

**TOXICOLGICAL IMPACT OF CHLORPYRIFOS AND
AMELIORATIVE POTENTIALS OF VITAMIN E ON NUCLEAR
ABNORMALITIES IN ERYTHROCYTES OF *Heteroclarias***

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ABSTRACT

*Chlorpyrifos is commonly used as an agricultural insecticide which is extremely toxic to non-target organisms like fish. The effect of chlorpyrifos and ameliorative potentials of vitamin E on nuclear abnormalities in the erythrocytes of **Heteroclarias** was investigated. Juveniles **Heteroclarias** were exposed to varying concentrations (0, 1, 2, 3, 4 and 5 ppm) of chlorpyrifos for 96 hours and fed on varying concentrations (50, 250, 500 and 1000 mg/kg diet) of vitamin E for 28 days. The LC_{50} value was calculated as 2.4 ppm. After the exposure period, the fish were sacrificed and blood was collected for micronucleus assay. Different abnormalities such as micronucleated, binucleated, multinucleated and altered cells were evident in the erythrocytes of chlorpyrifos-exposed fish compared to control. The micronucleus induction and percentage frequency increased ($P<0.05$) as the concentration of chlorpyrifos increased, but, decreased ($P<0.05$) in the groups ameliorated with vitamin E. However, a gradual decrease occurred as the concentration of vitamin E inclusion in the diet increased with a great reduction ($P<0.05$) in the group fed on 500 mg vitamin E/kg diet. This study revealed that chlorpyrifos induced micronucleus formation, but, 500mg vitamin E/kg diet is capable of minimizing micronucleus formation in **Heteroclarias**.*

Key words: Amelioration, Chlorpyrifos, *Heteroclarias*, Micronucleus, vitamin E

INTRODUCTION

Appreciations of fisheries and aquatic ecosystems have been accompanied by increasing concern about the effects of growing human populations and modern agricultural technology on aquatic life and water quality (Liou *et al.*, 1996). Pesticides are washed from farm lands during rainfall through surface runoff into the water bodies and this may result to disruption of the ecosystem which induced stress in the aquatic organisms and lead to physiological alterations that could caused genetic damage. This genetic damage arises as a result of chromosome or spindle abnormalities that lead to micronucleus formation. Micronucleus (MN) assay is therefore an ideal monitoring system in the assessment of genotoxicity. Fish species serve as a major test organism in assessing environmental pollution because of their link to man in the food chain. *Heteroclarus* is a species of the catfish family with remarkable fast growth rate and ability to adapt to adverse condition (Ending and Kamstra, 2001). Among the commonest pesticides used in the control of pests is chlorpyrifos, but its ability to persist in the environment and bio accumulate in animal's tissues make it to have potential adverse effect on organisms. Several researchers have reported the genotoxicity of Chlorpyrifos on the induction of micronuclei and micronucleus frequency in the erythrocytes of different fish species such as *Heteropneustes fossilis* (Mohi-ud-Din *et al.*, 2011); *Channa punctatus* (Sarangi, 2012); *Cirrhina mrigala*, *Catla catla* and *Ctenopharyngodon idella* (Kousar and Javed, 2015) and *Labeo rohita* (Ghaffar *et al.*, 2015).

However, information on the genotoxicity of Chlorpyrifos on fish and ameliorative potentials of vitamin E is spares. Vitamin E has been used as an ameliorative agent to prevent tissues from oxidative stress that cause damage as a result of pollutants, and to rectify nuclear damage in fish (Ibrahim and Banaee, 2014; Mohammed *et al.*, 2014 and Olsvik *et al.* 2015), but none of these reported on *Heteroclarus*. Ameliorative potentials on *Heteroclarus* exposed to Chlorpyrifos were therefore investigated.

MATERIALS AND METHODS

Experimental Set Up

Juveniles of *Heteroclinus* (average weight of 21.4 ± 1.3 g and average length of 11.12 ± 0.8 cm) were purchased from TJ farm, Ogidi, Ilorin, Kwara State, Nigeria. The fish were conveyed to the Department of Zoology laboratory, University of Ilorin in a portable well aerated black tank containing water from the farm. The fish were not fed throughout the day to avoid indigestion as a result of stress which could cause mortality. They were held in large water baths of 40L capacity at a temperature of 24-25°C and acclimatized to the laboratory condition for 14 days in well aerated chlorine-free bore-hole water. During this period, they were fed with 2.0 mm commercial feed (Durante) at 4 % body weight (Meyer *et al.*, 1993). The toxicant was renewed every 2 days to maintain the toxicity level and waste materials were removed to prevent additional contamination. The aquaria were covered with mosquito-mesh nets to prevent fish from escape and predators. Feeding was suspended 24 h before the commencement of the experiment (USEPA, 1996).

Preparation of Toxicant

A dark brown liquid of commercial grade chlorpyrifos {0, 0-diethyl 0-3, 5, 6-trichloro-2-pyridinyl-phosphorothioate}, emulsifiable concentrate (EC) 20% with a trade name, perfect killer was purchased from an Agrochemical Company. The fresh stock solution was prepared by dissolving 2 ml of CPF into 100 ml of distilled water out of which the working solution had been taken. This stock solution is equivalent to 20 ml/l. The solution was stirred thoroughly for homogenous mixing.

Range Finding Test

The range finding test was conducted to determine the definitive concentration range for CPF testing as toxicant following the methods of Solbe (1995). This was done by introducing four varying volumes of (0.5ml, 1.0ml, 2.0ml and 4.0ml) out of the stock solution with the use of syringe into four separate test aquaria containing 10 litres of dechlorinated water each. The concentrations for the volumes of solution above was determined using dilution formula ($C_1V_1=C_2V_2$) to arrive at (1.0 ppm, 2.0 ppm, 4.0 ppm and 8.0 ppm) respectively. Five fish were introduced into each prepared concentration

to determine the range required. Both the mortality and survival were recorded in the remaining concentration and the range was determined. Feeding was stopped 24 hours before the commencement of the experiment (USEPA, 1996). Five concentrations were then ranged between the highest and the lowest concentration of the range finding test to include (1.0 ppm, 2.0 ppm, 3.0 ppm, 4.0 ppm and 5.0 ppm) and used for the acute test.

Chronic Test

The chronic test was carried out using one-tenth ($1/10^{\text{th}}$) of the LC_{50} concentration of the toxicant which was determined from the acute experiment. This LC_{50} value was 2.4 ppm. The concentration of CPF used for sub-lethal experiment was estimated to be 0.24 ppm. The experiment was divided into six different groups each with two replicates. Five out these six groups were exposed to the same concentration of CPF which is 0.24 ppm and the sixth group serves as control. Five different feeds were formulated with varying vitamin E inclusion level to ameliorate the nuclei damage that occurred as a result of CPF. The varying level of vitamin E per kg of each feed were; 0 mg, 50 mg, 250 mg, 500 mg and 1000 mg. Percentage composition of the normal feed formulated with 0 mg of vitamin E per kg of the feed is shown in Table 2. This normal feed was labeled as diet A. The ingredients were grinded, thoroughly mixed, pelleted, air-dried and fed to the fishes both in group A and B aquaria. Four other feeds were also formulated using the same formula. After mixing, vitamin E was then incorporated as 50 mg, 250 mg, 500 mg and 1000 mg per kilogram of each feed and labeled as B, C, D and E respectively. These four feeds were used to feed fishes in group C, D, E and F aquaria respectively.

Micronucleus Assay

Blood sample was taken randomly with the use of insulin syringe needle and a drop of the blood was smeared on the slide with three slides per group. The slides were fixed in absolute methanol for 20 minutes and left to dry at room temperature for 24 hours. The fixed slides were then stained with 5% Wright-Giemsa and 10% May-Grunward solution.

Scoring Criteria for Micronuclei

The criteria suggested by Carrasco *et al.* (1990) were used for identification of micronuclei: (a) the MN must be smaller than $1/3$ of the main nuclei. (b) MN

must be clearly separated from the main nuclei, and (c) MN must be on the same plane of focus and have the same color as of main nuclei. Cells with more than four MN were discarded to exclude apoptotic phenomena. From each slide, 1000 cells were scored under light microscope. A light microscope was used to scan the blood smears under oil immersion at x100. The number of micronucleated erythrocyte (MN) per 1000 erythrocytes for each fish was determined. The number of MN was expressed as number per thousand MN. Different cellular abnormalities such as micronucleus, binucleated cells, echinocytes and altered cells were observed. Photographs were taken using OLYMPUS microscope with the aid of AMSCOPE camera. The MN frequency was calculated as follows:

$$\text{MN \%} = \frac{\text{no of cells containing micronucleus}}{\text{Total no cells counted}} \times 100$$

Nuclear Lesions

To complement and standardize the MN assay, the nuclear lesions were scored in the periphery blood cells of *Heteroclaris* and its frequency was also calculated. Six types of nuclear lesions were observed: (a) cells with two nuclei were considered as bi-nuclei (BN), (b) cells with more than two nuclei were considered as multi-nuclei (MT), (c) deformed cells were considered as altered (A), (d) cells without nuclear membrane were considered as enuclei (E), (e) cells with spiny nuclear membrane were considered as echinocyte (EC), and (f) cells with nuclear membrane similar to kidney shape were recorded as kidney-shaped nuclei (K).

Water Quality Parameter

The water quality parameters such as temperature, conductivity, pH and Dissolved oxygen were monitored during the experimental period following the standard method of APHA (1995).

Composition of the Formulated Feed

The percentage compositions of the basal diets fed to the experimental fish are fish meal (30 %), soya bean (20 %), GNC (18 %), bone meal (0.4 %), maize corn (30 %), vitamin C (0.2 %), methionine (0.4 %), lysine (0.4 %), salt (0.2 %), premix (0.4 %).

Statistical Analysis

The experimental results were subjected to one way analysis of variance (ANOVA) and multiple range tests for differences between levels of treatments and to separate means using SPSS version 16. Test for significant differences were at 95% ($P < 0.05$) (Duncan, 1995).

RESULTS

During the exposure period, the test fish (*Heteroclaris*) exhibited serious behavioural changes such as erratic swimming, respiratory distress, gasping, increase in opercula ventilation, changes in colour and loss of balance compared to control. The water quality parameters were within the recommended standard (USEPA, 1996).

Micronucleus Induction and Frequency

After 96 h of exposure, the control group revealed normal erythrocytes, but varying abnormalities such as micronucleated, binucleated, multinucleated, altered cells, Enucleated, echinocyte and kidney-shaped nuclei were observed in the erythrocytes of *Heteroclaris* exposed to different concentrations of chlorpyrifos compared to control (Fig 1 a-e). Micronucleus induction and frequency in the erythrocytes of *Heteroclaris* in the group exposed to the lowest concentration of chlorpyrifos was minimal, while the highest micronucleus induction and frequency in the highest concentrations of chlorpyrifos was revealed (Fig. 3). A time dependent gradual increase in the micronucleus frequency was observed as the concentration and exposure period increased (Fig. 3). At the end of 28 days of amelioration, the erythrocytes of the groups of *Heteroclaris* exposed to chlorpyrifos only without amelioration recorded more induction of micronucleus with varying abnormal nuclear alterations such as binucleated, echinocyte, enucleated, altered cells, and kidney-shaped (Fig. 2 a-f).

While the erythrocytes of the groups exposed to chlorpyrifos and ameliorated with varying concentrations of vitamin E revealed less micronucleus induction (Fig. 2 a-f). The micronucleus induction and frequency decreased as the concentrations of vitamin E supplementation in the diet increased from 50 to 500 mg vitamin E/kg diet. However the group ameliorated with 1000 mg vitamin E/kg diet recorded a high ($P < 0.05$) induction of micronucleus and frequency (Fig. 4).

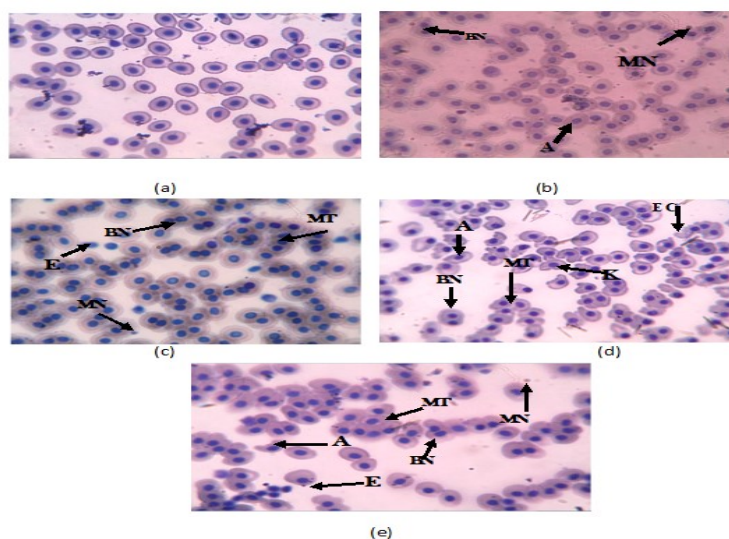


Fig. 1 (a-e): Micronucleus formation in erythrocytes of *Heteroclaris* exposed to varying concentrations of chlorpyrifos for 96hrs (H & E, X100). (a) normal cells in erythrocytes of the control group; (b) Altered cell (AL), Micronucleated (MN) and Binucleated (BN) in the erythrocytes of *Heteroclaris* exposed to 1.00 ppm of chlorpyrifos; (c) Binucleated (BN), Enucleated (EN), Multinucleated (MT) and Micronucleated (MN) in the erythrocytes of *Heteroclaris* exposed to 2.00 ppm of chlorpyrifos; (d) Altered (AL), Multinucleated (MT), Enucleated (EN), Echinocyte (EC), kidney-shaped (KD) and Binucleated in the erythrocytes of *Heteroclaris* exposed to 3.00 ppm of chlorpyrifos; (e) Altered (AL), Multinucleated (MT), Enucleated (EN) and Binucleated cell in the erythrocytes of *Heteroclaris* exposed to 4.00 ppm of chlorpyrifos.

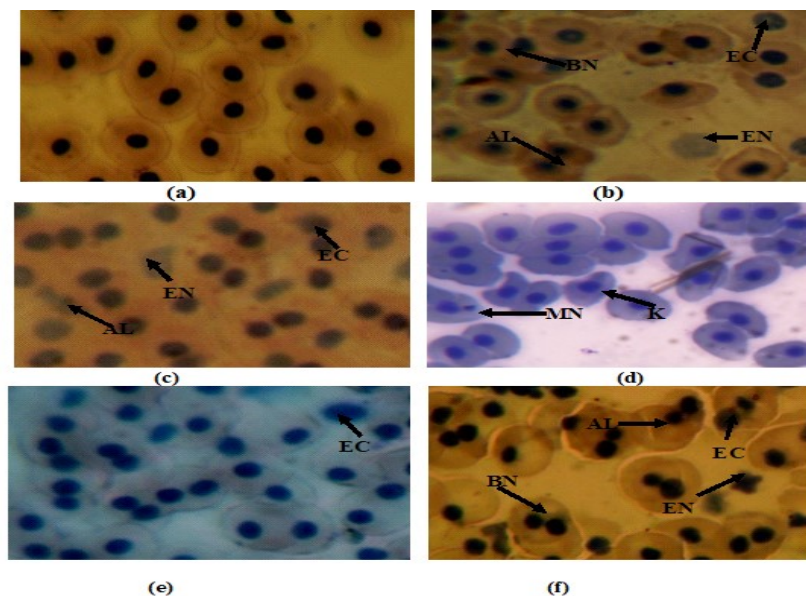


Fig. 2 (a-f): Micronucleus formation in the erythrocytes of *Heteroclaris* exposed to sub-lethal concentration of chlorpyrifos and ameliorated with varying concentrations of vitamin E for 28 days (H & E, X100).

(a) Normal erythrocytes in the blood of *Heteroclaris* in the control group fed on basal diet without toxicant, (b) Binucleated (BN), Echinocyte (EC), Enucleated (EN) and Altered (AL) in the erythrocytes of *Heteroclaris* exposed to 0.24 ppm of CPF only; (c) Echinocyte (EC), Enucleated (EN) and Altered (AL) in the erythrocytes of *Heteroclaris* exposed to 0.24 ppm of CPF and fed on 50 mg/kg diet of vitamin E; (d) micronucleated (MN) and Kidney-shaped (KD) in the erythrocytes of *Heteroclaris* exposed to 0.24 ppm CPF and fed on 250 mg/kg diet of vitamin E; (e) Echinocyte (EC) in the erythrocytes of *Heteroclaris* exposed to 0.24 ppm of CPF and fed on 500 mg/kg diet of vitamin E and (f) Binucleated (BN), Echinocyte (EC), Enucleated (EN) and Altered (AL) in the erythrocytes of *Heteroclaris* exposed to 0.24 ppm of CPF and fed on 1000 mg/kg diet of vitamin E.

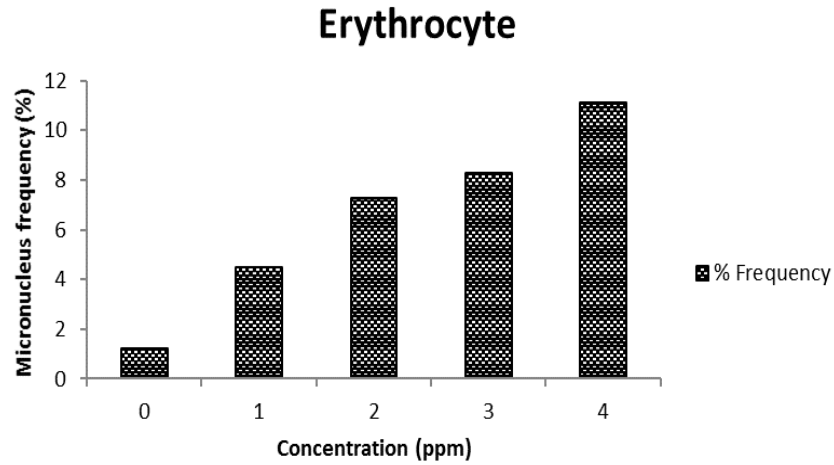


Fig 3: Mean percentage micronucleus frequency in the erythrocyte of *Heteroclaris* exposed to chlorpyrifos for 96 hr.

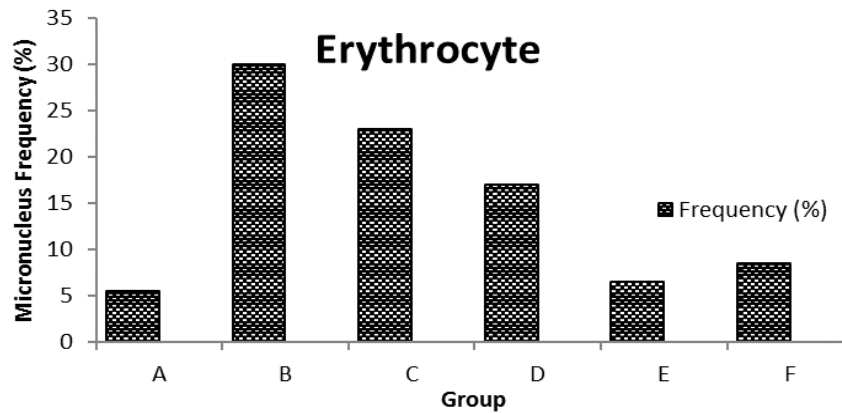


Fig 4: Mean Percentage Micronucleus frequency in erythrocytes of *Heteroclaris* exposed to sub-lethal concentration of chlorpyrifos and fed on varying concentrations of vitamin E for 28 days.

Nuclear Abnormalities Frequency

The percentage frequency of nuclear alterations increased as the concentration of chlorpyrifos increased. The commonest abnormalities in the group of *Heteroclaris* exposed to chlorpyrifos for 96 h are micronucleated, binucleated, multinucleated and altered cell. Among these abnormalities, binucleated cells (4.57) were recorded as the most frequent abnormalities, while the kidney-shaped nuclei (0.10) were the least frequent abnormalities (Table 1). After 28 days of amelioration, the most frequent abnormal cells were also recorded as the binucleated cell (15.44) with the least frequent abnormal cells as altered cells (8.10) in the group B exposed to sub-lethal concentrations of chlorpyrifos only. However, the frequency of the abnormalities reduced as the concentrations of vitamin E supplement in the diet increased, except in group F with the highest concentration of vitamin E/kg (1000 mg), but significant reductions ($P < 0.05$) in the frequencies of binucleated cells (2.20) and altered cells (1.60) occurred in group E that was fed on 500 mg vitamin E/kg diet (Table 2).

TABLE 1
Frequency of nuclear alterations in erythrocytes of *Heteroclarus* exposed to varying concentrations of chlorpyrifos for 96 hr.

Conc (ppm)	Micronucleated (%)	Binucleated (%)	Multinucleated (%)	Altered (%)	Enucleated (%)	Echinocyte (%)	Kidney-shaped (%)
0	0.30 ± 0.17 ^a	0.40 ± 0.06 ^a	0.27 ± 0.27 ^a	0.23 ± 0.03 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
1	1.10 ± 0.23 ^b	1.60 ± 0.18 ^b	1.45 ± 0.15 ^b	0.27 ± 0.15 ^a	0.00 ± 0.00 ^a	0.00 ± 0.03 ^{ab}	0.00 ± 0.00 ^a
2	1.17 ± 0.20 ^b	2.90 ± 0.06 ^c	1.00 ± 0.06 ^b	1.67 ± 0.20 ^b	0.00 ± 0.15 ^a	0.07 ± 0.15 ^b	0.00 ± 0.00 ^a
3	2.10 ± 0.23 ^c	2.77 ± 0.03 ^c	1.00 ± 0.29 ^b	1.47 ± 0.09 ^b	0.27 ± 0.45 ^b	0.07 ± 0.03 ^{ab}	0.00 ± 0.06 ^b
4	2.27 ± 0.20 ^c	4.57 ± 0.15 ^d	2.60 ± 0.17 ^c	1.70 ± 0.17 ^b	0.77 ± 0.00 ^a	0.27 ± 0.00 ^a	0.10 ± 0.00 ^b

Means within the same row with different superscripts are significantly different ($P < 0.05$). The values are representing the mean/1000 cells ± SE

NB:- Group A- fed basal diet without toxicant, Group B - exposed to 0.24 ppm of chlorpyrifos only; Group C - exposed to 0.24 ppm of chlorpyrifos and fed on 50 mg/kg diet of vitamin E; Group D - exposed to 0.24 ppm chlorpyrifos and fed on 250 mg/kg diet of vitamin E; Group E - exposed to 0.24 ppm of chlorpyrifos and fed on 500 mg/kg diet of vitamin E and Group F - exposed to 0.24 ppm of chlorpyrifos and fed on 1000 mg/kg diet of vitamin E.

TABLE 2

Percentage frequency of nuclear alterations in erythrocytes of *Heteroclaris* exposed to sub-lethal concentrations of chlorpyrifos and fed on varying concentrations of vitamin E for 28 days.

Groups	Micronucleated (%)	Binucleated (%)	Multinucleated (%)	Altered (%)	Enucleated (%)	Echinocyte (%)	Kidney-shaped (%)
A	0.00±0.00 ^a	0.01±0.03 ^a	0.030±0.00 ^a	0.030±0.17 ^a	0.00±0.00 ^a	0.07±0.03 ^a	0.00±0.00 ^a
B	1.70±0.00 ^b	15.40±2.02 ^d	1.93±0.55 ^b	8.10±3.23 ^c	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a
C	0.47±0.47 ^a	14.23±1.01 ^d	0.77±0.43 ^a	7.10±0.98 ^c	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a
D	0.07±0.03 ^a	10.00±1.04 ^c	0.77±0.32 ^a	4.97±1.41 ^b	0.00±0.00 ^a	0.20±0.16 ^b	0.00±0.00 ^a
E	0.07±0.03 ^a	2.20±0.09 ^b	0.27±0.09 ^a	1.60±0.58 ^{ab}	0.00±0.00 ^a	0.03±0.03 ^a	0.00±0.00 ^a
F	0.10±0.00 ^a	2.37±0.62 ^b	1.17±0.66 ^b	2.00±0.40 ^b	0.16±0.09 ^b	0.10±0.00 ^a	0.03±0.03 ^a

Means with the same superscripts in the same column are not significantly different ($P < 0.05$).

NB:- Group A- fed basal diet without toxicant, Group B - exposed to 0.24 ppm of chlorpyrifos only; Group C - exposed to 0.24 ppm of chlorpyrifos and fed on 50 mg/kg diet of vitamin E; Group D - exposed to 0.24 ppm chlorpyrifos and fed on 250 mg/kg diet of vitamin E; Group E - exposed to 0.24 ppm of chlorpyrifos and fed on 500 mg/kg diet of vitamin E and Group F - exposed to 0.24 ppm of chlorpyrifos and fed on 1000 mg/kg diet of vitamin E.

DISCUSSION

Heteroclaris showed behavioural changes against chlorpyrifos intoxication. These were loss of equilibrium, frequent surfacing, agitated and erratic swimming, loss activity and finally death. The behavioural alteration may occur as a result of nervous impairment due to blockage of nervous transmission among the system and various effector sites (Fryday *et al.*, 1996). Similar observations were made by Matsumura (1975) who reported that hyperactivity of fish on introduction to an unfavourable environment as the primary and principal sign of system failure due to pesticide poisoning which affected physiological and enzyme activities. Pal and Konar (1987) also observed disruption of the functioning of the nervous system of fish, resulting in slow and lethargic swimming, erratic movement and loss of equilibrium. *Heteroclaris* were also found to secrete mucous on the gill filament and body surface prior to death following chlorpyrifos poisoning. This phenomenon is evidently a symptom of the inflammatory reaction of the gill and body surface to the toxicant. Similar findings were made by many authors (Auta *et al.*, 2000; Svoboda *et al.*, 2003), who studied the effect of pesticides on different species. This is similar to the observations made by Talapatra and Banerjee, (2007) that induction of micronucleus and other nuclear lesions in fish is significantly different when compared with control. According to Carassaco *et al.* (1990) nuclear damage in erythrocytes might be from viral infections as a non genotoxic stressor. Cavas *et al.*, (2005) reported that individual or combined exposure to chemicals have shown morphological nuclear abnormalities in fish erythrocytes. Bakare *et al.*, (2005) said micronucleus formation and other nuclear lesions is said to be a consequence of genetic events. This result shows that all the abnormalities could be due to the effects of xenobiotics or process of cell division, which results in genetic imbalance and result in carcinogenesis later. This is similar to the studies conducted by Rodilla, (1993).

The frequency of nuclear alteration in the group A (control) might be due the stress and some other environmental factors as reported by Chaudhari and Saxena (2015) who carried out their work on *Channa punctatus*. Insignificant increase of damage frequency in group E as compared to group A might be as a result of ameliorative potential of vitamin E to correct nuclear damage which was reported by Olsvik *et al.* (2015). This result then reveals that vitamin E, at moderate level is capable of improving the immunity against some toxicants by inhibiting oxidative stress and correcting nuclear

deformity in fish as reported by Mohamed *et al.* (2014). This is similar to what was reported by Ibrahim and Banaee (2014) that toxicity of pesticides may be corrected with appropriate antioxidant based on their work on *Labeo rohita*. Also, the significant reduction ($P < 0.05$) in the nuclear damage of group E compared to the remaining treated groups equally shows the potential and moderate amount of vitamin E that can be used to rectify the nuclear effect of CPF on *Heteroclaris*. This suggests that, vitamin E only to a moderate level can modify chlorpyrifos toxicity in *Heteroclaris*. This is similar to what was reported on Atlantic salmon by Olsvik *et al.* (2015).

The present study has shown that runoff of Chlorpyrifos into the water bodies is highly genotoxic to *Heteroclaris* and as such poses a threat to the dependent human populace who rely on this fish species as a source of protein. It also revealed that only a moderate amount of vitamin E supplementation can restore genetic damage induced by chlorpyrifos near to the normal level.

Based on this study, it is recommended that the indiscriminate use of pesticides particularly chlorpyrifos in agricultural sector to control pests should be properly managed and monitored for health reason. Further study should be carried out to determine the magnification of genotoxicity of chlorpyrifos so as to prevent the wellbeing of the populace and also to find a way by which the nuclear damage can be properly and completely restored.

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