Micronucleus Test in Erythrocytes of *Cyprinus carpio*: A Sensitive Monitor for Aquatic Pollution

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Abstract
Micronuclei test is a system of mutagencity testing used for determining the pollution and chemicals causing changes in DNA fragments such as micronuclei in the cytoplasm of interphase cells. Damage caused on the DNA by genotoxic pollutants is the first consequence occurring in the aquatic organisms. The aquatic environment makes up the major part of our environment and resources, therefore its safety is directly related to the safety our health, thus, it was attempted to determine whether pollution affected the erythrocytes of fish *Cyprinus carpio* at the level of DNA by the means of micronuclei (MN) test. The test has been used successfully as a mutagenic assay. It is simple, reliable, sensitive, and it does not depend on any karyotypic characteristics. Fish were collected from locations that display differential environmental stresses. Organisms used in the MN test were collected from Dal Lake and Mansbal Lake. According to the results of the present study, frequency of MN was found higher in fishes from Dal water compared to Mansbal Lake. In conclusion, this study indicates that the micronuclei test gives sensitive results in monitoring the pollution, and thus it might be used as standard method in regular monitoring of pollution of water bodies.
Keywords: Fish, mutagenicity, micronucleus assay, erythrocyte

Introduction

Ecosystems are undergoing unprecedented alterations in their indices due to climate changes, biogeochemical cycles, and changes in land use and hydrology (Bogoni *et al.*, 2014). Metabolic processes in most aquatic environments are supported by debris and land materials contributing to the energetic stability and production of organisms. Thus, understanding changes in the dynamics of the matter transported to the riverbeds is particularly important to understand several processes (Kominoski and Rosemond, 2012). The contamination of surface waters, containing known and unknown compounds, could pose a serious public health and aquatic ecosystem threat (Claxton *et al.*, 1998). This increased the interest in studies for the evaluation of polluted water genotoxicity. In recent years several studies have evaluated the impact of agricultural and industrial effluents on river waters using different assays (Lemos and Erdtmann, 2000; Vargas *et al.*, 2001; Vigano *et al.*, 2002; Tagliari *et al.*, 2004; Ohe *et al.*, 2004; Ergene *et al.*, 2007 and Lemos *et al.*, 2007). Anthropogenic activities as sources of increased toxic substance content in aquatic systems are now common in Kashmir.

Aquatic biota is constantly exposed to great number of toxic substances during their lifespan both from the water and through aquatic food chain (Dar *et al.*, 2015). Studies reveal the fact that a number of chemicals contaminating the environment have carcinogenic or mutagenic effects. The major sources for the mutagenic and carcinogenic substances are industrial and agricultural activities (Bogoni *et al.*, 2014). Xenobiotics from these sources ultimately contacts the aquatic ecosystems. Although many hazardous substances exist in the water and sediment and they are accumulated by aquatic organisms and triggers DNA or cellular damage and even affects the ecosystem by passing through the tropic chain (Izquierdo *et al.*, 2003).

In recent years increasing concern about genotoxic pollution in water bodies has led to the development of many different mutagenesis test systems. Water and sediment samples can be tested for mutagencity under laboratory conditions using biological systems such as bacteria, yeast and plants (Minissi *et al.*, 1996 and Ergene *et al.*, 2007). The use of fish as bio-indicators of pollutant effects is being more and more used since fishes are very

sensitive to changes in their environment and play significant roles in assessing potential risk associated with contamination in aquatic environment of new chemicals (Dar *et al.*, 2015 and Nwani *et al.*, 2010); they are frequently used test organisms for studying cytotoxicity, water toxicity and genotoxicity. For example, at cellular level the micronucleus test on various fish tissues is among the most widespread assessments of genotoxicity in water (Ansari *et al.*, 2011 and Arkhipcuk and Garanko, 2005). Micronuclei (MN) test is one of the most reliable techniques used to determine genetic changes in the organisms. MN experiments is a fast method in detecting the chromosomal damage because it makes possible to determine the remaining chromosomes and broken chromosomes due to its several advantages such as (a) giving more objective results than other tests in detecting chromosomal impairments, (b) being easy to learn, (c) it does not require to count the chromosomes to investigate the chromatids and chromosomal damage hard to detect and see in the metaphase stage, (d) its preparation stage is fast and (e) it makes it possible to count thousands of cells, not hundreds of cells in each experiment (OECD, 2014). For all these reasons, the micronucleus test in fish erythrocytes seems to be promising test in environmental mutagenesis investigations (Al-sabti and Metcalfe, 1995).

In the present paper, micronucleus frequency in fish erythrocytes has been evaluated in *Cyprinus carpio* from fresh water environment characterized by different pollution levels, and compared with the values observed in erythrocytes of fish collected from less polluted water body. The aim of the present study was to validate the sensitivity of this test system and the suitability of bioindicators in environmental monitoring. As indicator species, *Cyprinus carpio* was chosen because of its ecology, wide distribution in fresh water environment of Kashmir, availability throughout the seasons, easy acclimatization in the laboratory conditions and commercial importance make this species as an excellent test specimen for geno-toxicological studies by comparing among different lakes. These considerations have prompted interest in the development of such techniques and its use as bioindicators for monitoring the genomic damage from environmentally hazardous contaminants in the aquatic environment.

Materials and Methods

Collection sites and sampling

The valley of Kashmir is situated in the middle of the Himalayas between the northwest and southeast (33°01′–35°00′N latitude and 73°48′–75°30′ E longitude) at an altitude ≥1500 m above sea level. The study was carried out in Dal Lake (34°07′ N, 74°52′ E), and Mansbal Lake (34°15′ N, 74°40′E). The Dal Lake is an urban lake that lies to the east of Srinagar city, at the foot of Zabarwan Hills, and is situated at an average elevation of 1,583 m (5,194 ft) above sea level with a maximum depth of 6 m (20 ft). Mansbal Lake is a rural lake situated at a distance of 32 km from Srinagar city. Its length and breadth are approximately 3.2 and 1 km, respectively. The lake is situated at the altitudinal zone of 1,585-1,600 m (5,200-5,200 ft) with a maximum depth of 13 m (43 ft). Recent studies have attested that the Dal lake has reached to the level of eutrophic condition, but the level of trophic state varies, with Dal lake being the most eutrophic and Manasbal Lake being the least nutrient enriched (Zargar *et al.*, 2012). *Cyprinus carpio* L. (Family: Cyprinidae and Order: Cypriniformes), was selected as the test organism. Live juvenile specimen, procured with the help of cast net from both the sites, were transported to the laboratory and subjected to a prophylactic treatment by bathing in 0.05% potassium permanganate ($KMnO_4$) for 2 min to avoid any dermal infection. Their average length and wet weight (±SD) were recorded as 18.12±0.62 cm and 77±6.782 g, respectively for Dal lake and 17.62±1.70 cm and 66.25±17.97 g, for Mansbal Lake.

Experimental procedures

Two main experiments were carried out. In the first one, blood samples were directly collected from caught fish. In the second experiment, fish were acclimated for a week in 60 L glass aquaria with well aerated water at 20°C. The specimen maintained in dechlorinated tap water and then exposed to Ethyl methanesulfonate (5 mg/L, concentration selection based on previous investigation; Cavas and Konen, 2008) were considered as the positive controls.

Micronucleus test

Peripheral blood samples were obtained by caudal vein puncture using heparinised syringe. Blood was immediately smeared on clean, grease free frosted glass slides. Slides were fixed in methanol for 10 min and left to air dry at room temperature and finally stained with 6% geimsa in Sorenson buffer(pH 6.9) for 20 min. MN were

identified and scored microscopically under 100 X in an Olympus microscope. Two thousand and five hundred erythrocytes were scored for each specimen to determine the frequency of micro nucleated erythrocytes. Slides were scored by a single observer using blind review. For MN scoring purpose, only non refractive small nuclei (>1/3 of the main nucleus) located close to the main oval nucleus of round erythrocytes with intact cytoplasm were considered (Schmid, 1975; Das and Nanda, 1986 and Ali *et al.*, 2009).To avoid intra specific differences related to fish size for each species, only adult specimens with similar sizes were sampled.

Results and Discussion

The obtained results are summarized in Tables 1 & 2 and Figure 1. Results reveal that the fish represent various degrees of sensitivity in monitoring genetic damage (especially clastogenic effect). The present results indicate that micronuclei frequencies were low in fish from Mansbal compared to that of Dal Lake. In the positive control experiments, the frequency of micro nucleated erythrocytes from fish treated with EMS was significantly higher in *C.carpio*.

This is indicated by variations in averages of the micronucleated cells among species at different locations. As previously mentioned by different limnologists these locations display differential environmental stress. These lakes were categorized according to approved international standards (OECD, 1982). As the valley is devoid of chemical factories nutrient input from domestic sewage define the main source of pollution. The marked difference in the water quality of the two lakes clearly depicts the influence of anthropogenic stresses on the lakes. These results are also in confirmation with the loading concept and support the findings of Pandit and Yousuf (2002).

Table 1: Length and weight of fishes collected at monitoring sites and scored for micronuclei

Sites	Length(cm) (mean±S.D)	Weight(g) (mean±S.D)
Dal	18.12±0.62	77±6.782
Mansbal	17.62±1.70	66.25±17.97

Table 2: Mean micronuclei counts for *C. carpio* from the two monitoring sites.

Sampling locations	No. of fishes observed	No. of cells observed (*NEA*)	MN frequencies (%)± SE	PC
Dal Lake	5	12500	0.15±0.007	0.14±0.08
Mansbal Lake	5	12500	0.08±0.06	0.21±0.08

NEA No. of erythrocytes analyzed; PC positive control

The contamination of aquatic environments poses serious consequences for the welfare of the organisms exposed because pollutants may induce mutations and cancer (Beyersmann and Hartwig, 2008). Chromosomal mutation is an important occurrence in carcinogenesis (Fenech, 2000). The presence of micronuclei represents a parameter for determining the extent of damage caused by an environmental agent to the process of cell division of the affected tissue (Vine, 1990) and reveals threats that cannot be detected through chemical or physical analyses. Cytogenetic methods are probably the most sensitive and effective means by which to detect the effects of genotoxins (Bogoni *et al.*, 2014).

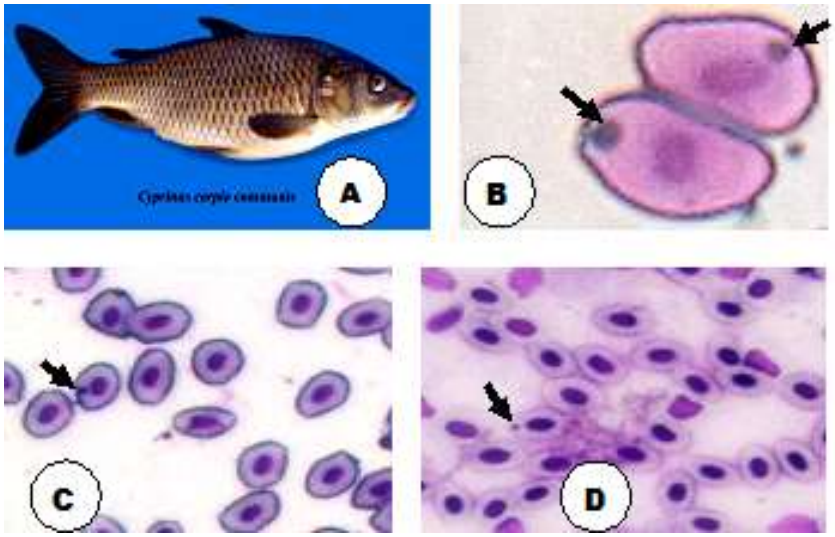

Figure 1: Photomicrograph showing *C.carpio* **(A)** and micronucleated erythrocyte **(B, C & D).**

Fish serve as sentinel organism for ecotoxicological studies because they play a number of roles in tropic web, accumulate toxic substances and respond to low concentration of mutagens. 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 (Nwani *et al.*,2010). Villela *et al.* (2006) and Al-Sabti and Metcalfe (1995) showed that fish are excellent subjects for the study of the mutagenic and/or carcinogenic potential of contaminants present in water samples since they can metabolize, concentrate and store waterborne pollutants. The sampling of peripheral blood is appropriate and sufficient for biomonitoring projects. Lyne *et al.* (1992) and Cesar and Célia (2000) demonstrate that *Tilapia rendalli* and *Oreochromis niloticus*, give different responses to genotoxic agents. According to clastogen and the species studied, the frequency of micronuclei may suffer important variations. Time-dependent responses have also been observed in amphibians exposed to radiation. Siboulet *et al.* (1984), Fernandez *et al.* (1993) and Ali *et al.* (2009) confirmed that the micronuclei frequencies may vary according to the season, the kind of pollution involved and the species of fish. It was concluded from these studies that fish can be used for estimating the genotoxic effects of water-borne pollutants.

Therefore, based on the results obtained, the main finding is that under the conditions and period of this study, the waters of the Dal lake induced genetic damage in erythrocytes of *C.carpio* because the frequency of MN was greater compared with the that of the Mansbal. Different references showed that MN can be affected by many factors such as age, sickness, species, feeding, chemical and physical agents and environmental conditions (Al-Sabti and Metcalfe, 1995; Saleh and Zeytinoglu, 2001). So to eliminate these factors, healthy, young and Active individual had been chosen from the same species, *C. carpio*.

The obtained results support the fact demonstrated by Kligerman (1982) that fish inhabi ting polluted waters have greater frequencies of micronuclei. The most remarkable result is that MN frequency appears to be strongly related to water quality of the different environments examined. The relationship between MN frequency and pollution levels observed in fresh water fish reflects what already observed by different authors in marine fish from coastal areas(Hose 1994), beside being in accordance with that observed by means of in situ exposure of rainbow trout to polluted riverine waters (Deflora *et al.*,1993). The presence of different pollutant in the waters of Dal Lake was evidenced by Zargar *et al.* (2012). On the contrary, the low MN frequencies observed in the Mansbal Lake lead to the conclusion that in this lake genotoxic agents are not detectable.

Discharging very low concentrations of chemicals, such as PAHs and trace metals (e.g., Cu, Zn, Pb, Mn, and Fe), into the environment may affect all levels of biological organization, from the molecule to the ecosystem. The duration of time from the moment of introduction of a contaminant into the environment until the very first

(harmful) physiological effects on the biota may vary between hours to decades (Everaarts *et al.*, 1998). Genotoxic chemicals may cause somatic and heritable mutations. Somatic mutations cause cellular damage that can ultimately result in disease. Such stress can reduce viability, survivability, and reproductive success. Heritable effects, such as deleterious germ-line mutations, also produce these effects. Subsequently, the genetic makeup of populations might be altered by the reduction of genetic variability, the increase of deleterious alleles, or the fixation of low-frequency alleles as the population becomes adapted to new environmental conditions (Bickham, 2000).

The results of the present investigation on the genotoxic potential of the polluted water of Dal lake suggested a serious concern about its potential danger to aquatic organisms, especially to fish, and indirectly to human beings. However, further studies are needed to explore the biological consequences of DNA damage in aquatic organisms due to deleterious effects of polluted water of Dal lake and to formulate future strategies for safeguarding aquatic organisms and environment.

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