Assessing the Genotoxicity Effect of a Commercial Chlorpyrifos Formulation in *Fejervarya limnocharis* Tadpoles (Anura: Dicroglossidae) Under Acute and Chronic Exposure

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Abstract

The potential for genotoxicity of pesticides is currently one of the world's concerns. Chlorpyrifos is the organophosphate active ingredient with the largest sales, but the potential for genotoxicity in amphibians is still not widely known. The purpose of this study was to assess the genotoxicity effect of a commercial chlorpyrifos formulation Dursban 200EC in *F. limnocharis* tadpole erythrocyte (Anura: Dicroglossidae) under acute and chronic exposure using by micronucleus assay. Acute and chronic toxicity tests consisted of negative control, positive control, and 0.4, 0.8, and 1 $\mu g.L^{-1}$ of chlorpyrifos with three replications. A toxicity test was carried out on ten tadpoles (Gosner 25) from artificial reproductions in each treatment. The results showed that the formulation of Dursban 200EC in low concentrations (0.4 $\mu g\ l^{-1}$) had the potential to induce DNA damage in erythrocytes of *F. limnocharis* tadpoles, and there was a positive correlation between chlorpyrifos concentrations and an increase in the frequency of MN. Erythrocytes exposed to chlorpyrifos in both acute and chronic treatment had significantly different MN frequencies between negative and positive controls, 0.4, 0.8, and 1 $\mu g.L^{-1}$ ($p<0.01$). Meanwhile, positive controls were not significantly different from 1 $\mu g.L^{-1}$ ($p>0.05$). However, the increase in the frequency of MN in chronic treatment was almost twice as high.

Keywords: Chlorpyrifos, *F. limnocharis*, Genotoxicity, Micronucleus Assay, Tadpoles

INTRODUCTION

The increase in world food demand has led to agricultural expansion, which is often accompanied by excessive and indiscriminate use of pesticides [1,2]. This phenomenon is known to have triggered chemical contamination in the agricultural environment and has been cited as one of the factors causing the decline in local and global amphibian populations [3-6]. Currently, organophosphate (OPs) pesticides are the most widely used pesticides in agriculture [7-9], while these pesticides have relatively no target specifications and are toxic to most non-target organisms, especially amphibians [10,11].

Chlorpyrifos is one of the most active non-systemic organophosphate compounds and is classified as a Class II (moderately toxic) pesticide that is most widely used in agriculture [12,13]. These insecticides act as inhibitors of acetylcholinesterase (AChE), which are involved in neurotransmission in muscles and nerve synapses [14,15], so that they can induce hyperexcitation that triggers paralysis, loss of respiratory control, and even death [10,16]. Chlorpyrifos is known to be toxic to most aquatic organisms [17], while the application of chlorpyrifos is often done carelessly and excessively so that chlorpyrifos residues are often detected in sediments or water surfaces [18]. Based on the evaluation, the residual chlorpyrifos from runoff, spraying, and spillage in streams and water bodies in the agro-ecosystem ranges from 0.01 to 0.1 $mg.L^{-1}$ [19-21] and in soils ranging from 0.011-0.063 $mg.kg^{-1}$ [9]. In addition, chlorpyrifos has also been shown to induce genotoxicity because it inhibits DNA and protein synthesis at the cellular level [6,22,23].

In contrast to many experimental studies of genotoxicity in rodents and fish, the genotoxicity information of chlorpyrifos in Anura is relatively scarce [24-26], whereas Anura is one of the aquatic organisms that have a high chance of being exposed to chlorpyrifos at all stages of its life [23,27,28]. The micronucleus (MN) test is one of the simplest, fastest, and most sensitive genotoxicity test methods, especially for aquatic organisms and amphibians [24,29]. The results of the evaluation of chlorpyrifos toxicity in Anura showed different and species-specific results [14, 15,23,30]. It indicates that additional studies on other species need to be carried out [14].

Fejervarya limnocharis (Anura: Dicroglossidae) is one of the Anura species that has great potential for exposure to chlorpyrifos, especially in the tadpole phase because it lives and breeds in shallow waters of the agroecosystems and is widespread in Asia where there are intensive agricultural activities [31-34].

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Therefore, the assessment of the genotoxicity effect of chlorpyrifos on *F. limnocharis* tadpoles is important to know because DNA damage from genotoxic stress can threaten the integrity of the organism [35], while the species is an integral component of the agroecosystems [34,36].

This study was aimed to assess the genotoxicity effect of a commercial chlorpyrifos formulation in *Fejervarya limnocharis* tadpole erythrocyte (Anura: Dicroglossidae) under acute and chronic exposure by using Micronucleus assay. This study was expected to be supporting data to determine the level of toxicity of chlorpyrifos to Anura.

MATERIAL AND METHOD

Ethical Clearance

All experiments involving animals were performed following ethical standards. The University of Brawijaya ethics committee evaluated and approved the study protocol with the registration number of ethical clearance, 092-KEP-UB-2021.

Experimental Design

This study consisted of acute and chronic toxicity tests with working procedures adapted from literature [21,37-40]. The treatments in both tests consisted of control (NC and PC), 0.4, 0.8, and 1 $\mu g.L^{-1}$ chlorpyrifos with three replications at each concentration. The negative control (NC) consisted of tadpoles that were reared in dechlorinated water without any mixture, while the positive control (PC) used 5 $mg.L^{-1}$ cyclophosphamide (CF). The treatments were carried out on ten pooled random tadpoles (Gosner stage 25) for each repetition. The Gosner stage is a generalized system to describe the stages of embryonic and larva development in anurans [41]. A toxicity test was carried out on the test solution with a volume of 3L with natural light and dark cycle for each treatment. The acute toxicity test was carried out for 96 hours of treatment, while the chronic toxicity test was carried out until there were tadpoles in NC entering Gosner 46. The parameter evaluated in this study was the number of micronucleated erythrocytes from each treatment. In each treatment, five tadpoles were taken as evaluation samples, so that five preparations were obtained for each treatment.

Treatment Preparation

Test Organism

The *F. limnocharis* tadpoles were obtained from artificial reproduction using a method that referred to the research of Kurniawan *et al.* [42]. Adult *F. limnocharis* were collected from rice fields in Blayu Village, Wajak, Malang Regency, East Java, Indonesia (Loc. 8°06'55.62" S 112°44'23.87" E). The process of acclimatization to semi-natural habitats and artificial reproduction is carried out in the Laboratory of Ecology and Animal Diversity, University of Brawijaya.

The gonadal maturation process in female individuals was initiated by injecting the Trial Batch of 2000 IU hCG by Kings Lab at a dose of $1mL.kg^{-1}$ dissolved in 0.9% physiological NaCl. The injection is done intraperitoneally, and after 8-10 hours, the eggs are removed by the stripping method. Fertilization was carried out for 10-15 minutes by mixing eggs and sperm suspension from the crushed male frog testes. The fertilized eggs were then transferred to a plastic container measuring 53 × 38 × 17 cm containing 10 L of dechlorinated water equipped with aeration at a water temperature of 20-25°C. The egg mass was allowed to hatch, and two days after hatching, the tadpoles were given feed in the form of boiled spinach and boiled egg yolk that had been mashed. Tadpoles that have reached Gosner stage 25 [41] are then randomly selected from the rearing tank as test animals.

Test Solution

Commercial grade chlorpyrifos with the trademark Dursban 200 EC containing 200 $g.L^{-1}$ chlorpyrifos (PT Dow AgroSciences, Indonesia, RI. 01010119746) was purchased from a local pesticide dealer. Stock solutions with a concentration of 1 $mL.L^{-1}$ were prepared using dechlorinated tap water, while test solutions based on effective concentrations for acute and chronic toxicity tests were prepared by diluting the stock solution. The test solution for the positive control was prepared to dissolve 5 mg CF per one liter of dechlorinated water.

Treatment

Acute Toxicity Bioassay

An acute toxicity bioassay on *F. limnocharis* tadpoles was carried out for 96 hours. Five treatments were tested, consisting of negative control (NC), positive control (PC), and three levels of chlorpyrifos concentration (0.4, 0.8, and 1 $\mu g.L^{-1}$). NC consisted of tadpoles reared in dechlorinated water, while PC consisted of tadpoles reared in 5 $mg.L^{-1}$ CF solution. The treatment for each concentration and control was carried out on ten Gosner 25 tadpoles [35] with three replications. Samples of tadpoles that have been randomly selected are then

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transferred to a plastic container with 3 L of the test solution. During the experiment, the experimental animals were kept under controlled water conditions with a temperature of 24 ± 2°C, a median 7.1 pH, and a natural light/dark cycle.

Chronic Toxicity

The chronic toxicity test in this study consisted of five treatments which were divided into negative control (NC), positive control (PC), and three levels of chlorpyrifos concentration (0.4, 0.8, and 1 µg.L^{-1}). The test concentrations are obtained from the result of the preliminary test. The treatment was carried out in a plastic container containing 3 L of the test solution with ten Gosner 25 tadpoles [41] with three replications for each concentration. The period of chronic exposure was carried out from Gosner 25 to Gosner 46 [41].

During the experiment, all animals were kept under controlled water conditions with a temperature of 24 ± 2°C, a median pH of 7.1, and a natural light/dark cycle. Update of the test solution was carried out every nine days. The tadpoles were fed boiled organic spinach and boiled egg yolks that had been mashed three times a week during the exposure period up to Gosner 41. Tadpoles that already had forelegs (Gosner 42) were kept in tanks with an inclination of 10° so that there was a dry area. During this period, the tadpoles were not fed until the tail is completely absorbed because the tadpoles have obtained nutrition from the fat stored in the tail.

Data Collection

Micronucleus Assay (MN)

F. limnocharis tadpoles for each acute and chronic treatment were prepared in duplicate. At the end of exposure, a blood smear is made of the blood removed from the heart for each tadpole. The blood sample was placed on a glass slide and then smeared. Next, it was fixed with absolute methanol for 10 minutes, air-dried, and stained with Giemsa 6% for 20 minutes. Five smear preparations from each acute and chronic treatment were then observed using a light microscope (Olympus, Type CX21LEDFS1-1-2, oil immersion lens, 100/1.25) to determine the frequency of micronuclear cells (MN‰). The number of count cells is 1000 cells from five fields of view. The calculation results obtained are then calculated as cells per 1000 (‰) with the following formula [24]:

$$MN\ ‰ = \frac{number\ of\ micronucleus}{total\ number\ of\ cell\ counted} \times 1000$$

Data Analysis

The data were statically analyzed using the SPSS program with One Way of Variance Analysis (ANOVA) and were continued to the Post Hoc test with the p-value <0.05.

RESULT AND DISCUSSION

Erythrocytes of *F. limnocharis* Tadpole

The mature erythrocytes of the *F. limnocharis* tadpoles were elongated oval in shape with the structure and position of the nucleus visible so that the presence or absence of fragments in the cytoplasm can be easily identified. Micronuclei (MN) induced by commercial formulations of chlorpyrifos in erythrocyte cells were generally dot-shaped separated from the main nucleus with shapes and sizes that vary between cells. Most of the erythrocytes exposed to chlorpyrifos had a single micronucleus (Fig. 1A) while some cells had more than one micronucleus (Fig. B) and some had larger micronuclei sizes.

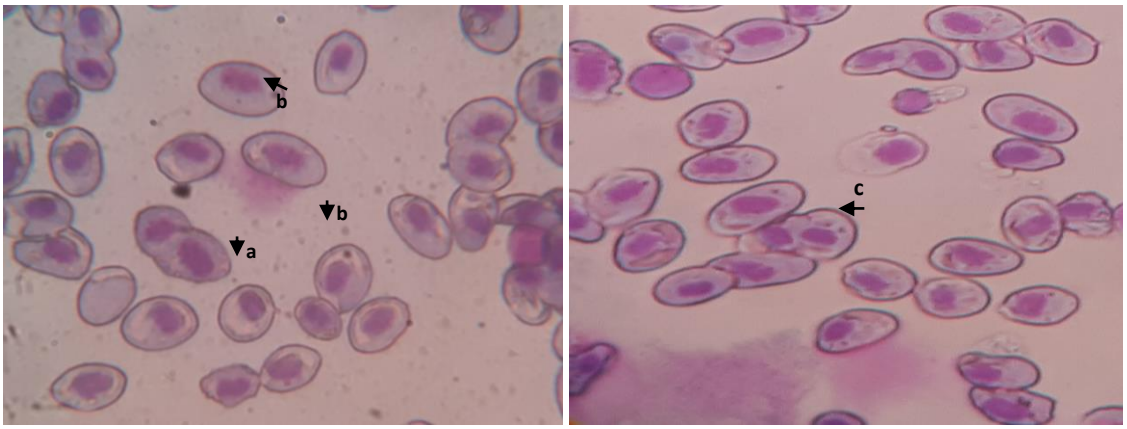

Figure 1. Erythrocytes of *F. limnocharis* tadpole exposed to the commercial organophospate insecticide Chlorpyrifos (1 µg.L^{-1}). Blood smear stained with Giemsa, 1000x . a) Normal erythrocytes, b) Single micronucleus, c) Double micronucleus.

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Micronucleus (MN) analysis is widely used as a method to evaluate the genotoxicity effect of pollutants on several organisms, especially amphibians, by detecting the presence or absence of small chromosomal fragments produced by clastogens or chromosomes [24-26]. The formation of MN in amphibian tadpole erythrocytes that were exposed to pesticides has been well demonstrated in previous studies [43,44], while the micronucleus was an indicator of genetic damage [24,25]. The results of research by Bhatnagar *et al.* [45] stated that exposure to chlorpyrifos was able to induce changes in cell morphology, the presence of nuclear anomalies such as damage to the nucleus, and the presence of large micronuclei. In the study of Yin *et al.* [25], exposure to chlorpyrifos of the tadpoles *Bufo bufo gargarizans* showed the presence of nuclear pyknosis, two-nucleated erythrocytes, and changes in erythrocyte cell morphology.

Micronucleus Frequency

Acute Exposure

The results of the genotoxicity test from acute exposure showed the potential for low doses of commercial chlorpyrifos formulations to induce DNA damage in erythrocyte cells of *F. limnocharis* tadpoles and a positive correlation between chlorpyrifos concentrations and increased micronuclear frequency (MN‰). MN frequency of all treatment groups consisting of positive control (57.2 ± 3.8‰), 0.4 µg.L^{-1} (40.8 ± 3.7‰), 0.8 µg.L^{-1} (48.8 ± 2.3‰), and 1 µg.L^{-1} (59.6 ± 8.8‰) was significantly different ($p<0.01$) with the negative control group (18 ± 4.9‰). Meanwhile, the increase in the frequency of MN in the positive control was not significantly different by 1 µg.L^{-1} ($p>0.05$) (Fig. 2).

This study showed that acute exposure to a commercial formulation of chlorpyrifos in low concentrations (0.4 µg.L^{-1}) induced DNA damage in erythrocytes of *F. limnocharis* tadpoles. Chlorpyrifos exposure to *Bufo bufo gargarizans* tadpoles with a concentration of 0.16; 0.32 and 0.64 mg.L^{-1} [25] and 10, 100, 200, and 400 mg.L^{-1} in the tadpole *O. carvalhoi* [13] for 96 hours showed a significant increase in micronuclear frequency after being compared with negative controls [13,24,25]. These results indicated that *F. limnocharis* tadpoles had a higher sensitivity to chlorpyrifos than the results of previous studies on *B. b. gargarizans* and *O. carvalhoi* tadpoles.

This variation is most likely caused by differences in the resistance of the test species [25]. The difference in sensitivity of commercial chlorpyrifos between these species confirms the idea of a species-specific possibility. Thus, the tadpoles of *F. limnocharis* species may be more sensitive to the toxic effects of chlorpyrifos in lower concentrations when compared to other species in the literature.

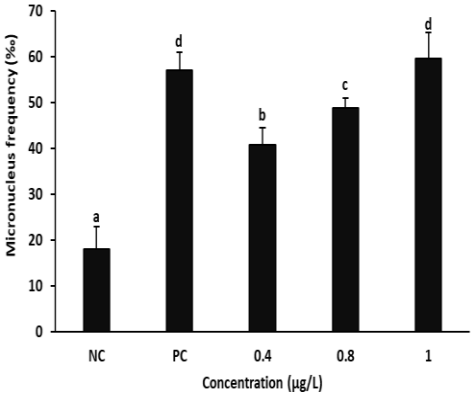

Figure 2. Micronucleus frequency (‰) of *F. limnocharis* tadpoles exposed to different chlorpyrifos concentrations under acute exposure. The a, b, c and d letters showed a significant difference compared to the control group (NC) ($P<0.05$) according to the ANOVA test at each concentration

Chronic Exposure

Long-term exposure of the commercial formulation of chlorpyrifos showed the potential to induce DNA damage with the formation of micronuclei in erythrocytes of *F. limnocharis* tadpoles after 36 days of exposure. The results of the genotoxicity test showed a positive correlation between the concentration of chlorpyrifos and an increase in the frequency of micronuclei (MN). The MN frequency of individuals in the negative control group (32.8 ± 6.6‰) had significantly different results with the positive control (119 ± 9.6‰), 0.4 µg.L^{-1} (92.8 ± 5.2‰), 0.8 µg.L^{-1} (113 ± 6.4‰) and 1 µg.L^{-1} (121 ± 8.1‰) ($p<0.01$). The increase in the frequency of MN in the positive control was not significantly different from the 1 µg.L^{-1} treatment ($p>0.05$) (Fig. 3).

The results of long-term exposure with the same concentration as acute exposure showed an almost twofold increase in the frequency of MN compared to the results of acute exposure. All concentrations of chlorpyrifos assay can induce much higher micronucleus counts compared to controls [44]. Yin *et al.* [25] reported that exposure to chlorpyrifos in Anura could significantly increase the frequency of MN in proportion to the increase in concentration

and duration of exposure. Similar results have been reported by several other studies in humans [45], fish [46], and tadpoles [47].

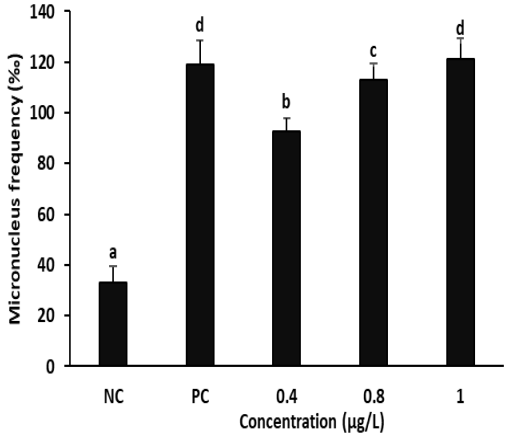

Figure 3. Micronucleus frequency (‰) of *F. limnocharis* tadpoles exposed to different chlorpyrifos concentrations under chronic exposure. The a, b, c and d letters a significant difference compared to the control group (NC) ($P<0.05$) according to the ANOVA test at each concentration.

In summary, the commercial formulation of chlorpyrifos (Dursban 200EC) could cause DNA damage in erythrocyte cells of *F. Limnocharis* tadpoles. The mechanism of DNA damage triggered by chlorpyrifos can be through oxidative stress mechanisms or the mitochondrial pathway [48-50]. MN and morphological changes of the erythrocyte nucleus can trigger genetic mutations, metabolic damage, pathology, and pathophysiological conditions so that it can affect or reduce physical conditions, species survival, and loss of local diversity. *O. carvalhoi* tadpoles were susceptible to genetic changes caused by the use of the commercial formula chlorpyrifos (Klorpan®) [13]. Thus, the results of this study further show that chlorpyrifos can induce micronucleus and DNA damage in tadpole erythrocytes. Therefore, it is possible that the results could support further genotoxic studies in other Anura species and for the understanding of sustainable agricultural models that do not compromise the sustainability of amphibian communities.

CONCLUSION

The genotoxicity assessment showed that the commercial chlorpyrifos formulation of Dursban 200EC had potential genotoxicity in *F. limnocharis* tadpoles. It is known that it can induce an increase in the formation of micronuclei, even at the lowest concentration ($0.4\ \mu g.L^{-1}$). There was a positive correlation between the concentration of chlorpyrifos and an increase in the frequency of micronuclei (MN) in both the short-term and long-term treatment. As this is the first study to examine the genotoxicity effect of the chlorpyrifos on *F. limnocharis* tadpoles, it establishes baseline data that can be used as general reference values for future investigations involving this species and other anuran tadpoles.

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