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Mutagenic and genotoxic assessment of atrazine-based herbicide to freshwater fish *Channa punctatus* (Bloch) using micronucleus test and single cell gel electrophoresis

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ABSTRACT

The mutagenic and genotoxic effect of 'Rasayanzine', an atrazine herbicide, was carried out in fish *Channa punctatus* using micronucleus and single cell gel electrophoresis (SCGE) assays. Three sub-lethal concentrations of the test material *viz.*, SL-I (1/5th LC_{50} = ~8.48 mg L^{-1}), SL-II (1/8th LC_{50} = ~5.30 mg L^{-1}) and SL-III (1/10th LC_{50} = ~4.24 mg L^{-1}) were calculated using LC_{50} value and the fish specimens were exposed to these concentrations. Erythrocyte and gill cells were sampled on day 0, 1, 3, 5, 7, 14, 21, 28 and 35 of exposure for assessment of micronuclei induction in blood erythrocytes and DNA damage using SCGE assay in both blood erythrocytes and gill cells. Significant effects ($p < 0.01$) for both concentration and time of exposure were observed in treated fish. Micronuclei induction in erythrocytes was highest (8.4 mg L^{-1}) on day 7 of exposure. The highest level of DNA damage in the SCGE was observed in both tissues on day 5 at all concentrations followed by gradual non-linear decline. This study further confirmed that the micronucleus and SCGE assays are useful in determining potential genotoxicity of water pollutants and might be appropriate as part of monitoring program.

1. Introduction

The use of herbicides to control weed has been recognized as a part of agricultural practices throughout the world. Unfortunately, the indiscriminate use of these herbicides to improve agricultural production and yield may have impacts on non-target organisms especially aquatic lives and their environment. Atrazine [6-chloro-N-ethyl-N′-(1-methylethyl)-triazine-2, 4-diamine] is one of the most commonly used herbicides found in the rural environments though its use has been controversial (currently banned in the EU but not in US and other countries). It is widely used in corn, sorghum, sugarcane, pineapple crops and to some extent in landscape vegetation. It has relatively high water solubility (32 mg L^{-1}), which aids in its infiltration into ground water (Orme and Kegley, 2004). Rated as moderately toxic to aquatic species, atrazine is mobile in the environment and is among the most detected pesticides in streams, rivers, ponds, reservoirs and ground water (Battaglin et al., 2003, 2009; Scrubner et al., 2005). Atrazine concentrations of 20 μg L^{-1} have been commonly detected in surface water runoff, while concentrations

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as high as 700 $\mu g L^{-1}$ have been reported (Selim, 2003). Due to low persistence of atrazine herbicide, repeated application are practiced for the control of weeds in agricultural field. As a result of this, large quantities of the herbicide find their ways into water bodies. The indiscriminate use of this herbicide, careless handing, accidental spillage or discharge of untreated effluents into natural water ways have harmful effects on the fish population and other aquatic organisms and may contribute to long term effects in the environment. Sublethal effects may occur at levels of exposure less than 2 $mg L^{-1}$ of atrazine during long-term exposure with biochemical and histopathological alteration of fish tissue (Neškovic et al., 1993).

Fish can serve as bio-indicators of environmental pollution and can play significant roles in assessing potential risk associated with contamination in aquatic environment since they are directly exposed to chemicals and mutagens resulting from agricultural production via surface run-off or indirectly through food chain of ecosystem (Cavas and Ergene-Gözükara, 2005; Lakra and Nagpure, 2009). 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).

Several ecotoxicological characteristics of *Channa punctatus*, such as wide distribution in the freshwater environment, noninvasive, availability throughout the seasons, presence of 32 well-differentiated diploid chromosome number, commercial importance, ease of blood collection and acclimatization to laboratory conditions, make this species an excellent test specimen for toxicity studies (Kumar et al., 2009).

Several studies have shown that the micronucleus (MN) test and single cell gel electrophoresis (SCGE) are two sensitive, rapid and extensively used methods in the detection of mutagenic and genotoxicity of chemicals and xenobiotics under field and laboratory conditions (Jha, 2004; Ateeq et al., 2005; Cavas and Ergene-Gözükara, 2005; Pandey et al., 2006; Talapatra et al., 2006; Sharma et al., 2007; Ergene-Gözükara et al., 2007; Nagpure et al., 2007; Ali et al., 2008, 2009; Xiao et al., 2008; Ventura et al., 2008; Kumar et al., 2009). One of the advantages of MN and SCGE assays are that both can be used for the simultaneous assessment of DNA damage in many tissues from the same animal and for the comparison of their responses under identical treatment condition. The formation of MN may be related to anaphase chromosomal delays characterized by a bad functioning in the spindle or due to the presence of acentric chromosomic fragments (Al-Sabti and Metcalfe, 1995). The SCGE has been considered as sensitive rapid and reliable method of quantitatively measuring DNA damage in eukaryotic and prokaryotic cells (Cotelle and Ferard, 1999; Bajpayee et al., 2005). It is increasingly being used in testing of substances such as industrial chemicals, biocides, agrochemical, food additives and pharmaceuticals for genotoxicity testing (Brendler-Schwaab et al., 2005). The assay is favored among other cytogenetic methods (viz. chromosome aberrations, sister chromatid exchange and micronucleus test) used for the detection of damage (Buschini et al., 2003) as it is capable of detecting wide variety of DNA damage such as DNA strand breaks (Sharbel, 2004). Thus, these techniques can be used in combination for screening genotoxic effect of chemicals and for investigating the implications of DNA damage and its recovery in the sentinel fish species.

These biomarkers have also opened a broad perspective in aquatic toxicology as fish erythrocytes and gills are constantly being exposed to environmental pollutants. Many workers have supported and demonstrated the relevance of fish erythrocytes for MN and SCGE in ecotoxicological studies (Schultz et al., 1993; Al-Sabti and Metcalfe, 1995; Abd-Allah et al., 1999; Gustavino et al., 2001; Sumathi et al., 2001) due to their easy sampling and not required cell dissociation (Ateeq et al., 2005; Cavas and Ergene-Gözükara, 2005). Fish blood is particularly favored because it comprises 97% erythrocytes, thus ensuring great homogeneity of cells for SCGE studies. In the present study, gill cells were also used for SCGE assay as they are prone to injury caused by chemicals and xenobiotics. Gill cells are the most appropriate target organ that is directly and constantly exposed to the DNA damaging chemicals dissolved in water (Dzwonkowska and Hubner, 1986).

The mutagenic and genotoxic properties of atrazine have been studied using a variety of assays in the past but the results were contradictory (Clements et al., 1997; Kligerman et al., 2000; Garaj-Vrhovac and Zeljezic, 2000; Tennant et al., 2001; Freeman and Rayburn, 2004; Ventura et al., 2008). Hence, hazardous effects of this herbicide are a matter of great concern due to human exposure consequent to wide use all over the world. In the present study, attempts have been made to investigate the mutagenic and genotoxic effects of atrazine herbicide using MN and SCGE assays in erythrocyte and gill cells of *C. punctatus* exposed *in vivo*.

2. Materials and methods

2.1. Experimental fish specimen and chemical

Freshwater air-breathing fish *C. punctatus* (Bloch; Family: Channidae, order: Perciformes) were caught from nearby ponds and lakes with the help of local fishermen. The specimens had an average (±SD) weight and length of 13.30 ± 0.70 g and 10.02 ± 0.72 cm, respectively. Fish specimens were subjected to a prophylactic treatment by bathing twice in 0.05% potassium permanganate ($KMnO_4$) for two min to avoid any dermal infections. The fishes were then acclimatized for two weeks under laboratory conditions in semi-static systems. They were fed boiled eggs, minced goat liver and poultry waste materials during acclimatization. The fecal matter and other waste materials were siphoned off daily to reduce ammonia content in water. For the present study, commercial formulations of atrazine (50% WP) with the trade name "Rasayanzine" manufactured by Krishi Rasayan Exports Ltd., India was purchased from the market.

2.2. Determination of sub-lethal concentrations

Acute toxicity was conducted to determine the 96 h LC_{50} value of atrazine with definitive test in semi-static system in laboratory as per standard methods (APHA, AWWA, WPCE, 2005). The range finding test was carried out prior to the definitive

test to determine the concentration of the test solution. For the test, the atrazine was dissolved in distilled water, filtered and added to the aquarium following the method of Pluta (1989). The test water with herbicide was changed after every 48 h by replacing with the fresh atrazine solution in order to counterbalance decreasing herbicide concentrations because of its hydrolysis in water. Hydrolysis of atrazine is rapid under acidic or basic conditions but slower at neutral pH. In the definitive test, a set of 10 fish specimens were randomly exposed to each of the atrazine concentrations (viz. 25, 32, 39, 46, 53 and 60 mg L^{-1}) and the experiment was set in triplicate to obtain the LC_{50} value of the herbicide for the species. The LC_{50} value of test chemical in *C. punctatus* was determined by probit analysis method (Finney, 1971) for 24, 48, 72 and 96 h. Based on the 96 h LC_{50} value, three sublethal test concentrations of atrazine were determined and the fish specimens were exposed to these concentrations for the assessment of its mutagenicity and genotoxicity.

2.3. *In vivo exposure experiment*

The fish specimens were exposed to the three sublethal concentrations of test chemical in a semi-static system with the change of test solution after every 48 h of exposure. The exposure was continued up to 35 days and the tissue sampling was done at intervals of 1, 3, 5, 7, 14, 28 and 35 days at the rate of five specimens per sampling interval for MN and SCGE assays. The specimens maintained in tap water separately were considered as negative control. An additional set of specimens was separately exposed to cyclophosphamide (4 mg L^{-1}) for positive control (Cavas and Ergene-Gözükara, 2005) in MN assay. However, for SCGE assay, the erythrocyte and gill cells sampled from *C. punctatus* on day 1 were treated *ex vivo* with 100 μM H_2O_2 for 10 min at 4 °C and the average value of DNA damage in five specimens was used as positive control. Similarly, the value of negative control was also based on average DNA damage in five specimens on day 1.

On each sampling interval, ten specimens (five each for MN and SCGE) were sampled from each atrazine treated group. MN test was only performed on blood erythrocytes while both blood erythrocytes and gill cells were processed for SCGE. The blood was collected from the caudal vein of fish by puncture technique using heparinized syringe. Sampling of the negative and positive control was also done for comparison. The physicochemical properties of test water, namely temperature, *p*H, conductivity, dissolved oxygen, and total hardness were analyzed at the beginning of experiment and on each sampling day using standard methods (APHA, AWWA, WPCE, 2005).

2.4. *Micronucleus assay*

Peripheral blood samples obtained from the caudal vein were smeared on clean, grease free, one end frosted glass slides. Slides were fixed in methanol for 10 min and left to air-dry at room temperature and finally stained with 6% Giemsa in Sorenson buffer (pH 6.9) for 20 min. After dehydration through graded alcohol and clearing in xylene, slides were mounted in DPX (distyrene, plasticizer and xylene). From each slide, 1500 erythrocyte cells were scored under light microscope (Leitz Wetzlar Germany, Type 307 – 083.103, oil immersion lens, 100/1.25). The criteria used for the identification of MN were their size smaller than one-third of the main nucleus, no attachment with the main nucleus, and same color and intensity as the main nucleus. The MN frequency was calculated as

$$\%\,\mathrm{MN} = \frac{\text{Number of cells containing micronuclei}}{\text{Total number of cells counted}} \times 100$$

2.5. *Single cell gel electrophoresis*

The alkaline single cell gel electrophoresis or comet assay (CA) was performed as a three-layer procedure (Singh et al., 1988) with slight modifications (Klaude et al., 1996). The gill tissue was removed with the help of scissor and homogenized in ice-cold homogenization buffer [1X Hanks' balanced salt solution (HBSS), 20 mM EDTA, 10% dimethyl sulfoxide (DMSO), pH 7.0–7.5] followed by centrifugation at 3000 rpm at 4 °C for 5 min. The cell pellet was then suspended in chilled phosphate buffered saline (PBS). Viability of both the erythrocytes and gill cells was evaluated by the trypan blue exclusion test method (Anderson et al., 1994) and the cell suspensions showing viability >84% were further processed for SCGE according to the method described by Ali et al. (2008).

Two slides per specimen were prepared and 25 cells per slide (250 cells per concentration) were scored randomly and analyzed using an image analysis system (Komet-5.5 Kinetic Imaging, UK) attached to a fluorescent microscope (Leica) equipped with appropriate filters. The parameter selected for quantification of DNA damage was percent tail DNA (% tail DNA = 100 – % Head DNA), as determined by the software.

2.6. *Statistical analysis*

The percentage of MN frequency among different exposure times and concentrations were compared using the Mann–Whitney test. One-way analysis of variance (ANOVA) was employed using SPSS software (Standard Version 11.5 SPSS Inc.) to compare the mean differences in % tail DNA, estimated by SCGE assay, among different tissues, concentrations, and exposure intervals. A p value less than 0.01 was considered statistically significant.

3. Results

3.1. *Physicochemical properties of the test water*

The test water temperature varied from 24.3 to 25.5 °C, the pH values ranged from 7.3 to 8.0 while the dissolved oxygen concentration varied from 6.6 to 7.9 mg L^{-1} during the experimental period. The conductivity of the water ranged from 260 to 300 μM cm^{-1} while the total hardness and total alkalinity ranged from 200 to 230 mg L^{-1} and from 275 to 320 as $CaCO_3$, respectively.

3.2. *Acute toxicity bioassay and sublethal concentrations*

In the acute toxicity bioassay, a concentration dependent increase and time dependent decrease was observed in the

Table 1 – Median lethal concentration of atrazine (mg L^{-1}) (with 95% confidence intervals) depending on exposure time in *C. punctatus* ($n = 10$ each in 3 replicates).

Lethal concentration	Exposure time (h)			
	24	48	72	96
LC_{10}	43.04[a] (36.66–46.87)	37.38[b] (33.42–40.11)	34.66[c] (31.32–37.06)	33.19[c] (29.92–35.53)
LC_{50}	64.05[a] (57.86–79.92)	49.10[b] (46.63–51.94)	44.41[c] (42.25–46.63)	42.38[c] (40.28–44.46)
LC_{90}	95.32[a] (77.41–160.76)	64.48[b] (59.51–73.55)	56.90[c] (53.32–62.72)	54.11[c] (50.84–59.32)

Values with different alphabet superscript differ significantly ($p < 0.01$) between exposure time within lethal concentration as determined by one-way analysis of variance (ANOVA).

mortality rate, such that as the exposure time increased from 24 to 96 h the concentration of the test material, required to kill the fish, was reduced. In the bioassay, the LC_{50} values (with 95% confidence limits) of atrazine were found to be 64.05 (57.86–79.92), 49.10 (46.63–51.94), 44.41 (42.25–46.63) and 42.38 (40.28–44.46) mg L^{-1}, respectively, in *C. punctatus* for 24, 48, 72 and 96 h exposure time (Table 1). Accordingly, the three sublethal test concentrations were calculated as SL-I (1/5th LC_{50} = ~8.48 mg L^{-1}), SL-II (1/8th LC_{50} = ~5.30 mg L^{-1}) and SL-III (1/10th LC_{50} = ~4.24 mg L^{-1}) at which the fish specimens were exposed for assessment of genotoxicity and mutagenicity of atrazine.

3.3. *DNA damage and induction of micronucleus*

The DNA damage measured as % tail DNA in the erythrocyte and gill cells of the control and treatment groups (Table 2, Figs. 1a–d and 2a–c) indicated that the fish specimens exposed to the positive control and different concentrations of atrazine exhibited significantly higher DNA damage ($p < 0.01$) in their tissues than the negative control specimens. The values of % tail DNA in negative and positive control were recorded as 3.85 (±0.53) and 6.05 (±0.45), respectively, in erythrocytes and 4.07 (±0.54) and 7.02 (±1.02) in gill cells (Table 2). The DNA damage in both tissues was found to be dose and time dependent with the highest DNA damage observed at SL-I concentration, followed by SL-II and SL-III concentrations. The study also showed that the highest DNA damage was observed on day 5 at all concentrations in both the erythrocyte and gill tissues. After attaining the peak, there was a gradual non-linear decline, in general, in DNA damage with the advancement of the experiment.

With regard to the variation in DNA damage between the tissues, the gill exhibited comparatively higher DNA damage ($p < 0.01$) at SL-I than the blood erythrocytes between day 3 and 14 (Table 2, Fig. 2a). With SL-II, the gill cells also exhibited significantly ($p < 0.01$) higher DNA damage than the blood cells from day 3 to 7 (Fig. 2b), whereas no significant variation in the DNA damage was observed between the tissues at SL-III concentration (Table 2).

The result of MN analysis in erythrocytes of *C. punctatus* at different concentrations and durations indicated significant induction ($p < 0.01$) of MN in the fish specimens due to atrazine exposure than compared to the control group (Table 3). MN induction was significantly higher in the positive than the negative control from day 5 to day 21. Increase in the concentration of the herbicide resulted in higher induction of MN with the highest frequency recorded at SL-I on day 7 (0.823). At SL-II and SL-III, the MN formation was highest on day 5 (0.672) and day 14 (0.221), respectively. There was a gradual non-linear decline in MN frequency after peak attainment for all the concentrations.

Table 2 – Mean ± SE% tail DNA in erythrocytes and gill cells of *C. punctatus* exposed to different concentration (mg L^{-1}) of atrazine ($n = 250$ cells/concentration were scored).

Exposure time (days)	Erythrocytes			Gills		
	8.48	5.30	4.24	8.48	5.30	4.24
1	4.51 ± 0.34^{aA}	4.22 ± 0.36^{aA}	3.91 ± 0.50^{aA}	5.10 ± 0.62^{Aa}	4.74 ± 0.33^{aA}	4.04 ± 0.46^{aA}
3	$8.94 \pm 0.37^{bB*}$	$6.82 \pm 0.81^{bB*}$	$5.64 \pm 0.90^{aA*}$	$10.52 \pm 0.56^{aC*}$	$9.81 \pm 0.37^{aB*}$	$6.33 \pm 0.80^{aB*}$
5	$16.54 \pm 0.44^{aC*}$	$14.55 \pm 0.37^{bC*}$	$13.96 \pm 1.20^{aC*}$	$19.5 \pm 0.33^{bD*}$	$17.64 \pm 1.51^{aC*}$	$16.31 \pm 1.25^{aC*}$
7	$14.68 \pm 1.27^{aCD*}$	$13.97 \pm 1.20^{aC*}$	$13.84 \pm 1.11^{aC*}$	$17.40 \pm 1.43^{aD*}$	$16.25 \pm 1.26^{aC*}$	$16.13 \pm 0.54^{aC*}$
14	$12.13 \pm 0.86^{aD*}$	$11.95 \pm 1.52^{aCD*}$	$11.88 \pm 1.22^{aC*}$	$14.47 \pm 0.58^{bE*}$	$13.88 \pm 0.92^{aD*}$	$11.76 \pm 0.83^{aD*}$
21	$11.26 \pm 1.41^{aD*}$	$10.63 \pm 2.40^{aD*}$	$7.52 \pm 0.97^{abB*}$	$11.23 \pm 0.83^{aC1*}$	$10.78 \pm 0.85^{aE*}$	$7.69 \pm 0.78^{aB*}$
28	$6.64 \pm 0.99^{aB*}$	5.13 ± 0.73^{bA}	3.85 ± 0.53^{aA}	$6.91 \pm 1.02^{aB*}$	$7.49 \pm 1.10^{aB*}$	5.50 ± 0.91^{aA}
35	$6.29 \pm 0.76^{aB*}$	4.54 ± 0.57^{aA}	4.36 ± 0.71^{aA}	$6.56 \pm 0.78^{aB*}$	4.55 ± 0.32^{aA}	4.52 ± 0.78^{aA}

The mean %tail DNA in negative control was 3.85 (±0.53) and 4.07(±0.54), respectively, in erythrocytes and gill cells, whereas the same were 6.05(±0.45) and 7.02(±1.02) in positive control. Values with different alphabet (lowercase) superscripts differ significantly ($p < 0.01$) between tissues within concentration and exposure duration. Values with different alphabet (uppercase) superscript differ significantly ($p < 0.01$) between exposure durations within concentration and tissue. Values with * indicate significant ($p < 0.01$) difference between negative control and atrazine treated groups within tissue and exposure duration.

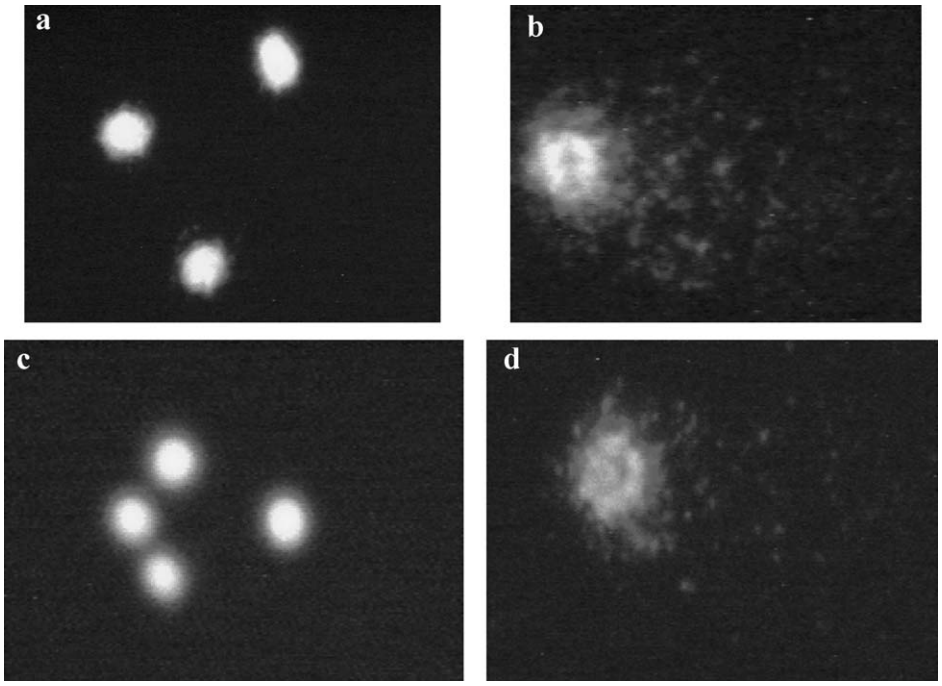

Fig. 1 – (a) Control, (b) atrazine exposed gill cells; (c) control, and (d) atrazine exposed erythrocyte cells.

4. Discussion

Acute toxicity data has been used to derive water quality guidelines for regulatory measures (Sunderam et al., 1994). The results of the LC_{50} at 96 h exposure time show that the toxicity of atrazine for *C. punctatus* is both time and concentration dependent, thus, accounting for the differences in the values obtained at different concentrations and time of exposure. However, some other researchers have shown that exposure time is not significant in LC_{50} determination for fish (Lakota et al., 1989). The estimated LC_{50} value observed in this study was higher from the results obtained by Bathe et al. (1973), Neškovic et al. (1993) and Hussein et al. (1996) who reported 16.0, 18.8 and 9.37 mg L^{-1} for *Lepomis macrochirus* (Bluegill sun fish), *Cyprinus carpio* and *Oreochromis niloticus*, respectively, exposed to atrazine. Toxicity of the chemicals to aquatic organisms has been reported to be affected by age, size, health and type of species (Abdul-Farah et al., 2004). Physiological parameters like quality, temperature, pH, dissolved oxygen and turbidity of water, amount and kind of aquatic vegetation, concentration and formulation of chemical and its exposure also greatly influence such studies (Gupta et al., 1981; Young, 2000). However, the large variation in safe levels, determined by different methods, has resulted in controversy over it acceptability (Buikema et al., 1982; Pandey et al., 2005).

We applied the alkaline SCGE to evaluate total DNA strand breaks in the erythrocyte and gill cells of *C. punctatus* exposed *in vivo* to different sublethal concentrations of atrazine for 35 days. The long-term genotoxicity studies can be important approach for achieving greater insight into the organisms DNA repair ability and other protective mechanisms for excreting the toxic chemicals.

The results showed that the frequencies of % tail DNA damage for all concentrations of atrazine tested in both tissues were significantly higher ($p < 0.01$) than the negative control and, thus, indicated the genotoxic potential of the herbicide to aquatic organisms. A concentration and time dependent increase in DNA single strand breaks in the form of comet induction followed by a time-dependent decrease in DNA damage was observed. The induction of DNA damage as observed in the present study is in accordance with the findings of Ahmed et al. (2008) who reported apoptic cell death of *C. punctatus* due to arsenic toxicity. Our results are also in agreement with the findings of Clements et al. (1997) for the erythrocytes of some species of frogs after atrazine (AATREX Nine-O) exposure and those of Garaj-Vrhovac and Zeljezic (2000) for employees who were occupationally exposed to atrazine herbicide. The time dependent decrease in DNA damage might indicate repair of damaged DNA, loss of heavily damaged cells or both (Banu et al., 2001; Miyamae et al., 1998). Another possible explanation could be the gene activation of metabolizing enzymes such as cytochrome p450 in various tissues that provides a defensive mechanism against persistent organic pollutants (Wiegand et al., 2001). Similar repair mechanism was observed when the same fish species (*C. punctatus*) were exposed to endosulfan (Sharma et al., 2007) and malathion (Kumar et al., 2009).

The DNA damage detected in this study could have originated from DNA-single-strand breaks, DNA double-strand breaks, DNA adducts formations and DNA–DNA and DNA–protein cross links (Mitchelmore and Chipman, 1998) resulting from the interactions of herbicides or their metabolites with DNA (Fairbrairn et al., 1995).

Differential DNA damage in blood erythrocyte and gill cells in the fish specimen exposed to atrazine could be explained by the number of the alkali-labile sites, being variable in DNA

Table 3 – MN frequencies in blood erythrocytes of *C. punctatus* exposed to atrazine at different concentrations and exposure times ($n = 15{,}000$ cells/sampling interval/concentration).

Concentration ($mg\,L^{-1}$)	% MN frequencies (Mean ± SE)						
	1	3	5	7	14	21	28	35
NC	0.021 ± 0.02^{a1}	0.033 ± 0.009^{a1}	0.042 ± 0.008^{a1}	0.044 ± 0.006^{a1}	0.032 ± 0.009^{a1}	0.030 ± 0.019^{a1}	0.022 ± 0.002^{a1}	0.012 ± 0.001^{a1}
PC	0.142 ± 0.016^{a1}	0.065 ± 0.008^{a1}	0.143 ± 0.004^{b1}	0.164 ± 0.006^{c1}	0.133 ± 0.007^{bd1}	0.091 ± 0.002^{b1}	0.064 ± 0.003^{a1}	0.033 ± 0.001^{a1}
4.24	0.031 ± 0.019^{a1}	0.08 ± 0.016^{ab1}	0.172 ± 0.07^{ab2}	0.150 ± 0.061^{ab1}	0.221 ± 0.062^{b1}	0.093 ± 0.025^{ab1}	0.101 ± 0.025^{ab1}	0.030 ± 0.019^{a1}
5.30	0.041 ± 0.016^{a1}	0.111 ± 0.057^{ac1}	0.672 ± 0.099^{b3}	0.373 ± 0.030^{c2}	0.254 ± 0.066^{cd2}	0.122 ± 0.043^{ad1}	0.102 ± 0.043^{ad1}	0.074 ± 0.019^{a1}
8.48	0.112 ± 0.025^{a1}	0.221 ± 0.088^{a2}	0.612 ± 0.095^{bc3}	0.823 ± 0.080^{c3}	0.271 ± 0.137^{ab2}	0.182 ± 0.077^{a2}	0.141 ± 0.047^{a2}	0.133 ± 0.066^{a1}

Values with different alphabetic (lowercase) superscripts differ significantly ($p < 0.01$) between exposure durations within concentration. Values with different numeric superscripts differ significantly ($p < 0.01$) between concentrations within exposure duration. NC = Negative control, PC = Positive control.

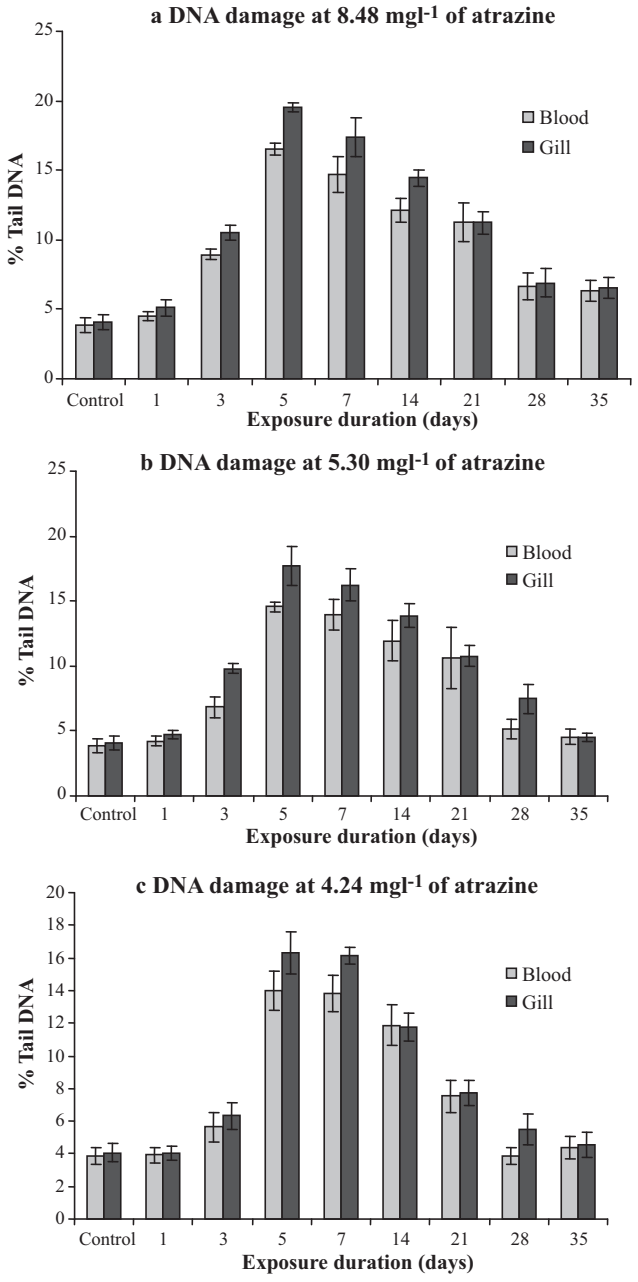

Fig. 2 – DNA damage in blood and gill cells at: (a) 8.48, (b) 5.30, and (c) 4.24 mg L^{-1} concentrations of atrazine.

from different tissues and by different cell types having very different background levels of DNA single-strand breaks due to variation in excision repair activity, metabolic activity, antioxidant concentrations and other factors (Lee and Steinert, 2003). In general, the gill cells showed significantly ($p < 0.01$) higher DNA damage in comparison with the blood erythrocytes in the atrazine treated fish. The higher DNA damage in the gill cells could be explained as these cells are directly and constantly exposed to the DNA damaging chemicals dissolved in water (Dzwonkowska and Hubner, 1986) whereas blood erythrocytes receive chemicals when they enter the circulatory system. The suitability of gill tissues for genotoxicity studies has also been demonstrated earlier using the shellfish (Sasaki

et al., 1997). The observed tissue-specific response may also be due to physiochemical activities distinctive to these organs, with respect to either the activation or detoxification of pollutants or the repair of different types of strand breaks.

A concentration and time dependent increase followed by time dependent decrease in MN induction observed in our result is similar to that reported by Ali et al. (2008) and Kumar et al. (2009) in the same fish species exposed to chlorpyrifos and malathion, respectively.

Further, the maximum MN frequency was reached on day 7 (0.823) at the highest (SL-I) concentration whereas for SL-II and SL-III concentrations, the MN formation was highest on day 5 (i.e. 0.672 and 0.172, respectively). Al-Sabti and Metcalfe (1995) demonstrated that maximal MN induction normally occurred at one to five days post -exposure, which agrees with our results for SL-II and SL-III concentrations. The maximal MN observed on day 7 at the highest concentration may probably be due to high production of reactive oxygen species (ROS) resulting to cell apoptosis. ROS and oxidative stress have been demonstrated to be triggers of apoptosis (Shen and Liu, 2006). Oxidative stress is caused by an imbalance between the production of ROS and an organism's ability to detoxify them or repair the resulting damage. The free radicals formed in this process can damage macromolecule like DNA. Oxidative stress may also be due to the depletion of cellular glutathione (GSH) content below the critical level which prevents the conjugation of xenobiotics like atrazine to GSH and thus enables them to freely combine covalently with cell proteins (Yamano and Morita, 1995). However, organisms are equipped with interdependent cascades of enzymes (superoxide dismutase (SOD) and catalase (CAT)) to alleviate oxidative stress and repair damaged DNA, produced during normal metabolism or due to exposure to xenobiotics. This may explain why the DNA damage and the frequency of MN declined after day 7 of exposure. Cyclophosphamide has been used widely as a positive control because of its alkylating activity. Cyclophosphamide itself is not genotoxic but undergoes complex metabolic activation by mixed function oxidases, which result in genotoxic metabolites (Colvin and Chabner, 1990).

Although our tested atrazine concentration is higher than the recommended maximum concentration level (MCL) of $5\,\mu g\,L^{-1}$ in natural freshwater (USEPA, 2006), it has been found in much higher concentration than the freshwater life standard in most rivers, pools and other aquatic environments impaired by humans (Ritter, 1990, Battaglin et al., 2009) thus raising serious concern about its potential dangers to aquatic organisms. The results of this study emphasized the importance of the peripheral blood MN assay and suggest its broader application as an early biological marker of exposure of fish to clastogenic pollutants in the aquatic environments.

5. Conclusion

The current study, thus, indicated that the MN and SCGE assays are sensitive tools for evaluating the mutagenic and genotoxic effects of atrazine in different fish tissues. A significant increase ($p < 0.01$) in micronuclei and percentage of DNA damage were recorded with the increase in exposure time and concentration of the atrazine pesticide. Comparison of DNA damage between the tissues showed that the gill cells were more sensitive than the blood erythrocytes. Based on the results presented, we may suggest judicious and careful use of atrazine herbicide to guard against genetic hazards to aquatic ecosystems and also to human population.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Ethical statement

All experiments were carried out in accordance with the guidelines for the care of experimental animals as approved by the National Bureau of Fish Genetic Resources, Indian Council of Agricultural Research, Canal Ring Road, PO Dilkusha, Lucknow (UP) 226 002, India.

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