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Fish eco-genotoxicology: Comet and micronucleus assay in fish erythrocytes as in situ biomarker of freshwater pollution

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ABSTRACT

Owing to white meat production *Labeo rohita* have vast economic importance, but its population has been reduced drastically in River Chenab due to pollution. Atomic absorption spectrophotometry showed a merciless toxicity level of Cd, Cu, Mn, Zn, Pb, Cr, Sn and Hg. Comet assay results indicated significant ($p < .05$) DNA fragmentation in *Labeo rohita* as 42.21 ± 2.06%, 31.26 ± 2.41% and 21.84 ± 2.21% DNA in comet tail, tail moment as 17.71 ± 1.79, 10.30 ± 1.78 and 7.81 ± 1.56, olive moment as 13.58 ± 1.306, 8.10 ± 1.04 and 5.88 ± 0.06, respectively, from three different polluted sites on the river. Micronucleus assay showed similar findings of single micronucleus induction (MN) as 50.00 ± 6.30‰, double MN 14.40 ± 2.56‰, while nuclear abnormalities (NA) were found as 150.00 ± 2.92‰. These higher frequencies of MN induction and NA were found to be the cause of reduction of 96% of the population of this fish species in an experimental area of the River Chenab. This fish species has been found near extinction through the length of the river Chenab and few specimens in rainy seasons if restored by flood, may die in sugarcane mill season. Due to sweeping extinction *Labeo rohita* showed the highest sensitivity for pollution and could be used as bioindicator and DNA fragmentation in this column feeder fish species as a biomarker of the pollution load in freshwater bodies.

1. Introduction

Waste disposal from industry and urban structures in Asian rivers has resulted in deposition of a variety of new toxic chemicals and organic compounds. Such activities have endangered the existence of ecosystems and their inhabitants. Changes in genome caused by genotoxic agents led to mutations and pose a burden to the populations of fish species. Toxicants those induce genetic damage involve everlasting monitoring and before time detection (Villela et al., 2006). The unremitting input of toxicants into the freshwater bodies has led to the advancement in techniques for evaluation and monitoring the fate of such ecosystems (Rand, 1995). Fishes are marvelous model animals for genotoxicological studies and provide early warnings for toxicants induced environmental alterations and degradations (Pawar, 2012). According to Harshbarger and Clark (1990) fish species may be used to estimate the possible effects of toxicants to produce carcinogenic and teratogenic effects in human.

Singh et al. (1988) founded a most economical and sensitive technique under alkaline (pH > 13) conditions for the detection of genetic damage at cellular level, the comet assay having sensitivity for detecting minimum intensity of DNA fragmentation and require a small number of blood cells per fish specimen (Tice et al., 2000). Other most promising and accepted method used for cytogenetic damage is the micronucleus (MN) assay. Measurement of cytogenetic damage by MN presented an incredibly important assay in detection of pollution stress and load in aquatic ecosystems resulting in the decline of populations of particular species (Dixon et al., 2002; Baršienė et al., 2013). Micronucleus test along with nuclear abnormalities is extensively applied method among currently available assays due to its proven suitability for fish species (Cavas and Ergene-Gozukara, 2003, Kirschbaum et al.,

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2009). The micronucleus test detects both aneugenic and clastogenic effects and have the ability to identify the genotoxicity of a wide range of toxic compounds (Heddle et al., 1991). Nuclear abnormalities (notched nuclei, blebbed, lobbed, budding, fragmenting nuclei and bi nucleated cells) are considered as high-quality indicators of cytotoxicity (Kirschbaum et al., 2009; Ayllon and Garcia-Vazquez, 2000, 2001).

Indian major carp, *Labeo rohita* is present in the river system of the Indian subcontinent and this species is also cultured in freshwater ponds (Mahboob et al., 2009). This study was aimed to find the cause of extinction of *Labeo rohita* in the experimental area of the river and to adapt these assays to *Labeo rohita* blood to prove this column feeder species as a reliable indicator of freshwater pollution load and habitat degradation.

2. Materials and methods

2.1. Study area

River Chenab receives vast amount of toxic industrial and domestic wastes disposed (31.570°N & 72.534°E Bhawana, Faisalabad, Punjab, Pakistan) by Chakbandi Main Drain (Fig. 1). This waste water holds genotoxic and cytotoxic chemicals from a variety of industries situated in Faisalabad city and is well sufficient for disparaging change in water productivity by changing the physicochemical parameters of River Chenab. This habitat degradation has resulted in retarded growth of aquatic organisms, including fish species like *Labeo rohita*. 170 km stretch of the river was selected for the estimation of pollution at downstream Chakbandi Main Drain. For this purpose, water analysis and fishing were performed from three experimental sites (R1, R2 and R3) along the river. Two sites U1 and U2 upstream Chakbandi Main Drain was selected as a control and samples were polled and designated as U.

2.2. Sampling of fish species

Specimens of *Labeo rohita* were collected by using gill nets and drag nets from highly polluted water of the River Chenab from the area of Thatta Muhammad Shah (Site R1), Bela Reta (Site R2), and Bandimahni Beg (Site R3). Sampling campaigns were performed though out the year twice in a month. Farmed fish were also used as a reference for wild (polluted) and wild (non-polluted) for the estimation of genotoxicity. Farmed fish was collected from the Fish Seed Hatchery, Faisalabad and divided into two groups. One group of farmed fish was treated with colchicine and designated as "positive control" and untreated group as a control (negative control). The weight of the fish specimens collected from each point ranged from 800 to 1150 g. Fish blood (2cc) was collected just after catch from the caudal vein near the ventral fin of each specimen in heparinized tubes. After bleeding each wild fish was released to the river. Four years were spent collecting data regarding ecogenotoxicology and population dynamics.

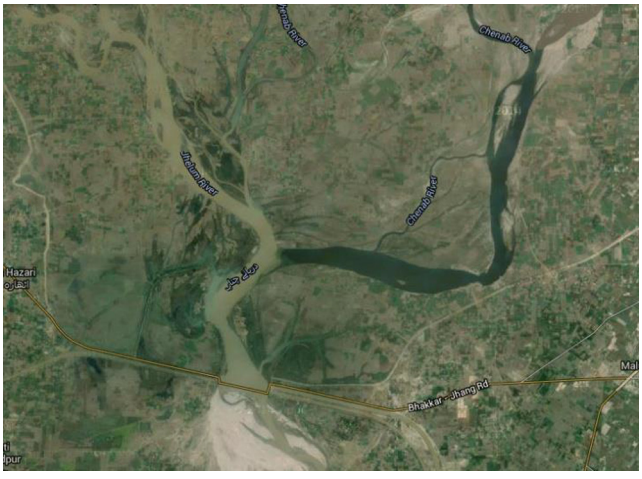

Fig. 1. Joining of the River Jhelum (left) and the River Chenab (right) at Head Trimu Jhang (31.5676°N, 72.6565°E). There is clear difference in the water of both rivers. The dark black color of the River Chenab is due to the polluted industrial and sewage wastes (Google map source).

2.3. Water analysis

River and the drain water samples were collected in polypropylene bottles and analyzed for selected heavy metals (Sb, Pb, Cr, Mn, Zn, Cd, Cu and Hg) and other water quality parameters (Boyd, 1981). The concentration of each metal was detected by heavy metal kits (Merck) and atomic absorption spectrophotometry (APHA, 1998).

2.4. Comet assay

Two µl of fresh blood was spread and sandwich between two layers, one of low melting agarose (0.5%) and other layer of normal melting agarose (0.6%) on frosted microscopic slides. The gel was then polymerized on ice. After solidification of agarose slides were dipped in lysis buffer (100 mM Na_2EDTA, 10 mM Tris-HCl, 2.5 M NaCl, 1% sodium sarcosinate, 1% Triton X-100 and 10% Dimethyl Sulphoxide) for one hour at 4 °C. For DNA unwinding slides were placed in the electrophoresis buffer (pH 10, 1 mM Na_2EDTA and 0.3 M NaOH) for 20 min and then placed for electrophoresis (20 V and 300 mA) for 30 min. Slides were then placed in Tris-HCl buffer at 25 °C for neutralization. Slides were stained with ethidium bromide (10%) and visualized by fluorescent microscopy (Dhawan et al., 2009).

2.5. Micronucleus assay

Fish blood was smeared on clean and oven dried microscopic slides. These blood smear slides were air dried at 25 °C for two hours and then fixed in cold Corney's fixative for five minutes and were again fixed in methanol for ten minutes and left to air dry at 25 °C for 1 h. Slides were stained for 30 min in 10% aqueous Giemsa and washed in double distilled water and again let them air dry. 35 fish specimens were analyzed for each experimental site for a total of 35,000 erythrocytes/fish sample. For positive control, blood from the farmed specimens was subjected to colchicine treatment. For each fish specimen five slides were prepared. The frequencies of micronucleus induction in erythrocytes were scored at T1200x magnification. Erythrocytes in fish blood with intact nuclear abnormalities were also scored by following protocol adopted by Alink et al. (2007) and Obiakoret al. (2010).

2.6. Statistical analysis

Data were statistically analyzed by the one-way analysis of variance while variance was considered significant at $P < .05$. The results represent mean along with standard error. Duncan's multiple range test was used to compare the means. Statistical analyses were executed by using the program SPSS 9 for the PC. Image analyses for DNA damages were performed by using TriTek Comet Score™ Freeware 1.6.1.13.

3. Results

Water quality parameters (WQPs) analyzed in this study proved the acute level of toxicity and high pollution load in this section of

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