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Assessment of Genotoxicity of Methyl Parathion in Zebrafish by Use of the Micronucleus Test

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***Abstract*— It was aimed to assessment the genotoxic potential of methyl parathion (MP) on zebrafish (*Danio rerio*), by using micronucleus test. Twenty adult zebrafish were exposed to three different concentrations (1, 1.5, 3 mg/L) of MP for 96 h. In addition to negative controls, cyclophosphamide monohydrate (4mg/L) was used as a reference genotoxic agent for positive controls. Peripheral blood samples were collected from all of the specimens and stained with acridine orange. The immature, polychromatic (PCEs) and mature, monochromatic (MCEs) erythrocytes were identified by their staining intensity and scored. The ratio of PCEs/MCEs were calculated, analyzed and compared. In parallel with increased concentrations of MP, the micronucleated PCEs were increased in number, while a decline in the ratio of the PCEs/MCEs was recorded. The results revealed that MP has a short-time genotoxic effects on zebrafish.**

***Index Terms*— Methyl parathion, *Danio rerio*, genotoxicity, micronucleus test, acridine orange,**

I. INTRODUCTION

Methyl parathion (MP ,*o*,*o*-dimethyl *o*-4-nitrophenyl phosphorothioate) is an organophosphorus pesticide, which is used worldwide in agriculture. MP often contaminates water resources, and affects non-target organisms such as fish; moreover, it is directly used in aquaculture to eliminate aquatic larvae of predator insects [1] Beyond its well-known mode of action via acetylcholinesterase inhibition, MP has some hazardous effects on fish such as an increase in food consumption and energy demand [2], [3], and induction of oxidative stress [1]. As being a mutagenic agent, MP is listed in the HazDat database of United States Environmental Protection Agency (EPA), and classified as most toxic (Toxicity Category I).

Analysis of micronuclei and other nuclear deformities in peripheral blood samples is a basic technique to reveal genotoxic effects of chemicals; and widely used in fish [4]. Although the determination of aberrations in metaphase figures of fish chromosomes exhibits some problems [5], [6]; micronuclei formations in peripheral blood samples can be observed easily by different methods [6], [7], and micronucleus assay (MN) has some noticeable advantages such as rapidity and simplicity [7], [8], [9], [10], [11].

When compared to routine staining procedures such as May-Grunwald's Giemsa, it was reported that the MN assay was more sensitive when acridine orange (AO) was used [12]. AO is a nucleic acid-specific ultraviolet fluorochrome and stains both of DNA and (bright yellowish-green) and RNA (orange/reddish), on a typically green cytoplasmic background [4].

The aim of this investigation was to use MN test by AO staining to determine whether MP give raise to nuclear abnormalities in peripheral blood samples of zebrafish.

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II. MATERIAL AND METHOD

A. Experimental Design

Twenty-five adult zebrafish (Danio reiro) were purchased from commercial dealers and acclimated in 20L aquaria for two weeks under natural photoperiod. The water temperature was maintained at 27±0.3ºC. Physicochemical parameters were noted as pH 6.7–7.5, dissolved O2 6.0–7.5 mg L− 1, hardness 25–30 mg L− 1 (as CaCO3) and conductivity 65–72 µS cm− 1. Fishes were fed once daily with commercial fish food (Sera-San). Specimens were randomly divided into four experimental (TG1-4) and one control (CG) groups. TG1-3 were exposed to different concentrations (1, 1.5 and 3 mg/L) of methyl parathion (Paramet, Adeko Tarım), TG4 was exposed to 4 mg/L cyclophosphamide monohydrate (Sigma), a reference genotoxic agent. Test durations were designed as 24, 48, 72 and 96 h. After anaesthetized with MS222 (Sigma-Aldrich), peripheral blood samples were collected with heparinized syringes from tail puncture.

B. Fluorescent Staining

After diluting with fetal bovine serum (FBS; Sigma), blood samples was smeared on glass slides. Three slides were prepared for each of the specimen. Slides were air-dried overnight, fixed with absolute methanol for 10 min, stained with 0.003% acridine orange in phosphate buffered saline (pH 6.8) for 2–3 min, immediately before the microscopical obsevation.

C. Cells and Micronuclei Scoring

Immature, polychromatic (PCEs) and mature, monochromatic (MCEs) erythrocytes were differentiated under Leica DM3000 fluorescence microscope by following the classification. 1000 cells of each slide were counted and imaged by DFC 290 imaging system.

D. Calculation of Immature Erythrocyte (PCE) Frequency and Micronucleated PCE Frequencies

The frequencies (%) of PCEs and micronucleated PCEs were calculated by using the following equations:

PCE frequency=[PCEs/(PCEs+MCEs)]x100

Micronucleated PCE frequency=[Micronucleated PCEs/(PCEs+MCEs)]x100

E. Calculation of Immature Erythrocyte (PCE) Frequency and Micronucleated PCE Frequencies

The data were analyzed by Kolmogorov–Smirnov test. Two-way analysis of variance and a least significant difference (LSD) tests were also used respectively for discrimination the interactions between MP concentrations and exposure time. The p value was accepted as 0.05.

III. RESULTS AND DISCASSION

The nuclei of mature erythrocytes (MCEs) were elipsoid in shape and bright green in color. Immature erythrocytes (PCEs) were identified with their round, relatively large nucleus. Due to their RNA contains, cytoplasm of PCEs were observed as pale red (Fig 1).

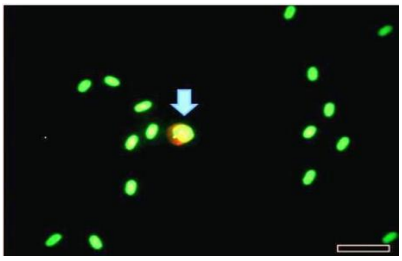

Figure 1. Immature Erythrocyte (PCE) (Arrow)

Micronucleus frequencies of PCEs of *D. rerio* treated with cyclophosphamide and MP were shown in Table 1. When compared to CG2, which was treated neither cyclophosphamide nor MP; both of the treatments were caused an increase in micronucleus frequencies of PCEs. For all of the TGs, this increase was noted as statistically significant ($P < 0.01$), and depended to MP concentration and exposure time. However, the PCE/MCE ratios of TGs were decreased with respect to CG2, as summarized in Table 2. Dose–duration interactions were also found to be statistically significant in all of the groups treated ($P < 0.01$).

GROUPS	TREATMENTS	CONCENTRATIONS	% MICRONUCLEATED PCE FREQUENCIES (MEAN ±SD)			
			24h	48h	72h	96h
CG1	Cyclophosphamide	4mg/L	3,8 ± 0,30	4,7 ± 0,35	4,8 ± 0,40	5,3 ± 0,35
CG2	-	-	0,8 ± 0,20	0,9 ± 0,30	0,9 ± 0,23	1,2 ± 0,25
TG1	MP	1mg/L	1,3 ± 0,23	1,6 ± 0,34	1,9 ± 0,37	2,1 ± 0,34
TG2	MP	1.5 mg/L	2,4 ± 0,22	3,2 ± 0,32	3,8 ± 0,46	4,2 ± 0,25
TG3	MP	3 mg/L	2,9 ± 0,30	3,6 ± 0,36	4,3 ± 0,39	5,6 ± 0,22

Table 1. Micronucleated PCE frequencies in control and test groups. ($p < 0.01$)

GROUPS	TREATMENTS	CONCENTRATIONS	% PCE VALUES (MEAN ±SD)			
			24h	48h	72h	96h
CG1	Cyclophosphamide	4mg/L	4,8± 0,23	4,0 ± 0,32	3,5 ±0,25	3,2 ± 0,27
CG2	-	-	5,4± 0,20	5,2± 0,41	5,0±0,24	4,9± 0,34
TG1	MP	1mg/L	4,9± 0,10	3,7± 0,30	3,9±0,34	4,0± 0,36
TG2	MP	1.5 mg/L	4,8± 0,30	3,4 ± 0,24	3,8 ±0,36	4,0 ± 0,36
TG3	MP	3 mg/L	4,6 ± 0,32	4,2 ± 0,52	3,6±0,27	3,9 ± 0,27

Table 2. PCE values in control and test groups

Cytotoxicity and genotoxicity can be monitored using different assays. Among the microscopical methods used to identify genotoxic effects of chemicals, MN assay was stood out with its properties noted as simplicity and rapidity [5], [8], [9], [10], [11]. As reported by Criswell et al. [13], the main disadvantage of routine MN assays is the presence of artifacts that they may raise false positive or false negative results. Together with these detriments, the most known staining methods for bright-field microscopy e.g. May-Grunwald's Giemsa, were noted as inefficient and time-consuming [4]. Although it is not routinely used, fluorescence microscopy based on AO staining can successfully remove these kinds of handicaps. Our findings are strongly confirmed that AO staining verifies to be less time wasting and more sensitive than classical techniques, moreover, it offers a better contrast to identify the micronuclei, since artefacts are not stained. Our data are in a great accordance with the reports of Costa and Costa [4], Polard et al. [12], Criswell et al (13), Çavaş and Ergene-Gözükara (14), and Çavaş [15]. As suggested formerly (16), PCEs are more sensitive for AO than MCEs, and AO could be used for determination of toxic effects of chemicals with any reservation.

Besides methodological arguments, present study demonstrated that MP has a cytotoxic potential in erythrocytes of D. rerio. MP exposition was highly effective in all of the TGs, when compared to CG2.

Although a depressed micronucleus frequency was detected in a previous field study [17], it is well known that so many chemical agents and/or pollutants cause genotoxicity. For example, erythrocytic nuclear abnormalities were increased in peripheral blood samples of Anguilla anguilla exposed to benzo[a]pyrene, dehydroabietic acid and bleached kraft paper mill effluent

[18]. Metronidazole was also noted as an inducer for micronucleus formation in Oreochromis niloticus [13]. However, informations about the cytotoxic effects of pesticides on fish are limited and only a few studies were performed. Costa and Costa [4] were revealed a striking increase in MN frequencies of erythrocytes of cadmium injected Sparus aurata. Lambda-cyhalothrin treatments on Cheirodon. interruptus interruptus [19] and Garra rufa [20] were also caused an increase of the MN frequency in erythrocytes.

The decrease of PCE/MCE ratio would also be accepted an indicator for mutagen-induced cytotoxicity [21]. In MP treated D. rerio, PCE/MCE ratios were decreased in parallel with increasing concentrations and durations.

It had been previously revealed that [17] PAH contamination was raised a decrease in PCE ratio in peripheral blood of fish. In past decades, some chemicals and pollutants such as metronidazole [6]; benzo[a]pyrene, dehydroabietic acid and bleached kraft paper mill effluent [18] were also noted as inducer of reduction of %PCE. More recently, Çavaş [15] reported a pronounced decrease in %PCE values of Carassius auratus exposed to mercury chloride and lead acetate. As noted in this report, the effects of both of the chemicals were depended on dose and time.

Our findings are in parallel with this consideration, and it is clear that MP is not only genotoxic, but also cytotoxic in zebrafish. In D. rerio, MP treatment caused a significant decrease in the PCE/NCE ratios especially for 72h exposition. From this point of view, it has been strongly proposed that MP is not only genotoxic, but also cytotoxic agent at least for D. rerio.

As a conclusion, the erythropoietic cytotoxicity is a very suitable key for monitoring survey aimed to environmental protection, and micronuclei analyses by using AO staining are the effective and affordable method for determinations of mutagenic activities. Moreover, it is clear that MP could be a destructive pollutant for aquatic environments. All of the hematopoietic processes of fish could be affected by genotoxic and cytotoxic potential of MP.

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