

Expression of Testicular Estrogen Receptor Alpha in Rats Exposed to Subchronic Inhalation Exposure of Transfluthrin

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ABSTRACT

Endocrine Disrupting compounds (EDCs) are exogenous materials that can interfere with the functions of the endocrine system. Research has demonstrated that pyrethroid compounds affect endocrine function. *Transfluthrin* belongs to the pyrethroid group that is widely used. These compounds could interact with estrogen receptor α and may increase the expression of estrogen receptor α in rat testis. **Aim:** to determine the effect of subchronic inhaled *transfluthrin* exposure on the expression of estrogen receptor α (ER α). **Method:** we used 35 adult male Wistar rats as experimental animals, which were randomly divided into 5 groups (n=7 per group): negative control group (rats without treatment), solvent control group (rats exposed to n-hexane solvent), and three treatment groups of subchronic inhaled *transfluthrin*: 0.1 mg/ml (treatment 1), 0.2 mg/ml (treatment 2) and 0.4 mg/ml (treatment 3), treated for 60 days. The immunohistochemical (IHC) staining was used to assess the expression of ER α . **Result:** there was a tendency for an increase in ER α expression with the addition of a *transfluthrin* dose. ER α in negative control group, solvent control group, treatment 1 group, treatment 2 group, and treatment 3 group was $16.75 \pm 5.01 \%$; $16.64 \pm 13.00 \%$; $19.31 \pm 6.52 \%$; $25.63 \pm 7.08 \%$, and $28.79 \pm 20.31 \%$; $p > 0.05$, respectively. There was a weak positive correlation between the dose of *transfluthrin* with ER α expression ($r = 0.38$, $p = 0.025$). **Conclusion:** subchronic inhalation exposure of *transfluthrin* in rats showed a tendency to increased expression of ER α (dose-dependent). There is a weak positive correlation between the dose of *transfluthrin* with ER α expression.

Keywords: Transfluthrin, subchronic inhalation, endocrine-disrupting chemicals, expression of testicular estrogen receptor α

INTRODUCTION

The debate and controversy about Endocrine-Disrupting Chemicals (EDCs) starting from the publication of an article entitled Our Stolen Future by Colborn *et al.* in 1996.^[1] EDC definition according to U.S. EPA (2009) is "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process were originally thought to exert actions primarily through nuclear hormone receptors."^[2]

Pesticide is a chemical compound that is frequently used. There are about 1844 pesticide compounds that have been commercialized in the

US. One of the many forms of pesticide that is often used in the household are anti-mosquitos and spy pesticides. We found many pieces of evidence concerning the effect of chemical compounds in pesticides on human health. A compound of pesticide from the pyrethroid group shows estrogenic activities and can cause sexual dysfunction in male rats.^[3] Contributes to a variety of hormonal disturbances^[2,4,5] and is thought to increase the expression of α estrogen receptors in male rats.^[6]

Table 1. Classification of EDC/ Pyrethroid/ Transfluthrin^[7]

Organ halogen	Pesticides	Heavy Metals
Dioxin and furan,	Organophosp	Cadmium
PCB, PBB,	hates	mercury

OctachloroBenzen e,	pyrethroid DDT	etc.
Hexachlorobenzen e,	Cyclodien Carbamate	
Pentachlorophenol	Fumigant etc.	

EDC, Endocrine-Disrupting Chemicals; PBB, Polybrominated biphenyls; PCB, polychlorinated biphenyls; DDT, dichloro-diphenyl-trichloroethane

Mechanism action of EDC: [7]

1. Assemble hormone so that it can bind appropriately to that hormone receptor; for example, *xenoestrogen*
2. Stimulate hormone receptor
3. Inhibit hormone to bind with its receptor
4. Diminish the hormone by stimulating hormone destruction or elimination
5. Disrupt the enzyme for hormone degradation activity
6. Directly or indirectly impair the hormone

As the usage of pyrethroid is getting higher, there have been some reports of change in physiological activity in mammalian animals exposed to this compound. It caught special attention to pyrethroid pesticide in the last few years.^[8]

Pyrethroid pesticide is the synthetic analog of pyrethrin, which naturally occurs in Chrysanthemum flowers. Although synthetic pyrethroid is made based on the chemical structure and biological activities of *pyrethrin*, this chemical modification results in a more toxic and hard to degrade compound in the environment.^[9] An example of a compound in the pyrethroid group is transfluthrin.^[10]

Transfluthrin is a relatively volatile substances and can serve as an inhalation agent and dermatologically contact agent.^[2] For many years, transfluthrin was considered as a safe insecticide. Nevertheless, as a synthetic compound in the environment, transfluthrin can interact with estrogen receptors (ERs).^[11] ERs regulate the gene expression towards estrogen exposure. ERs signaling can occur in a ligand-dependent or ligand-independent manner. The target gene for estrogen is regulated by the genomic pathway, by directly interacting with ER-DNA or by an attracting mechanism in ER which will sustain the DNA through other transcription factors, and non-genomic pathway where the estrogen exposure will cause an activation from kinase cascade

signals.^[12] In an experimental study, pyrethroid was proved to cause testicle disturbances.^[11] If the exposure happens during sexual development, it can cause impaired sexual differentiation.^[13]

The purpose of this study was to determine the effect of subchronic transfluthrin exposure to the expression level of α estrogen receptors in the testicle of Wistar strain *Rattus norvegicus*. An understanding of the effect of transfluthrin exposure per inhalation can be the guideline of sample control that is included in endocrine disruption substances in the environment. It will increase the awareness of the endocrine effect of the environment.

METHODS

Experimental study with a total of 35 adults male rats (*Rattus norvegicus* Wistar strain) as experimental animals which were divided into 5 groups. Each group consists of 7 rats consisting of negative control group (rats without intervention), dissolved control group (solvent inhalation n-hexane), transfluthrin inhalation group with dosage of 0.1 mg/ml (intervention 1), 0.2 mg/ml (intervention 2), 0.4 mg/ml (intervention 3). Duration of transfluthrin inhalation exposure was 60 days.

The study was conducted in Universitas Brawijaya, Faculty of Medicine, Malang. The animal in this study is *Rattus norvegicus* Wistar strain, young male, bodyweight ± 200 grams, 2.5-3 months old with randomization sampling. This study was done for 6 months and had been approved by the ethical committee in Universitas Brawijaya, Faculty of Medicine, Malang. The animal study was given a standard diet, consisted of *Comfeed* PARS 53%, wheat flour 23.5 % and 23.5 %, and were adapted for 2 weeks.

Transfluthrin Exposure

Transfluthrin used was purified, research-grade transfluthrin with n-hexane solvent. Every day, rats were kept in inhalation cage and sprayed with nebulizer until no more substance left in

the nebulizer. This process was repeated every day using solvent exposure and transfluthrin exposure, inside a different tube to prevent contamination. After that, the rats were observed for 10 minutes for any response to the exposure. Rats that had seizures were returned to the cage to prevent injury and followed

with other rats. The device was cleaned after every exposure.

Research Variables

The independent variables in this study was subchronic exposure to transfluthrin solvent (n-hexane), transfluthrin in a 0.1 mg/ml; 0.2 mg/ml; 0.4 mg/ml dosages. The dependent variable was the level of α estrogen receptor expression (ER α). The confounding variable in this study was controllable variables: species, age, stress, space, diet, and infection, also uncontrollable variables, such as genetics and metabolism.

Testicle Extraction

On the 60th day, all Wistar rats were anesthetized with intraperitoneal ketamine. Surgical procedures and perfusion with PBS pH 7.4 were done to clean the organ from the blood. After that, the testicles were taken and kept in formalin for 24 hours, and then the paraffin block was done.

Expression of Estrogen Receptor α (ER α) Measurement

Expression ER α was measured with immunohistochemical staining (bs-0725R) and observed visually in every 10 view field with a light microscope, 400x magnification on the brown-colored nucleus in testicle's tubules seminiferous and interstitial cells. The analysis was done using the immunoRatio JPEG 2000 program, with a scale ratio in (%).

Statistical Analysis

The data was presented in means and standards deviation. Uncoupled numerical comparative data was analyzed with the One-Way ANOVA/Kruskal-Wallis test, with significant *p-value* if $p < 0.05$. *Pearson* correlative test was used to evaluate the correlation between transfluthrin dose and expression of ER α . All technical data processing results were analyzed by computerization using Statistical Product and Service Solution software, IBM SPSS Statistics 20 with a significance level of 0.05 ($p = 0.05$) and a confidence level of 95% ($\alpha = 0.05$).

RESULTS

Expression of Estrogen Receptor Alpha (ER α)

The expression of ER α in rat's testicle can be seen from the brown-colored nucleus in

seminiferous tubules and interstitial space using IHC staining and observed every 10 viewing field of a light microscope, and analyzed using the *immunoRatio JPEG 2000 virtual slide microscope* program (Figure 1).

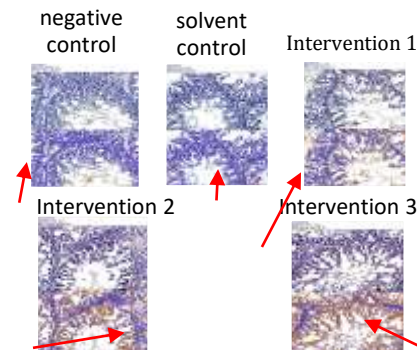


Figure 1. The immunohistochemical observation of ER α expression in rats' testicles using light microscope in 400x magnification. The higher the transfluthrin dose, the darker the brown color of the nucleus.

The mean ER α expression in each group is presented in Figure 2. The highest score is 28.79% in intervention group 3, which was given 0.4 mg/ml transfluthrin exposure. The lowest score is 16.74% in the negative control group, which was given no intervention.

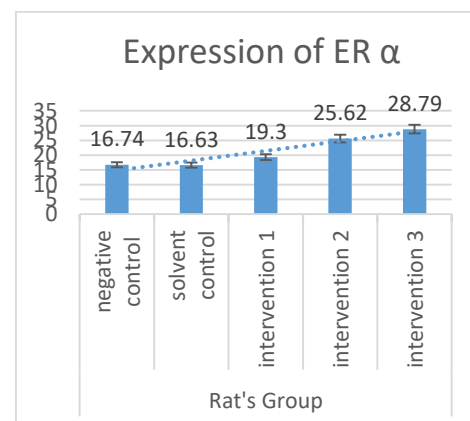


Figure 2. The mean of ER α expression in each group showed increased ER α expression in a *dose-dependent* manner.

There are two rats in this study that had some seizure: one from group P2 (P2-3) that was given 0.2 mg/ml inhaled transfluthrin on the 22nd day and one from group P3 (P3-4) which was given 0.4 mg/ml inhaled transfluthrin on the 30th day until the last day of the study. Both samples had repeated generalized seizures for 1-2 minutes after inhaled transfluthrin exposure, and after the seizure stopped, the rats continued to regular activity.

There was one death in group P1 (P1-2), which was given 0.1 mg/ml transfluthrin. This rat was found injured and dead outside the cage on the

14th day. New rat did not replace the dead rat because it will affect the result of the study.

One sample was found wounded and deceased outside of the cage on day 14, and this sample was from the group P1 (P1-2), treated with 0.1 mg/ml transfluthrin. The deceased sample was not replaced by a new rat because the addition might affect the result of the research.

Statistical analysis of the study data as done using data normality test: *Shapiro-Wilk test* presented in **Table 2** and variance homogeneity test. It was followed by a numerical comparative hypothesis test of uncoupled data using the Kruskal-Wallis test (**Table 3**). Data normality test of ER α expression, using the *Shapiro-Wilk test*, showed a normal data distribution with $p > 0.05$ (**Table 1**).

Table 1. Expression of ER α for each groups

Group	<i>p-value*</i>
Negative control	0.618
Solvent control	0.059
Intervention 1	0.238
Intervention 2	0.554
Intervention 3	0,149

Shapiro –Wilk test, Tests of Normality

After that, the homogeneity test using the Levene test showed a homogenous sample with $p = 0.005$, there was a variance difference among each group (**Table 2**).

Table 2. Difference mean of ER α expression between group

	ER α expressio n	<i>p</i>
Negative control (6)	16.74 \pm 5, 012	<0.22 9*
Solvent control (7)	16.64 \pm 13 .00	
Intervention 1 (6)	19.31 \pm 6. 52	
Intervention 2 (7)	25.63 \pm 7. 08	
Intervention 3 (7)	28.79 \pm 20 .31	

*Data presented as mean \pm SD. *Kruskal-Wallis test*

Using the Kruskal-Wallis test, the *p-value* was > 0.05 . It concludes that there is no significant difference between ER α expression level among rats intervention groups (negative control, solvent control, intervention 1, intervention 2, intervention 3).

Pearson correlation test showed that the correlation between ER α expression and transfluthrin dose was significant. Pearson correlation test score of 0.388 showed a positive correlation with a low correlation strength (**Table 3**). The higher the transfluthrin dose, the higher the ER α expression level.

Table 3. Correlation between Transfluthrin Dose and ER α expression

Transfluthrin dose		ER α expression
	R	0.388*
	<i>P</i>	0.003
	N	20

**Pearson correlation test*

DISCUSSION

Arieska *et al.*^[14] conducted a study where Wistar rats given subchronic transfluthrin per inhalation showed an increase in ER α in seminiferous tubules. In this study, the researcher evaluated the effect of subchronic transfluthrin per inhalation exposure to estrogen receptors α in the testicles (seminiferous tubules and *interstitial cells*).

In this study, we had a solvent control group in which n-hexane solvent was used and the negative control group that received no intervention. We want to evaluate the effect of n-hexane solvent to the ER α expression in the testicles. On the ER α expression level graphics, the value between the solvent control group and negative control group was almost the same. The concentration that was related to mortality in animal study was about 11.000 ppm.

No study had reported endocrine effects after inhaled n-hexane exposure in experimental animals.^[15] N-hexane is metabolized in the liver. The initial reaction was oxidation by isozyme cytochrome P-450 into hexanol, especially 2-hexanol. The effect of n-hexane solvent probably caused seizures in rats because this exposure was known to have toxic effects on the central and peripheral nervous system in experimental animals.^[15,16]

This study used a lower transfluthrin dose than the recommended dose that causes no toxicity. Rats that were given inhaled transfluthrin exposure for 13 weeks (6 hours/day) showed that the NOAEC (*No Observed Adverse Effects Concentration*) safe dose was 47,6 mg/m³. Transfluthrin had inhaled LC₅₀ (*Lethal Concentration*) >513 mg/m³ in rats.^[9] This study used inhaled transfluthrin dose of 0.1 mg/ml; 0.2 mg/ml and 0.4 mg/ml that was equivalent with 0.16 mg/m³; 0.32 mg/m³ dan 0.8 mg/m³.

Transfluthrin is a member of the pyrethroid group, which is an endocrine disruptor. Pyrethroid and its metabolite disrupt nuclear hormone receptors with an unclear interaction. The chemical compounds have *mimicking effects* and can interact with the ER.^[17]

In this study, we found no significant difference between estrogen receptor α and rats intervention groups, showed by a comparative analysis study using Kruskal-Wallis test with $p=0.229$.

Our study showed increased ER α level in the testicles in the intervention group, which were given transfluthrin exposure, compared to the control group. It might reflect the regulation effect of the nuclear receptor^[18] and could serve as a coactivator or *mimicking effect* that bound with the ERs.^[17]

Nuclear receptor regulation can occur through the binding with its ligands, such as hormones, vitamins, metabolite products, and intermediate and also *xenobiotics*.^[18] Pyrethroid, like transfluthrin, is a *xenobiotic*.^[19] The characteristics of nuclear receptors are that when there is no binding between the ligand and its receptor, the nuclear receptor inside the nucleus will not come out to the nuclear surface. Nuclear receptors have to bind with its ligand with a high affinity. Some conditions that affect the ligand in nuclear receptor regulation are: ^[18]

1. Precursors availability
2. Synthesis
3. Secretion
4. Activation (*prohormone to active hormone*)
5. Deactivation (*active hormone to inactive hormone*)
6. Elimination (*hepatic, renal clearance*)

(Table 3) showed a weak positive correlation between increased transfluthrin dose

and ER α expression with correlation value $r= 0.388$ and $p = 0.003$. This result confirmed the immunohistochemical description, which showed that the higher the transfluthrin concentration (0.1 mg/ml; 0.2 mg/ml and 0.4 mg/ml), the higher the ER α expression. This data showed that level of ER α expression was dose-dependent. Insilico study proved that transfluthrin had an affinity effect on ERs with the binding affinity of -9 kcal/mol.^[19]

Our study had some limitations include a small sample number, the inhalation methods that used only the inhalation chamber because of limited source in the laboratory. The recommended methods for toxicity test nose-only inhalation, which is more accurate. This study should have measured the chromatography gas to evaluate the transfluthrin concentration in the inhalation chamber. This study also should have examined the histopathological anatomy of the testicles to identify the changes from transfluthrin exposure, to support the theory. We hoped that this data could be the basis for the next environmental epidemiology and endocrinology study development.

In conclusion, subchronic inhaled exposure of transfluthrin among rats showed a tendency to increased expression of ER α by the addition transfluthrin dose (dose-dependent). There is a weak positive correlation between the doses of transfluthrin with ER α expression.

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Conflict of Interest

There is no conflict of interest in this experimental study.

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