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Cytogenotoxicity of azo dye Reactive Red 120 (RR120) on fish *Catla catla*

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Abstract

The textile industries play a major role in the economy of India. In addition to this, they exert acute and/or chronic effects on environment by discharge of effluent in the environment. This study was designed to assess the cyto genotoxicity of Reactive Red 120 (RR120) on freshwater fish *Catla catla*, using DNA damage in gill cells and blood cells as sensitive biomarkers. For this, fingerlings were exposed to three sublethal concentrations of RR120 (0.35, 0.7, 3.5 mg L^{-1}). Samples (gills and peripheral blood) were collected and analyzed at fixed intervals (10, 20 and 30 days) and were compared with those of the control. Some nuclear changes were observed, such as micronuclei (MN), nuclear buds (NB), fragmented-apoptotic (FA) and bi-nucleated (BN) cells in both peripheral blood cells and gill cells. All the abnormalities showed dose- and time-dependent increase and were higher in the gill cells. The results clearly revealed the most significant change for MN, followed by FA, BN and NB in both samples. Hence, RR120 has a potential to damage DNA in cells and exerts genotoxic impact on fish. These findings also suggest the possible toxicity of RR120 to humans by direct or indirect exposure.

Key words: azo dye, *Catla catla*, genotoxicity, micronuclei, RR 120.
Abbreviations: BN, bi-nucleated cells; FA, fragmented-apoptotic; MN, micronuclei; NB, nuclear buds; RR 120, Reactive Red 120.

Introduction

Increasing industrialization and urbanization lead to environmental pollution. The discharge of toxic effluents from various industries adversely affects the aquatic ecosystem (Puvaneswari et al. 2006). Approximately more than ten thousand synthetic dyes are available commercially. Among these, azo dyes are widely used in the textile industry because of their good brightness, low cost, ease of application and a wide range of colours (Padhi 2012). Approximately up to 10 – 15% of dyes are discharged out in the form of effluents during the dyeing process. Very low concentration of azo dye (even less than 1 mg L^{-1}) can be highly visible in water and interfere with penetration of light and affect aquatic flora and fauna (Patial, Kaur 2013). Most of the azo dyes are non-biodegradable, potentially toxic, mutagenic and carcinogenic to living organisms, aquatic animals and humans (Rinde, Troll 1975; Ogugbue, Oranusi 2006; Padhi 2012).

Fish have been considered as an efficient and effective model to evaluate toxic, mutagenic and carcinogenic potential of pollutants (Monteiro et al. 2010). Among the fish models used for toxicity screening of aquatic environment, the cyprinid *Catla catla* was chosen for this study because of its common availability in India throughout the year, and its considerable economic importance as an edible species and since it has been proved to be a sensitive indicator of environmental stress (Tilak et al. 1981; Anbumani, Mohankumar 2011).

Micronucleus (MN) assay is one of the most sensitive and promising tests to check the water quality and to verify the genotoxic potential of pollutants when discharged in aquatic environment (Kuniyoshi, Braga 2014). This test has served as an index of cytogenetic damage over 30 years due to its sensitivity (Fenech et al. 2003). In the present study, micronuclei assay was used to assess the genotoxicity of commercial Reactive Red 120 dye in *C. catla*. There is not much data on the effect of commercial dyes on the genetic integrity of aquatic organisms like fish. In addition to MN, which indicates unrepairable DNA damage, some other nuclear changes like nuclear bud (NB), bi-lobed nuclei (BN), fragmented apoptotic cells (FA) are also considered as sensitive indicators to assess cytogenotoxic impacts of toxins (Sreedevi, Chitra 2014).

Previous studies show a good record on genotoxic effects of various contaminants on different species of fish (Deepa et al. 2011; Kousar, Javed 2015; Javed et al. 2016). Very few studies are available on azo dye toxicity on MN induction. Some results have reported earlier on effects of synthetic dye C.I. Acid violet 66 and C.I. acid red 217 dye on *Oncorhynchus mykiss* (Marlsca et al. 1992). Similar results are reported on Prussian carp exposed to chlorotriazine reactive azo red 120 dye (Al-sabti 2000). Cytogenotoxicity of azo dye acid blue-113 to *Channa punctatus* has been also reported recently (Kirandeep et al. 2015). It was recently reported that different concentrations of RR 120 induced

MN formation and nuclear abnormalities in *Catla catla* (Jagruti 2015).

The present study was aimed to determine genotoxic impact of three sub-lethal doses of azo dye RR 120 using micronuclei (MN) and other nuclear alterations like NB, BN and FA, in two different target tissues (peripheral blood cells and gill cells), in a tissue-specific dose and time dependent study on *Catla catla* as a test model.

Materials and methods

Test species and husbandry

Catla catla fingerlings of 12 ± 2 cm length and weight 22 ± 5 g were obtained from a fish seed farm at Sivan, Gujarat, India. They were first disinfected with 0.1% $KMnO_4$ and acclimatized in laboratory conditions for two weeks. During acclimatization, fish were kept in continuous aerated chlorine-free tap water in aquarium (150 L). They were fed daily with commercially available trout food at 5% of their body weight. Fingerlings were not fed for 24 h prior to the experiment. Water quality was measured and maintained during acclimatization and throughout the experimental period as described in APHA (American Public Health et al. 2005). Some physicochemical parameters of the water were as follows: temperature, 25 ± 2 °C; pH 7.4; hardness 120 mg L^{-1} (as $CaCO_3$); alkalinity 125 mg L^{-1} (as CaCO3); and dissolved oxygen concentration 6.2 to 6.8 mg L^{-1}.

Test chemical

The azo dye used in the present study is C.I. Reactive Red 120 (RR120) [$C_{44}H_{24}Cl_2N_{14}Na_6O_{20}S_6$] was obtained from a local source and used directly for experimental purpose. A stock solution was prepared by dissolving accurately weighed dye in distilled water to the concentration of 50 mg mL^{-1}. It was stored it at 4 °C temperature. The experimental concentrations were obtained by diluting the stock solution in accurate proportions with dechlorinated water.

Experimental design

Based on LC_{50} value (35 mg L^{-1}), three sublethal concentrations (0.35, 0.7, 3.5 mg L^{-1}) were selected for the exposure (Finney 1971). Healthy 70 acclimatized fingerlings were divided into seven groups (10 in each) and exposed to above mentioned sublethal concentrations of RR120 for 30 days. One group (n = 10) was exposed to dye free dechlorinated tap water as a control. Peripheral blood and gill samples were collected three times on the same day at fixed intervals (10, 20 and 30 days) from the day of each exposure and analyzed for MN.

Micronucleus test

The MN test was performed according to standard protocol (Al-Sabti, Metcalfe 1995). In brief, after exposure at fixed interval, peripheral blood samples were collected directly from caudal vein of each fingerling (exposed and control) in triplicate. Smears were prepared, air dried, fixed in methanol for 5 min and stained for 10 min with 10% Giemsa. Stained slides were then air dried and examined using light microscope under 100X. Microphotographs were taken and documentation was done for micronuclei.

Gills were dissected from control fish as well as exposed fingerlings in triplicate at 10, 20 and 30 days after exposure. Micronuclei smears were prepared following the standard air-drying Giemsa staining technique (Yunis, Barr 1966). In brief, gills were cut into small pieces and homogenized in 0.56% hypotonic KCl solution and kept as such for 20 to 25 min. These homogeneous mixtures were then centrifuged at 1000 to 1500 rpm for 10 min. Supernatants were discarded and the pellets were fixed in child Carnoy's fixative. Three to four changes of fixative were given but the last change was in a small amount of fixative. This suspension was dropped on clean dry slides and air dried. Air dried smears were stained in 10% Giemsa solution for 10 to 20 min. Stained slides were then air dried and examined using a light microscope under 100×. Microphotographs were taken and documentation was done for micronuclei.

For the scoring of micronuclei, adopted criteria were as follows: MN should be separated from or marginally overlap with the main nucleus as long as there is a clear identification of the nuclear boundary. Micronuclei should have similar staining as the main nucleus. Micronuclei should be on same plane of focus as the main nuclei (Fenech et al. 2003). The frequency of micronuclei in each tissue was established by counting the number of micronuclei in at least 1000 cells per slide under 100×. Frequency of micronuclei was calculated by using the following formulae:

Micronucleus frequency (%) = *Number of cells with micronuclei* / *Total number of cells counted* × 100.

Statistical analysis

Data from the micronucleus test were expressed as mean±SD. Statistical analyses were calculated in triplicate performed using the computer software 'SPSS'. $p < 0.05$ was considered to be the level of significance. Statistical significance in the frequencies of micronuclei and nuclear alterations between exposed and control groups after each dose and duration of exposure were evaluated.

Results

The sublethal concentrations of RR120 used in the present study induced a significantly higher number of MN and NAs in both peripheral blood and gills compared to the control (Fig. 1 to 4). The frequency of MN and NAs in all the dye-treated groups was significantly different from control ($p < 0.05$). Frequency histograms of the damage grade in control and dye-treated group are shown in Fig. 1 and 3. Further, the induction of MN and NAs increased significantly with higher dye concentration and time of exposure. Fig. 1 shows the graphical representation of frequency of MN and NAs

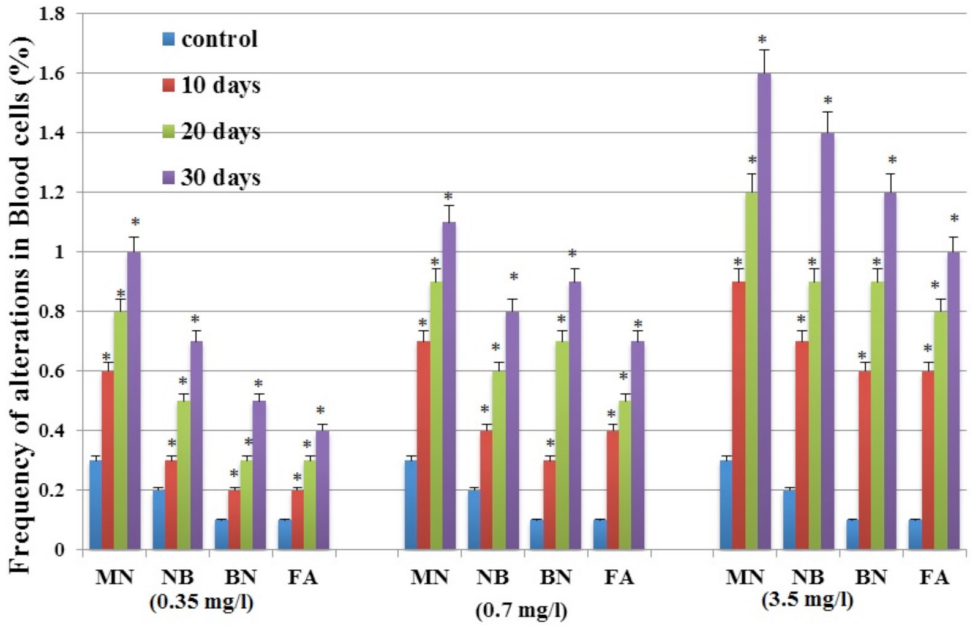

Fig. 1. Comparison of the frequency of MN, NB, BN, and FA induction (±SD) in erythrocytic alteration in *Catla catla* fingerling after three different RR120 exposure treatments (0.35, 0.7, 3.5 mg L–1) compared with the controls after 10, 20 and 30 days. Asterisks (*) indicate significant ($p < 0.05$) difference compared to control.

induction in peripheral erythrocytes of dye exposed *C. catla* (Fig. 2). The frequency of MN and NAs increased over time in all concentrations (10 < 20 < 30 days) for gills (Fig. 3). Similarly sublethal concentrations led to greater effect (0.35 < 0.7 < 3.5 mg L^{-1}). In lower concentrations MN and NAs had lower frequency. The maximum MN frequency was recorded at 3.5 mg L^{-1} concentration of RR120 at 30 days after exposure. MN and NAs frequency increased in a dose- and time-dependent manner.

The frequencies of MN, FA, BN and NB were also significantly higher in all experimental groups when compared to the control one (Fig. 2, Fig. 4). However, effect on MN was more significant in all concentrations at all time intervals, than for FA, BN, and NB in both samples (Fig. 1, Fig. 3).

Sub-lethal concentrations of RR120 evidently increased the micronuclei frequencies in both tissues in a dose- and time-dependent manner, compared to the respective controls. Fig. 3 presents the frequency of MN and NAs (FA, BN, NB) in gill tissue after exposure to RR120. There was remarkable and gradual increase in all markers including MN, FA, BN, and NB frequency in gill cells of dye exposed fingerlings (Fig. 3, Fig. 4).

A comparison between the micronucleus frequency in both tissues revealed higher micronuclei frequencies in gill cells than in erythrocytes cells. The observation that RR120 caused a greater increase in micronuclei frequency in cells of gills may be due to direct contact of gills with the test chemical in solution and therefore, higher exposure to the *in situ* concentration of the compound.

The present study found that RR120 induced the micronuclei formation in *C. catla*. The results revealed that commercially available reactive red dye is genotoxic to fish at all doses tested in a dose-dependent manner.

Discussion

An attempt was made to assess the time- and dose-dependence of tissue specific genotoxic effect of RR120 azo dye in *C. catla* fingerlings. When fish were exposed to higher concentrations of pollutants, nuclear and cellular

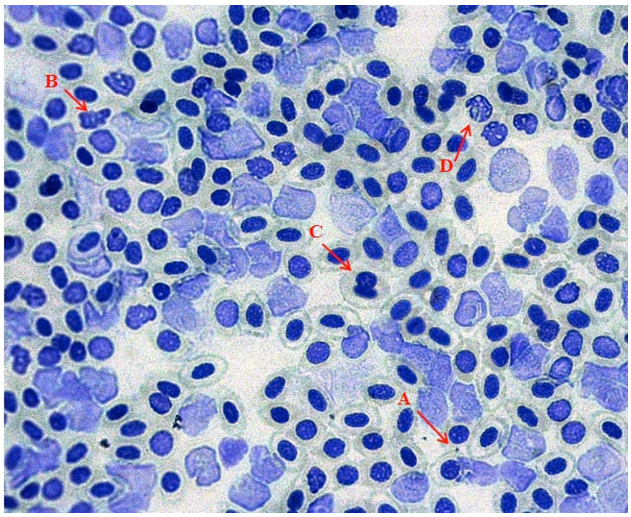

Fig. 2. Representative images of various erythrocytic cellular abnormalities in fish *Catla catla* exposed to azo dye RR120. A, micronuclei; B, nuclear bud; C, bi-nucleated cells; D, fragmented-apoptotic cells (100×).

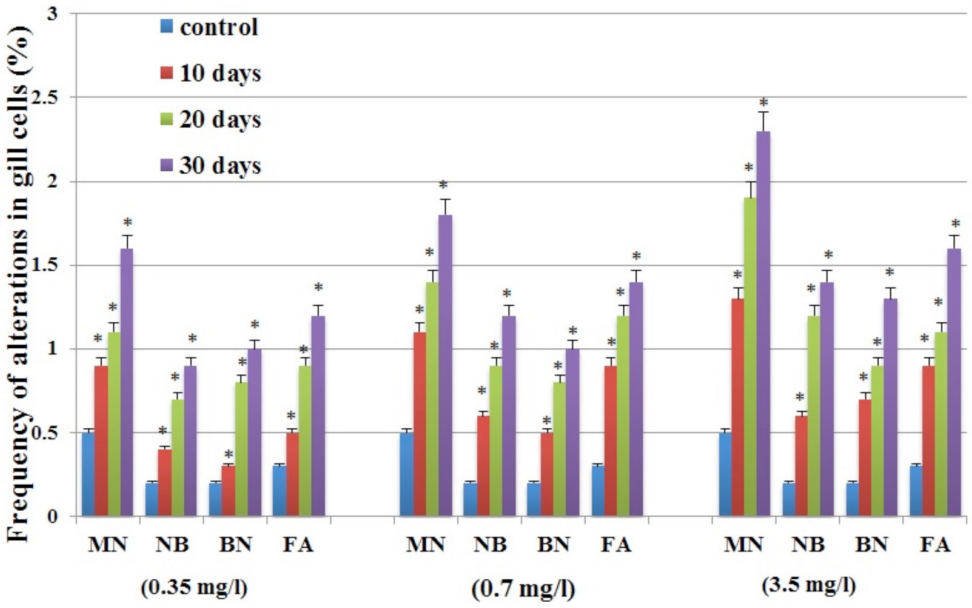

Fig. 3. Comparison of the frequency of MN, NB, BN, and FA induction (±SD) in gill cells alteration in *Catla catla* fingerling after three different RR120 exposure treatments (0.35, 0.7, 3.5 mg L^{-1}) compared with the controls after 10, 20 and 30 days. Asterisks (*) indicate significant ($p < 0.05$) difference compared to control.

abnormalities were observed in all the tissues. MN is the result of chromosome breaks (or mitotic anomalies), which are normally extruded along the main nucleus and their presence suggests their origin at a more recent cell cycle (Chandra, Khuda-Bukhsh 2004). Genotoxicity bioassay using micronuclei induction therefore helps to evaluate toxicity of minute quantities of pollutants especially in situations when there is no mortality. In the present study the selected anomalies (MN and NAs) were at higher frequency in gill cells as compared to peripheral blood. Gills are the first target organ that comes direct in contact with the contaminants, compared to other tissues of the body. Therefore, gills exhibit extreme cellular, biochemical and histopathological changes as compared to other organs of the body (Shimizu et al. 2007).

A dose dependent significant increase in MN clearly hints towards a clastogenic nature of the dye, as MN are believed to be derived from chromosome fragments. Induction of MN actually represents un-repairable and higher damage to DNA (Shimizu et al. 2007).

Only a few studies have been published on the effect of some textile dyes inducing micronuclei in fish erythrocytes. Our findings were supported by some recent and previous studies. Rainbow trout (*Oncorhynchus mykiss*) exposed to Acid violet 66 and Acid Red 217 exhibited a significant increase in erythrocyte MNs following 30 days of exposure (Marlasca et al. 1992; Marlasca et al. 1998). Cytogenotoxicity of Acid Blue-113, azo dye was also concentration dependent and caused increased MN frequency along with NAs in all studied target tissues of *Channa punctatus* (Kirandeep et al. 2015). Al-Sabti used micronucleus induction in fish erythrocytes to study the genotoxicity of chlorotriazine Reactive Azo Red 120 textile dye in aquatic ecosystems (Al-Sabti 2000). Determination of genotoxic effect of azo dye C.I. RR 120 on fish *C. catla* to studied by Avni and Jagruti (2016). Similar study was carried out on fish *Oreochromis niloticus* (Cavas, Ergene-Gozukara 2003).

The present study indicated that the commercially available Reactive Red 120 dye is potentially genotoxic to fish. The study can be used as a baseline to assess its toxic effects at chromosomal and/or gene levels. The anonymity in the chemical structure, purity and other manufacturing processes makes these compounds difficult for evaluation.

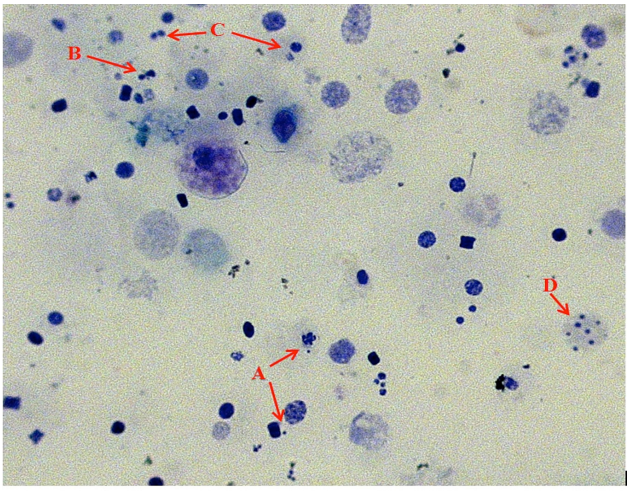

Fig. 4. Representative images of various Gills cellular abnormalities in fish *Catla catla* exposed to azo dye RR120. A, micronuclei; B, nuclear bud; C, bi-nucleated cells; D, fragmented-apoptotic cells (100×).

Nevertheless, continued usage of commercial dyes with such ambiguous molecular toxicity can cause impact to aquatic system and hence invites attention.

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