

Full Length Research Paper

Evidence of micronuclei in fish blood as a biomarker of genotoxicity due to surface run off agricultural fungicide (Propiconazole)

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Micronuclei, nucleoplasmic bridges and nuclear buds are biomarkers of genotoxic events and chromosomal instability. In laboratory, these genome damages can be measured easily. The measurement of cytogenetic alterations *in-vivo* has considered an initial step in the risk assessment procedures by genotoxic agents. In fishes, micronucleus assay has shown useful *in vivo* techniques for genotoxicity testing, and potential for *in situ* monitoring of water quality. This paper evaluates the genotoxic effects of fungicide in fish erythrocytes, with emphasis on the induction of micronuclei formation. The binucleate/mononucleate cells ratio in peripheral erythrocytes exposed to propiconazole (1.11 and 2.23 mg/L) has also been used to evaluate the time-dependent response. Micronucleus frequencies induced by fungicide is significantly greater than their respective control ($p < 0.05$) for the fish species *Clarias batrachus* throughout all treatment periods. This paper is directed to assisting laboratories in the development of micronucleus test for assays of genotoxic potential of chemicals.

Key words: Micronucleus assay, propiconazole, genotoxicity, binucleate/mononucleate cells.

INTRODUCTION

Propiconazole (PCZ) is a trizole group fungicides which was introduced in mid 1970s classified as category III and IV. Trizole containing fungicides have been used as an antifungal in agriculture for controlling pest and also for increasing food crops (Vanden Bossche et al., 1989; Warmerdam, 2008). Trizole, belonging to demethylation inhibitors (DMI) group with rapid acropetal systemicity acts on the pathogen inside the plant to stop disease development by interfering with sterol biosynthesis in fungal cell membrane. The trizole fungicides inhibit one specific enzyme C14-demethylase, which plays a role in sterol production. It has a shorter half-life and lower

bioaccumulation, but announcing effects on the aquatic ecosystems may arise from spray drift or surface run-off (Konwick et al., 2006). It has been reported that it undergoes transformation of secondary metabolites in terrestrial mammals (Chen et al., 2008). Series of studies show that the trizole fungicides alter the metabolic pathways, cell signaling, cell growth pathways, cell cycle genes and other transcriptional factors (Bruno et al., 2009; Nesnow et al., 2009; Li et al., 2010). In body tissues, it metabolite into 1,2,4 trizole, trizole alanine, trizole acetic acids, trizole pyruvic acid and trizole lactic acid. Of these metabolites, 1,2,4 trizole and trizole alanine

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are the main metabolites which have toxic effects. 1,2,4-triazole is an isomeric chemical compound which have 5-membered ring of 2 C-atom and 3 N-atom. Due to the presence of lone pair electron on N₂-atom, it has specific toxicity. Therefore, because of its widespread use, toxicity evaluations have been performed in this studies.

In aquatic organisms, the micronucleus test (MNT) is one of the most applicable techniques for identifying the genomic alterations. This assay targets interphase cells of any proliferating cell population regardless of its karyotype. In environmental biomonitoring programme, this technique has been adapting because of its simplicity. The purpose of this paper is to know, whether the used fungicide has genotoxic property or not in reference to aquatic organisms.

There are several events of formation of micronuclei (MN) and other nuclear lesions in blood such as fragments caused by mis-repair of DNA breaks or unrepaired DNA breaks or mal-segregation of whole chromosomes at anaphase, hypo-methylation of repeat sequences in centromeric and peri-centromeric DNA, defects in kinetochore proteins or assembly, dysfunctional spindle and defective anaphase checkpoint genes, dysfunction in the error free homologous recombination DNA repair pathways and defect in enzymes of non-homologous end joining pathways in organisms (Fenech, 2007; Fenech and Crott, 2002; O'Donovan and Livingston, 2010; Srivastava and Singh, 2014).

Other important mechanisms, which induce MN formation in blood of acentric fragments, include simultaneous excision repair of damage or inappropriate base incorporated in complementary DNA strands (e.g., uracil) (Fenech and Crott, 2002).

Irrespective of the aforementioned reports, the reasonable mechanisms that lead to MN formation is not yet complete. In genomics, the specific gene expression patterns responsible with the formation of MN, is still rudimentary (Iarmarcovai et al., 2008). In the present study, the formations of MN frequencies have been studied with the exposure of fungicide, PCZ.

MATERIALS AND METHODS

PCZ (CAS No. 60207-90-1) has been purchased from Syngenta Ltd. from India, a technical grade pesticide. Other chemicals such as Giemsa stain has been purchased from Indian market. Fresh-water fish *Clarias batrachus* were obtained from local hatchery (Chhappy Hatchery). Fish had an average weight of 40.01 g and average length 17.26 cm. Fish were fed with commercial fish food, and acclimatized under laboratory conditions for 2 weeks, containing de-chlorinated tap water (pH = 7.6 alkalinity, 150 mg/L CaCO₃, dissolve oxygen (DO) = 7.03 mg/L, temperature = 22°C). The photoperiod used 12 h/12 h dark/light. Aquaria used in study was made of glass and had constant aeration. Physical dimensions of aquarium had 100×40×40 cm, and a 120 L capacity.

Fishes were divided into 4 groups and each group containing 10 individual fishes. Group 1: negative control (only tap water was used), Group 2: positive control (4 mg/L cyclophosphamide; Sigma), Group 3: 1.11 mg/L PCZ, Group 4: 2.23 mg/L PCZ according to pre test (Srivastava and Singh, 2013).

Blood smear slides were prepare by the method of Das and Nanda (1986) with some modifications. Peripheral blood samples were obtained from caudal vein of fish from 24, 48, 72 and 96 h. Blood smears were prepared from each group of randomly selected fishes. At each assessment, 2500 cell/fish were analyzed (7500 erythrocytes for each group). Slides just dried in air, fixed in absolute methanol for 10 to 15 min, and stained in Giemsa (pH 7.0) for 1 to 2 h. The average frequencies of abnormalities were determined for each group as presented in Table 1. MN frequencies (MN‰) were calculated as follows:

$$\text{MN (\%)} = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \times 1000$$

Then the mean ± standard error for each group was calculated. Student's t-test was employed for comparison of control and experimental animals (Sokal and Rohlf, 1973).

RESULTS

The results of micronucleus and nuclear abnormalities in peripheral erythrocytes of *C. batrachus* is as shown in Table 1 and Figure 1. In both groups which were receiving fungicide as well as in the positive control group, frequencies of MN and other nuclear abnormalities were observed significantly higher $p < 0.05$ when compared with negative control group. In Group 3, which was receiving 1.11 mg/L PCZ, increased frequencies of MN were found in erythrocyte after 48 h in comparison to 24, 72 and 96 h with respect to negative control. Group 4,

receiving 2.23 mg/L, showed the same results in all periods, but the frequencies of MN and other nuclear anomalies were higher than Group 3 fishes. In positive control group, the results were also similar. In both Groups 3 and 4 and also in positive control, the frequencies of lobbed nuclei (LN), notched nuclei (NN), nuclear buds (NB) and nucleoplasmic bridge (NPB) were found to significantly increased after 24 and 48 h. NN and NPB increased after 24 and LN and NB increased after 48 h (Table 1).

DISCUSSION

The results of the present study show the induction of MN and other nuclear anomalies in peripheral erythrocytes of *C. batrachus* when exposed to different concentrations of

Table 1. Frequencies of formation of micronuclei and nuclear anomalies.

Assay	Exposure period	Group 1	Group 2	Group 3	Group 4
		Average frequency	Average frequency	Average frequency	Average frequency
NN	24 h	1.06±0.03	8.0±0.12*	5.2±0.10*	6.8±0.12*
LN	"	0.66±0.009	8.2±0.12*	4.93±0.09*	6.53±0.11*
NB	"	0.66±0.009	9.06±0.14*	4.4±0.07*	7.6±0.13*
NPB	"	0.93±0.02	6.9±0.11*	3.86±0.06*	4.93±0.09*
Micronuclei	"	0.66±0.009	34.8±0.25*	23.46±0.20*	30.13±0.25*
NN	48 h	0.66±0.009	6.8±0.11*	4.26±0.06*	5.2±0.07*
LN	"	1.46±0.04	11.33±0.15*	8.13±0.12*	9.2±0.14*
NB	"	1.46±0.04	12.26±0.16*	6.0±0.10*	10.93±0.15*
NPB	"	0.66±0.009	5.6±0.10*	3.33±0.05*	4.8±0.06*
Micronuclei	"	1.46±0.04	42.4±0.30*	32.0±0.27*	34.93±0.30*
NN	72 h	0.68±0.01	4.53±0.08*	2.86±0.04*	2.66±0.04*
LN	"	0.93±0.02	7.33±0.10*	5.06±0.07*	5.46±0.08*
NB	"	0.94±0.02	7.46±0.10*	3.73±0.07*	5.06±0.08*
NPB	"	0.4±0.006	4.93±0.09*	1.73±0.05*	2.53±0.07*
Micronuclei	"	0.93±0.02	35.2±0.25*	21.73±0.18*	24.66±0.21*
NN	96 h	0.67±0.01	2.93±0.08*	1.33±0.03*	1.46±0.04*
LN	"	0.66±0.009	5.86±0.10*	3.33±0.06*	3.86±0.05*
NB	"	0.94±0.02	6.93±0.11*	3.20±0.04*	4.66±0.06*
NPB	"	0.4±0.006	4.66±0.08*	1.33±0.03*	2.13±0.06*
Micronuclei	"	0.67±0.01	32.8±0.22*	17.86±0.17*	20.86±0.20*

*p < 0.05 Significant value. NN: Notched nuclei, LN: Lobed nuclei, NB: Nuclear bud, NPB: Nucleoplasmic bridge.

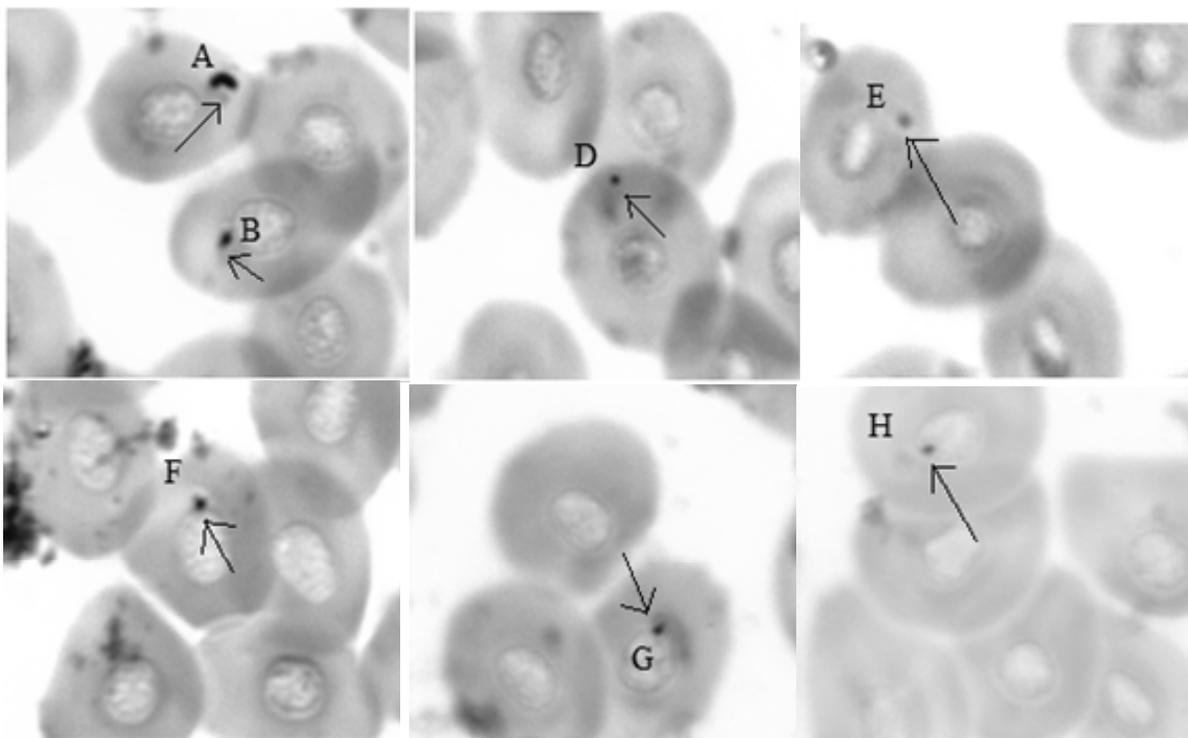


Figure 1. Micronuclei (MN) in blood of fish *Clarias batrachus* after treatment with Propiconazole. A = notched nuclei, B, F, H, and G = lobed nuclei, and D and E = micronuclei.

fungicide PCZ. PCZ is a triazole group fungicide. It has 1H-1,2,4-triazole group at 4-position, 1,3-dioxolane moiety and an aryloxymethyl group and also nitrogen containing hetero-aromatic ring. In blood and tissue, it metabolites into 1, 2,4-triazole alanine, is the main metabolite of triazole containing fungicides. Metabolites of triazole also produce many N₂-containing substances that might be affected on enzymes activity and also nucleic acids (Srivastava and Singh, 2013).

Increasing concentration of nitrogenous metabolites in blood affects the series of enzymatic systems in fish and induces the formation of reactive oxygen species (ROS). Production of reactive intermediates such as ROS, are highly toxic cause DNA lesion in fish blood (Pandey et al., 2011). DNA lesion can be different in shape and size such as in bud form (attached to the main nucleus), lobed form (evagination of nuclear membrane), or in bridge form, however, each anomalies are the signal of cytogenetic damages in fish that could be resulted with failures in cell division, cell death process with mutagenicity (Bombail et al., 2001; Pacheco and Santos, 2002).

After different exposures of fungicide, the formation of MN, NB, NPB and other anomalies were observed. It has been suggested that gene amplification via the breakage-fusion-bridge cycle could cause formation of nuclear anomalies during the elimination of amplified DNA from the nucleus (Shimizu et al., 1998). Abnormal cell division due to the blocking of cytokinesis can also cause binucleation that may lead genetic imbalance in the blood (Rodilla, 1993). Formation of these anomalies in fish blood clearly indicate that PCZ has high genotoxic potency and insight of socio-economic purpose; it is very harmful for aquatic ecosystem. However, the well-documented and reasonable significant associations with an increase in the occurrence of MN and exposure periods are still unknown (Bolognesi et al., 2006). Although the significant increases in erythrocyte MN in fish after 48 h was recorded, whereas some workers have demonstrated the increased frequencies of MN at 72 h. These variations may also be a direct consequence of the sensitivity behavior and the niche of the species, as the number of MN in the cells of fish may be variable (Graziela et al., 2010). Due to chemical pollutants, the low amount of DNA per cell, the large numbers of small chromosomes and the low mitotic activity in many fish species impaired the metaphase analysis of chromosomal damage and sister chromatid exchanges are demonstrated (Pavlica et al., 2000).

Conclusion

It is evident that multiple mechanisms can lead to the formation of MN. MN frequency provides a useful index of accumulated genetic damage during the lifespan of the cells. MN expression strongly depends on the mitotic activity in the studied tissue, which in turn depends on a

complex interaction of biotic and environmental parameters such as temperature, salinity and food availability. The MN frequencies in blood evaluate the kinetics of cytogenetic alterations under fungicides influence. So based on the present study, it was concluded that the fungicide PCZ has great potential to alter the DNA model. Therefore, the extensive use of these fungicides in field crop and its surface run-off at near water bodies should be avoided.

Conflict of interest

The authors declare that there are no conflicts of interest.

REFERENCES

- Bolognesi C, Perrone E, Roggieri P, Pampanin DM, Sciuotto A (2006). Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. *Aqua. Toxicol.* 78:93-98.
- Bombail VAWD, Gordon E, Batty J (2001). Application of the cometand micronucleus assays to butterfish (*Pholis gunnellus*) erythrocytes from the Firth of Forth, Scotland. *Chemosphere* 44:383-392.
- Bruno M, Moore T, Nesnow S, Ge Y (2009). Protein carbonyl formation in response to propiconazole-induced oxidative stress. *J. Proteome. Res.* 8:2070-2078.
- Chen PJ, Moore T, Nesnow S (2008). Cytotoxic effects of propiconazole and its metabolites in mouse and hepatoma cells and primary mouse hepatocytes. *Toxicol. In Vitro* 22:1476-1483.
- Das RK, Nanda NK (1986). Induction of micronuclei in peripheral erythrocytes of fish, *Heteropneustes fossilis* by mitomycin C and paper mill effluents. *Mutat. Res. Lett.* 175:67-71.
- Fenech M (2007). Cytokinesis-block micronucleus cytome assay. *Nat. Protoc.* 2:1084-1104.
- Fenech M, Crott JW (2002). Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes-evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. *Mutat. Res.* 504:131-136.
- Graziela S, Eliana Mara FLR, Any CA, Eliana CV, Monica LA, Cintia MRO (2010). Genotoxic effects in erythrocytes of *Oreochromis niloticus* exposed to Nanograms-per-liter concentration of 17 β -Estradiol (E₂): An assessment using micronucleus test and comet assay. *J. Water Air Soil Pollut.* 218:353-360.
- Iarmarcovai G, Bonassi S, Botta A, Baan RA, Orsière T (2008). Genetic polymorphisms and micronucleus formation: a review of the literature. *Mutat. Res.* 658:215-233.
- Konwick BJ, Garrison AW, Avant JK, Fisk AT (2006). Bioaccumulation and biotransformation of chiral triazole fungicides in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 80:372-338.
- Li ZH, Velisek J, Roman G, Ping Li (2010). Use of hematological and plasma biochemical parameters to assess the chronic effects of a fungicide propiconazole on a freshwater teleost. *Chemosphere* 83:572-578.
- Nesnow S, Ward W, Moore T, Ren H, Hester SD (2009). Discrimination of tumorigenic triazole conazoles from phenobarbital by transcriptional analyses of mouse liver gene expression. *Toxicol. Sci.* 110:68-83.
- O'Donovan P, Livingston, DM (2010). BRCA1 and BRCA2: Breast/ovarian cancer susceptibility gene products and participants in DNA double strand break repair. *Carcinogenesis* 31:961-967.
- Pacheco M, Santos M (2002). Naphthalene and beta-naphthoflavone effects on *Anguilla L.* hepatic metabolism and erythrocytic nuclear abnormalities. *Environ. Int.* 28:285-293.
- Pandey AK, Nagpure NS, Trivedi SP, Kumar R, Kushwaha B (2011). Profenofos induced DNA damage in freshwater fish, *Channa*

- punctatus* (Bloch) using alkaline single cell gel electrophoresis. *Mutat. Res.* 726:209-214.
- Pavlica M, Klobucar GIV, Vetma N, Erben R, Papes D (2000). Detection of micronuclei in hemocytes of zebra mussel and great ramshorn snail exposed to pentachlorophenol. *Mutat. Res.* 465:145-150.
- Rodilla V (1993). Origin and evolution of binucleated cells and binucleated cells with micronuclei in cisplatin-treated CGO cultures. *Mutat. Res.* 300:191-281.
- Shimizu N, Itoh N, Utiyama H, Vahl GM (1998). Selective entrapment of extra chromosomally DNA by nuclear budding and micro nucleation during the S-phase. *J. Cell. Biol.* 140:1307-1320
- Sokal RR, Rohlf FJ (1973). *Introduction of biostatistics* WH Freeman and Comp San Francisco.
- Srivastava P, Singh A (2013). Study of *in vivo* Effects Caused by Metabolites (1,2,4-Trizole Alanine) of Steroid-Inhibitor Fungicide on Aquatic Life (Fish). *J. Aquac. Res. Dev.* 4(4):183.
- Srivastava P, Singh A (2014). Statistical analysis of hydrological properties and genetic toxicity of Maheshara Lake. *J. Ecol. Nat. Environ.* 6(4):159-165.
- Vanden Bossche H, Marichal P, Gorrens J (1989). Biochemical approaches to selective antifungal activity, Focus on azole antifungals. *Mycoses.* 32:35-52.
- Warmerdam MA (2008). California Department of Pesticide Regulation Summary of Pesticide Use Report Data 2008 Indexed by Chemical p 547.