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The Sublethal Genotoxic Effects of Environmental Pollutants of Etofenprox on Zebrafish *(Danio rerio)*

Çevresel Kirletici Etofenproks'un Zebra Balıklarında *(Danio rerio)* Subletal Genotoksik Etkileri

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ABSTRACT

In this study, zebrafish (*Danio rerio*), which is a model organism in ecotoxicological research, was used to determine the sublethal effects of etofenprox on aquatic ecosystems. Non-ester synthetic pyrethroid etofenprox (2-(4-ethoxyphenyl)-2-methylpropyl 3-phenoxybenzylether) can be taken into the body either by direct water or indirectly with rainwater and surface waters of pest control programs. Experimental groups were exposed to etofenprox for 48 and 96 hours at the 96th hour LC_{50} 1/10 (8.1 µg/L) and 1/100 (0.81 µg/L) dose. In order to evaluate genomic oxidative DNA damage, whole body zebra fish were homogenized and DNA isolation was performed. DNA samples are then hydrolyzed and the oxidative damage was measured by commercial kit as EIA. Compared to the control group, low and high doses of 8OHdG in both groups were high. DNA damage level was found to be statistically significantly higher in both doses compared to the 96th hour group exposed to high and low dose etofenprox and the 48th hour group exposed to etofenprox. As a result, it is suggested that the sublethal concentrations of etofenprox has acute genotoxic effect in zebra fish and causes tissue damage and related with the duration of exposure repair mechanisms may be effective.

Keywords: Etofenprox, zebra fish, DNA damage, environmental pollutants

ÖZ

Bu çalışmada ekotoksikolojik araştırmalarda model organizmalardan olan zebra balığı *(Danio rerio)*, etofenproksun sucul ekosistemler üzerindeki öldürücü etkilerini belirlemek için kullanılmıştır. Ester olmayan sentetik piretroid etofenproks (2-(4-etoksifenil)-2-metilpropil 3-fenoksibenzileter phenoxybenzylether), haşere kontrol programları ile direkt su aracılığı ile ya da dolaylı olarak yağmur suları ve yüzey suları ile vücuda alınabilir. Deney grupları 96. saat LC_{50} değeri 1/10 (8.1 µg/L) ve 1/100 (0.81 µg/L) dozunda etofenproksa 48 ve 96 saat boyunca maruz bırakılmıştır. Oksidatif DNA hasarını değerlendirmek için tüm vücut zebra balıkları homojenize edilerek DNA izolasyonu yapıldı. Daha sonra DNA örnekleri hidrolize edilerek, oksidatif hasar 8-hidroksi-2'deoksiguanozin (8OHdG, ng/g doku) olarak enzim immun yöntem ile ölçülmüştür. Kontrol grubu ile karşılaştırıldığında her iki grupta, düşük ve yüksek her iki dozda 8OHdG düzeyleri yüksek gözlendi. DNA hasar düzeyi 96. saat yüksek ve düşük doz etofenproksa maruz bırakılan grup ile 48. saat etofenproksa maruz kalan grup ile karşılaştırıldığında her iki dozda istatistiksel olarak anlamlı yüksek bulundu. Sonuç olarak subletal konsantrasyonlarda etofenproksa maruziyetin zebra balıklarında akut genotoksik etki gösterdiği ve doku hasarına yol açtığı, maruziyet süresinin devamı ile tamir mekanizmalarının etkin olabileceği düşünülmektedir.

Anahtar kelimeler: Etofenproks, zebra balığı, DNA hasarı, çevresel kirletici

INTRODUCTION

The wide spread distribution and toxic nature of pesticides may have a serious impact on the aquatic environments and can reach to human beings through food web. The extensive use of insecticides has increased the incidence of pollution of the whole environment and the most significant effects can be seen on the contaminated water ecosystems. Etofenprox (1-[[2-(4-ethoxyphenyl)-2-methylpropoxy]methyl]-3-phenoxybenzene, CAS Registry Number: 80844-07-1), non-ester pyrethroid against broad spectrum of pests, used in agricultural pest control, forestry,

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animal health and public health against many insect pests, especially for *Lepidoptera, Hemiptera, Coleoptera, Diptera, Thysanoptera* and *Hymenoptera* (1). Like other pyrethroids, the mode action of etofenprox is disrupting the Na channel functions in the nervous system following direct contact or ingestion. The concentration of 0.03-1.2 kg/hectare can be used for 14 days changing from country to country, vegetation and the formulation (2,3). It can reach to aquatic resources directly vector control through run off treated areas and rain water (4).

Although etofenprox is one of the most used insecticide, the studies are limited to acute toxic effects on some aquatic species. The 96-h LC_{50} values of etofenprox for different aquatic vertebrates *Lepomis macrochirus*, *Oncorhynchus mykiss*, *Oreochromis niloticus*, *Tilapia zilli* and *Danio rerio* were calculated as 13 µg/L, 2.7 µg/L, 8.4 mg/L, 5 mg/L and 0.079 mg/L, respectively (5-7). For aquatic invertebrates, DeLorenzo and Leon (8) were found 96-h LC_{50} values 0.89 µg/L for larvae and 1.26 µg/L for adults of *Palaemonetes pugio*. 96-h LC_{50} values were determined as 0.41 µg/L on *Astacus leptodactylus* (9).

Exposure of the organisms to sublethal concentrations of chemicals can cause stress as resulting changes in biochemical, histological, genotoxic and physiological responses. Aquatic vertebrates were preferred as model organisms in toxicological studies due to the similar responses that can occur in higher vertebrates (humans) (10). Zebrafish (*Danio rerio*) is one of the most recommended test species and an eminent model vertebrate organism in multidisciplinary use with a number of existing test protocols. The zebrafish genome shares a high degree of sequence similarity to that of humans. Approximately 70% of genes associated with diseases in humans have functional homologs in the zebrafish (11). Zebrafish are also good bioindicator organisms as a toxicological model for the determination of genotoxic and histopathological effects.

The disruption in the water molecule structure and the breaking in intramolecular bonds (H-OH) occur due to the formation of free radical groups ($H^{\bullet}$, $OH^{\bullet}$, H^{+} and OH – groups). The most important oxygen-free radical causing damage to the basic biomolecules (proteins, membrane lipids, and DNA) is the hydroxyl radical ($HO^{\bullet}$) (12). The interaction of $OH^{\bullet}$ with the nucleobases of the DNA strand, such as guanine which is a highly polar molecule, leads to the formation of its nucleoside deoxyguanosine (8-hydroxy-2-deoxyguanosine, 8-OHdG) which is the predominant form of free radical-induced oxidative lesions, and has therefore been widely used as a biomarker for oxidative stress. Guanine might interact with its surroundings, especially with other polar molecules in the cell in a stronger way that makes it a potential threat to cellular damage (13).

This study was aimed to evaluate the oxidative DNA damage to the whole body of zebra fish after exposure to two sublethal concentrations for 48 and 96 hours.

MATERIAL and METHODS

Test Organism

Adult zebra fish (*Danio rerio*, n=112) model organisms on ecotoxicological studies, were used to determine the sublethal effects of etofenprox on aquatic ecosystems. The fish were obtained from local breeder. The mean length of adult zebrafish was 3.59±0.67 cm.

Acclimatization and Test Concentrations

Fish were acclimated to laboratory conditions for two weeks before the experiments; maintained in spring water. The fish were stocked as 14 fish/8 aquariums. They were fed ad libitum with commercial fish feed. Feeding was stopped 24 h before starting the experiments. The protocol (Gazi University GU.ET-17.029) for using zebra fish in the experiments was reviewed and approved by the Gazi University (Ankara, Turkey) Animal Experiments Local Ethical Council. Guiding principles for experimental procedures found in Gazi University Council and Declaration of Helsinki of the World Medical Association regarding animal experimentation were followed in the present study. Standardized OECD and Turkish National regulation for static bioassays were applied.

Test Chemical and Experimental Design

Technical grade (95.5%) etofenprox (Shenzhen Co. Ltd., Shenzhen, Guangdong, China) was donated by the Insecticide Testing Laboratory of Hacettepe University, Ankara and stored at +4°C. The first stock solution was prepared by adding 1.02 g of etofenprox to 100 mL dimethyl sulfoxide (DMSO) then diluting that as stock solution 2 with DMSO as a ratio off 1/100. Dosing solutions were prepared from this stock solution 2 by diluting with DMSO. The 1/10 (8.1 µg/L high dose, HD) and 1/100 (0.81 µg/L,

low dose, LD) of 96 h LC_{50} value were applied for 48 and 96 h for zebrafish. Control groups (Control and DMSO added control) were also conducted under same conditions. All aquaria were aerated during the experiments except for the dosing instance.

Tissue DNA Oxidation Assay

For the measurement of oxidative DNA damage (lesions/10^6 DNA nucleosides), after the whole tissue of zebra fish genomic DNA were extracted by MO BIO (UltraClean Tissue and Cells DNA Isolation Kit, Cat No: 1233-250) DNA extraction kit, it was denatured by heating at 95°C for 3 min and then cooled on ice. 100 µL, 2 mM DFAM and 20 mM acetate buffer (pH=5) were added to the denaturated DNA. DNA content was analyzed spectrophotometrically at 260 nm and then hydrolyzed to nucleotides by incubation with 4µl of 3.3 mg/mL suspension of nuclease P1. The Tris–HCl buffer (pH=8.5) was added to the mixture and hydrolyzed to the corresponding nucleosides by incubation with calf intestine alkaline phosphatase for 1 h at 37°C. After adding acetate buffer and 50 mM EDTA/10 mM DFAM solution, the mixture was filtered through a 0.22-lm Millipore filter unit (UltraFree, Bedford, MA) and then centrifuged at 10.0009 g for 20 min at 4°C. Oxidative damage was analysed by commercial kit Cayman DNA/RNA Oxidative Damage as EIA (Catalog No: 589320) (14, 15).

Statistics

The data are expressed as mean ± standard error (SEM). After assessing data normality distribution and homogeneity of variances, parametric tests of Student's t-test were used for differences between groups. When these assumptions were not met, nonparametric Mann-Whitney U and Kruskal-Wallis H tests were used.

RESULTS

Oxidative DNA damage as 8-hydroxy-2'deoxyguanosine (ng/g tissue) was statistically significantly increased at 48 hours etofenprox exposed groups ($P<0.05$), however no difference was observed for 96 h at both exposed groups compared to controls. The mean of the 48-hour 8-OHdG values of HD (8.1 µg/L) and LD (0.81 µg/L) etofenprox exposed group were found to be statistically significantly higher (2829.20 ± 235.48, 2558.07 ± 289.37 ng/g tissue respectively) compared to control group (1780.43 ± 47.70 ng/g tissue) ($p<0.05$). The results were shown in Figure 1.

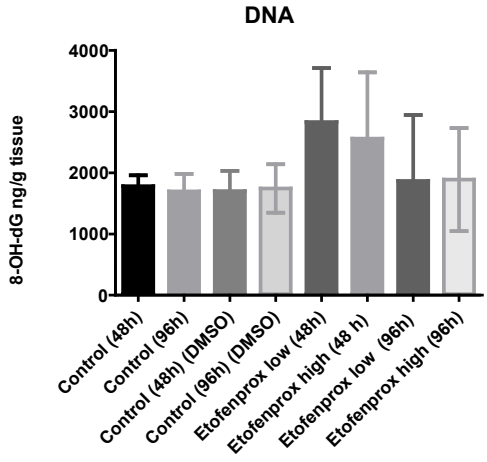

Figure 1. DNA-RNA damage as 8-hydroxy-2'deoxyguanosine (ng/g tissue) after exposed to 8.1 and 0.81 µg/L etofenprox for 48 and 96-h.

DISCUSSION

In this study, zebra fish were exposed to sublethal doses of 8.1 µg/L and 0.81 µg/L of etofenprox for 48 and 96 hours and following the DNA isolation from the whole tissue homogenates the product of oxidative DNA damage was determined as 8-hydroxy-2-deoxyguanosine (8-OHdG, ng/g tissue). One of the most important results obtained was that the mean 8-OHdG levels of the low and high dose groups were significantly higher than the control group after 48 hours of exposure. Besides, after 96 hours of exposure, there was a statistically significant decrease in low and high dose groups compared to 48-hour groups, but no difference was observed compared to control groups. The 8-OHdG levels were determined after 96 h of exposure suggest that the adaptive or devastating mechanisms against radicals are active throughout the long term exposure despite damage observed at acute phase. Besides antioxidant mechanisms may have a role to overcome the radical effects. These results are important in order to suggest about the ecotoxicological effects of etofenprox on DNA.

Etofenprox is a broad-spectrum insecticide that affects the nervous system of insects after ingestion or by direct contact. It is used in agriculture, horticulture, viticulture, forestry, animal and public health practices against different organisms. It is absorbed in small amounts by plant roots

and has low translocation in the plant. It is widely used to fight against malaria. Taking in to account the public health aspects, it can be transmitted to humans by direct application or by impregnation of fabrics (1,5).

In recent years, synthetic pyrethroid and neonicotinoid pesticides with low toxicity are preferred for public health and agricultural purposes instead of dichloro diphenol trichloroethane (DDT) and similar pesticides which were prohibited had long lasting effects in the environment. In addition to their toxicity to target insects, they also show toxic effects on some aquatic organisms (including fish species consumed by humans). Since some toxic effects may impair the genetic structure of organisms, they both adversely affect the reproduction of the population and also disrupt the ecological balance. Water pollutant pesticides exposed at low concentrations do not cause a significant deterioration in the external structure, but can cause damage at the gene level, tissue-organ levels and may affect the biochemical parameters. Aquatic organisms are exposed to insecticides as a result of non-focal contamination from agriculture in natural surface waters; the other species of the ecosystem, and therefore the food web is affected by this environmental pollution up to the carnivore fish from the highest trophic level. The major routes of insecticides and other pesticides from agricultural areas to neighboring streams, lakes and ponds are surface run off, drainage, groundwater, wind drift and atmospheric transport (16). The toxic and genotoxic effects of this pollution on exposed organisms can be much more risk than the adverse health effects of a single compound in controlled experimental conditions, due to factors such as the presence of multiple compounds, bioaccumulation and long-term degradation due to sediment. Despite the lack of significant agonistic activity of etofenprox in different studies conducted at gene level (17), the observation of antiestrogenic and thyroid hormone antagonist activity suggested that etofenprox and other pyrethroids may have multiple mechanisms of action (18). In resistance tests using World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) bio-methods, high levels of resistance to etofenprox have been described as a reduction in target sensitivity, similar to DDT (19). Cross-resistance between insecticides in different organisms has been reported, as well as resistance to oxidative mechanism (Cytochrome P450) (20,21). Hojo et al. found etofenprox had a stimulating effect on liver tumor in rats and increased reactive oxygen species production in microsomes isolated from the livers of etofenprox treated rats. Besides the thiobarbituric acid-reactive substances levels and 8-OHdG content also significantly increased in all of the etofenprox treated groups (22).

In agricultural workers exposed to the chronic effect of pesticides; disorders of the liver, kidney and muscles have been observed, neurological disease, cancer and as well as many genetic damages (23). Also recent studies have suggested oxidative stress as one of the mechanisms for the adverse health effects of pesticides exposure that the alteration of the physiological balance bring to the excess of oxidant species, resulting in severe damage to cellular components and macromolecules, especially the DNA (24).

In our previous study we determined the antioxidant enzyme activities that could be neither increased nor decreased levels related with exposure time and dose of carbamate pesticides and concluded about the necessity of control the doses of pesticide levels around the environment and avoid reaching them to water supplies emphasis about the rapid tissue specific metabolic effects (25). In this study, our findings showed the genotoxic effects of etofenprox on zebra fish, which could be suggested the pesticide possess a potential mutagenic and genotoxic effects on organisms depending on the widespread usage of the it all around the world. Etofenprox was found to be very highly toxic to zebrafish, a non-target organism, even in sublethal concentrations.

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