide	53,478
Lambda-cyhalothrin + Thiamethoxam	Insecticide	26,453
Paraffin oil	Insecticide	24,226
Aluminum phosphide	Insecticide	19,536
Acephate	Insecticide	17,423
Chlorpyrifos-methyl	Insecticide	15,360
Emamectin benzoate	Insecticide	9,873
Chlorantraniliprole	Insecticide	8,816
Bifenthrin + thiamethoxam	Insecticide	8,650
	Total	365,610

Elaborated with data from DGSA-MGAP 2017.

Table 1. List of active ingredients with the highest import volume for each main class.

Active ingredients	US EPA status	EC status	PANNA	PPBD
2,4 DB Butyl ester (H)	Not classified	Approved	Suspected	Suspected
2,4-D, dimethylamine salt (H)	Not classified	Approved	Suspected	n/d
Acephate (I)	Not classified	Not approved	Suspected	Yes
Acetochlor (H)	Restricted use	Not approved	Suspected	Suspected
Aluminum phosphide (I)	Restricted use	Approved	No	n/e
Azoxystrobin + cyproconazole (F)	Not classified	Approved/approved	No/no	No/suspected
Bifenthrin + thiamethoxam (I)	Not classified	Approved/approved	Suspected/no	Yes/no
Captan (F)	Not classified	Approved	Suspected	Suspected
Chlorantraniliprole (I)	Not classified	Approved	No	No
Chlorothalonil (F)	Restricted use	Approved	Suspected	Suspected

Active ingredients	US EPA status	EC status	PANNA	PPBD
Chlorpyrifos ethyl (I)	Restricted use	Approved	Suspected	Suspected
Chlorpyrifos-methyl (I)	Restricted use	Approved	No	Suspected
Copper oxide (F)	Not classified	Approved	No	No
Emamectin benzoate (I)	Restricted use	Approved	No	n/d
Folpet (F)	Not classified	Approved	No	n/e
Glyphosate, ammonium salt (H)	Not classified	Approved	No	n/d
Glyphosate, dimethylammonium salt (H)	Not classified	Approved	No	n/d
Glyphosate, isopropylamine salt (H)	Not classified	Approved	No	No
Glyphosate, potassium salt (H)	Not classified	Approved	No	No
Lambda-cyhalothrin + thiamethoxam (I)	Restricted use	Approved/approved	Suspected/no	No/no
Mancozeb (F)	Not classified	Approved	Suspected	Suspected
Mancozeb + metalaxyl (F)	Not classified	Approved/approved	Suspected/no	Suspected/no
Metolachlor (H)	Restricted use	Not approved	Suspected	Suspected
Paraffin oil (I)	Not classified	Approved	No	No
Paraquat (H)	Restricted use	Not approved	No	No
S-Metolachlor (H)	Restricted use	Approved	Suspected	Suspected
Sodium metabisulfite (F)	Not classified	Not approved	No	n/d
Sulfur (F)	Not classified	Approved	No	n/e
Tebuconazole (F)	Not classified	Approved	Suspected	n/e
Triflumuron (I)	Not classified	Approved	No	No

Suspicion of endocrine disruption effects according to the PAN Pesticide Database (PANNA) and the Pesticide Properties Database (PPDB) is noted. If the active ingredient is not found in any database, it is reported as n/d.

n/e: reported without evidence.

The substances are listed in alphabetical order. F-fungicide, H-herbicide, I-insecticide.

Table 2. List of active ingredients according to the categorization of the US EPA and the European Commission (EC).

The status regulatory according to international agencies as well as the potential to interfere with the endocrine system functioning were analyzed and the results are presented in **Table 2**.

From the regulatory point of view, a total of 18 compounds present a status "Approved," 7 "Approved with restricted use" and 5 "Not approved." The last status regulatory includes three herbicides, one fungicide and one insecticide. Whereas in relation to potentially endocrine disrupting compounds, six fungicides, five herbicides and five insecticides were identified. The analysis of effects on endocrine system was complemented with information from several scientific articles [9, 10, 23–26] and according to the crop protection compound priorities are: acetochlor, chlorpyrifos methyl, mancozeb, metolachlor and tebuconazole (**Table 3**).

Active ingredients	References	Effect
2,4 DB butyl ester (H)	PPDB	
2,4-D, dimethylamine salt (H)	PANNA; Cocco [23]; McKinlay et al. [25]; Mnif et al. [9]	2,5
Acephate (I)	PANNA; PPDB; McKinlay et al. [25]; Mnif et al. [9]	1,2,5
Acetochlor (H)	PANNA; PPDB; Cocco [23]; McKinlay et al [25]; Mnif et al. [9]	1,2
Azoxystrobin + **cyproconazole** (F)	PPDB; McKinlay et al. [25]; Mnif et al. [9]	1,3,5
Bifenthrin + thiamethoxam (I)	PANNA; PPDB; McKinlay et al. [25]	1,2
Captan (F)	PPDB; McKinlay et al. [25]; Mnif et al. [9]	1
Chlorothalonil (F)	PPDB; McKinlay et al. [25]; Mnif et al. [9]	5
Chlorpyrifos ethyl (I)	PANNA; PPDB	3,5
Chlorpyrifos-methyl (I)	PPDB; Morales y Rodríguez [24]; McKinlay et al. [25]; Mnif et al. [9]; Marx-Stoelting et al. [10]; Ewence et al. [11]	5
Lambda-cyhalothrin + thiamethoxam (I)	PANNA; Morales y Rodríguez [24]; Ewence et al. [11]	1
Mancozeb (F)	PANNA; PPDB; Cocco [23]; Morales y Rodríguez [24]; McKinlay et al. [25]; Mnif et al. [9]; Marx-Stoelting et al. [10]; Ewence et al. [11]	2
Mancozeb + metalaxyl (F)	PANNA; PPDB; Cocco [23]; Morales y Rodríguez [24]; McKinlay et al. [25]; Mnif et al. [9]; Marx-Stoelting et al. [10]; Ewence et al. [11]	2
Metolachlor (H)	PANNA; PPDB; Cocco [23]; Mnif et al. [9]	5
S-Metolachlor (H)	PANNA; PPDB; Cocco [23]; Mnif et al. [9]; Ewence et al. [11]	5
Tebuconazole (F)	PANNA; McKinlay et al. [25]; Mnif et al. [9]; Marx-Stoelting et al. [10]; Ewence et al. [11]; Ventura et al. [29]; Yang et al. [30]	1,3,5

Target effect metabolism is included as follows: 1-estrogen, 2-thyroid hormones, 3-aromatase, 4-pregnane receptor, 5-androgen. The substances are listed in alphabetical order.

Table 3. List of active ingredients suspected of generating endocrine disruption effects in at least one of the consulted databases (F-fungicide, H-herbicide, I-insecticide).

3. Geographical areas potentially affected by endocrine disrupting compounds

Several of the crop protection compounds identified as endocrine disruptors are applied in different crops, widely distributed within the territory. Therefore, were analyzed the spatial impacts combining the area occupied by each crops and the recommended doses (**Tables 4** and **5**). The crops considered were: grasslands (2,500,000 ha), soybean (1,140,000 ha), wheat (215,000 ha), barley (190,000 ha), rice (164,400 ha), corn (83,000 ha), sorghum (67,000 ha), fruit and citrus trees (17,000 ha), vegetables (14,190 ha) and sugarcane (7,600 ha) [21] **Figure 1**.

According to estimated loads, the crop protection compounds priority: 2,4 butyl ester, chlorpyrifos, 2,4-D, dimethylamine salt, acetochlor, tebuconazole, chlorpyrifos methyl, metolachlor and S- metolachlor. Considering only the agricultural lands (without grasslands), 60% of them are occupied for soybean and are mainly concentrated at the west littoral zone (Rio Negro, Soriano and Flores Department), represented in **Figure 1**, like the land use rain-fed crops.

Active ingredients	Crops	Recommended dose	Units
2,4 Butyl ester (50%) (H)	1, 3–4	2–2.5	L/ha
2,4-D, dimethylamine salt (50%) (H)	1, 3–7, 11	0.6–3	L/ha
Acephate (75%) (I)	2	0.5–1	kg/ha
Acetochlor (H)	2, 6, 11	0.8–3.8	L/ha
Azoxystrobin + cyproconazole (200/80) (F)	2–5	0.2–0.4	L/ha
Bifenthrin + thiamethoxam (6%/13%) (I)	2	0.20	L/ha
Captan (80%) (F)	9–10	0.8–1.5	kg/ha
Chlorothalonil (72%) (F)	8–10	1.5–5	L/ha
Chlorpyrifos (50%) (I)	1–4, 6–7, 9	0.3–2.5	L/ha
Chlorpyrifos-methyl (I)	1, 3–4	0.35–1	L/ha
Lambda-cyhalothrin + thiamethoxam (I)	2–7	0.05–0.25	L/ha
Mancozeb (80%) (F)	8–10	1–5	L/ha
Mancozeb + metalaxyl (F)	9	2–3	kg/ha
Metolachlor (H)	2, 6–7	0.8–1.6	L/ha
S-Metolachlor (90%) (H)	2, 6–7	0.8–1.6	L/ha
Tebuconazole (25%) (F)	2–5	0.5–2	L/ha

The substances are listed in alphabetical order. F-fungicide, H-herbicide, I-insecticide.
Source: SATA Guide.
1-pastures, 2-soybean, 3-wheat, 4-barley, 5-rice, 6-corn, 7-sorghum, 8-citrus, 9-vegetable, 10-fruit, 11-sugarcane.

Table 4. Crops and recommended doses of the compounds cataloged as suspect of generating endocrine disruption effects.

Active ingredients	Recommended average dose	Units	Total cultivated area (ha)	Estimated AI added (L or kg)
2,4 Butyl ester (50%) (H)	2,3	L/ha	2,905,000	**6,536,250**
2,4-D, dimethylamine salt (50%) (H)	1,8	L/ha	3,227,000	**5,808,600**
Acephate (75%) (I)	0,8	kg/ha	1,140,000	855,000
Acetochlor (H)	2,3	L/ha	1,230,600	**2,830,380**
Azoxystrobin + cyproconazole (200/80) (F)	0.3	L/ha	1,709,400	555,555
Bifenthrin + thiamethoxam (6%/13%) (I)	0,2	L/ha	1,140,000	228,000
Captan (80%) (F)	1,2	kg/ha	26,387	30,345
Chlorothalonil (72%) (F)	3,3	L/ha	27,000	87,750
Chlorpyrifos (50%) (I)	1,4	L/ha	4,209,187	**5,892,862**
Chlorpyrifos-methyl (I)	0,7	L/ha	2,905,000	**1,960,875**
Lambda-cyhalothrin + thiamethoxam (I)	0,2	L/ha	1,859,400	278,910

Active ingredients	Recommended average dose	Units	Total cultivated area (ha)	Estimated AI added (L or kg)
Mancozeb (80%) (F)	3,0	L/ha	27,000	81,000
Mancozeb + metalaxyl (F)	2,5	kg/ha	14,187	35,468
Metolachlor (H)	1,2	L/ha	1,290,000	**1,548,000**
S-Metolachlor (90%) (H)	1,2	L/ha	1,290,000	**1,548,000**
Tebuconazole (25%) (F)	1,3	L/ha	1,709,400	**2,136,750**

For the estimation, the average dose reported was used. The total sown area corresponds to the sum of the crops in which the compound is used. The substances are listed in alphabetical order. F-fungicide, H-herbicide, I-insecticide.

Table 5. Estimation of formulated applied according to hectares sown in the agricultural year 2016/2017.

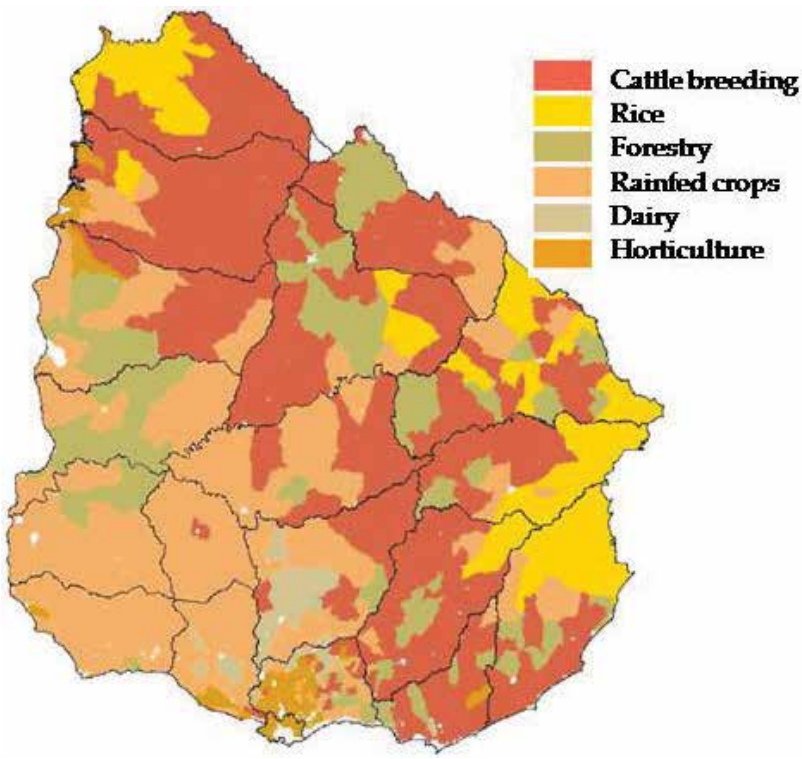


Figure 1. Uruguay regionalization according to the land use.

4. Scope and perspectives

The agricultural intensification and expansion are global processes associated with growing worldwide demands (food, feed, fiber and fuel), and these are highly dependent on external additions of nutrients and crop protection compounds [27, 28]. In Uruguay, these processes began from 2000, mainly in the west littoral zone with the inclusion of soybean in agricultural

sequences under no-tillage. Currently, more than 2 million hectares are destined to agriculture and approximately 50% correspond to soybean crops. In addition, the imported volumes of agrochemicals significatively increase, particularly herbicides (10,200,404 kg AI in 2017).

Considering the herbicides, insecticides and fungicides being more used, the doses/application numbers recommended and the agricultural area (crops and grasslands), we have estimated that in 2017, 15 million L of herbicides, 8 million L of insecticides and 750,000 L of fungicides were added. Several of them, as stated by the European and US regulatory agencies, have a status "Approved with restricted use" (7) or "Not approved" (5). In addition, PAN Pesticide Database (PANNA) and Pesticide Properties Database (PPDB) classified as "Suspected" interferes with the endocrine system functioning and four of these are: acephate, acetochlor, chlorpyrifos ethyl and metolachlor. On the other hand, although the aforementioned regulatory agencies confers tebuconazole the "Approved" status, it is one of the fungicides more used (2.136.750 L in 2017) and was reported as endocrine disruptor in PANNA and PPDB database, and by several authors [9–11, 25, 29, 30]. These last five compounds are used in the soybean cropping, and the bigger surfaces occupied by this crop are located around the two most important river basins in the country (Uruguay and Negro river).

On the other hand, it is important to highlight that the available information at National level on residues of crop protection compounds is basically for export products and some foods for internal market. While as data about environmental concentration (soil, water or biota) are scarce, environmental surveillance programs are not carried out.

According to our review about the crop protection compounds used in the agricultural systems in Uruguay, this activity is a potential source of endocrine disruptors. One of the first actions tending to reduce the environmental risk associated with the use of these compounds is to replace acephate, acetochlor, and metolachlor by other active ingredients. In the same way and in function of the scientific evidences, it is necessary to establish monitoring programs for determining environmental levels of chlorpyrifos and tebuconazole, as well as to assess the potential human health and wildlife risks. Finally, we consider that the west littoral is the zone with the highest risk associated with exposure to endocrine disrupting compounds (hot spot area), principally the Rio Negro and Soriano Department.

Conflict of interest

None.

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Chapter 4

Interactions between Bisphenol S or Dibutyl Phthalates and Reproductive System

Irfana Liaqat

Additional information is available at the end of the chapter

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Abstract

Endocrine disrupting chemicals (EDCs) adversely affect animals and human beings. This attracted the researchers in the previous decade to explore the possible association of these chemicals. However, among various studies, very limited data is available to explain the link between EDCs and reproductive tract outcome. One reason is that many potential EDCs and their probable mechanisms and underlying causes have not been studied so far. Bisphenol S (BPS) is used as an alternative of bisphenol A, after the worse effects of this bisphenol. Similarly, dibutyl phthalate (DBP) is the least studied of its group. Dibutyl phthalate is widely used in polyvinyl plastic products. The current chapter aims to explore the possibly association of these two chemicals with animals and humans.

Keywords: BPS, DBP, phthalates, reproductive system

1. Introduction

Endocrine disrupting chemicals (EDCs) are the exogenous agents that can disturb the synthesis, metabolism, and the action of endogenous hormones; also they affect the androgenic, antiandrogenic, and thyroid mechanism [1]. The "US Environmental Protection Agency" defined the endocrine disrupting chemicals as exogenous agents that affect the synthesis, transport, binding action, metabolism, and elimination of hormones required for homeostasis, development, and reproduction [2]. The idea of EDCs was formulated after their exposure studies and adverse effects on humans and wild animals [3]. The impact of these chemicals was examined on the embryonic and reproductive development of male and female reproductive systems [4, 5]. The exposure of EDCs showed long-term negative effect on animal development and

health [6]. Endocrine disrupting chemicals are one of the possible causes of reproductive problem.

Nowadays, plastics are used in most of the products, the harmful chemicals, bisphenols and phthalates, leach out into the environment. These chemicals attract the attention of regulatory agencies, scientific community, and general public due to its high production and uses [7].

2. Bisphenol S (BPS)

Due to the adverse effects of bisphenol A (BPA) on public health, certain alternative chemicals were replaced in consumer products. One such replacement, bisphenol S (BPS), is currently used in thermal receipts, consumer paper products, baby bottles, and personal care products, foodstuffs, and canned foods. BPS is chemically and structurally compassionate to bisphenol A. It is an organic compound with formula $C_{12}H_{10}O_4S$. It has two phenol functional groups on either side of sulfonyl group. It is commonly used in curing fast drying epoxy resin adhesives. Bisphenol S was made in 1896 and is presently used in consumer products. Bisphenol S is an analog of bisphenol A and it supersedes bisphenol A in variety of ways. Bisphenol S becomes endocrine disruptor in the existence of hydroxyl group on benzene ring. Bisphenol S also has endocrine disruptor properties. Bisphenol S is present in thermal receipts paper, plastics, and indoor dust. Scientific and public knowledge of negative consequences affiliated to bisphenol S disclosure have increased [8].

BPS is a more stable chemical compound with low biodegradability as compared to BPA [9]. This is alarming that these properties of BPS compared to BPA lead to higher burden on living organisms [10]. Due to this cause, BPS is measured as "regrettable substitution" of BPA. The esteem increases in BPS production and use in plastic industry will unfortunately spread this chemical to the level as for BPA [11]. BPS replaced BPA in almost every consumer goods, for example, clearing products, resins, and electroplating solvents [12, 13], in canned food stuff [14]. Increase consumption of BPS and its discharge in environment-noticed health hazard to human, aquatic life, and environmental risks [15].

The existence of BPS was determined in waste water, fluvial water, and indoor dust [4, 9, 16]. The humans are exposed to BPS through ingesting dust, recycled products, dietary exposures, and dermal contact [9]. Although BPS was not studied broadly, many studies indicate the estrogenic properties of this compound in genomic as well as membrane-associated estrogen signaling. To date, studies of BPS in mammals are limited, and there are very few studies investigating the effects of exposure on behavior [11]. The limited data is available dealing with the interaction of BPS with biological organisms. The studies indicate that BPS is capable to mimic the hormones and interact with its certain receptors including estrogen and androgens [17] and serum proteins [12]. The exposure of BPS changes the aromatase expression that is a major enzyme of the estrogen pathway [18]. In vivo studies demonstrate the effect of BPS, postnatal low and high dose exposure causes reproductive dysfunction including changes in gonads morphology and androgen level. BPS affects the reproductive and neuroendocrine pathway during embryonic development. The mechanism of action may involve the thyroid and estrogen receptors. This also changes the expression of genes involved in above pathways

[19]. The studies on cell cultures showed that BPS affects cells mutagenically, genotoxically, and cytotoxically [20, 21]. BPS exposure also disrupts the signaling pathway of apoptosis, so it may cause gametes cascades leading toward cell death and altered cell cycle [22, 23].

Consumer quest for bisphenol A products lead to the supersession of bisphenol A with other related compound including bisphenol S [12, 24]. Biomonitoring studies excavate that human manifestations are likely to distribute about 97% individual in US, have noticeable level of bisphenol S metabolites in urine [11]. The estimation from these urinary concentrations urge that daily exposure in the range of 0.3–2 ug/day, although these exposure will likely revolt as the displacement of bisphenol A in various consumer goods, also increase [25].

Like bisphenol A, human vulnerability to BPS seems to grow mostly by exposure through the skin absorption [26] and ingestion by plastic leaches [27]. There is also confirmation that bisphenol S vulnerability can affect body weight and neuro-behaviors in developmentally exposed male offspring [28].

The increase in urbanization and industrialization results in massive release of certain chemicals including bisphenols and phthalates into the surrounding and environment. These chemicals cause adverse effects on human beings, mainly reproductive system, endocrine disruption, and decline in life quality. The effect of potential hazards of BPS in human depends upon the exposure level. As its use is not regulated, it is difficult to mention the consumables that contain and leach this compound. It is often used as an alternative of BPA in "BPA free" products including plastic bottles and printing paper [11]. BPS is introduced in industry as safe substitute of BPA. However, little is available regarding the adverse effects of BPS on humans and mammals. Currently, few studies were carried out to study the role of BPS as endocrine disrupting chemical. Approximately, over and above "18 billion pounds" of phthalates are used every year worldwide [29]. Phthalates and its metabolites, after leaching from its product, were detected in environment [30], in saliva [31], and in urine [29] samples of human both children and adult. The children get exposure of DBP in mother's womb, breast feeding, and medical devices during neonatal care [31]. To date, the exposure of DBP is studied on fertility, development of female and male reproductive tract, sexual maturation, prenatal and postnatal effect, pregnancy, and tumor in animals and human beings.

3. Dibutyl phthalate (DBP)

The modern use of plasticizers has extensively increased the industrial and social well-being of the resident of both developing and under-developed countries. At the same time, they are very harmful for the living organisms, if taken inside the body through any source. Plasticizers or dispersants are the additive chemicals that are used to increase the plasticity or decrease the viscosity of a material. These substances alter the physical properties of certain products. These are available in different forms either liquids with low volatility or may be even solids. They make plastic products more flexible by decreasing the attraction between polymer chains. Among, more than 30,000 different substances have plasticizing properties. Of all, these plasticizers approximately 50 are commercially used to make various products [32].

Phthalates were used in 1930s for the first time to replace unpleasant odor camphor. Phthalate ester is colorless, odorless, nonvolatile, and potentially nontoxic plasticizers. Due to these properties, they are considered as consumer friendly plasticizers [33]. Phthalates are used mainly for manufacturing medical supplies, including blood storage bags and intravenous solution containers, food containers, food packaging materials, children's toys, curtain, bowls, raincoats, car interiors, floor tiles, food wraps, fabrics, and plastic products. Approximately, 3 million tons of phthalates are produced per annum around the globe. Dibutyl phthalate (DBP) is the most commonly used phthalate, fulfill about 40% of total phthalate use. Blood storage bags usually have a high content of 20–40% (DBP). The primary source of exposure to DBP is through contaminated food [34].

The diesters of similar phthalic acids constitute the phthalates, and these are commonly used plasticizers in polyvinyl chloride (PVC) plastics to make them flexible, durable, and soft [35]. The PVC is added in building materials, children's products, toys, clothing, intravenous fluid bags, infusion sets, blood bags, food packaging, and some medical devices. Humans are exposed to these phthalates mainly through foods as these are used in food processing, wrapping, and packing material [36]. The dibutyl phthalates are used as plasticizers in plastics, solvent in dyes, cosmetics, and other care products. DBP is also used in latex adhesives as a component [37]. Bisphenols and phthalates are known for weak estrogen properties and act as EDCs due to their capability to contest with steroid hormone binding to its receptors. The "National Toxicology Program-Center for the Evaluation of Risks to Human Reproduction" broadly reported and reviewed the impact of phthalates on human health [38]. There are adequate evidences and studies on mice and rodent models showing that the exposure of DBP causes reproductive and development toxicities. Moreover, the genital tract disorder was observed in human infants after prenatal exposure of phthalates [39]. The profound effect of DBP exposure was observed on the development of male reproductive system during acute period of late gestation (sexual differentiation). The similar phenotypic variations of prenatal exposure of DBP was observed in male rats, comparable to human disorders including decrease sperm count, hypospadias, and cryptorchidism (Martino-Andrade and Chahoud [40]). Both low and high concentrations of phthalates showed antagonistic and synergistic activities, respectively [41].

Dibutyl phthalate (DBP) is an odorless oily liquid. It may be colorless or yellow to faint. The chemical formula of DBP is $C_{16}H_{22}O_{4,}$ with molecular weight of 278.35 g/mol. Dibutyl phthalate (DBP) is also known as di-n-butyl phthalate and is widely used as plasticizers that belong to the class of phthalate esters (PAEs). DBP is a plasticizer used in most plastics and present in water, air, soil, plants, and animals. Some adverse effects with long-term exposure are linked with this plasticizer. As a plasticizer, they are used in polyvinyl chloride (PVC); dibutyl phthalate is found in cloves. DBP was added to the California Proposition 65 (1986) list of suspected teratogens in November 2006. It is a suspected endocrine disruptor. In some nail polishes, DBP is also present as an active ingredient. DBP is soluble in alcohol, ether, and benzene. It can easily penetrate the soil and contaminate groundwater and nearby streams. It is combustible, though it may take some effort to ignite. It is used in paints and plastics and as a reaction media for chemical reactions. It has an excellent stability to light. It emits acrid smoke and fumes, when heated to decompose it [42] (**Figure 1**).

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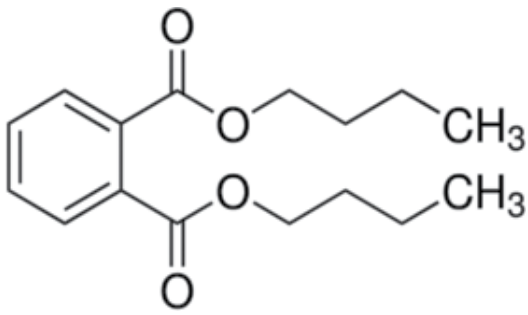

Figure 1. Chemical structure of dibutyl phthalate (DBP).

It does not break down in water but can break down in soil as a free chemical. The various stages of its release are: production, distribution, processing, use, incineration, and disposal. In the environment, high concentrations are mostly present in nearby production and processing sites of waste water and surface water nearby. It is also present in sediment, soil, and in aquatic and soil-dwelling organisms near to sources; its highest level is present in air around PVC-processing plants. Dibutyl phthalate is classified as Group D, not categorized as human carcinogenic agent by Environmental Protection Agency (EPA) [41].

DBPs are well-known endocrine-disrupting chemicals (EDCs) that have health risks to both animals and humans. Endocrine disrupting chemicals are exogenous substances that affect the endocrine system of the body and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife. The occurrence of human disease increases by chronic exposure to DBP. It is banned in the United States, European Union, and many other countries; however, it is still present in our environment, as in water, its concentration is sufficient to affect development and reproduction in aquatic organisms. So, DBP as an endocrine-disrupting chemical also causes human health risks [42]. Studies revealed that the male reproductive system is mainly affected by exposure to DBP. It causes serious developmental disorders like spermatogenesis dysfunction, hypospadias, and cryptorchidism insufficient sperm motility [43]. The high dose of DBP has harmful effect on testis of mice. Several abnormalities regarding testis were observed like loss of spermatogenesis, abnormal level of serum hormones, and anomalous development of testis and epididymis [44]. The chronic exposure of DBP causes the reduction in weight of pubs and litter in both animals and humans [45]. During sexual development, even the short-term exposure to DBP can cause adverse permanent changes in the reproductive system of rats. DBP reduces the sperm count even for several months after the end of exposure to it. It reduces the male anogenital distance; decrease in the weight of both ventral prostrate and bulbocavernosus muscle was also clearly seen. The adverse changes in the mammary gland development were also noted clearly in the adulthood and puberty of rats [46].

Human exposure to DBP may take place, through its presence in the environment including workplace and consumer products. Workers are generally exposed through the air they inhale or through dermal contact. Plastic toys and baby equipment are the sources of children exposure to this chemical. A relatively low concentration of DBP is also determined in the

breast milk [47]. Humans are daily exposed to DBP through contaminated food, contaminated water, dermal contact, and by ingestion. In human, DBP decreases anogenital distance (AGD), affects pubertal development, disrupts sperm motility, and reduces sperm count. Continuous exposure of this plasticizer to humans decreases the number of sperms and suppresses spermatogenesis. It also reduces testosterone biosynthesis and disrupts the androgen:estrogen ratio in human embryos. DBPs have antiandrogenic-like properties and have a great role in hypospadias and cryptorchidism in humans [33].

The effect of DBP on sexual maturation was examined either through estrous cycle or vaginal opening. In animal models, the effect of low and high dose was observed in experimental trials. The highest dose used in these studies was 750 mg/kg/day, while the lowest dose was 0.5 mg/kg/day. Similarly, the duration of exposure was from postnatal day to twenty-first day, or the adult trial period from 10 to 45 days. Moreover, in humans, this study includes those people, who were directly exposed with the metabolites of phthalates [48]. Few previous studies on mice suggested that DBP had no effect on sexual maturity when exposure was given during gestation, weaning, or nursing period. However, some studies report delay in onset of vaginal opening, estrous cycle, and afterward in sexual maturation [49]. Few epidemiological studies also suggest that DBP exposure was not associated with sexual maturity [50]. In male offspring, the prenatal and postnatal exposures of phthalate were linked with reduced androgenic activity. Therefore, the level of phthalates in infants was associated with the exposure to mother. These results in hypospadias, decrease in anogenital distance, and endogenous hormones [51].

Studies confirmed the presence of dibutyl phthalate in the rat bile after oral administration, while in intestine, a fraction of dose was absorbed intact, indicating the nondegradable property of DBP [52].

The studies on the presence of phthalate esters in the blood of individuals who had ingested food that had been in contact with flexible plastics, suggested that levels of dibutyl phthalate observed in the blood were much higher than prior to eating food in the plastic packaging system. Results revealed that in blood, dibutyl levels were 0.35 ppm in comparison to an average value of 0.02 ppm before the use of plastic packaging system [43].

It is accumulated in viscera being rich in fat, like liver, kidney, and could overcome physiological barriers to penetrate testes. The accumulations of DBP exposed through dermal route as compared to the oral route and most of DBP was metabolized in 2 or 3 days [53].

DBP is metabolized along the same or parallel pathways for unsaturated fats indicated by the in vitro studies with pancreatic lipase. However, rats given DBP orally excreted the monobutyl ester as the principal metabolite in the urine with phthalic acid as the secondary metabolite. It is concluded that early life phthalate exposure may enhance the chance of allergic sensitization and atopic disorders [8].

Animal studies with mice exposed orally to dibutyl phthalate have suggested developmental effects, like reduced fetal weight, decreased number of viable litters, and birth defects (neural tube defects). In addition, oral animal studies reported the reproductive effects, like decreased spermatogenesis and testes weight. Structural degeneration in the epididymis

and deferens also caused by dibutyl phthalate administration studied in the mice exposed orally to this, parallel to dose evaluation and RSV can reverse these changes with its protective effects [54].

In the embryos of zebra fish, acetylcholinesterase activity was considerably inhibited. These results suggest that DBPs have the potential neurotoxicity in zebrafish embryos [55].

Dibutyl phthalate had been extensively used and its exposure in children has been thought to be one of the reasons causing a tendency of advanced pubertal timing in girls. As puberty starts from hypothalamic gonadotropin-releasing hormone, its release is controlled by several factors including neurotransmitter kisspeptin (Kiss 1) and its receptor G protein-coupled receptor (GPR54). So earlier pubertal timing in females and both neonatal and prepubertal periods are inducing by DBP and its exposure [56].

The cytokine secretion is also influenced by investigated phthalate monoester from monocytes/macrophages similar to that of the diesters. However, the effect of the monoester was different in T cells as compared to the diesters. The influence of the phthalates on the cytokine secretion did not seem to be a result of cell death. Thus, results indicate that phthalates influenced both human innate and adaptive immunity in vitro. Therefore, cell differentiation, regenerative and inflammatory processes are observed to be influenced by phthalates in animal in vitro studies [57].

The evaluation of phthalates exposure on pubertal development is rarely studied. Few previous studies support that high exposure of phthalates was associated with delay in puberty, although some controversies do exist [58]. In animal studies, decrease in mother and fetal weight and implantation losses was observed after exposing with DBP. Similarly, administration of high dose during gestation period badly affects the pregnancy and mother health [46]. Moreover, a study on female rat demonstrates that higher dose exposure make them unable to become pregnant. Inversely, at lower dose (50 mg/kg/day) exposure, the decrease in mother weight and increase in pregnancy loss were observed. However, some studies stated no effect of higher dose (500 mg/kg/day) on pregnancy, implantation, and serum progesterone level during gestation exposure of DBP [40, 59, 60]. The mode of action of DBP is poorly studied; but decreased in progesterone titer was measured in pregnant rats exposed with higher dose (1500 mg/kg/day) of DBP, however, no change was observed in estradiol level [59]. This suggests that DBP may affect the level of hormones required for pregnancy maintenance indirectly through circulation. A study on female rats suggests no effect of DBP on ovarian histology, ovary weight, number of follicles, serum luteinizing, and follicle stimulating hormone and mating behavior during lactation period exposure [60]. Thus, this concludes that female reproductive system is insensitive to the toxic effect of DBP. Although, certain animal experimental studies are lacking, but this is concluded that phthalates exposure is toxic to reproductive system. However, the adverse effects were observed at higher dose exposure as compared to lower dose.

DBP also affects kidneys of mice. The exposure to this chemical causes oxidative stress in renal fibroblast and tubular epithelial cells, which leads to the dysplasia of kidney and renal fibroblast [40]. Acute administration of DBP induces significant injuries in the kidneys and

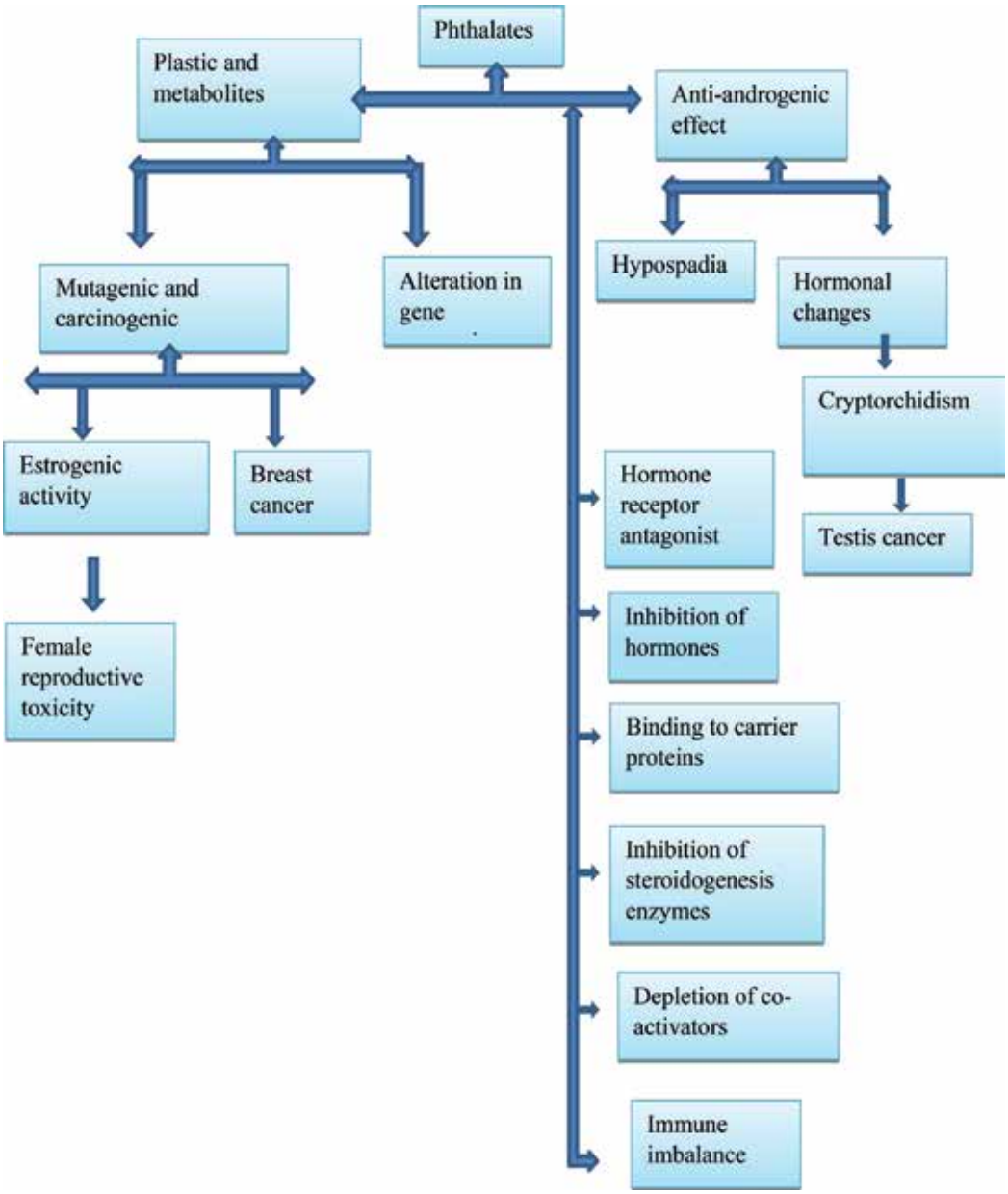

Figure 2. Schematic representation of phthalates effects on reproductive system.

liver of mice. The DBP alters the renal and hepatic cell structure by raising uric acid, blood urea level, lactate dehydrogenase, creatinine, etc. [61].

The prenatal exposure of DBP causes dysplasia in kidney of newborn offspring, while sever renal fibrosis in young adults. However, the mechanism of these disorders remains enigmatic.

Both androgen receptors and fibroblast growth factors (Fgf10) and their receptors (Fgfr2) recognized to be significant for renal development. The anti-androgenic properties of DBP played significant role in the pathological phenomena of prenatal exposure [62].

In a study of prenatal exposure on language development in boys and girls showed no notable effect on girls. However, in boys, this was significant for phthalates metabolites exposure [63].

A study on embryological development of zebrafish demonstrates that DBP causes proteomic changes leading toward the metabolic disorder and affects the networks of embryo development. Moreover, the concentration used in the study was higher than the actual in drinking water due to acute and chronic effects of DBP in experimental trials. This study concludes that DBP induces considerable changes in molecular mechanism of development and metabolism [64].

The effect of DBP was demonstrated on human prostate lymph node/adenocarcinoma epithelial cells (LNCap) to investigate the influence of eno-estrogen on prostate. The effect was also studied on cell viability along with 17β-estradiol. In the same study, the expression of genes involved in cell cycle was also studied. This study examined that the interaction of DBP with estrogen receptor was different from the estradiol. The exposure of DBP changes the gland physiology and ultimately causes the down regulation of cell cycle [65].

The mammary glands are influenced by hormones. The pre-pubertal and adult exposure of BPS was studied in female mice with low dose. Age- and dose-specific effects were associated with the mammary tissues when exposed with BPS and this effect was different from the other bisphenols [66].

The biological effect and mechanism of action of BPS was studied on cell models. The BPS binds with the estrogen receptors in a different way from BPA [67].

The alternative use of BPS was studied on zebrafish development. The study concludes that at concentration of 100 μg/L, showed same effects as were of BPA; so, BPS is also harmful for ecosystem and health and can be used with great care and limitation [68] (**Figure 2**).

4. Conclusion

From current review of literature, it is concluded that the increase use of plastic products enhances the phthalates in environment. The epidemiological studies of human as well experimental trials on animal models investigated the adverse effect of BPS and DBP at lower and higher doses. The reproductive system of male and female are at higher risk of exposure to these chemicals. In females, reduced size of mammary glands, degeneration of ovaries, immature follicles, and pubertal disorders were observed. While in males, decrease in sperm count, damage to sperm duct, and reduced testis was examined in various animal models.

5. Future directions and recommendations

The use of BPS as an alternative to BPA is not safe as it showed similar effect. This should be used with precaution and limitation. Similarly, the use of DBP should be restricted. There should be legislation on its use in various plastic products specially used in baby milk bottles, toys, dermal, and personal products. Many large scale studies are needed to investigate its adverse effect.

Conflict of interest

The author does not have any conflict of interest.

Abbreviations

AGD	anogenital distance
BPA	bisphenol A
BPS	bisphenol S
DBP	dibutyl phthalate
EDCs	endocrine disrupting chemicals
EPA	Environmental Protection Agency
Fgf	fibroblast growth factors
GPR	G protein-coupled receptor
Kiss 1	kisspeptin
LNCap	lymph node carcinoma epithelial cells
PAEs	phthalate esters
PVC	polyvinyl chloride

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Chapter 5

Biotransformation of Bisphenol A and Its Adverse Effects on the Next Generation

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Additional information is available at the end of the chapter

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Abstract

Although we are exposed to many chemical substances in routine daily life, the body has metabolic systems capable of detoxifying and eliminating these chemicals. Bisphenol A (BPA) is an endocrine disrupter of great concern because of its estrogenic activity, but studies have indicated no severe adverse effects in adult rodents exposed to BPA due to metabolic detoxification. BPA is metabolized by glucuronidation mediated by phase II enzymes such as UDP-glucuronosyltransferase. Numerous recent studies in rodents have indicated that maternal BPA exposure causes adverse effects in offspring. It was also shown that bisphenol analogs are efficiently absorbed via the oral route and distributed to the reproductive tract in pregnant rats, with its residue capable of crossing the placental barrier in the late stage of gestation. Both animal and human studies have demonstrated that BPA and the BPA metabolite BPA-GA are detectable in fetal and amniotic fluid, suggesting the presence of a placental transfer mechanism. In this review, we discuss the pharmacokinetics of BPA, particularly its (1) metabolism and disposition in the intestine, (2) metabolism and disposition in the liver, and (3) transfer from maternal tissues to the fetus.

Keywords: bisphenol A, UDP-glucuronosyltransferase (UGT), multidrug resistance-associated protein (MRP), organic anion-transporting polypeptide (Oatp), xenobiotic-metabolizing enzymes (XMEs), liver perfusion, β-glucuronidase

1. Introduction

Bisphenol A (BPA; 2,2-bis[4-hydroxyphenyl]propane) is an industrial chemical widely used in the manufacture of polycarbonate plastics and epoxy resin liners for aluminum cans [1–7]. BPA is an endocrine-disrupting chemical (EDC) that has been demonstrated to affect

reproductive organ development [7–9], brain development [10–15], metabolic diseases [16], and postnatal behavior [17–19]. These adverse effects are thought to be due to disturbed signaling mechanisms involving estrogen, androgen, and thyroid hormone.

BPA introduced into the body orally must pass through the gastrointestinal tract and liver before arriving at target tissues such as the uterus, testes, or fetus. To elucidate the mechanism responsible for the adverse effects of BPA, it is essential to clarify the fate of the compound during its passage through the hepatointestinal pathway. The hepatointestinal pathway serves as a protective barrier against a variety of potentially harmful chemicals due to the activity of potent xenobiotic-metabolizing enzymes (XMEs), which can be classified into three main categories [20]. The first category consists of phase I enzymes, mainly of the cytochrome P450 (CYP) family [21]. Most drugs are metabolized by CYPs, either during detoxification or due to the activation of a pathway for an inactive prodrug. The second XME category consists of what are referred to as phase II enzymes [22–24]. These enzymes usually conjugate phase I products but can also conjugate other intermediate compounds and intracellular substrates, such as steroids and bilirubin. The third category of XMEs consists of drug transporters, which are membrane-bound proteins involved in drug uptake or excretion [25, 26].

Detoxification enzymes have been shown to play a pivotal role in the elimination of ingested chemicals from the intestinal wall and liver. In rat liver, BPA is metabolized by phase II enzymes via glucuronidation, which is mediated by UDP-glucuronosyltransferase (UGT, Enzyme Classification 2.4.1.17), primarily the UGT2B1 isoform [27]. Glucuronidation is a major elimination process that converts lipophilic substrates to hydrophilic molecules that are readily excreted via the bile and urine [20, 28]. Glucuronidation is the main pathway by which BPA is metabolized to a hydrophilic form lacking estrogenic activity. We were the first to report that BPA is highly glucuronidated in rat liver [27]. To clearly elucidate the metabolism and disposition of BPA in the body, we thought it was important to conduct an investigation at the tissue level, and we therefore carried out tissue perfusion experiments.

In this chapter, we discuss the metabolism and disposition of BPA, particularly in the gastrointestinal tract and liver, as well as why the fetus is so easily affected by BPA. The investigation specifically focused on the following: (1) metabolism and disposition of BPA in the intestine, (2) metabolism and disposition of BPA in the liver, and (3) transfer of BPA from maternal tissues to the fetus.

1.1. Metabolism and disposition of BPA in the intestine

This section focuses on the absorption and metabolism of BPA in the intestine. For a thorough investigation of the metabolism of BPA in the intestine, we adopted a method using a segment of everted intestine (**Figure 1A**) [29]. We find that in the intestine of Sprague-Dawley rats exposed to BPA, (1) most of the compounds absorbed by the intestine are glucuronidated within the intestinal wall and (2) the resulting GA is preferentially eliminated into the mucosal side of the small intestine and the serosal side of the colon (**Figure 1B** and **C**).

These results suggest that the proximal intestine plays a highly protective role against ingested BPA. BPA was highly glucuronidated during its passage through the lumen of the rat intestine, with most of the compounds being excreted to the mucosal side as BPA-GA, which

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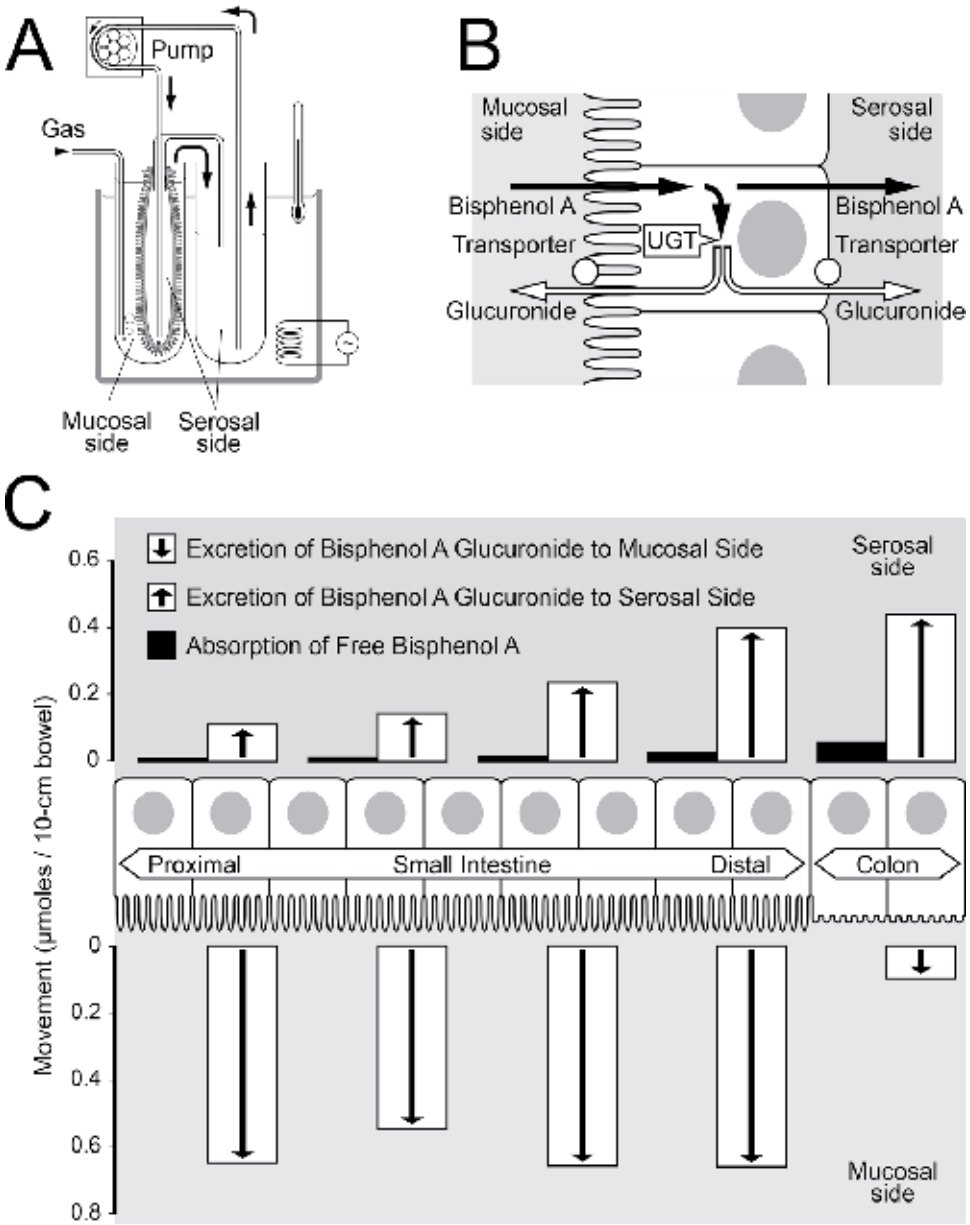

Figure 1. Overview of the metabolism and disposition of BPA in the intestine. (A) Schema of method using a segment of everted intestine. (B) Metabolism and disposition of BPA in small intestinal mucosal cells. BPA was highly glucuronidated through the lumen of the rat intestine. (C) Summary of BPA metabolism in the intestinal tract. Mucosal excretion of BPA-GA greatly exceeded serosal excretion in the proximal intestine; however, the direction of elimination was reversed in the colon.

is low in estrogenic activity (**Figure 2B**) [30]. This was particularly evident in the proximal jejunum, where mucosal excretion of BPA-GA greatly exceeded serosal excretion. Therefore, it appears that the proximal jejunum defends against the potentially adverse effects of orally

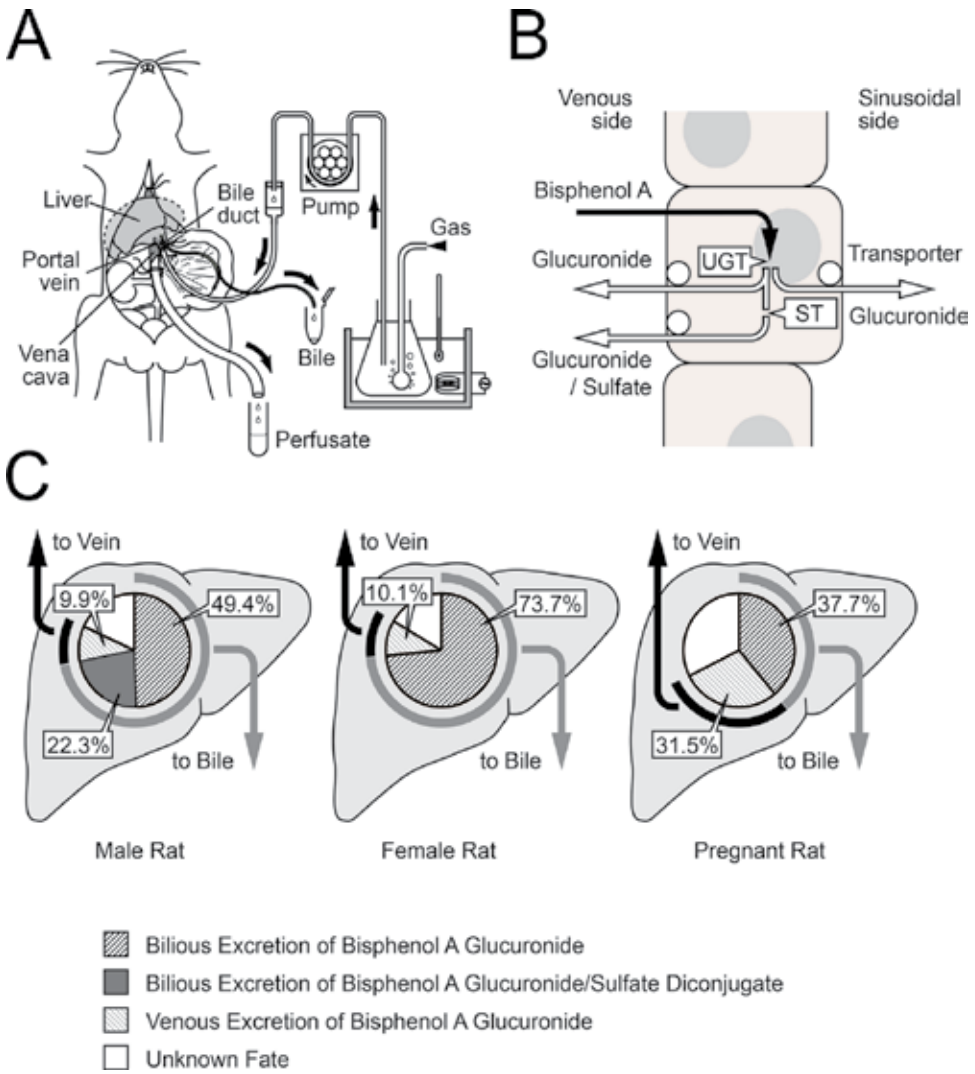

Figure 2. Overview of the metabolism and disposition of BPA in the liver. (A) Schema of the liver perfusion method. (B) Metabolism and disposition of BPA in hepatocytes. (C) Summary of BPA metabolism in the liver. BPA is conjugated primarily to monoglucuronide and partially BPA-glucuronide/sulfate diconjugate in males, but otherwise almost no diconjugate was detected in female rats. Most metabolites were excreted into the bile, but during pregnancy, bilious excretion of BPA-GA decreases, and reciprocally, venous excretion increases.

introduced BPA by limiting entry of the free compound into the bloodstream and curtailing exposure to the middle and distal parts of the intestine. These results are in line with previous reports of low exposure to the compound in association with the oral intake of BPA [31–36]. Comparing the concentration-time profiles of BPA in the blood of F344 rats exposed to the compound intraperitoneally with those of rats exposed orally, Pottenger et al. [31] found that oral administration results in lower exposure to unconjugated BPA. In light of these findings, the diminution of exposure to unconjugated BPA on oral administration may be ascribed to

the high degree of glucuronidation of the compound in the proximal intestine, which is the foremost barrier to damage from oral administration. We previously reported that BPA glucuronidation in the liver is mediated by UGT2B1, an isoform of UDP-glucuronosyltransferase, and that this isoform is not expressed in rat intestine [27]. Generally, the UGT2B family glucuronidates steroid hormones [37]. In humans, several UGT isoforms are known to conjugate BPA. UGT2B15 reportedly has the highest activity, with lower activities reported for recombinant UGT1A1, UGT1A3, UGT1A9, UGT2B4, and UGT2B7 [38]. The results of a recent analysis of a mouse cell line in which all UGT 2B genes were deleted suggest that members of the UGT1 family play a major role in BPA glucuronidation [39], which in turn suggests that the UGT1 family also plays a major role in intestinal BPA glucuronidation.

Although BPA-GA was excreted into the mucosal side of the small intestine, the direction of elimination was reversed in the colon, where excretion was into the serosal side (**Figure 2C**). ATP-dependent transporters have been described as mediating the transport of GA-conjugated compounds across the cell membrane [40]. In rat liver, a member of the ATP-binding cassette (ABC) transporter family, namely multidrug resistance-associated protein (MRP), is capable of mediating transmembrane excretion of a wide range of amphipathic compounds, including bilirubin-, estrogen-, and xenobiotic-GA [41]. In rat intestine, MRP2, localized in the apical domain of enterocytes, is distributed in the proximal intestine [42], and MRP3, localized in the basolateral domain, is distributed mainly in the ileum and colon [43]. Intriguingly, the apical and basolateral directions of BPA-GA excretion in the present study paralleled the distribution patterns of MRP2 and MRP3, respectively. Other reports have indicated that MRP2 is highly expressed in the proximal intestine, whereas MRP3 and MRP4 are highly expressed in the colon [44]. As MRP3 and MRP4 are expressed in the basolateral domain of the liver and intestine [45], the supposition may be made that the elimination direction of BPA-GA is governed by the distribution of an organic anion transporter system such as MRP.

As large amounts of BPA-GA are eliminated from the lumen, the excreted GA would presumably flow into the distal intestine with the luminal contents. In the colon, GA would most likely be deconjugated by lumen bacterial β-glucuronidase, an enzyme known to generate toxic and carcinogenic substances [46]. Deconjugation by lumen bacterial β-glucuronidase is known to be involved in the reactivation of an antitumor compound derived from irinotecan [47]. Furthermore, as excreted BPA-GA is deconjugated by bacterial β-glucuronidase in the cecum, free BPA is detected only in the colon and feces [48]. In light of these previous findings, the notable absorption and transport of unconjugated BPA to the serosal side of the rat colon observed in this study suggests that deconjugated BPA is eventually reabsorbed by the colon.

Generally, the paramount issue in studies of the adverse effects of BPA concerns oral exposure to the chemical in low doses [7, 49]. Although Rubin et al. [50] described adverse effects in rat offspring after maternal administration, other studies have found no adverse effects [51, 52]. Therefore, the toxicity of low doses of BPA remains controversial. We believe that an animal's sensitivity to ingested BPA reflects the conditions inside the intestine (e.g., the luminal contents and composition of the bacterial flora). Further studies are required to clarify the correlation between the catalytic reactivation of BPA-GA by the luminal flora and any resulting adverse effects.

1.2. Metabolism and disposition of BPA in the liver

Because environmental estrogens introduced orally are absorbed by the gastrointestinal tract and consequently the liver before being distributed throughout the body, it is important to trace their fate before they reach the reproductive organs. This section focuses on the metabolism and disposition of BPA in the liver. To facilitate thorough investigation of the metabolism of BPA in the liver, we adopted a liver perfusion assay in a previous study (**Figure 2A** and **B**) [53–55]. The results showed that (1) in Sprague-Dawley rats, most BPA absorbed by the intestine is likely glucuronidated in the liver, (2) the resulting BPA-GA is excreted into the bile and venous blood, and (3) in pregnant rats, there is a slight but significant decrease in bilious excretion of BPA-GA, which results in a reciprocal increase in venous excretion (**Figure 2C**).

These findings are in line with those from a study reporting that BPA added to the culture medium of isolated hepatocytes is highly metabolized to BPA-GA [56]. Moreover, previous studies of BPA pharmacokinetics and metabolism in rats provided evidence that the major metabolite in the plasma is BPA monoglucuronide conjugates [31–33]. Therefore, glucuronidation is a major pathway of BPA metabolism in the liver. After glucuronidation, the conjugates must be excreted from the hepatocytes into the bile and venous blood. Intriguingly, in male rats, approximately one-fourth of infused BPA was eliminated as a GA/sulfate diconjugate, whereas this diconjugate was virtually absent in female rats (**Figure 2**). Suiko et al. reported that BPA is conjugated with sulfate by several forms of human sulfotransferase [57]. We recently reported the results of rat liver perfusion experiments in which we found that BPA is conjugated primarily to monoglucuronide; in males, we found that diconjugate (GA/sulfate diconjugate) production occurs under conditions of high-dose BPA exposure [53]. These findings agree with those from a previous study in which BPA added to the medium of isolated hepatocytes was metabolized into both monoglucuronide and diconjugate; moreover, almost no diconjugate was detected in female rats [56]. BPA sulfoconjugation is mediated by phenol sulfotransferase isoforms of the SULT1 family [58]. One member of the SULT1 family, SULT1A1, exhibits a high conjugation activity toward BPA [59]. The expression level of SULT1 family enzymes is estimated to be higher in male than in female rats [60].

During pregnancy, the bilious excretion of BPA-GA decreases, and reciprocally, venous excretion increases. A wide variety of drug conjugates are transported by members of the ABC transporter family known as glutathione-S-conjugate export pumps. MRP2, a pump that is expressed primarily in the canalicular membrane of hepatocytes, transports drug GA to the bile [45]. Both the hepatic expression and function of MRP2 decrease in pregnant rats [61]. Regarding sinusoidal excretion, MRP1 and MRP3 have been shown to mediate chemical-GA transport [62–64], and the expression of MRP3 is attenuated in pregnancy [61]. Together with our previous results regarding liver perfusion in Eisai hyperbilirubinemic rats [54], these findings give rise to the view that a low expression of MRP2 in pregnancy limits the transport rate of BPA-GA into the bile and that sinusoidal transport systems such as MRP1 and MRP3 compensate by transporting GA to the venous blood.

1.3. Transfer of BPA from the maternal side to the fetus

Venous BPA-GA excreted from the liver enters the systemic blood circulation. Pottenger et al. [31] showed that BPA-GA can be detected in the urine after administration of

BPA; therefore, BPA-GA is excreted into the urine. However, certain organs, such as the lungs, small intestines, and placenta, show a high β-glucuronidase activity [65, 66]. BPA-GA can be cleaved in these organs, and it can be predicted that the resultant BPA moves to the lower organs supplied by the bloodstream. In the placenta, β-glucuronidase activity leads to fetal exposure to BPA. Kushari and Mukherjea [67] reported that placental β-glucuronidase activity is present during early gestation in humans, which is a highly vulnerable period for the developing fetus. An important concern, however, is that previous investigators reported that the placenta exhibits a minimal glucuronidation activity [68, 69]. We also demonstrated that BPA-GA is transported to the fetus following uterine perfusion and that BPA-GA and deconjugated BPA can be detected in the fetus and amniotic fluid due to a high deconjugation activity and vulnerable drug metabolism in the fetus [70]. After oral administration of 10 mg/kg 14C-BPA to GD16.0 rat mothers, Domoradzki et al. found that BPA-GA was concentrated in the fetus [32]. Kurebayashi et al. also detected radioactivity in GD18 fetal tissues 24 h after oral administration of 14C-BPA to pregnant rats, but they found no radioactivity in GD13 or GD15 fetuses [35]. Therefore, BPA-GA may be transferred across the placenta to the fetus by placental transporters that mediate the transfer of essential endogenous physiologic estrogenic compounds (**Figure 3**).

BPA is highly glucuronidated through the lumen of the rat intestine. In the intestine, serosal excretion of BPA metabolite is probably BPA-GA, and partially free BPA may be also transported into the portal vein. In the liver, BPA is conjugated primarily to monoglucuronide and

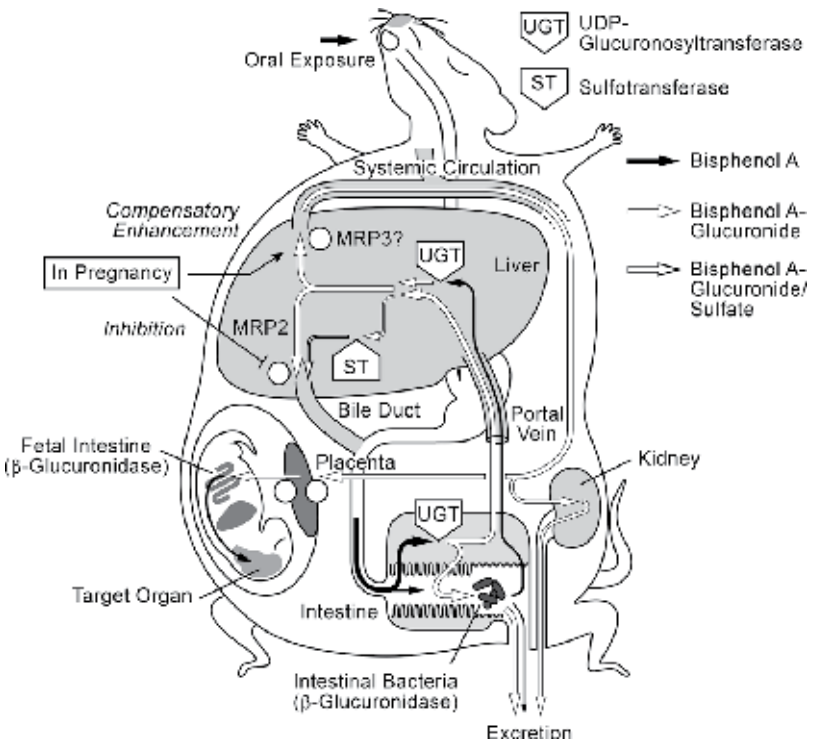

Figure 3. Illustration of the metabolism and disposition of BPA during pregnancy.

partially BPA-glucuronide/sulfate diconjugate in males. During pregnancy, bilious excretion of BPA-GA decreases, and reciprocally, venous excretion may increase through MRP. BPA-GA remaining in systemic blood circulation is metabolized by placental or fetal β-glucuronidase, and the resultant BPA would permeate the fetal tissues. MRP, multidrug resistance-associated protein; UGT, UDP-glucuronosyltransferase; ST, sulfotransferase.

Some members of the Oatp [71–73] and Mrp [74, 75] transporter families are known to transport conjugates of steroid hormones such as DHEAS and 17β-estradiol-GA, suggesting that BPA-GA is transported across the placenta by these transporters. In light of the studies cited above and our present results, we surmise that if BPA-GA remaining in systemic blood circulation is metabolized by placental or fetal β-glucuronidase, the resultant BPA would permeate the fetal tissues (**Figure 3**). Due to a low UGT2B1 expression in fetal rat liver, we also reported that this metabolic system is weak in the fetus [70, 76, 77]. Numerous recent studies in rodents have found that maternal BPA exposure causes adverse effects in the offspring [17, 78–84]. In light of these findings, the present results suggest that the risk of BPA exposure to the fetus is high, despite preservation of BPA glucuronidation in the maternal liver.

2. Conclusion(s)

Many reports have suggested that human health may be affected by exposure to even low levels of BPA, especially during the gestation period. However, the detailed mechanisms of BPA's effects remain unknown. To further elucidate the mechanism governing the detrimental effects of EDCs on target organs, it is essential to clarify both the metabolism and elimination pathways of such chemicals in the body. However, BPA is highly glucuronidated in the intestine and liver, and the resultant formation of BPA-GA prevents a complete understanding of metabolism and disposition by facilitating deconjugation during enterohepatic circulation and systematic circulation in the body. Given that exposure to BPA could adversely affect the fetus in pregnant animals, it is critical that further work be done to determine the fate of venous GA compounds in the complete BPA pathway before excretion.

In modern society, we are continually exposed to many chemical substances. We have to deal with all of these chemicals to ensure good health. Many studies of the effects of chemical substances have focused only on terminal mechanisms. We originally developed the prominent drug metabolism systems to eliminate various chemicals in the process of evolution. The various mechanisms that determine the effects of EDCs can only be productively discussed after a more complete understanding of their metabolism systems is achieved. At that point, new precautions to avoid the risks of adverse effects could be developed.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 6

Androgen Receptor Plays a Vital Role in Benomyl- or Carbendazim-Induced Reproductive and Developmental Toxicity and Endocrine-Disrupting Activity in Rats

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Additional information is available at the end of the chapter

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Abstract

Benomyl and its metabolite carbendazim were reported to induce reproductive and developmental toxicity and endocrine-disrupting activity in rats. The exactly underlying mechanism of reproductive and developmental toxicity and endocrine-disrupting activity still remain unclear. Based on our unpublished data it showed that the antiandrogen flutamide can completely recover the reproductive and developmental toxicity including embryolethality induced by benomyl and carbendazim in rats. This manuscript aimed to review and generalize the results based on our previous reports. Androgen receptor might play an important role in benomyl- and carbendazim-induced reproductive and developmental toxicity and endocrine-disrupting activity. The evidences were (1) androgen- and androgen receptor-dependent mechanisms are possibly involved in carbendazim-induced toxicity; (2) carbendazim exposure in utero displays a transient and weak androgenic effect and reduces flutamide antiandrogenicity in male rats; (3) antagonistic effect of flutamide on the carbendazim-androgenic effect on mRNA and protein levels; (4) benomyl and carbendazim exhibit an androgenic effect, leading to increase weight of ventral prostate and seminal vesicles and uterine fluid retention in young adult rats. The molecular underlying mechanism of reproductive and developmental toxicity and endocrine-disrupting activity induced by benomyl and carbendazim through androgen receptor need to be further investigated.

Keywords: benomyl, carbendazim, reproductive and developmental toxicity, endocrine-disrupting activity, rats

1. Introduction

As reported carbendazim (methyl-2-benzimidazole carbamate) is used to be a systemic fungicide [1]. Both carbendazim and its parent benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate] are exhibiting low acute toxicity [2]. In contrast, carbendazim and benzimidazole chemicals induced severe reproductive and developmental toxicity in rodents [3–6]. Reports showed that carbendazim and benomyl exhibited testicular toxicity such as sloughing of immature spermatids [7, 8], inhibition of microtubule assembly [9], seminiferous tubular atrophy [10], and testicular atrophy and infertility [11] in male rats. Both carbendazim and benomyl induced developmental toxicity in rodents. Prenatal treatment of carbendazim to rats during pregnancy exhibited embryonic death, growth retardation, and developmental abnormalities including exencephaly, microphthalmia and hydronephrosis in offspring [3, 12]. Treatment of benomyl to pregnant rats induced craniocerebral and systemic malformations such as cleft palate, hydrocephalus, and exencephaly in offspring of male and female rats [13]. In contrary to the more reports available on the reproductive and developmental toxicity of carbendazim and benomyl, studies for endocrine-disrupting activity or mode of action of the these two fungicides remains unclear. The earlier report on endocrine activity for carbendazim might be Rehnberg et al. (1989). They showed that administration of male rats with carbendazim raised testosterone concentration and the levels of androgen binding protein in the interstitial and seminiferous tubule fluid, meaning an association between endocrine disruption activity and carbendazim toxicity [14]. Recently Rama et al. (2014) reviewed and reported that carbendazim induced reproductive toxicity and possible hormonal effects in rats [15]. They reviewed the previous reports and generalized that carbendazim have androgenic effects acting directly in the androgen receptors and/or increasing the expression of androgen receptors. Some chemicals were reported to increase or decrease AR expression. Bisphenol A was reported to increase AR expression [16] while di-n-butyl phthalate (DBP) [17, 18] and sodium valproate [19] decrease it. We found out that it is common for estrogen receptor agonist and androgen receptor antagonist but not for androgen agonist in pesticides. Based on the chemical structure it seems to determine the AR agonist or antagonist. The degree of increase or decrease of AR expression might be depended on the chemical structure, which shared with nature ligand dihydrotestosterone. This manuscript would like to combine our unpublished data and previous studies to infer that androgen receptor plays an important role in benomyl- and carbendazim-induced reproductive and developmental toxicity and endocrine-disrupting activity in rats.

2. Previous studies of reproductive and developmental toxicity and endocrine-disrupting activity induced by benomyl or carbendazim

2.1. Endocrine-disrupting activity in carbendazim-induced reproductive and developmental toxicity in rats

2.1.1. Materials and methods

(1) Animals and related preparation

Both male and female SD rats, 3–4week old, were obtained from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. All rats were kept in specific-pathogen-free

animal facility in Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Taichung, Taiwan. The animal rooms were kept on a 12-h light and dark cycle, 23 ± 2°C, and 50 ± 10% relative humidity. When they were transported to animal room, the rats were quarantined for at least 1 week and opened on the bases of enough body weight gain and without clinical signs of disease of injury. Both carbendazim and benomyl with 99% pure were a gift from Sinon Co., Taichung, Taiwan. These two pesticides were suspended in polyethylene glycol 200 and treated to animals orally by gavage in a volume of 2 ml/kg body weight, once daily. In reproductive toxicity studies, male rats were administered with these two pesticides and/or flutamide for 28 days. In developmental toxicity studies, both male and female rats were administered with 200 mg/kg carbendazim or 100 mg/kg benomyl for 28 days. Then the female rats were mated with male within each treatment group for 14 days. No treatment was carried out during the mating period. Pregnant dams with plug detected were kept to deliver the offspring at term and conception rates were calculated. All rat offspring were weaned at 21 days postnatal and then fed up to 6 week old [20].

(2) Organ and tissue weight, morphology, and histopathological examination

Both testis and epididymis were weighed by right and left sides. The incidence of abnormal morphology was recorded. The half of testis or epididymis each was fixed in 10% neutral phosphate-buffered formalin solution for subsequent histopathological examinations. Tissues of testis and epididymis were processed by standard histopathological processes and stained using haematoxylin and eosin for light-microscopic examinations. Both testis and epididymis sections were stained with the Giemsa staining periodic acid-Schiff methods and then counterstained using haematoxylin as reported in Simoes and Schoning [21]. Both testis and epididymis histology were evaluated and histopathological findings were scored according to Oakberg [22] and Hess [23].

(3) In vitro androgen receptor binding assay

The ligand binding assay was processed to determine the concentration of androgen receptor in rat tissue according to Nonneman et al. [24]. The ligand [1, 2, 3, 5, 6, 7-3H(N)]-5α-androstan-17β-ol-3-one (dihydrotestosterone, 5α-DHT) (110–150 Ci/mmol) was obtained from NEN Life Science Products, Inc., Boston. Nonlabelled 5α-DHT was obtained from Sigma Chemical Company, St. Louis, MO, and recrystallized from ethanol prior to use. Both rat testis and epididymis were homogenized in ice-cold low-salt TEDG buffer, pH 7.4, consisting of 10 mM Tris, 1.5 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, and 1 mM each of dithiothreitol, phenylmethylsulfonyl fluoride, and sodium molybdate as described by Hardy et al. [25]. Tissue homogenates were centrifuged at 30,000 × g for 1 h and the supernatant were processed to use as the low-salt extract. Before analysis, the endogenous steroids were removed from the low-salt extract by incubation with dextran-coated charcoal. The binding of [3H]-5α-DHT to androgen receptor of testis and epididymis extract each was determined by competitive inhibition binding using nonlabelled 5α-DHT. Charcoal-treated testicular and epididymal extract each was incubated with 1 nM [3H]-5α-DHT at 4°C for 24 h. The nonspecific binding was carried out with incubating the extract with 100-fold excess nonlabelled 5α-DHT. Unbinding [3H]-5α-DHT was isolated from the binding steroid by adding the extract to packed hydroxyapatite in the low-salt TEDG buffer. Mixture were incubated for 30 min with several mixings and then centrifuged at 600 × g for 3 min at 4°C. After that the supernatant was aspirated. An aliquot of the packed HAP was washed 4 times with ice-cold 50 mM Tris buffer, pH 7.3. In determination of total binding,

the binding [3H]-5α-DHT was extracted from HAP with ethanol and counted for radioactivity using a Beckman model LS6000 TA liquid scintillator. For specific binding of testis and epididymis extracts they were determined by subtracting nonspecific binding from total binding and corrected for protein concentration. The protein concentration was determined according to Lowry et al. [26]. Analysis of effect of carbendazim on androgen receptor binding, specific binding of [3H]-5α-DHT to testis extract was carried out with incubation of the charcoal-treated testis extract with [3H]-5α-DHT in the presence of carbendazim at 4°C for 24 h. Incubation mixtures were carried out to the same procedures as before in the androgen receptor binding assay.

(4) Statistical analysis

All these data were expressed as mean ± SE. All data were processed to analysis of variance followed by Student's t-test. The level of significance was set at $p < 0.05$.

2.1.2. Abstract

This study aimed to investigate the endocrine-disrupting activity of carbendazim-induced reproductive and developmental toxicity in rats. The male rats were co-treatment with 675 mg/kg carbendazim and 50 or 100 mg/kg flutamide, an androgen receptor antagonist, once daily for 28 days decreased testis weight induced by treatment with carbendazim alone. Co-treatment of carbendazim and flutamide blocked losses of spermatozoa and cell morphology and decrease of sperm concentration induced by carbendazim. An important evidence for endocrine disrupting activity induced by carbendazim and benomyl was that premating treatment of male and female rats with 200 mg/kg carbendazim for 28 days resulted in androgenic effects including incomplete development of uterine horn, enlargement of urethra, absence of vagina, and induction of seminal vesicles in female offspring, without significant effects in male offspring. Also, premating treatment with 100 mg/kg benomyl, the parent compound of carbendazim, produced incomplete development of uterine horn and absence of vagina in female offspring and induced testis and epididymis atrophy in male offspring. When male rats were treated with 25, 50, 100, 200, 400, and 800 mg/kg carbendazim for 56 days androgen receptor concentrations were increased in testis and epididymis with dose dependent. Furthermore, additions of 5, 50, and 500 M carbendazim to testis extract from untreated rats substituted binding of [3H]5 -dihydrotestosterone to androgen receptor with concentration dependent. This study illustrated that reproductive toxicity exhibited by carbendazim is relieved by an androgen receptor antagonist flutamide in male rats and developmental toxicity of the fungicide shows androgenic properties in female offspring. The authors concluded that androgen- and androgen receptor-dependent mechanisms are quite possibly involved in carbendazim-induced toxicity.

2.2. Antagonistic and synergistic effects of carbendazim and flutamide exposures in utero on reproductive and developmental toxicity in rats

2.2.1. Materials and methods

(1) Animals and related preparation

Both male and female rats were obtained from the National Laboratory Animal Center, Taipei, Taiwan. All rats were kept in specific pathogen-free animal facility in Taiwan Agricultural

Chemicals and Toxic Substances Research Institute, Taichung, Taiwan. All animal rooms were kept under a 12-hour light and dark cycle, 23 ± 2°C, and 50 ± 10% relatively humidity. All animal had access *ad libitum* to reverse osmosis water and rodent chow (LabDiet® 5001, PMI Nutrition International, LLC, Brentwood, MO, USA). When the animals were transported to the animal room, all rats were quarantined for at least 1 week and opened on the basis of enough body weight and without clinical signs of disease or injury. Female rats were mated with male within each same treatment group for 14 days. Gestation day (GD 0) was defined as the day that sperm was detected in vagina of the mated female. Allocating animals to treatment groups was finished on the basis of body weight randomization to ensure unbiased weight distribution across groups. Dams and offspring were kept in polycarbonate cages on Laboratory Animal Bedding (TCP Chipsi Heimtier Steu, Germany) until weaning postnatal day 21 (PND 21), at which the test animals were housed, up to 5 per cage, by sex and treatment until necropsy on PND 56. All male and female offspring were euthanized by CO_2 asphyxiation and processed to subsequent postmortem examination [27].

(2) Treatment and dose design

Both carbendazim and benomyl with 99% pure were a gift from Sinon Co., Taichung, Taiwan. All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. Carbendazim, benomyl, or flutamide each was suspended in corn oil and treated to animals orally by gavage in a volume of 2.5 mL/kg body weight, once daily. Groups of five rats from GD 0 to 20, were treated with carbendazim at 6.25, 12.5, and 25 mg/kg; benomyl at 25, 50, and 100 mg/kg; or flutamide at 0.6, 2.5, and 10 mg/kg. Also, rats were co-treated with 25 mg/kg carbendazim and 0.6, 2.5, and 10 mg/kg flutamide or co-treated with 100 mg/kg benomyl and 0.6, 2.5 or 10 mg/kg flutamide. Female rats were checked daily for clinical signs of toxicity. Female body weight and food consumption were measured daily throughout dosing and lactation period. All rat offspring were weaned at PND 21 and fed up to 8-week-old. All rat organ weights were determined on PND 21. Conception rate on GD 21 and 22, proportion of pups born alive on PND 1, proportion of pups surviving to weaning on PND 21, and sex ratio on PND 56 were measured and recorded.

(3) Determination of androgen-dependent reproductive development effects

What they determined for androgen-dependent reproductive end points were signs of clinical toxicity, anogenital distance (AGD), male and female pup weight, retention of areolae and/or nipples, malformations of external genitalia, testicular descent, preputial separation, vaginal opening, and organ weight and malformation on PND 56 [28, 29]. All pups were counted and examined for signs of clinical toxicity on PND 0 and were individually identifievd by tail-labeling on PND 21. All pups with AGD, and live male and female offspring weights were measured on PND 2, 22, and 42. Day of completion of preputial separation (PPS) and body weight in PPS of male offspring during PND 40 and 50 were also measured. Day of onset of vaginal opening (VO) and body weight in VO of female offspring during PND 30 and 45 were measured. End points of gross morphology of reproductive organs, nipple retention, abnormal testis and epididymis, hypospadias, underdevelopment of prostate or/and seminal vesicle, absent prostate or/and seminal vesicle, bladder stone, and underdevelopment of levator ani bulbocavernosus muscle in male offspring were determined on PND 56.

(4) Necropsy of rats

All pups were weaned on PND 21. Rats were euthanized by CO_2 asphyxiation. All body and organ/tissue weights including liver, kidneys, adrenals, uterus, ovaries, thyroids and number of implantation sites were measured on PND 21.

(5) Necropsy of F1 offspring

Both male and female offspring on PND 56 were euthanized by CO_2 asphyxiation and blood was collected via trunk. After blood collection, the ventral surface of offspring was shaved for counting the number of nipples. External genitalia, including the scrotum, prepuce, and penis of male offspring and vaginal of female offspring were visually inspected. End points of gross internal examination of the reproductive tract such as inspection of the testes, epididymides, prostate, seminal vesicles, levator ani bulbocavernosus muscle, and penis were measured. Also, the liver, kidneys, adrenal glands and thyroids were grossly examined and weighed. Body and organ weights such as testes, epididymides, prostate, seminal vesicles with fluid, levator ani bulbocavernosus muscle, and penis, liver, kidneys, adrenals and thyroids were collected. All examined tissues were fixed in 10% neutral buffered formalin, processed, sectioned, and stained with haematoxylin and eosin.

2.2.2. Abstract

Both carbendazim (methyl 2-benzimidazolecarbamate) and benomyl are reported to exhibit reproductive and developmental toxicity in male rats. This study was mainly to detect the ability of carbendazim exposure *in utero* to alter androgen-dependent development indicators in rat offspring and measure the effects of antiandrogen flutamide on the carbendazim-induced reproductive and developmental alterations. All pregnant female rats were administered with 6.25, 12.5 or 25 mg/kg carbendazim, 25, 50 or 100 mg/kg benomyl, and 0.6, 2.5 or 10 mg/kg flutamide by gavage once daily from gestational day 0 to 20. Also, group of female rats was co-treated with 25 mg/kg carbendazim or 100 mg/kg benomyl and 0.6, 2.5, and 10 mg/kg flutamide. The results showed that the various treatments decreased the survival rates of pups on PND 1 and 21. For male offspring, 12.5 and 25 mg/kg carbendazim increased AGD, an androgen-dependent indicator, on PND 2. Also, benomyl increased AGD of offspring. Co-treatment with 25 mg/kg carbendazim with 0.6, 2.5, and 10 mg/kg flutamide relieved the androgenic effect on AGD induced by carbendazim. The androgenic effects of AGD induced by carbendazim and benomyl on AGD were reversible on PND 22 and later. Carbendazim had no effects on other androgen-dependent indicators such as testis and epididymis malformations, hypospadias, nipple retention, and organ weights of seminal vesicle and levator ani bulbocavernosus muscle on PND 56. Quite surprisingly, carbendazim antagonized the antiandrogenic effects on these indicators induced by flutamide cotreatment. For female offspring, carbendazim exhibited synergistic effects on the flutamide cotreatment-mediated increases of organs weights in liver and kidney on PND 56. No significant effects on female reproductive organs were induced by carbendazim. These findings suggested that carbendazim exposure *in utero* exhibited a transient and weak androgenic effect and reduces flutamide antiandrogenicity in male rats. These two fungicides enhance flutamide-mediated increases of liver and kidney weight in female rats. The antagonistic and synergistic interactions between carbendazim and flutamide *in utero* need to be further investigated.

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2.3. Carbendazim-induced androgen receptor expression antagonized by flutamide in male rats

2.3.1. Materials and methods

(1) Animals and related treatments

Male SD rats with three-week-old were obtained from the National Laboratory Animal Center, Taipei, Taiwan. All rats were kept in a specific-pathogen-free animal facility in the Taiwan Agricultural Chemicals and Toxic Substances Research Institute (TACTRI) in Taichung. The animal rooms were sustained at a 12-hour light and dark cycle, 23 ± 2°C and 50 ± 10% relative humidity. When the animals were transported to the room, the rats were quarantined for at least 1 week and were available for test only when they exhibited enough body weight gain and no clinical signs of disease or injury. Carbendazim with 99% pure was obtained from Sinon Co. (Taichung, Taiwan). Flutamide (FLU) and the other chemicals were obtained from Sigma (St. Louis, MO, USA), unless otherwise stated. The pesticide was suspended in corn oil and orally administered to five rats in each group once a day by gavage at a volume of 2.5 mL/kg body weight. Male rats (322 ± 15 g) were randomly assigned to each treatment group. In order to carry out the time- and dose-dependent tests, the protocol included two treatment-duration and dosages. The first one was as follows: The doses of carbendazim were 0, 25, 50, 100, 200, 400 and 800 mg/kg/day for 56 days. The doses of flutamide were 0, 6.25, 12.5, 25, 50 and 100 mg/kg/day for 28 days. In mixed doses, the rats were co-treated with 675 mg/kg/day of carbendazim and 0, 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days. The second one was as follows: The doses of carbendazim were 0, 6.25, 25, 100 and 400 mg/kg/day for 7 days, while the doses of flutamide were 0, 0.78, 3.13, 12.5 and 50 mg/kg/day for 7 days. The rats in the co-treatment group were given either 400 mg/kg of carbendazim and 0, 0.78, 3.13, 12.5 and 50 mg/kg/day of flutamide, or 50 mg/kg/day of flutamide and 0, 6.25, 25, 100 and 400 mg/kg/day of carbendazim for 7 days. All animal care and experimental procedures were approved by the Institution Animal Care and Use of Committee (IACUC) of TCATRI [30].

(2) Immunohistochemical (IHC) evaluation

The testes tissues of three groups of rats were tested: (1) 0, 25, 50, 100, 200, 400 and 800 mg/kg/day of carbendazim for 56 days; (2) 0, 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days; and (3) co-treatment with 675 mg/kg/day of carbendazim and 0, 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days. Testes from the following test groups were fixed in 10% neutral buffered formalin for 1 week. The tissues were then dehydrated with increasing concentrations of ethanol, cleared in toluene and embedded in paraffin. Sections were cut into 5-mm slices and deparaffinized, hydrated and treated with 0.3% H_2O_2 in PBS (pH 7.6) for 30 min to block endogenous peroxidase activity, and finally treated with a protein-blocking solution (5% goat serum diluted in phosphate-buffered saline). These steps were followed by heating the sections in a microwave oven for antigen retrieval using a 0.01 M citrate buffer solution (pH 5.5). Tissue sections were immunostained with rabbit anti-AR (N-20, Santa Cruz Biotechnology, Inc., CA, USA), which was diluted 1: 250 in phosphate-buffered saline and 0.25% bovine serum albumin and maintained at room temperature overnight. The tissue sections were then developed with a streptavidin-HRP kit (Chemicon IHC Select® CA, USA), using diaminobenzidine as the

chromogen, and were counterstained with haematoxylin. All images were optimized by using an inverted microscope (Leica, Wetzlar GmbH, Germany). To quantify the relative amount of AR protein in the IHC, 200 nucleus stained per field in a slide, 5 fields per slide, 5 slides per dose were counted. The intensity of AR protein stained in nucleus was graded as (0, negative), + (1, mild), ++ (2, moderate), +++ (3, intense), ++++ (4, more intense) or +++++ (5, very intense). The measurements were control group adjusted and the values were statistically analyzed.

(3) Reverse transcription-polymerase chain reaction (PCR)

Testes (n = 5) from the following treatment groups were stored at −80°C for 7 days. Total RNA was extracted with an RNeasy® Mini Kit (QIAGEN, TAIGEN Bioscience Corporation, Dusseldorf, Germany) according to the protocol provided by the manufacturer. For the reverse transcription (RT) reaction, 3 mL of total RNA was used from the individual rats of each group. The RT-PCR reactions in this study were carried out with SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA polymerase kits from Invitrogen (Cat. No. 12574–026) in DNA Engine® & DNA Engine Tetrad® Peltier Thermal Cyclers (PTC-200, MJ Research, Incorporated, Massachusetts 02451 USA). For AR mRNA amplification, the primers were designed to amplify a 570-bp fragment (forward, 5′-TGCTGCCTTGTTATCTAGTCTCA-3′; reverse, 5′-ACCATATGGGACTTGATTAGCAG-3′) (annealing temperature, 60°C; the number of cycles, 24, 26 and 28; product size, 570 bp). PCR was subsequently performed using an optimized protocol of between 24 and 28 cycles. Each cycle consisted of the following: 94°C, 30 s; 60°C, 30 s and 72°C, 45 s. For b-actin mRNA amplification, the primers were designed to a 359-bp fragment (forward, 5′-CTGTGCCCATCTATGAGGGTTAC-3′; reverse, 5′-AATCCACACAGAGTACTTGCGCT-3′) (annealing temperature, 60°C; the number of cycles, 24, 26 and 28; product size, 359 bp). PCR was subsequently performed using an optimized protocol of between 24 and 28 cycles. Each cycle consisted of the following: 94°C, 30 s; 60°C, 30 s and 72°C, 45 s. PCR products were resolved in a 1.2% agarose gel and stained with ethidium bromide, and DNA bands from triplicate reactions were quantified using a FOTO/Analyst® Investigator System (Fotodyne Incorporated, Hartland, WI, USA). The PCR products for β-actin served as an internal standard.

(4) Western blot

A Polytron PT3100 homogenizer (Kinematica AG, Littau, Switzerland) was used to examine frozen testicular tissues from the following treatment groups. Tissues of testes from the first protocol were homogenized for a few seconds in an M-PER® Mammalian Protein Extraction Reagent (Cat. No. 78505, Pierce). The homogenates were then centrifuged at 105,000 × g for 1 h at 4°C. The supernatants were aliquoted and stored at −86°C before use. Before western blotting, protein contents were measured by BCA protein assay (Cat. No. 23225, Pierce). Equal amounts of protein were loaded onto each polyacrylamide gel. The antibody dilutions were 1: 200 for the anti-AR antibody (N-20, Santa Cruz Co., CA) and 1: 5000 for the horseradish peroxidase conjugated goat anti-rabbit IgG (AP132P, Chemicon International). For each treatment group, five samples were analyzed in two separate blots. Total protein extracts from the testicular tissue were denatured and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% polyacrylamide. The proteins were transferred to nitrocellulose membranes. The membranes were then blocked for non-specific binding and incubated with polyclonal primary

antibodies for AR (N-20, Santa Cruz Co., CA) and β-actin (AP132P, Chemicon International). After incubation with primary antibody, the membranes were incubated with horseradish peroxidase-linked anti-goat IgG secondary antibody and visualized on film exposed to enhanced chemiluminescence (VisualizerTM Western Blot Detection Kit, Millipore, MA, USA). The relative amount of protein in the resulting immunoblot bands was estimated by measuring the optical densities of the bands on exposed films using a FOTO/Analyst® Investigator System (Fotodyne Incorporated, WI, USA). The measurements were background adjusted and the values were statistically analyzed. Protein for ß-actin served as an internal standard.

(5) Hormone analysis

Serum luteinizing hormone (LH) (RPN 2562, Amersham, UK), follicular stimulating hormone (FSH) (RPN 2560, Amersham, UK; AE R004, Biocode, Belgium), 17b-estradiol (E_2) (Cayman Chemical., Ann Arbor, MI, USA) and testosterone (T) (Cayman Chemical., Ann Arbor, MI, USA) levels were determined using the relevant EIA systems. The serum samples collected from rats treated with 0, 6.25, 25, 100 and 400 mg/kg/day of carbendazim for 7 days and 0, 0.78, 3.13, 12.5 and 50 mg/kg/day of flutamide for 7 days were directly applied to the well in the kit and measurements were taken according to the procedure described by the manufacturer.

(6) Statistical analysis

The values of AR in Western blot and RT-PCR were normalized against b-actin. All results were statistically analyzed with the t-test and $p < 0.05$ was considered statistically significant. The other data were expressed as mean ± SE. Data were subjected to ANOVA followed by *t-test*. The level of significance was set at $p < 0.05$.

2.3.2. Abstract

Carbendazim was widely used as a fungicide, and it was reported to exhibit reproductive and developmental toxicity. This study aimed to detect the expression of androgen receptor caused by carbendazim and the antagonistic effect of flutamide. Groups of five rats were treated with carbendazim, flutamide or a combination of both to determine androgen receptor mRNA, immune activity and protein expression. Carbendazim increased androgen receptor mRNA with dose dependent, while flutamide, an androgen receptor antagonist, blocked it. When co-treatment with carbendazim and various flutamide doses it decreased the androgen receptor mRNA dose dependent. In contrast, co-treatment with flutamide and various carbendazim doses increased the androgen receptor mRNA with dose dependent. In the immunohistochemistry (IHC) and Western blot (WB) analyses it showed that carbendazim increased androgen receptor activity particular in rat testes with dose dependent, while flutamide decreased it. Moreover, treatment with carbendazim or flutamide for 7 days raised testosterone and follicular stimulating hormone concentrations in the serum of male rats with dose dependent, which might involve the disruption of the androgen receptor. Despite the fact that we need to examine the underlying mechanism of androgen receptor involved in the reproductive toxicity and endocrine-disrupting activity exhibited by carbendazim and its parent, benomyl, we should first discuss how to take advantage of flutamide antagonism on

carbendazim-produced reproductive and endocrine disrupting activity possibly in human. This study concluded that carbendazim exhibited androgen receptor expression in mRNA and protein levels, while flutamide antagonized it. As we know this is the first report on the antagonistic effect of flutamide on the carbendazim-androgenic effect on mRNA and protein levels. This study would give a light way to illuminate the mechanism of carbendazim- and chemical-produced developmental toxicity and endocrine disrupting activity.

2.4. Detecting benomyl and its metabolite carbendazim inducing androgenic activity in rats by using uterotrophic and Hershberger assays

2.4.1. Materials and methods

(1) Chemicals

The following materials were obtained: testosterone propionate (TP, purity ≥97%), Sigma-Aldrich Co. (Buchs, Switzerland); 17β-estradiol (E_2, purity ≥98%), flutamide (Flu, purity ≥97%), and corn oil (0.9 g/mL), Sigma Chemical Co. (St. Louis, MO, USA); carbendazim (Mbc, purity ≥99%), and benomyl (Ben, purity ≥99%), Sino Co. (Taichung, Taiwan, ROC) [31].

(2) Animals, experimental conditions, castration (Cast) and ovariectomy (OVX) procedures

The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Taiwan Agricultural Chemicals and Toxic Substances Research Institute. Four-week-old male and female SD rats were purchased from the National Laboratory Animal Center, Taipei, Taiwan. All rats were accustomed at least to the laboratory environment for 3 weeks before test. During the experiment period, all rats were kept, two to three per cage, in suspended aluminum cages with stainless-steel wire-mesh front and floor under the controlled conditions, containing a temperature of 21 ± 2°C, a relative humidity of 40–70%, a frequency of ventilation of more than 10 air exchanges per hour, and a 12-h light/dark cycle. Drinking water and pellet rodent diet were available *ad libitum*. Male rats at 7 weeks of age underwent Cast procedure. Under ether anesthesia an incision was made in the scrotum and both testes and epididymis were removed with ligation of blood vessels and seminal ducts. Chemical treatment was not initiated until 30 days later to allow for complete recovery from surgical stress. Ovariectomy (OVX) procedure for female rats was operated about 7 weeks of age under ether anesthesia by opening the dorsolateral abdominal wall at the midpoint between the costal inferior border and the iliac crest, a few millimeters from the two lateral margins of the lumbar muscle. On the abdominal cavity, the ovaries were located. On an aseptic field, the ovaries were physically removed from the abdominal cavity. A tie was operated between the ovary and uterus to control bleeding and the ovary was detached by incision above the tie at the junction of the oviduct and each uterine horn. Following the surgery, females were acclimated for 30 days to allow for recovery from operation stress and to monitor the estrus cycle to confirm the success of OVX. Only those animals in the diestrus or metestrus phase were used in the experiments.

(3) Study design and clinical examination

This study was designed according to standardized test guidelines, including OECD test 440 [32], USEPA OPPTS 890.1600 [33], OECD test 441 [34] and USEPA OPPTS 890.1400 [35], with

modification of endpoints. **Table 1** shows the treatment conditions for uterotrophic assay (estrogenic and antiestrogenic/estrogenic) and Hershberger assay (androgenic and anti-androgenic/androgenic) in young adult rats (275 ± 15 g). Each experimental group consisted of six animals. Test and reference substances were suspended or dissolved daily in vehicle (corn oil). Daily dosages of E_2 and TP were 2.5 ml/kg body weight (BW) administered via oral gavage and 0.5 ml/kg BW administered via subcutaneous injection. Oral gavage was selected because it is one of the potential exposure routes of test chemicals in humans. For all experiments, clinical signs, BW and weights of liver and kidneys were assessed as indices of systemic toxicity. Clinical signs including any abnormal behavior were recorded twice a day for each animal.

(4) Assessment of antiestrogenicity/estrogenicity in young adult rats

A 10-day uterotrophic assay using OVX rats was performed to determine if benomyl, carbendazim and flutamide interfere with estrogen receptor-mediated mechanisms. For assessment of antiestrogenicity/estrogenicity, 5 mg/kg/day E_2 was administered daily, as a reference estrogen, by subcutaneous injection on the dorsal surface, as previously described [32, 33] with modified dosage. Benomyl, carbendazim or flutamide was administered to OVX or E_2-treated OVX rats by oral gavage for 10 days. A previous study has shown that AR antagonist flutamide blocks the androgenic effect induced by carbendazim [27]. To investigate the effects of AR agonists benomyl and carbendazim (50, 100, 200, 400, and 800 mg/kg/day) and antagonist flutamide (6.25, 12.5, 25, 50, and 100 mg/kg/day) on estrogenic activity in rats, these chemicals were administered to OVX rats by oral gavage for 10 days. The dose levels of benomyl, carbendazim or flutamide have been previously described [20]. One day after the final administration, rats were euthanized by blood withdrawal from the abdominal femoral artery under light ether anesthesia and exhaust ventilation to maintain the airborne concentrations of vapors below their respective threshold values. Uterus with fluid, vagina, thymus, thyroid, liver, lung, adrenal glands, kidneys and bladder were examined for gross lesions and then dissected and weighed after careful trimming to remove fat and other contiguous tissues in a uniform manner.

(5) Assessment of antiandrogenicity/androgenicity in young adult rats

A 10-day Hershberger assay using male rats was performed to determine if benomyl, carbendazim and flutamide interfere with AR-mediated mechanisms. For assessment of antiandrogenicity/androgenicity, 5 mg/kg/day testosterone propionate (TP) was administered daily, as a reference androgen, by subcutaneous injection on the dorsal surface as previously described [34–36] with modified dosage. Benomyl, carbendazim or flutamide was administered to Cast or TP-treated Cast rats by oral gavage for 10 days. Dosages of 50 and 100 mg/kg/day benomyl, carbendazim or flutamide [20] were administered as antagonist control for anti-androgenicity in young adult rats, as previously described. One day after the final administration, rats were euthanized by blood withdrawal from the abdominal femoral artery under light ether anesthesia and exhaust ventilation to maintain the airborne concentrations of vapors below their respective threshold values. The reproductive accessory glands/tissues (prostate, seminal vesicles with coagulating glands, levator ani plus bulbocavernosus muscles, and penis), as well as thymus, thyroid, lung, liver, adrenal glands, kidneys, bladder, and scrotum, were examined for gross lesions and dissected. All tissues were carefully trimmed to remove fat and weighed.

Dose (mg/kg/day)	Uterotrophic assay[6]				Hershberger assay[6]			
	Estrogenic		Anti-estrogenic/ estrogenic		Androgenic		Anti-androgenic/ androgenic	
Treatment group[1]	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Control (intact)		+[7]				+		
Control (OVX) [2]		+				-[8]		
E_2 (sc) [3]	—	—	+	+	—	—	—	—
Control (Cast) [4]		—				+		
TP (sc)[5]	—	—	—	—	—	—	+	+
Corn oil (oral)	+	—	+	—	+	—	+	—
Benomyl (Ben)								
50	—	+	—	+	—	+	—	+
100	—	+	—	+	—	+	—	+
200	—	+	—	+	—	—	—	—
400	—	+	—	+	—	—	—	—
800	—	+	—	+	—	—	—	—
Carbendazim (Mbc)								
50	—	+	—	+	—	+	—	+
100	—	+	—	+	—	+	—	+
200	—	+	—	+	—	—	—	—
400	—	+	—	+	—	—	—	—
800	—	+	—	+	—	—	—	—
Flutamide (Flu)								
6.25	—	+	—	+	—	—	—	—
12.5	—	+	—	+	—	—	—	—
25	—	+	—	+	—	—	—	—
50	—	+	—	+	—	+	—	+
100	—	+	—	+	—	+	—	+

[1]All treatment groups were treated with 6 male or 6 female rats.

[2]OVX: ovariectomy.

[3]E_2 (sc): 17β-Estradiol, 5 mg/kg/day (subcutaneous).

[4]Cast: castrated.

[5]TP (sc): testosterone propionate, 5 mg/kg/day (subcutaneous).

[6]Comparison pairs for uterotrophic and Hershberger assays are as follows, respectively: treatment group 1 vs. treatment group 2; treatment group 3 vs. treatment group 4.

[7]+: with.

[8]–: without.

Table 1. Study design for uterotrophic and Hershberger assays.

(6) Statistical analysis

Data are expressed as mean ± SD. BW and organ weights were subjected to ANOVA followed by student's t-test. The level of significance was set at $p < 0.05$.

2.4.2. Abstract

The both benomyl and carbendazim are widely used systemic fungicides. It has been shown that benomyl and carbendazim induce endocrine-disrupting activity, resulting in reproductive and developmental toxicity, as well as androgen receptor (AR) gene expression in rats. The aim of this study was to link AR induction by benomyl and carbendazim, observed in our previous reports, with the results of Hershberger and uterotrophic assays. In an uterotrophic assay, neither benomyl nor carbendazim, except at 800 mg/kg/day, affected weight of uterus and vagina when compared to the ovariectomized control rats. Co-treatment with 17β-estradiol (E_2) and 200 mg/kg/day benomyl or co-treatment with E_2 and 200, 800 mg/kg/day carbendazim significantly increased uterine weight when compared to treatment with E_2 alone in an uterotrophic assay. This uterotrophic activity might be mediated through AR. Treatment with flutamide alone or in combination with E_2 had no effect on uterine weight. In the Hershberger assay, treatment with 50 and 100 mg/kg/day benomyl increased weight of ventral prostate plus seminal vesicles. Carbendazim or flutamide alone exhibited no effect on reproductive accessory gland weight. Co-treatment with testosterone propionate (TP) and 50 or 100 mg/kg/day carbendazim, but not benomyl, significantly increased the weight of ventral prostate plus seminal vesicles. Co-treatment with TP and 50 or 100 mg/kg/day flutamide significantly decreased these reproductive accessory gland weights when compared with TP alone. Based on our previous report, carbendazim increases mRNA and protein expression of AR in testis, epididymis and prostate and antagonizes the reduced tissue weights of seminal vesicle and prostate of male offsprings induced by in utero exposure to flutamide in rats. This infers that benomyl and carbendazim increase the weight of ventral prostate plus seminal vesicles through induction of AR expression. Moreover, according to a previous report, TP, an AR agonist, induces fluid retention in uterus by exhibiting androgenic activity, similar to that of benomyl and carbendazim, in an uterotrophic assay. Based on these results, benomyl and carbendazim exhibit an androgenic effect, leading to increased weight of ventral prostate and seminal vesicles and uterine fluid retention in young adult rats. The exact mechanisms require further investigation.

3. Future work and recommendations

OECD takes much effort to promote adverse outcome pathways (AOP) methodology. Androgen receptor-mediated reproductive and developmental toxicity and endocrine disrupting activity would be a novel AOP. It is an approach to support the use of a mode (and/or mechanism) of action basis for understanding the adverse effects of chemicals and other stressors. AR mediated reproductive and developmental toxicity and endocrine disrupting activity would be a novel future application. Specific molecular signals of AR mediated effects would be the future work.

4. Diagram/schematic figure

In the respect of chemical structure, benomyl and carbendazim shared the same C and D ring structure with the natural ligand, dihydrotestosterone (**Figure 1**). We made a schematic labeling of the benomyl, carbendazim mimicking the main ligand interaction features of the natural ligand, dihydrotestosterone, with the androgen receptor referred to the previous report by Tamura et al. (2003) [37].

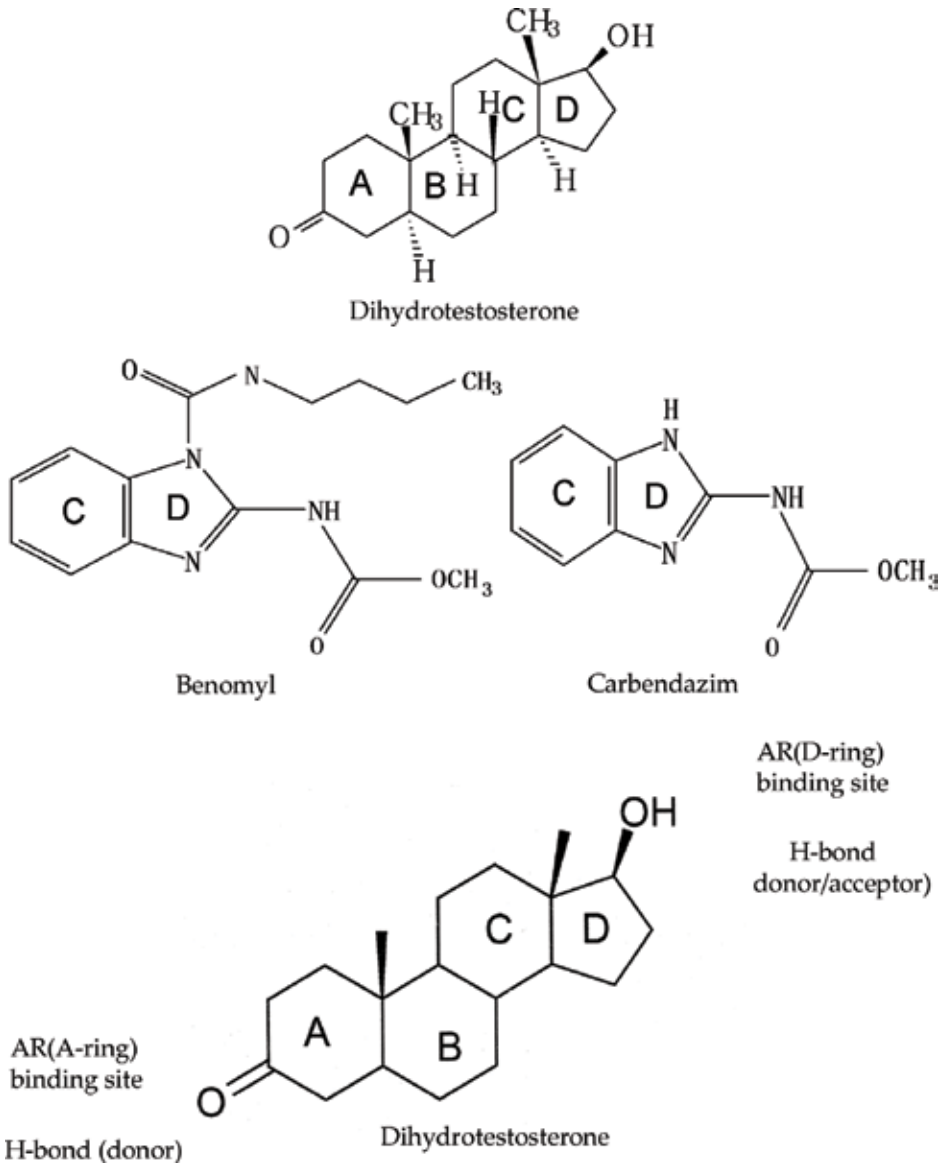

Figure 1. Schematic labeling of the benomyl, carbendazim mimic the main ligand interaction features of the natural ligand, dihydrotestosterone, with the androgen receptor.

5. Conclusions

Based on the previous study firstly it proved that reproductive toxicity produced by carbendazim is relieved by an androgen receptor antagonist in male rats and developmental toxicity of the pesticide showed androgenic properties in female offspring. We concluded that androgen- and androgen receptor-dependent mechanisms are quite possibly complicated in carbendazim-produced toxicity. Secondly findings show that carbendazim exposure *in utero* displays a transient and weak androgenic effect and reduces flutamide antiandrogenicity in male rats. Thirdly we concluded that antagonistic effect of flutamide was on the carbendazim-androgenic effect on mRNA and protein levels. The results would help us to illustrate the mechanism of carbendazim- and chemical-induced developmental toxicity and endocrine-disrupting activity. Fourthly benomyl and carbendazim exhibit an androgenic effect, leading to increased weight of ventral prostate and seminal vesicles and uterine fluid retention in young adult rats.

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Notes/Thanks/Other declarations

No

Abbreviations

LD_{50}	lethal dose with 50% mortality statistically
GD 0	gestation day, day of vaginal plug detected
PND	postnatal day
AGD	anogenital distance
PPS	preputial separation
VO	vaginal opening
AR	androgen receptor

OVX	ovariectomy
TP	testosterone propionate
OECD	Economic Co-operation and Development

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Chapter 7

Endocrine Disruptors and Reproductive Health in Males

Tomas Jambor, Hana Greifova, Jana Bistakova and Norbert Lukac

Additional information is available at the end of the chapter

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Abstract

Nowadays, endocrine-disrupting chemicals are considered to be one of the main causes of the ever-increasing occurrence of problems with male fertility. These compounds of natural or anthropogenic origin are omnipresent in the environment and organisms are exposed to them practically nonstop through the air, water, food, and occupationally. Endocrine disruptors have the ability to mimic effects of reproductive hormones and demonstrably can interfere with the endocrine system leading to reproductive disorders at different levels, and considering male reproductive functions, most of the impacts are performed by the breakdown of estrogen- or androgen-mediated processes. A significant body of evidence based upon laboratory or wildlife animal experiments and meta-analysis of semen studies in men indicates that exposure to endocrine disrupting compounds is associated with male reproductive malfunctions, including impairment of spermatogenesis followed by reduced semen quality parameters (sperm concentration, motility, and morphology). Alkylphenols, bisphenol, and phthalates are substantial components of many products with which people come into contact daily. This brief review will emphasize on the possible effects of alkylphenols, bisphenol, and phthalates on the male reproductive system, and current research efforts related to these substances mainly in the context of two main processes taking place in testicular tissues—steroidogenesis and spermatogenesis.

Keywords: male, reproduction, steroidogenesis, spermatogenesis, alkylphenols, bisphenols, phthalates

1. Introduction

Over the last years, many epidemiological studies have been observing worrisome trends in the incidence of human infertility rates. Increasing prevalence of congenital

abnorm-alities such as hypospadias and cryptorchidism has also been confirmed by numerous reports. Male fertility generally relies on the quantity and quality of spermatozoa, sufficient activity of Leydig cells, and a proper hormonal balance. Infertility is a widespread problem defined as the inability to conceive after one year of unprotected intercourse. In many cases, there are no obvious signs of infertility. Substantial part of the problem is the disruption of essential cellular processes responsible for normal reproductive functions [1, 2]. Given the short time, genetic changes cannot explain such alterations. We may assume that they only reflect on persistently adverse changes in the environment or in lifestyle. However, it cannot be ignored that some individuals may be more susceptible or resistant to these adverse effects than others, indicating that genetic factors do play key roles [3]. Enormous production and release of industrial chemicals into the environment has led the scientific community to hypothesize that current pollutants may irrefutably disrupt health conditions, leading to extensive damages to physiological functions. In fact, a huge number of chemicals have been found to interact with the endocrine system of different animals in laboratory studies and there is an increasing number of reports on the endocrine disruption in wildlife [4]. Endocrine disruptors (EDs) are an extremely heterogeneous group of ubiquitous synthetic substances, environmental pollutants, and commercial products. They are able to alter functions of the endocrine system, inhibit critical cellular processes, increase the risk of hormone-dependent malignancies, and may result in a wide array of adverse health effects. The term endocrine disruption has been adopted by the vernacular of scientists, toxicologists, and appears here to stay [5, 6]. There are many varied sources of EDs. Typical human exposure occurs with respect to the environmental contamination of the food chain, contact with contaminated household dust, and during the use of personal care products. Other EDs are used as industrial lubricants, solvents and high amounts of EDs were found in household products, pesticides, herbicides, detergents, beverage and food storage containers, metal cans, epoxy resins, etc. Many textiles contain contaminants, such as flame-retardants, including tetrabromobisphenol A and polybrominated diphenyl ethers [7]. Although a chronic exposure to ED takes place through the skin contact or inhalation, the major source are food products. Some experimental studies assume that plastic packaging is the largest source of EDs in the human diet. Repeated exposure of food – contact materials to UV light, acid or alkaline contents and heat may cause polymers to breakdown into monomers as phtha lates, which then leach into the food or beverages [8]. Other by-products such as alkylphenols, bisphenols, polychlorinated biphenyls, dioxins or phthalates are ubiquitous and there is a growing concern that living in an ED-contaminated environment may initiate adverse health effects. Detection of ED residues in human serum, seminal plasma, and follicular fluid has raised concerns that environmental exposure to EDs may be affecting human fertility [9]. Nowadays, some of EDs have been banned or otherwise removed from the industrial processes years ago. On the other hand, these are persistent in the environment throughout many years. A wide range of industrial PCB compounds may be still found in pronounce quantities in the environment, although their manufacture was banned in 1977 [10]. Indeed, humans and wildlife are continually exposed to copious potentially hazardous substances that are released into the environment at an alarming rate.

2. Male reproductive system as a major target of EDs

In this context, possible adverse effects of EDs have been taken into focus, both regarding the effects of EDs on the male reproductive system and with respect to its differential susceptibility towards these compounds. Although there has been an effort to list and rank all possible EDs, the number of evaluated chemicals remains limited. Such information and associated concerns regarding the ubiquitous presences of EDs in the environment have sparked discussions regarding the need for strategies to assess and regulate chemicals with endocrine disrupting properties in order to protect human and wildlife health. During the last years, some epidemiological studies have been comparing an increase in the incidence of male reproductive disorders in many countries. The results showed that the global average sperm count dropped by half and that the sperm motility/viability significantly decreased. In addition, many types of reproductive tract abnormalities were observed in several countries [12]. Several experimental studies have found associations between poor semen quality and increased levels of EDs in the environment [13, 14]. EDs may disrupt not only spermatogenesis, by interfering with germ cells and sperm-supporting cells, but may also affect steroidogenesis occurring in Leydig cells. Many researchers have focused on the potential sources of EDs and their pathological consequences on reproductive health as well as ethnologies in the environment.

2.1. Alkylphenols and their impact on steroidogenesis and spermatogenesis

As we mentioned before, environmental exposure to EDs may adversely affect human and wildlife reproductive functions. Many environmental contaminants including alkylphenols are widely used in the preparation of agrochemicals, industrial and household detergents, paints, and plastics [15]. Alkylphenol ethoxylates, a class of nonionic surfactants, are microbially degraded into alkylphenol diethoxylates and alkylphenol monoethoxylates. These are subsequently degraded into alkylphenols (4-octylphenol; 4-nonylphenol) and along with other subproducts, are known to persist in the environment for a long time [16]. Alkylphenols are endocrine-disrupting agents with native estrogen-like structure and show estrogenic activity. This activity is mediated through the binding of these environmental estrogens to estrogen receptors. Previous studies suggested that estrogenic activity of alkylphenols is linked to a tertiary branched α-carbon and the length of the side chain at that position. Therefore, many experimental studies have investigated estrogen receptor binding and subsequent pathological changes in male reproductive functions. The mechanism also involves interaction with steroidogenic enzymes, transport proteins, and cell signaling processes. However, little is known about the direct effect of alkylphenols on the steroidogenic enzymes (3β-HSD and 17β-HSD) and gene expression [17].

2.1.1. Nonylphenol

One of the most commonly used alkylphenol is nonylphenol (NP). Due to its wide usage, a large amount of nonylphenol is widespread in the environment, especially into water sources. Vazquez-Duhalt et al. [18] have been convinced that the concentration of 0.1 μg/L evokes a

public health risk. Based on this knowledge, several studies have investigated the potential impact of NP on male reproductive functions.

Ying et al. [19] demonstrated that nonylphenol's isomers had different effects on the release of steroid hormones in rat Leydig cells. However, all experimental doses had an unfavorable impact. Specifically, the inhibitory effect of p363-NP isomer was found to be as much as 1.26 times higher than the others. The results imply that the effects of different nonylphenol isomers on the testosterone production do not appear to be completely mediated through the estrogen receptor α or β. For the steroidogenesis, ensured by Leydig cells is an essential conversion of cholesterol into various steroid classes, where 3β-HSD, 17β-HSD, and StAR are responsible for the rate-limiting step. PCR analysis showed that the decrease of testosterone production may be explained by the drastic inhibition of StAR and 3β-HSD gene expression. In a recent study, Wu et al. [20] demonstrated that NP increased testosterone production in rat Leydig cells. The concentration of 127.5 μM NP stimulated the steroidogenic process by elevating the activity of $P450_{scc}$ and stimulating protein expression of StAR. During the same experiment, trypan blue assay was performed. The authors observed the cytotoxic effect of the highest doses of NP (425 μM). Lower experimental doses (42.5–127.5 μM) used in this study had no cytotoxicity until 4 h cultivation. In a previous study, Jambor et al. [21] evaluated the potential impact of NP on the biosynthesis of steroid hormones, cell viability, and ROS production. The production of steroids, specifically dehydroepiandrosterone, androstenedione, and testosterone was reduced following exposure to NP after 44 h of *in vitro* cultivation. Furthermore, the treatment to NP caused a significant intracellular accumulation of ROS in mice Leydig cells. Majdic et al. [22] reported that NP has an inhibitory effect on P450c17, which is essential in the testosterone synthesis. Several studies demonstrated that NP treatment increased apoptosis of testicular cells, including germ and Sertoli cells [23, 24]. According to Han et al. [25], the highest experimental concentration of NP (250 mg/kg/day) may significantly increase the number of apoptotic cells following *in vivo* exposure of male rats. Recent evidence also confirms that NP exposure rapidly increases the apoptosis of Sertoli cells in a dose-dependent manner *in vitro.* The results of flow cytometric analysis indicate that the proportion of apoptotic cells was significantly increased at 20 and 30 μM of NP [26]. *Gap junctional intercellular communications (GJIC)* were shown to be present between adjacent TM4 Sertoli cells [27]. An important role of *GJIC* is to regulate cell growth and differentiation and it is also critical for coordinating steroidogenesis and spermatogenesis. *Gap junctions* are pores composed of connexins (Cx). Several reports indicate that Cx43 is essential for normal testicular functions [28]. Aravindakshan and Cyr [29] showed that the exposure to NP dramatically inhibited *GJIC.* A significant reduction was observed at 10 μM of NP (almost 80%). The effect of NP on the Cx43 expression was dose- and time-dependent. Time-response analyses in which cells were exposed to 10 μM NP indicated that there was a decrease in Cx43 after 24 h. Exposure of TM4 cell line to NP resulted not only in a decrease in the CX43 levels but also a progressive effect on the level of renewal of the connexins, or on their synthesis, or both was confirmed. In addition, epidemiological studies have reported numerous other adverse effects of nonylphenol on the reproductive system, including reduced testis weights, spermatozoa abnormalities, and a decreased sperm production [30, 31].

NP is considered to be an endocrine disrupting compound which could be involved in declines of both quantity and quality of spermatozoa in adult men [32, 33]. A lot of experiments show an

in vitro NP inhibition of sperm motility and viability [34, 35], while *in vivo* studies confirm spermatotoxicity, spermatogenesis failure, reduced sperm counts and motility, seminiferous tubule degeneration including decreased diameters of seminiferous tubules, lumen and epithelial thickness leading to testicular atrophy [36], and abnormalities in sperm morphology following NP exposure [37, 38]. Huang et al. [39] observed detrimental activity of NP on prepubertal Sprague–Dawley male rats under *in vivo* and also under *in vitro* condition, when the animals were treated with 25–100 mg/kg/day for 30 consecutive days by an intraperitoneal injection of NP. NP exposure induced the sperm toxicity, resulting in cell damage and reproductive disorders and initiated oxidative stress, disturbed the PI3K/AKT/mTOR pathway, induced apoptosis and autophagy, and caused developing reproductive damage *in vivo* and *in vitro.* Uguz et al. [40] designed an *in vitro* study with epididymal rat sperm, observed NP-induced (250–500 μg/mL; 1–4 h exposure) impairment of sperm motility, and a decreased mitochondrial membrane potential which probably plays a key role in the malfunction of spermatozoa. Another *in vitro* experiment with ram and boar spermatozoa provides similar results, when exposure of both sperm types to 250 and 500 μg/mL was harmful to progressive motility, percentages of ram and boar sperm with high mitochondrial membrane potential decreased significantly following exposure to concentrations ≥250 μg/mL. Unlike chromatin integrity, which did not seem to be changed after NP administration, there was a dose-dependent activity of NP on the acrosomal integrity of both species at as low as 1 μg/mL for boar sperm and 10 μg/mL for ram sperm 35]. Lukac et al. [41] used a cell model of bovine spermatozoa to determine the effect of NP (1, 10, 100, and 200 μg/mL) on the motility and viability of spermatozoa during several time periods. The results showed a decreased spermatozoa motility and viability in all experimental samples following the addition of NP after 6 h of exposure. The effects of NP were also evaluated in frozen-thawed bull spermatozoa, when the cells were exposed to concentrations of NP at doses 1, 10, 100, 250, and 500 μg/mL. Sperm parameters were assessed at cultivation times of 0, 1, 2, 3 and 4 h and both motility and mitochondrial membrane potential of sperm cells decreased at concentrations ≥250 μg/mL. In addition, the acrosome reaction was induced even at the lowest concentration of NP [42]. Ergun et al. [43] showed that 100 μg/mL NP induced apoptosis by causing DNA breaks in bovine spermatozoa. Vitellogenesis is a sensitive biomarker of xenoestrogen exposure *in vitro* and *in vivo* and vitellogenin is considered to be a key in indicating the presence of xenoestrogens in the environment, as these chemicals have been found to induce the production of this yolk protein in males leading to the impairment of male sexual organ development and disruption of male fertility [44]. NP is estrogenic also to aquatic organisms and experiments related to fish and amphibians have shown that NP is able to induce vitellogenin in the gonads, violating the development of the embryo and larvae, and results in a strikingly skewed sex ratio in aquatic organism via modulating the effects of sex hormones [45]. NP has been connected with the development of different types of sexual dysgenesis in the laboratory and wild fish [46, 47]. Feng et al. [45] investigated the *in vivo* and *in vitro* effects of NP on the motility parameters and fertilizing ability of *Bufo raddei* during amplexus and fertilization period. Based on the results, ROS induced via NP and NP itself was associated with the decrease of the fertilization rate, when *in vitro* assays showed a direct exposure of sperm to NP with a significant impairment of motility, integrity, and increased ROS levels. Negative correlations were observed between motility of spermatozoa and corresponding ROS concentrations, but the level of NP that admittedly affected spermatozoa in this study (200 μg/L) was about 2.5 times of the highest NP level found in natural aquatic environments (0.065–83 μg/L).

2.1.2. Octylphenol

Numerous reproductive issues such as an increased incidence of testicular cancer, lower spermatozoa activity, and disruption of the steroidogenic process have been related to exposure to alkylphenols. One of the greatly widespread alkylphenols is octylphenol (OP). It is used as a component of emulsifiers, detergents, paints and many other synthetic products. Nowadays, OP is mainly present in sediments, surface waters, and even drinking water. Due to its relative stability and hydrophobic properties, OP is bioaccumulated in various tissues and poses a large health risk for the organism [48–50]. It has been reported that certain doses of OP may negatively affect cellular processes such as steroidogenesis and spermatogenesis essential for a normal development and functions of the male sex. However, there are still limited information about the mechanism, through which OP affects biosynthesis of steroid hormones. Some experimental studies have hypothesized that OP may directly modulate the activity of steroidogenic enzymes. Murono et al. [51] documented that exposure to 2000 nM OP affected the testosterone production in rat Leydig cells. In response to the experimental dose, testosterone levels significantly increased after 2, 4, and 8 h cultivation, when compared with the control. Exposure to shorter periods (0.5 and 1 h) were also examined; however, the weak increase at these times was not statistically significant. The increase in hormone production was not associated with changes in cAMP levels and it did not involve the estrogenic activity (binding) to the estrogen receptors. Furthermore, higher testosterone secretion was not the consequence of inhibiting 5α-reductase activity in Leydig cells. Although these results did not describe signaling pathways, it is necessary to identify the potential mechanisms through which intermediate stages of steroidogenesis may be affected. Some epidemiological studies imply that the inhibiting effects of OP on the steroidogenesis are mediated through the potential of OP to generate ROS and inhibit testosterone secretion. Cytochrome $P450_{scc}$ and P450c17 are essential in converting cholesterol to testosterone in Leydig cells. During the steroidogenic process, ROS are produced by electron leakage outside the electron transfer chains and these radicals may cause lipid peroxidation to inactivate P450 enzymes [52]. Several reports evaluated the potential effects of OP on the steroid hormone synthesis [51, 53]. According to Kotula-Balak et al. [54], independently of the incubation time, high doses of OP significantly inhibited the progesterone production in mice MA-10 cells. Inhibition in progesterone levels was significantly higher in the experimental groups cultivated with OP for 3 h than in cells incubated for 12 h. This can be related to the restoration of Leydig cell steroidogenic function within the time of culture. Decreased progesterone production could be mediated through the inhibition of 3β-HSD since it was reported that estradiol inhibits the progesterone level via the disruption of the 3β-HSD function. Murono et al. [55] investigated the impact of OP on the biosynthesis of steroid hormones in rat Leydig cells *in vitro.* The authors reported a biphasic effect, where the lower experimental doses (1 and 10 nM) increased the testosterone production by approximately 10–70% above the control group, whereas higher concentrations (100 and 200 nM) decreased the testosterone level progressively. The inhibitory effect of OP was also evaluated by Nikula et al. [53]. Inhibition of testosterone secretion by 4-*t*-octylphenol in cultured mice Leydig cells has been suggested to occur at the 17β-HSD step. It has also been reported that the gestational exposure of pregnant rats to OP decreases the amount and activity of the P450c17 steroidogenic enzyme in male

offspring and SF-1 (steroidogenic factor) involved in the gonad development and expression of steroidogenic enzymes [56]. Based on the evidence gathered from the literature, it seems possible that inhibited functions of a male reproductive system might be mediated not only through the disruption of steroidogenic enzymes but also via the direct toxic effect of OP, resulting in a lower cell viability and apoptosis. Qian et al. [50] evaluated the cytotoxic effect of OP (30–60 μM) in rat Sertoli cells after a 24 h exposure. Cell viability was significantly reduced at 40, 50, and 60 μM OP. Additionally, the highest experimental dose decreased the Sertoli cell viability in a time-dependent manner with a significant decrease following a 12 h cultivation. The cytotoxic effect of OP is strongly dependent on the experimental doses. Jambor et al. [57] evaluated the *in vitro* effect of 4-OP on mice Leydig cell viability. The results showed a greater viability at 1, 2.5 and 5 μg/mL of 4-OP following 44 h of cultivation. Kotula-Balak et al. [54] illustrated marked differences in the Leydig cell morphology after OP treatment. Mice Leydig cells exposed to experimental doses of OP (10^{-4} to 10^{-6} M) grew in a small group and 60% of cells showed nucleus shrinkage, cytoplasm vacuolization and membrane floating, while the control cells were formed as a monolayer with an epithelioid shape and abundant cytoplasm. Conversely, lower concentrations of OP did not markedly affect the morphological structure of exposed cells. In the recent years, a link was confirmed linking OP and the increased incidence of male reproductive dysfunction. The ability of OP to affect spermatogenesis has been the subject of much investigation. Spermatozoa abnormalities, a decreased sperm motility and lower spermatozoa viability are current problems mediated through OP exposition. Of the alkylphenols examined for their ability to act as an estrogen compound, octylphenol has been observed to be vastly effective, showing approx. one thousandth of the estrogenicity when compared to a strong estrogen 17β-estradiol [58]. Exposure to OP extremely inhibits the testicular function as exhibited by a reduced size of the testes, reduced androgen concentrations, and a negatively affected spermatogenesis. Similarities in the activity of OP and those noticed after the addition of 17β-estradiol indicate that OP exerted its effect to impair the testes in an estrogenic-like manner on the hypothalamus and/or anterior pituitary gland to arrest the gonadotropin secretion [59]. OP is also believed to support the reduction in sperm quantity in men resulting in male infertility and it has been defined as a potential reason of reproductive tumorigenesis [60]. It has also been reported that OP shows a toxic potency on cultured prespermatogonia and Sertoli cells [61]. In addition, it is proved that OP is able to generate ROS which are cytotoxic compounds resulting in oxidative damage associated with damage to biomolecules such as membrane lipids and DNA in sperm cells [62]. Adverse effects of OP on male reproductive functions in pubertal rats were evaluated by Herath et al. [63], when 50-day-old rats in the OP group received daily injections of the xenoestrogen at a concentration of 3 mg/kg. After 5 weeks of exposure, the epididymal sperm motility and sperm head counts were determined with reduced sperm counts resulting from a decreased plasma testosterone, but without significant effects of OP on the sperm motility parameters. The potential *in vivo* genotoxic activity of OP in adult male Wistar albino rats was studied by Ulutas et al. [64], when animals received OP oral doses of 125 and 250 mg/kg for 4 weeks. Possible genotoxic effects of OP were evaluated as comet parameters including tail length and tail moment with significant differences in both tested parameters only in the case of animals treated with the highest dose of OP. Peng et al. [65] also provide results of a combined genetic toxicity of OP along with NP in male mice following a peritoneal injection of nonylphenol-octylphenol (50,

100, and 200 mg/kg). The effect on the DNA damage in the testicular cells and sperm deformation rate after the exposure were measured using the comet assay and sperm morphologic test. Within the examined doses of 100 and 200 mg/kg, the quantity of the comet cells in the testes cells was increased. The DNA migration length was also significantly increased as OP-NP elevated and the rate of sperm deformation was higher following exposure to the tested chemicals too [66]. OP was also examined in the context of the biochemical composition of the seminal fluid and production of the viviparous eelpout (*Zoarces viviparus*) and the investigation was carried out at the time of spawning. After 10 days of exposure to OP, a decline in the gonadosomatic index was observed following the milt volume with a spermatocrit increase. The histological investigation manifested that OP impaired the lobular composition, including the Sertoli cells. In most of the OP-exposed individuals, trapped sperm cells in parts of the seminiferous lobules and the sperm ducts were observed. OP also affected the biochemical composition of the seminal fluid with elevated concentrations of the tested parameters such as magnesium, calcium, and total protein, meanwhile values of free amino acids were decreased in the exposed fish [67]. Movement characteristics are always the most important parameters in the evaluation of semen quality. Spermatozoa motility represents the primary characteristic in the assessment of male fertility and it is a fundamental premise for a successful fertilization. Motility parameters are closely linked to the mitochondrial activity of spermatozoa as these organelles play a key role in the energy provision by production of ATP [68]. Lukacova et al. [69] confirmed a decline of bovine sperm motility, progressive motility, and mitochondrial activity after exposure to 1–200 μg/mL OP during several time periods (0, 2, 4, and 6 h). Interestingly, the values of intracellular superoxide production revealed a slight decline of the superoxide concentration at the dose of 1 μg/mL when compared to the control group and conversely, doses 10, 100, and 200 μg/mL of OP increased the concentrations of superoxide in bovine sperm. Thus, in general, the effects of alkylphenols on the testicular function are not clearly defined yet and their effect may be attributed to the concentration, estrogen-mimicking activity, and time of exposure.

2.2. Bisphenols and their impact on steroidogenesis and spermatogenesis

Exposure to xenoestrogens such as bisphenols has been shown to cause adverse effects on male reproductive system in humans and numerous animal species. As typical endocrine disruptors, bisphenols are one of the most studied xenoestrogens in the field of male reproductive system. A survey of the Pubmed database provides more than 10,000 articles on the topic, including epidemiological as well as experimental studies. The overwhelming majority of bisphenols is used as stable components of household products, epoxy resins, inner surface of food metallic cans, dental sealants, and for myriad additional synthetic products. Many of us are mostly confronted by bisphenols through gastrointestinal exposure (food packaging) and dermal exposure (paper money and paper products). It is well known that increased concentration of bisphenols was detected in urine, milk or sweat and over 90% of human population is daily exposit to bisphenol A. Subsequent bioaccumulation and kinetic properties may adversely affect the overall health [70, 71]. Nowadays, bisphenols have been associated with a variety of human diseases, specifically kidney and cardiovascular diseases, obesity, developmental defects, and reproductive disorders. Recent studies indicate a direct link

between the incidence of male reproductive dysfunction and rising concentrations of bisphenols in the environment. A decrease in semen quality was the first reported alteration and from this moment on an informative expansion was launched on the potential consequences of bisphenol exposure [72]. Several reports demonstrate a direct effect of bisphenols on the biosynthesis of steroid hormones. Negative effects of bisphenol A (BPA) have been reported in both *in vivo* and *in vitro* studies, where the steroidogenic enzymes were recognized as primary targets. Downregulation of the expression levels of *CYP11A* and *CYP17A* has been observed primarily, resulting in the decline of testosterone synthesis [73]. The altered levels of testosterone may cause subsequent reproductive dysfunction by interfering with the feedback regulatory mechanisms. Another serious effect by which bisphenolic compounds perform their adverse impact on the male reproductive cells are disruption of the brittle balance between the antioxidant capacity of cells and prooxidants in testicular tissues, which is linked to the increased risk of oxidative stress development resulting in the arrest of spermatogenic processes, production of abnormal sperm cells, and impairment of normal existing sperm cells in the reproductive tract [11]. Oxidative reactions may lead to the decline of spermatozoa quality, as observed by the decrease of spermatozoa motility, velocity, and viability values. Moreover, bisphenol exposure could also result in the depletion of ATP metabolism and damage to the genetic material by sperm DNA fragmentation [74].

2.2.1. Bisphenol A

Lan et al. [75] evaluated the effects of BPA on two steroidogenic enzymes (*CP11A1; CYP19*) essential for the normal steroidogenic process. According to the PCR analysis, the endogenous gene expression in both was upregulated by BPA at 100–1000 nM. Another steroidogenic enzyme, *CYP17*, involved in the testosterone synthesis was also measured. The results showed that BPA did not affect *CYP17* protein expression significantly. However, the authors hypothesized that the balance of steroid hormones may be affected. This was confirmed in the next part of the study, where the testosterone production was slightly decreased at 1–100 nM BPA following a 24 h exposure. The next steroidogenic enzyme responsible for the conversion of pregnenolone to progesterone is 3β-HSD. Ye et al. [76] reported a significant inhibition of the 3β-HSD activity in rats and humans. Human 3β-HSD was more sensitive to BPA's inhibition than the rat enzyme. The authors also evaluated the effects of BPA on the testosterone production in rat Leydig cells. Experimental doses of 10 and 100 μM markedly decreased the testosterone generation. Importantly, evidence exists that exposure to BPA *in utero* may reduce the neonatal serum testosterone level [77]. In summary, although BPA directly affects the steroidogenic genes, it is clear that BPA disrupts the hormone synthesis and contributes to reproductive disorders. Because of an increased concern over the safety of BPA, European Union has banned its use in plastic bottles for infants. The viability of Leydig cells is a significant indicator for a sufficient production of steroid hormones. Lan et al. [75] illustrated a dose-dependent effect of BPA on this parameter in the MA-10 cell line. The data show a decrease in the cell viability (1–200 μM) following a 24 h cultivation *in vitro*. However, significant differences were recorded only with respect to the highest dose of BPA (200 μM). Goncalves's et al. [78] study showed a decrease in the Leydig cell viability upon the exposure to BPA. The authors found out that experimental doses above 1 μM inhibited the cell viability

following a 24 h incubation compared to the control. Nonetheless, the viability of TM3 cell line did not decrease significantly even after a 48 h exposure at concentrations below 50 μM. De Freitas et al. [79] observed a significant reduction in the viability of human Sertoli cells after the cultivation with 10 μM BPA for 48 h.

Nowadays, there are many epidemiological studies which evaluated the effect of bisphenols on the spermatozoa or spermatogenesis. Observable changes were recorded in the spermatozoa motility, spermatozoa viability, and DNA integrity. *In vivo* experiments with adult male rats indicated that the low concentration of BPA (2 μg/kg body weight) administered orally can effectively inhibit spermatogenesis via disruption of the biosynthesis of reproductive hormones resulting in the meiosis inhibition of sperm cells and induction of the Fas/FasL pathway with a subsequent apoptosis. Declining amounts of testosterone were followed by a reduction of sperm quantity [80]. Evidence showed an obvious link between increased urine levels of BPA and reduced values of the sperm concentration what can be attributed to the disturbed processes of spermatogenesis following BPA exposure. Harmful effects of BPA on the spermatogenesis observed in experimental animals are also in agreement with an epidemiological study focused on the impact of BPA on exposed human males. Reduced spermatozoa count, indicating a primary association between BPA exposure and production of sperm cells were attributed to increased values of BPA in urine when men with high urine BPA levels had more than three times lower sperm concentration and viability; however, no correlation was observed between the urine BPA concentrations and semen volume or abnormal sperm morphology compared to subjects without the presence of BPA in the urine [60]. Also, other *in vitro* studies revealed a direct effect of BPA exposure on the sperm quality. Singh et al. [81] used in his *in vitro* study chicken sperm to determine environmentally relevant concentrations of BPA (0.18, 0.37 and 0.74 mM) related to motility, fertilizing ability, live sperm percentage, and mitochondrial membrane potential after 30 min of BPA treatment. The results showed that 0.74 mM BPA is able to compromise sperm functions in the case of all analyzed parameters leading to the decline of sperm fertilizing ability. Data obtained from *in vitro* experiments by Lukacova et al. [82] refer that BPA has negative effects on bovine spermatozoa motility in different doses (1, 10, 100, and 200 μg/mL). The results showed that BPA has the ability to reduce the values of mitochondrial activity and spermatozoa motility, causing mitochondrial damage as evidenced by the increased values of intracellular superoxide. Spermatozoa motility parameters were significantly decreased in experimental groups exposed to concentrations of BPA higher than 100 μg/mL. In experimental mice, the motility analyzed following 6 h of *in vitro* treatment with 0.0001, 0.01, 1, and 100 μM BPA, the number of motile sperm cells was also reduced in the case of dose of 100 μM BPA [83]. Administration of different BPA concentrations (0.6, 4.5, and 11.0 μg/L) demonstrated an impairment of motility in fish spermatozoa too [84].

2.2.2. Bisphenol alternatives

More stringent global regulations of BPA production and the use have led to the development of alternative bisphenol compounds [85]. A few years ago, researchers have begun to deal with potential properties of 4,4′-dihydroxydiphenylsulphone (BPS) or 4,4′-dihydroxydiphenlymethane (BPF). Both are presently not regulated and are used without restriction. Additionally,

currently available toxicological data are scarce and the information about their potential impact is limited. Nowadays, studies reported the effects of BPS via genomic mechanisms using extremely high concentrations but there are still no studies evaluating the *in vivo* toxicity. Although BPS is less likely to leach from plastic packaging with heat, it does still escape the polymer in small quantities under the normal use. Chen et al. [86] showed that 40 μM BPS had a 15-fold lower genomic estrogenic activity than BPA. Only a few studies have evaluated BPS at low concentration ranges likely to be present in foods, wildlife or humans. Eladak et al. [87] used the mouse FeTA model to illustrate the effects of BPS and BPF on the testosterone synthesis. Results from the present study showed that BPF has a similar dose-response effect as BPA with a significantly decreased amount of testosterone starting from 1000 nmol/L. On the other hand, BPS had even a more potent inhibitory effect than BPA. Indeed, 100 nmol/L BPS significantly reduced the testosterone production after 3 days of treatment. Authors also compared the effects of 10,000 nmol/L BPA, BPS, and BPF on specific gene expression in mice Leydig cells. All bisphenol alternatives reduced the expression of key genes involved in steroidogenesis such as *Star, hsd3βa CYP17a1*, expect *CYP11a1*. In addition, the expression of Lhcgr (the gene encoding the LH/CG receptor) was also decreased. This is one of the few reports that suggest harmful consequences on the reproductive functions in humans and rodents. According to Ji et al. [88], BPS is able to reduce the level of testosterone as well as *CYP17*a and *17β-HSD* mRNA levels in zebrafish. It must be noted that the binding activity of BPS and BPF to estrogen receptor (α; β) is, respectively, 5-or 10-fold lower than that of BPA in the HELN cells [89].

Effect of BPS exposure on oxidative stress, generation of ROS, and impairment of DNA integrity of rat sperm cells under the *in vitro* condition and daily sperm production and sperm DNA damage under the *in vivo* condition was examined in the study of Ullah et al. [90]. Spermatozoa were cultivated along with BPS at doses of 0.5, 1, 10 and 100 μg/L and the analyses showed that the highest concentration of BPS initiated ROS generation, induced peroxidation of membrane lipids, altered superoxide dismutase concentrations, and increased the incidence of DNA fragmentation in the sperm cells. The *in vivo* part of this study revealed that adult rats exposed to concentrations of 0.5, 5, 25, and 50 μg/kg/day for 28 days demonstrated a decline in daily sperm production with rising values of DNA damage occurring in spermatozoa observed in experimental animals treated with the highest dose (50 μg/kg/day) of BPS; however, the motility parameters were not inhibited. Similarly, treatment with 50 μg/kg/d lead to the development of oxidative stress in the testes and impaired reproductive functions in rats [91]. An earlier study on zebrafish embryos focusing on the developmental exposure to BPS was performed to examine the reproduction potential and hormonal balance in adult individuals. Embryos of zebrafish were treated and bred in the presence of various doses of BPS (0, 0.1, 1, 10, and 100 g/L) for 75 days. Following that period, adult males and females were paired for next 7 days in fresh water and subsequently the impact on individual development, reproduction, plasma vitellogenin, sex steroids, and thyroid hormone rates were examined. The results showed skewed sex ratio in favor of females and decreased values of body length and weight in males exposed to 100 g/L of BPS. The gonadosomatic index showed reduced values in fish at tested concentrations ≥10 g/L of BPS. In both males and females, a significant stimulation in plasma vitellogenin level was noticed at ≥10 μg/L of BPS and also thyroxine and

triiodothyronine levels were significantly decreased at 10 and 100 μg/L of BPS in males. Sperm count was also reduced in the experimental groups exposed to 10 and 100 μg/L of BPS [92]. In other studies, cytotoxic, genotoxic [93], and mutagenic [94] effects of BPS in different cell models were documented. It is proved that the exposure to BPS can violate the cellular signaling path in the apoptotic and viability ways, which is why it is possible to expect a reaction of BPS with pro-apoptotic and signaling cascades observed also in the sex cells resulting in the affected cell cycle and apoptosis [95]. Nowadays, further research is required to elucidate the effects of bisphenols on the male and female reproductive system.

2.3. Phthalates and their impact on steroidogenesis and spermatogenesis

Numerous environmental contaminants have hormonal or anti-hormonal actions that interfere with endocrine homeostasis of individuals. As we mentioned above, the group of endocrine disruptors is very heterogeneous and phthalates, as ubiquitous chemical compounds are widely used as plasticizers in children's plastics toys, food packaging, medical tubing, certain cosmetics, shampoos, soaps, and many others household products [96]. Early experimental studies found a low level of phthalate toxicity in rodents, but nowadays, a high extent of carcinogenicity, teratogenicity or testicular atrophy has been widely confirmed. Recent studies have verified that phthalates are capable to affect many physiological mechanism and functions, especially within the reproductive system. Moreover, disorders linked to reproductive toxicity may appear in early life stages, puberty, and some of them may manifest in adulthood. The Department of Health and Human Services estimated that daily human consumption of commonly used phthalates diethylhexyl phthalate (DEHP) revolves around 5.8 mg and monoethylhexyl phthalate (MEHP) ranges from 3.26 to 4.15 in males and 2.93 to 3.51 in females. On the other hand, DEHP is metabolized by intestinal lipases to MEHP, which is glucuronized and excreted from the organism with minimum tissue accumulation [97, 98]. According to its toxicological profile, MEHP seems to be 10-fold more potent in its toxicity to Leydig and Sertoli cells in comparison to DEHP, suggesting that DEHP is the pretoxin which acts via metabolizing into MEHP [99]. Several toxicological reports suggest that DEHP and MEHP disrupt reproductive development and now it is established that these phthalates inhibit the biosynthesis of steroid hormones in Leydig cells at different developmental stages. *In utero* exposure to phthalates has been shown to reduce male fertility potential in rats. Subsequent postnatal changes preceded an inhibition in Leydig cell function, including lower levels of testosterone. Many authors suggest that phthalates exert their effect via multiple mechanism of action such as the peroxisomes proliferator-activated receptors, estrogen receptors or yet unidentified mechanism.

2.3.1. Diethylhexyl phthalate (DEHP)

Akingbemi et al. [100] investigated the ability of DEHP to affect the biosynthesis of steroid hormones in rat Leydig cells. Pubertal rats were exposed to 1, 10, 100, and 200 mg/kg/day DEHP for 2 weeks. The highest experimental dose (200 mg/kg/day) DEHP caused a 77% decrease in the activity of 17β-HSD and reduced the testosterone production to 50% of the control. Paradoxically, prolonged time of cultivation to 28 days resulted in significant increases in the testosterone secretion capacity and in serum LH levels. A few years later, Akingbemi et al. [101] evaluated the

potential effects of DEHP on isolated rat Leydig cells *in vitro*. When compared to the control, mRNA levels of PCNA and cyclin D3 were expressed at statistically higher levels of proliferation following treatment. Additionally, estradiol levels were elevated by as much as 50% above the control group and aromatase gene expression was also higher in DEHP exposed cells. Several recent investigations have shown that DEHP disrupts the reproductive system of the male rat in an antiandrogenic manner. In the present study, Parks et al. [102] explored the antiandrogenic action of DEHP and MEHP as well as alterations in the testosterone production. Maternal exposure at 750 mg/kg/day caused a significant reduction in the testosterone levels. In addition, Liu et al. [103] performed gene expression profiling following *in utero* exposure to phthalates and observed a decline in levels of steroidogenic enzymes (*CYP11a1; CYP17a1*) and lipid transport (StAR). However, the exact mechanism of action is not fully clear. The negative impact of DEHP on the male reproductive system has been related to their monoester metabolite MEHP. It has been shown that this endocrinologically active phthalate may negatively affect the testes and more specifically suppress Leydig cells functions [104].

2.3.2. Monoethylhexyl phthalate (MEHP)

Dees et al. [105] reported that MEHP inhibits androgen production in MA-10 Leydig cells. By using different MEHP concentrations over a longer time interval (24 and 2 h), the authors have demonstrated that even at low experimental doses MEHP inhibits the steroid production (a 50% inhibition was observed at 10 μM), induces morphological changes such as mitochondrial swelling and vesiculation of the Golgi apparatus. Conversely, at 100 and 300 μM doses, this inhibition was not seen. Thus, it is possible that the absence of any effect may be mediated through an unidentified mechanism, distinct to the mechanisms responsible for the inhibition of steroid production. In the next *in vitro* study, Jones et al. [106] exposed the primary culture of Leydig cells to MEHP (1 mM) for 2 h. A moderate decrease in testosterone production was shown which correlated with the changes in the cell ultrastructure. Treatment with MEHP confirmed mitochondrial swelling with the loss of matrix granules, reduction in the number of Golgi apparatus and dilatation of the smooth endoplasmic reticulum. Svechnikov et al. [107] also confirmed the inhibitory effect on steroidogenesis in rat Leydig cells. The result showed significantly lower testosterone levels (57–62% inhibition) in exposed cells (250 μM MEHP) after 24 h incubation when compared with the control group. In order to determine whether the inhibition of testosterone secretion was due to the disruption of StAR, the authors decided to monitor the expression of this protein by Western blotting. A marked decrease in StAR expression was observed after 24 h incubation. In addition, the activity of 5α-reductase, an enzyme synthesizing the potent androgen dihydrotestosterone, was dramatically inhibited in immature Leydig cells. The dysfunction of Leydig cells is postulated to have a direct association with androgen-dependent parameters of sexual development. Nevertheless, it is necessary to determine whether the effects of chronic DEPH or MEHP exposure are reversed or mitigated when exposure is terminated.

Numerous studies have evaluated the testicular toxicity of phthalates in different experimental models and showed that spermatozoa and spermatogenesis were one of the main targets of their actions. Kasahara et al. [96] indicate associations between DEHP administration and increased production of ROS and selectively decreased GSH and ascorbic acid in the testis with a consequent induction of rat sperm cell apoptosis leading to testicular atrophy after *in vivo* DEHP exposition. More specifically, the results provided by Li et al. [108] when male rats were fed

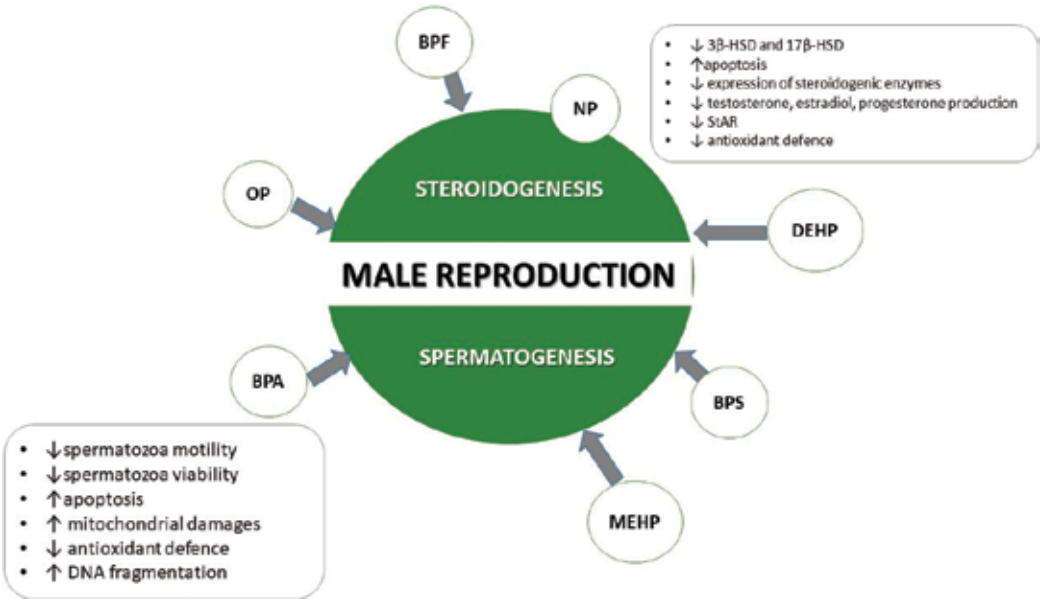

Figure 1. The effects of selected EDs on the male reproductive system.

DEHP for 2 weeks. The result was that the spermatogenesis became disrupted with decreased spermatocytes and spermatids counts and in addition, DEHP (20, 100, 500, to 1000 mg/kg) appeared to inhibit DNA replication. This resulted in the induction of the mitochondrial apoptotic pathways and overgeneration of ROS. Also, elevated activity of superoxide dismutase, reduced activity of glutathione peroxidase, and increased values of malondialdehyde after exposure to 500 mg/kg/day of dibutyl phthalate in the epididymis support the importance of oxidative stress as a major mechanism of phthalate action [109]. Likewise, the response to oxidative stress indicates an increased expression of mitochondrial peroxiredoxin and cyclooxygenase-2 in germ cells after phthalate treatment [110]. Apoptosis of germ cells has also been proposed as a potential effect of phthalates on male reproduction based on the results reporting an increased membrane localization of Fas and apoptic cells [111, 112]. One essential trace element necessary in spermatogenesis is zinc and even a slight deficiency of zinc has been observed to arrest spermatogenesis in both mice and humans [113]. Earlier studies examined phthalate-induced modifications in metabolism of zinc after treatment with high doses of phthalates with reduced testicular zinc concentrations [114], a decline of zinc half-life in the testes [115] and increased excretion of zinc in urine after phthalate exposure [114, 116]. The next schematic figure **(Figure 1)** summarizes final findings.

3. Future directions and recommendations

Probably, research is just at the very start of a long journey to refine understanding of the principal mechanisms of toxicity related to endocrine disruptive compounds and the range of influence of these hormonally active substances to the human and environmental health in the

context of male reproduction. Society will definitely continue to use these materials because of their undeniable benefits and primary we have to aim future investigation on testing and development of chemicals to maintain healthier, safe, and more sustainable world for next generations and on evolve suitable strategies of remediation of EDs. Progress in the experimental area of endocrine disruptors effects provides rich lessons that can be usable in other fields of science, as well as in the future missions in toxicology and environmental health.

This still controversial and live topic has already improved research of toxicology and risk assessment and has moved it into certain radically different trends. Further improvement in this field including reproductive biology rests in modern technology, such as toxicogenomics, which can study precursor changes on the level of cells and biological molecules and thus offer understanding of dose and time-dependent responses in more detail. Moreover, the increased usage of human, rather than animal, cell models keep a promise for intensify issues of human relevance. However, reality is that new questions are asked while previous issues associated with impact of EDs on male reproductive organs and behavior persist. The most important fields of investigation for better understanding of how EDs affect functions of tissues involved in male reproductive physiology are associated especially with questions such as why are some tissues, time periods, and even organisms more resistant to EDs exposure; how EDs effect in model organisms and cells translates to human exposure to EDs. There is also need for more studies with aim on syndromes and EDs contribution to development of multiple symptoms at once. The summary of some EDs affecting male reproductive system is presented in **Table 1**. There is also necessity to interpret specific cell culture responses in the context of whole-organism physiology, ideally that of humans. It is well known that endocrine system

Chemicals	Cellular effects	Source/applications	Study
Aldrin	Competitive binding to androgen receptors; ↓weight of testes; ↓ 3β-HSD and 17β-HSD; ↓spermatozoa MOT;	Insecticide, groundwater	Lemaire et al. [117] Chatterjee et al. [118] Das Neves et al. [119]
Alachlor	Competitive binding to estrogen and progesterone receptors; no effects on testosterone production; ↓ spermatozoa MOT and viability;	Herbicide	Mikamo et al. [120] Gizard et al. [121]
Bisphenols	Estrogenic and anti-androgenic affinity; ↓ 3β-HSD and 17β-HSD; ↑apoptosis; ↓ sperm MOT, viability and concentration;	Plasticizers, epoxy resins, dental sealants,	Eladak et al. [87] Lukacova et al. [82] Akingbemi et al. [122] Ahmed [123]
DDT and metabolites	Competitive binding to androgen receptors, activation of androgen-sensitive cells proliferation; ↓ expression of steroidogenic enzymes; ↓ testosterone, estradiol, progesterone production;	Pesticides, insecticide	Tapiero et al. [124] Tesier and Matsumura [125] Castellanos et al. [126]

Chemicals	Cellular effects	Source/applications	Study
Mono/Di-(2-ethylhexyl) phthalate	↓17β-HSD; ↓ StAR expression, ↑ mitochondrial damages; ↑ ROS; ↓ antioxidant defense; ↑spermatozoa apoptosis;	Plasticizers, cosmetics, food packaging	Akingbemi et al. [100, 101] Svechnikov et al. [107] Dees et al. [105]
Alkylphenols	↓3β-HSD, 17β-HSD, StAR; ↑ ROS production; ↓ cell viability; ↑ apoptosis; ↓ spermatozoa MOT and viability; ↑ DNA fragmentation,	Cosmetics, pesticides, paints, food packaging's,	Jambor et al. [21, 57] Lukacova et al. [69, 82] Diemer et al. [127] Haavisto et al. [128]

3β-HSD, 3beta-hydroxysteroid dehydrogenase; 17β-HSD, 17beta-hydroxysteroid dehydrogenase; MOT, motility; StAR, steroidogenic acute regulatory protein; and ROS, reactive oxygen species.

Table 1. Summary of some EDs affecting male reproduction.

mediates reactions on distant tissues and cells. Therefore, research that focuses only on isolated components of endocrine system or target tissues may provide incomplete information. Essential principles of toxicokinetics should be part of key studies related to impact of EDs on specific structures of organisms.

4. Conclusion

In recent years, a growing incidence of EDs has led scientific community to show how these substances may affect the male reproductive system. The *in vitro* evaluation of steroidogenesis and spermatogenesis are necessary for the screening potential of reproductive toxicants such as alkylphenols, bisphenols, phthalates, and many others. The mechanism of their negative effect is by diverse but one important endpoint is reduced processes, essential for normal reproductive functions. This review has demonstrated that certain groups of EDs may directly or indirectly interfere with the biosynthesis of steroid hormones and spermatogenesis via different mechanisms of action. Dysfunction of these processes may cause an incomplete masculinization, suppressed libido, reduced steroidogenic capacity, develop various malformations in spermatozoa and subsequently totally inhibit the reproductive potential of humans and animals. It must be noted that further studies are required to understand the effects of EDs on the male reproductive functions and their contributions to male sub- or infertility.

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Conflict of interest

The authors declare no conflicts of interest.

Abbreviations

AR	androgen receptor
AhR	aryl hydrocarbon receptor
cAMP	cyclic adenosine monophosphate
ER	estrogen receptor
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
ROS	reactive oxygen species

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Chapter 8

Rapid, High-Throughput Detection of Endocrine Disrupting Chemicals Using Autobioluminescent Cellular Bioreporters

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Additional information is available at the end of the chapter

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Abstract

Overexposure to endocrine disruptor chemicals (EDCs) can result in serious health problems, yet they are commonly found in everyday items such as pesticides, personal care products, nutritional supplements, and plastics. The U.S. Environmental Protection Agency, along with other such agencies from around the world, have therefore mandated that new approaches be designed to screen these products for the presence of EDCs. However, despite the presence of several types of extant EDC detection assays, there still exists a backlog approaching 87,000 chemicals currently awaiting screening. Autobioluminescent detection systems, which utilize cellular bioreporters capable of autonomously modulating bioluminescent signals without the need for external stimulation or investigator interaction, provide an attractive means for addressing this backlog because of their reduced performance costs and increased throughput relative to alternative assay systems. This chapter reviews the variety of existing EDC detection assays and evaluates the performance of a representative autobioluminescent estrogen-responsive EDC bioreporter to provide an overview of how autobioluminescence can be used to improve EDC detection using *in vitro* assay systems.

Keywords: bioreporter, autobioluminescence, high-throughput analysis, endocrine disruptor, estrogen, luciferase

1. Introduction

The human endocrine system is an interconnected, finely tuned network of glands that produce hormones responsible for health and well-being from the time of conception until death. Chemicals classified as endocrine disruptors (EDCs) interfere with the production, release, transport, and/or action of these hormones and cause imbalances that are suggested to result in significant negative health impacts such as infertility, premature puberty, obesity, diabetes, heart disease, and breast, prostate, testicular, thyroid, endometrial, and ovarian cancers [1]. These chemicals, which are present in a variety of sources including pesticides, cosmetics, and plasticizers, number in the tens of thousands (**Figure 1**) [2].

The potential adverse effects of EDCs on human, wildlife, and ecosystem health have received significant worldwide attention from the scientific community, regulatory agencies, and the general public. Unfortunately, the uncertainties inherent to understanding the true health consequences of EDC exposure have fostered significant controversy, and the lay person is besieged with an extensive collection of 'facts' when attempting to grasp the fundamental content of the EDC problem. One only needs to Google bisphenol-A (BPA) to appreciate the informational complexity surrounding a chemical suspected of being an endocrine disruptor. Capitalizing on the difficulties posed by this situation, a multitude of companies have formed to evaluate how the compounds that make up everyday items such as pesticides, personal care products, nutritional supplements, and plastics can imbalance the delicate regulation of normal endocrine function in humans and wildlife.

There are currently over 500 contract testing service companies in the U.S. alone that are dedicated to performing assays for the chemical, pesticide, and personal care products industries, and this industry is expected to continue growing year-over-year at an annual rate of 13.5% [3].

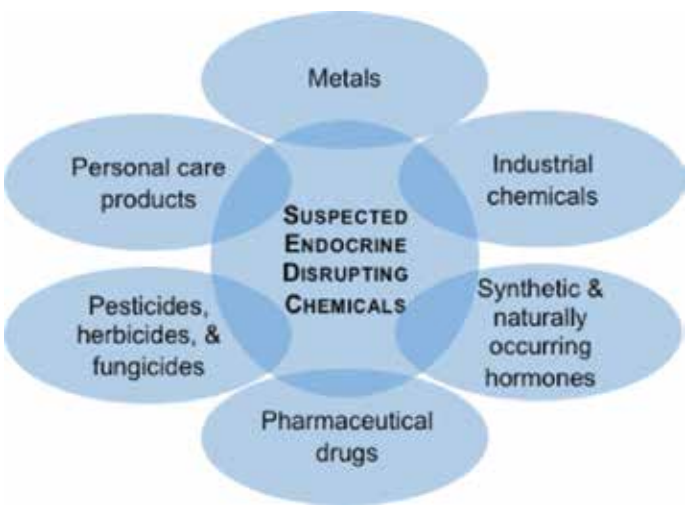

Figure 1. Tens of thousands of chemicals are suspected of having the potential to interfere with the endocrine system, resulting in adverse health effects in people and wildlife.

To improve throughput and decrease costs, these companies have adapted a two-tiered screening format, with Tier 1 consisting of *in vitro* assays aimed at identifying those chemicals that have the potential to interact with the endocrine system, and Tier 2 re-screening those compounds that test positive using *in vivo* assays to define their endocrine-related effects and obtain dosage-relevant information. Unfortunately, despite their societal importance, these tests remain biologically, logistically, and economically challenging. Tier 1 testing of chemicals for potential EDC activity is estimated to cost from $100,000 to $250,000 per chemical, with Tier 2 testing requiring upwards of 1,200 experimental animals and costing $1.2–$2.5 million per chemical [4, 5]. The majority of these costs will be borne by the chemical manufacturing industry, which then trickles down as increased prices at the consumer level. Furthermore, many of the common Tier 1 assay formats employed by these companies use non-human cell lines that can obscure bioavailability data [6, 7], require the use of radioactive materials that necessitate dedicated use areas and specially trained personnel [6–8], rely on expensive analytical equipment [8, 9], or do not meet the U.S. Environmental Protection Agency's (EPA) full testing requirements [3].

Realizing the deficiencies of these screening programs, and receiving considerable pressure from the public to reduce the use of animals for EDC testing, the U.S. EPA, with stakeholder input from the NIH National Institute of Environmental Health Sciences (NIEHS), has established the Endocrine Disruptor Screening Program for the twenty-first century (EDSP21) [10]. The goal of EDSP21 is to replace the current battery of Tier 1 tests with less expensive and faster high-throughput assays that can reduce the number of compounds that unnecessarily move forward to Tier 2 testing. This focus on improving the characterization of chemicals during Tier 1 screening is paramount to controlling costs, as mischaracterizations (i.e., false positives) during the Tier 1 stage magnify the costs of downstream Tier 2 screening, with a chemical's progression through multiple phases of Tier 2 screening only to be classified as negative for EDC activity representing a very poor return on investment. With the current chemical backlog approaching 87,000 chemicals [11], and considering the conventional scientifically acceptable false positive error rate of 5%, under current Tier 1 testing formats a minimum of 4350 chemicals will likely mistakenly proceed toward Tier 2 screening at a cost of approximately $8 billion.

The use of autobioluminescent EDC cellular bioreporters represents an attractive means to overcome the limitations of existing Tier 1 screening platforms and address the needs of the EDSP21 program. Autobioluminescence, with is defined as the ability to self-initiate the production of a luminescent signal using only endogenously supplied substrates to perform the enzymatic reactions necessary for signal generation [12], can reduce the number of required assay steps, eliminate the need for superfluous reagent costs, maintain human bioavailability relevance through the use of human cellular hosts, and increase throughput by minimizing hands-on performance time and employing automated processing and detection systems [13]. These benefits are made possible by the autonomous functionality of the synthetic luciferase gene cassette (*lux*) that controls the autobioluminescent phenotype. To enable autonomous EDC detection, *lux* cassette expression is regulated by a yeast upstream activating sequence (UAS), which is itself activated by a hybrid Gal4 transcriptional activator. Expression of this activator is, in turn, governed by the binding of an EDC to

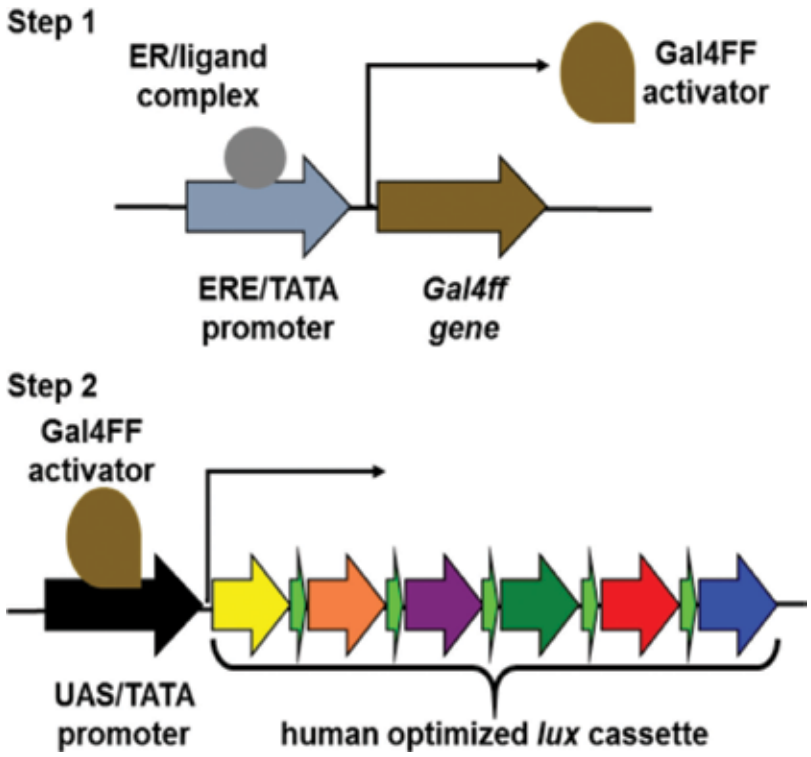

Figure 2. Functional schematic of an estrogenic compound-responsive autobioluminescent induction system. Step 1: *Gal4ff* expression is induced upon estrogenic compound exposure. Step 2: The *lux* cassette is then activated through stimulation of the UAS/TATA promoter by the *Gal4FF* transcriptional activator. Androgenic compound induction proceeds similarly.

an upstream estrogen (ERE; pictured) or androgen (ARE) response element (**Figure 2**). The use of this EDC-responsive promoter system within a human cell can therefore signal EDC bioavailability while simultaneously providing information regarding the timing, magnitude, and duration of the resulting effect. Using the detection of estrogenic compounds as an example, this chapter will provide an overview of how these autobioluminescent cellular bioreporters function in this role relative to alternative, traditional Tier 1 EDC sensor platforms and the advantages and disadvantages they provide for addressing the needs of the EDSP21 program.

2. Requisite endocrine disrupting chemical detection parameters

The U.S. EPA [14] and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [15] have established performance requirements for all EDC detection assays. These performance requirements ensure that the assays can function efficiently enough to identify the presence of EDCs at levels believed to be impactful to human and environmental health. However, rather than mandating that an assay demonstrates predetermined responses

Compound	Log PC_{50}	Log PC_{10}	Log EC_{50}	Hill Slope	Concentration
17β-estradiol	-11.4 ~ -10.1	< -11.0	-11.3 ~ -10.1	0.7 ~ 1.5	$10^{-14} \sim 10^{-8}$ M
Cortisol	-9.6 ~ -8.1	-10.7 ~ -9.3	-9.6 ~ -8.4	0.9 ~ 2.0	$10^{-12} \sim 10^{-6}$ M
17α-Methyltestosterone	-6.0 ~ -5.1	-8.0 ~ -6.2	N/A	N/A	$10^{-11} \sim 10^{-5}$ M
Corticosterone	N/A	N/A	N/A	N/A	$10^{-10} \sim 10^{-4}$ M

PC_{50}—concentration which induces a response at 50% of the maximal positive control response.
PC_{10}—concentration which induces a response at 10% of the maximal positive control response.
EC_{50}—half maximal effective concentration.
EC_{50}—half maximal effective concentration.

Table 1. EPA guidelines for the successful detection of endocrine disruptor chemicals in human cells.

Compound	Average EC_{50} Value	Required Detection Range
Levonorgestrel	0.984 nM	0.689 ~ 1.279 nM
Cortisol	0.043 μM	0.030 ~ 0.056 μM
Hydroxyflutamide	41.5 μM	29.0 ~ 54.0 μM
Flutamide	N/A	Undetected

Table 2. ICCVAM average EC_{50} value guidelines required for the successful detection of androgenic compounds.

across all known EDC compounds, these organizations require that the assay respond appropriately to treatment with serial dilutions of representative strong, weak, and very weak agonists, and that they do not respond to an appropriate negative control. To be considered successful, estrogenic detection assays must meet the U.S. EPA metrics presented in **Table 1** and androgenic detection assays must meet the ICCVAM metrics presented in **Table 2**.

3. Non-autobioluminescent detection assay formats

There are five *in vitro* assay formats, other than autobioluminescence, that are used for EDSP21 Tier 1 screening [16] (**Table 3**). All of these assays are well-established, having been initially developed as early as the 1960's. Although their performance is reproducible and reliable, each is subject to a number of detriments that limit their utility for low-cost, high-throughput EDC detection with high human relevance [6–9, 14].

For instance, in the estrogen receptor (ER) binding assay, cytosol must be isolated from the uteri of rats that have undergone ovariectomy prior to collection of the uterine tissue. These animal subjects are ovariectomized 7–10 days before harvesting the uterine tissue, with each test chemical requiring the use of approximately 19 subjects. Once the uteri have been harvested, they are homogenized and centrifuged to isolate ER-containing cytosol. Before conducting the assay, saturation radioligand binding assays using various concentrations of radioactively labeled 17β-estradiol added to each batch of cytosol are performed to first

Assay	Detection Format	Direct human bioavailability?	Multi-day?	Requires Animal Subjects?
Estrogen receptor (ER) binding assay	Radiological	No	Yes	Yes
Androgen receptor (AR) binding assay	Radiological	No	Yes	Yes
Aromatase assay	Radiological	Yes	No	No
Steroidogenesis assay	Metabolite quantification	Yes	Yes	No
Estrogen receptor transactivation assay (ETRA)	Luminescence	Yes	Yes	No

Table 3. The five traditional ***in vitro*** tier 1 EDC detection assays used in EDSP21.

validate that there are sufficient ER concentrations and to confirm that the receptor is functioning with appropriate affinity. Only after this series of preliminary steps are the actual assays run. During the assay, radioactively labeled 17β-estradiol, uterine cytosol, and test chemical are combined and must undergo a 16–20 h incubation at 4°C in the dark. Following incubation, hydroxyapatite is added, and multiple washings are performed before a final elution with ethanol and measurement of radioisotope activity in a liquid scintillation counter [7]. Similarly, the complementary androgen receptor (AR) binding assay follows the same intricate assay steps as the ER binding assay, but begins with the collection of rat ventral prostate tissues using subjects that are castrated ~24 h prior to assay initiation. Similar to the ER binding assay, this inclusion of approximately 19 animal subjects per test chemical results in increased moral, economical, and logistical concerns [6].

Like the ER and AR binding assays, the aromatase assay also uses radioactively labeled chemicals as detection targets. In addition, assay performance also requires the use of controlled substances, and therefore necessitates specialized waste disposal. Although these attributes do not directly hinder assay performance, they add cost and increase the logistical hurdles underlying assay execution. However, the tradeoff for the use of these chemicals is an increased throughput. Under standard conditions, the aromatase assay can be completed with only 6–8 person hours per run. This makes the aromatase assay a more attractive format for companies concerned with personnel costs. Another advantage of the aromatase assay is that it uses human recombinant microsomes as the detection vehicle, which provides additional human bioavailability relevance compared with the use of animal tissues in the ER and AR binding assays [8].

Unlike the above-mentioned assays, the steroidogenesis assay uses a human adrenocortical carcinoma cell line as its detection vehicle, which provides direct information on the human-relevant effects of compound exposure. However, while this represents a significant advantage, it also comes with the drawback that the cells must remain exposed to the test

chemical for 48 h, making this one of the longer duration assay formats. Further complicating the throughput of the assay is the detection method, which uses liquid chromatography positive atmospheric pressure photoionization tandem mass spectroscopy (LC/APPI-MS/MS) to measure the hormone concentrations in the medium as the assay endpoint. While this provides exquisite levels of sensitivity, the equipment required to perform these measurements is relatively expensive and requires highly-skilled technical personnel for operation. This limits the performance of this assay format to only those labs large enough to justify the associated operational costs [9].

The estrogen receptor transactivation assay (ERTA), also uses a human cell line as its detection vehicle. In this case, the assay leverages a human cervical cancer cell line containing a firefly luciferase reporter gene that emits a bioluminescent signal when chemicals bind to and activate the estrogen receptor. The bioluminescent output of this format makes it an attractive option because it does not require specialized equipment or skilled personnel to perform. The cells for this assay are simply plated in microtiter plates, the test chemical is added, and the plates are incubated for 20–24 h. Following incubation, the luciferase assay reagent is then added to each well to lyse the cells, and bioluminescence is measured. While this assay format is among the most simplistic to perform, the multi-day performance period and the need for requisite sample destruction concurrent with the addition of an exogenous activation chemical impart concerns relating to throughput, performance costs, and the potential interaction of the activating chemical with the compound under study. Nonetheless, the ETRA remains a popular choice for EDC detection due to its many advantages relative to the alternative assay formats [14].

4. Autobioluminescent detection assay formats

4.1. Advantages

Autobioluminescent assays systems address the backlogging problems endemic to EDSP21 because they utilize human cellular hosts as their detection vehicles, their signal generation is fully performed by these host cells without the need for external stimulation, their resulting reporter signal does not require cellular destruction or interfere with cellular metabolism, they are capable of self-regulating bioluminescent production throughout EDC exposure, and they maintain the same output format (luminescent production) as the commonly used ERTA [13]. Because these same output and detection vehicle formats are maintained, autobioluminescent assay systems share the advantages of providing direct human bioavailability information and not requiring specialized equipment or skilled personnel to perform. However, unlike the ERTA, the bioreporter cells used in autobioluminescent assays do not require lysis and therefore remain viable for an unlimited number of repeated or fully continuous measurements. This allows cytotoxicity measurements to be taken on control wells within each plate at any time point desired and eliminates the need for duplicate plate preparation. Since all data are obtained in real-time, the assay intervals employed in autobioluminescent assays can be shortened or lengthened on-the-fly based on the results being obtained, which provides an increased level of flexibility when working with previously uncharacterized compounds

(**Figure 3**). In addition, the detection equipment used to perform the ERTA can be used to perform autobioluminescent assays, so no change in equipment infrastructure is required [17].

This continuous imaging ability of autobioluminescence provides higher levels of data acquisition than the alternative assay formats and is more amenable to high-throughput use. This results in a significant cost savings of approximately 87% per assay relative to the ERTA, which has the lowest performance costs of the alternative assay types. For example, under moderate throughput conditions a 96-well microtiter test plate can be used to accommodate triplicate replicates of four test chemicals and their associated controls. Using this testing format, it would require 21,750 96-well plates to characterize the existing backlog of 87,000 chemicals that are pending under EDSP21 [11]. Based on existing market costs for technician time and chemical reagents [18, 19], it would cost approximately $1.5 billion (USD) to process all of these compounds. However, the reduced performance costs of the autobioluminescent assay format, which result primarily from a reduction in technician hands-on time and removal of the need to purchase an activating chemical substrate, reduces these costs to approximately $191,000 (USD), representing a savings of approximately $1.3 billion (USD).

4.2. Performance and EDC detection abilities

To evaluate the utility of autobioluminescence's repeated interrogation approach, autobioluminescent T47-D cells were seeded in triplicate into multi-well plates and incubated under standard growth conditions for 24 h. After this time, the medium was removed, cells were washed once with phosphate buffered saline (PBS), refreshed with EDC-free medium, and

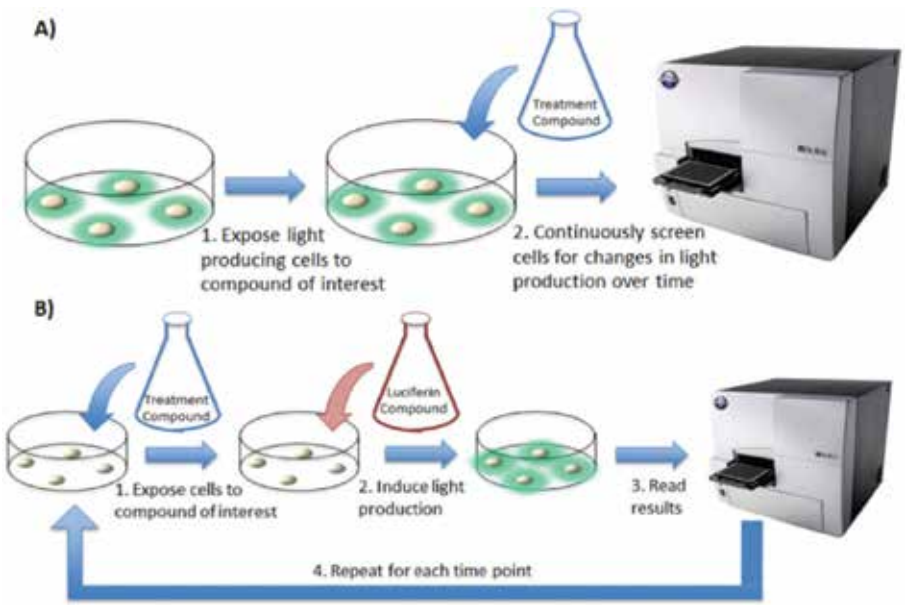

Figure 3. (A) The continuous signal generation of autobioluminescent assays allows for uninterrupted, real-time, high-throughput monitoring of cell activity across consecutive time points. This increases flexibility relative to (B) the ERTA, which only generates single time point snapshots of cellular activity.

supplemented with 17β-estradiol at concentrations of 0 pM (control), 0.1 pM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, or 100 nM. Autobioluminescent measurements were then obtained every 24 h for 6 days using an IVIS Lumina imaging system with a 10 min integration time. Increased autobioluminescent signals relative to untreated control cells were observed by day 3 for all treatments ≥1 pM, although this trend was only maintained throughout the full 6 day assay period at treatment levels ≥10 pM. A dose–response relationship was observed between 17β-estradiol treatment levels and autobioluminescence, with an EC_{50} value of 10 pM (**Figure 4**). Similar results were obtained using the alternative MCF-7 breast cancer cell line, which could detect 17β-estradiol at concentrations of both 1 and 10 nM through the significant ($p < 0.05$) induction of an autonomously-regulated autobioluminescent signal compared to both background light detection and the signal generated cells treated only with vehicle controls (**Figure 5A**).

Notably, the autobioluminescent production from both of these breast cancer cell lines displayed a relatively low signal-to-noise ratio, likely due to their natural expression of estrogen receptors and EDC transporters. To overcome this limitation, the system was re-created in the naturally ER-negative HEK293 human kidney cell line and co-transfected with human estrogen receptor alpha. This allowed for expression of the system without interference from native EDC uptake and processing pathways and significantly reduced the level of background autobioluminescent production in the absence of EDC stimulation, as well as

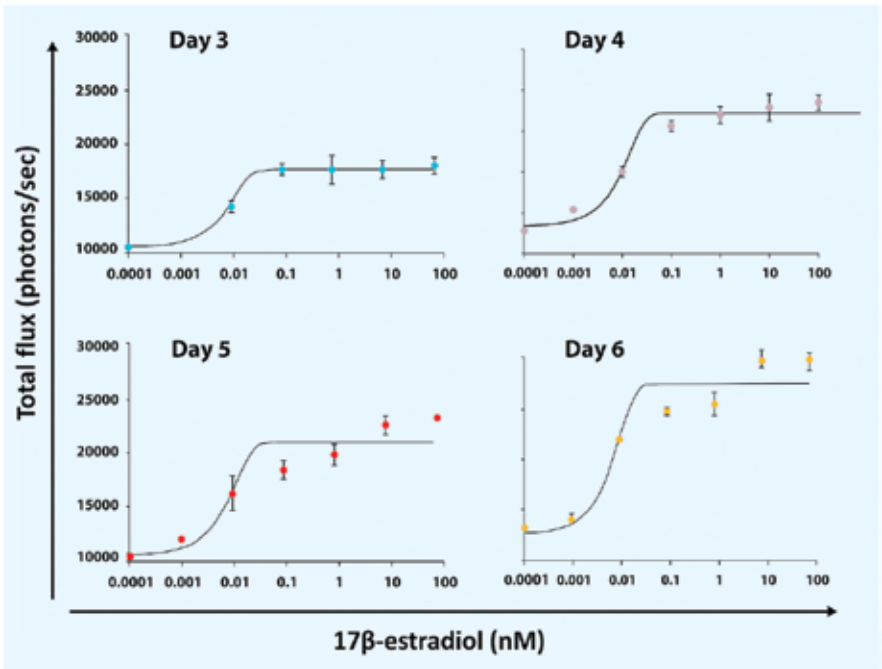

Figure 4. Using repeated measurements of T47-D breast cancer cell line samples, the autobioluminescent assay format allowed dose/response relationships between autobioluminescence and EDC treatment levels to be determined for each day that showed a significant increase compared to negative control cells.

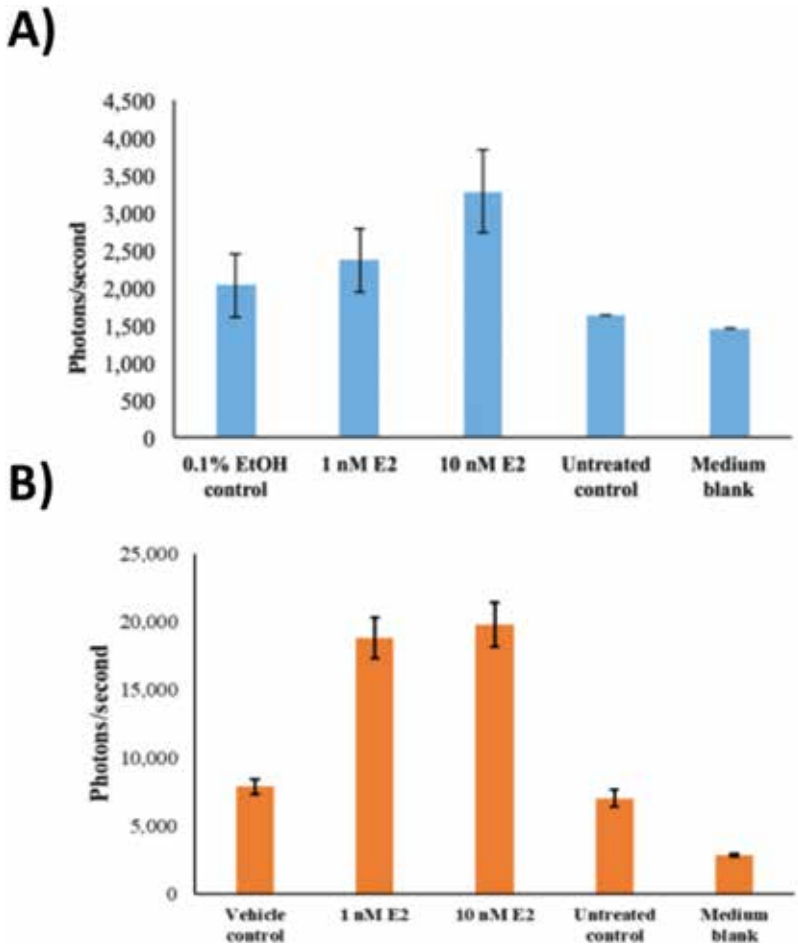

Figure 5. (A) An autobioluminescent MCF-7 cell line was capable of fully autonomous 17β-estradiol detection but displayed a low signal-to-noise ratio. (B) Re-created HEK293 cell line expressing human estrogen receptor alpha and the autobioluminescent reporter construct allowed for fully autonomous 17β-estradiol detection with an improved signal-to-noise ratio.

increasing the signal-to-noise ratio during positive detection events (**Figure 5B**). Using this system design, EDC-responsive autobioluminescent HEK293 bioreporters were able to detect an array of representative EDCs at levels relevant to the requirements of EDSP21 (**Table 4**).

This bioreporter similarly proved to be effective for the detection of other commonly encountered EDCs, such as synthetic hormones, synthetic industrial compounds, phytoestrogens, and fungicides (**Table 5**). These detection capabilities are especially promising given that the autobioluminescent system can be scaled to allow for robotic integration. This would allow cell plating, dosing, and reading to be fully automated. Since the addition of exogenous substrate or sample manipulation post-treatment is not required, this system reduces assay complexity and facilitates rapid detection using automated systems. Given its advantages relative to the existing assay formats (**Table 6**), autobioluminescence represents an attractive alternative assay for potential high-throughput Tier 1 screening of the EPA's current chemical inventory list.

Compound	Measured EC_{50} Value
17β-estradiol	7.9 pM
17α-estradiol	290 pM
17α-methyltestosterone	1 μM
Corticosterone	Negative

Table 4. When expressed in HEK293 cells, the estrogen compound-responsive autobioluminescent reporter system detected an array of representative EDCs within the EPA detection guidelines.

Compound	Compound Class	Measured EC_{50} Value
Diethylstibestrol	Synthetic hormone	12 pM
Bisphenol A	Synthetic compound	460 nM
Daidzein	Phytoestrogen	470 nM
Fenarimol	Fungicide	10 μM

Table 5. The autobioluminescent HEK293-based estrogenic compound-responsive bioreporter was found to be an efficient and simplistic means for the detection of a wide variety of compounds with known estrogenic effects.

Method	Uses non-human cells?	Requires chemical substrate?	Requires radioactive substances?	Requires specialized personnel?	Requires analytical equipment?	Scalable for high-throughput screening?
ER/AER binding	Yes	No	Yes	Yes	No	No
ETRA	No	Yes	No	No	No	No
Aromatase	No	No	Yes	Yes	No	No
Steroidogenesis	No	No	No	Yes	Yes	No
Autobioluminescence	No	No	No	No	No	Yes

Table 6. Summary of the observed advantages and disadvantages of the autobioluminescent EDC detection format relative to alternative tier 1 screening methods.

5. Future directions and recommendations

While autobioluminescent assays have the potential to significantly improve the throughput and cost effectiveness of Tier 1 EDC detection, they are currently in their infancy. Of the tested methods, only the HEK293-based autobioluminescent assay format was capable of producing data with similar performance metrics to the incumbent screening procedures. It is clear that the utility of the autobioluminescent assay format will need to expand to additional cell types and to the detection of androgenic compounds in order to fully address the bioavailability and health effects of EDCs. Similarly, while this work screened the performance of

the HEK293-based estrogen-responsive bioreporter against a variety of EDCs and associated controls, it will be necessary to validate the performance of this assay format at the levels of scale required for commercial use. Therefore, the development of additional bioreporter cell types and their validation at scale using automated assay preparation, performance, and detection equipment is recommended as a next step in the maturation of this assay format. If autobioluminescent assays can perform reliably under these conditions while maintaining a similar level of performance to that observed from the HEK293-based estrogen-responsive bioreporter, they will prove a valuable tool for Tier 1 EDC detection.

6. Conclusions

Tier 1 *in vitro* assays are the front line in EDC detection. However, the limitations of traditional assay formats, which use non-human cell lines that can obscure bioavailability data [6, 7], require the use of radioactive materials that necessitate dedicated use areas and specially trained personnel [6–8], or rely on expensive analytical equipment [8, 9], are currently incapable of handling the sheer number of compounds that must be screened. Autobioluminescent assays, such as the HEK293-based estrogen-responsive bioreporter assay presented here, are uniquely positioned to overcome the limitations of existing assay formats by autonomously generating bioluminescence in response to target chemical or chemical class bioavailability. The use of these reporter systems allows bioluminescent responses to be linked to EDC detection for reagent-free, fully automated screening at a fraction of the cost of existing assays, providing a promising route toward addressing the existing EDC compound screening backlog.

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Conflict of interest

S.R., G.S., and D.C. are board members in the for-profit entity 490 BioTech.

Abbreviations

AR	Androgen receptor
ARE	Androgen response element
BPA	bisphenol-A

EC_{50}	Half maximal effective concentration
EDC	Endocrine disruptor chemical
EDSP21	Endocrine Disruptor Screening Program for the twenty-first century
EPA	U.S. Environmental Protection Agency
ER	Estrogen receptor
ERTA	Estrogen receptor transactivation assay
ERE	Estrogen response element
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
LC/APPI-MS/MS	Liquid chromatography positive atmospheric pressure photoion ization tandem mass spectroscopy
lux	Synthetic luciferase gene cassette
NIEHS	NIH National Institute of Environmental Health Sciences
PC_{10}	Concentration inducing a response at 10% of the maximal positive control response
PC_{50}	Concentration inducing a response at 50% of the maximal positive control response
PBS	Phosphate buffered saline
UAS	Upstream activating sequence

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The World Health Organization estimated that each year there are more than 13 million deaths caused by environmental causes. Exposure to endocrine disrupting chemicals (EDCs) during development may cause long-term health outcomes. This book, Endocrine Disruptors, includes eight chapters that illustrate potential endocrine-disrupting activities in water, sediments, crops, animals, and humans. This book assesses the relationship between the EDCs and development, reproduction, or obesity. Finally, detection of the levels of EDCs by autobioluminescent cellular bioreporters is discussed. Scientists, physicians, neuroendocrinologists, neurotoxicologists, and lay readers who have engaged in EDC studies or practice will discover that this book offers insight into all areas of EDC research.

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