Oestradiol Potentiates the Effects of Certain Pyrethroid Compounds in the MCF7 Human Breast Carcinoma Cell Line

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Summary — Pyrethroids are the most widely used insecticides for indoor pest control, so human exposure to them is common. The main target of pyrethroids is the nervous system, but their endocrine disrupting capabilities may also be of toxicological concern. In the present study, the proliferation of the breast cancer cell line, MCF7, was studied after a 7-day exposure to various concentrations of pyrethrin, permethrin and cypermethrin. The effects of oestradiol and the combined effects of oestradiol (0.10nM) and pyrethroids (0.1–100μM) on MCF7 cell proliferation were also evaluated. Proliferation and cell toxicity were studied by measuring the ATP content with a luminescence method, and mitochondrial metabolic enzyme activity with the WST-1 test. In the ATP test, low concentrations (0.1–1μM) of pyrethroids in co-exposure with oestradiol caused a clear statistically significant increase in the proliferation of MCF7 cells. This was evident when compared to the proliferative effect caused by 0.1nM oestradiol alone. High concentrations were cytotoxic, and the greatest cell toxicity was that of cypermethrin, which has a cyano group in its molecular structure.

Key words: *cell proliferation, cypermethrin, endocrine disruption, MCF7 cell line, oestradiol, permethrin, pyrethrin.*

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Introduction

Natural pyrethrins and their synthetic homologues form an important group of insecticides, with a worldwide commercial production. Their use to control harmful insects in agriculture and gardening is considerable, and human exposure to pyrethroid compounds is common. The main advantages of pyrethroids are low mammalian toxicity and rapid biodegradation. The soil does not absorb pyrethroids, but they are of toxicological concern in natural water ecosystems.

Natural pyrethrin is an extract from the flowers of *Chrysanthemum* spp., and it is a mixture of chemically related pyrethrins. It was used extensively until World War II. Since then, many synthetic pyrethroids with better light stability, activity and safety have been developed. Some of the synthetic compounds differ significantly from the original structure of natural pyrethrins. Depending on their chemical structures, pyrethroids can produce various symptoms. Pyrethroid compounds are neurotoxins, and cause neuronal hyperexcitability in the central nervous system. The primary target for pyrethroid compounds is the sodium channel in the neural cell membrane (1, 2).

Hormonal homeostasis has also been shown to change following exposure to pyrethroids. Colborn and coworkers (3) have listed pyrethroids as endocrine-disrupting chemicals. Endocrine disrupters are of special environmental concern. Exposure to certain endocrine disrupters can alter the endocrine system, increase the risk of endocrine diseases and disorders, and affect development in both humans and wildlife (3, 4). Hormones and hormone-like compounds are already effective at low dose levels. Exposure to hormonally active agents may represent a risk at extremely low levels, particularly during the sensitive periods of fetal development. Thus, it is not surprising that there is evidence of a strong association between fetal death due to congenital anomalies and residential exposure to pyrethroids (a 5-fold risk during weeks 3–8 of pregnancy; 5). Earlier discussions have mainly focused on the neurotoxic characteristics of pyrethroids, but other mechanisms of pyrethroid toxicity should be given greater consideration (6).

In the present study, we investigated the cytotoxicity and interaction with oestradiol of various commercial pyrethroid products: pyrethrin (no alpha-cyano group, Biospray S), permethrin (no alpha-cyano group, Biokill) and cypermethrin (with the alpha-cyano group, Ripcord) in MCF7 human breast carcinoma cell cultures.

Materials and Methods

Test compounds

Three commercial pyrethroid products, namely, Biospray S (Kemira, Helsinki, Finland), Biokill (Jesmond, Zurich, Switzerland) and Ripcord (American Cyanamid Company, USA), were used as test compounds. Biospray S contained an active compound, 100g/l pyrethrin, Biokill contained 2.5g/l permethrin, and Ripcord 100g/l cypermethrin, with an 820g/l mixture of xylene and petrol.

Materials

75cm^2 Nunc (Roskilde, Denmark) flasks (Cat. No. 156472) and Nunclon 96-well plates (Cat. No. 167008) were used for the cell cultures. The cell culture reagents were as follows: Dulbecco's modified Eagles's medium:nutrient mixture F12 Ham (DMEM/F12) medium (high glucose with 2.5mM L-glutamine), with phenol red; antibiotic/antimycotic solution (Gibco; Paisley, UK); fetal bovine serum (FBS; Gibco); dextran-coated charcoal serum (DCC; Gibco); insulin (Sigma); and 17-β oestradiol (Sigma). All the reagents used were of analytical grade. Black microwell plates for luminometric measurements came from ThermoLabsystems. The ThermoLabsystems (St Louis, MO, USA) bioluminescent assay for ATP was used.

Culture of MCF7 cells

The human breast carcinoma cell line, MCF7, was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). This epithelial-type cell line originates from a mammary gland adenocarcinoma. MCF7 cells were cultured according to the instructions of the ATCC, in high-glucose DMEM/F12 medium with 10% FBS and 10ng/ml insulin. The cells were split 1:4 when nearly confluent (80%). MCF7 cells were seeded at a density of 5000 cells/well on 96-microwell plates, in 100ml medium per well, and grown in DMEM/F12 medium with 100U/ml penicillin, 100μg/ml streptomycin, 10ng/ml insulin, and 5% DCC serum. The cells were then grown for 24 hours at 37°C in a humidified incubator with 5% CO_2.

Exposure of MCF7 cells

Natural pyrethrin (Biospray S), permethrin (Biokill) and cypermethrin (Ripcord) were suspended in the growth medium. The calculation of the final concentrations (1–100μM) of natural pyrethrin, permethrin and cypermethrin was based on the concentrations of active compounds in the commercial mixtures. The test substances were added to the wells, and the cells were grown for a further 7 days. Co-exposure to oestradiol was studied with 0.1nM oestradiol. The exposure was stopped by adding 10% trichloroacetic acid into the wells. The plates were deep-frozen at –75°C.

The bioluminescence assay for ATP

The bioluminescence method uses the luciferase enzyme, which catalyses the formation of light from ATP and luciferin (7–9). The plates were thawed, and 25μl of cell suspension from each well was moved onto black microplates (Thermo-Labsystems). When measuring the total ATP, 100μl of a mixture (1:5) of Tris-acetate buffer and ATP-monitoring reagent was added into the wells. The plates were shaken, and the luminescence intensity was measured with a ThermoLabsystems Luminoskan Ascent luminometer, with an integration time of 1000ms.

WST-1 test

The WST-1 test is a colorimetric assay for the quantification of cell proliferation and cell viability. The evaluation of cell proliferation is based on the cleavage of the tetrazolium salt, WST-1, by the mitochondrial dehydrogenases in viable cells (10, 11). MCF7 cells were cultured and exposed to the test compounds in a similar way to that used for the ATP measurement. After exposure for 7 days, 10μl of WST-1 cell proliferation reagent was added to 100μl of the medium in each well, and the plate was shaken for 1 minute. The plates were incubated for 50 minutes in a humidified atmosphere at 37°C, and the absorbances were measured at 450nm with a Multiskan MS (Labsystems, Vantaa, Finland).

Statistics

The determinations were repeated five times at each dose level, with three tests in each determination. The mean ± SEM of 15 independent tests were calculated at each concentration. Statistical differences between treated cells and controls were evaluated by using one-way ANOVA with Dunnett's *post hoc* test (GraphPad Prism).

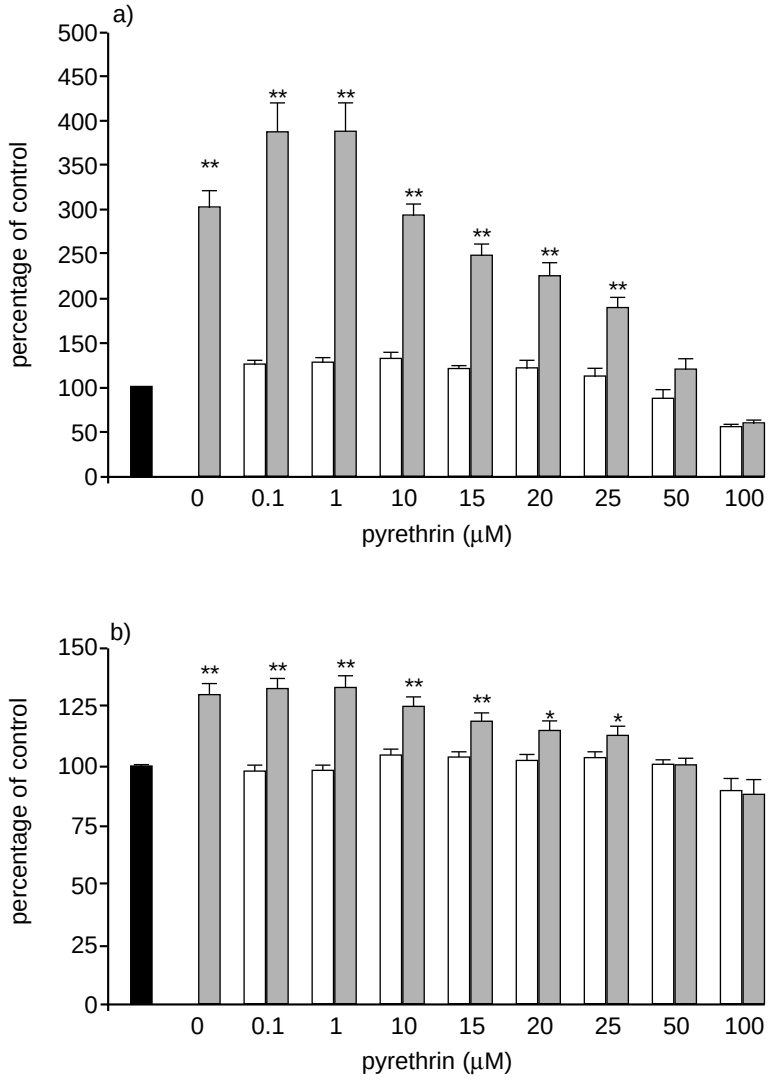
Results

In this study, MCF7 human breast carcinoma cells were exposed for 7 days to three commercial pyrethroid compounds (pyrethrin, permethrin and

cypermethrin) with and without co-exposure to 0.1nM oestradiol. Cypermethrin contains an alpha-cyano group, while pyrethrin and permethrin do not. Cell proliferation was evaluated by determining the total ATP content of the cells with a luminometric method, and cytotoxicity by determining mitochondrial metabolic activity with the WST-1 test.

In the co-exposure experiment with oestradiol (0.1nM) and various concentrations of pyrethroids, there was a clear increase in MCF7 cell proliferation (Figures 1–3). Exposure to 0.1μM pyrethrin with 0.1nM oestradiol increased the total ATP to 388%, compared to the control (no oestradiol, no pyrethroid compound). Without oestradiol, the ATP content after 0.1μM pyrethrin exposure was 127% compared to the control. Corresponding results for cypermethrin were 373% (with oestradiol) and 125% (without

Figure 1: The effects of pyrethrin and a combination of pyrethrin and oestradiol on cellular ATP content and mitochondrial metabolic enzymes (WST-1) in the MCF7 cell line



a) ATP; b) WST-1.

□ = *pyrethrin (Biospray S);* ▒ = *pyrethrin (Biospray S) + 0.1nM oestradiol;* ■ = *control, with no pyrethrin or oestradiol.*

The normalised values are compared with the control (medium only, taken as 100%).

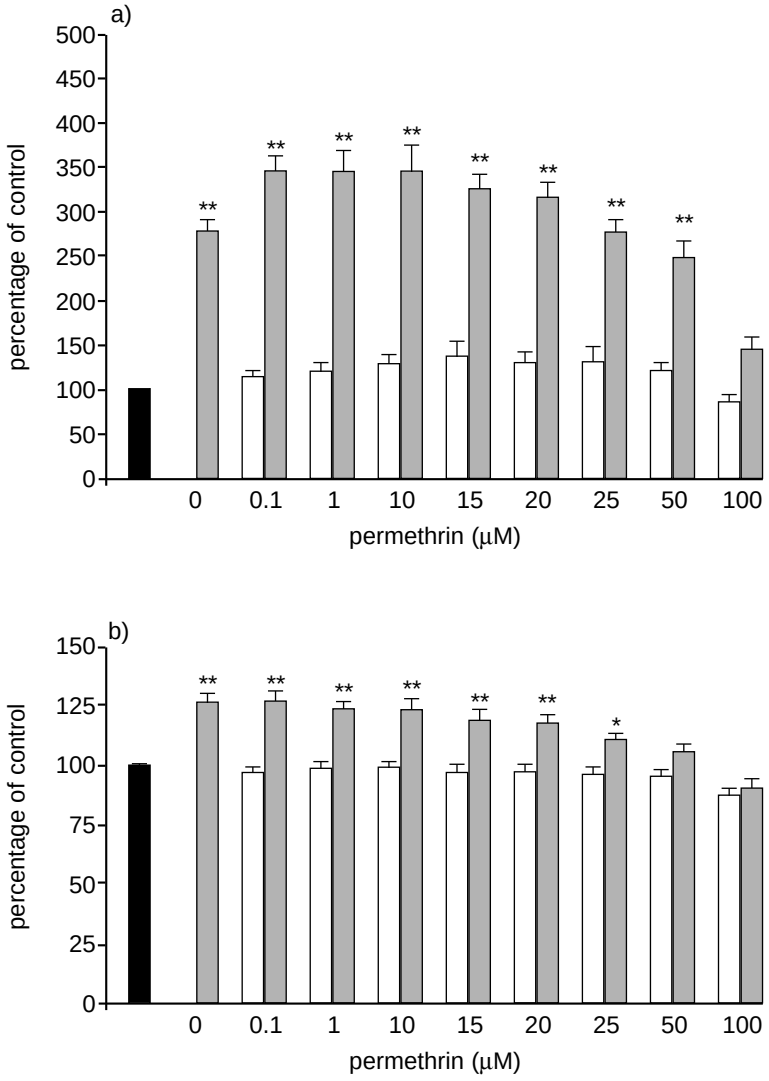
**p < 0.05; **p < 0.01.*

oestradiol), and for permethrin, 346% (with oestradiol) and 116% (without oestradiol). Compared to the control (no oestradiol, no pyrethroids), oestradiol alone increased the cell proliferation to 280% in the ATP test and to 130% in the WST-1 test.

Without oestradiol, pyrethroids did not cause any significant increases in proliferation at low concentrations (Figures 1–3). High concentrations were cytotoxic, and the greatest cell toxicity was that of cypermethrin in both tests, starting at the 10μM concentration. Correspondingly, the cytotoxic effect started at the 50–100μM concentrations of pyrethrin and permethrin.

To illustrate the effects of pyrethroids on the cell proliferation caused by oestradiol, the ATP and WST-1 results from the co-exposure of pyrethroids and oestradiol were compared with the effect of

Figure 2: The effects of permethrin and a combination of permethrin and oestradiol on cellular ATP content and mitochondrial metabolic enzymes (WST-1) in the MCF7 cell line



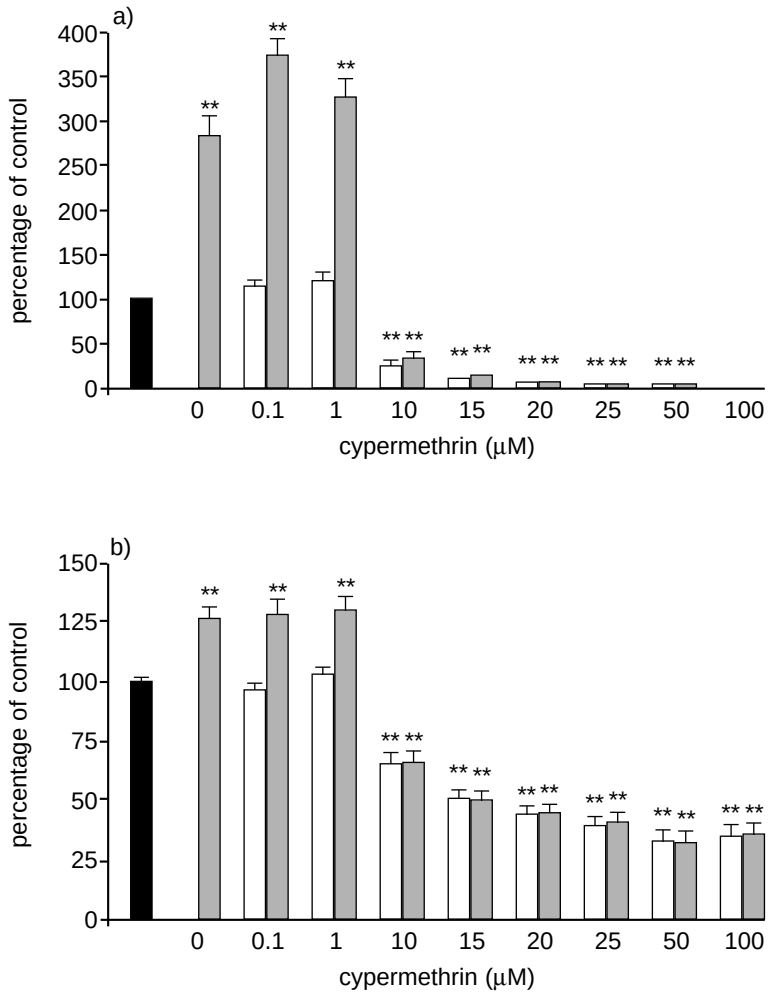
a) ATP; b) WST-1.

□ = *permethrin (Biokill);* ▒ = *permethrin (Biokill) + 0.1nM oestradiol;* ■ = *control, with no permethrin or oestradiol.*

The normalised values are compared with the control (medium only, taken as 100%).

**p < 0.05; **p < 0.01.*

Figure 3: The effects of cypermethrin and a combination of cypermethrin and oestradiol on cellular ATP content and mitochondrial metabolic enzymes (WST-1) in the MCF7 cell line



a) ATP; b) WST-1.

□ = *cypermethrin (Ripcord);* ▒ = *cypermethrin (Ripcord) + 0.1nM oestradiol;* ■ = *control, with no cypermethrin or oestradiol.*

The normalised values are compared with the control (medium only, taken as 100%).

**p < *0.01.*

0.1nM oestradiol only (Table 1). This comparison showed that pyrethroids significantly increased the proliferation of MCF7 cells at low concentrations, according to the ATP results. Pyrethrin and cypermethrin caused a significant increase in ATP content at the 0.1–1μM concentrations, and permethrin at the 0.1–10μM concentrations. In the WST-1, the corresponding effect was not seen. For detecting both cell proliferation and cell toxicity, measurement of the ATP content of the cultured MCF7 cells was more sensitive than measurement of mitochondrial enzyme activity with the WST-1 test.

Discussion

Pyrethroids are the most common insecticides in use for indoor and agricultural purposes. They are considered safe because of their low accumulation and effective biodegradation. However, there is evidence of chronic and irreversible injuries caused by pyrethroid intoxication. Symptoms in humans have even appeared more than two years after intoxication (12, 13). The endocrine disruption capability of pyrethroids has raised major environmental concern in the past few years (17, 18). There is evi-

dence of the effects of pyrethroids on the regulation of thyroid hormone and androgen functions. Furthermore, pyrethroids at high concentrations seem to inhibit the binding of testosterone to receptors by a competitive mechanism (17, 19–25).

We studied three chemically different pyrethroid compounds, namely, pyrethrin (natural pyrethrin) and two synthetic pyrethroids (permethrin and cypermethrin). Pyrethrin is a mixture of active constituents in pyrethrum extracts. Cypermethrin differs from permethrin and pyrethrin in having an alpha-cyano group in its molecular structure. The alpha-cyano group is usually connected with the toxicity of the pyrethroid in question. Toxicity to the central and peripheral nervous systems is caused by alterations in the permeability of the sodium channels and subsequent disturbances in the transfer of nerve impulses. Pyrethroids without an alpha-cyano group generally show the weakest toxic effects (14). In our previous studies, we have found that cypermethrin inhibits the activity of synaptosomal ATPases after *in vitro* exposure, more than pyrethroids having no cyano group in their molecules (15, 16). In the present study with the MCF7 cell line, the most cytotoxic compound was cypermethrin, both in the ATP test and in the WST-1 test.

In addition to specifically neurotoxic pyrethroid mechanisms, various other cytotoxic effects have been detected. They can affect the regulation of the cell cycle, and, consequently, mitotic cell cycle progression (27). Pyrethroids also have genotoxic effects (6, 28–30). They can induce chromosomal aberrations, sister chromatid exchanges and micronuclei in human lymphocyte cell cultures (6, 28), although, in human *in vivo* studies, no induction of chromosomal aberrations has been found (31, 32). The results with various cells *in vitro* may vary, due to differences in the regulation of cell growth in individual cell types. Interaction with hormone regulation in certain cells may dominate and overcome other changes.

In the present study, all three pyrethroid compounds considerably increased the proliferative effect of 0.1nM oestradiol alone, when applied at low concentrations together with oestradiol to the MCF7 cell cultures. The most effective synergists at low concentrations were pyrethrin (Biospray S) and cypermethrin (Ripcord). This synergistic effect may be of importance, because human blood concentrations of oestradiol vary between 0.08nM and1.6nM in women and 0.03nM and 0.2nM in men. This could mean that the normal blood oestradiol concentration may be able to enhance the effects of pyrethroids on steroid-regulated cells. In earlier studies, pyrethroids have been shown to have oestrogenic potential (17, 18, 26). Their structural components show similarity to oestrogen metabolites and polyphenols, which might

Table 1: Cell proliferation detected with the ATP and WST-1 tests after 7-day exposure of MCF7 cultures to 0.1nM oestradiol and various concentrations of pyrethrin, permethrin and cypermethrin

Dose (μM)	ATP test			WST-1 test		
	Pyrethrin	Permethrin	Cypermethrin	Pyrethrin	Permethrin	Cypermethrin
0	100.0 ± 1.3	100.0 ± 1.5	100.0 ± 3.0	100.0 ± 2.9	100.0 ± 1.1	100.0 ± 0.7
0.1	128.1 ± 4.4**	124.1 ± 3.5**	132.5 ± 3.4**	102.0 ± 2.4	100.2 ± 2.3	94.5 ± 7.1
1	127.7 ± 3.9**	123.2 ± 3.0**	116.3 ± 3.3**	102.3 ± 3.3	97.8 ± 2.0	103.3 ± 2.8
10	99.3 ± 4.3	123.6 ± 8.5**	12.8 ± 2.0**	96.3 ± 2.6	97.7 ± 2.2	52.8 ± 2.6**
15	85.3 ± 5.1	116.3 ± 3.0	5.4 ± 0.8**	91.2 ± 2.4	94.5 ± 3.2	41.1 ± 2.8**
20	80.2 ± 7.5*	113.6 ± 5.5	3.3 ± 0.7**	88.5 ± 2.6*	94.5 ± 1.9	36.9 ± 3.0**
25	69.1 ± 7.1**	99.9 ± 4.1	2.2 ± 0.7**	86.9 ± 2.3**	88.7 ± 2.2**	34.1 ± 3.8**
50	44.5 ± 5.9**	89.8 ± 5.6	0.7 ± 0.2**	77.7 ± 2.0**	84.4 ± 2.8**	28.2 ± 4.1**
100	22.5 ± 2.0**	51.1 ± 4.0**	0.3 ± 0.1**	68.0 ± 3.9**	72.9 ± 3.5**	31.0 ± 4.9**

Comparisons were made with the effect of 0.1nM oestradiol (taken as 100%).

*p < 0.05; **p < 0.01.

Mean ± SEM, n = 9.

explain their oestrogenic effects (27). In our study, pyrethrin, permethrin and cypermethrin enhanced cell proliferation in the oestrogen-regulated breast cancer cell line MCF7.

In conclusion, our results show that pyrethroids have a clear effect on cell proliferation and the viability of MCF7 cell cultures. At low concentrations (< 10mM), pyrethroids increased the oestradiol-induced proliferation in MCF7 cell culture detected with the ATP test. At higher concentrations, cytotoxic effects were detected.

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