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Micronuclei Induction by Genotoxic Effects of Methyl-S-Demeton on Peripheral Blood Erythrocytes of *Cyprinus carpio*.

Md. Niamat Ali, Hilal Ahmad Ganaie* and Zeenat Nisar
Molecular Biology Research Laboratory, Centre of Research for Development, University of Kashmir, Hazratbal, Srinagar-190006, India
*Corresponding author: Email hilalganie@hotmail.com

ABSTRACT

Methyl-S- demeton, a pale yellow oily liquid with a penetrating smell, is a systemic and contact organophosphate insecticide used to control *Thysanoptera, Hymenoptera* and *Homoptera* in fruits, cereals, ornamentals and vegetables. To evaluate the Methyl-S-Demeton mediated genotoxicity on local fish of Kashmir, the micronucleus analysis was performed on peripheral erythrocytes of adult specimens of *Cyprinus carpio communis* and *Cyprinus carpio specularis* collected from the Dal Lake. Fishes were divided in two groups i.e. the control and the experimental groups. The experimental groups for each fish species were divided into three sub groups based on the selected doses of each insecticide. After treatment with each dose of insecticide, the frequency of micronuclei in all experimental groups was examined at the durations of 24, 48 and 72h. Three sub lethal concentrations of Methyl-S-Demeton, 2 ppm, 4 ppm and 6 ppm were used and it was observed that all these concentrations were able to induce micronucleus formation in erythrocytes of both fish species. Both dose and time dependent increase in micronucleus frequency was observed in treated fish species and a peak value detected at 72 h, higher concentration of Methyl-S-Demeton clearly showed a higher incidence of micronucleated peripheral erythrocytes.

Keywords: Methyl-S- Demeton, genotoxicity, *Cyprinus carpio communis, Cyprinus carpio specularis,* micronucleus test.

INTRODUCTION

Aquatic environment pollution is a serious and governing problem. Inspite of legislation limiting the disposal of toxic chemicals, pollution of aquatic environments still occurs (Fleeger *et al*., 2003). Chemical contaminants with genotoxic and carcinogenic potential in the aquatic environments are a serious concern because they constitute a threat to aquatic as well as terrestrial life. These considerations have prompted interest in the development of techniques and bio indicators for monitoring genomic damage from hazardous contaminants in the aquatic environments (Dixon and Wilson, 2000). Among current genotoxicity test systems, the assessment of micronuclei is commonly used for evaluating structural and numerical chromosomal aberrations induced by clastogenic and aneugenic agents (Celic *et al*., 2003). Micronucleus analysis was originally developed for mammals, but it has been successfully adapted for use in aquatic organisms especially fish (Cavas and Ergene-Gozukara, 2005). Because fish species constitute a vertebrate model, they have been widely used as a model organism in aquatic genotoxicity studies. Fish are considered sentinel organisms in a health assessment of aquatic environments (Dixon *et al.,* 2002). They have a great commercial and recreational value. Micronuclei have been induced in fish exposed to genotoxic substances under laboratory conditions and field conditions (Al-Sabti, 1991; Al-Sabti and Metcalfe, 1995; Russo *et al*., 2004). Micronuclei (MN) are produced from fragments or entire chromosomes that lag in cell division because of a lacking or a damaged centromere or defect in cytokinesis. These small secondary structures of chromatin are surrounded by membranes, located in the cytoplasm and have no detectable link to cell nucleus (MacGregor, 1991; Seelbach *et al*., 1993; Zoll-Moreux and Ferrier, 1999).

The run-off of insecticides from the agricultural fields to aquatic water bodies comprises a major part of aquatic pollution. This water pollution caused by such toxic chemicals is a matter of great human concern and warrants their testing for potential

genotoxic effect. During last few years, fishes have attracted much attention as laboratory animals for this type of study and attempts have been made to examine the peripheral erythrocytes in fish for the occurrence of micronuclei and using the information in a monitoring system for potential genotoxicity of an agent proposed (Hooftman and Raat, 1982; Manna *et al.,* 1985). However the protocol is yet to be standardised with different substances and in different piscine species.

In present study the incidence of micronucleus in the peripheral erythrocytes of *Cyprinus carpio communis* and *Cyprinus carpio specularis* treated with Methyl-S-demeton was analysed. The aim of the study was to assess the micronucleus test from the blood smear of fishes for detecting the possible genotoxic effect of Methyl-S-demeton which is formulated as an emulsifiable concentrate and used as a spray on cereals, fruits, ornamentals, vegetables and particularly used in agricultural fields of Kashmir against woolly aphids.

MATERIALS AND METHODS

Collection Site and Fish Collection

Adult specimens of *Cyprinus carpio communis* and *Cyprinus carpio specularis* were collected from the Dal Lake with help of local experienced fisherman and then these collected fish were transported to the laboratory in specially designed container having oxygen supply. After collection, fish specimens were acclimated for 45 days at 28^0C prior to trials (Anitha *et al*., 2000). Specimens were kept in propylene troughs with each 5-6 individuals/50 L of water. Water was kept O_2 saturated by aeration. The troughs were cleaned daily, and the water along with the insecticide was renewed to keep the concentration constant throughout the test period of 96 h. Water quality of the test solution was determined according to the standard procedures (APHA, 1998). The control fish were kept in experimental water (pH=7.3 ± 0.6; dissolved oxygen = 7.3 ± 0.4 ppm; free CO_2 = 5.8 ± 0.4; alkalinity = 106 ± 6.8 ppm) without adding these insecticides,

keeping all other conditions constant. Fish were fed daily with commercial feed at least one hour prior to the replacement of the water.

Selection and dosage of Methyl-S- demeton:

Commercial grade formulations of Methyl-S- demeton were used because only commercial preparations are used in agriculture. The commercial grade of Methyl-S-demeton was obtained from G.M.Shah pesticides (Srinagar), manufactured from Bhopal pesticides Ltd. (Bhopal India).

On the basis of literature data (LC_{50} values for each insecticide), three sub lethal concentrations of this insecticide was selected for the experiment as shown in table:

Organophosphate Insecticide	CAS	Concentration 1	Concentration 2	Concentration 3
Methyl-S- demeton	S-[(2 (ethylthioethyl] O, O - dimethyl phosphorothioate.	2 ppm	4 ppm	6 ppm

Experimental Design:

In the experimental design, for the micronucleus assay fishes were divided in two groups i.e. the control and the experimental groups. The experimental groups for each fish species were divided into three sub groups based on the selected doses of insecticide. All groups had equal no. of fishes i.e., 5 fish per group in 50 L propylene troughs. After treatment with each dose of insecticide, the frequency of micronuclei in all experimental groups was examined at the durations of 24, 48 and 72h. Five fish specimens were used for each duration and at each concentration.

Analysis of micronucleus:

This micronucleus test was performed on peripheral blood according to the published protocols (Schmid, 1975; Hooftman and Raat, 1982; Al Sabti, 1986) with slight modifications as under:

Fishes were killed with a slight blow on the head region. Chemical treated and control

fish were cut in caudal region and smears of the peripheral blood were made on grease free clean glass slide. The simple hematoxylin and Eosin staining procedures of Pascoe and Gatehouse (1986) was employed with slight modification. The methanol fixed, air dried, smears were stained in filtered Mayer's Hematoxylin solution for approx. 5-10 minutes. The slide were then washed and rinsed in Scott's tap water substitute until the nuclear colour change (from red to blue) was completed as confirmed by microscopic examination. After completion of this blueing process, the slides were then washed in 30%, 50%, 70% and 90% alcohol. The slides were then stained in filtered Eosin solution for approximately 5 seconds or more. They were then again washed in 90% alcohol followed by washing in absolute alcohol. After dehydration, the slides were cleared in xylene and mounted using DPX. The slides were then examined using a simple light microscope (Olympus CX21) under low (600X) and high magnification (1000X).

Scoring of micronucleus

For each concentration and each duration five specimens were used and from each fish 6 slides were studied and 1200 cells (about 200 erythrocytes per slides) were scored under 600X magnification. Small non refractive, circular or ovoid chromatin bodies, displaying the same staining and focussing pattern as main nucleus, were scored. Particles with colour intensity higher than that of the main nuclei were not counted as micronuclei. Other nuclear abnormalities were also studied as classified by Carrasco *et al*., (1990). Briefly, cells with two nuclei were considered as binuclei. Blebbed nuclei present a relatively small evagination of the nuclear membrane, which contains euchromatin. Nuclei with vacuoles and appreciable depth into a nucleus that does not contain nuclear material were recorded as notched nuclei.

The slides were carefully studied and various morphological peculiarities of the nuclear material were examined under light Trinocular microscope (Leica DMLS2) for accurate scoring of micronucleus.

Statistical analysis

Statistical analysis of data to verify the significant differences in the incidence of micronucleus between treated and control groups at 0.05 and 0.01 level of significance was performed using non-parametric criteria, Mann-Whitney U test to analyse the frequency of micronuclei. To ensure statistical accuracy, only cells with one micronucleus were considered, while rarely occurred two micronuclei and other nuclear abnormalities were eliminated from the counts. All the statistical calculations were done with the help of statistical software Minitab, V11.

RESULTS

The effect of Methyl-S- demeton on micronucleus induction was studied in Cyprinus *carpio specularis* and *Cyprinus carpio communis* following exposure to three sub-lethal different concentrations of (2 ppm, 4 ppm and 6 ppm) at 24h, 48h and 72 h. A dose response relationship was observed between the frequency of micronucleated erythrocytes and Methyl-S- demeton concentrations for *Cyprinus carpio specularis* and *Cyprinus carpio communis.* The peak frequency of micronucleated erythrocytes occurred at 72h exposure.

In *Cyprinus carpio specularis,* the percentage of single micronuclei (0.03 ± 0.01 of control) increased to 1.11 ± 0.37 from low to high concentrations by 24h and continued to increase by 1.92 ± 0.38 and 2.79 ± 0.56 in longer exposures (Table 1). Statistical analysis showed that all micronuclei frequencies significantly differ from controls ($P < 0.01$)

In *Cyprinus carpio communis* the incident of single micronucleus (0.03 ± 0.01) increased to 1.25 ± 0.356 from lower to higher concentration after 24hours and this value continued to increase by 2.18 ± 0.46 and 3.36 ± 0.37 after 48h and 72h respectively (Table 2). A statistically significant difference was observed among all treated groups in relation to control group ($P < 0.05$).

Table.1: Micronucleus frequency (%) in peripheral blood erythrocytes of *Cyprinus carpio specularis* exposed to different concentrations of methyl-S-demeton

Treatment	Concentration	MN frequencies (%)		
		24 h	48 h	72 h
		Mean ± SD	Mean ± SD	Mean ± SD
Control	-	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Methyl-S- demeton	2ppm	0.04 ± 0.02	0.11 ± 0.02**	0.70 ± 0.61**
	4ppm	0.39 ± 0.05**	0.50 ± 0.16**	0.80 ± 0.11**
	6ppm	1.12 ± 0.37**	1.92 ± 0.38*	2.79 ± 0.5**

(Mann-Whitney U test)** P < 0.01

Table 2. Micronucleus frequency (%) in peripheral blood erythrocytes of *Cyprinus carpio communis* exposed to different concentrations of methyl-S-demeton

Treatment	Concentration	MN frequencies (%)		
		24 h	48 h	72 h
		Mean ± SD	Mean ± SD	Mean ± SD
Control	-	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
Methyl-S- demeton	2 ppm	0.04 ± 0.01	0.138 ± 0.01*	1.95 ± 0.84*
	4 ppm	0.92 ± 0.01*	1.7 ± 0.42*	2.32 ± 0.70*
	6 ppm	1.25 ± 0.02*	2.18 ± 0.46*	3.36 ± 0.37*

(Mann-Whitney U test)* P < 0.05

DISCUSSION

Regulations in many countries are beginning to limit point source discharges of toxic chemicals into water resources, however, historical and current industrial and urban discharges are still responsible for high concentration of toxic substances in aquatic environments (Richards *et al*., 2000).The potential genotoxic effects in aquatic organisms exposed to Phorate are poorly understood. Many contaminants present in aquatic environments not only endanger the survival and physiology of the organisms but also induce genetic alterations which may lead to mutations and cancer (Russo *et al*., 2004). Future generations can be effected by reduced fitness and embryonic viability, along with genetic disorders (Kurelec, 1993). Insecticides may lead to changes in the blood biochemical parameters and haematological profile of fish which can be investigated as biomarker in pollution monitoring (Mushigeri & David, 2005; Banaee, *et al*., 2008; Kavitha and Rao, 2009). Fish are often used as sentinel organism for

ecotoxicological studies because they play a number of roles in the trophic we accumulate toxic substances and respond to low concentration of mutagens (Cavas and Ergene-Gozukara, 2005). Therefore, the use of fish biomarkers as indices of the effects of pollution, are of increasing importance and can permit early detection of aquatic environmental problems (Lopez-Barea, 1996; Van Der Oost *et al.*, 2003). The frequencies of micronuclei in fish *Cyprinus carpio communis and* of *Cyprinus carpio specularis* from the world famous Dal Lake were observed in the study. Several studies have evaluated the genotoxic effects of methyl-S-demeton. Test results indicate that methyl-S-demeton is mutagenic. Mutagenic studies shows effects on sex chromosomes in fruit flies treated with 80 mg/kg of methyl-S-demeton. Microbes also mutated when exposed to 5 mg/kg of methyl-S-demeton (Smith, 1993). *In vitro*, methyl-S-demeton induced reverse gene mutations in *S. typhimurium* strains TA1530 and TA1535, but not in several other strains, such as TA1531, TA1532, TA1534, *hisC117*, and *hisG46* (Hanna and Dyer, 1975). Tested both with and without metabolic activation, methyl-S-demeton (purity: > 98%) was positive in *S. typhimurium* strains TA100 and TA1535 and negative in strains TA98 and TA100 (Herbold, 1980) while an overall positive result was reported for a 50.2% formulation using the same four strains (Herbold, 1979). Testing in several *E.coli* strains without metabolic activation resulted in positive results in strain WP2 *uvrA* only (not tested with metabolic activation) (Hanna and, Dyer, 1975). Methyl-S-demeton as a 53% formulation in xylene did not induce mutations in *S. cerevisiae* strains S138 and S211a when tested in the presence or absence of an S9 mix (Hoom, 1983). Methyl-S-Demeton induced recessive lethal mutations in *D. melanogaster* (Hanna and Dyer, 1975). A test for forward gene mutations in cultured mouse lymphoma L5178Y cells was positive at doses of 50-500 µg/mL in the presence and absence of metabolic activation (Cifone, 1984). In the present study the increased incidence of micronuclei was observed in peripheral erythrocytes of fish exposed to 2ppm, 4 ppm and 5 ppm of methyl-S-demeton.

In the present study the genotoxicity of Methyl-S-Demeton was tested for the

induction of micronucleus formation in peripheral erythrocytes of freshwater fish *Cyprinus carpio specularis* and *Cyprinus carpio communis*. The results revealed significant induction of micronuclei in the peripheral erythrocytes ($P < 0.01$ and $P < 0.05$) of *Cyprinus carpio specularis* and *Cyprinus carpio communis.* The appearance of interspecific differences observed could be due to the specificity of DNA repair, cell turnover time, physiological peculiarities, contaminate uptake or biotransformation in the fish species studied. A significant difference in the micronucleus incidence among treated and control groups were observed. The peak frequency of micronucleated erythrocytes was observed at 72 h after exposure. The length of the cell cycle critical to micronuclei formation depends upon the time needed to replicate DNA and perform nuclear division. In man and mice the duration of the cell cycle has been well documented.

The present study reveals micronucleus assay has a great potential for detecting clastogenic substances in aqueous media. Fish micronuclei assays represent a sensitive mean of measuring genotoxic activity in the laboratory. However, the accuracy of such assays in fish depends on improvements in certain parameters such as the number of cells scored, a better understanding of biochemical responses of fish to xenobiotics and knowledge of the erythrocyte cell cycle.

REFERENCES

Al-Sabti, K. 1991. *Handbook of Genotoxic Effects and Fish Chromosomes*. Kristoff, Ljubljana, Slovenia: Joseph Stephan Institute. 221 Pp.

Al- Sabti, K. 1986. Comparative micronucleated erythrocyte cells induction in three cyprinids by five carcinogenic, mutagenic chemicals. *Cytobios,* **47:** 147-154.

Al-Sabti, K and Metcalfe C.D. 1995. Fish micronuclei for assessing genotoxicity in water. *Mutat Res***. 343**, 121-135.

Anitha, B., Chandra, N., Gopinath, P. M and Duraira, J. G. 2000. Genotoxicity

evaluation of heat shock in gold fish (*Carassius auratus*). *Mutation Res*. **469**:1-8.

APHA/AWWA/ WPCF 1998. Standard method for examination of water and wastewater. 20th ed. APHA, Washington.**1**: 6-139.

Banaee, M., Mirvagefei, A. R, Rafei, G. R., and Majazi A.B. 2008. Effect of sub-lethal Diazinon concentrations on blood plasma biochemistry. *Int. J. Environ. Res*., **2**: 189-198.

Carrasco K, Tilbury KL, Mays MS., 1990. Assessment of the piscine micronuclei test as an *in-situ* biological indicator of chemical contaminants effects. *Can J Fish Aquat Sci***. 47:**2123-2136.

Cavas, T. and Ergene-Gozukara, S. 2005. Micronucleus test in fish cells: a bioassay for *in situ* monitoring of genotoxic pollution in the marine environment. *Environ.Mol. Mutagen***. 46**, 6470.

Cavas, T. and Ergene-Gozukara, S., 2005. Genotoxicity evaluation of metronidazole using the piscine micronucleus test by acridine orange fluorescent staining. *Environ Toxicol Phar* **19**: 107-111.

Cifone, M.A. 1984. Mutagenicity evaluation of E 154 in the mouse lymphoma forward mutation assay. Kensington MD, USA: Litton Bionetics, Inc, cited in FAO 90

Dixon, D.R., Pruski, A.M., Dixon, L. R. J and Jha, A.N. 2002. Marine invertebrate eco-genotoxicology: a methodological overview. *Mutagenesis:* **17:** 495-507.

Dixon, D.R and Wilson, J,T. 2000. Genetics and marine pollution. *Hydrobiologia* **420:** 29-43.

Fleeger, J.W., Carman, K.R and Nisbet, R.M. 2003.Indirect effects of contaminants in aquatic ecosystems. *Sci Total Environ* **317**: 207-33.

Hanna, P. J. and Dyer, K. F. 1975. Mutagenicity of organophosphorus compounds in

bacteria and Drosophila. *Mutat Res*, **28**: 405-20.

Herbold, B.E. 1979. 25/154 Salmonella/microsome test to evaluate for point mutation. Leverkusen, FRG: Bayer AG, cited in WHO97.

Herbold, B.E, 1980. 25/154 Salmonella/microsome test to evaluate for point mutation. Leverkusen, FRG: Bayer AG, cited in FAO85 and WHO97.

Hooftman, R.N and de Raat, W. K. 1982. Induction of nuclear anomalies (micronuclei) in peripheral blood erythrocytes of Eastern mudminnow *Umbra pygmaea* by ethyl methanesulphonate. *Mutation res.***104:**147-152.

Hoorn, A,J,W. 1983. Mutagenic evaluation of E 154 50 VL (in xylene) batch no. 808 204 103, content 53.1% in the reverse mutation assay with *Saccharomyces cerevisiae* strains S138 and S211a (final report). Veenendaal, the Netherlands: Litton Bionetics, cited in FAO85.

Kavitha, P and Rao, V. J. 2009. Sub-lethal effects of profenofos on tissue-specific antioxidative responses in a Euryhyaline fish, *Oreochromis mossambicus*. *Ecotoxicology and Environmental Safety*. **72:** 17271733.

Kurelec, B 1993.The genotoxic disease syndrome. *Mar Environ Res* **35**: 341-348.

Leverkusen, FRG: Bayer AG; cited in FAO 85 and WHO 97.

Lopez-Barea, J. 1996. Biomarkers to detect environmental pollution. *Toxicol. Lett***. 88:** 79.

MacGregor, J. T 1991.Micronuclei as an index of cytogenetic damage: past, present and future. *Environ Mol Mutagen* **18**: 277-291.

Manna, G. K., Banerjee, G and Gupta, S 1985. Micronucleus test in the peripheral erythrocytes of the exotic fish, *Oreochromis mossambica*, *Nucleu*s, **28**:176-179.

Mushigeri, S.B. and David, M. 2005. Fenvalerate induced changes in the Ach and associated AchE activity in different tissues of fish *Cirrhinus mrigala* (Hamilton) under lethal and sub-lethal exposure period. *Environmental Toxicology and*

Pharmacology, **20:** 6572.

Pascoe, S and Gatehouse, D. 1986. The use of a simple haemotoxylin and eosin staining procedure to demonstrate micronuclei with rodent bone marrow. *Mutat Res*. **164**: 237-243.

Richards, J.P., Glegg. A.G and Cullianane, S 2000. Environmental regulation: industry and the marine environment. *J Environ Manage* **58**:119-134.

Russo, C., Rocco, L., Morescalchi, M. A and Stingo V. 2004. Assessmrnt of environmental stress by the micronucleus test and the comet assay on the genome of teleost populations from two natural environments. *Ecotoxicol Environ Saf.* **57**:168-174.

Schmid, W. 1975. The micronucleus test. *Mutat Res*. **31**: 9-15.

Seelbach, A., Fissler, B., Strohbush, A and Madle, S. 1993. Development of a modified micronucleus assay in vitro for detection of aneugenic effects. *Toxicol In vitro* **7:** 185-193.

Smith, G. J. 1993: Toxicology and pesticide use in relation to wildlife: Organophosphorus and carbamate compounds. Boca Raton, FL IV 171Pp

Van Der Oost, R., Beyer, J and Vermeulen, N. P. E. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharm*. **13:** 57149.

Zoll-Moreux, C and Ferrier, V. 1999. The Jaylet test (newt micronucleus test) and the micronucleus test in Xenopus: two in vivo tests on Amphibian evaluation of the genotoxicity of five environmental pollutants and of five effluents. *Water Res.* ***33***: 2301-2314.