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DETERMINATION OF ARSENIC INDUCED NUCLEAR ABNORMALITIES IN PERIPHERAL BLOOD ERYTHROCYTES OF CARPS USING MICRONUCLEUS TEST

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ABSTRACT

Genotoxic effects of arsenic, in-terms micronuclei (MN) frequency and frequency of other nuclear abnormalities (ONA), on four carp species (*Labeo rohita*, *Cirrhina mrigala*, *Catla catla* and *Ctenopharyngodon idella*) were studied at constant water temperature (30 ºC), pH (7.5) and total hardness (300 mgL^{-1}). Acute toxicity of arsenic in-terms of 96 hr LC_{50} and lethal concentration for fish was determined and then all fish species were exposed to four sub-lethal (17, 25, 33 and 50% of 96 hr LC_{50}) concentrations of arsenic, separately, for a period of 30 days. As the fish blood erythrocytes are nucleated therefore, selected for determination of genotoxic effects of arsenic. After metal exposure, frequency of (MN) and ONA were observed. All the four fish species showed significantly variable sensitivity to arsenic. Among four fish species, *Cirrhina mrigala* showed significantly higher micronuclei frequency with the mean value of 37.59 ± 12.49%, followed by that of *Labeo rohita*, *Catla catla* and *Ctenopharyngodon idella*. Mean frequency of other nuclear abnormalities was significantly higher in erythrocytes of *Cirrhina mrigala*, followed by that of *Catla catla*, *Ctenopharyngodon idella* and *Labeo rohita* with the mean values of 27.68 ± 10.22, 17.52 ± 11.02, 15.54 ± 4.40 and 11.36 ± 5.73%, respectively.

Key words: Fish, Arsenic, Acute toxicity, Micronuclei, Other nuclear abnormalities, Peripheral blood erythrocytes.

INTRODUCTION

Contamination of aquatic ecosystem due to metals is a universal issue of high magnitude as these metals have severe toxic effects on aquatic organisms (Raposo *et al*., 2009; Kousar and Javed, 2014). Continuous exposure of fish to higher concentrations of metals can induce various health hazards (Javed, 2012; Naz and Javed, 2013). Metals existing in aquatic habitats of Pakistan (Rauf *et al*., 2009) gained attention because of their toxicity, accumulation (Javed, 2004) and other toxic impacts on freshwater fish and consequently on physiological health of human beings (Mudgal *et al*., 2010). To determine the toxicity of metals, various tests have been developed. Among them, micronucleus test is considered as standard method to assess the clastogenic effects of toxic metals on fish blood (Obe *et al*., 2002). It is simple, very sensitive and most reliable test widely used to examine the biological impacts of aquatic pollutants (Bombail *et al*., 2001). Study of micronucleus formation and sister chromatid exchange is very helpful for the detection of genotoxicants in the aquatic environments. Micronuclei, found alongwith normal nuclei, are smaller than the normal nuclei that are created during anaphase due to lagging chromosomes (Parveen and Shadab, 2012). Binuclei are formed as a result of abnormal cell division. After karyokinesis the process of cytokinesis is blocked that resulted in the formation of binuclei. Such abnormal cell divisions are considered to result in genetic imbalance in cells leading to carcinogenesis (Cavas and Ergen, 2005).

Bio-monitoring is a potential way for the detection of toxicants that have adverse effects on environment as well as human health also (De Silva *et al*., 2003). Being a vertebrate model, fish is accessible to detect the major threats of genotoxicants (Diekmann *et al*., 2004). Moreover, fish blood erythrocytes are the most suitable for DNA damage analyses since peripheral blood shows the comprehensive health status of the organism. Concerning this problem, blood cells in fish have attained huge attention as their erythrocytes are nucleated and, therefore, most suitable for the study of nuclear abnormalities in blood (Costa *et al*., 2011).

Pakistan is among the countries where only 1% of industrial water is treated before its discharge into natural water bodies thus facing acute fresh water pollution problems (Khan *et al*., 2012). Main reason of aquatic pollution is heavy discharge of untreated waste water into the rivers and streams of Pakistan (Kousar and Javed, 2015) that adversely affect the fresh water fisheries (Jabeen and Javed, 2012). The indigenous fish species, in particular, are on the verge of extension in the rivers of Pakistan due to metal ion pollution problem (Rauf *et al*., 2009). Comparative toxicity studies are needed to identify the effects of metals pollution on the fish and their tolerance limits to devise proper strategies for their conservation in the natural habitats. In addition to that it is crucial to study the chronic effects of arsenic

on fish DNA. This work will help sustainable conservation of fresh water fisheries in Pakistan.

MATERIALS AND METHODS

In another study (phase I), 96-hr LC_{50} and lethal concentration of arsenic for *Labeo rohita*, *Cirrhina mrigala*, *Catla catla* and *Ctenopharyngodon idella* was determined (Kousar and Javed, 2014).

During phase II, experiments were conducted to determine the frequency of MN and ONA in blood erythrocytes of fish, exposed to four different sub-lethal concentrations of arsenic. For this purpose experiments were conducted with 150-day old fish (*Labeo rohita*, *Cirrhina mrigala*, *Catla catla* and *Ctenopharyngodon idella)* in glass aquaria under controlled laboratory conditions. The 96-hr LC_{50} value determined in previous experiment (Kousar and Javed, 2014) was used to obtain the four different sub-lethal concentrations viz. 17%, 25%, 33% and 50% 96-hr LC_{50} of arsenic. Fish were exposed to four different sub-lethal concentrations viz. 17%, 25%, 33% and 50% of 96-hr LC_{50} of arsenic in glass aquaria, separately, for 30 days. Fish (10 for each sub-lethal concentration) with following average wet weights and total lengths, after being acclimatized to laboratory conditions, were kept in glass aquaria with three replications for each treatment:

Fish Species	Average Wet Weights (g)	Average Total Lengths (mm)
Labeo rohita	14.47±0.43	110.33±2.95
Cirrhina mrigala	11.28±0.67	101.53±1.17
Catla catla	19.66±0.24	121.42±2.40
Ctenopharyngodon idella	10.58±0.33	99.78±1.63

For comparison to the treated group, fish grown in metal free tap water was used as negative control. Continuous aeration was supplied to all fish aquaria through capillary system. All toxicity trials were conducted under constant water hardness (300 mgL^{-1}), pH (7.5) and temperature (30°C). Fish were fed to satiation with feed containing ingredients fish meal, corn gluten (30 % CP), cotton seed meal, rice polish, wheat flour, oil (sun flower) and vitamin and mineral mixture (34% DP and 3.00 Kcal/g DE) twice daily throughout the experimental period. The test media were replaced weekly and toxicant concentration was maintained up to required level.

Micronucleus Test: After 30-day exposure of arsenic, blood samples were taken from caudal vein of fish. A drop of blood was smeared on clean glass slide. After the smear was air dried, fixed in methanol for 10 minutes. After fixing, Giemsa stain was used to stain the blood erythrocytes (Heddle, 1973). Duplicate slides were prepared from each arsenic concentration exposed fish species, separately, and examined for scoring nuclear changes and micronucleus frequencies under oil emersion (100 X) lens.

Scoring of cells with micronuclei and other nuclear abnormalities: Through blind scoring number of all types of nuclear abnormalities counted on coded slides. Total 2,000 blood cells (1000 / slide) with undamaged cell membrane and nuclear membranes were examined, separately, for each fish specimen. Cells including micronucleated cells, bi-nucleated cells, dumbbell shaped nuclei, blebbed, notched and de-shaped nuclei were evaluated (per 1,000 cells) by observing at a 1,000X magnification using binocular microscope (Labomed CX_{R3}).

For scoring of micronuclei, a criterion devised by Fench *et al*. (2003) was adopted. According to these criteria the diameter of micronuclei must be smaller than the 1/3rd of main nucleus. Micronuclei must have staining patterns similar to main nucleus. Micronuclei should be on same plane of focus as the main nuclei. Frequency of micronuclei was calculated by using the following formula:

$$\text{Micronucleus Frequency (\%)} = \frac{\text{Number of micronucleated cells}}{\text{Total number of cells counted}} \times 100$$

Statistical Analysis: Analysis of variance and comparison of mean values were performed to find out the significant differences among variables. To compare the frequency of MN and ONA between control as well as treated fish groups, Mann-Whitney U-test was performed. The relationships among various parameters were also established by using regression analyses (Steel *et al*., 1996).

RESULTS

Frequency of MN and ONA in peripheral blood erythrocytes of fish

***Labeo rohita*:** *Labeo rohita* exposed to four sub-lethal concentrations of arsenic exhibited significantly higher mean micronuclei frequency of 41.09 ± 0.66% at 50% arsenic LC_{50}, followed by that of 33%, 25%, positive control, 17% arsenic LC_{50} and negative control. However, the difference between positive control and 17% LC_{50} to induce micronuclei was statistically non-significant.

Frequency of binucleated cells remained significantly ($p < 0.05$) higher (2.97 ± 0.08%) at 50% LC_{50} exposure. However, positive control and 50% LC_{50} treatment showed statistically non significant difference for their ability to cause nuclear abnormality in terms of blebbed nuclei (Table 1).

***Cirrhina mrigala*:** Arsenic exposure at various sub-lethal concentrations, to *Cirrhina mrigala* induced significantly ($p < 0.05$) variable frequency of micronuclei in their peripheral erythrocytes. Significantly higher mean frequency of 50.44 ± 1.77% was recorded at 50% LC_{50} exposure while the frequencies of micronuclei varied significantly among treatments with the mean values of 45.50 ± 0.18, 30.67 ± 0.29, 23.75 ± 0.07, 13.94 ± 0.91 and 0.70 ± 0.01% at exposure concentrations of 33%, 25%, 17% LC_{50}, positive and negative controls, respectively.

The ONA in peripheral blood erythrocytes of *C. mrigalla* varied significantly ($p < 0.05$) due to various arsenic exposure concentrations, positive and negative control. The 50% of arsenic LC_{50} caused significantly higher frequency of blebbed, notched nuclei and deshaped cells while frequency of dumble shaped nuclei was significantly ($p < 0.05$) higher due to 25% of arsenic LC_{50} (Table 1).

***Catla catla*:** Arsenic exposure, at various sub-lethal concentrations, to *Catla catla* caused significantly ($p < 0.05$) variable frequency of MN. The highest micronuclei frequency of 39.28 ± 0.40% was occurred due to 50% arsenic LC_{50} exposure. Frequency of micronuclei, dumble shaped and binuclei were significantly ($p < 0.05$) higher due to 50 % arsenic LC_{50}.

Frequency of blebbed, notched nuclei and deshaped cells were significantly higher due to positive control treatment. However, frequency of MN and ONA appeared significantly ($p < 0.05$) lower in the peripheral blood erythrocytes of negative control fish (Table 1).

***Ctenopharyngodon idella*:** In *Ctenopharyngodon idella*, arsenic at 50% LC_{50} exposure caused significantly ($p < 0.05$) higher mean micronuclei frequency of 33.11 ± 0.99% while it was significantly lowest (2.26 ± 0.03%) in the erythrocytes of negative control fish. Positive control treatment caused significantly ($p < 0.05$) higher

Table 1. Frequency (% ± SD) of micronuclei and other nuclear abnormalities in peripheral blood erythrocytes of fish exposed to various concentrations of arsenic.

	-ve control	+ve control	17% of LC_{50}	25% of LC_{50}	33% of LC_{50}	50% of LC_{50}
Labeo rohita						
Micronuclei frequency (%)	4.23±0.07^{e}	12.08±0.23^{d}	11.56±0.12^{d}	17.50±0.16^{c}	31.01±0.74^{b}	41.09±0.66^{a}
Binucleated cells	0.00±0.00^{e}	2.03±0.14^{b}	0.20±0.03^{d}	1.25±0.10^{c}	1.98±0.04^{b}	2.97±0.08^{a}
dumble shape nucleus	0.53±0.02^{e}	2.22±0.44^{c}	0.25±0.01^{f}	1.40±0.05^{d}	3.56±0.02^{a}	3.66±0.05^{b}
Cells with blebbed nucleus	0.44±0.13^{e}	4.06±0.13^{a}	0.85±0.04^{d}	2.40±0.22^{c}	3.90± 0.05^{b}	4.06±0.08^{a}
Cells with notched nucleus	0.39±0.22^{d}	2.32±0.21^{b}	1.01±0.02^{c}	2.70±0.13^{a}	2.91±0.^{07a}	2.77±0.18^{a}
Deshaped cells	0.49±0.24^{d}	2.90±0.23^{ab}	1.21±0.11^{c}	3.05±0.03^{a}	2.37±0.12^{b}	2.92±0.10^{ab}
Cirrhina mrigala						
Micronuclei frequency (%)	0.70±0.01^{f}	13.94±0.91^{e}	23.75±0.07^{d}	30.67±0.29^{c}	45.50±0.18^{b}	50.44±1.77^{a}
Binucleated cells	0.00±0.00^{d}	0.89±0.04^{b}	1.50±0.12^{a}	0.89±0.07^{b}	0.00±0.00^{d}	0.44±0.13^{c}
dumble shape nucleus	0.40±0.01^{f}	2.38±0.19^{d}	1.00±0.18^{e}	7.11±0.11^{a}	3.50±0.14^{c}	4.82±0.17^{b}
Cells with blebbed nucleus	0.20±0.03^{e}	2.38±0.11^{c}	2.00±0.21^{d}	6.67±0.17^{b}	7.00±0.04^{a}	7.02±0.08^{a}
Cells with notched nucleus	0.35±0.16^{e}	3.92±0.20^{d}	4.50±0.08^{c}	5.33±0.06^{b}	4.00±0.10^{d}	12.72±0.16^{a}
Deshaped cells	0.95±0.11^{f}	8.63±0.07^{d}	10.35±0.11^{b}	5.33±0.12^{e}	9.00±0.01^{c}	17.54±0.13^{a}
Catla catla						
Micronuclei frequency (%)	0.99±0.40^{f}	15.03±0.12^{d}	6.70±0.03^{e}	17.10±0.27^{c}	29.89±0.17^{b}	39.28±0.40^{a}
Binucleated cells	0.00±0.00^{f}	2.55±0.47^{c}	0.95±0.08^{e}	1.94±0.22^{d}	2.76±0.05^{b}	3.86±0.11^{a}
dumble shape nucleus	0.15±0.02^{f}	4.82±0.05^{c}	0.75±0.03^{e}	3.23±0.15^{d}	5.05±0.34^{b}	9.89±0.06^{a}
Cells with blebbed nucleus	0.40±0.01^{f}	8.65±0.13^{a}	1.80±0.05^{e}	2.24±0.03^{d}	3.10±0.11^{c}	5.79±0.24^{b}
Cells with notched nucleus	0.30±0.00^{f}	7.27±0.42^{a}	1.20±0.10^{e}	2.78±0.08^{d}	4.90±0.07^{c}	5.16±0.21^{b}
Deshaped cells	0.84±0.03^{e}	9.78±0.22^{a}	0.85±0.12^{e}	2.83±0.11^{d}	4.22±0.31^{c}	6.76±0.08^{b}
Ctenopharyngodon idella						
Micronuclei frequency (%)	2.26±0.03^{f}	31.75±0.52^{b}	8.35±0.33^{e}	18.85±0.19^{d}	28.46±0.17^{c}	33.11±0.99^{a}
Binucleated cells	0.15±0.01^{f}	3.13±0.03^{a}	0.80±0.12^{d}	1.10±0.05^{b}	0.60±0.09^{e}	0.96±0.01^{c}
dumble shape nucleus	0.25±0.12^{e}	5.48±0.05^{a}	2.19±0.10^{c}	2.80±0.32^{b}	2.09±0.03^{d}	2.79±0.42^{b}
Cells with blebbed nucleus	0.44±0.04^{f}	3.33±0.04^{d}	2.78±0.32^{e}	3.90±0.18^{c}	4.87±0.37^{b}	5.49±0.76^{a}
Cells with notched nucleus	0.29±0.03^{f}	6.65±0.08^{a}	1.89±0.02^{e}	2.50±0.05^{d}	3.48±0.22^{c}	4.81±0.20^{b}
Deshaped cells	0.74±0.01^{f}	4.35±0.12^{c}	3.18±0.09^{e}	3.70±0.19^{d}	4.87±0.18^{b}	7.36±0.08^{a}

Means with similar letters in a single row are statistically non-significant at $p < 0.05$.

frequency of binuclei, dumble and notched nuclei with the mean values of 3.13 ± 0.03, 5.48 ± 0.05 and 6.65 ± 0.08, respectively (Table 1).

Table 2 shows the mean frequency of MN and ONA induced in blood erythrocytes of four fish species. Among four fish species, *Cirrhina mrigala* showed significantly ($p < 0.05$) higher frequency of MN with the mean value of 27.50 ± 18.85 %, followed by that of *Ctenopharyngodon idella* (20.46 ± 12.90%), *Labeo rohita* (19.58 ± 13.81%) and *Catla catla* (18.17 ± 14.29%). Mean frequency of all other nuclear abnormalities was significantly higher in peripheral erythrocytes of *Cirrhina mrigala*, followed by that of *Catla catla*, *Ctenopharyngodon idella* and *Labeo rohita* with the mean values of 21.81 ± 13.12, 17.47 ± 13.09, 14.49 ± 7.67 and 10.14 ± 6.07%, respectively (Table 2). The DNA damage, in-terms of MN frequency and frequency of ONA, in all the four fish species showed significantly ($p < 0.05$) higher dependence on arsenic concentration (Table 3). The partial regression coefficients for *Labeo rohita*, *Cirrhina mrigala*, *Catla catla* and *Ctenopharyngodon idella* were positive and highly significant at $p < 0.01$. The high value of R^2 for each fish species predicts significantly high reliability of these regression models (Table 3).

Table 2. Induction of micronuclei and other nuclear abnormalities (mean ± SD) in peripheral blood erythrocytes of various fish species due to arsenic exposure.

Frequency (%)	Fish Species			
	Labeo rohita	***Cirrhina mrigala***	***Catla catla***	***Ctenopharyngodon idella***
Micronuclei	19.58±13.81^{c}	27.50±18.85^{a}	18.17±14.29^{d}	20.46±12.90^{b}
Other nuclear abnormalities	10.14±6.07 ^{d}	21.81±13.12^{a}	17.47±13.09^{b}	14.49±7.67^{c}

Means with similar letters in a single row are statistically non-significant at $p < 0.05$.

Table 3. Relationship between exposure concentration of arsenic and frequency of nuclear abnormalities induced in peripheral blood erythrocytes of fish.

	Fish Species	Regression Equation		SE	r/MR	R^2
Micronuclei Frequency (%)	***Labeo rohita***	= -3.36+3.06	**Concentration	0.197	0.980	0.960
	Cirrhina mrigala	= 11.76+3.75	**Concentration	0.377	0.943	0.889
	Catla catla	= 7.24+9.60	**Concentration	0.703	0.974	0.948
	Ctenopharyngodon idella	= -0.45+3.27	**Concentration	0.376	0.940	0.883
Other nuclear abnormalities (%)	***Labeo rohita***	= 0.72+1.11	**Concentration	0.203	0.865	0.748
	Cirrhina mrigala	= 6.82+2.72	**Concentration	0.342	0.929	0.863
	Catla catla	= -6.55-7.58	**Concentration	0.161	0.998	0.996
	Ctenopharyngodon idella	= 5.82+1.40	**Concentration	0.058	0.992	0.984

SE: Standard Error; **r**: Multiple Regression Coefficient; **R^2**: Coefficient of Determination; ****** Highly Significant at $p < 0.01$.

DISCUSSION

In contaminated waters, significant increase in metallic ions toxicity was reported by Foulkes (2000). It is observed that the toxicity of various metallic ions to fish species is related to their rate of transformation to the bio-available form (Muniz and Oliveria-Filho, 2006). Micronucleus test in fish has been commonly employed for detection and estimation of biological impacts of water pollutants on genotoxic damage in fish (Ergene *et al*., 2007; Parveen and Shadab, 2012). Micronuclei are cytoplasmic chromatin containing bodies made up of broken fragments of chromosomes or from the chromosomes that could not be incorporated into daughter nuclei (Fagr *et al*., 2008). Moreover, determination of nuclear abnormalities in erythrocytes of fish serves as an index of cytotoxicity (Mahboob *et al*., 2013). Previous literature indicated that arsenic genotoxicity is linked with the production of reactive oxygen species that cause oxidative stress resulting in DNA damage (Vahter, 2009; Dopp *et al*., 2010). Yadav and Trivedi (2009) assessed the mutagenic potential of arsenic trioxide, mercuric chloride and copper sulphate using fish, *Channa punctata*. Arsenic appeared more toxic than other two metals as it caused significantly higher frequency of chromosomal abnormalities in fish at sub-lethal concentration. Nuclear abnormalities can be considered as excellent indicators of genotoxic damage and therefore, they may complement micronuclei scoring in routine genotoxicity surveys.

Results of present investigation revealed significantly variable mean frequency of MN and ONA induced in peripheral erythrocytes of four fish species. Among four fish species, *Cirrhina mrigala* appeared significantly ($p < 0.05$) more sensitive to arsenic and showed significantly ($p < 0.05$) higher frequency of micronuclei, followed by that of *Labeo rohita*, *Catla catla* and *Ctenopharyngodon idella*. Mean frequency of all other nuclear abnormalities was significantly higher in peripheral erythrocytes of *Cirrhina mrigala*, followed by that of *Catla catla*, *Ctenopharyngodon idella* and *Labeo rohita*. Micronucleus test showed inter-species variations in micronuclei frequency as reported by Kim and Hyun (2006), Barbosa *et al*. (2010) and Kumar *et al*. (2010). These variations may occur due to dissimilar metabolic indices as well as DNA repair ability of different fish species. Genotoxic damage may also show dependence on the toxicants involved and fish species exposed to that toxicant (Ali *et al*., 2008). It is evident from the literature that the variability in genotoxic response of various chemicals is mainly related to metabolism and other pharmacokinetic factors in animals (Al-Sabti and Metcalfe, 1995).

Result obtained from this experiment showed that arsenic trioxide act as clastogenic chemical that cause different chromosomal abnormalities in fish in concentration dependent manner. The DNA damage, in-terms of micronuclei frequency and other nuclear abnormalities, in all the four fish species showed significantly higher dependence on arsenic concentration. Various metallic ions act as valuable genotoxins at particular concentrations just because of their ability to bind to thiol groups and cause instability in the spindle formation in the blood cells (Patra *et al*., 2004). Ahmed *et al*. (2011) observed the genotoxic effects of arsenic on *Orechromis mossambicus*. He reported concentration dependent increase in MN frequency in the erythrocytes of fish. In *Channa punctatus*, the frequency of MN was found significantly higher due to arsenic exposure as compared to control fish (Patowary *et al*., 2012). According to Kumar *et al*. (2013) the genotoxic potential of arsenic at different exposure concentrations in *Channa punctatus* and *Carassius auratus* varied significantly. He observed concentration dependent increase in the frequency of micronucleated erythrocytes was dose dependent in both *Channa punctatus* and *Carassius auratus*. The DNA damage, determined in-terms of micronuclei frequency (%) and frequency of other nuclear abnormalities (%) showed significantly direct relationships with the arsenic concentrations. Summak *et al*. (2010) reported significantly positive correlation between concentration of metal and frequency of nuclear abnormalities in *Oreochromis niloticus* also.

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