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Evaluation of micronuclei and other nuclear abnormalities as genotoxic assays in Common carp, Cyprinus carpio exposed to sublethal malathion doses

Masarat Jan[1*], Md. Niamat Ali[2], Farooz Ahmad Bhat[3] ,Nighat un Nissa[4]

[1,2]Cytogenetics and Molecular Biology Research Laboratory,Centre of Research for Development (CORD),University of Kashmir, srinagar, J&K, (India)

[3]Sher-E-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKAUST K), J&K, (India)

[4]Parasitological Research Laboratory, Department of Zoology, University of Kashmir, Srinagar,J&K, (India)

ABSTRACT

Malathion is an organophosphorus insecticide extensively used in agriculture and houses to control diversity of insects counting aphids, beetles, scales and pill bugs. Malathion can have an effect on fish in different ways as behavioral, reproductive, enzymes and hormonal, developmental, growth, physiological, cellular and genetic effects. This study aimed to evaluate the genotoxic effects of sublethal doses of malathion in peripheral erythrocytes of Cyprinus carpio using genotoxicity assays. The genotoxicity of malathion in Cyprinus carpio was confirmed by frequency of micronucleus in peripheral erythrocytes and a range of nuclear abnormalities after 24, 48, 72 and 96 hours. Three sub-lethal concentrations of malathion 2 ppm, 4 ppm and 6 ppm were used, and it was observed that all these concentrations were proficient to stimulate micronucleus formation in erythrocytes of Cyprinus carpio. All the treated group of specimens had higher frequency of erythrocytes with MN and nuclear anomalies (NAs) as compared to controls.

***Key Words:Cyprinus carpio,* erythrocyte, malathion, micronuclei, nuclear abnormalities**

I. INTRODUCTION

The aquatic ecosystem is the larger constituent of natural environment which is in front of the threat of diminishing genetic base and biodiversity due to disorganized use of pesticides.[1] For centuries pesticides have been used in agriculture to enhance food production by eradicating unwanted insects and controlling disease vectors.[2] Pesticide residues reach the aquatic environment where it poses considerable toxicological risks to a myriad of non target organisms [3], and finally finding their way to the food chain threatening the ecological balance and biodiversity of nature.[4] Most of the chlorinated compounds used in the past are replaced by organophosphorus compounds (OPS) because the insistence of later in the environment is short. Eventually,

alterations in the chemical composition of natural aquatic environments can affect the freshwater fauna, mainly fish, which are of great economic importance to humans. [5 and 6]

Malathion is a non systemic, broad spectrum organophosphate insecticide. It was one of the most primitive organophosphate insecticides developed (Introduced in 1950). Malathion(O,O-dimethyl-S,1,2-bis ethoxycarbonyl ethylphosphorodithioate; CAS number 121-75-5) is an organophosphorus insecticide extensively used in agriculture and houses to control diversity of insects counting aphids, beetles, scales and pill bugs. Apart from target specimens, non-target animals including fish are greatly affected by these pesticides. [7] .Malathion can affect on fish in different ways as behavioral, reproductive, enzymes and hormonal, developmental, growth, physiological, cellular and genetic effects [8], but the information about genotoxic potential of malathion in aquatic organisms is scarce.[9] Many reports have shown that malathion, even at a low concentration, troubles fish. The negative effects of malathion on growth parameters, haematological properties, swimming ability, and the depletion of some biochemical parameters (glycogen, cholesterol, and total protein content) have been recognized [10, 11 and 12], and its oxidative damage to gold fish has been reported. [13]

It is believed that the fish posses the same biochemical pathways to deal with the toxic effects of endogenous and exogenous agents as do mammalian species.[14,15 and 16].Besides, causing mortality, these pollutants can cause genotoxicity in aquatic organisms which can show the way to harsh consequences in fishes like growth of tumors.[17].These changes in the genetic material of organisms can be detected by using genotoxicity test system. These studies will present information that would be important for formulation of strategies and preparation with reference to maintenance of aquatic ecosystems and can be accepted to industries and extra agencies for recognition.

The micronucleus test (MNT), one of the mainly accepted tests of environmental genotoxicity, has served as an indicator of cytogenetic damage. [18, 19 and 20]. Schroder (1966), [21] for the first time studied the formation of micronuclei in mammalian bone marrow cell, afterward this assay was developed by Schmid (1975) [22] in mammalian systems. Like mammalian species, MNT has also been adopted to study genotoxicity in fishes. It is one of the simplest, reliable, least pricey and fasttest system for both clastogenic (chromosome break and construction of acentric fragments) and aneugenic (chromosome lagging and effects on spindle) effects. [23,24] Micronuclei (MN) are cytoplasmic chromatin masses with the look of small nuclei that come up from chromosome fragments or from intact whole chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis. [25]

Al Sabti (1986) [26] tested several chemicals (aflatoxin B1, arochlor 1254, benzidene, benzo(a)pyrene, and 20-methylchloanthrene) for their ability to induce micronuclei under laboratory conditions in three cyprinids viz, Common Carp, *Cyprinus carpio*, Tench, *Tinca tinca*, and Grass carp, *Ctenopharyngodon idella*.. Svobodava *et al*. (1997)[27] premeditated the effect of malachite green on *Cyprinus carpio* by using micronuclei test.

Campana *et al*. (1999) [28] evaluated genotoxicity of the pyrethroid lambda- cyhalothrin via the micronuclei test in erythrocytes of the fish *Cheridon interruptus interruptus*.

For the determination of genotoxic effect in fish, the micronucleus test together with the study of abnormal shape of nuclei serves as an appropriate measure by which the presence or absence of genotoxins can be detected in water. The detection of MN and NAs in fish aid us to estimate the situation of water quality as well as the wellbeing of a particular species and at all possible risk it might have after consumption. [29] Since micronucleated piscine erythrocytes have been proved to be sensitive indicators of genetic damage, the basis of our study was to evaluate the cytogenetic (clastogenic or aneugenic) effects of malathion in *Cyprinus carpio* fish using the MN and Nuclear abnormalities (NAs) tests.

II. MATERIALS AND METHODS

2.1 Experimental fish specimen and chemicals

Cyprinus carpio (Family: cyprinidae and order:cypriniformes) was selected as test organism. Live juvenile specimen, procured with the help of hand nets from pollution free area of Dal Lake were transported to the laboratory and subjected to a prophylactic treatment by bathing in 0.05% potassium permanganate for 5 mins to elude dermal infection. The fish had an average weight of 49.06± 1.40 g and an average length of 14.46 ± 0.80 cm. Fish were fed with commercial fish food at 2% bodyweight per day and acclimatized under laboratory conditions for 3 weeks in polypropylene troughs each with 8-10individuals/50 L containing dechlorinated tap water (pH: 7.85; alkalinity: 154mg/LCaCO3; DO: 6.70 mg/L; temperature: 28°C) before the experiment. The photoperiod used provided 12/12 h dark/light. Water was kept O_2 saturated by aeration. The troughs were cleaned daily and the water as well as the pesticide was renewed to keep the concentration constant throughout the test period of 24, 48, 72 and 96 hrs. Control fish were kept in dechlorinated tapwater without any treatment. Only healthy, active fish starved for 24hr were used for the experiment and they were allowed no food during treatment procedures.

Malathion (EC 50%) manufactured by Albata Biotech (I) Pvt. Ltd was used during the present course of study. The commercial grade preparation is a yellow coloured liquid containing 50% active ingredients and the rest is constituted by inactive ingredients. Malathion was dissolved in test water to attain the stock solutions. These solutions were more diluted to obtain the experimental concentrations in the polypropylene troughs. On the basis of literature data (LC50 values for malathion), three sub-lethal concentrations (2ppm, 4ppm and 6ppm) of malathion were then chosen for the experiment.

2.2. Experimental Design

Tests were carried out in four batches:

(i) Control-10 fishes were maintained in water without malathion;

(ii) (ii) (iii) and (iv) **Experimental**-10 fishes were kept in each of the trough contaminated with different sub-lethal concentration of malathion (2ppm, 4ppm and6 ppm) lower than LC50. Environmental conditions were

alike in all the four cases.Water of each tank containing toxicant was changed daily to eliminate faecal matter and waste metabolite of fish and to retain the required concentration of pesticide. After treatment with insecticide, the frequencies of micronuclei and nuclear abnormalities in all experimental sub-groups were examined at four durations (24, 48, 72 and 96h) and at each concentration.

2.3. Measurement of NA, MN

The smear of peripheral blood drawn from the caudal vein with a heparinized syringe, was prepared and well-dried slides were stained in 10% Giemsa solution (Stock solution diluted with Sorensen's buffer at pH 6.8) for 30 min following the method of Schmidt W. Two thousand cells per animals (1000 cells per slide) were scored for micro-nuclei and nuclear anomalies examined under the light microscope using 1000× magnification to find out the frequencies of micronucleus and nuclear abnormalities. Erythrocytes were scored to determine the frequency (‰) of notched, lobed, and blebbed nuclei and micronuclei. NAs were classified according to Carrasco *et al*. (1990). [30] 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. 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 for thescoring of micronuclei, the following criteria were adopted from Al-Sabti and Metcalfe (1995).[31]

III. RESULTS

The genotoxicity of malathion in *Cyprinus carpio* was confirmed by frequency of micronucleus in peripheral erythrocytes and a range of nuclear abnormalities after 24, 48, 72 and 96 hours. Three sub-lethal concentrations of malathion 2 ppm, 4 ppm and 6 ppm were used, and it was observed that all these concentrations were proficient to stimulate micronucleus formation in erythrocytes of *Cyprinus carpio*. Micronuclei (MN) induced by malathion in the peripheral erythrocytes were generally dot shaped and were close to the main nucleus with size and shape varies between cells. Number of cells with one MN were found to be more in number whereas cells with two MN were very less. No cell with three to five MN was observed.It was observed that some erythrocytes exhibited nuclear anomalies like blebbed, notched,lobed nuclei. The frequency of micronuclei and nuclear anomalies of various kinds in the peripheral erythrocytes in *Cyprinus carpio* exposed to different concentrations of malathion for varying periods of time and in control is summarized in **TABLE 01 and TABLE 02** respectively. All the treated group of specimens had higher frequency of erythrocytes with MN and nuclear anomalies as compared to controls. The frequency of micronuclei in peripheral erythrocyte amplified progressively with the increase in the period of exposure and/or concentration of the malathion. The maximum induction of micronuclei was observed on day 4 at the highest concentration (6ppm).The fishes were exposed to

three sub-lethal concentrations of 2 ppm, 4 ppm and 6 ppm. The percentage of single micronuclei in *Cyprinus carpio* (0.10 ± 0.05 of control) increased to 1.35 ± 0.38 from low to high concentrations after 24h and continued to increase by 1.40±0.33 and1.72 ± 0.21 and then 1.81±0.22 respectively after 48h, 72h and 98 h exposure is shown in **TABLE 01.** In all the groups getting malathion dose significantly higher frequencies of NAs were observed when compared to the control. The frequencies of lobbed nuclei, blebbed nuclei, and notched nuclei were considerably higher in all experimental groups when compared to the control group. Nuclear abnormalities frequencies were found to be significantly after 96 hrs compared to other exposure days. On the whole, similar to the results obtained for MN, NA frequencies in erythrocytes increased dose- and time-dependently in all treatment groups.

Table 1 Micronuclei frequencies (%) in blood erythrocytes of *Cyprinus carpio* exposed to three concentrations of malathion.

Treatment	**Exposure(hrs)**	**MN Frequencies (%) Mean±SD**			
		24h	**48h**	**72h**	**96h**
Control	----------	0.10±0.05	0.09±0.05	0.16±0.03	0.19±0.02
Malathion	**2ppm**	0.31±0.10	0.44±0.16	0.59±0.09	0.49±0.21
	4ppm	0.66±0.15	0.87±0.27	0.92±0.26	0.98±0.23
	6ppm	1.35±0.38	1.40±0.33	1.72±0.21	1.81±0.22

Table 2

Nuclear abnormalities (%) in blood erythrocytes of *Cyprinus carpio* exposed to three concentrations of malathion.

Exp.(hrs)	**Treatment**	**Nuclear Abnormalities. Mean (%) ±SD**			
		NT	**BL**	**LB**	**Total NAs**
24hr	**2ppm**	1.04±0.21	0.54±0.15	0.63±0.15	0.73±0.26
	4ppm	1.70±0.14	1.36±0.20	0.87±0.09	1.31±0.41
	6ppm	1.96±0.29	1.64±0.16	1.11±0.17	1.57±0.42
48h	**2ppm**	1.38±0.16	0.96±0.21	0.72±0.23	0.93±0.39
	4ppm	1.68±0.27	1.62±0.18	0.9±0.14	1.4±0.43

	6ppm	2.05±0.33	1.72±0.21	1.19±0.17	1.65±0.43
72hr	**2ppm**	1.47±0.14	0.94±0.21	0.94±0.04	1.11±0.30
	4ppm	1.84±0.35	1.48±0.02	0.93±0.13	1.41±0.45
	6ppm	2.29±0.09	2±0.12	1.32±0.18	1.87±0.49
96hr	**2ppm**	1.63±0.33	1.4±0.88	0.88±0.13	1.30±0.38
	4ppm	2.12±0.19	1.49±0.04	0.92±0.10	1.51±0.60
	6ppm	1.98±0.36	1.71±0.63	1.28±0.15	1.65±0.35

Exp:exposure time in hours. NT:notched nuclei. BL:blebbed nuclei LB:lobbed nuclei NAs:nuclear abnormalities

IV. CONCLUSION

Organophosphorus compounds or organophosphates (OPs) form a big faction of chemicals used over the past 60 years for protecting crops, livestock, human health and as combat agents. OPs are the most widely used pesticides worldwide and their metabolites are prevalent across different populations. [32, 33 and 34]. On the other hand, these pesticides lack specificity, and it has been confirmed that they are also enormously toxic to non target species, including mammals, birds and aquatic organisms The unwanted short-term effects of contact to these chemicals have been studied commonly in the nervous system, which is their main target.[35] These pesticides can get to natural waters either by way of transfer of the chemicals from the soil or by direct spraying of the target organisms. Ultimately, alterations in the chemical composition of natural aquatic environments can affect the fresh water fauna, particularly fish, which are of great economic importance to humans. [36,37] It is known that in fish organophosphate pesticides are neurotoxic and they inhibit acetylcholinesterase activity with consequent disruption of nervous functions, thereby interfering with some of the vital physiological functions.[38] Fish have been largely used to estimate the quality of aquatic systems as bioindicators of environmental pollutants.[39] Micronucleus (MN) formation as well as induction of nuclear abnormalities are considered to be the result of genotoxic events in fish.[40, 41] Fish and aquatic invertebrates have been considered to be competent and cost effective model systems for studying the toxic, mutagenic, and carcinogenic potential of pollutants [42] due to their ability to metabolize, concentrate, and store water-borne pollutants.[43] Malathion has been reported to be more toxic to insects and fish than to mammals due to lack of hydrolytic enzymes in the former.[44] Numerous authors have reported that malathion insecticide induce NAs and MN in erythrocytes of fish. In *Channa punctatus* a progressive increase in the percentage of micronuclei is found with increase in the intensity of exposure to malathion. [45] While studying the genotoxic effects of sublethal doses of cadmium in peripheral erythrocytes of *Oreochromis niloticus* using micronucleus (MN) and nuclear abnormalities (NAs) tests the MN and NA frequencies in peripheral erythrocytes of fish increase in relation to both the time and dose applied. [46] The technical-grade malathion has the potential to generate chromosomal changes, including chromosome aberrations and MN induction in test animals. [47] A significant difference in frequency and distribution of MN was observed between the malathion exposed workers and

control workers.[48] In the present study, we evaluated effects of malathion on fish *C.carpio* using the MN and NA tests as genetic points. In the present study, low concentrations of malathion considerably induced MN and NA frequencies in erythrocytes of *C.carpio*. The frequency of micronuclei as well as nuclear anomalies in the present study considerably increased from 24 to 96 h post treatment. All test concentrations of malathion used in the present study induced a significantly higher number of MN compared to the control. Further, the MN induction and different nuclear anomalies increased significantly with the progression of the concentrations. A concentration dependent increase and time-dependent decrease in MN induction due to chlorpyrifos exposure has been reported earlier in *Channa punctatus.* [49] Although, the MN test has been found to be a sensitive test to estimate genotoxic compounds in fish under restricted conditions as an index of cumulative exposure, [50] it might undergo variations according to clastogen, test organism, and the life cycle of the cells.[51] Studies on MN induction in humans and animals indicated malathion to be genotoxic. Quite a lot of toxicants have been seen to cause micronuclei formation in fish. Al-Sabti and Hardig (1990) [52] observed micronuclei in perch found in Baltic Sea infected with pulp wastewater, while Poongothai *et al*. (1996) [53] reported micronuclei in five different fish species collected from sewage polluted water. An increase in the frequencies of cells with MN was found in *Salmo trutta* in downstream region of River Trubia where heavy metal pollutants are added in high concentration from an old military factory. [54]

Our results also showed that the exposure of fish to concentrations of malathion induced prominent levels of nuclear abnormalities. Several authors have reported that chemicals induce nuclear abnormalities in different tissues of fish. [55, 56 and 57] A large number of chemicals may obstruct in the DNA synthesis of an exposed organism, which could result in nuclear abnormalities. [58,59] Even though the mechanisms accountable for NAs have not been fully explained, these abnormalities are considered to be indicators of genotoxic damage and, therefore, they may complement the scoring of micronuclei in routine genotoxicity surveys. [60, 61 and 62] In conclusion, the results of the present study established the induction of genotoxic damage as exposed by the micronucleus and nuclear abnormalities assays on fish under exposure to malathion. However, additional studies are required to elucidate the mechanism of mutagenic activity of malathion.

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