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# Note

# Use of comet assay for genotoxicity assessment in fishes from Gomti River

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## ABSTRACT

An increasing number of genotoxic chemicals are being released into the aquatic environment, posing serious damage to our rich aquatic biodiversity and also indirectly to human health. In view of the above, attempts have been made to explore the feasibility of application of comet assay for biomonitoring of fish from polluted sites of River Gomti in Lucknow (Uttar Pradesh), which has been exposed to heavy pollution during the recent years. For comet assay, blood samples of two fish species found in the river namely *Labeo rohita* and *Puntius puntius*, were tested. Blood samples from fishes of the same species collected from the fish ponds of the National Bureau of Fish Genetic Resources (NBFGR), Lucknow were taken as control for comparison. The samples were then subjected to comet assay for assessing DNA damage. Significant differences were observed with respect to the two important comet parameters *viz.*, percentage tail DNA and tail length as compared to control. The results indicated the sensitivity and suitability of comet assay for '*in situ*' biomonitoring programmes to explore genotoxicity of environmental pollutants.

Aquatic exosystems are subjected to low-level and long-term exposure of increasing number of new chemicals released continuously (Maccubin and Earsing, 1990; Folmer *et al*., 1993). Many of these chemicals have the ability to interact with DNA and can lead to neoplasms (De Flora *et al*., 1991; Bhaskaran *et al*., 1999), gene mutations (Maccubin *et al*., 1991) or genetic disease syndromes (Kurelec, 1993) in the aquatic organisms, particularly fishes. Aquatic pollution may eventually affect human health too, through consumption of contaminated fishes, besides drinking water directly.

River Gomti, with a stretch of about 940 km from Pilibhit to Jaunpur, is one of the major rivers of Uttar Pradesh (UP). The riverine ecosystem is seriously exposed to pollution due to industrialization and urbanization during the past few decades (Singh *et al*., 2005a, b). The unabated pollution over the years has also adversely affected aquatic organisms, especially fishes. Recent ecological survey, conducted by the National Buraeu of Fish Genetic Resources (NBFGR), revealed that about 20 fish species of the river are threatened (personal communication, 2003), which might be due to the effect of high pollution levels in the river.

Fish serve as important biological indicators of water quality and can highlight the potential dangers of new chemicals introduced in the aquatic environment (Powers, 1989; Bailey *et al.*, 1992). They also respond to toxicants in a manner similar to higher vertebrates (Al-Sabti and Metcalfe, 1995) and can play different roles in the trophic web such as, bioaccumulators of environmental pollutants and biotransformers of xenobiotics through cytochrome P450-dependent oxidative metabolism, besides responding to mutagens at low concentrations (Goksoyr *et al*., 1991). Further, DNA repair has been reported to be slower in fishes than in mammals (Espina and Wesis, 1995). Therefore, they can be used as sentinel organisms for genotoxic studies (Landolt and Kocan, 1983).

The genotoxic effects of environmental pollutants can be monitored using a broad range of both *in vitro* and *in vivo* biomarker assays (Landolt and Kocan, 1983). Comet assay is gaining popularity owing to its sensitivity for detecting low levels of DNA damage [0.1 DNA break per $10^9$ Da (Gedik *et al*., 1992)], the requirement of small number of cells per sample, flexibility, low cost, ease of application and the short time needed to complete a study.

In view of the above, an attempt has been made to explore the utility of comet assay in detection of genotoxicity in fish of polluted sites of River Gomti at Lucknow and its further use as a suitable biomarker for environmental biomonitoring using fish as bioindicators.

For the present study, one species of Indian major carp namely, *Labeo rohita* and a minor carp species, *Puntius puntius* were selected to investigate the genotoxic effects

of pollutants in Gomti river. Four fish specimens each of *L. rohita* and *P. puntius* were collected from the polluted sites of River Gomti in the heart of Lucknow city, with the help of local fishermen. The fish specimens measured 10-12 cm in length and 12-15 g in weight. The blood samples were collected aseptically from the caudal veins in heparinzed syringes and were transported to the laboratory in an ice box and processed immediately for comet assay. For comparison, blood samples were collected from the fishes of the same species maintained in the NBFGR fish ponds and used as control. Hydrogen peroxide (35.2 µg) was used as positive control for comet assay by exposing the blood cells *in vitro* (Hellman *et al.*, 1999; Kammann *et al.*, 2000). The water samples were also collected from River Gomti for estimating the concentration of two heavy metals, *viz*., cadmium and chromium, and two pesticides *viz*., endosulfan and malathion.

Before processing for comet assay, cell viability test was carried for all the blood samples using trypan blue exclusion method (Anderson *et al.*, 1994; Henderson *et al*., 1998). The percentage of viability was calculated as number of unstained cells x 100 / total number of cells counted.

The blood samples were processed for single cell gel electrophoresis or comet assay, as per the method suggested by Singh *et al*. (1988), with minor modifications. In brief, about 15 µL of cell suspension (approx. 20,000 cells) was mixed with 80 µl of 0.5% low melting point agarose and the mixture was layered on slides (one end frosted), previously coated with a layer of 200 µl normal agarose (1%). Finally the slides were coated with a third layer of 100µl low melting point agarose. After solidification of gel, the slides were immersed in lysing solution [2.5 M NaCl, 100m M $Na_2$ – EDTA, 10 mM Tris, pH 10, with 10 % DMSO and 1% Triton X- 100 added fresh] for 1 h at 4 ºC for cell lysis. After lysis, slides were placed in a horizontal gel electrophoresis tank containing fresh alkaline electrophoresis buffer [300 mM NAOH, 1 mM $Na_2$ – EDTA, pH 13.5] and left in the solution for 20 min. at 4ºC for DNA unwinding and conversion of alkali – labile sites to single strand breaks. Electrophoresis of blood cells was conducted at 4ºC for 15 min. using 25 V (0.8 V/cm) and 300 mA. After electrophoresis, the slides were neutralized gently with 0.4 M Tris buffer at pH 7.5 followed by staining with ethidium bromide (20 µg/ml).

Comet cells were evaluated to assess the extent of DNA damage due to single strand breakages of DNA molecules resulting in a "comet with tail" formation during electrophoresis. The slides were scored for DNA damage by an image analysis system (Kinetic Imaging) attached to a fluorescence microscope equipped with appropriate filters. DNA damage of blood cells was quantified by two important comet parameters *viz.* percentage tail DNA and tail length. Two slides per specimen were prepared and 25 cells per slide were scored and thus a total of 200 cells per species were scored for estimation of % tail DNA and tail length.

One-way analysis of variance (ANOVA) was used to compare the mean differences in % tail DNA and tail length between specimens from control and Gomti River samples.

The analysis of river water samples revealed presence of mercury (1.19 ppb), cadmium (3.00 ppb) and endosulfan (0.006 ppb), however, organophosphate pesticide malathion was not detected.

In both the species collected from Gomti River as well as in control specimens, viability of the whole blood cells observed by trypan blue exclusion method exceeded 95% and hence all the blood samples were found suitable for performing comet assay. In *L. rohita*, a significantly higher ($p<0.01$) extent of DNA damage was observed in the blood cells of fishes from Gomti River as compared to the control group. The values of % tail DNA and tail length in Gomti River fishes were 24.71% and 66.56 µm as compared to 10.18% and 22.26 µm in control specimens, respectively (Table 1, Fig. 1 and 2). In *P. puntius*, similar findings were observed with estimated % tail DNA and tail length values of 20.59±0.61 and 59.63±1.42, respectively, in specimens from River Gomti, which was significantly ($p<0.01$) higher than the control (Table 1, Fig. 1 and 2).

Species-specific variations have been observed between *L. rohita* and *P. puntius* for percentage tail DNA and tail length, which were significantly higher ($p<0.01$) in former as compared to the latter (Table 1 and Fig.1). Thus, the comparison of comet parameters between two species from Gomti River indicated increased susceptibility of *L. rohita* to pollutants in the river as compared to *P. puntius*.

Among the various techniques so far used to assess the genotoxicity of environmental pollutants, the comet assay has been reported to be sensitive, rapid and economic to evaluate DNA damage (as single strand breaks) and repair

Table 1. Extent of DNA damage in erythrocytes (N= 200 cells) of *L. rohita* and *P. puntius* measured by % Tail DNA (± S.E).

| Species | % Tail DNA | | Tail length (ìm) | |
|---|---|---|---|---|
| | Control | River Gomti | Control | River Gomti |
| *Labeo rohita* | $10.18\pm0.959^{a}$ | $24.71\pm0.744^{b1}$ | $22.66\pm1.893^{a}$ | $66.56\pm0.775^{b1}$ |
| *Puntius puntius* | $8.60\pm0.749^{a}$ | $20.59\pm0.616^{b2}$ | $36.27\pm2.650^{a}$ | $59.63\pm1.415^{b2}$ |

Values with different superscripts differ significantly ($p<0.01$) between control and river specimens within species. Values with different numeric differ significantly ($p<0.01$) between species within river specimens.

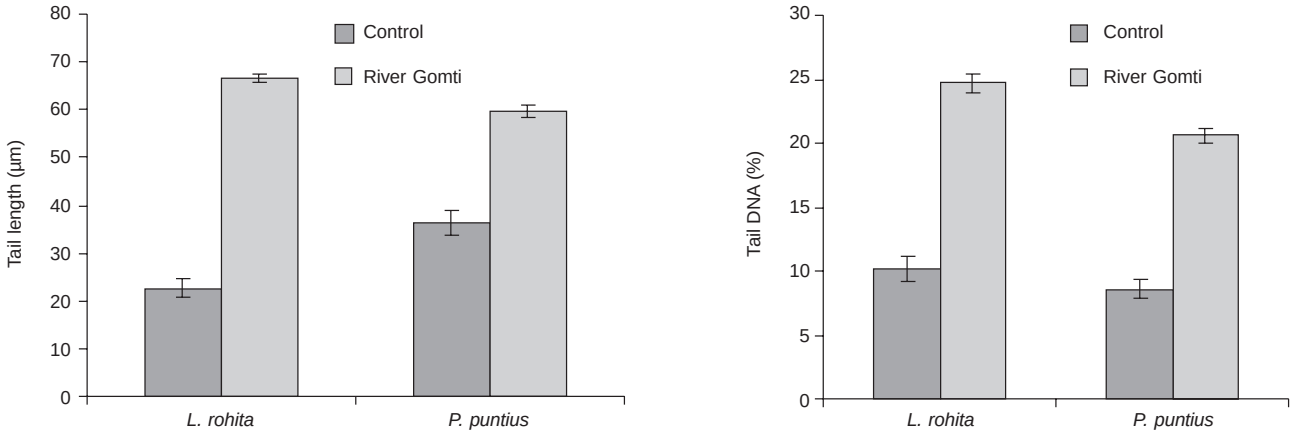


Fig. 1. Extent of DNA damage in blood cells of fishes.

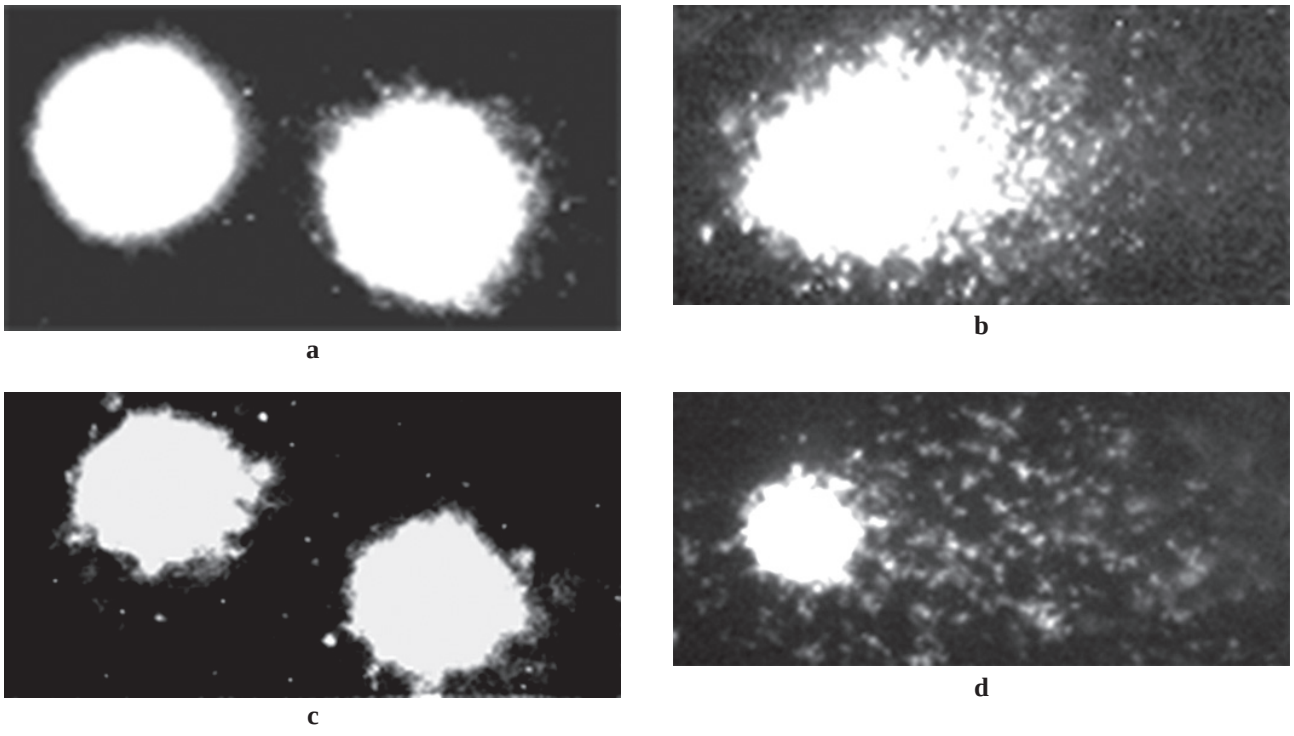


Fig. 2. Comet images of fish erythrocytes:

(a) Control cell of *L. rohita* (b) DNA damaged cell of *L. rohita* (c) Control cell of *P. puntius* (d) DNA damaged cell of *P. puntius*

in both proliferating and non-proliferating cells (Rojas *et al.*, 1999). Pandrangi *et al.* (1995), using comet assay, reported a greater extent of DNA damage in erythrocytes of bullheads (*Ameius nebulosus*) and carp (*Cyprinus carpio)*, collected from seven different sites, as compared to control. Similarly, Rajaguru *et al*. (2003) also measured the genotoxicity of a polluted river using comet assay in erythrocytes of *C. carpio*. Further, as comet assay does not depend on chromosomal characteristics or cell division and since the effect of genotoxic agents are often tissue and cell type specific, it can be ideally suited as a non-specific biomarker of genotoxicity in fish and other aquatic species (Pandrangi *et al*., 1995). The present study has indicated the suitability of comet assay in the field of environmental biomonitoring using fish as a model system and offers a rapid screening system for '*in situ*' biomonitoring programmes to explore genotoxic potential of environmental contaminants.

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