Chapter 9

# The Pragmatic Strategy to Detect Endocrine-Disrupting Activity of Xenobiotics in Food

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Additional information is available at the end of the chapter

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**Abstract**

Endocrine-disrupting activity induced by xenobiotics might pose a possible health threat. Facing so many chemicals, there is an issue on how we detect them precisely and effectively. The whole embryo culture (WEC) test, an ex vivo exposure lasting 48 hours with rat embryos of 10.5 days old, is used to detect prenatal developmental toxicity. We extended the WEC function to detect the endocrine-disrupting activity induced by environmental chemicals. Results showed that in the development of rat embryo, basically 17ß-estradiol, triiodothyronine, triadimefon, penconazole, and propiconazole exhibited no significant effect on yolk sac circulatory system, allantois, flexion, heart caudal neural tube, hindbrain, midbrain, forebrain, otic system, optic system, olfactory system, maxillary process, forelimb, hind limb, yolk sac diameter, crown-rump length, head length, and developmental score. In the immunohistochemistry, the positive control of 17ß-estradiol showed positive effect for its receptor expressions. These three triazoles induced expressions of ER$\alpha$ and ERß in WEC. This result basically meets the mode of action that triazoles were designed to disrupt the synthesis of steroid hormone. Here we gave a strategy to detect possible endocrine-disrupting activity induced by xenobiotics in food. This strategy is quick to initiate the whole rat embryo culture with 10.5 days to detect the hormone receptors such as androgen, estrogen, thyroid, aromatase activity and its related receptors.

**Keywords:** whole embryo culture, xenobiotic, receptors, ex vivo, in vivo, endocrine-disrupting activity

## 1. Introduction

As we know, there are many pesticides identified as endocrine disruptors, but the degree of endocrine-disrupting activity (EDA) is different [1–5]. The different disrupting activities are

involved in pesticide management. Because the potential endocrine-disrupting pesticides should be prohibited, low EDA will be accepted under the control of below maximum residue level (MRL). The development of new pesticide is based on its chemical functional groups for pests including fungicides, insecticides, herbicides, and others. Due to the objective of pest control of diseases, insects, and weeds, the side effect of pesticides will be appropriately managed in order not to pose risk to the human and environment. It is reported that 105 pesticides could be listed in the endocrine-disrupting chemical (EDC) group (**Table 1**) [6–54]. Among these 105 pesticides, 31% are fungicides, 21% herbicides, and 46% insecticides; some of these were withdrawn from use several years ago; even a little still can be detected in the environment such as dichloro-diphenyl-trichloroethane (DDT) and atrazine in some countries.

EDCs focused on interfering with endogenous hormones possible by binding to and activating various hormone receptors including estrogen, androgen, thyroid receptors, and aromatase enzymes and mimic the hormone or enzyme activities including agonistic and antagonistic actions. Basically, EDA is mainly related to the reproductive and developmental toxicity. Also the major endocrine pathways would be hypothalamus-pituitary-gonadal and hypothalamus-pituitary-thyroid, and the involving hormones are estrogen, androgen, and thyroid. The Organization for Co-operation and Development (OECD) test guidelines for reproductive and developmental toxicity and EDA are listed in **Table 2** [55, 56]. United States Environmental Protection Agency (US EPA) test guidelines for reproductive and developmental toxicity and EDA are as follows. Guidelines are 870.3550 reproduction/development toxicity screening test, 870.3650 combined repeated dose toxicity with the reproduction/development toxicity screening test, 870.3700 prenatal developmental toxicity study, 870.3800 reproduction and fertility effects, and 870.6300 developmental neurotoxicity study. USEPA Series 890 endocrine disruptor screening program test guidelines are isolated from OPPTS 870 Series. The final endocrine disruptor screening program test guidelines are generally intended to meet testing requirements under Toxic Substances Control Act (TSCA); Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA); and Federal Food, Drug, and Cosmetic Act (FFDCA) to determine if a chemical substance may pose a risk to human health or the environment due to the disruption of the endocrine system. Group A—EDSP Tier 1 and Group B—EDSP Tier 2 test guidelines are listed in **Table 3**.

The main shortcomings of above guidelines are that they are expensive and time-consuming and the need of a lot of number of laboratory animals. It is reported that cost and the minimum number of laboratory animals are requested for applying OECD test guidelines to test toxicity to reproductive and developmental toxicity. **Table 2** shows the cost and minimum number of laboratory animals [55, 56]. Besides, the associated bioethical and social concerns are becoming a challenge. Nowadays, the common knowledge of using laboratory animals is reduce, refine, and replace (3Rs). Facing these situations, we should take cheap and reliable alternatives to screen the reproductive and developmental toxicity and EDA and decide the next steps for necessities of toxicity tests.

It is reported that a widely used technique for screening prenatal developmental toxicity is by monitoring organogenesis during gestational days (GD) 10–12 [57]. In support to whole rat embryo culture (rat WEC), a variety of morphological endpoints is integrated in the total morphological score (TMS) [58]. When applying the TMS in rat WEC, effects of pesticides on

| Pesticides | EDC related | Pesticides | EDC related |
|---|---|---|---|
| 2,4-D (H) | AR [6] | Heptachlor (I) | ER, AR [25, 46] |
| Acephate (I) | Hypothalamus [7] | Hexaconazole (F) | Aromatase activity, estrogens, androgens [20] |
| Acetochlor (H) | ER, TR [8, 9] | Isoproturon (H) | Pregnane X cellular receptor [5] |
| Alachlor (H) | ER, PR [10, 11] | Iprodione (F) | Aromatase activity, estrogen [2] |
| Aldicarb (I) | 17 Beta-estradiol, progesterone [10, 12] | Linuron (H) | AR, TR [25, 47] |
| Aldrin (I) | AR [13] | Malathion (I) | TR [10, 48] |
| Atrazine (H) | Androgen, aromatase activity, estrogen, luteinizing hormone, prolactin [10, 14–17] | Methiocarb (H) | Androgen, estrogen [2] |
| Bendiocarb (I) | Estrogen effect [10] | Methomyl (I) | Aromatase activity, estrogen [2, 10] |
| Benomyl (F) | Estrogen, aromatase activity [18] | Methoxychlor (I) | Estrogenic effect, AR, pregnane X cellular receptor [10, 11, 13] |
| Bioallethrin (I) | Estrogen-sensitive [19] | Metolachlor | Pregnane X cellular receptor [5] |
| Bitertanol (F) | Aromatase activity, estrogens, androgen [20] | Metribuzin (H) | Hyperthyroidism, somatotropin [49] |
| Bupirimate (F) | Pregnane X cellular receptor [5] | Mirex (I) | Estrogen effect [10] |
| Captan (F) | Estrogen action [21] | Molinate (H) | Reduction of fertility [10] |
| Carbaryl (I) | Estrogen effect [10] | Myclobutanil (F) | Estrogen, androgen, ER, AR, aromatase [20, 21, 35] |
| Carbendazim (F) | Estrogen and aromatase activity [18] | Nitrofen (H) | Estrogen, androgen [21] |
| Carbofuran (I) | Progesterone, cortisol, estradiol, testosterone [22] | Oxamyl (I) | Estrogen effect [10] |
| Chlorothalonil (F) | Androgen-sensitive [23] | Parathion (I) | Melatonin, gonadotrophic hormone [10] |
| Chlordane (I) | ER [10], AR [13] | Penconazole (F) | Estrogenic effect, aromatase activity, estrogens, androgens [20, 35] |
| Chlordecone (I) | AE, ER [21, 24, 25] | Pentachlorophenol (H, F, I) | Estrogenic, androgenic affect [10] |
| Chlorfenvinphos (I) | Estrogen effect [26] | Permethrin (I) | Estrogen-sensitive [19, 29] |
| Chlorpyrifos methyl (I) | AR [27] | Phenylphenol (F) | Estrogen [50] |
| Cypermethrin (I) | Estrogenic effect [28, 29] | Prochloraz (F) | Pregnane X cellular receptor, AR, ER, AhR, aromatase activity [2, 5, 36, 51] |
| Cyproconazole (F) | Aromatase activity, estrogens, androgens [20] | Procymidone (F) | AR [25] |
| DDT and metabolites (I) | AR, androgen-sensitive, ER, PR [13, 23, 24, 30] | Propamocarb (F) | Aromatase activity, estrogen [2] |
| Deltamethrin (I) | Estrogenic activity [2] | Propanil (H) | Estrogen [52] |

| Pesticides | EDC related | Pesticides | EDC related |
|---|---|---|---|
| Diazinon (I) | Estrogenic effect [31] | Propazine (H) | Aromatase activity, estrogen [15] |
| Dichlorvos (I) | AR [2] | Propiconazole (F) | Estrogen, aromatase activity, androgens [20, 35] |
| Dicofol (I) | Androgen synthesis, estrogens synthesis, ER [17, 21] | Propoxur (I) | Estrogenic effect [10] |
| Dieldrin (I) | AR, estrogenic effect, ER [2, 13, 24, 32] | Prothiophos (I) | Estrogenic effect [31] |
| Diflubenzuron (I) | Pregnane X cellular receptor [5] | Pyridate (H) | ER, AR [21] |
| Dimethoate (I) | Thyroid hormones, insulin, luteinizing hormone [33, 34] | Pyrifenox (F) | Estrogen [35] |
| Diuron (H) | Androgen action [17] | Pyriproxyfen (I) | Estrogenic effect [31] |
| Endosulfan (I) | AR, estrogenic effect, ER, aromatase activity [2, 13, 30, 32] | Resmethrin (I) | Sex hormone [40] |
| Endrin (I) | AR [13] | Simazine (H) | Aromatase activity, estrogen [15] |
| Epoxiconazole (F) | Aromatase activity, estrogen, androgens [20, 35] | Sumithrin (I) | Estrogen-sensitive, progesterone [19, 39] |
| Fenarimol (F) | Androgenic action, aromatase, pregnane X cellular receptor [2, 5, 36] | Tebuconazole (F) | Aromatase activity, estrogens, androgens [20] |
| Fenbuconazole (F) | Thyroid hormones, pregnane X cellular receptor [5, 10] | Tetramethrin (I) | Estrogen [53] |
| Fenitrothion (I) | AR, estrogens [21, 37] | Tolclofos-methyl (I) | ER [36] |
| Fenoxycarb (I) | Testosterone [38] | Toxaphene (I) | Estrogen-sensitive, corticosterone [10, 32] |
| Fenvalerate (I) | Estrogen-sensitive, progesterone [18, 39] | Triadimefon (F) | Estrogenic effect, aromatase activity, androgens [21] |
| Fluvalinate (I) | Human sex hormone, progesterone [40, 41] | Triadimenol (F) | Estrogenic effect, aromatase activity, androgens [20, 21] |
| Flusilazole (F) | Aromatase activity, estrogens, androgens [20] | Tribenuron-methyl (H) | Estrogenic effect [2] |
| Flutriafol (F) | Estrogen [35] | Trichlorfon (I) | Thyroid function [54] |
| Glyphosate (H) | Aromatase activity, estrogens [42] | Trifluralin (H) | Pregnane X cellular receptor, steroid hormone [11] |
| HCB (F) | Thyroid hormone, androgen [43, 44] | Vinclozolin (F) | AR, pregnane X cellular receptor, steroid hormone [2, 11, 25] |
| HCH (lindane) (I) | Estrous cycles, luteal progesterone, insulin, estradiol, thyroxine, AR, ER, PR [33, 45] | | |

I, insecticides; F, fungicides; H, herbicides

**Table 1.** The summary of reported endocrine disruptor pesticides and their related EDC activity.

the embryonic toxicity could be investigated with qualitative and quantitative endpoints. As we know, azoles are antifungal agents for clinical and agricultural use. Penconazole, propiconazole, and triadimefon were most common triazole pesticides in Taiwan. A report

| OECD guideline | Topic | Animals | Estimated cost (€) |
|---|---|---|---|
| 414 | Prenatal development toxicity | 784 | 63,100 (rats) 92,500 (rabbits) |
| 416 | Reproductive toxicity in two generations | 3200[a] | 328,00 |
| 421 | Screening test for reproductive and developmental toxicity | 560 | 54,600 |
| 422 | Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test | 412 | 92,000 |
| 426 | Neurodevelopmental toxicity study | 1400 | 1100 |

Data came from Rovida and Hartung [55]; Sogorb et al. [56].
[a]All the animals including discarded pups.

**Table 2.** Economical cost and number of animals needed to apply the OECD guidelines for testing reproductive toxicology.

| OPPTS 890 series | Topic |
|---|---|
| Group A—EDSP Tier 1 | |
| 890.1100 | Amphibian metamorphosis (frog) |
| 890.1150 | Androgen receptor binding (rat prostate) |
| 890.1200 | Aromatase (human recombinant) |
| 890.1250 | Estrogen receptor binding |
| 890.1300 | Estrogen receptor transcriptional activation (human cell line HeLa-9903) |
| 890.1350 | Fish short-term reproduction |
| 890.1400 | Hershberger (rat) |
| 890.1450 | Female pubertal (rat) |
| 890.1500 | Male pubertal (rat) |
| 890.1550 | Steroidogenesis (human cell line—H295R) |
| 890.1600 | Uterotrophic (rat) |
| Group B—EDSP Tier 2 | |
| 890.2100 | Avian two-generation toxicity test in the Japanese quail |
| 890.2200 | Medaka-extended one-generation reproduction test |
| 890.2300 | Larval amphibian growth and development assay (LAGDA) |

**Table 3.** USEPA Tier 1 and Tier 2 test guidelines.

showed that triazole chemicals antagonized the aromatase, which transfer testosterone into 17ß-estradiol in mammals. Triazole chemicals were designed to disrupt the Cyp51 enzyme, which catalyzes the conversion of lanosterol to ergosterol on the fungal cell membrane, and led to cell death when attacked [59]. Though in the respect of mammalian systems Cyp51 is less

sensitive to azoles, it was still critical for the sterol biosynthesis pathway and might be related to the thyroid function. In this study, we will take triazoles penconazole, propiconazole, and triadimefon as an example for the alternative of endocrine-disruptor screening.

## 2. Materials and methods

### 2.1. Animals

The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Taiwan Agricultural Chemicals and Toxic Substances Research Institute. Five-week-old male and female Wistar rats were purchased from BioLASCO (Taipei, Taiwan, ROC). The rats were acclimated to the laboratory environment and reared under a controlled temperature (21 ± 2°C), humidity (40–70%), frequency of ventilation (at least 10/h), and alternating 12 hour cycles of light and darkness. The rats were administered a pellet rodent diet and water ad libitum until they were sacrificed. At 12 weeks of age, the 4 male and 20 female rats were allowed to mate with 2 males to 2 females per day. Gestation day (GD) 0 was defined as the day that sperm was observed in the vagina of the female following mating.

### 2.2. Chemicals

Materials were obtained from the following manufacturers: DMSO (dimethyl sulfoxide), T3 (triiodothyroxine), Tria (triadimefon), Penc (penconazole), and Prop (propiconazole). All these chemicals with 97% pure at least were purchased from Sigma Chemical Co. (St. Louis, MO).

### 2.3. Rat whole embryo culture

Five-week-old female and male rats were purchased and reared in the first animal house breeding room until 11–12 weeks of age. Two males and two females were bred in the same cage. The female rats were examined for vaginal plugs on the next day. The occurrence was considered as successful breeding. From the date of pregnancy to the 10.5th day, the embryos were dissected. Reichert's membrane was removed according to the method described by Andrews et al. [60] and Dimopoulou et al. [61], and the embryos containing the intact yolk sac placenta and the urinary membrane were removed and randomly placed in a 4 mL culture medium HBSS solution containing 50 IU of penicillin G/mL and 50 μg streptomycin/mL. The sample was added to a 25 T culture flask containing filter-sterilized rat serum and subjected to complement deactivation and cultured in a constant temperature incubator at 37°C for 48 hours. The culture solution was initially inflated with a mixed gas of 5% $O_2$, 5% $CO_2$, and 90% $N_2$ for 1 minute, and after about 16 hours of culture, 10% $O_2$, 5% $CO_2$, and 85% $N_2$, inflated for 1 minute, and were cultured until the 24th hour. Inflate for 1 minute with 20% $O_2$, 5% $CO_2$, and 75% $N_2$. Each treatment dose was inflated for 1 minute at 40% $O_2$, 5% $CO_2$, and 55% $N_2$ at 40 hours, and the embryos were measured for growth, development, and morphology at the end of 48 hours of culture. Embryonic development was modified according to Brown and Fabro [62], and the evaluation included embryo growth traits and developmental

stages, which were considered death if the embryonic yolk sac circulation system or the heart stopped beating. Finally, the carcass head-tail length, developmental grade, head length, number of body segments, and yolk sac diameter were analyzed by t-test and related measurements according to statistical methods; death and abnormal embryos were determined by chi-square. Half of the evaluated embryos were preserved in neutral formalin solution for immunostaining, and the other half were stored in PBS for WB analysis to detect antibody responses related to hormone receptor or enzyme antibodies including AR, ER$\alpha$, ERß, TR$\alpha$, TRß, and aromatase.

### 2.4. Pesticide treatment and evaluation of embryo morphology

This study aimed to investigate the effect of these three pesticides on estrogen receptor (ER$\alpha$ and ERß), thyroid receptor (TR$\alpha$ and TRß), and aromatase activities in whole rat embryo culture (rat WEC) on gestation day (GD) 10.5. The concentrations of WEC were 3.1E-5, 6.2E-5, and 1.2E-4 M of penconazole, propiconazole, and triadimefon. The culture period was 48 hours. After culture the embryo morphology was assessed according to the TMS system [62], we graded the endpoint as no effect (−), little effect (±), effect (+), and potential effect (++). After evaluation of embryo development, it was fixed in formalin or kept in HBSS for immunohistochemistry (IHC) and western blot (WB), respectively.

### 2.5. Immunohistochemical (IHC) evaluation

The embryos were treated by penconazole, propiconazole, and triadimefon with concentrations of 3.1E-5, 6.2E-5, and 1.2E-4 M. Embryos from control and pesticide treatments were fixed in 10% neutral buffered formalin for 1 week. The embryos were then dehydrated with increasing concentrations of ethanol, cleared in toluene, and embedded in paraffin. All the 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 minutes to block endogenous peroxidase activity and finally treated with a protein-blocking solution (5% goat serum diluted in phosphate-buffered saline). All 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), anti-ER (MC) antibody (Santa Cruz Co., CA), TR$\alpha$ (C0345), TRß (C0346) (Assay Biotechnology Co. Sunnyvale, CA), and aromatase (SM2222P)(Acris Antibodies, Inc., San Diego, CA), 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 hematoxylin. All images were optimized by using an inverted microscope (Leica, Wetzlar GmbH, Germany). To quantify the relative amount of activity of ER, TR, and aromatase in the IHC, 200 nuclei stained per field in a slide, 5 fields per slide, and 5 slides per dose were counted. The intensity of AR, ER, TR, and aromatase proteins 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.

### 2.6. Western blot

The embryo homogenates were then centrifuged at 3000 × g for 30 minutes 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 (N-20), ER$\alpha$ (MC-20), ERß (H-150) (Santa Cruz Co., CA), TR$\alpha$ (C0345), TRß (C0346) (Assay Biotechnology Co. Sunnyvale, CA), and aromatase (SM2222P) (Acris Antibodies, Inc., San Diego, 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 embryo homogenates 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), ER$\alpha$ (MC-20), ERß (H-150) (Santa Cruz Co., CA), TR$\alpha$ (C0345), TRß (C0346) (Assay Biotechnology Co. Sunnyvale, CA), aromatase (SM2222P) (Acris Antibodies, Inc., San Diego, CA), and ß-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.

### 2.7. Statistical analysis

The values of ER, TR, and aromatase in western blot were normalized against ß-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 $p < 0.05$.

## 3. Results

In the development of rat embryo, 17ß-estradiol ($E_2$), triiodothyronine ($T_3$), triadimefon, penconazole, and propiconazole exhibited no significant effect on yolk sac circulatory system, allantois, flexion, heart caudal neural tube, hindbrain, midbrain, forebrain, otic system, optic system, olfactory system, maxillary process, forelimb, hind limb, yolk sac diameter, crown-rump length, head length, and developmental score (**Tables 4–6**; **Figure 1**).

| Treatment | Yolk sac circulatory system | Allantois | Flexion | Heart | Caudal neural tube | Hindbrain | Midbrain |
|---|---|---|---|---|---|---|---|
| DMSO | 3.1 ± 0.3 | 4.0 ± 0.0 | 2.0 ± 0.8 | 2.6 ± 0.7 | 3.7 ± 1.3 | 2.8 ± 0.8 | 2.8 ± 0.8 |
| E2 | 3.0 ± 0.8 | 4.0 ± 0.0 | 3.0 ± 1.8 | 2.8 ± 0.5 | 4.3 ± 1.0 | 2.0 ± 1.2 | 2.5 ± 1.0 |
| T3 | 3.0 ± 0.0 | 4.0 ± 0.0 | 2.5 ± 2.1 | 2.0 ± 1.4 | 3.0 ± 0.0 | 3.0 ± 0.0 | 3.0 ± 0.0 |
| Triadimefon | | | | | | | |
| L | 2.5 ± 0.6 | 4.0 ± 0.0 | 3.0 ± 0.8 | 3.0 ± 0.0 | 4.0 ± 0.0 | 1.0 ± 0.0 | 3.0 ± 0.0 |
| M | 2.8 ± 0.5 | 4.0 ± 0.0 | 2.5 ± 0.6 | 3.0 ± 0.0 | 4.3 ± 0.5 | 2.5 ± 1.0 | 2.5 ± 1.0 |
| H | 3.0 ± 0.0 | 4.0 ± 0.0 | 1.8 ± 1.0 | 2.8 ± 0.4 | 4.0 ± 0.9 | 2.7 ± 0.8 | 2.7 ± 1.0 |
| Penconazole | | | | | | | |
| L | 3.7 ± 0.6* | 4.0 ± 0.0 | 3.7 ± 1.2* | 3.0 ± 0.0 | 4.3 ± 1.2 | 3.0 ± 0.0 | 3.0 ± 0.0 |
| M | 3.6 ± 0.6* | 4.0 ± 0.0 | 2.7 ± 0.6 | 3.0 ± 0.0 | 4.0 ± 0.0 | 3.0 ± 0.0 | 3.0 ± 0.0 |
| H | 3.4 ± 0.5 | 4.0 ± 0.0 | 3.0 ± 1.9 | 2.4 ± 0.9 | 3.8 ± 0.8 | 2.2 ± 1.1 | 2.4 ± 0.9 |
| Propiconazole | | | | | | | |
| L | 3.0 ± 0.0 | 4.0 ± 0.0 | 3.0 ± 2.0 | 3.0 ± 0.0 | 3.7 ± 0.6 | 2.3 ± 1.2 | 2.3 ± 1.2 |
| M | 2.8 ± 0.4 | 3.8 ± 0.4 | 2.4 ± 0.9 | 3.0 ± 0.0 | 4.0 ± 0.7 | 2.8 ± 0.4 | 2.8 ± 0.5 |
| H | 3.0 ± 0.8 | 4.0 ± 0.0 | 2.5 ± 1.7 | 3.0 ± 0.0 | 3.8 ± 1.0 | 3.0 ± 0.0 | 3.0 ± 0.0 |

All pesticide concentrate are 3.1E-5 M (low concentration, L), 6.2E-5 M (middle concentration, M), and 1.2E-4 M (high concentration, H). Dimethyl sulfoxide, DMSO; 17ß-estradiol, E2; and triiodothyronine, T3. E2 and T3 concentrations, 1.2E-4 M.
*$P < 0.05$.

**Table 4.** Effect of treatment with triazole pesticides on some developmental scores of rat embryo culture of day 10.5 for 48 hours.

In the immunohistochemistry (IHC), the 17ß-estradiol (ER$\alpha$ and ERß) positive control showed the respective results of receptor expressions. Our results showed that penconazole, propiconazole, and triadimefon induced expressions of ER$\alpha$ (**Figure 2**) and ERß (**Figure 3**) in WEC. This result basically meets the mechanisms of triazoles designed to disrupt the synthesis of steroid hormone. Also, results showed that penconazole, propiconazole, and triadimefon induced expressions of TRß (data not shown), but not in TR$\alpha$ (data not shown) with WEC. The relationship among TRß and AR and ER still needs to be investigated. Also, we need to study the antagonistic effects by adding the antagonists for the receptor expression. These three pesticides did not affect significantly AR (data not shown) and aromatase activity (data not shown). In the western blot (WB) data, these three pesticides did not affect significantly AR, ER$\alpha$, ERß, TR$\alpha$, TRß, and aromatase expressions in WEC (data not shown). The difference between IHC and WB induced by these three pesticides might be the sensitivity of detecting method. WB needs some embryos for the protein quantitative, while IHC can detect activity in an embryo.

| Treatment | Forebrain | Otic system | Optic system | Olfactory system | Branchial bars | Maxillary process | Mandibular process |
|---|---|---|---|---|---|---|---|
| DMSO | 2.7 ± 0.7 | 1.8 ± 0.4 | 2.8 ± 1.3 | 1.5 ± 0.7 | 1.4 ± 0.5 | 0.9 ± 0.3 | 2.0 ± 0.0 |
| E2 | 2.8 ± 1.3 | 2.0 ± 0.8 | 3.0 ± 1.4 | 1.0 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0 | 2.0 ± 0.0 |
| T3 | 3.0 ± 0.0 | 1.5 ± 0.7 | 2.5 ± 2.1 | 1.5 ± 0.7 | 1.5 ± 0.7 | 1.0 ± 0.0 | 2.0 ± 0.0 |
| Triadimefon | | | | | | | |
| L | 2.8 ± 0.6 | 1.5 ± 0.6 | 3.5 ± 1.0 | 1.8 ± 0.5 | 1.3 ± 0.5 | 1.0 ± 0.0 | 2.0 ± 0.0 |
| M | 2.3 ± 1.0 | 1.5 ± 0.6 | 3.3 ± 1.0 | 1.0 ± 0.0 | 1.5 ± 0.6 | 1.0 ± 0.0 | 2.0 ± 0.0 |
| H | 2.7 ± 1.0 | 1.7 ± 0.5 | 3.3 ± 0.8 | 1.5 ± 0.5 | 1.2 ± 0.4 | 1.0 ± 0.0 | 2.0 ± 0.0 |
| Penconazole | | | | | | | |
| L | 3.3 ± 0.6 | 1.7 ± 0.6 | 3.3 ± 1.2 | 1.7 ± 0.6 | 1.3 ± 0.6 | 1.0 ± 0.0 | 2.0 ± 0.0 |
| M | 3.7 ± 0.6 | 1.7 ± 0.6 | 4.0 ± 0.0 | 1.7 ± 0.6 | 1.7 ± 0.6 | 1.0 ± 0.0 | 2.0 ± 0.0 |
| H | 2.6 ± 1.5 | 2.0 ± 1.0 | 3.2 ± 1.8 | 1.2 ± 0.8 | 1.2 ± 0.4 | 1.0 ± 0.0 | 2.0 ± 0.0 |
| Propiconazole | | | | | | | |
| L | 2.7 ± 1.5 | 1.7 ± 1.2 | 3.3 ± 2.1 | 1.3 ± 0.6 | 1.3 ± 0.6 | 1.0 ± 0.0 | 2.0 ± 0.0 |
| M | 2.8 ± 0.4 | 1.4 ± 0.5 | 2.6 ± 1.1 | 1.2 ± 0.4 | 1.2 ± 0.4 | 1.0 ± 0.0 | 2.0 ± 0.0 |
| H | 3.3 ± 0.5 | 1.5 ± 0.6 | 2.5 ± 1.7 | 1.8 ± 0.5 | 1.0 ± 0.0 | 1.0 ± 0.0 | 2.0 ± 0.0 |

All pesticide concentrate are 3.1E-5 M (low concentration, L), 6.2E-5 M (middle concentration, M), and 1.2E-4 M (high concentration, H). Dimethyl sulfoxide, DMSO; 17ß-estradiol, E2; and triiodothyronine, T3. E2 and T3 concentrations: 1.2E-4 M.

**Table 5.** Effect of treatment with triazole pesticides on some other developmental scores of rat embryo culture of day 10.5 for 48 hours.

| Treatment | Forelimb | Hind limb | Yolk sac diameter (A) (mm) | Yolk sac diameter (B) (mm) | Crown-rump length (mm) | Head length (mm) | Developmental score |
|---|---|---|---|---|---|---|---|
| DMSO | 0.7 ± 0.5 | 0.7 ± 0.5 | 6.4 ± 1.2 | 5.7 ± 1.0 | 5.2 ± 1.1 | 1.9 ± 0.6 | 38 ± 7 |
| E2 | 0.8 ± 0.5 | 0.8 ± 0.5 | 6.6 ± 1.4 | 5.2 ± 1.7 | 4.4 ± 1.4 | 2.2 ± 0.7 | 38 ± 8 |
| T3 | 1.0 ± 0.0 | 1.0 ± 0.0 | 5.8 ± 0.1 | 4.9 ± 1.6 | 4.0 ± 1.8 | 1.7 ± 0.8 | 38 ± 6 |
| Triadimefon | | | | | | | |
| L | 0.5 ± 0.6 | 0.8 ± 0.5 | 4.8 ± 1.0 | 4.8 ± 0.6 | 4.9 ± 0.4 | 1.7 ± 0.3 | 40 ± 3 |
| M | 0.5 ± 0.6 | 1.0 ± 0.0 | 5.0 ± 0.7 | 5.0 ± 0.7 | 5.4 ± 1.0 | 1.9 ± 0.3 | 38 ± 2 |
| H | 0.7 ± 0.5 | 0.8 ± 0.4 | 4.7 ± 0.9* | 5.3 ± 1.2 | 4.9 ± 0.9 | 1.8 ± 0.5 | 39 ± 4 |
| Penconazole | | | | | | | |
| L | 0.7 ± 0.6 | 1.3 ± 0.6 | 7.1 ± 1.7 | 6.4 ± 1.6 | 6.0 ± 1.0 | 2.5 ± 0.6 | 43 ± 4 |
| M | 1.0 ± 0.0 | 0.7 ± 0.6 | 6.9 ± 0.5 | 5.7 ± 1.1 | 5.8 ± 0.7 | 3.0 ± 0.5 | 43 ± 3 |
| H | 0.6 ± 0.5 | 1.0 ± 0.7 | 6.3 ± 1.2 | 6.2 ± 1.2 | 4.6 ± 1.7 | 2.1 ± 1.1 | 39 ± 9 |

| Treatment | Forelimb | Hind limb | Yolk sac diameter (A) (mm) | Yolk sac diameter (B) (mm) | Crown-rump length (mm) | Head length (mm) | Developmental score |
|---|---|---|---|---|---|---|---|
| Propiconazole | | | | | | | |
| L | 0.7 ± 0.6 | 0.7 ± 0.6 | 6.0 ± 1.4 | 5.4 ± 0.8 | 5.0 ± 0.4 | 2.1 ± 0.7 | 38 ± 9 |
| M | 0.6 ± 0.5 | 0.6 ± 0.5 | 4.2 ± 0.8* | 4.5 ± 1.0* | 4.5 ± 1.1 | 1.8 ± 0.4 | 40 ± 5 |
| H | 0.7 ± 0.6 | 0.8 ± 0.5 | 5.5 ± 2.2 | 4.8 ± 0.5 | 4.2 ± 1.4 | 1.9 ± 0.8 | 38 ± 6 |

All pesticide concentrate are 3.1E-5 M (low concentration, L), 6.2E-5 M (middle concentration, M), and 1.2E-4 M (high concentration, H). Dimethyl sulfoxide, DMSO; 17ß-estradiol, E2; and triiodothyronine, T3. E2 and T3 concentrations: 1.2E-4 M.
*$P < 0.05$.

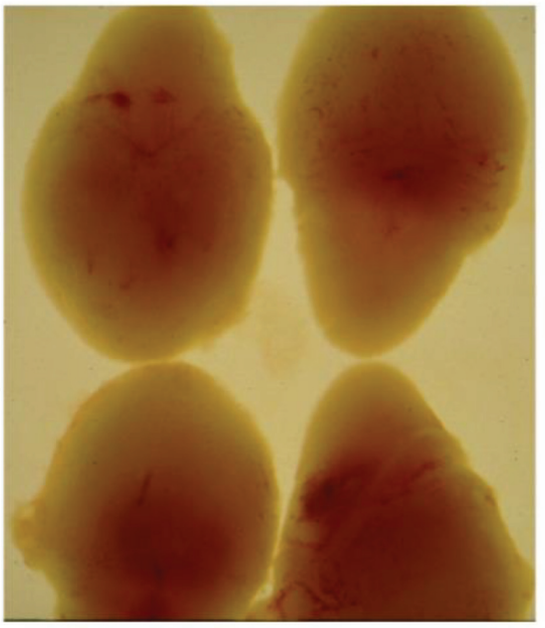
**Table 6.** Effect of co-treatment with triazole pesticides on developmental parameters and scores of rat embryo culture of day 10.5 for 48 hours.

## 4. Discussion

WEC was used to study the prenatal developmental toxicity induced by environmental chemicals including phthalate and methoxyacetic acid [63, 64], aliphatic amides [65], and triazole pesticides [66, 67]. In respect of the 3Rs principle of animal study, WEC is an alternative to screen the potential of prenatal developmental toxicity of environmental compounds. Although ex vivo exposure of WEC was used limitedly without metabolisms of chemicals, most chemicals exhibited their action by parent compound. In this study, we found that in combination with IHC and WB, WEC will be a robust way to detect the endocrine-disrupting activity induced by environmental chemicals. In this study, we used WEC to detect the important receptors including AR, ER$\alpha$, ERß, TR$\alpha$, and TRß and enzyme aromatase activity potential induced by triadimefon, penconazole, and propiconazole. There is one shortcoming of WEC to be addressed. Due to the small amount of embryo, WB is hard to quantify the proteins of hormone receptors. The solution to the problem is to pool the embryo treated by one dose and analyze it. Also, we knew that fortunately nowadays IHC quantification is available. Finally, we concluded that in combination with IHC and WB, WEC will be a robust way to detect EDCs in food.

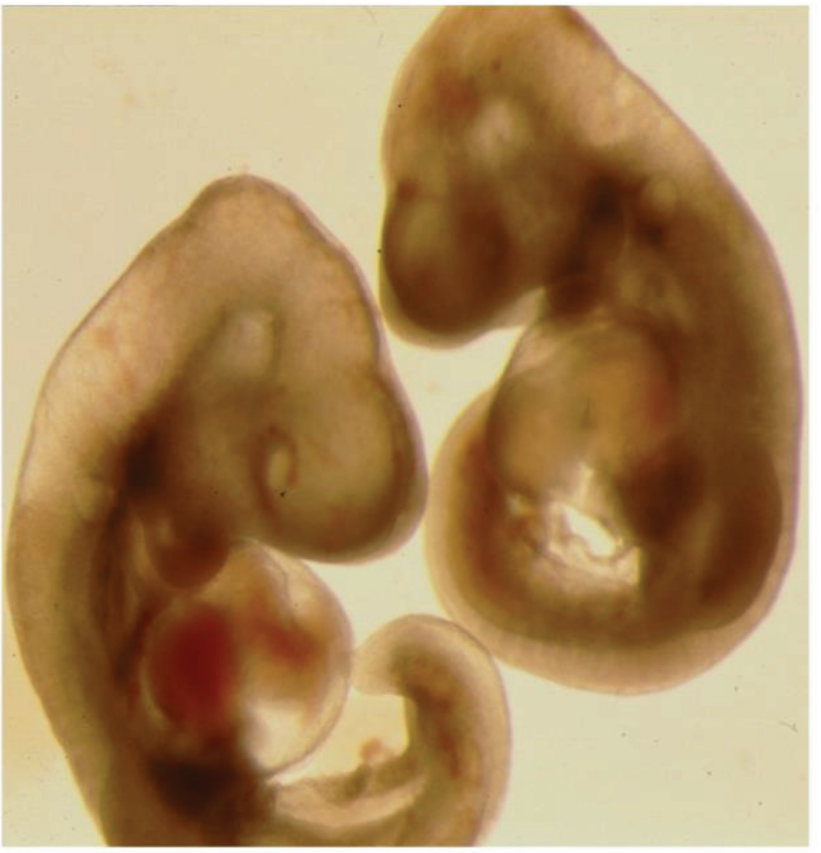
## 5. Future work and recommendations

In order to meet the 3Rs including reduction, refine, and replace and precise risk assessment, adverse outcome pathway (AOP) is extensively developed by OECD. By tier screening for EDCs, the molecular initiating event (MIE), key event (KE), key event relationship (KER), and adverse outcome (AO) will be studied. As the guideline stated, the AOP framework made clear the mechanisms from MIE, KE, and KER to AO will meet the criteria of 3Rs of the animal study and provide a quick and precise way to regulatory protection goals and decision-making.



Day 10.5 embryo

Embryo after culturing for 48 hrs



Embryo after culturing for 48 hrs and tearing down the yolk sac

**Figure 1.** The rat whole embryo culture.

http://dx.doi.org/10.5772/intechopen.81030

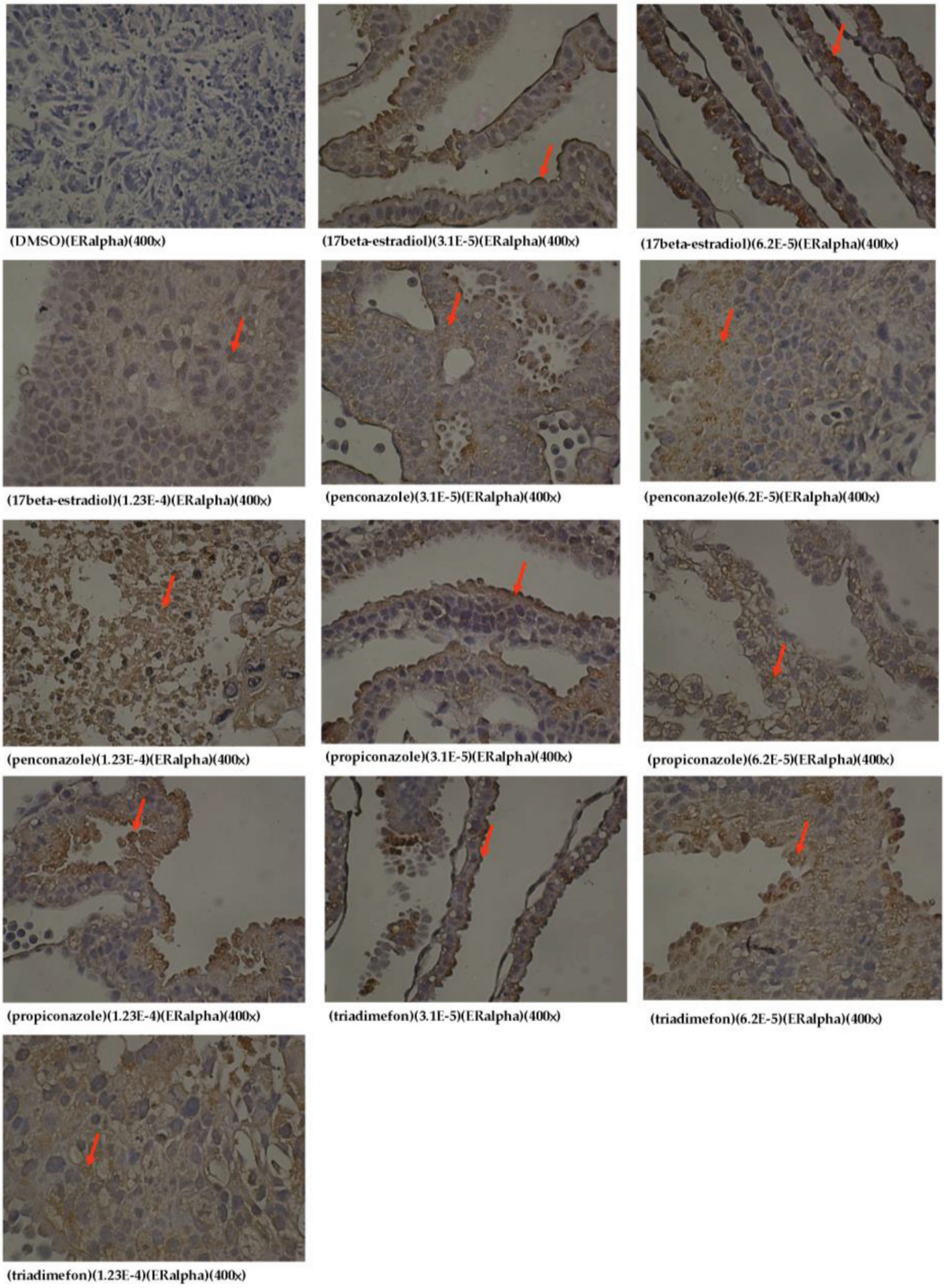



**Figure 2.** Effect of penconazole, propiconazole, and triadimefon on ERalpha activity in WEC.

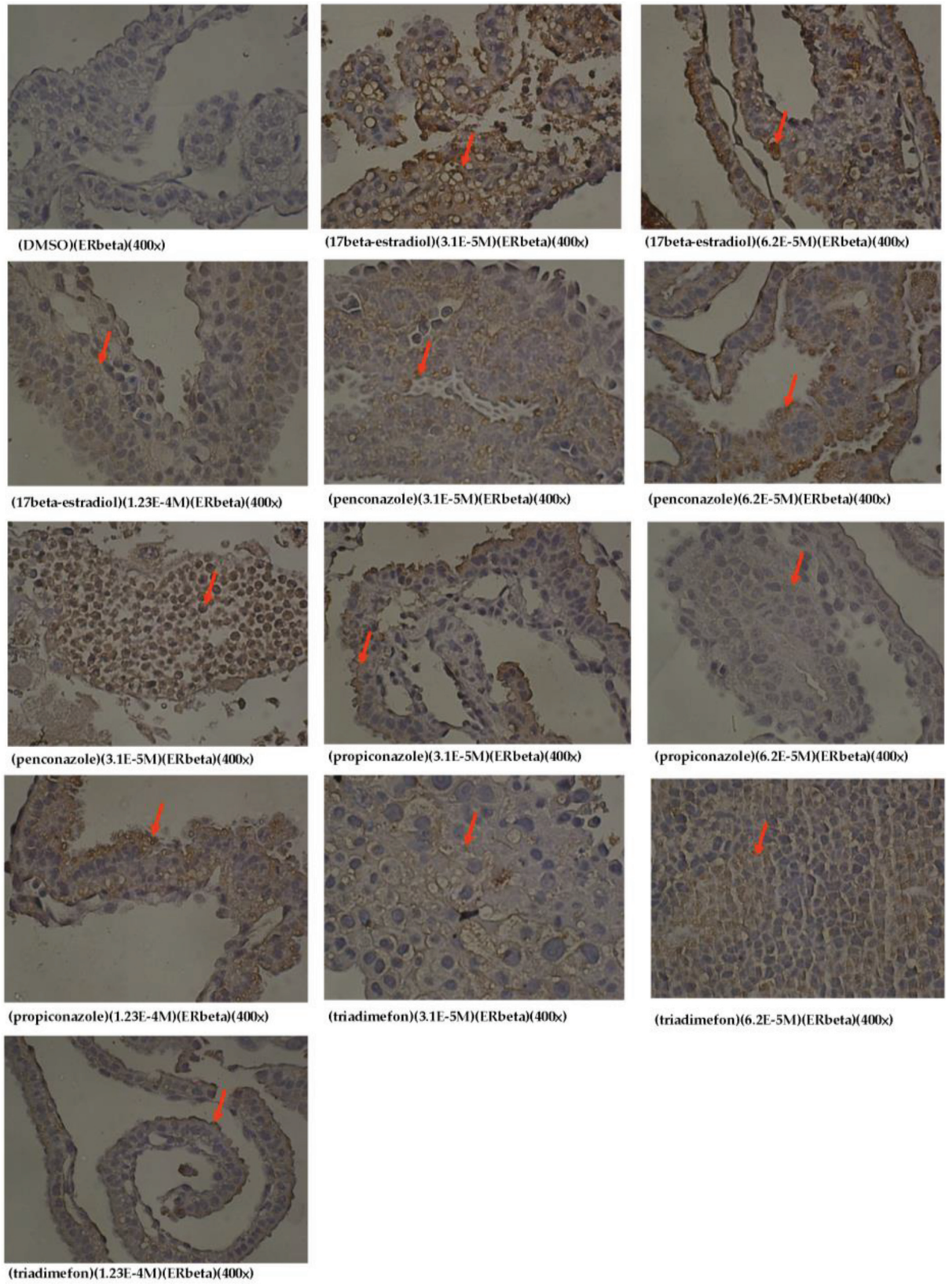



**Figure 3.** Effect of penconazole, propiconazole, and triadimefon on ERbeta activity in WEC.

http://dx.doi.org/10.5772/intechopen.81030

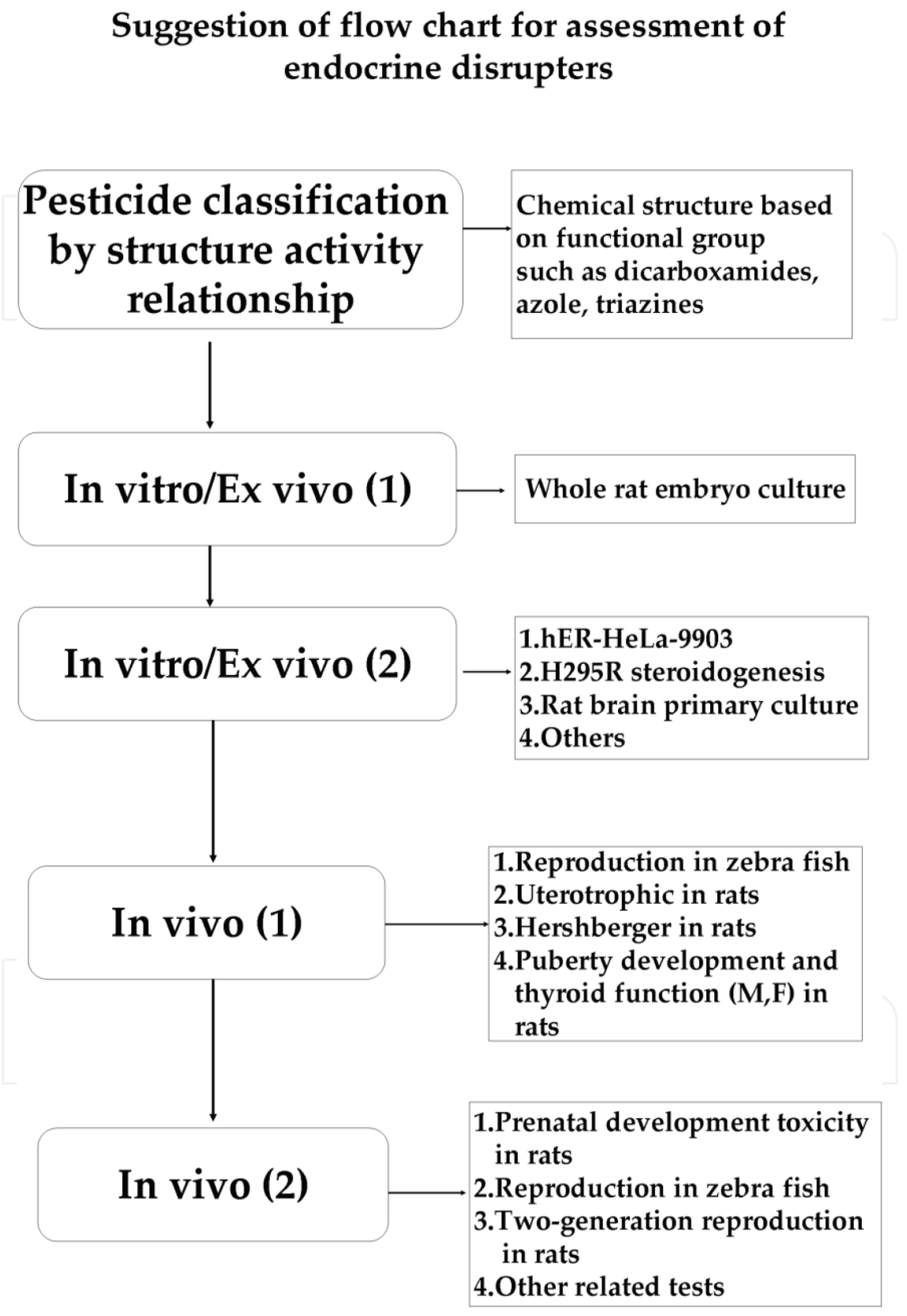



**Figure 4.** Suggestion of flow chart for assessment of endocrine disrupters.

The overall weight of evidence (WoE) and level of certainty underlying the inference and extrapolation will in turn dictate the most suitable application of the AOP.

## 6. Diagram/schematic figure

The pragmatic strategy to detect EDA of xenobiotics in food is to take a tier screening. **Figure 4** showed the suggestion of flow chart for assessment of endocrine disruptors. Basically rat embryo culture could be the first screening method except for chemical structure-activity relationship.

## 7. Conclusions

Penconazole, propiconazole, and triadimefon significantly induced the estrogen receptor expressions. It seems that WEC can be used as a robust method of endocrine-disrupting screening for estrogen receptors.

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## Conflicts of interest

The authors declare no conflicts of interest.

## Abbreviations

| | |
|---|---|
| MRL | maximum residue level |
| EDCs | endocrine-disrupting chemicals |
| OECD | Organization for Economic Co-operation and Development |
| OPPTS | The Office of Prevention, Pesticides, and Toxic Substances |
| rat WEC | whole rat embryo culture |

| | |
|---|---|
| AR | androgen receptor |
| ER$\alpha$ | estrogen receptor alpha |
| ERß | estrogen receptor beta |
| TR$\alpha$ | thyroid receptor alpha |
| TRß | thyroid receptor beta |
| IHC | immunohistochemistry |
| WB | western blot |

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