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Induction of micronuclei and nuclear abnormalities in erythrocytes of mosquito fish (*Gambusia affinis*) following exposure to the pyrethroid insecticide lambda-cyhalothrin

Fulya Dilek Gökalp Muranli, Utku Güner*

Department of Biology, Trakya University, Faculty of Science, 22030 Edirne, Turkey

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ABSTRACT

In the present study the induction of micronuclei (MN) and nuclear abnormalities (NA) in erythrocytes of mosquitofish (*Gambusia affinis*) (Baird & Girard 1853) was studied. Fish were exposed to three different concentrations of lambda-cyhalothrin (LCT) (1×10^{-4} μg/l, 2×10^{-4} μg/l, 4×10^{-4} μg/l) for periods of 6, 12, 24, and 48 h. NA (notched, lobed, blebbed nuclei), MN, bi-nucleated cells, and the ratio of polychromatic erythrocytes (PCEs) to normochromatic erythrocytes (NCEs) were evaluated to assess genotoxicity and cytotoxicity. LCT significantly induced MN and NA in erythrocytes of *G. affinis*. The PCE/NCE ratio was also decreased after 24- and 48-h treatments of 4×10^{-4} μg/l LCT. The results show that LCT has genotoxic and cytotoxic potential on *G. affinis*.

1. Introduction

Applications of large amounts of pesticides on agricultural areas contribute to the presence of toxic substances in the environment. These chemicals can find their way into the water reservoirs, streams and rivers, thus producing an adverse impact on the aquatic biota, including fish [1]. Pyrethroids are synthetic forms of pyrethrins, which are widely used for control of various insect pests. They are extremely toxic to aquatic organisms, including fish, invertebrates, and amphibians [2–5]. Many pyrethroids may have potentially deleterious effects at sub-lethal levels [6–9]. The lipophilicity of pyrethroids facilitates their rapid access to the various tissues and thus leads to a high affinity of these pesticides to the central nervous system [10]. Lambda-cyhalothrin (LCT) is a synthetic type-II pyrethroid with a broad spectrum of insecticidal and acaricide activity used in a variety of applications to control a wide range of insect pests, including aphids, Colorado beetles, and butterfly larvae. It may also be used for structural pest management or in public health applications to control insects [11]. Many studies have revealed cytotoxic and genotoxic effects of LCT in mammalian test systems [12–14]. Because fish have a poor ability to metabolise such xenobiotics, these pesticides become relatively more toxic to fish species [15] as compared with species of mammals and birds [16,17]. Toxic effects of formula-grade pyrethroid insecticide LCT on fish species have been demonstrated [18–20].

Pyrethroids can enter the aquatic environment during agricultural use, by drift during forest-spraying procedures, and by direct spraying of water bodies. The presence of genotoxins—even in low doses—concerns aquatic and non-aquatic species through the food chain and via drinking-water [21]. It is therefore important to assess the genotoxic and cytotoxic activity at low concentrations of chemicals.

The micronucleus (MN) assay has been used as a measure of genotoxicity in fish under laboratory and field conditions [22–26]. The formation of nuclear abnormalities (NA) such as lobed, blebbed, and notched nuclei described by Carrasco et al. [27] has been reported in fish erythrocytes as a consequence of exposure to environmental and chemical contaminants with cytotoxic, genotoxic, mutagenic or carcinogenic activity. However, the mechanisms responsible for such abnormalities have not yet been described. Micronuclei (MN) are formed during cellular division, and they reflect cytogenetic effects, i.e. loss of chromosomal fragments or whole chromosomes that are not included in the main nucleus following anaphase. The micronucleus test in fish has the potential to detect clastogenic and aneugenic effects of environmental agents in aqueous media. Because teleost erythrocytes are nucleated, MN have been scored in fish erythrocytes as a measure of clastogenic activity [28].

Several authors have identified NA including blebbed, lobed, and notched nuclei and bi-nucleated cells as possible indicators of genotoxicity [29–33]. Although the mechanism responsible for the

* Corresponding author. Tel.: +90 284 235 28 26x1194; fax: +90 284 235 40 10.
E-mail addresses: fulyadilek@trakya.edu.tr (F.D. Gökalp Muranli), uguner@trakya.edu.tr (U. Güner).

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formation of NA has not been fully explained, these abnormalities are considered to be indicators of genotoxic damage and therefore may complement the scoring of MN in routine genotoxicity surveys.

This study was undertaken to determine NA, MN, and cytotoxic activity (ratio of polychromatic erythrocytes [PCEs] to normochromatic erythrocytes [NCEs]) of LCT in mosquito fish (*Gambusia affinis*). These widespread, easily obtainable fish are used for biological control of mosquitoes. They are also used for many experimental tests and readily adapt to laboratory conditions. In the present study, we investigated genotoxic and cytotoxic effects of low concentrations of LCT at different exposure periods on erythrocytes of *G. affinis*.

2. Materials and methods

2.1. Chemicals

Concentrations of the pesticide were chosen according to a previous study in which the 96-h LC_{50} of LCT was found to be 1.107 μg/l [34]. Lower concentrations than the LC_{50} dose were chosen (1×10^{-4} μg/l, 2×10^{-4} μg/l, 4×10^{-4} μg/l) at which the animals did not show signs of reduced survival. The commercial product LCT (Tekvando 5 EC) was used as the test substance. The study compound LCT, CAS chemical name alpha-cyano-3-phenoxy-benzyl(Z)-(1S,3S)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2-2-dimethylcyclopanecarboxylate, was obtained from Safa Agro Konya, Turkey. Cyclophosphamide (CP) (CAS no.: 6055-19-2 SIGMA) was used as positive control at a concentration of 5 mg/l.

2.2. Experimental animals

G. affinis (Ordo, *Cyprinodontiformes*; Family, *Poeciliidae*) were obtained from the Güllapoğlu Pond (41°38′45″N, 26°37′21″E) in Edirne, Turkey, by use of a fish trap. The fish were transferred to our controlled laboratory and kept in continuously aerated glass aquaria (100 l) for two weeks before the experiment, in flowing dechlorinated (active-carbon filtered) and aerated Edirne city tap-water (Güllapoğlu Pond water: Ca^{2+} 40 mg/l, pH 8.0, 14 °C). The temperature, oxygen content, and pH of the aquarium water were monitored daily.

2.3. Experimental design

Before the experiment, fish were acclimatized in an aquarium (100 l) of well-aerated water at 20–21 °C. Fish were then placed in aquaria containing tap water (negative control) and three different concentrations of LCT and CP for 6-, 12-, 24-, and 48-h exposure periods. Five fish were tested for each concentration and exposure period.

2.4. Measurement of NA, MN, and PCE/NCE

Slides were prepared according to Ueda et al. [35]. Briefly, peripheral blood samples were obtained from the caudal vein of the specimens and smeared on clean slides. Cells were dried overnight, fixed with absolute methanol for 5–10 min, and stained with acridine orange (AO; 0.01 g/100 ml) in Sorensen's phosphate buffer. Three slides were prepared from each fish, and 2000 cells were observed from each fish. Erythrocytes were scored under 100× magnification to determine the frequency (‰) of notched, lobed, and blebbed nuclei, micro- and bi-nucleated cells, and PCE/NCE [27]. The slides were coded and randomized prior to scoring for MN, NA, and PCE/NCE ratios.

NA were classified according to Carrasco et al. [27]. Blebbed nuclei represent a relatively small evagination of the nuclear membrane, which contains euchromatin. Nuclei with evaginations larger than those of the blebbed nuclei, which could have several lobes, were classified as lobed nuclei. Nuclei with vacuoles and appreciable depth into a nucleus that did not contain nuclear material were recorded as notched nuclei. Small, non-refractive, circular, or ovoid chromatin bodies showing the same staining pattern as the main nucleus were considered micronuclei [28]. Only MN—one-fifth or one-third the diameter of the main nucleus—that were in the same plane of focus and were of the same colour, texture, and refraction as the main nucleus and clearly separated from it, were counted. Decreases in the proportion of PCE/NCE were considered as indicators of induced cytotoxicity [36]. PCE frequency was calculated as follows, according to Pacheco and Santos [37]:

$$\text{PCE frequency } (\%) = \frac{\text{No. PCEs}}{\text{No. PCEs} + \text{NCEs}} \times 100$$

The weight and length of the specimens (mean ± SD) were 0.14 ± 0.1 g and 23.9 ± 3.5 mm.

2.5. Statistical analysis

The frequencies of MN and NA were expressed per 1000 cells (‰). The statistical significance of the differences in mean values between exposure and control groups was determined with Student's *t*-test, and the differences between exposure periods were determined with the Tukey test, at the $p < 0.05$ level.

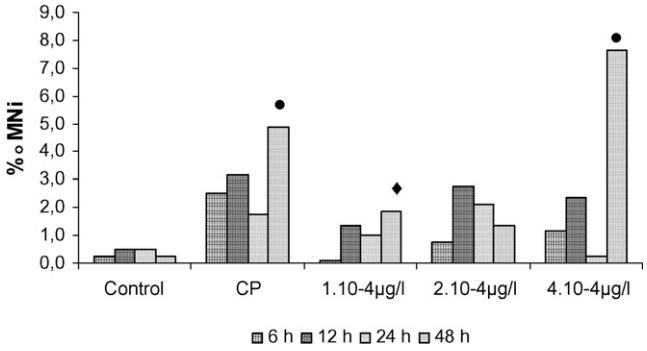

Fig. 1. Percentage of micronucleated erythrocytes after exposure to three concentrations of LCT during four different time periods in erythrocytes of *G. affinis*. Symbols show significance of MN induction according to exposure periods. Significantly different from: (♦) 6-h exposure period; (●) 6- and 24-h exposure periods.

3. Results

Table 1 summarises the frequencies of MN, NA, and PCE/NCE determined in different treatments. Low concentrations of LCT significantly induced MN and NA in erythrocytes of *G. affinis*. Although LCT did not induce MN after 6 h of exposure, all the concentrations of this pesticide significantly induced MN after the 12- and 48-h exposure periods. After 24 h, only the 2×10^{-4} μg/l concentration induced MN in erythrocytes of *G. affinis*. NA were increased after 2 and 4×10^{-4} μg/l LCT for the 6-h exposure period and after 1, 2, and 4×10^{-4} μg/l LCT for the 12- and 48-h exposure periods. Moreover, it was seen that the 24-h exposure to 2×10^{-4} μg/l concentration significantly induced NA, just like the MN induction for the same exposure period. After 24 and 48 h, the concentration of 4×10^{-4} μg/l LCT decreased the PCE/NCE ratio in erythrocytes and revealed its cytotoxic effect.

Figs. 1 and 2 show the results of MN and NA induction according to concentration and exposure periods. The negative control did not show any change according to exposure period in both graphs. The frequency of MN was increased at 12 h and had decreased at 24 h. At 48 h, CP and 4×10^{-4} μg/l LCT increased the frequency of MN (Fig. 1). This increase at 48 h is significantly different from same exposure at 6 and 24 h. In Fig. 2, 1×10^{-4} and 4×10^{-4} μg/l LCT concentrations decreased NA frequencies, although this was not significant. Increases of NA after 4×10^{-4} μg/l at 12 h and after 2×10^{-4} μg/l at 24 and 48 h are significant in their exposure periods.

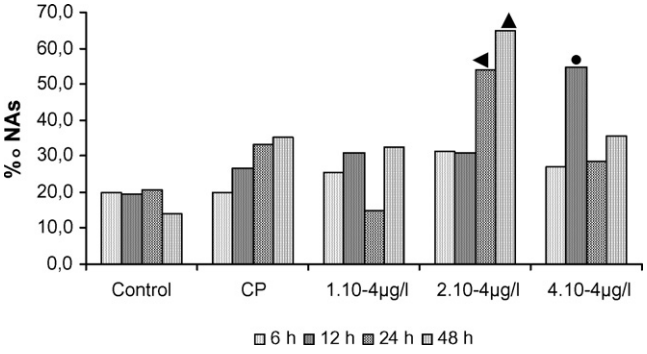

Fig. 2. Percentage of NA after three concentrations of LCT during four different time periods in erythrocytes of *G. affinis*. Symbols show significance of NA induction according to exposure periods. Significantly different from: (◄) 12-h exposure period; (▲) 6- and 12-h exposure periods; (●) 6- and 24-h exposure periods.

Table 1
Frequencies of MN and NA, and PCE/NCE ratio after exposure to 1, 2, and 4×10^{-4} μg/l LCT concentrations during four different exposure periods in erythrocytes of *G. affinis*.

Treatment	Conc.	MN/1000 erythrocytes (mean ± S.E.)			
		6 h	12 h	24 h	48 h
Control	–	0.25 ± 0.14	0.5 ± 0.29	0.5 ± 0.28	0.25 ± 0.14
CP (mg/l)	5	2.5 ± 0.64*	3.18 ± 0.47**	1.75 ± 0.82*	4.87 ± 0.66***
Lambda-cyhalothrin μg/l	1×10^{-4}	0.12 ± 0.12	1.38 ± 0.24*	1 ± 0.68	1.84 ± 0.29*
	2×10^{-4}	0.75 ± 0.48	2.75 ± 1.10*	2.13 ± 0.31**	1.38 ± 0.38*
	4×10^{-4}	1.17 ± 0.39	2.38 ± 0.37**	0.25 ± 0.14	7.63 ± 2.71*
Treatment	Conc.	NAs/1000 erythrocytes (except MN) (mean ± S.E.)			
		6 h	12 h	24 h	48 h
Control	–	20.0 ± 0.88	19.5 ± 3.83	20.75 ± 1.48	14.04 ± 4.28
CP (mg/l)	5	19.75 ± 1.97	26.5 ± 3.99	33.17 ± 8.97	35.25 ± 5.12*
Lambda-cyhalothrin μg/l	1×10^{-4}	25.37 ± 4.03	30.87 ± 1.56*	14.88 ± 5.3	32.33 ± 5.63*
	2×10^{-4}	31.12 ± 1.7***	30.87 ± 3.41*	53.88 ± 7.23**	64.75 ± 7.24***
	4×10^{-4}	27.14 ± 2.23**	54.62 ± 4.65***	28.38 ± 11.52	35.53 ± 1.99**
Treatment	Conc.	PCE/NCE			
		6 h	12 h	24 h	48 h
Control	–	2.29 ± 0.31	2.25 ± 0.7	1.77 ± 0.18	3.02 ± 0.54
CP (mg/l)	5	4.57 ± 1.17	3.55 ± 0.79	6.53 ± 1.54	0.57 ± 0.23**
Lambda-cyhalothrin μg/l	1×10^{-4}	2.11 ± 0.65	4.27 ± 0.99	4.24 ± 1.21	2.85 ± 0.23
	2×10^{-4}	3.54 ± 0.15	15.05 ± 0.5	7.2 ± 1.39	9.57 ± 2.41
	4×10^{-4}	5.67 ± 3.73	5.69 ± 2.07	0.89 ± 0.3*	0.47 ± 0.27**

* $p \leq 0.05$.
** $p \leq 0.01$.
*** $p \leq 0.001$.

The positive control CP significantly induced MN formation, but it significantly induced NA formation only at 48 h. The 4×10^{-4} μg/l concentration caused a higher frequency of MN than the positive control at 48 h. NA at 2 and 4×10^{-4} μg/l were significantly higher than the positive control at all times.

4. Discussion

Various chemical exposures have shown morphological nuclear abnormalities (NA) in both human and fish cells [38,39]. Micronucleus (MN) formation as well as induction of nuclear abnormalities were considered to be the consequence of genotoxic events in fish [37,24]. Several authors have reported that pyrethroid insecticides induce NA and MN in erythrocytes of fish. Cabagna et al. [40] demonstrated that a commercial formulation of cypermethrin (pyrethroid) induced MN formation in tadpoles of *Odontophrynus americanus* (amphibian). A commercial form of deltamethrin increased MN frequency in erythrocytes of *Tilapia rendalli* [41]. Ansari et al. [42] indicated that deltamethrin induced MN and NA in erythrocytes of the freshwater fish *Channa punctata*. In the present study, low concentrations of LCT significantly induced MN and NA frequencies in erythrocytes of *G. affinis*. The observed abnormalities, which were higher in number than the positive control, may be a result of experimental conditions (temperature, pH, and Ca^{2+} concentration). Also, Mauck et al. [43] stated that LCT is more toxic at cooler temperatures.

The results of the present study are similar to those of Campana et al. [44], who reported that LCT is a genotoxic agent in erythrocytes of the fish *Cheirodon interruptus interruptus*, and are in accordance with those of Çavaş and Ergene-Gözükara [45], who showed that LCT treatment caused an increase in the frequency of micronucleated erythrocytes in the fish *Garra rufa* at concentrations of 0.01 and 0.05 μg/l.

In the present study, the response to treatment with LCT diminished at 24 h, although increased responses were seen at 12 and 48 h. Similarly, in the study of Campana et al. [44] time variations in the MN frequency were observed in erythrocytes of *Cheirodon i. interruptus* after LCT exposure. The researchers explained this variation as to be related to the blood-cell kinetics and erythrocyte replacement. Although their study and the present study are similar, we observed different variations in MN frequency after different exposure times. These variations may be a result of species differences and may depend on genetic factors, the assay used, or environmental effects. Information about the induction and frequency of MN in hematopoietic tissues of *G. affinis* is lacking. These variables may affect the responses of fish erythrocytes to chemical agents at different time intervals.

In the study by Çavaş and Ergene-Gözükara [30], after exposure of textile-mill effluent on *Oreochromis niloticus*, the frequency of MN and NA in erythrocytes decreased with time as the dosage increased. These differences in the MN frequency with time seem to be related to cell kinetics and cell replacement. Similar time-related effects were observed in peripheral erythrocytes of fish exposed to mill effluents [46], river pollutants [26], and metallic mercury [47].

Under normal conditions, fish are usually able to keep the concentration of red blood cells relatively constant. Such a homeostasis results from a dynamic equilibrium between new formation (erythropoiesis) and destruction of erythrocytes. New erythrocytes are continuously entering the circulation, and effete erythrocytes are destroyed at the same rate [48]. Assessment of the PCE/NCE ratio can provide evidence of exposure to toxic substances. Such effects result from the inhibition of the division and maturation of nucleated erythropoietic cells. In this case, depression of the proportion of PCE occurs [49]. Reductions in the proportion of PCE/NCE are considered as indicators of mutagen-induced cytotoxicity [36]. Some studies indicate that a decrease of the PCE/NCE ratio reveals cytotoxic effects of some chemicals. Çavaş [50] showed that mercury chloride and lead acetate significantly reduced the PCE/NCE ratio in peripheral blood of *Carassius auratus auratus*. Pacheco and Santos [37] indicated that the PCE frequency decreased in the European eel (*Anguilla anguilla* L.) after exposure to benzo[*a*]pyrene and dehydroabietic acid. Çavaş and Ergene-Gözükara [51] analyzed peripheral blood samples obtained from *O. niloticus* and showed

a significant decrease in the PCE/NCE ratios after metronidazole treatment. In the present study, the PCE/NCE ratios of peripheral blood samples of *G. affinis* were significantly decreased after exposure to 4×10^{-4} μg/l LCT at 24 and 48 h. This significant decrease indicates cytotoxic effects of LCT on erythrocytes of *G. affinis*.

In conclusion, our data indicate that very low concentrations of commercial-grade LCT induced MN formation in erythrocytes of *G. affinis* and revealed genotoxic effects. In addition, LCT has a cytotoxic potential as revealed by a decrease in the PCE/NCE ratios in erythrocytes of *G. affinis*. Although large amounts of pyrethroid insecticides degrade in water and soil under field conditions [19], LCT exhibits high toxicity to aquatic organisms. These data may be significant whilst assessing long-term potential risks to the aquatic ecosystems.

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