RESEARCH PAPER | Bio-medical | Volume : 3 | Issue : 12 | Dec 2013 | ISSN - 2249-555X

Genotoxicity Evaluation of Bifenthrin Pyrethroid Insecticide to Freshwater Fish Danio Rerio Using the Alkaline Comet Assay in Gill Tissue

KEYWORDS: Comet assay; Bifenthrin technical; Freshwater fish Danio rerio; Gill tissue

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ABSTRACT *The DNA damaging potential of pyrethroid insecticide bifenthrin (technical grade- purity 95%) to the fresh water fish, Danio rerio, was assessed using the alkaline comet assay. Bifenthrin technical being insoluble in water was dissolved in DiMethylSulfOxide and tested at a maximum concentration of 3.18ug/L. Two fish from each group were sacrificed at the end of weeks 1, 2 and 3 following exposure and the gill tissues were processed for comet assay. A reversal group was maintained for period of 10 days without treatment and later sacrificed and processed similarly. Comets were scored visually based on their tail intensity (0 -Undamaged, 1-mild, 2-moderate, 3- severe and 4-extensive). Cells were categorized into various degrees of damage according to the extent of DNA migration. DNA damage was observed from the first week of exposure, which increased with duration of exposure. However, in the reversal group, the percentage of DNA damage was statistically lower suggesting effect on DNA repair. The study clearly demonstrated an elevated DNA damage in cells from gills of Danio rerio due to bifenthrin, which is reversible. Further, the results presented here show that gills can be used as a tissue to explore chemical induced DNA damage in fish*

1. Introduction

Bifenthrin (2-methyl-1, 1-biphenyl-3-y1)-methyl-3-(2-chloro-3, 3, 3-trifluoro-1-propenyl)-2, 2-dimethyl cyclopropanecarboxylate) is a pyrethroid insecticide that affects the nervous system.Bifenthrin is registered as a Restricted Use Pesticide (RUP) owing to its high toxicity to aquatic organisms. Bifenthrin is registered for use on greenhouse ornamentals orchards, nurseries, cotton and homes. It is available as an emulsifiable concentrate or a wettable powder. It is highly toxic to fish and is also an ATPase inhibitor. Aquatic vertebrates are much more sensitive to ATPase inhibitors than terrestrial vertebrates due to their high dependence on ATP synthesis in the gills to maintain osmotic balance. Genotoxicants have the ability to alter DNA and their effects may be particularly harmful as these agents can induce changes that may be passed onto future generations and have an impact on populations long after the original exposure (X. N. Verlecar, 2006). A more useful approach for assessing DNA damage is the single-cell gel (SCG) or Comet assay. During the last decade, this assay has developed into a basic tool for use by investigators interested in research areas ranging from human and environmental biomonitoring to DNA repair processes to genetic toxicology (R.R Tice, 2000). Comet Assay" or "Single Cell Gel Electrophoresis (SCGE) assay" is now considered a very important alternative for the cytogenetic tests; it is much less labour intensive, more rapid and less expensive. The use of the Comet Assay in eco-(geno-) toxicological studies has become more common as research on the importance of the genetic damage caused by pollutants is becoming increasingly important (Barbara *et al* 2004).

Bifenthrin is moderately toxic to mammals when ingested. Large doses may causeincoordination, tremor, salivation, vomiting, diarrhea, and irritability to sound and touch. The LD50, for bifenthrin is about 54 mg/kg in female rats and 70 mg/kg in male rats. The LD50 for rabbits whose skin is exposed to bifenthrin is greater than 2,000 mg/kg. Bifenthrin does not sensitize the skin of guinea pigs.

The mode of action and chemical characteristics of bifenthrin make it very effective against insects yet relatively benign to mammals and birds when applied properly (Mokrey and Hoagland, 1989). The photo degradation half-life for bifenthrin on soil was >100 days, which is considered, stable (Andrew Fecko (1999) Environmental Fate of Bifenthrin). Very high bioconcentration factor (¡Ö6000x, whole body, bluegill sunfish) combined with the persistence of bifenthrin in natural settings could lead to exposure risks for predatory birds and mammals that feed on aquatic organisms. In aquatic systems that have high sediment and/or organic carbon loads, the risks to non-benthic feeding aquatic species are somewhat mitigated by the affinity of pyrethroids, including bifenthrin, for suspended soil particulate and organic carbon (Muir et al., 1985). Bifenthrin adsorbs to soil and sediment, and has an especially strong affinity for soils with high organic carbon content. Sediment bound bifenthrin could contaminate surface water sources during runoff events. Genotoxicity is a very important aspect of risk assessment of chemicals. The Organization for Economic Cooperation and Development (OECD) emphasizes the use of a battery of genotoxicity assays for testing chemicals intended for registration and marketing. The genotoxic effects of environmental pollutants can be monitored using a broad range of both in vitro and in vivo biomarker assays. Comet assay is gaining popularity over other assays due to its sensitivity in detecting DNA damage, robustness, feasibility of application to any eukaryotic cell and is economical. (Andrade et al. 2004, Ateeq et al.2005, Al sabti et al 1995., Blasiak et al ,1998, Cavas et al. 2005, 2003a, 2003b, Clements et al., 1997). Bifenthrin is very highly toxic to fish, crustaceans and aquatic animals. The LC50 after a 96-hour exposure is 0.00015 mg/l for rainbow trout, 0.00035 mg/l for bluegill, and 0.0016 mg/l for

Daphnia. However, not much information is known on genotoxicity of bifenthrin in fish species. Available data on the mutagenic effects of bifenthrin is inconclusive (EXTOXNET PIP 1995); gene mutation studies show positivity, while other tests on bacteria and mammalian cells were negative (EPA's Pesticide Fact Sheet Database). Hence the present study was conducted to assess the genotoxicity of bifenthrin using fish species as a model. Comet assay was applied to study this end point. The suitability of the gill tissue to study the chemical induced DNA damage in fish was also assessed.

2. Materials and methods

2.1 Chemicals

Bifenthrin technical (purity 95%) was obtained from a commercial source. Low melting agarose, normal melting agarose and Tris base were purchased form Sigma-Aldrich (USA). All the other chemicals used for the comet were purchased

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from Merck (India).

2.2 Fish
Hatchery bred zebra fish *Danio rerio,* was procured from a commercial fish farm (Manimangalam, Kancheepuram district, Padappai, India) and quarantined for a period of one month in cement tanks in the laboratory . During quarantine and acclimatization the fish were fed with flake feed (Ingredients : High class fish meal, Plant Asthaxanthin, Animal proteins, Soya meal, Cereals, Fish oil, Multivitamins and Trace elements). Healthy fish were used for the study. The fish were acclimatized in glass aquaria for 10 days to the laboratory condition prior to the exposure. The weight and length was recorded and the mean value was found to be 0.521 g and 2.6 cm, respectively. The feed was withdrawn 24h prior to exposure. The physico-chemical parameters of the water used was recorded and found to be pH (7.3-7.6), dissolved oxygen (7.4- 7.8 mg/l), hardness (185 mg/l) (APHA, 1975) and temperature (27 ± 1°C).

2.3 Acute toxicity exposure and dose fixing
A static acute toxicity exposure was conducted for a period of 4 days with ten fish and the 96h LC50 was determined as 31.80 µg/l. 1/10th of the LC50 was calculated and used for the chronic exposure of 3 weeks followed by a 10 day recovery. Freshwater fish, *Danio rerio* was exposed to Bifenthrin technical spiked medium for a period of 4 weeks. Every week 2 fish from each group was collected for assessing the DNA damage by comet assay in gill tissue.

2.4 *In vivo* comet assay from gill
2.4.1 Sample preparation
The exposed fish were dissected and the gill tissue was collected, weighed and placed in ice cold PBS buffer. The gill tissue was homogenized with a micro pestle and allowed to settle. The supernatant which contained single cells was used for comet assay.

2.4.2 Slide preparation
Comet assay was essentially performed using the method of Singh *et al* with slight modifications. For the basal layer, 1% Normal melting agarose was prepared in Phosphate Buffered Saline (PBS); the slides were dipped in the agarose solution and air dried. About 20 µl of the cell suspension (Obtained in the previous step) was mixed with 80 µl Low Melting agarose (0.7% in PBS) and added to the basal agarose layer, covered with cover glass and allowed to solidify. This was followed by the addition of the third layer of agarose (100 µl 0.5% low melting agarose), cover slipped and allowed to solidify.

2.4.3 Lysis
After removal of cover slips, the slides were immersed in 50 mL cold lysing solution (2.5 M NaCl, 100 mM Na2 EDTA, 10 mM Tris, pH 10, 10% DMSO and 1% Triton X-100). The slides were kept in dark at 4°C for 1 hour in refrigerator. To avoid any additional DNA damage, the procedure was performed under dim light.

2.4.4 Alkaline unwinding, electrophoresis and neutralization
The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank for DNA unwinding for 20 minutes in the electrophoresis buffer (300 mM NaOH, 1 mM Na2 EDTA, pH >13). The electrophoresis unit was filled with the freshly prepared buffer approximately 0.25 cm above the slides. The cells were exposed to alkali for DNA unwinding. This was followed by electrophoresis at 18 V (0.7-1.0V/cm) 300 mA for 10 minutes at 4oC. After electrophoresis, the gel slides were washed in neutralizing buffer (0.4 M Tris-HCl, pH 7.5) for 15 min (5minutes x 3 washes).

2.4.5 Staining and scoring
The slides were stained with 75 µl of ethidium bromide (2µg/mL) and covered with a cover glass and the slides were screened for comets using a Carl Zeiss Axiostar fluorescence microscope (Carl Zeiss, Germany) at 400X magnification. The migrated cells resemble the comets with a head region containing undamaged DNA and a tail containing broken DNA. The amount of DNA able to migrate and the distance of migration indicate the number of strand breaks present in that cell and the extent of DNA migration indicates the level of DNA breakage in the cell. Cells with increased DNA damage display an increased migration of chromosomal DNA from the nucleus towards the anode. The cells are scored visually based on their tail intensities and categorized **(Fig. 2)** as 0 (Undamaged), 1 (mild), 2 (moderate), 3 (severe) and 4 (extensive). (Maluf and Erdtmann, 2000. Zang *et al.*, 2000, Zhong *et al.*, 2001). About 50 comet images were visually scored at random for each fish covering a total of 100 cells per group. The percentage of damage is calculated and is compared between the control and treated groups.

2.5 Statistics
TOXSTAT 3.5 Version (WEST, Inc. and Dave Gulley, Univ. of Wyoming; 2003, Central Avenue, Cheyenne, WY82001, USA) was used for statistical evaluation. Statistical comparison of DNA damage in the controls and treatment group was performed using the Dunett's test.

3. Results
Singh and Stephens (1997) suggested that counting 50 cells in a slide was sufficient to detect a significant level of DNA damage caused by chemicals in comet assay. The number of cells in each degree of DNA damage and DNA damage scores in control, solvent control and treated groups as arbitrary units (AU) has been presented in **Table 1**.

The arbitrary unit (AU) was used to express the extent of DNA damage and was calculated as follows:

$$4AU = \sum N_i i$$

$$i = 0$$

Where n is number of cells in damage degree i (0, 1, 2, 3, 4) (Feng *et al.*, 2004). DNA damage % was found to be high and statistically significant in the treated group with respect to the control. The solvent control also showed considerable damage and was also significant when compared to the control. However, the damage in the treated group was exceedingly higher than that recorded in the treated group. This indicates that, minus the solvent effect, the chemical (Bifenthrin) by itself, has the capacity to cause DNA damage. The results are expressed in the graph. **(Fig.1)** The damage started during the first weeks of exposure and increased in the subsequent weeks. However, in the reversal group the graph declined showing a repair process during the recovery period in the absence of the chemical. This suggests that the chemical Bifenthrin causes DNA damage in the gill tissue of fish; in addition it seems to have suppressed the repair systems in its presence.

4. Discussion
The present investigation provides newer information on the increased genotoxic effect of bifenthrin technical on the gill tissues via direct exposure. DNA damage in fish is used more often as a biomarker of the effects of pollutant exposure. DNA adducts have been studied in fish exposed to xenobiotics, as investigations of genotoxic compounds are deemed highly relevant (Bethanie Carney Almroth, 2008). Mitchelmore and Chipman (1998) recommended that DNA strand breaks, particularly as measured by the comet assay, act as a biomarker of genotoxicity in fish and other aquatic species. Biomarkers are used as early warning pollution monitoring tools to signal the onset of sublethal deleterious effects at the physiological, molecular, cellular or subcellular level therefore being able to provide evidence for changes at higher levels of the biological organisation (Barillet *et al*, 2005). Comet assay, the microelectrophoretic study of DNA damage in individual cells, was first described by Ostling and

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Johanson (1984). It can be applied to proliferating and non-proliferating cells and the cells of those tissues, which are the first sites of contact with mutagenic/carcinogenic substances. What makes this assay even more valuable is the specificity for detecting genotoxicity (Barbara *et al* 2004). Like in other tests, DNA effects induced due to cytotoxicity is a big issue. Data show that cytotoxic effects can be detected (dead cells show specific kinds of comets called "clouds") and distinguished from genotoxic effects, therefore, should have no confounding effects on results. Given its overall characteristics, this method has been widely used in several areas (Hartmann 1999). In this in vivo study, zebra fish was used for the purpose of measuring the effect of low levels of bifenthrin technical on fish. The results of this study demonstrate that gill tissues from zebra fish exposed to low levels of Bifenthrin technical for 3 weeks have increased DNA strand breaks representing extensive DNA damage and further during the reversal the damage was found to be less when compared with the control. The effect was time dependent and the cells were able to recover from the damage caused, in the absence of bifenthrin.

Bifenthrin being a fluorinated pesticide carries a fluorine group in it structure. Fluorine, is a known mutagen and also carcinogenic. It causes extensive chromosomal aberrations and hence the very presence of this group might have contributed to the genotoxic effect of bifenthrin. Bifenthrin, as such, has been reported to cause tumor in the urinary bladder. The present study proves the genotoxic potential of Bifenthrin. If the current observations are extended to animal studies, the exact mechanism of action of bifenthrin can be delineated. Accumulation of DNA damage may occur either through an increase in the number of DNA damaging events or a decrease in DNA repair. Although the DNA repair mechanisms in fish are not as efficient as those in mammals (Espina *et al.*, 1995., Wirgin *et al.*, 1998.,), they do exist. Saleha Banu *et al.* reported that, in erythrocytes of *Tilapia mosambica* treated with the pesticide Monocrotophos, the maximum increase in mean comet tail length was observed at 24 h and reductions in mean comet tail length were seen at 48 and 72 h. They also observed that by 96 h, values had returned to control levels at all doses, indicating repair of the damaged DNA and/or loss of heavily damaged cells. Although the current study did not use erythrocytes, but the gill tissue, a similar effect of recovering from DNA damage was observed. This observation proves the fact that fish possess repair systems for recovering from chemical induced DNA damages. The genotoxic potential of bifenthrin in its technical form has been identified. Despite the fact that bifenthrin is a Restricted Use Pesticide, the very use of the chemical needs to be carefully scrutinized with additional data on genotoxicity and other parameters associated with the safety of the ecosystem.

Table 1.
Number of cells in each degree of DNA damage and the DNA damage scores (in arbitrary unit, AU) in control and treated groups. Data from 4 slides; 2 slide/fish scored has been presented.

Week	Sample	No. of cells in each damage degree (mean ± SD)					Damage %	AU
		0	1	2	3	4		
1	Control	23.25 ± 0.96	1.50 ± 0.58	0.25 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	7.0	2.0
	Sol. con	20.75 ± 2.99	4.25 ± 2.99	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	17	4.25
	Treated	17.75 ± 1.71	4.00 ± 2.00	2.50 ± 1.00	0.75 ± 0.96	0.00 ± 0.00	29	11.25
2	Control	22.75 ± 1.26	2.25 ± 1.26	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.0	2.25
	Sol. con	20.50 ± 3.32	4.50 ± 3.32	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	18	4.45
	Treated	16.75 ± 2.99	5.25 ± 1.89	2.50 ± 3.32	0.50 ± 0.58	0.00 ± 0.00	33	7.8
3	Control	23.50 ± 2.38	1.50 ± 2.38	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.0	1.5
	Sol. con	21.00 ± 2.16	3.75 ± 1.89	0.25 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	16	1.44
	Treated	15.50 ± 2.89	4.25 ± 3.30	4.75 ± 2.06	0.50 ± 1.00	0.00 ± 0.00	38	12.06
Reversal	Control	23.25 ± 1.71	1.75 ± 1.71	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	7.0	1.75
	Sol. con	21.50 ± 2.38	3.50 ± 2.38	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	14	3.5
	Treated	18.25 ± 1.50	6.75 ± 1.50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	27	6.75

Control – untreated, Sol.con – Solvent control – DiMethylSulfOxide (DMSO), Treated – Bifenthrin technical - 3.18 µg/L

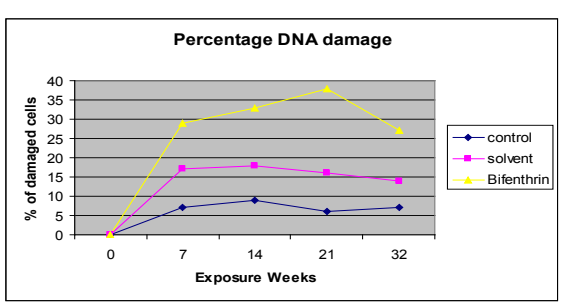

Figure 1: Graph showing the DNA damage percentage in various experimental groups.

Volume : 3 | Issue : 12 | Dec 2013 | ISSN - 2249-555X

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