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Endocrine Disrupting Investigations of Six Pesticides with Estrogen Receptor Binding Assays and Uterotrophic Effects in Rats

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Abstract

Lu, S. Y., and Tsai, W. R. 2018. Endocrine disrupting investigations of six pesticides with estrogen receptor binding assays and uterotrophic effects in rats. Taiwan Pestic. Sci. 5: 31-51.

The previous reports showed that estrogen receptor played an important role in reproductive toxicity in rats. Estrogen receptor disrupting is one of important endocrine disrupting (ED) issue. Due to the *in vitro* estrogen binding assay with radioisotope it was unfriendly to most of laboratories in spite of reducing animals. We aimed to make sure if the uterotrophic assay can cover the *in vitro* method for ED screening with six reported reproductive toxicity and ED pesticides including permethrin, endosulfan, cypermethrin, methyl parathion, benomyl and carbendazim. The results showed that in the estrogen receptor competitive binding assay the inhibition concentration with the half maximal inhibitory concentration (IC_{50}) were 141, 249, 523, 1022, 1413 and 4334 μM in permethrin, endosulfan, cypermethrin, methyl parathion, benomyl and carbendazim, respectively. This implied that permethrin, endosulfan and cypermethrin showed estrogen receptor affinity without identification of agonist or antagonist while methyl parathion, benomyl and carbendazim exhibited none to weak estrogenic. In the uterotrophic assay permethrin exhibited significantly antiestrogenic while endosulfan showed approaching estrogen receptor agonist. As the permethrin did cypermethrin exhibited significantly antiestrogenic activity while methyl parathion did not. Both benomyl and carbendazim showed estrogenic-like agonist. In comparison with endpoint of estrogen receptor binding and uterotrophic assay in these pesticides revealed that the coincidence existed in permethrin, cypermethrin, and methyl parathion

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and even in endosulfan. It seems that the coincidence of estrogen receptor binding and uterotrophic assay did not exist in benomyl and carbendazim. That is because the uterotrophic effect was induced by benomyl and carbendazim through androgen receptor activation referring to our previous reports. This study concluded that in terms of estrogen receptor affinity the order is permethrin＞ endosulfan＞ cypermethrin＞ methyl parathion ＞ benomyl＞ carbendazim. The final outcome should depend on the *in vivo* study, uterotrophic assay. Permethrin and cypermethrin exhibited anti-estrogenic activity while endosulfan showed weak estrogenic activity and methyl parathion did not. Benomyl and carbendazim increased uterus fluid through androgenic activity. Based on above we suggest that uterotrophic test not only can cover the *in vitro* estrogen receptor binding assay but show more friendly and robust than *in vitro* method as well.

Key words: Cypermethrin, Permethrin, Endosulfan, Methyl parathion, Benomyl, Carbendazim

Introduction

The final endocrine disruptor screening program test guidelines are generally intended to meet testing requirements under Toxic Substance Control Act (TSCA, USEPA), Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA, USEPA) and Federal Food, Drug and Cosmetic Act (FFDCA, USFDA) to determine if a chemical substance may pose a risk to human health or the environment due to the disruption of the endocrine system. The series 890[37] - endocrine disruptor screening program test guidelines tier I included amphibian metamorphosis (frog), *in vitro* androgen receptor binding (rat prostate), aromatase (human recombinant), *in vitro* estrogen receptor binding (rat uterus), estrogen receptor transcriptional activation (human cell line HeLa-9903), fish short-term reproduction, Hershberger (rat), female pubertal (rat), male pubertal (rat), steroidogenesis (human cell line - H295R) and uterotrophic (rat). Due to the different mechanisms of endocrine disrupting activity it is necessary to develop respective screening tests. In terms of estrogen receptor binding, *in vitro* estrogen receptor binding (rat uterus), estrogen receptor transcriptional activation (human cell line HeLa-9903) and uterotrophic (rat) might be used but the former two need to use radioisotope and maintain a stably cell line, respectively and this is not friendly to most of laboratories. In order to make sure if uterotrophic assay can determine the estrogenic binding activity as *in vitro* estrogen receptor

binding test did we compare the *in vitro* estrogen receptor binding and uterotrophic assay with the reported reproductive toxic or endocrine disrupting pesticides, cypermethrin [38, 13], permethrin [9, 7, 36], endosulfan [29, 5, 2, 14], methyl parathion [12, 25, 26, 31], carbendazim [6, 16, 17, 20, 21, 30] and benomyl [28, 33] to test.

Chemical structures of benomyl (Methyl 1-(butylcarbamoyl) benzimidazol-2-ylcarbamate) and carbendazim (methyl benzidazol-2-ylcarbamat) are fungicides while cypermethrin ((*RS*)-α-cyano-3-phenoxybenzyl (1*RS*)-*cis, trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate), endosulfan ((1,4,5,6,7,7-dexachloro-8,8,10-trinorborn-5-en-2,3-ylenebismethylene)sulfite), methyl parathion (*o,o*-dimethyl o-4-nitrophenyl phosphorothioate) and permethrin (3-phenoxybenzyl (1*RS*)-*cis*, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecar boxylate) are insecticides (Fig. 1). Though endosulfan is prohibited and methyl parathion is limited some times the smuggling cases might be took place in Taiwan [15]. Cypermethrin, permethrin, carbendazim and benomyl are common used in Taiwan. This study aimed to compare the six reported pesticides exhibiting reproductive toxicity and endocrine disrupting activity with *in vitro* estrogen receptor binding and uterotrophic assays and to determine if uterotrophic assay can cover the *in vitro* methods.

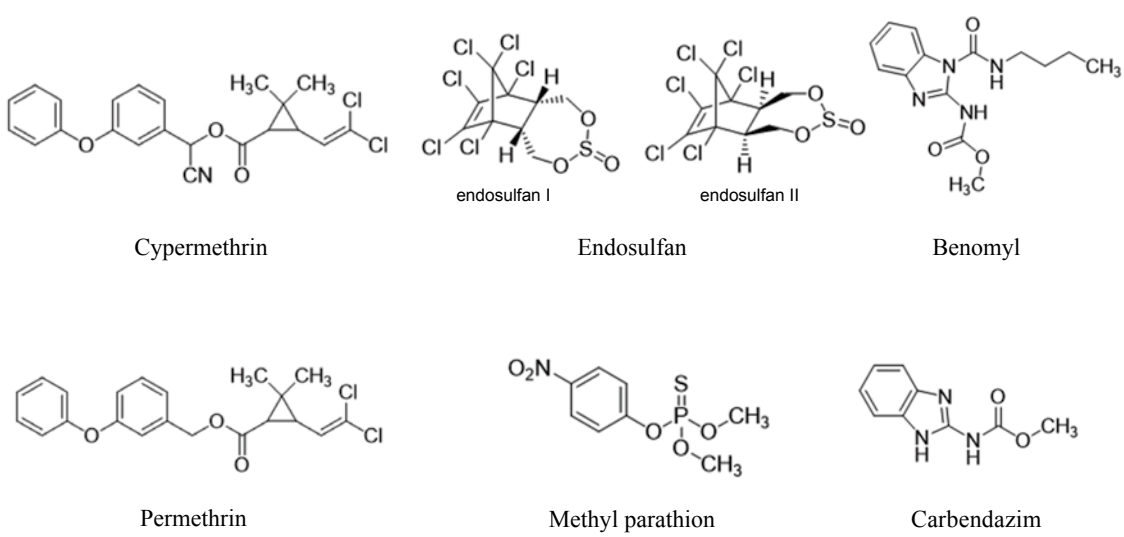

Fig. 1. Chemical structure of cypermethrin, permethrin, endosulfan, methyl parathion, benomyl and carbendazim.

Materials and methods

Materials were obtained from the following manufacturers: 17ß-estradiol (E_2, purity ≥ 98%), endosulfan (purity ≥ 97%), methyl parathion (purity ≥ 95%), and corn oil (0.9 g/mL), Sigma Chemical Co. (St. Louis, MO); permethrin (purity ≥ 96%), cypermethrin (purity ≥ 97%), carbendazim (MBC, purity ≥ 99%), and benomyl (purity ≥ 99%), Sino Co. (Taichung, Taiwan, ROC).

I. Animals and housing

The animal use protocol was reviewed and approved (13-TACTRI-IACUC-003) by the Institutional Animal Care and Use Committee (IACUC) of the Taiwan Agricultural Chemicals and Toxic Substances Research Institute. Female Wistar rats were purchased from the BioLASCO (Taipei, Taiwan, ROC). During the experiment, the rats were housed two to three per cage in suspended aluminum cages with stainless-steel wire-mesh front and floor under controlled environmental conditions, including a temperature of 24 ± 2℃, a relative humidity of 55 ± 10%, a frequency of ventilation of more than 10 air exchanges per hour, and a 12-h light/dark cycle (lighting period, 0800-2000). Drinking water and pellet rodent diet were available *ad libitum*. After quarantine period, all animals in good health based on clinical signs and body weights were selected for operations.

For estrogen receptor competitive binding assay, uterus cytosol was extracted from untreated female rat, 8 week-old. For estrogenicity assay, ovariectomy (OVX) for female rats was performed under Zoletil 50 anesthesia at about 7-week old by opening the dorsolateral abdominal wall at the midpoint between the costal inferior border and the iliac crest, a few millimeters from the lateral margin of the lumbar muscle. Within the abdominal cavity, the ovaries were located. On an aseptic field, the ovaries were physically removed from the abdominal cavity. A ligature was placed between the ovary and uterus to control bleeding and the ovary was detached by incision above the ligature at the junction of the oviduct and each uterine horn. After the surgery, females were acclimated for 30 days to release the operation stress and to monitor estrus cycling for confirmation whether OVX was performed successfully. In order to make sure that the OVX rats were successfully, the OVX rats should not go to the estrus cycle with proestrus and estrous. That is only those animals found to be diestrus or metestrus was used in the experiment.

II. Study design

For each experiment, each group consisted of five animals. Test and reference substances were suspended or dissolved daily in vehicles

(corn oil). According to OECD test guideline (OECD 414) the volume should not exceed 1 mL/100g body weight, except in the cae of aqueous solutions where 2 mL/100g body weight may be used. When corn oil is used as a vehicle, the volume should not exceed 0.4 mL/100g body weight. The daily amounts of administration were 2.5 mL/kg body weight for oral gavage and subcutaneous injections of E_2. The dose design was summarized in Table 1. Oral gavage was selected because it is one of the potential exposure routes of test chemicals for humans. For female rat, the amount administered for each animal was calculated based on body weights. For all experiments, clinical signs, body weight, weights of liver and kidneys were assessed as indices of systemic toxicity. Clinical signs including any abnormal appearance of behavior were recorded twice a day in each animal.

Table 1. Study design for the uterotrophic test[5)]

Dose (mg/kg/day)[4)]	Estrogenic		Anti-estrogenic/estrogenic	
Treatment groups[1)]	1[8)]	2[9)]	3[10)]	4[11)]
Control (intact)				
Control (OVX)[2)]	+[6)]	+	+	+
E_2 5 (sc)[3)]	-[7)]	-	+	+
Corn oil (oral)	+	-	+	-
Cypermethrin 15.6	-	+	-	+
Permethrin 5	-	+	-	+
Endosulfan 4	-	+	-	+
Methyl parathion 2.5	-	+	-	+
Benomyl 200	-	+	-	+
Carbendazim 200	-	+	-	+

1) All treatment groups contain 5 female rats.
2) OVX: ovariectomy
3) E_2 5 (sc): 17ß-estradiol, 5 mg/kg/day, subcutaneously
4) Dose (mg/kg/day): cypermethrin 15.6; permethrin 5; endosulfan 4; methyl parathion 2.5; carbendazim 200; benomyl 200.
5) Comparison pairs for uterotrophic assay is follows, respectively: treatment group 1 vs. treatment group 2; treatment group 3 vs. treatment group 4.
6) +: with
7) -: without
8) Group 1 (estrogenic- baseline): OVX with corn oil (oral)
9) Group 2 (estrogenic-by pesticides): OVX with pesticide treatment (oral)
10) Group 3 (estrogenic-positive): OVX with positive control E_2
11) Group 4 (anti-estrogenic/estrogenic): OVX with both E_2 and pesticide each

III. Estrogen receptor binding assay *in vitro*

A ligand binding assay was carried out to determine the concentration of estrogen receptor in untreated rat tissue following the procedures described by Nonneman et al. (1992)[27]. The ligand [2,4,6,7,16,17-^{3}H(N)]-17β-estradiol (E_2) (110-170 Ci/mmol) was purchased from NEN Life Science Products, Inc., Boston, MA. Nonlabeled 17β-estradiol was purchased from Sigma Chemical Company, St. Louis, MO and recrystallized from ethanol prior to use. Rat uterus was homogenized in ice-cold low salt-TEDG buffer, pH 7.4, consisted of 10 mM Tris, 1.5 mM EDTA, 10% glycerol, and 1 mM each of dithiothreitol at a ratio of 1:10 (w/v). The tissue homogenate was centrifuged at 100,000 × g for 1h and the resulting supernatant was subjected to use as the low-salt extract. Prior to analysis, endogenous steroids were removed from the low-salt extract by incubation with dextran-coated charcoal. Binding of [^{3}H] 17β-estradiol to estrogen receptor of uterus extract was. determined by competitive inhibition using nonlabeled 17β-estradiol. An aliquot of the charcoal-treated uterus extract was incubated with 1 nM [^{3}H] 17β-estradiol. Nonspecific binding was determined by incubating the extract with 100-fold excess nonlabeled 17β-estradiol at 4°C for 24h, during which time [^{3}H] 17β-estradiol bound to the unoccupied estrogen receptors. Unbound [^{3}H]17β-estradiol was separated from the bound steroid by adding the extract to packed hydroxyapatite (HAP) in the low salt-TEDG buffer. The mixture was incubated for 30 min with several mixings and then centrifuged at 600 × g for 3 min at 4°C. The supernatant was aspirated. The packed HAP was washed four times with ice-cold 50 mM Tris buffer, pH 7.3. For determination of total binding, the bound [^{3}H] 17β-estradiol was extracted from HAP with ethanol and counted for radioactivity using a Beckman Model LS6000 TA on line radioisotope detector. Specific binding was determined by substracting nonspecific binding from total binding and corrected for protein concentration in uterus extract. Protein concentration was determined according to the method of Lowry et al. (1951)[18] using bovine serum albumin as a standard.

For the analyses of effects of permethrin, endosulfan, cypermethrin, methyl parathion, benomyl, and carbendazim on estrogen receptor binding, specific binding of [^{3}H] 17β-estradiol to uterus extract was determined by incubation of the charcoal treated uterus extract with [^{3}H] 17β-estradiol in the presence of permethrin, endosulfan, cypermethrin, methyl parathion, benomyl, and carbendazim at 4°C for 24 h. The concentrations of all test chemicals were 0, 4, 40, 400 and 4000 μM

except permethrin (0, 4, 40 and 400 μM). Data of permethrin at 4000 μM was not available due to artificial mistake. The incubation mixture was subjected to same procedures in estrogen receptor binding assay as described above.

IV. Assessment for estrogenicity *in vivo*

A ten-day uterotrophic assay using ovariectomized rats was performed to determine if permethrin, endosulfan, cypermethrin, methyl parathion, benomyl and carbendazim interfere with ER-mediated mechanisms *in vivo*. These chemicals were administered to ovariectomized rats by oral gavage for 10 days. The dose levels of permethrin (5 mg/kg/day), endosulfan (4 mg/kg/day), cypermethrin (15.6 mg/kg/day), methyl parathion (2.5 mg/kg/day), benomyl (200 mg/kg/day), and carbendazim (200 mg/kg/day) were selected on the basis of the dose-finding study was described below. The dose-finding study of test chemicals was based on the one tenth of LD_{50}. The doses of test chemicals were permethrin (5, 10, 20 and 40 mg/kg/day), endosulfan (2, 4 and 8 mg/kg/day), cypermethrin (15.6, 31.2, 62.5 and 125 mg/kg/day), methyl parathion (1.25, 2.5 and 5 mg/kg/day), benomyl (25, 50, 100, 200, 400 and 800 mg/kg/day), and carbendazim (25, 50, 100, 200, 400 and 800 mg/kg/day) in the preliminary experiment. Endosulfan 8, cypermethrin 125, methyl parathion 5 mg/kg/day resulted in death in the preliminary test. Doses of permethrin 5, endosulfan 4, cypermethrin 15.6, methyl parathion 2.5, benomyl 200 and carbendazim 200 mg/kg/day showed the peak of estrogenic activity in the preliminary test. Based on the preliminary test we chose these doses to carry out the test. One day after the final administration, rats were euthanized by blood withdrawal from the abdominal aorta under light ether anesthesia, and then uterus with fluid, thymus, thyroid, liver, lung, adrenal, kidneys, and bladder were dissected and weighed after careful trimming to remove fat and other contiguous tissue in a uniform manner.

V. Statistical analysis

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

Results

I. Organ weight

Organ weights of thymus, thyroid, liver, lung, adrenal, kidneys, and bladder in treatments of sc corn oil + oral corn oil (intact), sc

corn oil + oral corn oil, sc corn oil + oral cypermethrin 15.6, sc corn oil + oral permethrin 5, sc corn oil + oral endosulfan 4, sc corn oil + oral methyl parathion 2.5, sc corn oil + oral benomyl 200, sc corn oil + oral carbendazim 200, sc E_2 5 + oral corn oil, sc E_2 5 + oral cypermethrin 15.6, sc E_2 5 + oral permethrin 5, sc E_2 5 + oral endosulfan 4, sc E_2 5 + oral methyl parathion 2.5, sc E_2 5 + oral benomyl 200 and sc E_2 5 + oral carbendazim 200 were weighed. Basically based on the designation of Group 1 vs. Group 2; Group 2 vs. Group 3, 4, 5, 6, 7, 8, 9; Group 9 vs. Group 10, 11, 12, 13, 14, 15 kidney weight was changed in most of groups. Weights of liver, thumus, thyroid and bladder were also changed in some groups. All these organ weights seem not to disturb the effect of pesticides on uterus weight because body weight was not significantly decreased (Table 2).

II. *Estrogen receptor competitive binding assay in vitro*

Fig. 1 is to show the structure of cypermethrin, permethrin, endosulfan, methyl parathion, carbendazim, and benomyl. Chemical structure plays an important role in competitive binding activity. We set up the standard curve of estrogen receptor competitive binding to make sure the testing system with uterus extract of untreated mature female rats. In the standard curve total count (TB), nonspecific binding (NSB) and specific binding (SB) increased with concentration-dependent manner (data not shown). It means that estrogen receptor can be detected in the uterus extract. In the estrogen receptor competitive binding assay the inhibition concentration (IC_{50}) with 50% of control were 141, 249, 523, 1022, 1413 and 4334 μM in permethrin (Fig. 2A) , endosulfan (Fig. 2C), cypermethrin (Fig. 3A), methyl parathion (Fig. 3C), benomyl (Fig. 4A) and carbendazim (Fig. 4C), respectively. Based on these data the order of estrogen receptor binding activity is permethrin > endosulfan > cypermethrin > methyl parathion > benomyl > carbendazim.

III. *Estrogenicity and antiestrogenicity in vivo*

We can not distinguish the estrogenicity from antiestrogenicity based on the estrogen receptor competitive binding assay. Because estrogen receptor binding only showed us the interaction between estrogen receptor of extract and test chemical *in vitro* we should further carried out to process the uterotrophic assay *in vivo*. In the uterotrophic assay ovariectomized rats significantly decreased the uterus weight with fluid when compared to the intact female rat (Fig. 2B, Fig. 5). Treatment with permethrin 5 mg/kg/day only for 10 days showed

Table 2. Absolute and relative organ weight at necropsy in the female rats

Treatments[1)]	BWf[3)] (g)	LuW[4)] (g/%)[2)]	LiW[5)] (g/%)	KW[6)] (g/%)	AW[7)] (g/%)	TuM[8)] (g/%)	TrW[9)] (g/%)	BlW[10)] (g/%)
1	341±10	2.1±0.1	10.9±0.7	2.8±0.7	0.08±0.00	0.37±0.06	0.034±0.008	0.13±0.01
		0.62±0.03	3.18±0.22	0.83±0.21	0.024±0.001	0.107±0.020	0.010±0.002	0.040±0.004
2	353±37	2.3±0.5	11.7±1.5	2.3±0.2*	0.07±0.01	0.29±0.10	0.026±0.005*	0.12±0.02
		0.66±0.17	3.32±0.42	0.64±0.05*	0.020±0.003*	0.081±0.021	0.007±0.001*	0.034±0.004
3	336±24	2.2±0.6	14.6±4.7	3.0±0.8*	0.07±0.01	0.28±0.11	0.022±0.003	0.16±0.04*
		0.64±0.16	4.31±1.21	0.90±0.20*	0.022±0.002	0.08±0.03	0.007±0.001	0.046±0.011*
4	330±52	2.2±0.6	14.2±4.1	3.1±0.8*	0.07±0.01	0.28±0.04	0.029±0.013	0.14±0.03
		0.67±0.20	4.41±1.42	0.98±0.33	0.022±0.004	0.09±0.02	0.009±0.003	0.042±0.006
5	339±21	2.0±0.8	15.8±4.7*	3.4±1.0*	0.09±0.01*	0.31±0.13	0.028±0.009	0.16±0.03*
		0.60±0.24	4.68±1.48*	1.01±0.32*	0.026±0.004*	0.09±0.04	0.008±0.003	0.046±0.009*
6	325±25	2.1±0.4	13.6±3.5	2.9±0.9*	0.08±0.01*	0.19±0.10*	0.033±0.014	0.15±0.04
		0.63±0.11	4.15±0.76*	0.88±0.20*	0.026±0.004*	0.06±0.03	0.010±0.003*	0.045±0.011*
7	338±35	1.9±0.4	15.0±5.3	2.9±0.7*	0.08±0.02	0.52±0.26*	0.030±0.006	0.14±0.02
		0.57±0.13	4.43±1.48	0.87±0.19*	0.023±0.007	0.16±0.08	0.009±0.002*	0.041±0.005*
8	330±33	1.9±0.4	12.9±3.9	2.9±0.6*	0.07±0.02	0.38±0.23	0.018±0.008	0.13±0.02
		0.58±0.14	3.88±1.07	0.89±0.15*	0.022±0.003	0.12±0.06	0.006±0.003	0.040±0.007
9	312±29*	2.2±0.5	13.3±1.7	2.3±0.4	0.07±0.01	0.13±0.09*	0.026±0.007	0.16±0.04*
		0.70±0.20	4.27±0.50*	0.75±0.06*	0.023±0.005	0.04±0.03*	0.008±0.002	0.051±0.014*
10	296±25	2.2±0.4	14.5±3.3	2.7±0.7	0.07±0.01	0.09±0.04	0.023±0.003	0.17±0.05
		0.75±0.09	4.87±0.82	0.93±0.24	0.023±0.006	0.03±0.01	0.008±0.001	0.058±0.019
11	299±8	1.4±0.1*	10.8±1.0*	2.2±0.1	0.07±0.00	0.05±0.02	0.029±0.007	0.13±0.03
		0.46±0.05	3.64±0.35	0.74±0.04	0.023±0.000	0.02±0.01	0.010±0.003	0.045±0.010
12	314±13	2.1±0.7	16.3±4.7	2.9±0.6*	0.07±0.02	0.06±0.02*	0.027±0.014	0.16±0.04
		0.67±0.22	5.24±1.59	0.92±0.20*	0.024±0.005	0.02±0.005*	0.009±0.004	0.050±0.011
13	307±23	1.8±0.4	14.4±3.1	2.9±0.6	0.07±0.02	0.06±0.04	0.029±0.007	0.15±0.02
		0.60±0.15	4.73±1.26	0.95±0.22*	0.024±0.006	0.02±0.012	0.009±0.002	0.050±0.007
14	288±34	1.6±0.4	16.4±5.0	3.0±0.9	0.09±0.02	0.16±0.12	0.026±0.007	0.15±0.03
		0.57±0.11	5.65±1.49	1.04±0.24	0.030±0.005	0.05±0.04	0.009±0.003	0.052±0.006
15	274±20	1.8±0.4	15.2±4.7	3.0±0.8	0.07±0.02	0.16±0.13	0.026±0.009	0.13±0.012
		0.64±0.14	5.56±1.70	1.10±0.30	0.025±0.007	0.06±0.05	0.010±0.003	0.046±0.005

[1)] Treatments:1.sc corn oil + oral corn oil (intact), 2.sc corn oil + oral corn oil, 3.sc corn oil + oral cypermethrin 15.6, 4.sc corn oil + oral permethrin 5, 5.sc corn oil + oral endosulfan 4, 6.sc corn oil + oral methyl parathion 2.5, 7.sc corn oil + oral benomyl 200, 8.sc corn oil + oral carbendazim 200, 9.sc E_2 5 + oral corn oil, 10.sc E_2 5 + oral cypermethrin 15.6, 11.sc E_2 5 + oral permethrin 5, 12.sc E_2 5 + oral endosulfan 4, 13.sc E_2 5 + oral methyl parathion 2.5, 14.sc E_2 5 + oral benomyl 200, 15.sc E_2 5 + oral carbendazim 200, OVX: treatment 2-treatment 15

[2)] Data are expressed as absolute (g)/relative (%)weight

[3)] BWf: body weight finally at necropsy

[4)] LuW: lung weight

[5)] LiW: liver weight

[6)] KW: kidney weight

[7)] AW: adrenal weight

[8)] TuM: thymus weight

[9)] TrW: thyroid weight

[10)] BlW: bladder weight

* $P < 0.05$(Group 1 vs. Group 2; Group 2 vs. Group 3, 4, 5, 6, 7, 8, 9; Group 9 vs. Group 10, 11, 12, 13, 14, 15)

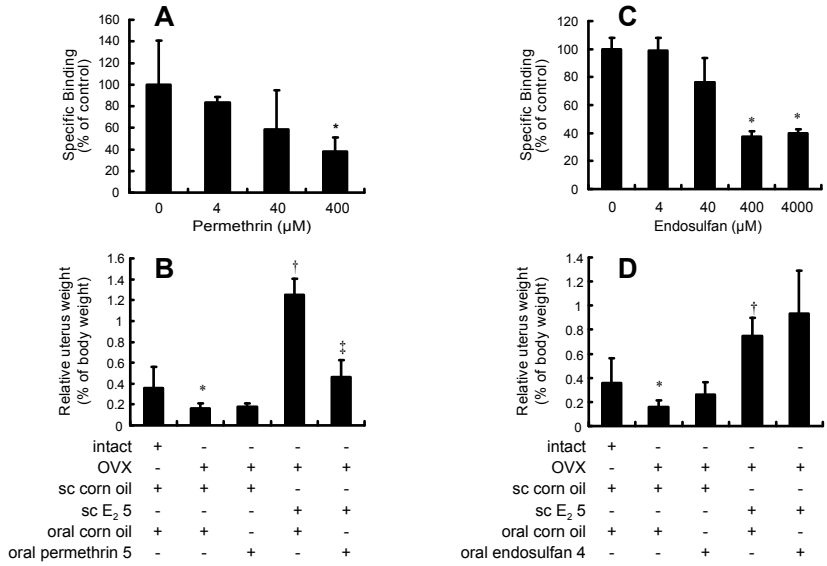

Fig. 2. Effects of treatment with permethrin (A and B) or endosulfan (C and D) on estrogen receptor binding *in vitro* and relative uterus weight *in vivo*, respectively. OVX: ovariectomy; sc: subcutaneously; E_2 5 (sc): 17β-estradiol 5 mg/kg/day.

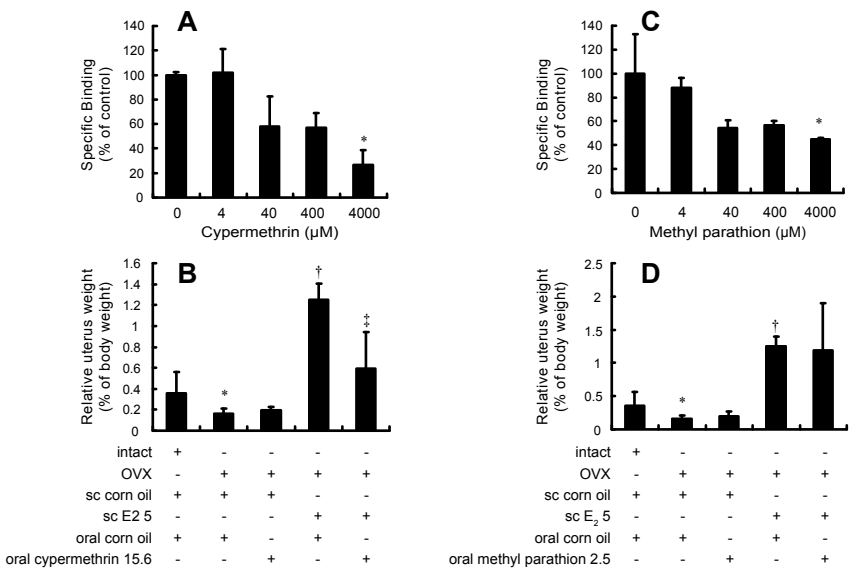

Fig. 3. Effects of treatment with cypermethrin (A and B) or methyl parathion (C and D) on estrogen receptor binding *in vitro* and relative uterus weight *in vivo*, respectively. OVX: ovariectomy; sc: subcutaneously; E_2 5 (sc): 17β-estradiol 5 mg/kg/day.

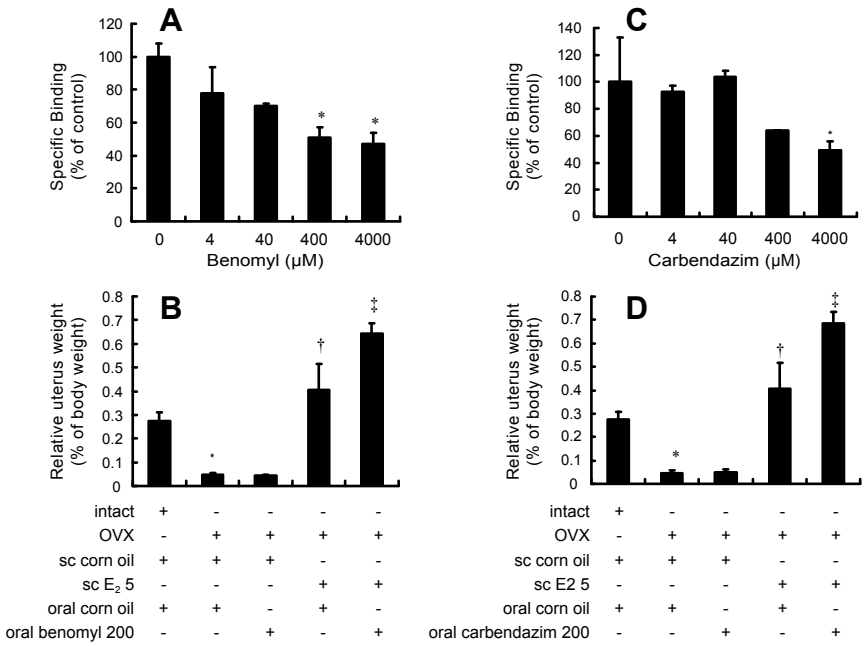

Fig. 4. Effects of treatment with carbendazim (A and B) or benomyl (C and D) on estrogen receptor binding *in vitro* and relative uterus weight *in vivo*, respectively. OVX: ovariectomy; sc: subcutaneously; E_2 5 (sc): 17β-estradiol 5 mg/kg/day.

no significant effect and treatment with E_2 5 mg/kg/day significantly increased the uterus weight with fluid (Fig. 2B, Fig. 6). Co-treatment with permethrin and E_2 significantly reduced the uterus compared to that treatment with E_2 only (Fig. 2B, Fig. 6). Treatment with endosulfan 4 mg/kg/day increased uterus weight though it is no significant (Fig. 2D, Fig. 5). Co-treatment with endosulfan and E_2 increased the uterus weight but it is not significant (Fig. 2D, Fig. 6). Treatment with cypermethrin 15.6 mg/kg/day showed no significant effect on uterus weight (Fig. 3B, Fig. 5). Co-treatment with cypermethrin and E_2 significantly decreased the uterus weight compared to that treatment with E_2 only (Fig. 3B, Fig. 6). Treatment with methyl parathion 2.5 mg/kg/day (Fig. 3D, Fig. 5) or cotreatment with methyl parathion and E_2 showed no significant effect on uterus weight (Fig. 3D, Fig. 6). Treatment with benomyl 200 (Fig. 4B, Fig. 5) or carbendazim 200 mg/kg/day (Fig. 4D, Fig. 5) showed no effect on uterus weight. Co-treatment with benomyl (Fig. 4B, Fig. 6) or carbendazim (Fig. 4D, Fig. 6) and E_2 significantly increased uterus weight compared to that treatment with E_2 only.

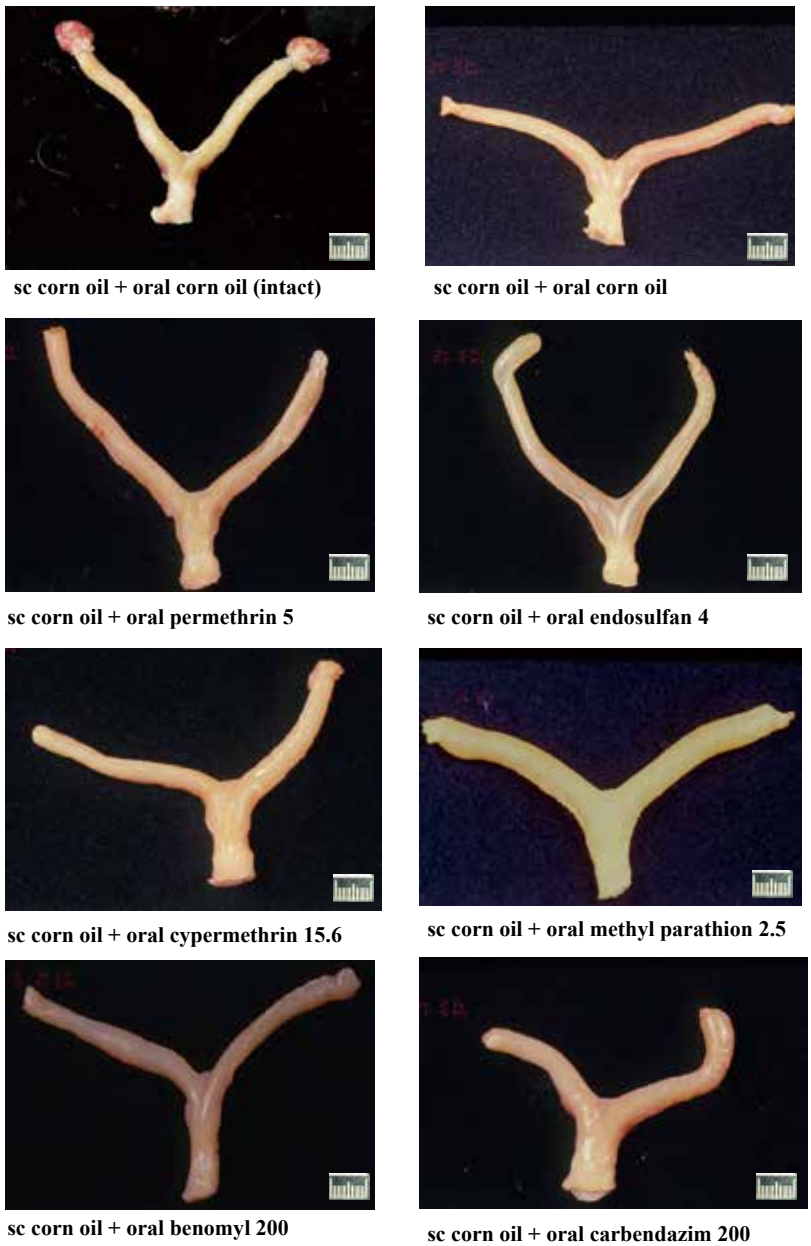

Fig. 5. Uterus appearance of treatment with permethrin, endosulfan, cypermethrin, methyl parathion, carbendazim and benomyl alone. Endosulfan increased uterus weight though it is not significant and the other five pesticides each showed no effect on uterus weight.

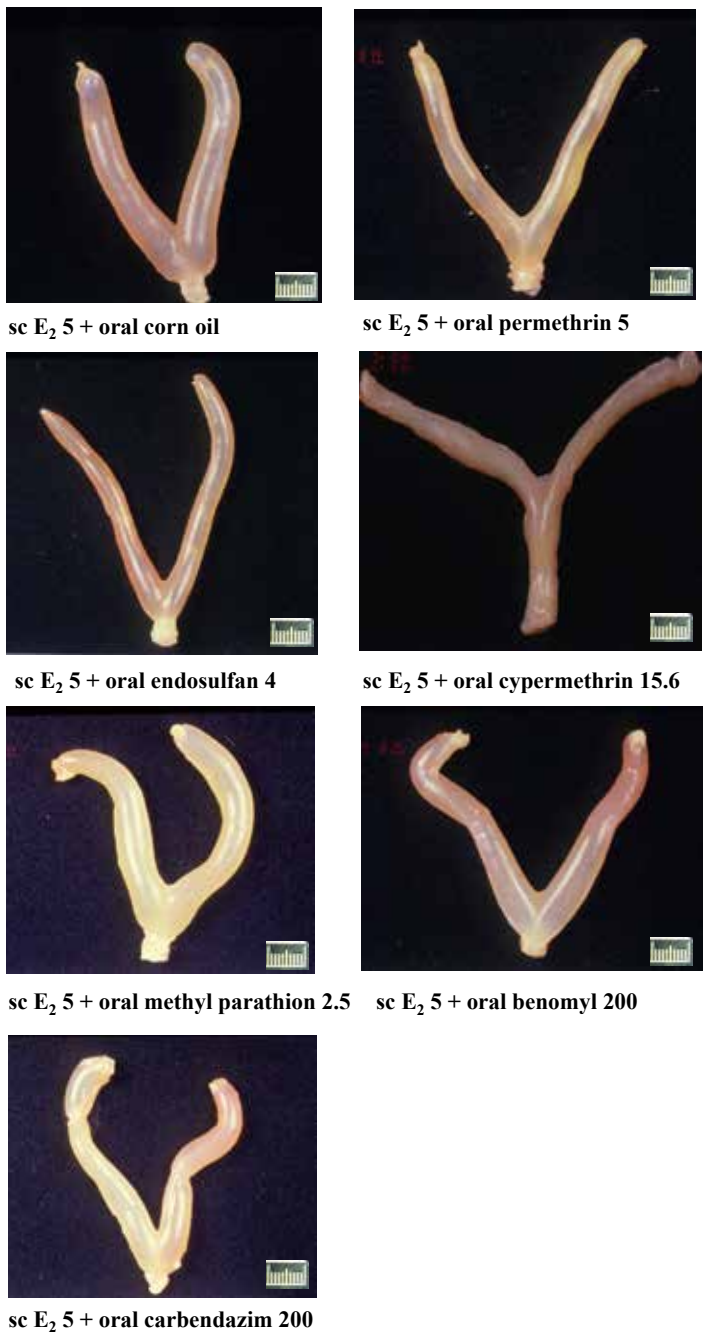

Fig. 6. Uterus appearance of co-treatment with permethrin, endosulfan, cypermethrin, methyl parathion, carbendazim or benomyl each and E_2.

Discussion

This study finished two findings. The first we inferred that permethrin and cypermethrin exhibited anti-estrogenic activity while endosulfan showed weak estrogenic activity and methyl parathion did not. Benomyl and carbendazim increased uterus fluid through androgenic activity. The second we confirmed that the high relationship between *in vitro* estrogen receptor binding assay and uterotrophic test with cypermethrin, permethrin, endosulfan, methyl parathion, carbendazim and benomyl. Based on above we concluded that uterotrophic test not only can cover the *in vitro* estrogen receptor binding assay but show more friendly and robust than *in vitro* method as well.

This study showed that cypermethrin and permethrin exhibited high affinity of estrogen receptor with low IC_{50} values *in vitro*. This result might be supported by Kakko et al. (2004) [8]. Kakko et al. (2004) [8] reported that oestradiol potentiates the effects of cypermethrin and permethrin. Also Jin et al (2004) [7] reported that permethrin enantiomers induced estrogen-responsive gene expression in embryo-larval zebrafish. In present uterotrophic test cypermethrin and permethrin decreased the uterus weight with anti-estrogenic activity. Previous reports concluded the same result of anti-estrogenic effect induced by permethrin but not cypermethrin in MCF-7 cell lines [9]. The only one inconsistent report on permethrin was lack of (anti-) androgenic or estrogenic effects in the Hershberger and uterotrophic assays [11]. To sum up *in vitro* assay for estrogen receptor usually only concluded the affinity of test substance if no estrogen positive was added. The affinity to estrogen receptor for test substance can not be identified as estrogen agonist or antagonist. In the present uterotrophic test for cypermethrin and permethrin showed the final outcome anti-estrogenic activity *in vivo*.

Also, previous reports reported the estrogen-related effects such as modulating gonadotropin synthesis via calcium homeostasis and ERK1/2 signaling in LßT2 mouse pituitary cells [13]. In addition to the estrogen receptor cyperemthrin inhibited interleukin-6-induced androgen receptor transactivation through signal transducer and activator of transcription 3 (STAT3) [38]. Furthermore, cypermethrin and permethrin exhibited anti-androgen receptor and anti-thyroid receptor ß in reporter gene assays [4]. Also, permethrin potentially disrupted the thyroid endocrine system in fish [36]. Cyperemthrin possess endocrine-disrupting potential *in vitro* that can be mediated via ER, AR and aromatase activities [10].

In the present study endosulfan showed

weak estrogen receptor agonistic effect both *in vitro* and *in vivo* assays though it is not significant. This result is supported by Li et al. (2013) [14]. The indirect related effect induced by endosulfan was to up-regulate telomerase reverse transcriptase (TERT) mRNA expression in MCF-7 cells [5]. Also, it was reported to exhibit anti-progestin, and anti-androgen in vitro bioassay [2].

In the present study methyl parathion showed low estrogen receptor affinity *in vitro* and no estrogenic effect *in vivo*. There is no report on endocrine disrupting activity induced by methyl parathion though there were some reproductive toxicity such as testis damage [26, 24], epididymis [31], male reproductive organ [25], uterine and placenta [12].

In the present study carbendazim and benomyl exhibited low affinity to estrogen receptor but showed estrogenic-like agonist with significant increasing uterus weight. There is no estrogen receptor agonist induced by these two pesticides. There were estrogen-related reports on benomyl. Benomyl was reported to inhibit AR expression in LNCaP prostate cell and prostate-specific antigen (PSA) in H295R adrenocortical carcinoma cell [33], and aromatase activity in H295R cell [33] and in human ovarian granulose-like tumor cell line (KGN) [28, 20]. In contrary to Robitaille et al. (2015) [33] report on AR inhibition, our previous report showed that benomyl and carbendazim induced androgenic activity in uterotrophic and Hershberger assays [17]. The discrepancy between Robitaille et al. (2015) [33] and Lu et al. (2015) [17] would be the test system. As we explained that *in vitro* estrogen receptor binding assay can not identify as agonist or antagonist [33] used H295R adrenocortical carcinoma cell to conclude the benomyl affinity to androgen receptor. In addition to the evidence of androgen receptor agonist, there are some reports to support the androgenic activity induced by benomyl and carbendazim [1, 3, 22, 23, 32, 33, 35].

Though the replacement, refinement and reduction are the trend of animal study, accurate experimental results is still an important issue. *In vitro* estrogen receptor binding assay can reduce the animal number but the radioisotope procedures need a lot of radioactive waste treatment. Generally speaking among the eleven in vitro and *in vivo* screening tests for endocrine disrupting of USEPA 890 series *in vitro* estrogen receptor binding assay and uterotrophic test have the common mechanisms.

Basically this study showed that there is high coincidence between *in vitro* estrogen receptor competitive binding and uterotrophic assays for these test pesticides. Also this study showed that estrogen and androgen receptors played an important role in reproductive toxicity in rats.

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Literature cited

1. Carter, S. D., Hess, R. A., and Laskey, J. W. 1987. The fungicide methyl 2-benzimidazole carbamate causes infertility in male Sprague-Dawley rats. Biol. Reprod. 37: 709-717.
2. Chatterjee, S., Kumar, V., Majumder, C. B., and Roy, P. 2008. Screening of some anti-progestin endocrine disruptors using a recombinant yeast based *in vitro* bioassay. Toxicol. *in Vitro* 22: 788-798.
3. Cummings, A. M., Ebron-McCoy, M. T., Rogers, J. M., Barbee, B. D., and Harris, S. T. 1992. Developmental effects of methyl benzimidazolecarbamate following exposure during early pregnancy. Fundam. Appl. Toxicol. 18: 288-293.
4. Du, G., Shen, O., Sun, H., Fei, J., Lu, C., Song, L., Xia, Y., Wang, S., and Wang, X. 2010. Assessing hormone receptor activities of pyrethroid insecticides and their metabolites in reporter gene assays. Toxicol. Sci. 116: 58-66.
5. Je, K. H., Kim, K. N., Nam, K. W., Cho, M. H., and Mar, W. 2005. TERT mRNA expression is up-regulated in MCF-7 cells and a mouse mammary organ culture (MMOC) system by endosulfan treatment. Arch. Pharm. Res. 28: 351-357.
6. Jeffay, S. C., Libbus, B. L., Barbee, R. R., and Perreault, S. D. 1996. Acute exposure of female hamsters to carbendazim (MBC) during meiosis results in aneuploid oocytes with subsequent arrest of embryonic cleavage and implantation. Reprod. Toxicol. 10: 183-189.
7. Jin, Y., Chen, R., Sun, L., Wang, W., Zhou, L., Liu, W., and Fu, Z. 2009. Enantioselective induction of estrogen-responsive gene expression by permethrin enantiomers in embryo-larval zebrafish. Chemosphere 74: 1238-1244.
8. Kakko, I., Toimela, T., and Tähti, H. 2004. Oestradiol potentiates the effects of certain pyrethroid compounds in the MCF7 human breast carcinoma cell line. Altern. Lab. Anim. 32: 383-390.

9. Kim, I. Y., Shin, J. H., Kim, H. S., Lee, S. J., Kang, I. H., Kim, T. S., Moon, H. J., Choi, K. S., Moon, A., and Han, S. Y. 2004. Assessing estrogenic activity of pyrethroid insecticides using *in vitro* combination assays. J. Reprod. Dev. 50: 245-255.
10. Kjeldsen, L. S., Ghisari, M., and Bonefeld-Jørgensen, E. C. 2013. Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity. Toxicol. Appl. Pharmacol. 272: 453-464.
11. Kunimatsu, T., Yamada, T., Ose, K., Sunami, O., Kamita, Y., Okuno, Y., Seki, T., and Nakatsuka, I. 2002. Lack of (anti-) androgenic or estrogenic effects of three pyrethroids (esfenvalerate, fenvalerate, and permethrin) in the Hershberger and uterotrophic assays. Regul. Toxicol. Pharmacol. 35: 227-237.
12. Levario-Carrillo, M., Olave, M. E. T., Corral, D. C., Alderete, J. G., Gagioti, S. M., and Bevilacqua, E. 2004. Placental morphology of rats prenatally exposed to methyl parathion. Exp. Toxicol. Pathol. 55: 489-496.
13. Li, F., Ma, H., and Liu, J. 2017. Pyrethroid insecticide cypermethrin modulates gonadotropin syntheisis via calcium homeostasis and ERK1/2 signaling in LßT2 mouse pituitary cells. Toxicol. Sci. 162: 43-52.
14. Li, Y., Luh, C. J., Burns, K. A., Arao, Y., Jiang, Z., Teng, C. T., Tice, R. R., and Korach, K. S. 2013. Endocrine-disrupting chemicals (EDCs): *in vitro* mechanism of estrogenic activation and differential effects on ER target genes. Enivron. Health Perspect. 121: 459-466.
15. Liberal Times Net. 2008. Check 19 tons of pseudo-pesticide case (In Chinese). Available at http://news.ltn.com.tw/news/local/ paper/235539/print
16. Lu, S. Y., Liao, J. W., Kuo, M. L., Wang, S. C., Hwang, J. S., and Ueng, T. H. 2004. Endocrine-disrupting activity in carbendazim-induced reproductive and developmental toxicity in rats. J. Toxicol. Environ. Health A 67: 1501-1515.
17. Lu, S. Y., Chen, M. C., Yuan, C. Y., Hsu, Y. H., and Tsai, W. R. 2015. Detecting benomyl and its metabolite carbendazim inducing androgenic activity in rats by using uterotrophic and Hershberger assay. Taiwan J. Agricul. Chem. Food Sci. 53: 235-250.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.
19. Moffit, J. S., Bryant, B. H., Hall, S. J., and Boekelheide, K. 2007. Dose-dependent effects of sertoli cell toxicants 2,5-hexanedione, carbendazim, and mono-(2-ethylexyl) phthalate in adult rat testis. Toxicol. Pathol. 35: 719-727.

20. Morinaga, H., Yanase, T., Nomura, M., Okabe, T., Goto, K., Harada, N., and Nawata, H. 2004. A benzimidazole fungicide, benomyl, and its metabolite, carbendazim, induce aromatase activity in a human ovarian granulose-like tumor cell line (KGN). Endocrinology 145: 1860-1869.
21. Nakai, M., Hess, R. A., Moore, B. J., Guttroff, R. F., Stader, L. F., and Linder, R. E. 1992. Acute and long-term effects of a single dose of the fungicide carbendazim (methyl 2-benzimidazole carbamate) on the male reproductive system in the rat. J. Androl. 13: 507-518.
22. Nakai, M., Hess, R. A., Matsuo, F., Gotoh, Y., and Nasu, T. 1997. Further observations on carbendazim-induced abnormalitites of spermatid morphology in rats. Tissue Cell 29: 477-485.
23. Nakai, M., Toshimori, K., Yoshinaga, K., Nasu, T., and Hess, R. A. 1998. Carbendazim-induced abnormal development of the acrosome during early phases of spermiogenesis in the rat testis. Cell Tissue Res. 294: 145-152.
24. Narayana, K. 2007. Methyl parathion induces the formation of symplasts by round spermatid fusion and alters the biochemical parameters in the testis. Morphologie 91: 173-179.
25. Narayana, K., Prashanthi, N., Nayanatara, A., Kumar, H. H., Abhilash, K., and Bairy, K. L. 2006a. Neonatal methyl parathion exposure affects the growth and functions of the male reproductive system in the adult rat. Folia Morphol (Warsz) 65: 26-33.
26. Narayana, K., Prashanthi, N., Nayanatara, A., Bairy, L. K., and D'Souza, U. J. A. 2006b. An organophosphate insecticide methyl parathion (o-o-dimethyl o-4-nitrophenyl phosphorothioate) induces cytotoxic damage and tubular atrophy in the testis despite elevated testosterone level in the rat. J. Toxicol. Sci. 31: 177-189.
27. Nonneman, D. J., Ganjam, V. K., Welshons, W. V., and Vom Saal, F. S. 1992. Intrauterine position effects on steroid metabolism and steroid receptors of reproductive organs in male mice. Biol. Reprod. 47: 723-729.
28. Ohno, K., Araki, N., Yanase, T., Nawata, H., and Iida, M. 2004. A novel nonradioactive method for measuring aromatase activity using a human ovarian grnulosa-like tumor cell line and an estrone ELISA. Toxicol. Sci. 82: 443-450.
29. Olea, N., Olea-Serrano, F., Lardelli-Claret, P., Rivas, A., and Barba-Navarro, A. 1999. Inadvertent exposure to xenoestrogens in children. Toxicol. Ind. Health 15: 151-158.
30. Perreault, S. D., Jeffay, S., Poss, P., and Laskey, J. W. 1992. Use of the fungicide carbendazim as a model compound to determine the impact of acute chemical

exposure during oocyte maturation and fertilization on pregnancy outcome in the hamster. Toxicol. Appl. Pharmacol. 114: 225-231.

31. Pesticide Manual Online. 2019. Pesticide Manual. British Crop Production Council (BCPC). Available at https://www.bcpc.org/my-account
32. Prashanthi, N., Narayana, K., Nayanatara, A., Chandra Kumar, H. H., Bairy, K. L., and D'Souza, U. J. A. 2006. The reproductive toxicity of the organophosphate pesticide *o, o*-dimethyl *o*-4-nitrophenyl phosphorothioate (methyl parathion) in the male rat. Folia Morphol. 65: 309-321.
33. Rehnberg, G. L., Cooper, R. L., Goldman, J. M., Gray, L. E., Hein, J. F., and McElroy, W. K. 1989. Serum and testicular testosterone and androgen binding protein profiles following subchronic treatment with carbendazim. Toxicol. Appl. Pharmacol. 101: 55-61.
34. Robitaille, C. N., Rivest, P., and Sanderson, J. T. 2015. Antiandrogenic mechanisms of pesticides in human LNCaP prostate and H295R adrenocortical carcinoma cells. Toxicol. Sci. 143: 126-135.
35. Sitarek, K. 2001. Embryolethal and teratogenic effects of carbendazim in rats. Teratog. Carcinog. Mutagen. 21: 335-340.
36. Spencer, F., Chi, L., and Zhu, M. X. 1996. Effect of benomyl and carbendazim on steroid and molecular mechanisms in uterine decidual growth in rats. J. Appl. Toxicol. 16: 211-214.
37. Tu, W., Xu, C., Jin, Y., Lu, B., Lin, C., Wu, Y., and Liu, W. 2016. Permethrin is a potential thyroid-disrupting chemical: *in vivo* and *in silico* evidence. Aquat. Toxicol. 175: 39-46.
38. United States Environmental Protection Agency. Series 890- Endocrine Disruptor Screening Program Test Guidelines. Available at https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-890-endocrine-disruptor-screening-program
39. Zhou, J. L., Ding, Z., Ge, X., Shi, Q. M., Wang, H. X., Chen, G., Li, M. X., Wang, H., Ju, Q., Wang, Q., Zhang, J. P., Zhang, M. R., and Xu, L. C. 2017. Cypermethrin inhibits interleukin-6-induced androgen receptor transactivation through signal transducer and activator of transcription 3. Toxicol. Mech. Methods 27: 717-722.

以體外雌激素受體結合與大鼠子宮激性測試評估六種農藥之內分泌干擾作用

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摘要

呂水淵、蔡韙任。2018。以體外雌激素受體結合與大鼠子宮激性測試評估六種農藥之內分泌干擾作用。臺灣農藥科學 5：31-51。

過去文獻顯示雌激素受體在生殖毒性中扮演重要角色，雌激素受體干擾乃內分泌干擾重要因子之一，由於體外受體結合測試雖可減少動物需求量但使用放射性標幟方法造成一般實驗室不便性，因此本試驗目的在探討以文獻報導具生殖毒或內分泌干擾作用之賽滅寧、百滅寧、安殺番、甲基巴拉松、貝芬替及免賴得等 6 種農藥進行兩種方法比對，是否大鼠子宮激性測試可涵蓋體外雌激素受體結合試驗結果。結果顯示，在體外雌激素受體結合競爭性試驗，抑制半數濃度值 (IC_{50}) 在百滅寧、安殺番、賽滅寧、甲基巴拉松、免賴得及貝芬替分別為 141、249、523、1022、1413 及 4334 μM，此意謂百滅寧、安殺番及賽滅寧較具雌激素受體親和作用，但無法辨別是促進或拮抗作用，而甲基巴拉松、免賴得及貝芬替則偏弱到無雌激素受體親和作用。在大鼠子宮激性試驗，百滅寧顯著拮抗雌激素作用而安殺番則接近促進雌激素作用。賽滅寧同百滅寧一樣顯著顯現拮抗雌激素作用，而甲基巴拉松則無明顯影響。免賴得與貝芬替均顯著顯現類似雌激素作用，比較各農藥在體外雌激素受體結合競爭性試驗與大鼠子宮激性試驗結果，百滅寧、賽滅寧、甲基巴拉松甚至安殺番在體外與體內試驗結果相符。至於免賴得與貝芬替在體外與體內試驗結果似乎不符，但參考過去文獻可知，免賴得與貝芬替在大鼠子宮激性作用乃因其雄性素激性作用所致。綜合上述結果，就與雌激素受體親和性而言，百滅寧＞安殺番＞賽滅寧＞甲基巴拉松＞免賴得＞貝芬替，但就活體內試驗結果，百滅寧與賽滅寧具拮抗雌激素受體作用而安殺番有弱促進雌激素受體作用，甲基巴拉松則無明顯作用，免

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賴得與貝芬替則透過雄性素受體誘發類雌激素受體作用，推論雌、雄激素受體在生殖毒性扮演重要角色。同時本研究也得到一結論，大鼠子宮激性可涵蓋體外以放射線標幟雌激素受體測試結果，增加以大鼠子宮激性試驗取代體外雌激素受體干擾篩選之友善與功能性。

關鍵詞：賽滅寧、百滅寧、安殺番、甲基巴拉松、免賴得、貝芬替